

# DIAS report



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## Health promoting compounds in vegetables and fruit

Proceedings of workshop in Karrebæksminde, Denmark, 6-8 Nov. 2002

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**Ministry of Food, Agriculture and Fisheries**

**Danish Institute of Agricultural Sciences**

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## Introduction

This book contains the papers presented at the workshop “Health promoting compounds in vegetables and fruit”, held 7-8 November 2002 at Smålandshavet Conference Centre, Karrebæksminde, Denmark

The subject of the workshop is the identification and utilisation of naturally occurring bioactive compounds with beneficial effects on human health was. In this context it focuses on the scientific state of the art; needs and opportunities for new discoveries; and the market situation, needs and opportunities for utilisation

### Objective

The workshop aims to provide an opportunity for scientists and companies to:

Present and discuss recent progress in relevant research on identification and understanding “new” bioactive compounds (primarily others than “traditional” minerals, fibres, vitamins and other antioxidants).

Present and discuss commercial opportunities for the food sector, including technical and market aspects. Technical aspects are e.g. plant breeding, plant cultivation, food processing and distribution as well as the use of plant material as ingredients in complex foods. Market aspects ranges from legislation in relevant countries to examples of and prospects for marketing of products with improved quality characteristics or documentation.

Establish relevant links and clusters among scientists and companies within this topic, in order to meet the challenges and opportunities of the developments taking place in the European Research Area.

### Background

It is well known that a high consumption of vegetables and fruit affords a protection against cancer, heart attacks and other important diseases. However, it has also become clear, that in developed countries it is not a simple question of deficiencies in known plant-derived vitamins, minerals and antioxidants. We simply don't know which components in plants are the most important for human health. Due to this, it is difficult to make further developments on the effects of food on health, It is also virtually impossible to predict if a change in practice regarding processing and distribution of food has consequences for consumer health.

One of the possible explanations is the hypothesis that plants contain other natural bioactive compounds, that provide benefits for health when present in the diet in relevant amounts, even though they are not essential nutrients. However, the methods normally used in nutrition research are not well suited to investigate compounds that are not essential for human health, and where the effects on human physiology are as

of yet only vaguely defined. Moreover, most bioactive plant compounds are known or suspected as toxicants. For these, often no studies have been made of their possible health promoting effects, and when this is done, particular attention must be made to concentration dependency. It is necessary to combine methods and results from research on herbal medicine

and on plant health with human nutrition research and studies on food processing. The most urgent need concerns preliminary studies to identify the compounds that have the greatest potential for improvements of the health value of food, and thus should be selected for further study.

### **Acknowledgements**

The workshop is supported by the Programme Committee for Research on Nutrition and Food (FELFO) of the Board of the Danish Research Councils, as part of a 3-year multidisciplinary project with two objectives:

To establish a network of scientists that can provide preliminary investigations of disease preventive effects of natural compounds in vegetables and fruit.

To make such a study of falcarinol in carrots.

This project comprises scientists from Danish Institute of Agricultural Science, Royal Veterinary and Agricultural University, Copenhagen National University Hospital and Technical University of Denmark.

Additionally the Øresund Food Network (ØFN) provided financial support for the workshop and distributed information about it. The working group for Food and Health selected “Bioactive compounds in vegetables, fruit and berries” as a priority subject to promote the food research in the Øresund area, covering Denmark and southern Sweden. This initiative comprises scientists from Danish Institute of Agricultural Science, the Universities of Lund, Århus and Copenhagen, the Agricultural Universities in Alnarp and Copenhagen, the Technical Universities in Lyngby and Lund, as well as the Danish Food Agency.

The workshop and its programme was organised by Kirsten Brandt and Björn Åkesson, with helpful input from Jóhanna Haraldsdóttir. The proceedings were collected and formatted by Tina Lillelund Hansen and Camilla Fjord, and Marian Grønbæk took care of registrations and payments.

# EU 6<sup>TH</sup> FRAMEWORK PROGRAMME FOR RESEARCH

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## Abstract

The 6<sup>th</sup> Framework Programme 2003-2006, which has an overall budget of €17.5 billion, is the EU's main instrument for funding of research in Europe. It is open to all public and private entities, large or small, in the EU and countries associated with the Programme.

The Programme has 7 key areas. In the key area Food Quality and Safety, activities will cover research, including, where appropriate, post-genomics research, relating to various aspects of the control of health risks and links between health and food.

Several instruments will be applied to implement the 6<sup>th</sup> Framework Programme, notably: Networks of excellence, Integrated projects, Specific targeted research projects, Specific research projects for SMEs and Specific support actions.

Acore can assist research institutes, universities, companies, etc. interested in participating in the 6<sup>th</sup> Framework Programme in various aspects of preparing and implementing a EU project. Acore is a team of consultants who serve clients requiring competencies within international project development, management and financing.

## Keywords

EU, research, financing.

## What is the 6<sup>th</sup> Framework Programme?

The 6<sup>th</sup> Framework Programme is the EU's main instrument for funding of research in Europe. The overall budget covering the four-year period 2003-2006 is €17.5 billion.

Seven key areas for the advancement of knowledge and technological progress have been identified:

- Life sciences, genomics and biotechnology for health
- Information society technologies

- Nanotechnologies and nanosciences knowledge-based multifunctional materials, and new production processes and devices
- Aeronautics and space
- Food quality and safety
- Sustainable development, global change and ecosystems
- Citizens and governance in a knowledge-based society

The first calls for proposals under the programme are expected end 2002-beginning 2003, with submission deadline 3 months later. Prior to that, input from the scientific community and industry through e.g. submission of expression of interest will be channelled into detailed work programmes and form the basis for the calls for proposals.

### **What does key area "Food Quality and Safety" cover?**

The text of the 6<sup>th</sup> Framework Programme says of the objective of the key area Food Quality and Safety that:

"The activities carried out in this area are intended to help establish the integrated scientific and technological bases needed to develop an environmentally friendly production and distribution chain of safer, healthier and varied food, including sea-food and to control food-related risks, relying in particular on biotechnology tools taking into account the results of post-genomic research, as well as to control health risks associated with environmental changes".<sup>1</sup>

Community activities will cover research, including, where appropriate, post-genomics research, relating to various aspects of the control of health risks and links between health and food:

(a) safer and environmentally friendly production and processing methods and healthier, nutritious, functional and varied foodstuffs and animal feed, based on systems such as integrated production, lower-input farming including organic agriculture, and the use of plant and animal sciences and biotechnologies;

(b) epidemiology of food-related diseases and allergies, including the impact of diet on the health of children and methods for the analysis of causes of food-related allergies;

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<sup>1</sup> L 232/12 29.8.2002 Official Journal of the European Communities EN

(c) impact of food, for instance new products, products resulting from organic farming, functional food, products containing genetically modified organisms and those arising from recent biotechnology developments on health;

(d) traceability<sup>TM</sup> processes all along the production chain, for instance relating to genetically modified organisms, including those based on recent biotechnology developments;

(e) methods of analysis, detection and control of chemical contaminants and existing or emerging pathogenic micro-organisms (such as viruses, bacteria, yeasts, fungi, parasites and new agents of the prion type including development of ante-mortem diagnostic tests for BSE and scrapie);

(f) impact of animal feed, including products containing genetically modified organisms and the use of sub-products of different origins for that feed, on human health;

(g) environmental health risks linked to the food chain (chemical, biological and physical), and combined exposures of authorised substances, including impact of local environmental disasters and pollution on the safety of foodstuffs, with emphasis being placed on cumulative risks, transmission routes to human beings, long-term effects and exposure to small doses, as well as the impact on particularly sensitive groups, and especially children.

€685 million has been set aside for the key area of Food Quality and Safety.

### **Which instruments will be used to implement the 6<sup>th</sup> Framework Programme?**

Several instruments have been developed for the implementation of the 6<sup>th</sup> Framework Programme. Below is given an outline of some of the actions.

#### *Networks of excellence*

Networks of excellence are designed to strengthen scientific and technological excellence on a particular research topic by networking together at European level the critical mass of resources and expertise needed to provide European leadership and to be a world force in that topic. This expertise will be networked around a joint programme of activities aimed principally at creating a progressive and durable integration of the research capacities of the network partners while at the same time advancing knowledge on the topic.

#### *Integrated projects*

An integrated project is designed to generate the knowledge required to implement the priority themes by integrating the critical mass of activities and resources needed to achieve ambitious clearly defined scientific and technological objectives. Each integrated project should be aimed either at increasing Europe's competitiveness or at addressing major societal

needs. The integrated project is therefore an instrument to support objective-driven research, where the primary deliverable is new knowledge.

*Specific targeted research projects*

These projects will aim at improving European competitiveness and meeting the needs of society or EU policies. They should be sharply focussed and will take either of the two following forms or a combination of the two:

- (a) RTD project designed to gain new knowledge either to improve or develop new products, processes or services or to meet other needs of society and EU policies
- (b) demonstration project designed to prove the viability of new technologies offering potential economic advantage but which cannot be commercialised directly.

Specific targeted research projects will be of a more limited scope and ambition, particularly involving smaller research actors and participants from candidate countries for the EU.

*Specific research projects for SMEs*

Two instruments will be available under this heading:

- (a) co-operative research projects undertaken by research institutions for the benefit of a number of SMEs on themes of common interest. Innovative SMEs in collaboration with research centres and universities may also carry out research.
- (b) collective research projects carried out for industrial associations or industry groupings in sectors where SMEs are prominent.

*Specific support actions*

Within the priority themes, specific support actions will be supported, e.g. conferences, seminars, studies and analyses, working groups and expert groups, operational support and dissemination, information and communication activities.

**How can Acore be of assistance?**

Acore is a team of consultants who serve clients requiring competencies within international project development, management and financing.

The business foundation of Acore consists mainly of international project counselling to European companies and organisations.

The company is situated in Brussels, the capital of Europe, close to the European Commission and the vast network of collaborators. With branches in both Copenhagen and Aarhus we serve clients in all of Denmark.

### *Project development*

Project development of partly financed projects (grants) requires an accurate assessment of a number of preconditions that must be described and presented to the offices of concession. Regardless of whether you deal with Danish or International Financing Institutions the same preconditions must be considered.

Acore has several years' experience in project development of partly financed projects – both in connection with large and complex projects as well as smaller and general projects.

Notably EU financed projects are a field of expertise within the company. We have been involved in the development of several successful research projects under the 5<sup>th</sup> Framework Programme.

Project development is, of course, closely associated with the elaboration of a funding application. Acore has in-depth experience with applications at all levels, not least in relation to applications for EU funding.

### *Project financing*

Acore assists clients in identifying relevant financing for projects, whether it is through grants, loans or investors. We help our clients expand contact with financing institutions and develop or adjust the projects to the preconditions laid down to obtain financing.

### *Project management*

Smaller projects as well as larger international projects need efficient management. Most often the time factor or the burden of the administrative tasks (especially in the case of EU projects) can pose a serious impediment to creating good results.

Acore can help companies with small and large projects. In relation to smaller projects the assignments can be administrative as well as day-to-day sparring, whilst those companies with larger, more complicated and internationally financed projects, need more assistance with the formal procedures and the maintenance of international contacts.

Furthermore, Acore offers general and tailor-made courses in project management with particular emphasis on international projects.

For further information on Acore, please see [www.acore.com](http://www.acore.com).

## **IS ANTIOXIDANT ACTIVITY OF FOODS IMPORTANT FOR HEALTH?**

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### **Abstract**

The present mini-review addresses the question whether there is a relationship between food antioxidants and health, e.g. prevention of ageing and disease. The negative outcomes in several long-term human intervention trials precludes a general positive answer to the question, however, it is still possible that specific antioxidants or combinations of antioxidants may have health preventive actions. The methodologies necessary to answer the questions experimentally are discussed and recent results from short-term intervention studies with antioxidant-rich food items are reviewed. It is concluded that oxidative damage is most often very localised at the molecular level and that the markers for oxidative damage therefore have to address very specifically the level of damage in structures important for health, e.g. lipoproteins or target organ DNA. More unspecific markers may also be useful, but they need to be validated against the disease endpoints in order to assess their usefulness. It is concluded that there is not presently much evidence to conclude that antioxidants in food are important to health but biobank-based biomarker studies with a nested case-control design may be useful in order to give a more definitive answer to the question of the relationship between antioxidant-rich foods and disease prevention.

### **Introduction**

The hypothesis that dietary antioxidants are preventive against chronic disease has a very strong appeal in the scientific community. This may be because it seems immediately plausible. It is very likely that ageing in humans is partly caused by free radical reactions just like ageing and rancidity of foods. We also know that radical reactions are involved in plaque formation in the intima of blood vessels. Furthermore, radicals may react with DNA to cause damage, some of which can lead to mutation, the putative initiating event in cancer. Thus, radical reactions are likely involved in ageing, atherosclerosis and cancer and consequently antioxidants at the right time and place should theoretically be able to partly prevent these conditions. The idea that the preventive action of fruits and vegetables towards chronic disease which has been observed in many epidemiological studies was caused by the high levels of antioxidants in these food items has given further plausibility to the antioxidant hypothesis. If the hypothesis was found to be generally true the preventive actions of

antioxidants would be immediately commercially exploitable, a fact that has probably helped funding of further research into the effects of antioxidants thereby lending economic support to research into the hypothesis. The most direct research has been directed towards the antioxidant minerals, vitamins and pro-vitamins in plant foods, including selenium, vitamins A, C, E and beta-carotene. However, human intervention studies with these nutrients have not given immediate support to the antioxidant hypothesis. Except for selenium-yeast the other antioxidant nutrients were either neutral or even harmful in controlled human studies (Combs, Jr. et al., 1997; Omenn et al., 1996; The Alpha- Tocopherol, 1994). Therefore, in its general formulation the hypothesis cannot be accepted. Increase in any antioxidant does not necessarily lead to decreased health risk. Some antioxidants may even be detrimental to health at dose levels above the nutritionally possible. However, it may still be true that specific antioxidants are preventive, or even that a general decrease in oxidative damage may decrease the risk of chronic disease. More likely, however, decreasing the damage at specific sites in the body may be truly preventive. The available methodology to evaluate such hypotheses consists of human and animal studies in combination with biomarkers for oxidative damage and for antioxidative defence. A brief review of the current evidence in favour of this modified antioxidant hypothesis will be given in the following sections.

### **Biomarkers for oxidative damage**

Radical or other oxidative attack on cells and other structures in the body may lead to damage to lipids, proteins and DNA. Any of these structures may therefore be used to monitor for damage provided damage is random. Results from animal studies and human biomarker studies indicate that markers of damage are not generally well correlated, indicating that damage may not be random. Since most radicals are rather short-lived we would expect that they tend to react close to the site where they are formed. It could be speculated that such sites might be regarded as epicenters for radical damage. Iron-containing proteins involved in oxygen reduction or transport would therefore seem to be potential epicenters generating oxidative damage. Peroxidases would be another potential localised site, whereas more general or background damage would be expected to result from the presence of free transition metal ions or from the presence of background levels of radioisotope decay in the body. We would also expect differences in radical formation at the organ level. The uncovered skin for example would be a site for random damage from UV-induced radical formation. The liver would be expected to have a higher level of radical formation from its high content of iron-containing cytochromes. Protective measures of the body also varies between organs, with skin pigment formation and high levels of antioxidative enzymes in erythrocytes and in liver as obvious examples. It may therefore be expected that large differences in radical formation and quenching capacity exists both at the organ level and at the subcellular level and that experimental studies are needed in order to get a picture of the real distribution of oxidative damage. Plants antioxidants which are absorbed into the body and which show high affinity for particular structures may therefore be able to decrease oxidative damage at such

particular sites. On the other hand, since antioxidants are redox active compounds they may also cause increased radical formation if they uncouple electron pathways in the body or if they chelate transition metals in such a way that they become more reactive like in the experimental Fenton oxidation systems. We cannot therefore *a priori* tell whether any plant derived antioxidant will be protective, harmful, or both, depending on the particular site in the body where we look for the effect.

#### *Protein oxidation markers*

Biomarkers rely on samples which can be obtained with minimal invasiveness, e.g. blood, urine, nailcuts, etc. Blood is rich in proteins and lipids and also contains potential sites for radical formation and destruction. The hemoglobin in the red blood cell, the cell membrane and the blood plasma surrounding it are therefore interesting sites to monitor for oxidative damage to proteins and lipids. In a study of carcinogen-induced oxidative damage in rabbits we have monitored damage in the form of oxidised lysine sites and oxidised proline sites in hemoglobin and oxidised lysines in plasma proteins in order to test whether plasma protein oxidation might be regarded as an echo of the radical formation in the red blood cell. We observed a very high correlation between damage to the two different amino acids in hemoglobin in the animals, but no such correlation existed between damage to lysine residues in hemoglobin and to lysine residues in the plasma proteins surrounding the erythrocyte (Dragsted, unpubl.). In a cross-sectional study of protein oxidation levels in a randomly selected danish cohort we found a similar lack of correlation and also lack of correlation to plasma MDA (Dragsted, unpubl.) indicating that other sources of radicals dominate protein oxidation in the plasma compartment. In a human study where fruits and vegetables were omitted from the diet over a period of 10 weeks we observed a similar time course in the change in protein oxidation in plasma and in erythrocytes, indicating that some factors are common to the two compartments. However, less than 10% of the oxidative damage in the two compartments was linked to this dietary change (Young et al., 2002). A similar weak relationship between these two blood compartments was observed in a study of the time course of protein oxidation in the rat over its entire lifespan (Daneshvar et al., 1997).

Different markers of protein oxidation also lead to different effects with the same antioxidant treatment. Ascorbate tends to increase oxidative damage to plasma protein lysine sites (Young et al., 1999; Young et al., 2000) whereas the formation of carbonyls in plasma globulins have been observed to decrease (Carty et al., 2000). Therefore redox active compounds may have dual actions even within the plasma compartment.

Thus, the interpretation of any change in protein oxidation must depend on the modified proteins in question. Since hemoglobin, globulin or albumin oxidation are not likely related to cancer or heart disease it is difficult to interpret the existing literature. It will be important in the future to develop protein oxidation markers which addresses the oxidation of specific proteins involved in chronic disease processes, e.g. apolipoprotein B.

*Lipid oxidation markers*

Lipid oxidation markers include *ex vivo* plasma lipoprotein lagtime measurements, plasma and lipoprotein MDA, and plasma or urinary isoprostanes. In a study on hyperlipidemic rabbits the level of MDA in HDL, LDL and VLDL fractions from plasma differed, and increased oxidation of LDL and VLDL was observed after dosing with probucol without change in the level of MDA in HDL (Lauridsen and Mortensen, 1999). When the *ex vivo* plasma lipoprotein lagtime was determined in the VLDL samples from these rabbits (kindly analysed by Birgit Mayer, Technical Univ. Graz, Austria) we observed a lag time close to 0 min, in accordance with the MDA determinations (Dragsted, unpubl.). However, when the relatively small changes in lagtimes and in lipoprotein or plasma MDA were compared in humans which had been depleted from fruit and vegetables there was no inverse correlation between these markers (Young et al., 2002).

In an intervention study with a parallel design, 18 female smokers and 16 non-smokers were initially depleted from carotenoid-rich dietary items for eight days and had then additional 300-400g of beta-carotene-rich, lutein-rich, or lycopene-rich vegetables to their diets sequentially for one week each. During the lycopene-rich dietary intervention LDL-oxidation lagtimes significantly increased in the non-smokers (Chopra et al., 2000). In a somewhat similar study, a 2-week intervention with carotenoid-rich fruits and vegetables in smokers and non-smokers of both sexes led to an increase in LDL-oxidation lagtimes in both groups, 14% in smokers and 28% in non-smokers. However, the effect was not significantly different in the two groups (Hininger et al., 1997). In an intervention study without control group, a two-week low carotenoid period was followed by daily consumption of 330 mL tomato juice, then by 330 mL carrot juice and then by 10 g of spinach powder, each for 2 weeks in 23 healthy men (smoking status not given). LDL lagtimes increased by 18% during the intervention with tomato juice but not during the other interventions (Bub et al., 2000). It is difficult to evaluate the uncontrolled results in the latter two studies since the effect may have been due to outside factors.

In a parallel dietary-controlled human intervention study with 123 healthy females given 9 servings per day of fruits and vegetables or basic diet low in plant foods over a period of 8 weeks there was no effect on plasma thiobarbituric acid reactive substances (TBARS), a relatively unspecific measure of MDA (Miller, III et al., 1998). In a study of carotenoid-rich vegetable juices, tomato juice for two weeks, as opposed to carrot- or spinach juice, reduced plasma TBARS (Bub et al., 2000). These authors did not provide the diet but used plasma carotenoids to assess that compliance was acceptable. In a similarly assessed 3-6 months study of carotenoid-rich vegetables and fruits in 29 healthy females plasma TBARS decreased marginally but significantly (Maskarinec et al., 1999).

In a two-week fruit and vegetable intervention study in 28 smoking females where the fruit and vegetable intake was increased from 5.6 to 12 servings (976g) per day, a reduction in urinary 8-isoprostane  $F_{2\alpha}$  but not in MDA was reported (Thompson et al., 1999). In a human 2x3 week cross-over intervention study with fruit and vegetable concentrates rich in antioxidants there was no effect on plasma 8-isoprostane  $F_{2\alpha}$  or on plasma MDA (van den

Berg et al., 2001). In studies on specific antioxidants, neither polyphenols from green or black tea consumption, vitamin E, vitamin C, vitamin C+E combinations, or isoflavonoids have been found to significantly affect the excretion of 8-isoprostane  $F_{2\alpha}$  in well-controlled short-term human intervention studies (Hodgson et al., 1999; Hodgson et al., 2002; Huang et al., 2002; Patrignani et al., 2000).

There are an insufficient number of studies comparing these markers with each other or with other lipid oxidation markers, including breath pentane or ethane and plasma lipid peroxide concentrations, however, the general impression lasts that the various lipid oxidation markers are not closely linked but that very strong effects in one marker tends to 'spill over' into the other markers. Antioxidant-rich interventions may affect these markers differentially and the most general trend is that markers of *ex vivo* lipoprotein lagtime often tends to show increases with foods rich in lipid soluble antioxidants in favour of a hypothesis that these foods may confer oxidative protection specifically to lipoproteins. We should be warned, however, by the fact that no relationship between *ex vivo* lipoprotein lagtime and a decreased risk of atherosclerosis has ever been shown to exist. In the ASAP study vitamins C plus E partially decreased atherosclerosis and at the same time decreased lipid oxidation (Porkkala-Sarataho et al., 2000; Salonen et al., 2000). Also autoantibodies to oxidised LDL has been found to be correlated to an increased progression rate in carotid atherosclerosis (Salonen et al., 1992). In contrast, animal studies point towards the opposite relationship with increased lipoprotein oxidation leading to a decrease in atherosclerosis (Lauridsen and Mortensen, 1999).

#### *Markers of oxidative damage to DNA*

Markers of oxidative damage to DNA include nuclear or urinary 8-oxo-deoxyguanosine, and the comet assay when used in conjunction with endonucleases which specifically cut DNA at oxidatively damaged bases. There is a standing controversy as to the level of steady-state oxidative damage in human cellular DNA (Collins et al., 1996) and a good correlation between 8-oxo-dG in cellular or urinary DNA obtained by different methods is still to be seen. No DNA is present close to an iron-oxygen system in the blood since erythrocytes have no nucleus and the oxidative damage observed in leucocyte DNA may not be representative of damage to DNA in other parts of the body. Oxidative damage to DNA is efficiently repaired by a range of DNA-repair systems, and some of these systems are sensitive to the level of induced damage and can be induced by factors which lead to increased oxidative stress. For instance the mRNA levels of both ERCC1 and OGG1 were recently found to be positively correlated to the average daily influx of sunlight in the previous 30 and 5 days, respectively. However, they were unaffected by intervention with 600g fruits and vegetables for three weeks (Vogel et al., 2002).

Administration of 300 mL freshly prepared green tea to smoking and non-smoking volunteers for three weeks was reported to decrease 8-oxo-dG in both white blood cells and urine as well as urinary MDA excretion, although no statistical analysis was presented (Klaunig et al., 1999). The decrease in urinary MDA but not in urinary 8-oxo-dG was reported to be most prominent in the smokers. Other antioxidants, including vitamin E, ascorbate and coenzyme Q10 were previously found not to affect overall DNA oxidation as

determined by 8-oxo-dG excretion (Porkkala-Sarataho et al., 2000; Priemé et al., 1997). On the other hand a range of strong effects of fruits, vegetables and antioxidant vitamin supplements on the level of leucocyte comets in volunteers have been reported (Collins et al., 1998; Collins et al., 2001; Duthie et al., 1996). We have not been able to reproduce these effects in an intervention study with 600g Fruits and vegetables during three weeks (Moller et al., submitted). Thus, the effects of antioxidant-rich foods on markers of DNA damage is controversial and none of the markers have been observed so far to be related to cancer risk. It is therefore too early to conclude on the preventive actions against cancer of antioxidants in foods.

## **Biomarkers for antioxidant defence**

### *Antioxidant enzyme markers*

The regulation of defence enzymes such as superoxide dismutase, catalase, glutathione peroxidase involved in removal of reactive oxygen species and of enzymes such as glutathione reductase involved in the regeneration of endogenous antioxidants can be regarded as markers of antioxidative defence. The levels of these markers are particularly high in the erythrocytes in support of the view that these cells are potential sources of radical formation in the body.

Although chemical and physical inducers of these enzymes are known (Alvarez and Boveris, 1993; Cowan et al., 1992; Stevens et al., 1988) the presence of dietary effects on the activation or induction of antioxidant enzymes is controversial. We have observed increased activity of glutathione peroxidase (Gpx) in erythrocytes after only one week of intervention with fruit juice or grape skin extract in healthy volunteers. Since circulating erythrocytes do not have genetic regulation this would indicate that post-transcriptional regulation may take place (Young et al., 1999; Young et al., 2000). In contrast Bub and coworkers found no effect on glutathione peroxidase of various vegetable juices in healthy individuals (Bub et al., 2000), whereas Bruce et al. even observed a significant decrease in erythrocyte Gpx in hyperlipidemic women after four weeks of intervention with a diet rich in fruits, vegetables and whole grains (Bruce et al., 2000). Consequently, it is still uncertain whether ordinary intake levels of fruits and vegetables can influence circulating or tissue levels of antioxidant enzymes and therefore whether dietary fruits and vegetables actually may confer disease protection by enzyme induction.

### *Antioxidant capacity markers*

There are very many assays which may be used to determine antioxidative capacity of plasma or other body fluids. Two of them, Trolox Equivalent Antioxidant Capacity (TEAC) and Ferric Reducing Capacity of Plasma (FRAP) have been extensively used (Benzie and Strain, 1996; Rice-Evans and Miller, 1994). TEAC and FRAP are sometimes reported to change in short-term human intervention studies with specific plant foods in controlled studies. Thus green tea (Benzie et al., 1999), tomato products with olive oil (Lee et al., 2000), whisky

phenolics (Duthie et al., 1998) or black tea without milk (Leenen et al., 2000) apparently increased FRAP postprandially, while diets rich in tomato products alone (Bub et al., 2000) or spinach (Castenmiller et al., 1999) did not. In previous studies we have not been able to note any effects on fasting values of TEAC or FRAP after fruit juice, grape skin extract or green tea extract intervention (Young et al., 1999; Young et al., 2000; Young et al., 2002). In the latter study we noted a postprandial effect on radical-induced plasma oxygen consumption. Thus, fruits and vegetables seem only rarely to affect plasma antioxidant capacity measures whereas food items with very high levels of plant phenols may have postprandial effects.

## Conclusions

In conclusion antioxidants in foods and foodstuffs may modulate the level of oxidative damage, however, the evidence that this modulation is related to a changed health risk is still lacking. The most compelling evidence so far comes from the ASAP study which indicates that combinations of antioxidants may retard the development of atherosclerosis, however, the study found effects in men only and was not lengthy enough to show a change in mortality resulting from the antioxidant effects.

Biobank-based biomarker studies with a nested case-control design may be able to validate the markers of oxidative damage and defence against some of the harder endpoints such as mortality from cancer or heart disease. This evidence will be necessary in order to answer more definitively the question posed as a title to this short review.

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## RECENT DEVELOPMENTS IN BIOAVAILABILITY OF FALCARINOL

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### Abstract

Falcarinol from carrot may have cancer-preventing properties. So it is important to know its bioavailability, to determine if the concentrations found in the human body correspond to those concentrations that appear to have beneficial effects *in vitro*.

Fourteen males received doses of 300, 600 and 900 ml of carrot juice for breakfast, containing 4, 8 and 12 mg falcarinol, respectively. The level of falcarinol was measured in plasma 10 times during an 8-hour period starting just before breakfast. All three doses resulted in rapid increases in plasma falcarinol, within half an hour after the meal. The values peaked after 2 hours with concentrations between 1 and 2.5 ng/ml plasma for each dose when averaged across subjects, coming almost down to baseline at 8 hours. This corresponds well with *in vitro* studies reporting positive effects on cell cultures in a concentration range of 0.5–50 ng/ml in the medium.

The results of the present study show that falcarinol from carrots is biologically available to humans, and can occur in concentrations that are relevant for the postulated beneficial effects. They thus demonstrate the relevance of further investigations of the potential health benefits of falcarinol.

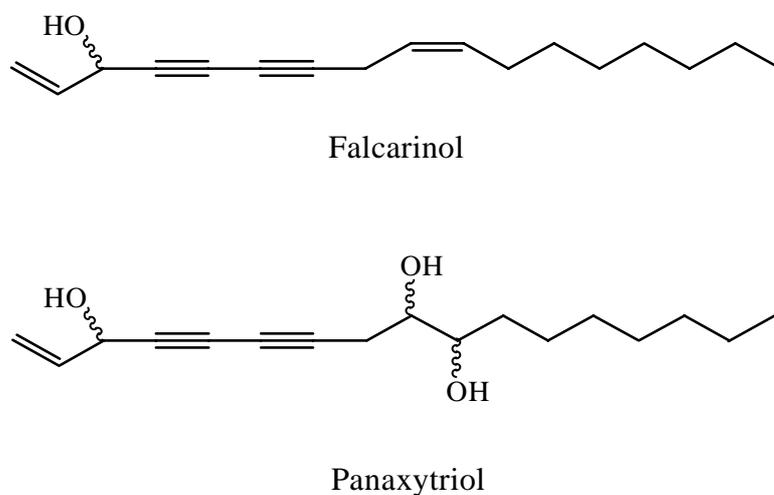
Key words: *Daucus carota*, carrot juice, falcarinol, bioavailability

### Introduction

Falcarinol is a polyacetylene present in carrots and some other root vegetables, as well as in ginseng. It is a biologically active compound showing cell stimulating as well as cell inhibiting and cytotoxic effects *in vitro*. Chemistry, occurrence and biological activity of falcarinol have already been described by Christensen *et al.* (this volume). The occurrence of falcarinol in a common vegetable like carrot, and the increasing number of reports from *in*

*vitro* studies documenting its potentially beneficial biological activity, raises the question whether man is able to absorb and utilise falcarinol from foods. No such information has been available up to now, neither from animal or human studies. However, for a closely related compound, panaxytriol (figure 1), a rat study on plasma response showed rapid uptake after a single oral dose of the pure compound (Saita *et al.*, 1994).

Bioavailability of a food component is considerably more complex than the availability of the pure compound, as it depends both upon its chemical form and physical structure in the food, and upon the matrix to which the compound may be bound. For example,  $\beta$ -carotene in some fruit is present as lipid droplets, whereas in carrots it is in a crystalline form, and in a dark green vegetable like spinach it is bound in a complex protein-fiber structure in the chloroplast



**Fig. 1.** Chemical structures of the polyacetylenes falcarinol and panaxytriol.

(Castenmiller & West, 1998). These differences have implications for bioavailability, which seems to be about four times higher for  $\beta$ -carotene in fruit than for  $\beta$ -carotene in dark-green leafy vegetables (de Pee *et al.*, 1998). For many nutrients only a small or moderate proportion of their content in foods is actually absorbed and thus biologically available. Heat treatment and other conditions, which disrupt cell structures or affect the binding of the compound to its food matrix, may change bioavailability substantially. Thus intake of lycopene from heat processed tomato juice resulted in a considerably higher plasma response than intake of lycopene from unprocessed juice (Stahl & Sies, 1992).

Bioavailability is typically first estimated by giving a single dose of the compound, or the food, followed by measurements of its concentration in blood plasma at certain time intervals. More thorough investigations may then follow, including balance studies or use of radioisotope labelled compounds.

### *Study on bioavailability of falcarinol from carrot juice*

The aim of the present study was to investigate whether falcarinol in carrots is absorbed by man, and to describe the time course and the dose-response relationship. Bioavailability was measured as plasma response following an oral dose.

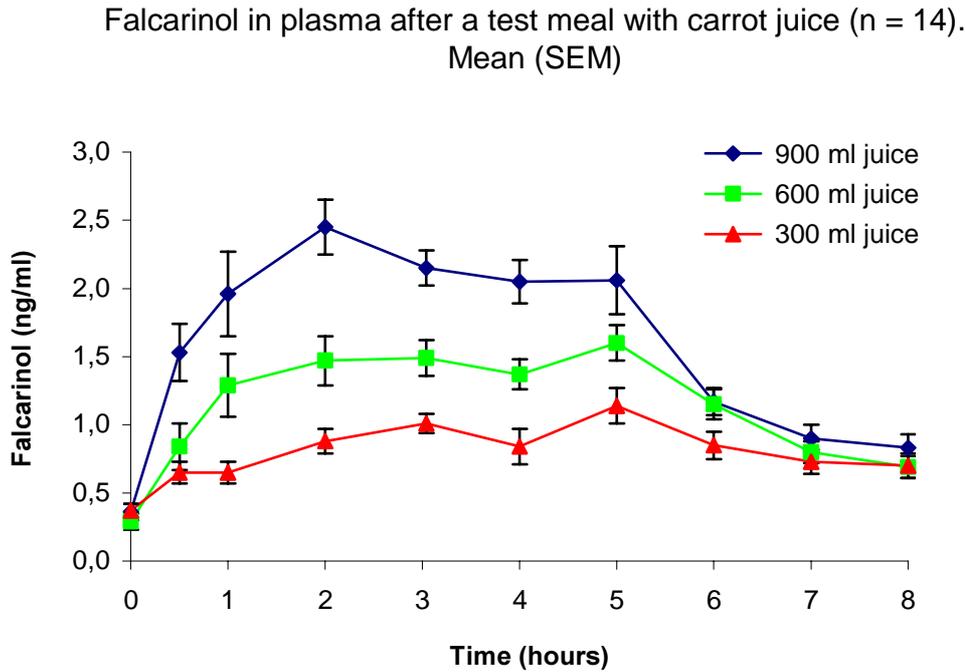
### **Materials and methods**

In a randomised cross-over study 14 young, healthy males ingested a breakfast meal containing 300, 600 or 900 ml carrot juice (containing 4, 8 and 12 mg falcarinol, respectively) together with standardised amounts of bread and butter. Fluid intake and energy intake in the meal were standardised to the 900 ml level by including an energy drink with carbohydrates. Prior to each of the test days subjects excluded all food items containing falcarinol from their diets for 2 days. A blood sample was drawn before the breakfast meal and at regular intervals thereafter (see figure 2). A standardised (falcarinol free) lunch was ingested 4 hours after the breakfast. The carrot juice was made at the institute and analysed for falcarinol content by HPLC using diode array detection. The order of the three juice doses was randomised.

For the quantification of falcarinol in plasma samples a sensitive LC–MS method was developed as (Hansen-Møller *et al.*, 2002): Plasma proteins were precipitated by addition of 2 parts of acetonitril to one part plasma. The extracted falcarinol was analysed on a Quattro LC LC/MS system using a reversed phase C18 column and a binary gradient solvent system consisting of 0.1% formic acid in water and acetonitril. The MS system was operated in the electro-spray positive mode. Falcarinol was detected using an MRM method with argon as collision gas and quantified using falcarinol as external standard.

### **Results**

The results of the study are presented in figure 2. An increase in plasma falcarinol was observed with all three doses, also the lowest dose with 4 mg falcarinol. The increase was rapid, within half an hour after the meal, and peak values were observed after 2 hours, with concentrations between 1 and 2.5 ng/ml plasma, depending upon the dose. The two lowest doses then showed a small second peak after the (falcarinol free) lunch meal ingested after 4 hours. A distinct decrease started after 5 hours, and at 8 hours the falcarinol concentration was almost down to baseline.



**Fig. 2.** Concentration of falcarinol in plasma as a function of time after ingestion of a breakfast meal with 300, 600 and 900 ml carrot juice, containing 4, 8 and 12 mg falcarinol, respectively.

Substantial inter-individual variation in response was observed. The maximum individual value measured with these doses was approximately 4 ng/ml plasma. In a small pilot study (n = 2) with larger intake of falcarinol (28 mg in 800 ml carrot juice) we observed individual peak values between 6 and 10 ng/ml.

## Discussion

This first study of the bioavailability of falcarinol in human subjects demonstrates that falcarinol in carrots is biologically available, and is carried with the blood stream to the tissues. This is the fundamental basis for doing further work on its fate in the body and its potential health effects and mechanisms of action (Christensen *et al.*, this volume).

With the doses included here, i.e. single doses of 4–12 mg falcarinol, mean plasma concentrations of 1.0–2.5 ng/ml were reached. These values should be viewed in comparison with results from *in vitro* studies, where positive effects were reported on cell cultures within a concentration level of 0.5–50 ng/ml in the medium (Christensen *et al.*, this volume). Obviously these concentrations are not directly comparable, but the values give an impression of the relative orders of magnitude.

The short elimination time of falcarinol from plasma, approximately 8 hours, raises the question about its fate in the body. Such an elimination may be due to uptake in tissues, to metabolic conversion (which may result in a more or a less bioactive compound), to

excretion through the urine or other routes, or to a combination of these. This list of possible explanations shows that the short elimination time of falcarinol from plasma may either be an indication of loss of its biological potential, or on the contrary an indication of increased biological activity and relevance. Investigation of these questions on kinetics and metabolism will require the use of radioisotope labelled falcarinol.

The results of the present study shows, that falcarinol from carrots is biologically available to man, and thus raise a number of new research questions. First of all, however, they demonstrate the relevance of investigating the potential health benefits of falcarinol further.

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## BIOACTIVE CARBOHYDRATES IN VEGETABLES AND FRUIT

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### Abstract

The colonic microflora is vital for the health of the human host. The survival and growth of these bacteria are highly dependent on the substrates available for degradation. The main source for this fermentation is indigestible carbohydrates which, in affluent societies, amounts to 20-60 g/day (Cummings J H and Macfarlane G T 1991). The fermentation products are mainly short-chain fatty acids (SCFA; acetic, propionic and butyric acid) and gases. Some acids, especially butyric acid, are in turn the main source of energy for the colonocytes, and have been reported to exert beneficial health effects on the host. Since the different SCFA have diverse physiological implications, the proportions of which these acids are formed are important. The distribution is dependent on both the microflora and substrate available.

### Introduction

The concept of bioactive carbohydrates has during the last decade come to be a very popular way to describe the new shades found in the carbohydrate nutrition research. Specific carbohydrates (e.g. glucoproteins and gangliosides) with a defined target are often referred to as bioactive in medical research. However, in the field of food science and nutrition, the definition of bioactive carbohydrates is no way near that sharp. The expression could of course go for all carbohydrates with beneficial health effects, which would include digestible carbohydrates with a low glycaemic index, or indigestible carbohydrates that are either resistant to fermentation or easily fermented. The broad appellation is, however, mostly used for highly fermentable carbohydrates giving specific short-chain fatty acid-patterns during the bacterial degradation in colon.

Increasing the intake of carbohydrates has been a part of the dietary recommendations for a long time. However, studies the last ten to twenty years have shown interesting nutritional differences between various carbohydrates, resulting in an even bigger reason to take the quality of the carbohydrates under consideration (Asp N-G 1996). The fact that the proportions of different carbohydrates (starch, sugar and dietary fibre) in the diet are highly variable is noteworthy, resulting in typical nutrient profiles for different communities around the world (Garrow J S and James W P T 1996).

The nutritional properties of carbohydrates are highly connected to their availability for digestion and absorption in the upper intestinal tract. The properties of both digestible and indigestible carbohydrates are important from a health perspective. The most well known source for indigestible carbohydrates is the dietary fibre. The nature of impact dietary fibre has on human physiology depends very much on the resistance against colonic fermentation. Insoluble dietary fibres, found to a great extent in brans and whole-grain cereals, are generally more resistant to fermentation, and will therefore act as bulking agents, thus reducing the transit time and the risk for constipation and eventually also colonic cancer (Harris P J and Ferguson L R 1993). Soluble dietary fibres, mainly found in fruits and vegetables are, on the other hand, more susceptible to bacterial degradation in the large intestine. During this fermentation short-chain fatty acids are formed, with possible effects on colonic health and glucose and lipid metabolism. The soluble dietary fibre polymers also exert effects by physical means in the upper gastrointestinal tract, where they increase the viscosity and in that way may diminish the absorption of digestible carbohydrates and bile acids. In recent years there has been an increasing interest for other indigestible carbohydrates than the dietary fibre. Examples of such carbohydrates are oligosaccharides, resistant starch and fructans. These act as a type of dietary fibre and are generally completely fermented in the colon.

### **Fermentation**

The large intestine is densely populated with over 400 species of bacteria in excess of  $10^{11}$  cells per gram of contents (Moore W E et al. 1978). The bacteria and their metabolic activity can affect the health of the human host in both positive and negative ways. Species that have the potential to cause diseases are designated as pathogenic. Bacteria that survive the passage through the gastrointestinal tract and have health-promoting effects on the host are, in contrast, often referred to as probiotic. Bacterial growth is stimulated through fermentation. Carbohydrates that increase the number and/or activity of a specific bacteria capable to improve the health of the host, are often referred to as prebiotics (Gibson G R et al. 1995).

The fermentation products, with carbohydrates as substrate, are mainly the SCFA; acetic, propionic and butyric acid and gases ( $H_2$ ,  $CO_2$  and  $CH_4$ ). The colon rapidly absorbs 90 % of the SCFA, stimulating water and sodium absorption (Ruppin H et al. 1980). A numerous of positive health effects have been proposed for some of these acids. Indigestible carbohydrates that exert beneficial physiological effects through the SCFA (formed during their fermentation) may be better referred to as bioactive.

## **Physiological implications of short-chain fatty acids**

The rate of fermentation varies in different regions of the large bowel. The short-chain fatty acid production in the caecum may be eight-fold higher than in the sigmoid or rectum (Macfarlane G T et al. 1992). The main destinations for the SCFA absorbed are muscle-, hepatic- and mucosal metabolism (Salminen S et al. 1998). SCFA constitute the main energy source of the colonic mucosa, of which butyric acid accounts for 70 % (Scheppach W 1994). Butyric acid is therefore a potent trophic agent on normal colonic mucosa under different experimental conditions (Medina V et al. 1998), and affects proliferation and differentiation of normal colonic cells (Schwartz B et al. 1998).

Butyric acid is most likely important in the prevention and treatment of colon diseases such as cancer (Medina V et al. 1998) and distal ulcerative colitis (Scheppach W et al. 1992). A possible mechanism of which butyric acid may increase gene expression and arrest cell growth, is that it inhibits histone deacetylase. Thus, it causes a hyperacetylation of histone H4, which facilitates the access of DNA repair systems (Archer S et al. 1998). The fact that butyric acid, also enhances apoptosis in colonic cancer cells, might contribute to the protective effects against colonic cancer (Aukema H M et al. 1997). It has further been suggested that the lowered pH caused by SCFA production, inhibits the bacterial breakdown of primary to secondary bile acids. Secondary bile acids is probably implicated as a promoting agent in the adenoma-carcinoma sequence of colorectal cancer (Christl S U et al. 1997).

Butyric acid may play a role in the pathogenesis of distal ulcerative colitis. The inflammation has shown to be ameliorated upon treatment of butyric acid irrigation (Scheppach W et al. 1992). It appears, furthermore, as patients with ulcerative colitis has an impairment in the oxidation of butyric acid (Chapman M A et al. 1994).

Most of the propionic acid escapes metabolism in the colonocytes but is instead highly cleared by the liver. Its presence in the liver seems to improve the glucose tolerance in healthy subjects (Thorburn A et al. 1993). The effect could be due to the inhibition of gluconeogenesis and increase of glycolysis, that has been shown with propionic acid in rat hepatocytes (Anderson J W and Bridges S R 1984). The acid has also been proposed to inhibit hepatic cholesterologenesis, but the results are not consistent. One mechanism suggested is that propionic acid inhibits the rate-limiting step (hydroxymethylglutaryl CoA reductase) in the synthesis of cholesterol (Chen W J et al. 1984). Propionic acid may also increase the faecal bile excretion, and thereby affect the enterohepatic circulation (Levrat M A et al. 1994). Further, rectal infusion of propionate has been shown to reduce serum lipid levels, by inhibition of acetate incorporation into triacylglycerols (Wolever T M et al. 1995). The fact that the substrate for fermentation affects the pattern of SCFA formed, makes it valuable to find foodstuff containing carbohydrates that, upon fermentation, gives a high production of especially butyric-, but also propionic acid.

## **Substrates for fermentation and short-chain fatty acid pattern**

The carbohydrates are the most important substrate for the colonic flora (20-40 g/day reach colon), but small amounts of protein (3-5 g/day) and fat may also reach colon for fermentation. Starch which, for different reasons, escapes digestion in the upper intestinal tract is commonly referred to as resistant starch (RS) (Champ M 1992). The three major forms of RS that is identified so far are retrograded starch (e.g. bread and boiled potatoes), raw starch granules (e.g. raw potatoes and unripe green bananas) and physically entrapped starch (e.g. legumes) (Liljeberg H 1995).

Other substrates for colonic fermentation are fructans such as inulin, which is mainly found in chicory roots, Jerusalem- and globe artichokes. Fructans are indigestible oligo- and polysaccharides consisting of fructose residues linked to a single glucose molecule. It has been reported that inulin and other fructo-oligosaccharides selectively stimulate the growth of *Bifidobacteria*, a genus of bacteria in the large bowel, considered beneficial to health (Gibson G R et al. 1995). The D-galactose-containing raffinose family oligosaccharides (RFO; raffinose, stachyose and verbascose) are highly fermentable and mainly found in legumes. Raffinose has, when given to rats, shown a high production rate of butyric acid (Nyman M and Björck I 1996).  $\beta$ -glucans, mainly found in oat bran, have also been reported to give a high rate of butyric acid upon fermentation, while guar gum generates high proportions of propionic acid. Acetate is formed in high proportions when pectin, e.g. found in apples, is fermented (Berggren A et al. 1993).

Until now research has focused on indigestible carbohydrates and lately resistant starch, as potential substrates for fermentation. However, there are reasons to believe that even some low molecular weight carbohydrates can escape digestion and reach the colon. Fructose may for example, when consumed in large amounts, be poorly digested and/or absorbed in many subjects, and thus constitute a substrate for fermentation (Truswell A S et al. 1988). Further, there is little knowledge about the digestibility of mono- and disaccharides in a botanically intact structure, as found in raw vegetables.

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## ANTICANCER EFFECTS OF BOSWELLIC ACIDS, THE COMPOUNDS ISOLATED FROM GUM RESIN OF *BOSWELIC SERRATA*, ON HUMAN COLON AND LIVER CANCER CELLS

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### Abstract

Boswellic acids are a type of pentacyclic triterpenic acids isolated from gum resin of *Boswellia serrata*, a large deciduous tree growing in hilly parts of India. The gum resin of *Boswellia serrata* has been used as an anti-inflammatory herb for hundreds of years and the effective composition was found recently to be boswellic acids. We studied the effects of the acids on cell proliferation and apoptosis in human colon and liver cancer cells. It was found that boswellic acids inhibited cell proliferation, reduced [<sup>3</sup>H] thymidine incorporation, increased sub-G1 peak in flow cytometry analysis, and induced apoptosis. The apoptosis was mediated via activation of caspase-8 and caspase-3 pathway leading to degradation of poly-(ADP-ribose)-polymerase (PARP). The anticancer effect varied with the type of the acids with the most effective one being acetyl-keto-boswellic acid, which was 3.5 fold more potent than camptothecin, a common anticancer drug. Boswellic acids were also found to activate alkaline sphingomyelinase, which is present in the intestinal mucosa and can inhibit proliferation of colon cancer cells. In conclusion, boswellic acids might be a promising candidate for chemoprevention of colon and liver cancers.

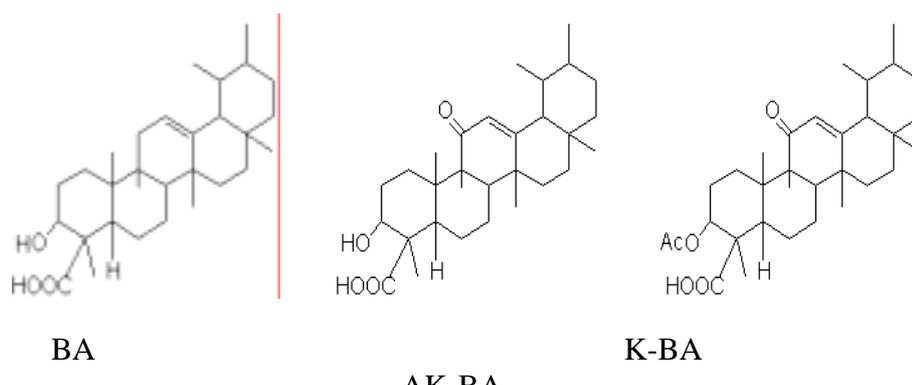
Key words: boswellic acids, cancer, proliferation, apoptosis, sphingomyelinase

### Introduction

The gum resin of *Boswellia serrata*, a kind of deciduous tree growing in the dry part of India, has been used for the treatment of arthritic diseases for hundreds of years. The pentacyclic triterpenic acids, named boswellic acids, in the gum resin of the tree are the main constituents for their anti-inflammatory property. Inhibition of leukotriene synthesis via suppressing 5-lipoxygenase and inhibition of leukocyte elastase are considered the main mechanisms underlying their anti-inflammatory effects (Safayhi *et al.*, 1992; Safayhi *et al.*, 1997). Companies in India and USA have provided the market with products in the form of capsules, yogurt, or cream (for topical application) containing boswellic acids for relieving arthritis, back pains and other joint problems. Other products containing a mixture of boswellic acids with other biologically active plant compounds such as curcuminoids, nextrutine, and salicin

have also been developed. It has been suggested that boswellic acids be used as food supplement for patients with inflammatory diseases. The herb is so far known to be of few side effect and less toxic, and is considered as a safe component.

Recently a few studies on brain tumours and leukemic cells indicated that boswellic acids might have antiproliferative effects (Glaser *et al.*, 1999; Shao *et al.*, 1998). Very recently, the acids have been used in clinical trials or experimental animal models for the treatment of Crohn's disease, ulcerative colitis and ileitis (Gerhardt *et al.*, 2001; Gupta *et al.*, 1997). Because inflammatory bowel diseases particularly chronic ulcerative colitis increase the risk of colon cancer, we studied the potential anticancer effects of the acids on both colon cancer HT29 cells and liver cancer Hep G2 cells. These two cell lines differ from location and degree of differentiation. Three types of boswellic acids were used, named  $\beta$ -boswellic acid (BA), 11-keto- $\beta$ -boswellic acid (K-BA) and acetyl-11-keto- $\beta$ -boswellic acid (AK-BA), which are the main constituents of *Boswellia serrata* extract. The structures of these acids are shown in Figure 1.



**Fig 1.** Structures of boswellic acids. Left panel:  $\beta$ -boswellic acid (BA), middle panel: 11-keto- $\beta$ -boswellic acid (K-BA) and right panel: acetyl-11-keto- $\beta$ -boswellic acid (AK-BA).

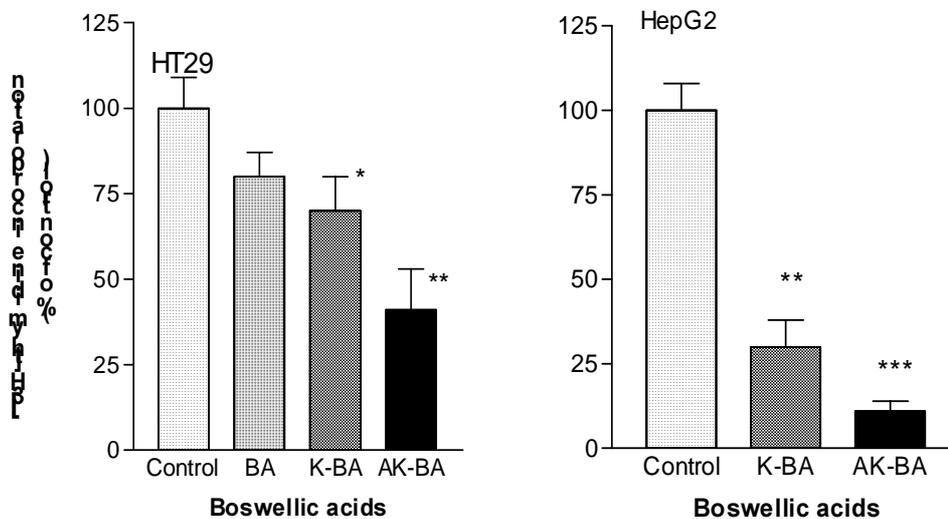
## Methods

The cells were cultured in RPMI-1640 medium with glutamine, 100 IU/ml penicillin, 10  $\mu$ g/ml streptomycin and 10% foetal calf serum and treated with boswellic acids at different concentrations. The cell proliferation and viability were determined by hydrolysis of tetrazodium salt (WST-1) to formazan. The DNA synthesis was determined by incorporation of [ $^3$ H] thymidine. The apoptosis was analysed by the enrichment of mono- and oligonucleosomes in cytoplasm using a Cell Death Detection ELISA kit and by flow cytometry. The degradation of PARP and expression of Fas were studied by Western blot. The activities of caspase-8, 9 and 3 were assayed by their abilities to hydrolyse specific substrates. Selective inhibitors of individual caspase and inhibitor of Fas were used to

identify the apoptotic pathway. Sphingomyelinase activity was determined by the measurement of hydrolysis of [ $^{14}\text{C}$ ]-sphingomyelin to ceramide and phosphocholine.

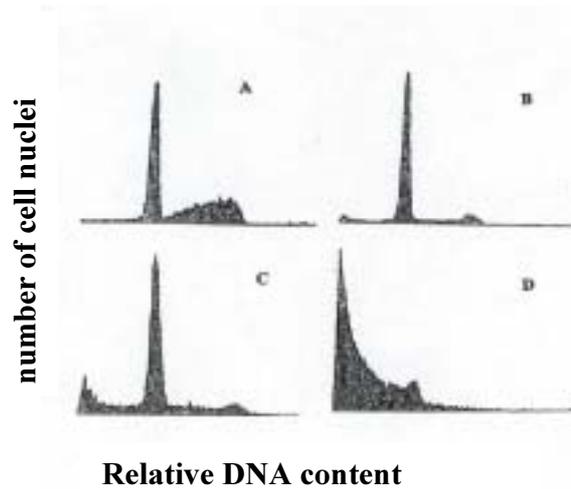
## Results and Discussion

When the colon cancer and liver cancer cells were incubated with boswellic acids, the incorporation of [ $^3\text{H}$ ] thymidine as well as the cell viability were inhibited. The effect of AK-BA was stronger than that of K-BA. BA was the least effective one (Fig. 2).

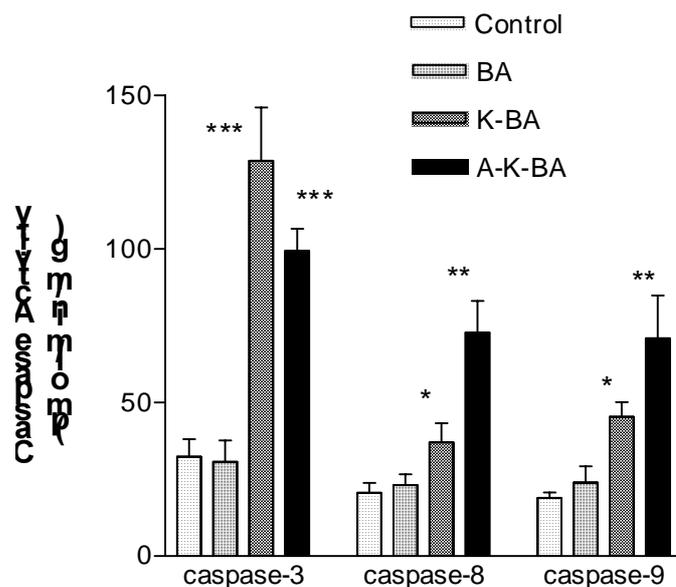


**Fig. 2.** Antiproliferative effects of boswellic acids on human colon cancer HT29 cells and liver cancer Hep G2 cells. The cells were treated with different boswellic acids (50  $\mu\text{M}$ ) for 24 hours. The incorporation of [ $^3\text{H}$ ]thymidine was determined.

AK-BA and K-BA also induced apoptosis in both colon and liver cancer cells after 24 hours incubation as measured by the levels of cytoplasmic DNA-histone complex. At 50  $\mu\text{M}$ , AK-BA increased apoptotic rate in colon cancer cells by 5 fold and in liver cancer cells by 6.5 fold. Flow cytometry analysis demonstrated that AK-BA and K-BA arrested the cell cycle at sub-G1 and increased the number of sub-G1 cells (Fig. 3). The effect of AK-BA was stronger than that of K-BA, whereas the effect of BA occurred only at the highest concentration.



**Fig. 3.** Apoptotic effect of boswellic acids on HT29 cells assayed by flow cytometry. The cells were treated with 100  $\mu$ M boswellic acid for 24 hours. The figure shows the profile of control (A), BA (B), K-BA (C) and AK-BA (D)



**Fig. 4.** Changes of caspase 3, 8 and 9 activities induced by boswellic acids in HT29 cells. The cells were treated with 100  $\mu$ M BA, K-BA and AK-BA for 24 hours.

In order to further confirm the apoptotic effects of the acids, the changes of three types of caspase activities were determined. As shown in Fig.4, after treatment of HT29 cells with K-BA and AK-BA, the activities of caspase-3, caspase-8 and caspase 9 increased significantly. Similar effect was demonstrated for Hep G2 cells. BA at the same concentration did not show significant stimulatory effect on the activities of the caspases. It is well-known that PARP is

cleaved by caspase-3 and the cleavage of PARP is regarded as a specific marker of apoptosis. We therefore also investigated the cleavage of PARP in HT29 cells after incubation with boswellic acids. We found that the intact form (113 kDa) of PARP was decreased and its 89 kDa cleaved fragment was increased by AK-BA incubation.

To evaluate the efficiency, the apoptotic effects of AK-BA on HT29 cells were compared with that induced by camptothecin. At 50  $\mu$ M, the formation of cytoplasmic DNA-histone complex and the activation of caspase 3 induced by AK-BA were more than 3-fold greater than those induced by camptothecin.

Apoptotic effect is one of the important properties for many anti-cancer drugs. Apoptosis occurs via two major different activation pathways in principle (Green, 1998; Green & Reed, 1998). One pathway involves changes in mitochondrial transmembrane potential, leading to the release of cytochrome c and activation of caspase-9. The other pathway starts with death receptor ligation or Fas/FasL interaction, followed by oligomerization of the receptor, recruitment of Fas-associated death domain protein (FADD), and activation of caspase-8. Both caspase-9 and caspase-8 are defined as initiator caspases and can in turn activate caspase-3, the executor of apoptosis. Cross communication exists between the two pathways, as caspase-8 may activate caspase-9 via Bid, a member of bcl-2 family. In the present work, we demonstrated that the activities of caspase-3, -8 and -9 were activated by boswellic acids, indicating that both apoptotic pathways were activated. To distinguish which pathway is initiating, different caspase inhibitors were employed. We found that either caspase-8 or caspase-3 inhibitor completely blocked the boswellic acid-induced apoptosis, while the caspase-9 inhibitor only partially inhibited it, indicating that apoptosis induced by boswellic acids in HT-29 cells was initiated by a pathway involving the activation of caspase-8, leading to the activation of caspase-3 and the cleavage of PARP. The activation of caspase-9 was likely to be a secondary consequence of the activation of caspase-8 through the cross communication between the two apoptotic pathways. Similar phenomena were also observed in Hep G2 cells.

As caspase 8 is closely linked to the apoptosis pathway initiated by interaction of Fas/FasL, we further examined whether activation of Fas would be important in mediating the effect of boswellic acids. We found that preincubation with ZB4, the antagonistic Fas antibody, blocked Fas-induced apoptosis but did not change the AK-BA induced apoptosis. AK-BA also had no effect on the expression of Fas in HT 29 cells as demonstrated by Western blot. The findings indicate that interaction of Fas/Fas ligand is not critically involved in boswellic acid-induced apoptosis.

Sphingomyelin (SM) is a type of phospholipid which is present in the diet such as milk, egg, meat, and fish. SM metabolism triggered by sphingomyelinase (SMase) has been considered a novel signal transduction pathway which generate multiple lipid messengers which can inhibit cell proliferation and induce apoptosis (Hannun & Linardic, 1993). Recently SM metabolism

has been implicated particularly in colon cancer development (Duan, 1998). A SMase with alkaline pH-optimum was previously discovered in the intestinal mucosa (Nilsson, 1969) and its activity was recently found to be decreased with 50-75% in human colon cancer (Hertervig *et al.*, 1999; Hertervig *et al.*, 1996). Purified alkaline SMase inhibited cell proliferation and DNA biosynthesis by 50 % in HT29 cells. Of interesting when colon cancer cells were incubated with AK-BA, the activity of alkaline SMase significantly increased accompanied by a reduction of SM in the cells. Thus activation of SMase at least contributes partly to antiproliferative effects of boswellic acids on colon cancer cells.

Our findings that boswellic acids have anti-proliferative and apoptotic effects in colon and liver cancer cells may have clinical implication. The chemopreventive effect of nonsteroidal anti inflammatory drug (NSAID) such as sulindac on colon cancer has been well documented (Shiff & Rigas, 1998). However, blockade of cyclooxygenase (COX) by NSAIDs, especially the blockade of COX-1, could cause multiple severe side effects such as the bleeding in the intestinal tract and impairment of renal function. In contrast, boswellic acids did not inhibit COX and their clinical safety has been demonstrated in the treatment of Crohn's disease, ulcerative colitis and bronchial asthma. Due to the low toxicity, the acids can be given orally or intrarectally in a high dose. Judged from these aspects above, whether boswellic acids can be a promising candidate for the chemoprevention of colon cancer might be an interesting topic for further investigation.

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## **ASSAY OF TOTAL ANTIOXIDANT CAPACITY IN VEGETABLES, FRUITS AND BERRIES**

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### **Abstract**

The intake of fruit, berries and vegetables is linked to reduced incidences of cardiovascular diseases and certain types of cancer. Several components may mediate these effects among them antioxidants and other bioactive compounds. In the development of processed foods with enhanced health effects, it is important to maintain and optimise the antioxidant content. For this purpose, new methods have been developed to measure the total antioxidant capacity (TAC) of food. In this paper studies on different methods for measuring the TAC in fruits, berries, vegetables and prepared products are summarised.

Key words: Antioxidant capacity, ABTS radical, ferric ion reduction, electrochemistry

### **Introduction**

Many vegetables, fruits and berries have a favourable macronutrient composition and many of them also have a high content of ascorbic acid and beta-carotene. There is also an increasing awareness that plant foods contain a large number of bioactive compounds many of which have antioxidant properties (Diplock et al., 1998, Ewald et al, 1999). Although analytical methods for individual compounds are necessary, there is also a need to establish simple methods to get an overall measure of the amount of antioxidants and their activity in different foods. Several principles for this have been advanced (Benzie, Strain, 1996; Re et al. 1999, Cao et al., 1993), but so far only few data are available on their application to different foods.

### **Methodological aspects**

Several vegetables, fruits and berries and also beverages, purées and other beverage ingredients were analyzed. The samples were homogenised in buffer and centrifuged. The measurement of water-soluble antioxidants was performed in the resulting supernatant. Moreover, the pulp resulting from centrifugation was extracted with acetone and TAC measured in the acetone extract.

For all methods, the TAC values were standardized in relation to the water-soluble analogue of alpha-tocopherol, Trolox and the results were expressed as  $\mu\text{mol}/\text{gram}$  of Trolox equivalent antioxidant capacity. One assay used was a modification of the FRAP procedure (Benzie, Strain, 1996), which measures the ferric ion reducing capacity. It is a spectrophotometric method based on the measurement of the formed coloured complex of ferrous ion and 2,4,6-tri(2'-pyridyl)-1,3,5-triazine. The second assay used was the ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] assay (Re et al., 1999). It is a spectrophotometric method based on the quenching of the ABTS radical cation, a blue/green chromophore, resulting in decreased absorbance after the addition of antioxidants.

In some experiments the ABTS and FRAP methods were compared using the same samples. Analysis of TAC in extracts from strawberries, cauliflower, carrots, tomatoes and carrots resulted in data, which were highly correlated ( $r=0.75-0.94$ ), and good agreement between the two methods was obtained for most matrices.

In other types of samples measurement of antioxidant capacity was made using electrochemical methods (Chen, 2002; Chen et al., 2002). Based on studies with cyclic voltammetry and rotating disk electrode linear sweep voltammetry a procedure for flow-injection amperometry was developed. It was performed in a flow-through electrochemical cell of the wall-jet type using a three-electrode system (Chen et al., 2002). Good agreement between data obtained by amperometry, the FRAP method and the ABTS method was obtained (Chen, 2002).

### **Studies on strawberries**

In strawberries differences in TAC could be seen among the ripening stage, among the varieties and between the water-soluble and the water-insoluble fractions. The water-soluble TAC was in the range  $5-9 \mu\text{mol}/\text{g}$  and the water-insoluble TAC was in the range  $3-14 \mu\text{mol}/\text{g}$ . Both the water-soluble and the water-insoluble TAC varied twofold within among cultivars and there was also some variation between the two investigated years within each cultivar. In previous studies by the ORAC procedure, strawberries were found to have the highest TAC value among twelve fruits tested (Wang *et al.*, 1996). Addition of vitamin C during strawberry jam preparation markedly increased TAC but there was also a considerable antioxidant capacity left in samples in which ascorbic acid had decayed (Viberg *et al.*, 2000). Among 27 fruits from Singapore markets tested with the ABTS method, only ciku (a tropical fruit) had higher TAC than strawberries (Leong and Shui, 2002) and recently strawberries among other dietary plants were screened with the FRAP method (Halvorsen *et al.* 2002). The TAC of strawberries in these other studies was in the range  $13-27 \mu\text{mol}/\text{g}$ .

## **Studies in processed berry and fruit products**

TAC was measured in fruit and berry beverages and purées available on the Swedish market. The highest TAC value was seen in raspberry purée and in a beverage based on black currant. The stability of TAC in some of the beverages and purées stored at room temperature was tested during 12 months and there were only a moderate decrease in TAC during the year. A screening of raw materials potentially useful for new beverages showed a wide range in TAC where rose hip, acerola, and sea buckthorn had the highest values. Fractions taken from the processing of the fruit and berries were also examined. The data showed several folded differences in TAC among berries and processed fractions and also between water-soluble and water-insoluble TAC.

## **Studies in peas**

For peas different varieties, harvested during two years in early and late season were studied. Thirty-five varieties of green peas (*Pisum sativum*) were analysed for TAC. In different varieties of blanched and frozen green peas, the water-soluble fraction had a total antioxidant capacity of 0.61(0.22)  $\mu\text{mol/g fw}$  (mean(SD)) and the water-insoluble fraction a value of 0.23(0.08)  $\mu\text{mol/g fw}$ . For the antioxidant capacity in the water-soluble extract, there was a significant difference among the genotypes and there was also a significant effect of the harvest year and harvest period. Also for the water-insoluble fraction, there were significant differences among genotypes and between harvest periods but not between harvest years. There was a significant correlation between the total antioxidant capacity in the water-soluble and water-insoluble fractions ( $r=0.41$ ;  $P<0.001$ ).

## **Conclusion**

The data show that the methods used are useful screening methods for TAC. Additional measurements are necessary to document the role and biological activity of individual antioxidants and other bioactive compounds. It is also important to extend the approach to meal and intervention studies using plant based foods and to measure TAC and other variables in blood plasma of the consumer (Önning et al., 1998).

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## **POSTHARVEST CHANGES IN THE CONTENT OF BIOACTIVE COMPOUNDS**

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### **Abstract**

Vegetables, fruit and berries are still living tissues with an active metabolism after harvest, but senescence processes are initiated. Senescence may be described as endogenously controlled degenerative processes. Postharvest changes in the content of bioactive compounds will be the result of different factors as senescence processes, genetics, structural origin of the plant part, metabolic rate, postharvest handling and preharvest factors. Three examples of postharvest compositional changes are presented; changes during storage of broccoli, storage of strawberries and storage of onion.

Keywords: senescence, ascorbate, glutathione, flavonoid, carotenoid, antioxidant

### **Factors influencing the postharvest changes**

Contrary to most other food, vegetables, fruit and berries are living tissues with an active metabolism. From the harvest at the producer and during the whole distribution chain to the consumer, different physiological processes take place continuously. The plant has some basic requirements that must be fulfilled to maintain life and metabolism. Sufficient energy must be available to supply both anabolic and catabolic processes with energy through respiration. Sufficient amount of water must be available to maintain concentrations of dissolved compounds within physiological limits and to transport substances in the plant. After harvest stored energy is consumed through respiration, and water is lost through transpiration, but since no new energy or water are supplied, the harvested plant parts have to live on the reserves (Wills et al., 1998). Furthermore, no additional mineral nutrients can be supplied from uptake by the roots, so only translocation within the plant part is available for biosynthesis.

After harvest senescence processes are initiated in the vegetable, fruit or berry. Senescence may be described as endogenously controlled degenerative processes ultimately leading to death of the tissue, organ or the whole plant (Noodén and Leopold, 1988). At the cellular level, degenerative processes can be seen, leading to deterioration of structures as membranes (Paliyath and Droillard, 1992) or organelles, as for instance chloroplasts (Hurkman and Kennedy, 1976). However, senescence does not seem to be a passive breakdown of different

structures, but an active energy-requiring process (Noodén, 1988). Mitochondrial structure and activity are persisting until very late stage in senescence. Inhibition of ATP-production does not cause senescence, but necrosis, and many processes connected with senescence require energy (Noodén, 1988). In addition, the course of senescence seems to be under genetic control. Although there is generally a progressive loss of protein content during senescence, certain RNA species increase while other decrease (Brady, 1988).

There are many suggestions of what event(s) in the plant that may initiate the senescence processes. Shortage of available energy or mineral nutrient starvation can be mentioned as some of the suggested causes (King and O'Donoghue, 1995; Noodén et al., 1997). Contrary to the lack of consensus of what initiates the senescence, there seem to be a general understanding that the rate of the senescence process, and thereby the length of the shelflife of the produce, is connected to the rate of metabolism. The metabolism is related to the respiration rate. Produce with low respiration rate can generally be stored longer (Wills et al., 1998).

The structural origin of vegetables and fruits has a strong influence of the changes that occur within the products during their postharvest period. Produce that before harvest function as storage organ (e.g. carrots or potatoes) are structurally different than for instance leaves with photosynthetic function (e.g. spinach or lettuce) (Kays, 1991). The structure as well as the content of different compounds are different, and therefore the changes that occur after harvest will be different, although many similarities can be found as well. The different origin of the harvested plant parts also means that the metabolic rate, and the respiration rate, is quite varying between different products.

The structural origin and the metabolic rate has a strong influence of the postharvest changes that occur in the produce, but the postharvest handling may also have a strong impact of the compositional alterations. Storage at lower temperature slows down respiration and other metabolic processes, and a higher relative humidity decreases the transpiration rate (Wills et al., 1998), leading to a slower rate of the senescence processes. On the other hand, bruising of the products or exposure of ethylene (a natural plant hormone that accelerate senescence) may increase the rate of the senescence processes and the compositional changes of the produce. Preharvest factors as cultivation practices (fertilization, irrigation etc.) or climate may also influence both composition, structure and metabolic status and therefore the postharvest changes.

### **Bioactive compounds in plants**

In recent years, there has been an increasing interest in nutrient value and content of bioactive compounds of the produce. Many of these bioactive compounds are antioxidants, which have been proposed to play an important role in the protection against the incidence of cancer and coronary heart disease (Rice-Evans and Miller, 1995; Williamson, 1996). Antioxidants can scavenge reactive oxygen species, which might have the potential to damage cell components as DNA, lipids and proteins (Gutteridge and Halliwell, 1994). Further, other effects of the bioactive compounds that might be associated with decreased risk of cancer have been

suggested, as affecting enzyme activity of cyclooxygenase-2 or inhibit oncogene expression (Fosslien, 2000; Ranelletti et al, 2000).

Like humans, plants have an antioxidative defence, consisting of both antioxidants and antioxidative enzymes (Alscher and Hess, 1993). During senescence, reactive oxygen species may be formed at a higher rate, and they have been implicated to play an important role in the senescence process (Thompson et al., 1987; Paliyath and Droillard, 1992; Olsson, 1995). As a result of senescence after harvest, antioxidant levels often decrease. However, an increase of the antioxidant levels, that might be a stress response or a part of a ripening process, can be found in some tissues (Lazan et al., 1987; Olsson, 1995; Wills et al., 1998). Apart from the antioxidants vitamin C and E, plants contain a large range of other bioactive compounds with antioxidative and other properties. Carotenoids, flavonoids and phenolic acids have received increased attention as possible health promoting compounds in vegetables and fruits (Rice-Evans and Miller, 1995; Macheix and Fleuriet, 1998) and many more will surely be found in the future.

### **Three examples of postharvest compositional changes**

In accordance with previous discussion, the postharvest changes in the content of bioactive compounds will be the result of different factors as genetics, structural origin of the plant part, metabolic rate, postharvest handling and preharvest factors. Three examples of postharvest compositional changes will be presented in this paper; changes during storage of broccoli, storage of strawberries and storage of onion.

#### *Postharvest changes in broccoli*

Broccoli is a very perishable vegetable, with an estimated shelflife of 1-2 weeks under optimal storage conditions. It has a relatively high respiration rate, and the large surface to volume ratio leads to a relatively high transpiration rate of the broccoli florets (Wills et al., 1998). The changes in the content of the antioxidants ascorbate and glutathione were investigated during storage of broccoli, along with the activities of some of the enzymes that participate in the ascorbate-glutathione cycle. The ascorbate-glutathione cycle provides a mechanism for recycling oxidised ascorbate and maintaining ascorbate in its active, reduced form (Noctor and Foyer, 1998). Although glutathione may be synthesised by humans, it has been suggested that it might also be absorbed by humans from food (Jones et al., 1992). Ascorbate content decreased in broccoli during storage at 12°C, and after 8 days of storage it was reduced by about 50 % compared to day 1, which was the first day it would be available for consumers at the retail market. The content of total glutathione (reduced and oxidized form) increased initially, followed by a decrease. The activity of two of the enzymes of the ascorbate-glutathione cycle, dehydroascorbate reductase and glutathione reductase, decreased during storage, calculated on dry weight basis. The capacity for enzymatic regeneration of ascorbate might be important for the maintenance of the nutrient value during the postharvest

period. Also the content of total carotenoids decreased during storage. Since ascorbate is one of the major antioxidants in plants, the decrease in content during storage might have implications for changes in the content of other bioactive compounds with antioxidative properties in broccoli.

### **Postharvest changes in strawberries**

Berries are probably the most perishable group of all vegetables and fruits and generally have high respiration rates. Strawberries have an estimated shelflife of only 1-5 days under optimal storage conditions (Wills et al., 1998). Changes in ascorbate content in strawberries during storage at 4°C were investigated, along with the content of ellagic acid and hydroxycinnamic acids. Ellagic acid has been suggested to have antimutagenic and anticarcinogenic effects (Clifford and Scalbert, 2000). Both flavonoids and hydroxycinnamic acids have strong antioxidant activity. Certain flavonoids have also been shown to inhibit some human enzymes, such as lipoxygenase and xanthine oxidase, which are potentially pro-oxidant (Parr and Bolwell, 2000). The content of ascorbate showed good retention during storage of strawberries, and no significant decrease could be found after the 72 hours of storage, which is in accordance with the results of Hägg et al., 1999. The content of chlorogenic acid decreased, while no significant changes could be found in the content of ellagic acid. As a group, berries and other fruit show specific changes after harvest (Goldschmidt, 1986). In some of the fruits, the ripening process continues after harvest, as well as senescence processes similar to some of those in vegetables. During ripening often changes in the content of soluble solids and organic acids can be found, sometimes accompanied by an increase in the content of carotenoids. Ripening can be considered as a process of senescence (Wills et al., 1998), but the function of the fruit in reproduction and spreading of seeds, may have lead to specific features of this process.

### **Postharvest changes in onion**

The onion is a bulb, an underground bud with storage function. It has an estimated shelflife of 12-28 weeks under optimal storage conditions, and a low respiration rate (Wills et al., 1998). The flavonol quercetin has been shown to inhibit cancer tumor initiation and promotion (Hertog and Katan, 1998). The content of the flavonol quercetin was investigated in 17 different varieties of onion, before and after five months of storage at 1-2°C. In the onions, the monoglucoside 4'-O-quercetin, the diglucoside 3,4'-O-quercetin and the quercetin aglycon were identified and analysed by HPLC. After storage, the content of total flavonols was higher in eight of the investigated varieties, lower in one variety, while no significant differences could be found in the other eight investigated varieties. The increase in total flavonols after storage was in average 24%. The content of the quercetin aglycon was low before storage, and it decreased below detection level in many varieties after storage. Despite the long storage time, good retention, or even increase in content of the flavonol, was shown in the different varieties.

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## **HYBEN VITAL® - A NEW ANTI-INFLAMMATORY AGENT WITH DOCUMENTED IMPACT IN HUMAN AND ANIMAL MODELS IS UNDER DEVELOPMENT FROM A SUB-TYPE OF ROSA CANINA L.**

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Even the old Romans knew that the *Rosa canina* rose hip had anti-inflammatory properties as Plini recommended rose hip as treatment after attack by a mad dog.

In the Nordic countries rose hip has been used the last centuries because of its content of vitamins. It is indeed interesting to note that the same plant although in two different time periods has been used for very different purposes.

This opens for discussion that sub-types of rose hip perhaps have different biochemical potency.

In the early nineties it was reported from Langeland, an island south of Funen, Denmark that a special sub-type of rose hip might have anti-inflammatory properties, as users with inflammatory diseases of different origin including osteoarthritis and rheumatoid arthritis claimed that the locally made dry rose hip powder alleviated pain and stiffness.

In 1995 the first attempt was made to investigate a powder made from the *Rosa canina* berries. It was easily shown in vitro that chemotaxis of neutrophil leucocytes as well as the oxidative burst response from the same cell fraction was inhibited by even low concentrations of the dry HYBEN VITAL® powder as used in this first test. The next study was performed in a group of volunteers at Kolding Hospital, Denmark. This study confirmed the initial in vitro studies. Moreover it was also shown that C-reactive protein, a marker of inflammation, was reduced as a result of treatment.

This preliminary research prompted a group of orthopaedic surgeons in Norway to run a strictly controlled, parallel, double-blind, placebo controlled, clinical trial in patients with osteoarthritis all on the waiting list for hip or knee replacement. Fifty patients were given five HYBEN VITAL® capsules each containing 0.5 g of the powder in the morning and a similar dose in the evening. Another group of fifty patients were given identical capsules containing inactive placebo of the same dose.

After 4 months of treatment the two groups were compared. The group treated with HYBEN VITAL® reported a significant decline in pain and stiffness, and when the surgeon measured the flexibility of joints he could measure that mobility had significantly improved.

At the same time a clinical trial on HYBEN VITAL® was running in Denmark. The design was double-blind, placebo controlled, randomized and cross-over which means that each patient in turn will receive both treatments, but the patient and the researchers do not know the sequence of treatment. This study also aimed at testing the impact of HYBEN VITAL® on pain and stiffness in patients with osteoarthritis. This study also aimed at testing any possible impact on the immune system and on the consumption of concomitant pain killing medication.

Each treatment period lasted three months. The study confirmed that pain and stiffness is reduced as a result of treatment with HYBEN VITAL®. Moreover it was again shown that the chemotaxis of neutrophil leucocytes and the level of the inflammatory marker C-reactive protein had declined. A new and very interesting aspect was that the consumption of Paracetamol and Opioid drugs often used as to reduce pain in osteoarthritis declined significantly by up to 50% as a result of treatment with HYBEN VITAL®.

## NATURALLY OCCURRING ACETYLENES IN COMMON FOOD PLANTS: CHEMISTRY, OCCURRENCE AND BIOACTIVITY

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### Abstract

It is well known that a high intake of fruit and vegetables protect against cancer and other important diseases. In order to explain the health promoting effects of fruit and vegetables focus has primarily been on vitamins, minerals and antioxidants but still the mechanisms for this protection is unknown. Many bioactive compounds with known effects on human physiology and disease have been identified through studies of plants used in traditional medicine. Some of these substances occur also in common food plants, and hence could play a significant role in relation to human health. Among highly bioactive compounds that occur in very common umbelliferous vegetables (e.g., carrots, celery and knob celery) are aliphatic C<sub>17</sub> acetylenes of the falcarinol-type. These acetylenes have shown to be highly toxic to fungi, bacteria, viruses, and mammalian cells, to display neurotoxic effects and to be responsible for allergic and irritant skin reactions. However, the effect of these acetylenes towards human cancer cells and their growth regulating properties towards mammalian cells indicate that they may also provide benefits for health. The present state of knowledge on the occurrence of naturally occurring acetylenes in common plant foods is presented, including their chemistry and bioactivity.

Key Words: Acetylenes, food plants, distribution, bioactivity, hormesis

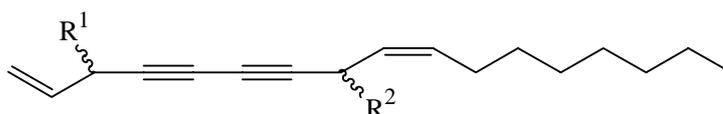
### Introduction

Through epidemiological investigations it is well known that a high consumption of vegetables and fruits protect against certain types of cancer and other important diseases (Block *et al.*, 1992; Greenvald *et al.*, 2001; Ness & Powles, 1997). In order to explain the health promoting effects of fruit and vegetables focus has primarily been on vitamins, minerals and antioxidants, but still we do not know which components are responsible for these effects of food plants (Block *et al.*, 1992; Greenvald *et al.*, 2001; Ness & Powles, 1997).

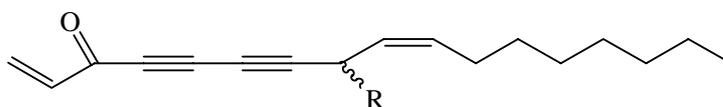
One of the possible explanations is the hypothesis that plants contain other bioactive compounds that provide benefits for health, even though they are not essential nutrients.

Plants contain a great number of different secondary metabolites, of which many are used in plant defence against e.g., insects, fungi and other microorganisms, and hence display biological activity. Many bioactive substances with known effects on human physiology and disease have been identified through studies of plants used in e.g., traditional medicine. Some of these compounds occur also in food plants, although they are normally considered undesirable in human food due to their “toxic” effects. However, the occurrence of low concentrations of “toxins” in plant foods may be an important factor in the search for an explanation of the beneficial effects of fruit and vegetables on human health. In the few cases where biologically active compounds have been investigated despite known deleterious effects, very interesting results have been achieved. Glucosinolates are for example goitrogenic, but until now also the best proven cancer preventing principles in vegetables (Hecht, 1999) and the potato glycoalkaloids with known deleterious effects on humans in high concentrations, have been shown to be protective against lethal infection with *Salmonella* infections in low concentrations as demonstrated in mice (Gubarev *et al.*, 1998).

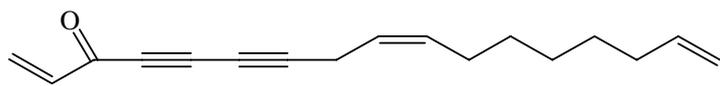
Acetylenes is a further example of a group of bioactive secondary metabolites, which have been considered undesirable in plant foods due to their “toxic” effects. Although some polyacetylenes are known to be potent skin sensitizers and irritants, and to be neurotoxic in high concentrations, they have also been shown to have a pronounced selective cytotoxic activity against cancer cells, and hence may have beneficial effects on human health. This paper highlights the present state of knowledge on the occurrence of naturally occurring acetylenes in common plant foods, including their chemistry and bioactivity.



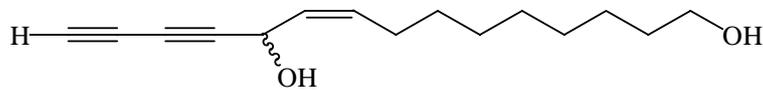
- 1 R<sup>1</sup> = OH, R<sup>2</sup> = H, Falcarinol (syn. panaxynol)
- 2 R<sup>1</sup> = R<sup>2</sup> = OH, Falcarindiol
- 3 R<sup>1</sup> = OCOCH<sub>3</sub>, R<sup>2</sup> = OH, Falcarinol-3-monoacetate



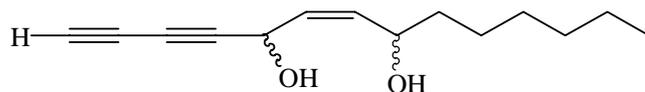
- 4 R = H, Falcarinone
- 5 R = OH, Falcarinolone



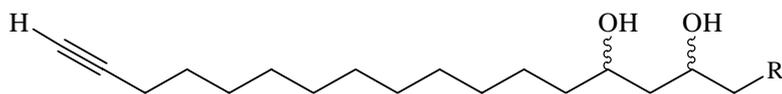
6 R = H, Dehydrofalcarinone



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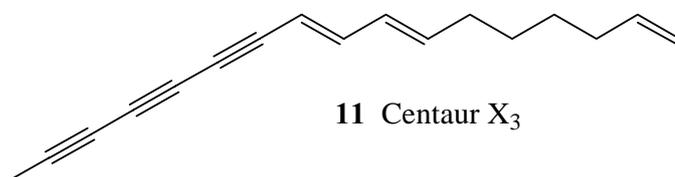


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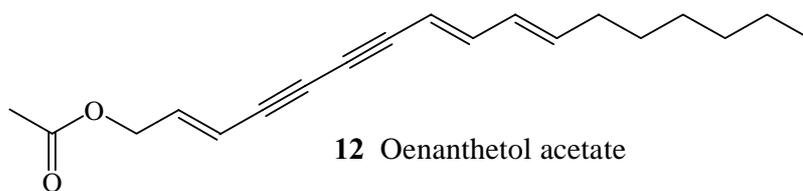


9 R = OH

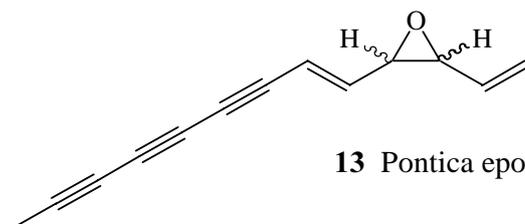
10 R = OCOCH<sub>3</sub>



11 Centaur X<sub>3</sub>



12 Oenanthetol acetate



13 Pontica epoxide

**Fig. 1.** Acetylenes found in common food plants.

**Table 1.** Naturally occurring acetylenes in the edible parts of common food plants.

Family/species	Common name	Plant portion used	Acetylenes	References
<b>Apiaceae</b>				
<i>Anethum graveolens</i> L.	Dill	L, S	<b>12</b>	Bohlmann <i>et al.</i> , 1973
<i>Anthriscus cerefolium</i> (L.) Hoffm.	Chervil	L, S	a	Bohlmann <i>et al.</i> , 1973
<i>Apium graveolens</i> L. var. <i>dulce</i>	Celery	L, St	<b>2, 4, 5</b>	Bohlmann <i>et al.</i> , 1973; c
<i>A. graveolens</i> L. var. <i>rapaceum</i>	Celeriac, knob celery	R	<b>2, 4, 5</b>	Bohlmann <i>et al.</i> , 1973
<i>Bunium bulbocastanum</i> L.	Great earthnut	R	<b>1, 4, 5, 12</b>	Bohlmann <i>et al.</i> , 1973
<i>Carum carvi</i> L.	Caraway	R, L, S	<b>2, 5</b>	Bohlmann <i>et al.</i> , 1973
<i>Chaerophyllum bulbosum</i> L.	Turnip rooted chervil	R, L	<b>4</b>	c
<i>Daucus carota</i> L.	Carrot	R	<b>1–3, 5</b>	Lund, 1992; Garrod <i>et al.</i> , 1978
<i>Pastinaca sativa</i> L.	Parsnip	R, L	<b>2, 4, 5</b>	Bohlmann <i>et al.</i> , 1973
<i>Petroselinum crispum</i> (Mill.) Nyman ex A. W Hill.	Parsley	L	<b>4, 5</b>	Bohlmann <i>et al.</i> , 1973; c
<i>P. crispum</i> (Mill.) Nyman ex A.W. Hill. var. <i>tuberosum</i>	Hamburg parley, parsley root	R, L	<b>1, 2</b>	Nitz <i>et al.</i> , 1990
<b>Asteraceae</b>				
<i>Cynara scolymus</i> L.	Artichoke	L, Fl	a	Bohlmann <i>et al.</i> , 1973; c
<i>Cichorium endivia</i> L.	Endive	L	<b>13, b</b>	c
<i>C. intybus</i> L. var. <i>foliosum</i>	Chicory	L, R	<b>13, b</b>	Rücker & Noldenn, 1991
<i>Helianthus tuberosus</i> L.	Jerusalem Artichoke	R	<b>6</b>	Bohlmann <i>et al.</i> , 1973
<b><i>Lactuca sativa</i> L.</b>	Lettuce	L	<b>11</b>	Bohlmann <i>et al.</i> , 1973
<b>Lauraceae</b>				
<b><i>Persea americana</i> Mill.</b>	Avocado	Fr	<b>9, 10</b>	Adikaram <i>et al.</i> , 1992
<b>Solanaceae</b>				
<i>Lycopersicon esculentum</i> Mill.	Tomato	Fr	<b>1, 2, 8</b>	De Wit & Kodde, 1981; Elgersma <i>et al.</i> , 1984
<i>Solanum melongata</i> L.	Eggplant, aubergine	Fr	<b>2, 7</b>	Imoto & Ohta, 1988

<sup>a</sup>Acetylenes detected but not identified. <sup>b</sup>Further acetylenes with a triyn-ene chromophore have been detected but not identified. <sup>c</sup>Unpublished results (Christensen, L. P.). L: leaves, Fl: flowers, Fr: fruits, R: roots, S: seeds.

## Acetylenes in common plant foods

### *Distribution and biosynthesis*

Acetylenes form a distinct group of relatively chemically reactive natural products, which have been found in about 24 families of the higher plants, although they seem to occur regularly in only seven families, namely Apiaceae (= Umbelliferae), Araliaceae, Asteraceae (= Compositae), Campanulaceae, Olacaceae, Pittosporaceae, and Santalaceae. Asteraceae is the best studied family and the majority of the naturally occurring acetylenes have been isolated from this plant family. Today more than 1400 different acetylenes and related compounds have been isolated from higher plants, including thiophenes, dithiacyclohexadienes, thioethers, sulphoxides, alkamides, chlorohydrins, spiroacetal enol ethers, furans, pyrans, tetrahydropyrans, isocoumarins, aromatic and aliphatic acetylenes. Despite the great variation in structure, feeding experiments with  $^{14}\text{C}$ - and  $^3\text{H}$ -labelled precursors have shown that the biosynthesis of acetylenes in general follow the same biosynthetic route, starting from unsaturated fatty acids, although there are a few examples of acetylenic compounds which are derived from carotenoids and other terpenoids (Bohlmann *et al.*, 1973; Christensen, 1992).

The acetylenes so far isolated from plant foods are all aliphatic acetylenes, of which the majority are of the falcarinol-type (Fig. 1) that is widely distributed in the Apiaceae and Araliaceae families (Bohlmann *et al.*, 1973; Hansen & Boll, 1986). The biosynthesis of polyacetylenes of the falcarinol-type follows the normal pathway for acetylenes starting from oleic acid as shown in Fig. 2.

Food plants so far known to contain acetylenes are listed in Table 1, and include important root crops such as carrot and celeriac, and fruits such as tomatoes and aubergines. However, some of the main reasons for the relative low number of food plants known to contain acetylenes are: (i) many food plants have not yet been investigated for acetylenes and (ii) the majority of food plants belong to plant families that do not normally produce acetylenes.

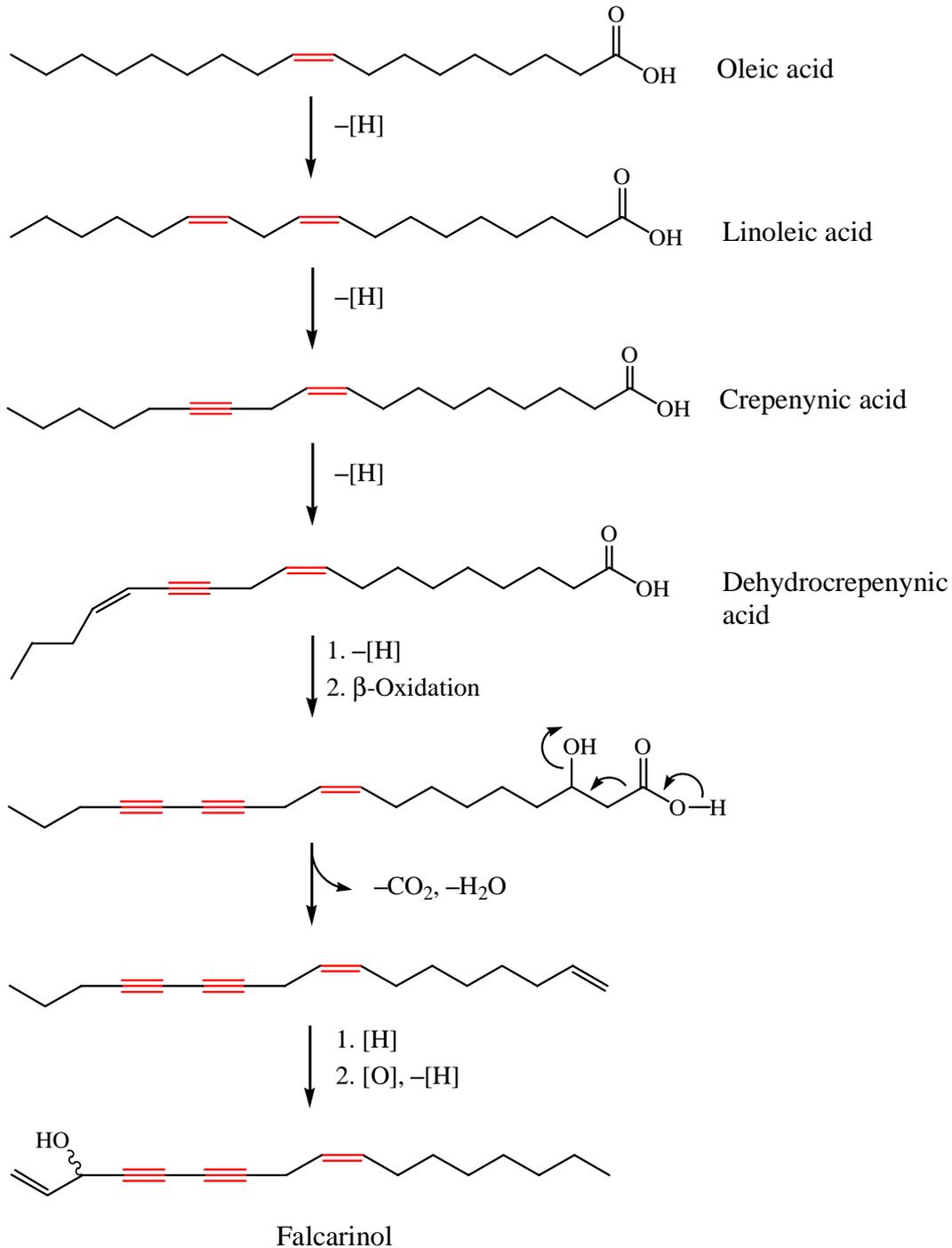
The biological activities of naturally occurring acetylenes have been studied intensively in the past 20 years and their activity to various organisms is now well documented. The toxicity towards a wide range of organisms suggest that they may have a protective role within the plant especially against insect predators and microorganisms.

### *Bioactivity*

#### *Antifungal activity*

Falcarinol (**1**) and falcarindiol (**2**) seem to have a defensive role in carrots, *Daucus carota* L., against invading fungi. Falcarinol inhibits germination of *Botrytis cinerea* spores and its concentration is greatly increased when carrots are infected with this fungus (Harding & Heale, 1981). *Botrytis cinerea* attacks carrots during storage, but not when they are fresh (Harding & Heale, 1980). Another fungi which attacks carrots during storage is *Mycocentrospora acerina* and it has been shown that falcarindiol is highly toxic towards this

fungi (Garrod *et al.*, 1978). Falcarinol and falcarindiol have also been identified as antifungal compounds in many other Apiaceae plant species inhibiting spore germination of different fungi in concentrations ranging from 20 to 200  $\mu\text{g/ml}$  (Christensen, 1998; Hansen & Boll, 1986). Polyacetylenes of the falcarinol-type seem to act as sort of pre-infectious compounds in the species producing them, and hence may play an important role in protecting these plants from fungal attack.



**Fig. 2.** Biosynthesis of polyacetylenes of the falcarinol-type according to Bohlmann *et al.*, 1973.

The families Solanaceae and Lauraceae do not normally produce acetylenes (Bohlmann *et al.*, 1973). However, when healthy tomato fruits and leaves (*Lycopersicon esculentum*, Solanaceae) are infected with leaf mould, which is due to the fungus *Cladosporium fulvum*, they accumulate the acetylenic phytoalexins falcarinol, falcarindiol and (6*Z*)-tetradeca-6-ene-1,3-diyne-5,8-diol (**8**) (De Wit & Kodde, 1981). These compounds are also detected in tomato plants upon infection with *Verticillium alboatrum* (Elgersma *et al.*, 1984). Whether healthy tomato fruits and leaves in fact contain small amounts of polyacetylenes, which undergo post-infectious increases in response to fungal attack, is not known. Also aubergines (*Solanum melongata*) of the Solanaceae family have been shown to be capable of producing polyacetylenes (**2**, **7**) when exposed to phytoalexin elicitors (Imoto & Ohta, 1988). Avocado anthracnose, caused by the fungus *Colletotrichum gloeosporoides* is a major disease factor to post-harvest rotting in avocado fruit (*Persea americana*, Lauraceae). Unripe fruit show no evidence of incipient decay lesions but the decay process may develop rapidly during ripening, indicating the presence of latent infection. This characteristic behaviour has been shown to be attributed to the presence of a significant amount of antifungal acetylenes (**9**, **10**) and related alkadienes which suppress the vegetative growth of the fungus. During ripening this natural antifungal activity is gradually lost presumably due to degradation of the active compounds.

The production of acetylenes in plant species belonging to a family where acetylenes are not normally produced is also known from less common food plants of the Leguminosae plant family (Christensen, 1998). Hence the production of antifungal acetylenes in food plants belonging to families that do not normally produce acetylenes could be a much more common phenomenon than first anticipated.

#### *Neurotoxicity*

The neurotoxic effects of some acetylenes have long been known. Acetylenes with this activity include the fish poisons ichthyothereol (**14**) and ichthyothereol acetate (**15**) (Cascon *et al.*, 1965), which occur regularly in the tribes Heliantheae and Anthemideae of the Asteraceae (Bohlmann *et al.*, 1973; Christensen, 1992). It has been suggested that the toxicity of the fish poisons **14** and **15** (Fig. 3) be due to their ability to uncouple oxidative phosphorylation and inhibiting ATP-dependent contractions (Towers & Wat, 1978).

The acetylenes oenanthotoxin (**16**) and cicutoxin (**17**) (Fig. 3) isolated from water-hemlock, *Cicuta virosa* L., spotted water-hemlock, *C. maculata* L., and from the hemlock water dropwort, *Oenanthe crocata* L. (Apiaceae) (Anet *et al.*, 1953; Konoshima & Lee, 1986; Wittstock *et al.*, 1995) are extremely poisonous causing violent convulsions and death, and they have been responsible for the death of numerous human beings and livestock (Anet *et al.*, 1953). Less well-known is the effects of falcarinol (**1**), which produces pronounced neurotoxic symptoms upon injection into mice with an LD<sub>50</sub> of 100 mg/kg whereas the related falcarindiol (**2**) does not seem to have any acute effect (Crosby & Aharonson, 1967). The



Allergic contact dermatitis from food plants of the Apiaceae are, however, rarely detected, probably due to their relative low concentrations of allergenic polyacetylenes compared to ornamental and wild plant species (Hausen, 1988).

#### *Anti-inflammatory, anti-platelet-aggregatory and antibacterial effects*

Falcarinol (**1**) have shown anti-inflammatory and anti-platelet-aggregatory effects (Teng *et al.*, 1989; Alanko *et al.*, 1994). It has been suggested that this pharmacological action is related to the ability of the compound to modulate prostaglandin catabolism by inhibiting the prostaglandin catabolizing enzyme PGDH (15-hydroxy-prostaglandin dehydrogenase) (Fujimoto *et al.*, 1998). Falcarinol and related C<sub>17</sub>-acetylenes have also shown antibacterial and antituberculosis activity (Kobaisy *et al.*, 1997). These pharmacological activities strongly suggests that falcarinol and related polyacetylenes have a positively effect on human health.

#### *Cytotoxicity*

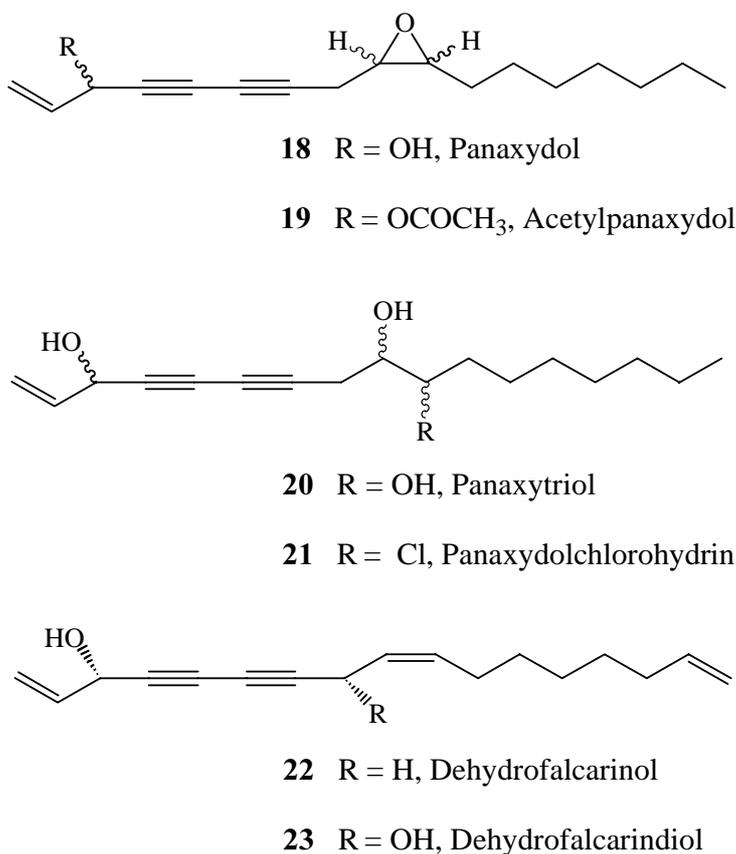
*Panax ginseng* C. A. Meyer (Araliaceae) is one of the most famous and valuable drugs in Asia. The active principles in *P. ginseng* have for many years been considered to be saponins (ginsenosides) and studies on the constituents of this plant have therefore mainly focused on these constituents. However, since the anticancer activity of petrol extracts of the roots of *P. ginseng* was discovered in the beginning of 1980s (Shim *et al.*, 1983), the lipophilic portion of this plant has been intensively investigated. This had led to the isolation and identification of several cytotoxic polyacetylenes (e.g., **1** and **18–21**) (Fujimoto & Satoh, 1988; Ahn & Kim, 1988; Ahn *et al.*, 1989; Matsunaga *et al.*, 1989; Matsunaga *et al.*, 1990). Falcarinol (**1**), panaxydol (**18**), and panaxytriol (**20**) have been found to be highly cytotoxic against numerous cancer cell lines (Ahn & Kim, 1988; Matsunaga *et al.*, 1989; Matsunaga *et al.*, 1990) showing the strongest cytotoxic activity towards human gastric adenocarcinoma (MK-1) cancer cells with an ED<sub>50</sub> of 27, 16, and 171 ng/ml, respectively (Matsunaga *et al.*, 1990). Falcarinol, panaxydol, and panaxytriol have also been shown to inhibit the cell growth of normal cell cultures such as human fibroblasts (MRC-5), although the ED<sub>50</sub> against normal cells was very high compared to that of cancer cells. In particular, panaxytriol did not inhibit the growth of MRC-5 cells by 50% even at concentrations higher than 700 ng/ml (Matsunaga *et al.*, 1990). The selective *in vitro* cytotoxicity of falcarinol, panaxydol, and panaxytriol against cancer cells compared to normal cells, indicate that they may be useful in the treatment of cancer. Furthermore, acetylpanaxydol (**19**) and panaxydolchlorohydrin (**21**) have been reported to be cytotoxic against leukemia (L1210) cells in very low concentrations (ED<sub>50</sub> = 30–50 ng/ml) (Fujimoto & Satoh, 1988; Ahn & Kim, 1988; Ahn *et al.*, 1989).

From the aerial parts of *Dendropanax arboreus* (L.) Decne. & Planchon. several aliphatic polyacetylenes have been isolated of which falcarinol, falcarindiol (**2**), dehydro-falcarinol (**22**) and dehydrofalcarindiol (**23**) were found to exhibit *in vitro* cytotoxicity against human tumour cell lines, with falcarinol showing the strongest activity (Bernart *et al.*, 1996). Preliminary *in vivo* evaluation of the cytotoxic activity of falcarinol, dehydrofalcarinol and dehydrofalcarindiol using a LOX melanoma mouse xenograft model demonstrated some

potential for *in vivo* anti-tumour activity of falcarinol and dehydrofalcarinol, with dehydrofalcarinol showing the strongest therapeutic effect (Bernart *et al.*, 1996).

As falcarinol, falcarindiol, and related C<sub>17</sub>-acetylenes are common in the Araliaceae and Apiaceae one might expect that more species within these families exhibit cytotoxic activity, including food plants. Furthermore it is interesting to note that the cytotoxic polyacetylenes dehydrofalcarinol and dehydrofalcarindiol are widely distributed in several tribes of the Asteraceae (Bohlmann *et al.*, 1973; Christensen, 1992).

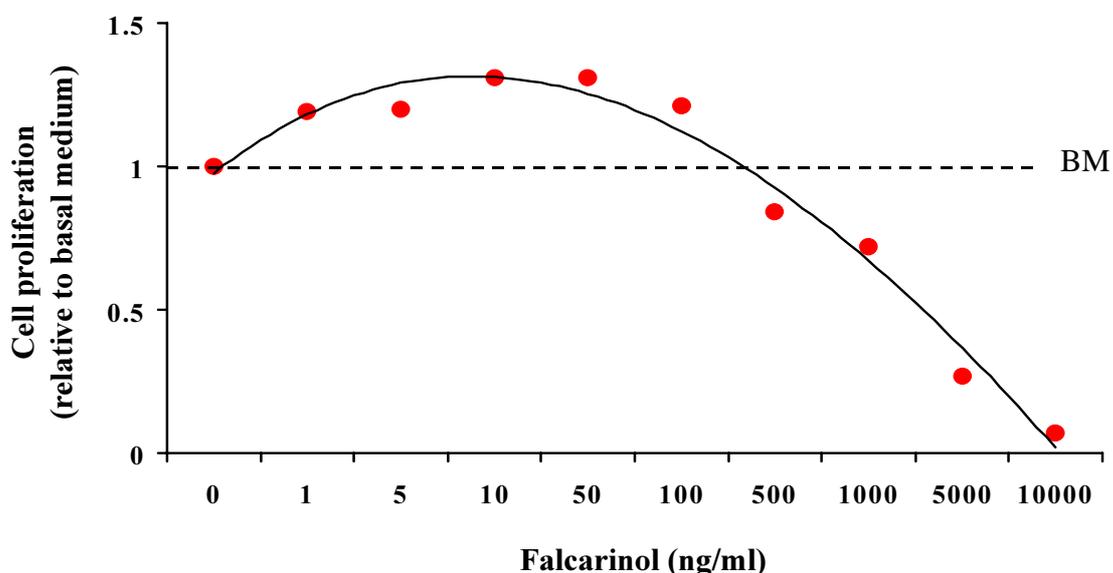
The strong selective cytotoxic activity of falcarinol and related C<sub>17</sub>-acetylenes towards different cancer cells indicates that they may be valuable in the treatment of different types of cancer, and could contribute to the health promoting properties of food plants that contain these compounds.



**Fig. 4.** Examples of highly cytotoxic polyacetylenes of the falcarinol-type isolated from medicinal plants.

*Chemical hormesis*

Cellular responses to bioactive components can be concentration-dependent, having stimulatory effects at low concentrations and inhibitory or toxic effects at high concentrations. This biological phenomenon is also known as chemical hormesis (Calabrese & Baldwin, 1998). The concentration-dependent bioactivity of falcarinol isolated from carrots has been investigated in a bioassay with primary mammary epithelial cells in collagen gels. The bioassay, which is stable and reproducible, is based on incorporation of tritiated thymidine into cell DNA as a measure of cell proliferation. This bioassay has previously been shown to be sensitive to a number of growth factors of the IGF, EGF, TGF, and FGF growth factor families as well as to other factors such as vitamin A and its metabolites (Purup *et al.*, 2000; Purup *et al.*, 2001). The response obtained with this bioassay cannot be directly related to an effect on human health but represent an *in vitro* system to investigate potency of different bioactive components.



**Fig. 5.** Effects of increasing concentrations of falcarinol on proliferation of primary mammary epithelial cells in collagen gel cultures relative to basal medium (BM).

The cellular responses to falcarinol were investigated over a wide range of concentrations (Fig. 5). Addition of 1 and 100 ng/ml of falcarinol stimulated cell proliferation, while higher concentrations of falcarinol (> 1000 ng/ml) inhibited cell proliferation compared to proliferation obtained in basal medium (BM). Maximal stimulation occurred between 10 and 50 ng/ml and half-maximal inhibition occurred at concentrations between 1000 and 5000 ng/ml. Maximal inhibition at 10000 ng/ml corresponded to more than 90% inhibition of cell proliferation. The results are fully in accordance with the hypothesis of chemical hormesis. This biphasic effect of falcarinol on cell proliferation therefore suggests, that falcarinol may

have concentration-dependent effects on human intestinal cells and human cancer cells. The effects of falcarinol towards MK-1 tumour cells at 30 ng/ml as described previously, therefore indicates that falcarinol can have inhibitory/toxic or stimulatory effects at the same concentration depending on the cell types. Therefore falcarinol appears to be one of the bioactive components in carrots and related vegetables that could explain their health promoting properties. This hypothesis is further supported by recent studies on the bioavailability of falcarinol in humans. When falcarinol was administered orally via carrot juice (35 mg falcarinol/L carrot juice) it was rapidly absorbed, reaching a maximum concentration in serum of about 12 ng/ml at 6 hours after dosing (Hansen-Møller *et al.*, 2002).

In contrast to falcarinol, no stimulatory or inhibitory effects were observed for  $\beta$ -carotene, the orange pigment in carrots and a potent antioxidant. This is surprising considering the wide range of concentrations (1–100000 ng/ml) to which the cells were exposed. Considering the fact that the primary epithelial cells used in the present bioassay are sensitive to a large number of growth factors, including A-vitamin metabolites, it is surprising that  $\beta$ -carotene did not affect cell proliferation. The absence of effect of  $\beta$ -carotene towards primary epithelial cells further supports the hypothesis that falcarinol, rather than  $\beta$ -carotene, is the primary cause of the beneficial effects of carrots on human health.

## Perspectives

Acetylenes, especially falcarinol and related polyacetylenes of the falcarinol-type, are likely to be responsible for a least a part of the beneficial effects of eating carrots and related vegetables. Although there appears to be a correlation between high intake of vegetables, containing highly bioactive polyacetylenes, and prevention of cancer and other severe diseases, this correlation has to be further established. Also other groups of bioactive compounds occurring in plant foods should be considered as possible contributors to the beneficial effects of fruit and vegetables on human health. Therefore, at present the most urgent need concerns preliminary studies to identify the compounds that have the greatest potential for improvements of the health value of food, and thus should be selected for further study.

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# **PRESENT REGULATIONS IN THE EU AND FUTURE TRENDS FOR MARKETING OF FOODS WITH ENHANCED HEALTH BENEFITS**

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## **Abstract**

Consumers are consistently confused about health claims made for foods. This is hardly surprising given the lack of clarity among those who are supplying information – industry, legislators and scientists. There is a danger that the driving force for diet related health claims will be marketing success and the likelihood of a particular approach being successful rather than realistic and substantiable claims. As a response to this, the EU have presented a draft proposal for the regulation of nutrition, functional and health claims for foods (DG SANCO/1832/2002). This initiative can form the basis of future legislation and will help to better define future directions and priorities. Nonetheless there will be increasing pressure on food producers and manufacturers to develop novel, healthy products. Our interpretation of the current priorities is that the key advances in functional food and health claims will be conservative in nature, limited in scope and will follow advances in pharmaceutical sciences. There will be public health issues that will encourage the development of functional foods in the context of reduction in morbidity and mortality, however, these are likely to focus more on general dietary advice rather than specific components present in foods.

**Keywords:** Functional food, market, legislation, future, claim

## **Introduction**

While the major driving force for the development of functional foods remains profitability, there are a number of other factors that impact upon the use and development of products in this area. In the paragraphs to follow, we will consider some of these and try to show how they might relate to future prospects.

One essential aspect is the intrinsic value of functional foods versus the cost required to develop them. In any case where clinical trials are required, the cost of the development of the food will rapidly outstrip the short-term value to the developing company. In addition, in many cases it is difficult to see how the intellectual property relating to a food can be protected. If the costs of making a claim regarding a functional food are prohibitive, then it is more likely that a more general claim backed by a considerable amount of marketing will be

the preferred route. At any rate, so far as a scientist working in the area of diet and health is concerned, funding for the development of claims for functional foods will inevitably be difficult to raise.

It is against this background that evidence for future trends must be assembled. There is, however, an alternative way of looking at health claims. In the past, contamination of foods (microbial or chemical) was a major problem. Currently non-communicable diseases are the largest diet-related public health problem. It has been estimated that, in certain parts of Europe, 25% of the population will have some form of cardiovascular problem before they retire. In addition, the incidence of hypercholesterolemia is increasing and may, together with increases in obesity, fuel the development of Type 2 diabetes in larger parts of the population. Positive food safety is about the prevention of disease and the improvement of health. This is a key aspect for the development of functional foods and, perhaps more importantly, to their enthusiastic adoption by population groups.

In this discussion we will seek to develop the theme of diet and functional foods as it relates to the food industry and the health of the consumer. In order to do this we will cover the following contributory factors:

- The nature of health claims and, in particular the scientific basis for them
- Current legislation relating to functional foods and nutrition in Europe and how it will impact upon industry and scientists.
- Future trends based upon historical analysis of the market for functional foods and the main drivers which influence it
- A new appraisal of the potential for functional foods to succeed in the future

The arguments expressed in the following do not (and should not) imply that there is no added value in foods or in the food industry, however, it is time for enlightenment which can lead to increased profitability for all stakeholders.

### **The nature of health claims**

Health claims fall into various categories. The lack of clarity concerning claims has been addressed most recently in projects funded by the EU as Concerted Actions. Three of these are of particular interest. The project FUFOSSE (EU FAIR CT95-572) sought to rationalise the various types of claims that can be made and to divide claims into different categories. The broad definition of a claim was felt to be divisible into two distinct types.

The first of these is the 'enhanced function' claim. This type of claim relates to a direct consequence of exposure on the consumer in terms of a measurable, beneficial effect on a body function whose enhancement leads to improved health. Clearly there is room for debate as to whether a particular change is truly beneficial and if so, over what type of time scale,

however, it is possible to envisage a series of tests which can be used to confirm (or otherwise) a particular claim. It is important to realise that this type of claim does not require *a priori* the presentation of a hypothesis to explain the effect nor does it require the use of predictive measures (commonly called markers or, since they generally relate to biological systems, biomarkers) to facilitate acceptance of the claim.

The second type of claim is one relating to 'disease reduction'. In this case the benefits of the food must be demonstrable for a future health state. In contrast to the previous example, a disease reduction claim, by its very nature, deals with future events. It is not concerned with curing existing disease (in contrast to conventional nutraceuticals) but rather with disease risk reduction. For this reason, predictive markers are essential. These markers should reflect the likelihood of disease being prevented by specific dietary components, however, it is broadly axiomatic that the further a marker is from a clinical outcome and the closer it is to the exposure event (either in temporal or mechanistic terms), the more difficult it is to relate the measurement to the endpoint. Consistently, the closer a marker is to a clinical consequence, the harder it is to define a precise relationship to a specific exposure.

The second EU funded project was the Biomarkers Concerted Action (EU FAIR CT 96/118), which sought to define the properties of biomarkers in the context of disease and health. In particular, this project concentrated on the separation of biomarkers into exposure and effect. These can be considered to be broadly similar to the two groups of markers defined by FUFUSE but arise from a different analysis of the problem. In addition, this project also considered in some detail the types of disease that diet might be expected to be able to make a contribution towards preventing and recognised the importance of epidemiology as a tool for delineating diet/health claims.

The third project, which is currently running, is the follow up to FUFUSE and has the acronym PASSCLAIM (QLK1-2000-00086). The underlying premise of this project is that, in order for consumers and other stakeholders to have confidence in health claims associated with food components, it is essential that the claims are underwritten by understandable and rigorous scientific evidence. Needless to say, a major part of this is the development of vigorous and clear (bio)markers to substantiate claims. While this is clearly of prime importance, it must be recognised that any claim should combine proof of exposure (bioavailability) with, if possible, mechanistic underpinning to support predictive measurements. In addition, it is important that food and dietary components are recognised as having synergistic effects in many instances. It is also important that common criteria should be developed for the identification, validation and use of diet related markers in human health.

The scientific basis for health claims is of prime importance but claims must also be clearly communicated to consumers, legislators and industry in a way that all stakeholders will be able to understand.

## **Current legislation**

There are clear EU rules on labelling and nutrition labelling of foods (Directive 2000/13/EC and Directive 90/496/EEC respectively). Claims must not mislead the consumer, however, there is room for interpretation particularly with regard to national differences and the use by industry of claims to support marketing. Codex rules do not permit the use of claims for food use to treat or cure a human disease. Within these strictures, however, Codex has issued guidelines for certain definitions including nutrient claims and nutrient function claims.

The EU draft document referred to above attempts to clarify the situation with regard to claims in specific areas. These include nutrition, health and functional claims. Both nutrition and functional claims are primarily concerned with nutrients. Health claims are those which are considered to describe a relationship between a category of food or a food or one of its constituents and health. Health claims will be able to include both 'enhanced function' and 'reduction of disease risk factor' claims. It is notable that, in the case of the latter, there is a requirement that the food or one of its constituents 'significantly reduces a major risk factor in the development of human disease'. This implies that epidemiological evidence in isolation will be insufficient and, furthermore, that risk factors should have been identified. This may lead to the exclusion of a range of disease states where there is insufficient information concerning mechanism of progression. The body responsible for the regulation of health claims and for administering the health claims register will be the European Food Safety Authority (EFSA). Certain types of claim are to be excluded. These include those made for psychological or behavioural functions since these are affected by a number of non-food factors.

There are two other important factors by which claims will have to be acceptable. Firstly, the claims must be understood by the consumer and secondly, the claim must relate to a reasonable level of consumption of the food. These criteria will directly affect the marketing of health-related foods and place strict limitations on what may be claimed on a food label.

There are a number of voluntary codes of practice for functional foods which are currently used in different European countries. The regulations are, in part, designed to harmonise these and to allow all consumers to have similar levels of protection regarding functional foods. One such initiative is the Joint Health Claims Initiative (JHCI) in the UK.

## **Future trends**

To date developments in the functional foods market have been made in products targeted at a relatively restricted range of health benefit categories i.e. heart, gut and bone health propositions. The global market estimate for these combined categories is currently around

\$9 bn, of which heart and gut health products account for \$3.5bn each and bone health products accounts for around \$2bn.

Heart health products dominate in Europe, reflecting concerns over the high levels of death and morbidity caused by the disease. The heart health category has been driven by innovations in a number of key ingredient application areas including phytosterols and stanols, omega-3 fatty acids, soya, and dietary fibre. Cholesterol spreads form the backbone of the heart health functional foods market and have been around since around 1999 when Unilever launched Take Control and Raisio introduced Benecol in the US. Benecol was launched into selected European markets later the same year but Unilever was not able to undertake the European launch of its Flora Proactiv product until 2000, after it had undergone a regulatory approval process. The heart benefit foods market is forecast to grow by about 40% by 2005, largely driven by the cholesterol-lowering spreads sector which has the backing of major advertising spends by the major multinationals. The impending tightening of claims regulation is unlikely to make any major impact on the sector in the short to medium term, as the claims, particularly in the area of cholesterol reduction have already been backed by considerable funding in terms of ensuring scientific substantiation. What seems likely however, is that significant new product innovation will be restricted to a few players and smaller companies will follow with me-too products probably without health claims. In the longer term, this main mean that the number of new innovative products for heart health falls.

Gut health products currently account for a global market of around \$3.5bn on a global basis with the major emphasis being on probiotics, prebiotics and dietary fibre. Probiotic dairy products have led product development, firstly in terms of probiotic yoghurts and more recently via dose delivery probiotic drinks, which now represent the fastest growing sector in the market. Probiotics and prebiotics are now being used increasing outside the dairy sector with significant innovation in terms of application in a broad range of products including bakery and cereal products and soft drinks. Whilst the scientific evidence remains patchy, on going research is adding further weight to the beneficial role of a range of ingredients in gut health. It is likely that there will be increasing complex ingredient combinations aimed at improving gut health in the future. The gut health area has to date relied heavily on the use of soft claims and on this basis the sector has achieved considerable market success. Envisaged tighter control of health claims through regulation is therefore forecast to have little impact on the growth prospects for the gut benefit foods with the health benefits of foods containing probiotics being acknowledged for centuries. The market is forecast to climb to \$4.5 billion by 2005.

Despite considerable product activity, the global market for bone benefit foods remains small and fragmented, estimated at just over \$2bn in 2000. Calcium enrichment has been the most popular route for product development to date but there is increasing interest in the application of other minerals, such as magnesium in bone health products, as well as prebiotics and ingredients such as soya isoflavones and trehalose. The market is expected to see continued market activity but is unlikely to enjoy the growth rates of some other

functional food categories, reflecting the already strong association among consumers that calcium rich products have with bone health. Nevertheless bone health is likely to attract increasing attention in the longer term with an ageing population and greater emphasis on the problems of bone health in older people. The market is likely to become more consolidated in the future and it is expected that a selected number of innovative products will reposition themselves with stronger bone health messages. The likely future requirement to permit only scientifically substantiated health claims may impact on levels of innovation in terms of ingredients other than calcium with bone benefit potential but it may be that we will see pharmaceutical companies willing to invest in this area of increasing health concern.

Growing consumer acceptance of the link between diet and health and an increasing consumer responsibility for their own health are likely to be key drivers in the functional foods market of the future. Diet-related diseases remain a cause of premature death in the western world and a major burden on overstretched resources. Improving diet is therefore an opportunity for western governments to reduce spiralling healthcare costs. The ageing population and a desire to remain healthy into old age are also likely to boost consumer interest in the functional foods market. In addition, we are enjoying increasing disposable incomes and this will increase consumers' willingness to purchase products that offer them significant health benefits.

Changes in claims legislation, however, are likely to have a significant effect on the shape of the future market. The high cost of claims substantiation in terms of clinical trials is likely to discourage all but the major food multinationals and pharmaceutical giants from developing innovative new functional foods products with health claims. The high level of risk in the market, reflected in the current high level of functional food product failures means that realistically, these are likely to be the only companies willing to invest significantly in the sector. Considerable financial support will clearly be needed for these products in terms of claims substantiation and marketing support if they are to succeed. This is likely to be a barrier to many small companies. They are likely to address the market in one of two ways. They may adopt a marketing approach, which relies on exploiting existing consumer knowledge of links between ingredients and health. In other words some companies may wait for other to raise awareness of an ingredient and follow with me too products that make no health claim. The use of women's press, etc. to communicate links between food ingredients and health is also likely to become more common. In this way, the market will become segmented into major brands with health claims and other products that just claim the presence of an ingredient. Long term growth in the sector may only happen if the major multinationals manage to achieve significant market success with their products. This may encourage the smaller to invest in developing truly novel product lines.

We also believe that there is likely to be increasing interest by the food industry in general foods for wellbeing, which will also be promoted without specific health claims. These are likely to be products aimed at dealing with lifestyle pressures such as foods for mental

performance foods, anti stress foods and also gender specific and lifestage foods. These products are likely to remain niche, however, coming largely from smaller manufacturers who may not be able to support them with significant advertising budgets.

In conclusion, we estimate that the key functional food product areas of heart, gut and bone health products are likely to expand in the short to medium term. Success, however, is likely to come from products ingredients that consumers already associate with various health propositions. Significant ingredient, product innovations and extensive use of health claims is only likely to be used by the major multinationals who will be the only companies able to support the development and marketing of the products effectively. Even they, however, will need to adopt a long term view in order to raise the consumer understanding and awareness of these products. Smaller companies are less likely to dedicate considerable resources to these high risk products and may rely on developing products with broader wellbeing propositions. These may appeal to consumers but are unlikely to have any significant impact in terms of improving health.

## FOOD HEALTH CLAIMS IN SWEDEN

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### Abstract

THE SWEDISH VOLUNTARY CODE on health-related claims in the labelling and marketing of food products was recently extended to “product-specific physiological claims” (abbreviated PFP in Swedish). The first expert panels have been set up by the SNF Swedish Nutrition Foundation for the required pre-marketing evaluation of the scientific documentation. The Assessment Board for Diet-Health Information (abbreviated BKH in Swedish) was established on 23 November 2001 and is now available for post-marketing assessment of particular marketing and labelling actions that have been questioned in relation to the Code.

### Different health-related claims

The original Swedish Code from 1990, revised in 1997, was limited to *generic claims* in two steps, related to well-established diet-health connections. These eight connections are: 1) *Obesity* – energy content, 2) *Cholesterol level in the blood* – fat quality or some soluble dietary fibre, 3) *Blood pressure* – salt (sodium chloride), 4) *Atherosclerosis* – blood cholesterol level/blood pressure, n-3 (omega-3) fatty acids in fat fish and fish products, 5) *Constipation* – dietary fibre, 6) *Osteoporosis* – calcium, 7) *Caries* – absence of sugars and other easily fermented carbohydrates, 8) *Iron deficiency* – iron content. Provided that the claims are made strictly in two steps and in the context of a healthy balanced diet, the original Code allows *generic reduction of disease risk claims*.

*Product-specific physiological claims* according to the extension of the programme means claims related to a specific physiological effect of the product itself, according to a 1998 report from SNF Swedish Nutrition Foundation and the organisations behind the programme (1,2). It corresponds to product-specific *enhanced function claims* as defined in reports from Codex Alimentarius (3) and Council of Europe (4), and would be classified as *innovative claims* according to the UK Joint Health Claims Initiative (5).

*Nutrient function claims* are defined by Codex and now also regarded as a kind of (generic) health claims (3).

The original Swedish Code is subject to evaluation, including reconsideration of the diet-health connections approved for generic claims. The process of such a revision has started. Rules for the use of nutrient function claims should then also be elaborated. Such claims are commented only in brief in the present Code.

**Information** about the Swedish Code can be found on the website of the SNF Swedish Nutrition Foundation  
[www.snf.ideon.se](http://www.snf.ideon.se)

## USING PLANT BREEDING TO OPTIMISE THE CONTENTS OF BIOACTIVE COMPOUNDS IN VEGETABLES

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### Abstract

The great interest in potential beneficial effects of plant substances on human health raises the question whether plant breeding might be used to optimise the contents of these compounds. Here glucosinolates and vitamin C in cabbage and carotenes and polyacetylenes in carrot are discussed. There is large genetic variation in these compounds, but it is not always known how they are inherited. It should also be noted that several of them are involved in the resistance mechanisms against pests and diseases. Plant breeders always must try to achieve the right balance between yield, quality and resistance.

Key words: Glucosinolates, vitamin C, carotenes, polyacetylenes

### Introduction

The vegetables are a large and heterogeneous group of plants containing a great number of natural substances. In addition to the primary compounds that play a fundamental role in the metabolism, a lot of secondary substances with less obvious functions exist. Almost certainly many of these are involved in the natural allelopathic defence systems against micro organisms, insects, other herbivores or even plants. Some secondary substances taste badly or are toxic to man. Other substances have proven to be beneficial due to an anticancerogenic or a heart-protective effect. With the help of plant breeding, the amount of unpalatable or unhealthy compounds might be reduced and the amount of compounds with a positive health effect on humans might be increased. It should, however, always be remembered that conflicts between breeding for quality and breeding for resistance might arise. Thus a reduction or increase of a certain compound to make the vegetable more 'healthy' might lead to a plant that is more susceptible to diseases, pests or weeds and thus a need for chemicals to avoid spoilage of the crop. Moreover, different compounds might act in a synergetic way both in the plant itself and in the human body.

### Bioactive compounds in *Brassica* vegetables

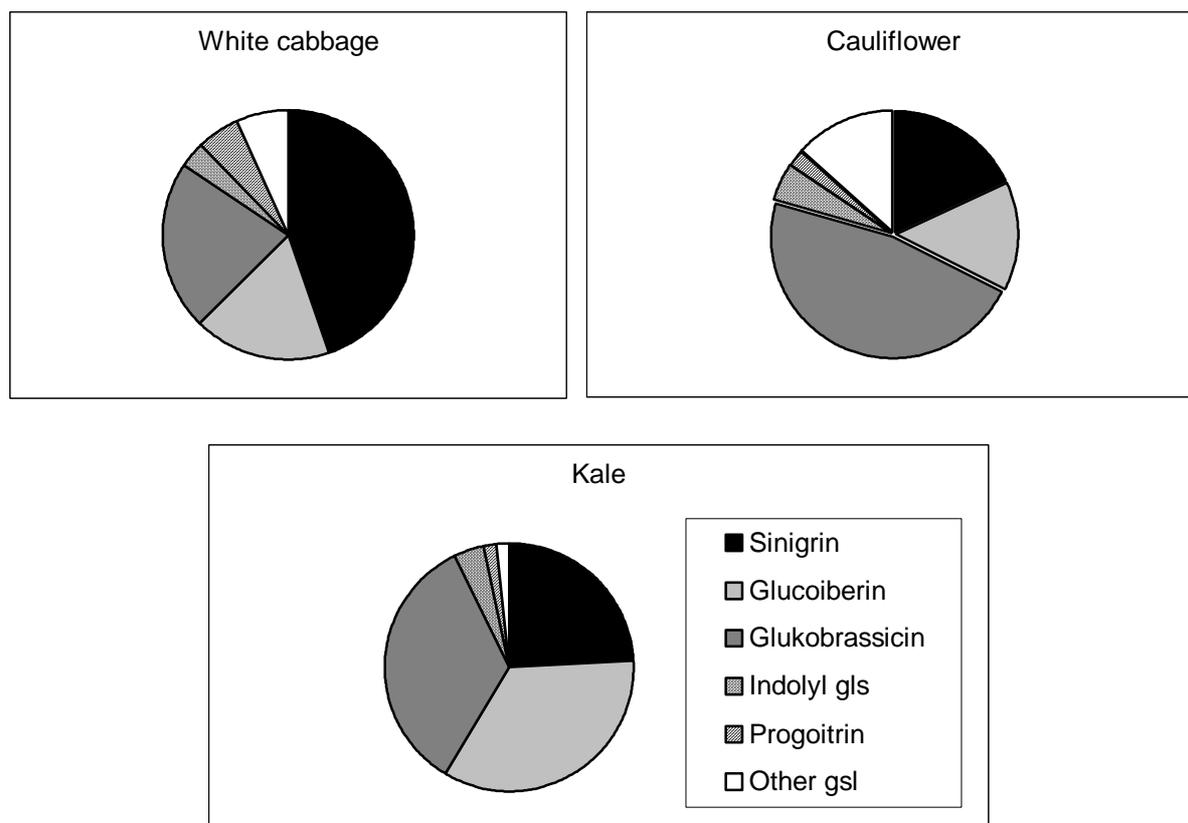
#### *Glucosinolates*

More than 100 glucosinolates occur in nature. They are sulphur-containing glycosides differing in their side chain R-groups. Glucosinolates are found in all *Brassica* vegetables but

the composition and the amount of the single glucosinolates vary. On average ca. 10-15 glucosinolates occur in a certain species but only between one and four glucosinolates are found in high concentrations. They are grouped as aryl, alkyl, aromatic, or indolyl glucosinolates according to the structure of the side chain. These compounds also vary between different parts of the plants (Kushad *et al.*, 1999).

The glucosinolates are vacuol-bound, and injury of the cells by insects or micro organisms results in hydrolysis by enzymes called myrosinases. Food preparation like chopping or too short blanching-time, also causes enzymatic breakdown of the glucosinolates to a mixture of volatile components (thiocyanates, isothiocyanates, nitriles and oxazolidiniones).

The most frequent glucosinolates in white cabbage are 2-propenyl glucosinolate (trivial name = sinigrin), 3-methylsulfinylpropyl glucosinolate (glucoiberin), and 3-indolylmethyl glucosinolate (glucobrassicin). Sinigrin is much less frequent in cauliflower and instead glucobrassicin is the dominating glucosinolate. Glucoiberin and glucobrassicin are the dominating glucosinolates in kale. All three species contain some 2-hydroxy-3-butenyl glucosinolate (progoitrin) (Figure 1).

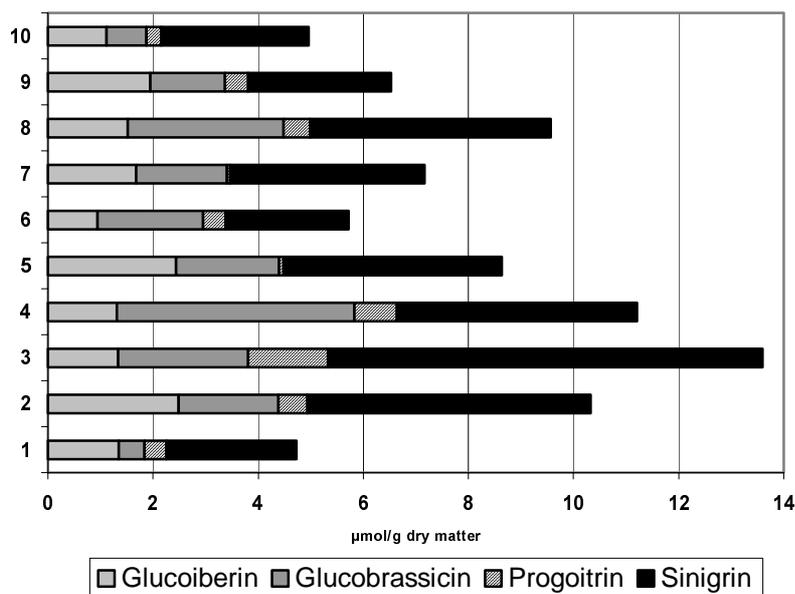


**Figure 1.** The glucosinolate profiles of white cabbage, cauliflower and kale grown in Sweden.

Some glucosinolates lower quality because they or their breakdown products have undesirable sensory or physiologic characters. Other glycosides are important precursors for flavour or of therapeutic interest (Rosa *et al.*, 1997). Both **sinigrin** and its degradation product, 2-propenyl-isothiocyanate, are bitter substances. The isothiocyanate also affects the protein, carbohydrate and carotene metabolisms in the human body and has been shown to be mutagenic. **Progoitrin**, which in itself is tasteless, produces a very bitter breakdown product, 5-vinyloxazolidine-2-thione. This component also interferes with the synthesis of thyroxin, which in extreme cases might lead to goitre.

Other glucosinolates have positive sensory effects. They are responsible for the special flavour and aroma of the different *Brassica* vegetables. Thus glucoiberin gives cauliflower its flavour while gluconasturtiin contributes to the special taste of white cabbage. Breakdown products from **glucobrassicin** and other **indolyl glucosinolates** might inhibit chemical and other types of cancer, which has been shown in animals. The isothiocyanates from **glucoraphanin** but also from **glucoiberin** are indirect antioxidants. They have proven to be potent inducers of phase II enzymes, which favour the antioxidative capacity of the cells (van Poppel *et al.*, 1999; Fahey & Talalay, 1999).

Breeding and selection of *Brassica* cultivars with more favourable glucosinolate profiles demands good knowledge of the presence of both desirable and undesirable glucosinolates and how these components are inherited. With today's knowledge of the effects on human health, new cultivars should have high levels of glucoraphanin, glucoiberin and indolyl glucosinolates and low levels of sinigrin and progoitrin. The levels of different glucosinolates show great variation between genotypes (Figure 2). Selection for higher or lower contents of certain glucosinolates is possible.



**Figure 2.** Variation in four glucosinolates in 10 cultivars of white cabbage grown in Sweden.

A conventional breeding program turned towards an optimisation of the entire glucosinolate profile is, however, not an easy task. Glucosinolate production is regulated by several genes and is very complex. The biosynthetic pathways are not fully investigated. One proposed way to change the glucosinolate pattern is via gene technology. By this technique one could take advantage of the genes coding for the substrate specific enzymes that control which types of glucosinolates that are to be synthesised. Today, only a few of these genes are available and there is also a lack of tissue specific promoters (Wallsgrave & Bennett, 1999). Moreover, consumers have strong objections towards genetically modified food.

It should also be noted that there is sometimes a risk that breeding for better quality of a crop might lead to greater susceptibility to attack by bacteria, fungi, insects etc. If the new cultivar needs more treatment with fungicides or insecticides some of its 'health profile' is lost. Several works have described glucosinolates and their hydrolysis products as part of the plant's defence against insects, but also as stimulants for feeding and oviposition. The effect depends on the developmental stage of the plant, the concentration of the glucosinolates and the insect species (Rosa *et al.*, 1997). Thus cultivars of white cabbage with very low levels of sinigrin are more susceptible to feeding by cabbage moth and cabbage butterfly than cultivars with high levels of sinigrin (Olsson & Jonasson, 1994).

In spite of some difficulties in breeding for a healthier glucosinolate profile in *Brassica* vegetables such efforts are recommended and breeding programs are already running.

### *Vitamin C*

In plants, vitamin C usually occurs as ascorbic acid although small amounts of its oxidized form, dehydroascorbic acid, could be present in the tissues. Vitamin C has many functional properties in the human body but cannot be synthesised by man. It is necessary for the protein of connective tissue in the skin, for wound healing, normal bone tissue and healthy teeth. The vitamin is further of great importance for the uptake of iron and folacin from the intestine and is a co-factor in enzymes. Vitamin C is also an antioxidant and it is thought to be involved in the immune defence (Davies *et al.*, 1991).

Sub-clinical lack of vitamin C has been and is more common than we think. The recommended daily intake in Sweden is 30-60 mg/day depending on age. Pregnant women need 70 mg/day and nursing women 90 mg/day. Smokers should have a daily intake of at least 140 mg (Abrahamsson, 1999).

*Brassica* vegetables have long been regarded as important sources for vitamin C and other vitamins. There are significant differences in ascorbic acid concentration between species (Table 1). In kale the level of ascorbic acid is three times higher than in white cabbage. The consumption of white cabbage is, however, much higher than that of kale.

**Table 1.** Ascorbic acid in various *Brassica* vegetables compared to other foodstuffs (Anon., 1996).

<b>Brassica vegetable</b>	<b>mg/100 g fresh weight</b>	<b>Other food</b>	<b>mg/100 g fresh weight</b>
White cabbage	36	Pasta and rice	0
Swede	36	Potato	11
Red cabbage	57	Orange and lemon	53
Cauliflower	73	Strawberries	66
Broccoli	83	Pepper (red)	200
Brussels sprouts	85	Black currant	210
Kale	120	Rose hips	270

Factors influencing the vitamin C level are genotype, soil conditions, and climate, maturity at harvest and storage conditions but also food preparation and processing. In addition, the vitamin level varies between different parts of the plants. In white cabbage the highest levels are found in the core.

The variation in ascorbic acid levels is considerable between cultivars of the same species. This is shown in table 2 with white and red cabbage grown under the same conditions.

**Table 2.** Variation in ascorbic acid level in cabbage cultivars from the collection of the Nordic Gene Bank and in new Swedish cultivars.

	<b>Number of samples</b>	<b>Variation in ascorbic acid (mg/100 g fresh weight)</b>	<b>Trial site</b>
White cabbage	44 old cultivars	23.4-50.9	Fyn, Denmark
	6 new cultivars	31.0-48.6	Skåne, Sweden
Red cabbage	6 old cultivars	39.3-58.7	Fyn, Denmark

Selection for genotypes high in vitamin C content is probably easy unless this character is linked to some undesirable traits. Up till now no cultivar has been marketed for its high vitamin C content. So many other traits have been regarded as more important than a high ascorbic acid level, and every new goal added to a plant breeding program costs money, time and effort. If the preferences of the market change there is good potential for breeding cabbage with an enhanced level of this vitamin. This would also mean that a crop produced in a Nordic country could become a good 'indigenous' source for vitamin C (Bruce, 2000).

## Bioactive compounds in carrot

### *Carotenes*

Carotenes give the orange colour of carrots. In this root crop, 20-25% of the total carotene amount consists of  $\alpha$ -carotene and 65-70% of  $\beta$ -carotene, while the rest has been identified as cis- $\beta$ -carotene,  $\gamma$ -carotene and lutein. Some carotenoids are converted to vitamin A in the human body. This vitamin is essential for proper growth and reproduction as well as for good eyesight.  $\beta$ -Carotene has potential for giving the largest vitamin A effect, as one molecule of beta-carotene should yield two molecules of vitamin A. One molecule of  $\alpha$ -carotene only yields one molecule of vitamin A. New evidence further supports the value of carotenes as antioxidants that may reduce the risk of cancer and cardiovascular disease (Pool-Zobel *et al.*, 1997).

The carotene level in carrots depends on root size and age as well as environmental conditions, but variation is primarily genetic. Since long, people have chosen the more orange roots and thus also received higher contents of carotenes. The differences in total carotene levels between genotypes are large (Table 3). This is also the case for the proportion of  $\alpha$ -/ $\beta$ -carotene. The possibility for increasing the carotene levels through plant breeding is thus large. 'High-carotene' varieties have been produced in USA without affecting any other culinary qualities (Simon, 1990).

**Table 3.** Variation in carotene levels ( $\mu\text{g}/100$  g fresh weight) in carrots grown in Sweden (figures of the American 'high-carotene' material from T. Nilsson, SLU, Alnarp, Sweden).

	$\alpha$ -Carotene		$\beta$ -Carotene	
	Variation	Mean	Variation	Mean
Cultivars (n=6)	1500-2900	2100	6900-11400	8750
Breeding material (n=76)	300-5100	2550	5300-14900	8000
American 'high-carotene' material	3800-9600	5900	9900-16300	12700

### *Polyacetylenes*

Carrots contain polyacetylenes like falcarinol and falcarindiol. Falcarinol has earlier been called 'carotatoxin' because it produces neurotoxic symptoms upon injections into mice. Falcarindiol has not shown this effect (Hansen & Boll, 1986). For many years, ginseng has been considered to be the most valuable drug in East Asia. Falcarinol extracted from its root has been shown to inhibit cancer cells. This made Danish researchers as well as other research groups interested in the effect of carrot falcarinol and -diol. They have also put up the question whether polyacetylenes and not carotenes cause the beneficial effects of carrot on health (Brandt *et al.*, 2001).

In carrot, the falcarindiol concentration decreases markedly from the root surface inwards. The falcarinol concentration is much lower than that of falcarindiol. It is significantly lower in the peel than in the phloem (Table 4).

**Table 4.** Variation in levels of polyacetylenes ( $\mu\text{g/g}$  fresh weight) in 16 carrot cultivars grown in Sweden (Olsson & Svensson, 1996).

Substance	In the peel		In the phloem	
	Variation	Mean	Variation	Mean
Falcarindiol	32-92	57	6-19	11
Falcarinol	1-5	3	3-12	6

Falcarindiol has anti-fungal properties *in vitro* against several fungi and *in vivo* against the fungus causing liquorice rot in carrot (Olsson & Svensson, 1996). Falcarinol has much less antifungal activity than the diol but shows some effect against the fungus causing grey mold in storage.

If polyacetylenes in carrot show to be potent protective compounds against cancer, plant breeding for higher contents of falcarinol and –diol in the roots of this vegetable could be of interest. Based on today's knowledge, an increased level seems to be favourable also for resistance to micro organisms. Much more work within this field is, however, needed. Studies of the heritability of the polyacetylenes are also lacking.

### Acknowledgement

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## **ZINAXIN GINGER-DERIVED BIOCOMPLEX – A GCP DOCUMENTED HERBAL PROVEN TO REDUCE JOINT PAIN AND STIFFNESS IN OSTEOARTHRITIC PATIENTS**

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**Summary of: Effect of a Ginger Extract on Knee Pain in Patients With Osteoarthritis:  
Altman RD, Marcussen KC; Arthritis & Rheumatism, 2001; vol 44: pp 2531-2538**

### **Objective**

To evaluate the efficacy and safety of a standardized and highly concentrated extract of 2 ginger species, *Zingiber officiale* and *Alpina galanga*, in patients with osteoarthritis (OA) of the knee.

### **Results**

In the 247 evaluated patients, the percentage of responders experiencing a reduction in knee pain on standing was superior in the ginger extract group compared with the control group (63% versus 50%;  $P=0.048$ ). Analysis of the secondary efficacy variables revealed a consistently greater response in the ginger extract group compared with the control group, when analyzing mean values: reduction in knee pain on standing (24.5 mm versus 16.8 mm;  $P = 0.005$ ) and reduction in knee pain after walking 50 feet (15.1 mm versus 8.7 mm;  $P = 0.016$ ). Change in global status and reduction in intake of rescue medication were numerically greater in the ginger extract group. Change in quality of life was equal in the 2 groups. Patients receiving ginger extract experienced more gastrointestinal (GI) adverse events than did the placebo group

### **Conclusion**

A highly purified and standardized ginger extract had a statistically significant effect on reducing symptoms of OA of the knee. There was a good safety profile, with mostly mild GI adverse events in the ginger extract group.

## **EFFECT OF QUERCETIN ON GENE EXPRESSION IN HUMAN CELLS AS MEASURED BY MICROARRAYS - A PILOT STUDY.**

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### **Abstract**

Quercetin is an important flavonoid in the human diet but there is a lack of knowledge concerning its mechanism of action. To gain new information, we studied its effect on cell proliferation and on gene expression in Caco-2 cells using DNA microarray technology. Using this procedure it is possible to measure the expression of several thousand genes in one run. Quercetin showed antiproliferative properties at a concentration of 250  $\mu\text{mol/l}$ . Results from the microarray analysis showed many up regulated and down regulated mRNAs indicating that quercetin affected gene expression.

### **Introduction**

Food components can affect physiological processes in the cell by many mechanisms. The regulation of the rate of synthesis of an individual protein is probably a major mechanism, which can be the result of an effect on the corresponding mRNA level. With a new miniaturised technology, DNA microarray, the expression of over 10000 human genes can be measured in one analysis. This very powerful tool can be used to find new genes, which are affected by different food components (Elliot, Ong, 2002). The identification of these genes is an important step in finding out the cellular mechanisms involved. Understanding of the cellular mechanisms would be helpful in the design of foods for optimal disease prevention.

Quercetin is part of the large family of flavanoids with over 4000 compounds. They are found in fruits, vegetables, nuts, seeds as well as in tea and wine. The basic structure of flavanoids consists of an *o*-heterocyclic ring fused to an aromatic ring with a third ring system attached at either C3 or C4. Quercetin is the main flavonol in our diet and occurs in many fruits and vegetables. It is particularly abundant in onion (0.3mg/g fresh weight) and tea (10-25 mg/L), but is also found in black currant, strawberries and broccoli (Scalbert, Williamson, 2000; Ewald et al., 1999). The aim of this study was to investigate if Caco-2 cells incubated with quercetin, exhibited changes in gene expression compared with control cells.

## Materials and Methods

The Caco-2 cell line, derived from a human colon adenocarcinoma, was maintained in serial passages in RPMI 1640 medium supplemented with 10% fetal calf serum, non-essential amino acids and antibiotics. The cell cultures were incubated at 37°C in a water-saturated atmosphere of 5% CO<sub>2</sub> in air. The cells used in the experiments were between passages 30-40. The effect of different quercetin concentrations on cell growth was monitored using the AlamarBlue method. For microarray experiments, Caco-2 cells were seeded in Petri dishes and after preincubation for 24 hours quercetin was added to a final concentration of 25 µmol/l. The cell cultures were then incubated for 24 hours. After washing, the cells were solubilized in Trizol and the RNA was isolated using an RNA isolation kit. The mRNA was converted in several steps to the corresponding cRNA that was hybridised to the Affymetrix human chip (HU95) at the Swegene Microarray Resource Centre at Lund University. The chips contained high-density, two-dimensional arrays of synthetic oligonucleotides on a 1.28 x 1.28 cm glass surface (Lipshutz et al., 1999).

## Results and Discussion

Cell proliferation was inhibited by quercetin treatment. TGF-β is a protein involved in the regulation of cell proliferation. TGF-β mRNA was up regulated in Caco-2 cells incubated with quercetin. TGF-β exerts its antiproliferative action by stimulating the production of the cyclin dependent kinase inhibitor p27 and this was also found in quercetin-treated cells where p27 mRNA was up regulated. p27 inhibits the kinase function of cyclin/cyclin dependent kinase complexes thereby inhibiting the G1/S transition. The antiproliferative activity of quercetin could in part be mediated by the increase of TGF-β expression.

The human gene for cytochrome P(1)-450 was up regulated 6-fold in Caco-2 cells treated with quercetin. Cytochrome P 450 enzymes catalyse a series of reactions whereby water-insoluble drugs or metabolites that would otherwise accumulate to toxic levels in cell membranes are rendered sufficiently water-soluble to leave the cell and be excreted in the urine. Cytochrome P 450 enzymes are also suggested to be involved in the metabolism of quercetin (Duthie, Dobson, 1999). Cytochrome P 450 may have been up regulated as a defence against high levels of quercetin in the cell.

Osteopontin mRNA was also found to be up regulated by quercetin. This glycoprotein is involved in bone resorption. Osteopontin is produced by osteoblasts under stimulation by calcitriol, binds tightly to hydroxyapatite and is involved in the anchoring of osteoclasts to the bone matrix (Reinholt et al., 1990). Osteopontin also has a role in cell signalling and adhesion and is secreted by most epithelial cells and many cancer cells.

The human urokinase-type plasminogen receptor, exon 7 was up regulated in Caco-2 cells when treated with quercetin. This receptor plays a key role in tumour invasion and metastasis and is associated with tumour aggressiveness (Muehlenweg et al., 2001).

Another up regulated mRNA in Caco-2 cells treated with quercetin, was the human phosphatidyl inositol 3-kinase homologue. It has a role in the receptor tyrosine kinase-signalling pathway and is thought to be important in regulating cell proliferation. Some cancer cells consistently express high levels of cyclin D2, which is an important protein for cell cycle progression. The high expression of cyclin D2 is mediated by the phosphatidyl inositol 3-kinase pathway, indicating that this pathway is of importance in some cancer forms (Deininger et al., 2001).

The mRNA for the integrin alpha-2 subunit was up regulated almost 4-fold in Caco-2 cells treated with quercetin. Integrins are cell surface receptors that mediate adhesion to the extracellular matrix and cell-cell interactions. Integrins are heterodimers consisting of an alpha and a beta subunit.

Among the genes down regulated by quercetin treatment was the human nerve growth factor (HBNF-1) mRNA and human 3 beta hydroxy steroid dehydrogenase/ delta-5-delta-4 isomerase (3-beta-HSD), which was down regulated more than 4 times. Nerve growth factor is required for neuron survival and is important for neuronal development. The 3 beta-HSD enzyme is essential for the biosynthesis of all active steroid hormones and catalyses the conversion of 3 beta-hydroxy-5-ene steroids to 3-oxo-4-ene steroids.

Sarcolectin, also down regulated by quercetin, is a lectin that is important in T-cell proliferation. It stimulates DNA synthesis in all immunocompetent cells and it partly regulates clonal expansion of T cells. Available data indicate that sarcolectin stimulates DNA synthesis in co-ordination with more specific growth factors and hormones, and that sarcolectin could play a role in tumour development (Kaba et al., 1999).

In response to tissue damage such as hepatic and renal injury, hepatocyte growth factor (HGF), an inactive single-chain precursor protein, is converted by HGF activator by limited proteolysis into an active heterodimeric molecule. Also the human mRNA for HGF activator like protein was down regulated in Caco-2 cells treated with quercetin.

### **Concluding remarks**

The results showed that quercetin affected the expression of a wide range of genes in Caco-2 cells. The genes affected by quercetin have many different functions, both inside and outside the cells. Some of the genes such as TGF- $\beta$  and human cyclin dependent kinase inhibitor p27 kip1 are involved in the regulation of cell cycle progression which is very interesting since

quercetin shows antiproliferative properties to Caco-2 cells (Kuntz et al., 1999). Other findings such as an up regulation of urokinase-type plasminogen receptor, exon 7 mRNA, are difficult to explain at present. Further studies are necessary to evaluate the health importance of these findings. The study also showed the wide potential applicability of the microarray technique to unravel the effects of food components.

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## **RETAINMENT OF PHENOLIC PHYTOCHEMICALS BY NEW TECHNOLOGICAL APPROACHES IN BERRY JUICE PROCESSING**

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### **Abstract**

Industrial production of berry juices comprise several steps intended to clarify the pressed juice. Current juice clarification practices include gelatin-silica sol treatment, vacuum filtration and other filtrations often requiring use of silica filter aids. These treatments result in 20 – 45 wt% decreases in the levels of phenolic phytochemicals in black currant juice and cherry juice. We examined the efficiency of high speed centrifugation, centrifugation prior to gelatin-silica sol addition, and other alternative procedures for clarification and haze prevention in sour cherry juice. High speed centrifugation (1000g – 35000g) reduced the turbidity significantly without compromising the phenols. Several of the other clarification strategies also showed promise. We also explored the applicability of a new Filtomat® thread microfiltration principle on semiprocessed black currant juice and cherry juice. This thread microfiltration retained the phenolic substances and successfully diminished the turbidity of both types of juices, notably of black currant juice. The data suggest that several alternative clarification strategies deserve further consideration in berry juice processing and that microfiltration with thread filters appears suitable as a novel technology for berry juice fining, especially for black currant juice filtration.

Key words: Clarification, microfiltration, black currant juice, cherry juice.

### **Introduction**

One of several quality criteria for berry juices, including those reconstituted from concentrates, is that the products are clear and neither contain sediments nor produce haze during storage. Industrial production of berry juices such as black currant juice, cranberry juice, grape juice and cherry juice therefore includes a number of steps intended to clarify the pressed juice. Hence, after the juice pressing operation, where the relatively turbid juice is expelled from the fruits, the juice is pasteurised, clarified, fined, centrifuged or filtered, aroma stripped, and filtered again before sterile transfer into cold storage tanks. Juice concentrates are furthermore evaporated prior to cold storage. The clarification and fining treatments

typically involve addition of pectinases and fining agents such as bentonite or gelatin-silica sol to remove cloud, sediments, and haze-active components. The juice clarification step usually includes a slow settling of the colloid flocs resulting from the treatment (Konja and Lovric, 1993). After the settling of colloids, the supernatant juice is centrifuged or filtered, while the viscous gelatin or bentonite induced precipitate is centrifuged or subjected to high-vacuum rotary filtration to recover some of the juice captured in the sediment slurry. Conventional filtration operations employed in large scale fruit juice processing, including vacuum rotary filtration and sludge frame filters, comprise use of filter aids such as kieselguhr, Perlite® or diatomaceous earth. These silica containing filter aids are considered as hazardous materials, and requires special handling and disposal, which is not only troublesome, but also inflict additional costs on the juice processing plant. Recently, we found that the conventional gelatine-silica sol clarification treatment followed by vacuum-filtration decreased the total phenol levels in cherry juice by 45%, with losses in the concentration of the principal anthocyanins by 11-13 %. With black currant juice, the total phenols decreased by 21 % and the contents of ascorbic acid, hydroxycinnamates, and the four major anthocyanins declined by 19-29 % (Meyer et al., 2002). Together with the filtering aid handling problems, these losses in phenolic substances create an incentive for the development of new strategies for berry juice clarification and filtration. This paper summarises some of our recent data obtained with novel, alternative approaches for clarification and filtration of black currant (*Ribes nigrum*) and cherry juices (*Prunus cerasus* L.) (Bagger-Jørgensen et al., 2002; Meyer et al., 2001). Our general aim is to develop improved and more gentle processing strategies for production of high quality fruit juices. In the work discussed here, specific emphasis was put on evaluating the impact on juice turbidity and haze formation as well as on retainment of phenolic phytochemicals.

## Experimental

### *Juice samples, thread filters, gelatin-silical sol*

Semiprocessed juice samples produced from sour cherries (*Prunus cerasus* L.) cv. Stevnsbær and blackcurrants (*Ribes nigrum*) cv. Ben Lomond were supplied by an industrial juice processor (Vallø Saft A/S, Vallø, Denmark). The Filtomat® thread filters were produced by Filtration Ltd. (Israel) and supplied by Gustaf Fagerberg A/S (Brøndby, Denmark). Gelatin (100 AB 30 acid bone gelatin) was obtained from SKW Biosystems (Boulogne Billancourt Cedex, France) and silica sol (Klar-Sol Super) was from Erbslöh Getränke Technologie (Geisenheim, Germany).

### *Centrifugation, clarification*

Impact of centrifugation on primary turbidity was tested by spinning unclarified cherry juice samples (100 mL) for 10 min. at 1000 – 35000 g and for extended time periods at 35000 g using a Sorvall, floor-model superspeed centrifuge (Buch & Holm, Herlev, Denmark). The influence of the centrifugation and gelatin-silica sol treatment sequence was evaluated with

centrifugation at 20000g for 20 min. Direct filtration was evaluated using lab-scale vacuum filtration through 50 µm nylon filters (Frisenette Aps, Denmark).

#### *Microfiltration with thread filters*

Filtrations were performed with batches of 2 L juices using a filter unit harbouring a single Filtomat thread filter® (details in Bagger-Jørgensen et al., 2002). The computer programme Modde (Umetri AB, Umeå, Sweden) was used to create and analyse the statistical design of the 2<sup>3</sup> factorial experiment with temperature (3-21 °C), flow (20-80 L/h) and filter pore size (3-20 µm) as test parameters. Significance of the results was established at  $p \leq 0.05$ .

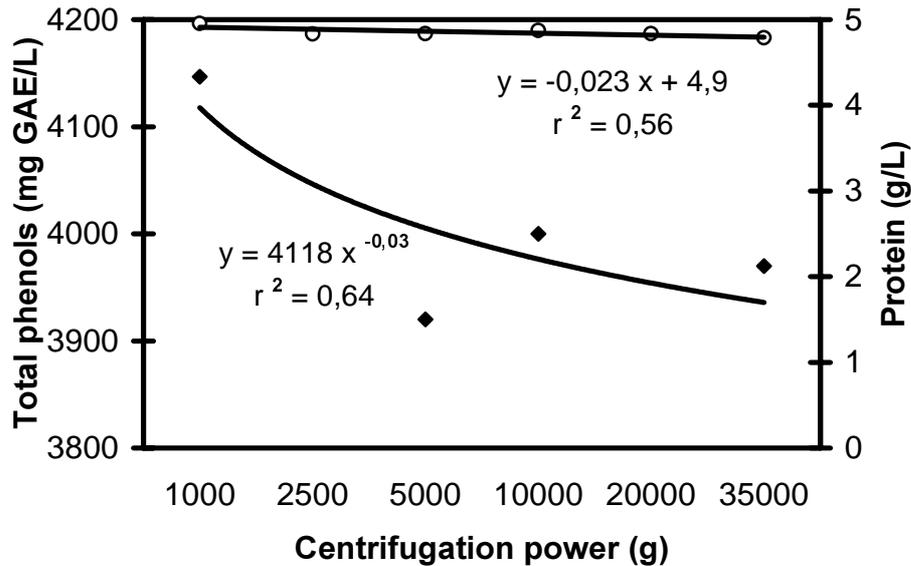
#### *Analyses*

The concentration of total phenols in juice samples was determined by the Folin-Ciocalteu procedure with total phenols expressed as mg/L gallic acid equivalents (GAE) (Singleton and Rossi, 1965). Protein was assessed using a Nitrogen Analyzer Macro N instrument (Elementar Analysensysteme GmbH, Foss Electric, Hillerød, Denmark); multiplication factor for protein 6.25. Turbidity in FNU (formazan nephelometric units) was measured by nephelometry at 90° light scattering, 860 nm, with a Nephla reader (Dr. Lange, Düsseldorf, Germany). 1 FNU equals 2.5 mg/L SiO<sub>2</sub>. Prior to measurement all juice samples were diluted to 3.0 °Brix.

## **Results and discussion**

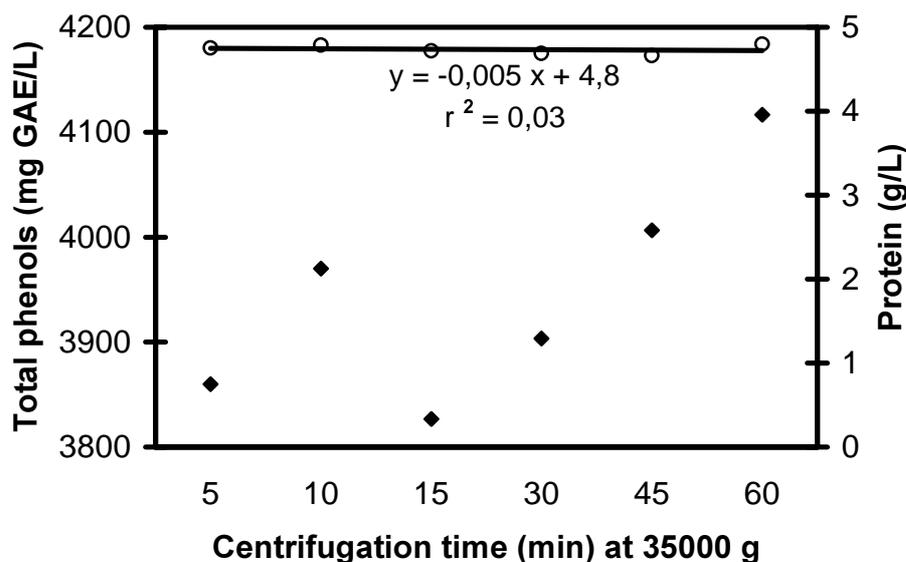
#### *Alternative clarification and fining treatments*

Direct centrifugation of freshly pressed cherry juice samples (100 mL) lead to significantly decreased turbidity with increasing centrifugation speed (g). When increasing the centrifugation speed from 1000 - 35000g, while keeping the centrifugation time constant at 10 min., the overall effect of centrifugation speed on juice turbidity could be described by a steep negative power function:  $Y = 3498 X^{-0.54}$  ( $r^2 = 0.95$ ), where  $Y$  is turbidity in FNU and  $X$  is centrifugation power in g. Extended high-speed centrifugation of cherry juice at 35000 g fitted a power function:  $Y = 49 X^{-0.58}$  ( $r^2 = 0.91$ ) (Meyer et al., 2001). By centrifugation for 10 min. at 20000 – 35000 g the lowest FNU levels obtained were from 14 – 18 FNU, but by extended centrifugation at 35000 g turbidity levels as low as 5 - 6 FNU were achieved (detailed in Meyer et al., 2001). Since the clarity criterion for red berry juices is a FNU of less than 5 (AIJN, 1996), this latter reduction corresponds to the level required for finished juice. The amounts of total phenols as well as protein content decreased only insignificantly with increased centrifugation speed (Fig. 1).



**Figure 1.** The effects of increased centrifugation speed (10 min. centrifugation) on levels of protein (O) and total phenols (◆) in the resulting supernatant cherry juice.

Extended centrifugation time at 35000g did not affect the protein content and gave total phenols in the range 3800 – 4100 in the cherry juice (Fig 2). Thus, neither increased centrifugation speed nor extended centrifugation time at 35000g compromised the levels of phenolic phytochemicals in the cherry juice. Obviously, centrifugation is an energy-expensive procedure, and at present, extended high-speed centrifugation beyond a few seconds may be difficult to achieve with the centrifuge equipment currently available in most large scale fruit juice processing plants. However, based on the data obtained in this work, we recommend that the economic and technological feasibilities of introducing pre-centrifugation treatments with longer centrifugation times at 5000-10000 g receive careful consideration in the design of novel approaches to fruit juice clarification in large scale, commercial plant operations.



**Figure 2.** The effects of extended centrifugation time (minutes) at 35000g on levels of protein (O) and total phenols (◆) in the resulting supernatant cherry juice.

Centrifugation of raw cherry juice at 20000g for 20 min. gave the same clarification effect as gelatin-silica sol treatment and both treatments reduced the total phenols by ~ 18 % relative to the level in the raw, freshly pressed juice (Table 1).

**Table 1.** Effects of centrifugation, gelatin-silica sol fining (G-K), and filtration on turbidity and phenols content in seminprocessed sour cherry juice. Data are given as average values obtained from at least duplicate treatments.

Juice treatment	Turbidity <sup>1</sup> (FNU)	Phenols <sup>2</sup> (GAE)
None	> 1300	4817
Centrifugation	17.9	3701
G-K + centrifugation	18.1	3957
Filtration nylon filter	63.1	4130
Filtration nylon filter + G-K + centrifugation	1.6	3834

<sup>1</sup> Coefficient of variation on mean < 3.4%. <sup>2</sup> Coefficient of variation on mean < 4.7%

However, a FNU value of ~18 indicates a turbid juice requiring further fining and filtrations, that may induce further losses in phenolics. Experimental filtration with nylon followed by gelatin-silica sol treatment also reduced the phenols by ~ 18 %, but immediately produced a clear juice of 1.6 FNU (Table 1). This level of clarity fits the generally used criterion that the

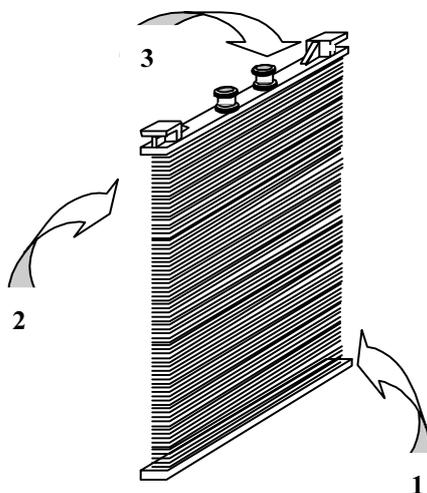
turbidity in the final juice is below 5 FNU. However, even though filtration prior to clarification treatment produced good results, the filtration was slow and may not be suitable in large scale processing. Further work is warranted to develop feasible, gentle filtration procedures for efficient clarification and high retainment of phytochemicals in large volume juice manufacture.

The available knowledge suggests that the turbidity in freshly pressed berry juices is mainly caused by suspended, proteinaceous pectin particles, but other polysaccharides (e.g. starch) and cell wall material may also contribute (Grassin and Fauquembergue, 1996; Sahin and Bayindirly, 1993; Weiss, 1987). In contrast, any possible sediments and postclarification haze - which forms during cold storage of single strength juice or juice concentrate - may result from mainly protein, polyphenols, and insoluble tannins (Siebert et al., 1996). We previously investigated the individual and interactive effects on cherry juice turbidity and haze formation during cold storage of combined treatments with a pectinase, two different proteases, and gallic acid addition (Meyer et al. 2001). The treatments resulted in very different juice turbidity ranging from 3.5 – 35 FNU in the different experiments. Both treatment of precentrifuged cherry juice with an acid protease (Novozym 89L) and co-addition of pectinase (Macer 8FJ) and gallic acid improved immediate juice clarity and diminished haze levels during cold storage of the juice at 2°C for 3 weeks. Pectinase treatment is assumed to induce electrostatic destabilisation of suspended, cloud-causing pectin particles (Endo, 1965), while the protease catalysed clarification apparently works by direct hydrolysis, and hence degradation, of haze-active protein molecules. Gallic acid addition has been shown to retard haze development in beer model solutions (Siebert and Lynn, 1998). A plausible hypothesis is that gallic acid blocks protein binding sites. In turn this blockage prevent the proteins from cross-binding with polyphenols and thus from entering into multimolecule protein-polyphenols crossbinding (Siebert and Lynn, 1998). Clarification and haze prevention by direct enzymatic removal of haze-active proteins, rather than unspecific gelatin-induced withdrawal of phenolics, appears a more sound approach to retain physiologically beneficial phenolic phytochemicals in fruit juices. Our current research priorities therefore include more detailed studies of the effects of protease clarification treatments on various berry juices as well as examination of the influence of such treatments on phenolic profiles and antioxidant activities of the juices.

#### *Thread microfiltration*

The Filtomat® thread filtration principle is increasingly used for water purification in the Mediterranean region. The individual filters, including the ones we tested, have a surface area of 0.01 m<sup>2</sup> and each consist of a plastic base with a polyester thread wound around it (Fig 3.). The main advantage of these thread filters is their easy rinsing, utilising the unique thread filtration design that permits removal of trapped particles within the filter structure by a patented automatic water spraying method (US Patent no. 5514270). When employed industrially, several thousand of such individual filters are mounted in a filtration unit. We

evaluated the performance of Filtomat® filters for filtration of industrially produced, semiprocessed cherry and blackcurrant juices (Bagger-Jørgensen et al., 2002).



**Figure 3.** Schematic representation of a Filtomat® thread filter: 1. Plastic support, 2. Thread layer, 3. Permeate outlet.

Thread microfiltration immediately reduced the turbidity of blackcurrant juice from 12.6 FNU down to a satisfactory level below 5 FNU independently of the combination of filtration parameters (Table 2).

**Table 2.** Summary of data obtained with Filtomat® thread filtration of cherry and black currant juices in a factorial design comprising combinations of temperature, flow, and filter pore size (adapted from Bagger-Jørgensen et al. 2002)

Exp. no.	FACTORS			RESPONSES			
	Temp. <sup>a</sup> (°C)	Flow <sup>b</sup> (L/h)	Pore size (µm)	black currant		cherry	
				Turbidity (FNU)	Phenols (g/L)	Turbidity (FNU)	Phenols (g/L)
1	3	20	3	3.2	4.29	25.7	2.99
2	20	20	3	3.8	4.28	27.1	2.84
3	3	80 (70)	3	4.1	4.29	25.8	2.96
4	20	80 (70)	3	4.5	4.25	30.4	3.02
5	3	20	10	3.9	4.39	29.3	3.08
6	20	20	10	4.3	4.32	27.7	2.97
7	3	80 (70)	10	4.4	4.33	26.5	2.79
8	20	80 (70)	10	4.9	4.37	30.4	3.02

<sup>a</sup> Black currant juice: temperature limits 3 and 21 °; cherry juice temp. limits: 3 and 19 °C.

<sup>b</sup> The values in parenthesis indicate the max. flow limit with cherry juice.

The turbidity of cherry juice (initial FNU 32.4) also decreased markedly after thread microfiltration, but the turbidity levels obtained did not define a completely clarified juice as turbidity ranged from 25 – 30 FNU (Table 2). With both juices the combination of parameters that most significantly decreased the turbidity was low temperature, low flow, and small pore size. Multiple linear regression analysis of the data revealed that the turbidity decrease in cherry juice was significantly better when filtration was performed at the lower (3 °C) than at the higher temperature (19 °C). As expected, the measured trans-membrane filtration pressure, indicating filtration performance, was highest at high flow rates on filters of small pore size (data not shown). Apart from a negative effect of small filter pore size on the phenolics in filtered black currant juice, as discussed below, none of the factors (temperature, flow, or filter pore size) produced main effects on the protein, sugar or phenols level nor the color assessed as concentration of anthocyanins (not all data shown here, for details see Bagger-Jørgensen et al., 2002). With cherry juice, high flow rate and elevated temperature exerted an interactive effect that significantly diminished the turbidity decrease as well as the decrease in phenolics. An explanation for the decrease in phenolics of black currant juice after filtration through small pore sized thread filters could be that the phenol compounds bound to, or were trapped with, other particles, which were retained by the Filtomat® filter. The results of several thread microfiltration experiments were comparable, if not better with respect to retainment of phenols and anthocyanins, than those obtained after ultrafiltration of the same blackcurrant and cherry juices in a large scale fruit juice processing plant (Bagger-Jørgensen et al., 2002). Besides this, industrial ultrafiltration often operates at high temperature, which increases solubility of turbidity causing substance and thereby boosts the post clarification haze development during cold storage.

## Conclusions

Several alternative clarification strategies to avoid losses in phytochemical components deserve further investigation in berry juice processing. Promising strategies include gentle filtration techniques, centrifugation as pre-treatment, and targeted enzymatic removal of haze-active components. The Filtomat® thread filtration principle appeared to be an efficient, filtration method for black currant juice clarification. For cherry juice, microfiltration with Filtomat® thread filters may prove useful as a gentle prefiltration treatment to partly remove turbidity-causing substances, but further treatments or filtrations appear necessary to obtain a fully clarified cherry juice. Further insight into the nature of the components and molecular interactions responsible for turbidity and haze formation in berry juices would provide an improved basis for development of more targeted methodologies for berry juice clarification and fining.

## Acknowledgments

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## HYBEN VITAL® - A SENSATIONAL SUCCESS STORY

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The production of HYBEN VITAL® rose hip (*Rosa canina* L.) powder originally started merely as a hobby 20 years ago by the now 81 years old Danish farmer, Erik “Farmer” Hansen based on his persistently maintaining many health benefits of rose hip.

He produced his powder from the shells *and* seeds (*Rosa canina pseudofructus cum fructibus*), gave it to friends and friends’ friends and received numerous encouraging user testimonials confirming his health benefit belief. By word of mouth the awareness of HYBEN VITAL® spread to such a surprising extent in Denmark that HYBEN VITAL® became the talk of the day.

In 1994 the demand took such dimensions that the hobby production subsequently had to be industrialised leading to establishment of a formalized holding-, production-, sales- and marketing entity in 1995 under leadership of Erik “Farmer” Hansen’s son, Torbjørn Hansen. He modernized and expanded the production facilities, obtained authorization by the Danish Ministry of Food and initiated professional research in an attempt to get scientific proof of his father’s persistent rose hip health benefit claims.

In 1995 the first promising *in-vitro* and open clinical trial results encouraged considerable investment in further research leading to the now existing long term standing scientific research cooperation with the Department of Clinical Microbiology of Rigshospitalet, the Department of Clinical Biochemistry of Copenhagen County Hospital in Gentofte, University of Copenhagen; The Royal Danish School of Pharmacy, the Danish Institute of Agricultural Science (DIAS), Institute of Clinical Research, Kolding and Institut für Sozialmedizin und Epidemiologie, Universitätsklinikum Charité, Humboldt Universität zu Berlin.

Realising the paramount importance of controlling stable supplies of rose hip berries of uniform high quality in 1997 induced the company to start cultivating its own raw material organically in standing research cooperation with the Danish Institute of Agricultural Science (DIAS). Today the company controls its own plantations in Denmark and Sweden. Up to now totally 135 hectares.

Next followed two GCP randomised, double-blind, placebo controlled clinical trials on patients with *osteoarthritis* – 100 patients in Norway and 100 patients in Denmark. \*The identical efficacy results of these two studies and other studies resulted in *peer reviewed* abstracts,

Oral presentations

posters and presentations on several international rheumatology and other medical congresses in Europe, China and the USA from 1997 to 2002. The study findings have been submitted for publication in respected international medical journals.

HYBEN VITAL® is patented in US. Other usage- and active substance patents pending in EU, US, Japan and other countries.

The researchers having subsequently succeeded in isolating the active constituent responsible for the inhibition of chemotaxis (patent pending) is now expected to pave the way for OTC approval.

**\* Abstracts available from Hyben Vital International ApS at request.**

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## RESVERATROL, A POTENTIAL CHEMOPREVENTIVE SUBSTANCE, CHANGES THE MORPHOLOGY AND ADHESION OF HUMAN COLON CANCER CELLS

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### Abstract

The naturally occurring compound resveratrol is a promising cancer chemopreventive agent. Resveratrol reduces growth of cancer cell lines derived from various tissues and prevents tumor development in animal models.

We have found that resveratrol reduces the growth of human colon cancer cells. Besides the inhibition of cell growth, resveratrol caused a 50 percent increase in mean cell diameter, changed the cell morphology and increased the cell adhesion. Elucidation of the mechanism for the modulation of morphology and growth inhibition is in progress.

Keywords: Resveratrol, inhibition of cell growth, cell adhesion, cell morphology, colon cells.

### Introduction

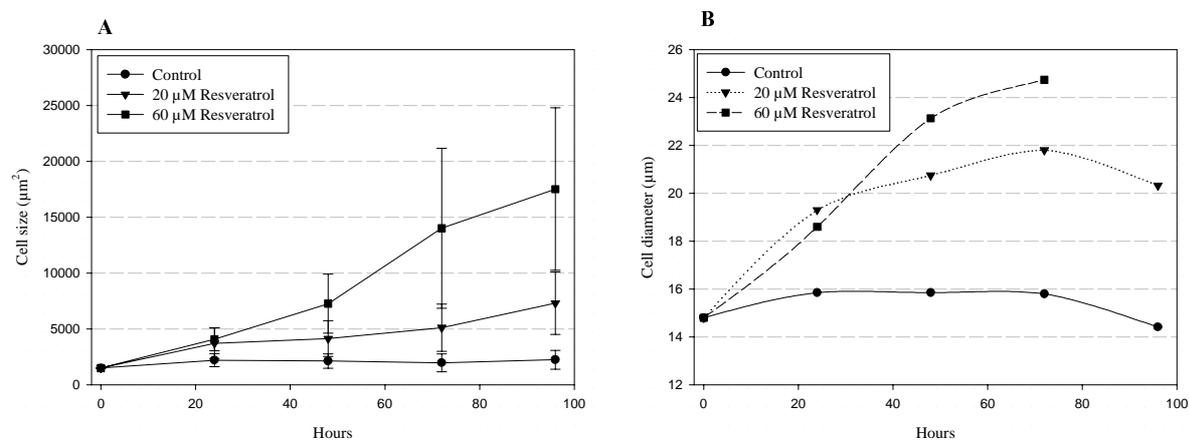
The human intake of resveratrol (*trans*-3,5,4'-trihydroxystilbene) is from grape, peanuts and wine and the levels of resveratrol increase in grapes in response to environmental stress. Since Jang *et al.* in 1997 reported that resveratrol acts as an anticarcinogen numerous of papers have focused on the different biological activities of resveratrol in relation to carcinogenesis:

i) Inhibition of various tumor markers in animal models (Jang *et al.* (1997), Schneider *et al.* (2001)), ii) Anti-inflammatory activity, iii) Inhibition of cyclooxygenase (COX) activity (Subbaramaiah *et al.* (1998)), iv) Agonist for the estrogen receptor (Bowers *et al.* (2000)), v) Inhibition of Ah-receptor and cytochrome P-450 1A activity (Ciolino and Yeh (1999)), vi) Counteraction of protein kinase C activation (Stewart *et al.* (1999)), vii) Counteraction of inhibition of gap junction intercellular communication (Nielsen *et al.* (2000)).

## Results

### *Inhibition of cellular growth*

The growth inhibitory activity of resveratrol was tested in human colon cells (DLD-1 and HCT-116). A dose- and time-dependent growth inhibition was observed. Fifty percent cell survival ( $IC_{50}$ ) was observed at 12  $\mu$ M and 25  $\mu$ M resveratrol in DLD-1 and HCT-116 cells, respectively, after 48 h exposure. The inhibition was reversible since removal of resveratrol from the medium after 48 h, results in cells resuming their normal exponential growth characteristics. Flow cytometry analysis show that DLD-1 cells treated with resveratrol for 48 h, accumulates in the S-phase at the expense of cells in the  $G_1$ -phase.



**Figure 1.** Resveratrol increase the cell area (A) and the diameter of the de-attached cells (B). Cells were exposed to vehicle, 20 or 60  $\mu$ M resveratrol for up to 96 hr. Every 24 h, the cells were photographed and trypsinized. The cell areas (A) were determined using the photographs, and the diameters (B) were determined using a coulter counter.

### *Increase in cell size*

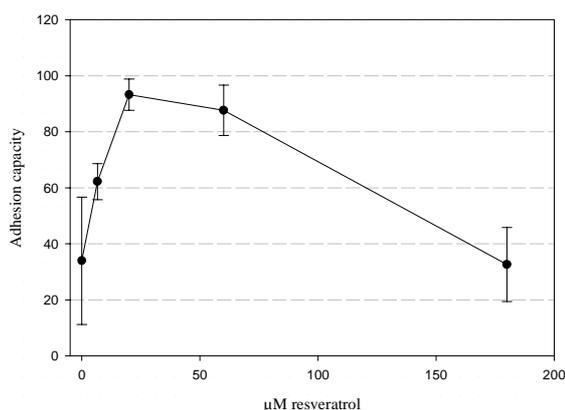
Concomitant with the growth retardation, we also observed that exposure to resveratrol increased the cell size and changed the cell morphology. The mean cell area increased time- and dose-dependently when cells were exposed to resveratrol (Figure 1A), and the mean cell area of cells exposed to 60  $\mu$ M resveratrol for 96 h were about 8 times larger than the control cells. The mean diameter of the de-attached cells also increases dose and time-dependently (Figure 1B).

### *Cell morphology*

When exposed to resveratrol, DLD-1 cells change their morphology, as they become more flattened. Staining of the actin cytoskeleton shows an increased number of filipodia.

### *Cell adhesion*

The changed cell morphology may indicate an increased adhesion. To verify this, the ability to trypsinize the cells, when treated with resveratrol, was investigated. DLD-1 cells were exposed to increasing concentrations of resveratrol for 48 h, treated with diluted trypsin for 120 min and counted. The adhesion capacity increased dose-dependently up to 20  $\mu\text{M}$  resveratrol, showing that resveratrol increases the cell adhesion to the plastic and likely to extra-cellular matrix.



**Figure 2.** Resveratrol increase the adhesion capacity up to 20  $\mu\text{M}$ . DLD-1 cells were treated with increasing concentrations of resveratrol for 48 h. The number of cells de-attached using trypsin was detected.

### *cDNA-array*

To identify the relevant gene expressions affected by resveratrol, we used cDNA-array analysis to identify the differentially expressed genes related to cell division and cell adhesion. The cDNA array led us to focus on the rearrangement of the actin and keratin cytoskeletons, as well as their anchoring through membrane-spanning integrins to the extra cellular matrix or neighboring cells.

### *Modulation of gene expression*

Human colon cancer DLD-1 cells were treated with resveratrol ranging in concentrations from 0 to 120  $\mu\text{M}$  for 48 hours, or treated with 60  $\mu\text{M}$  resveratrol for up to 48 hours. Total RNA was isolated and the expression of various cytoskeletal and cell adhesion genes analyzed by northern blot hybridizations.

The transcripts for integrin  $\beta 1$  and  $\beta 4$  encoding proteins, which in the intestinal epithelial cells are primarily associated with focal adhesion points and hemi-desmosomes, respectively, were up-regulated in response to resveratrol treatment in a concentration-dependent manner.

Likewise, transcript-levels of genes related to the focal adhesion protein scaffold and/or focal adhesion signaling, e.g. paxillin, zyxin, and cell adhesion kinase  $\beta$  were increased in cells treated with resveratrol for 16 hours onwards, whereas the mRNA-levels of others genes related to cell adhesion including focal adhesion kinase and vinculin were unchanged.

## Discussion

In human colon cancer DLD-1 cells, resveratrol induces several cellular responses, which likely are coupled: i) retardation of the cell growth, ii) change in the cell morphology and iii) increased cell adhesion.

Beside our data on the four different human colon cell lines, DLD-1, SW 480, HCT 116 (Wolter *et al.* (2001)) and LoVo, resveratrol has also been found to block the cell growth of other human colon cells: Caco-2 (Schneider *et al.* (2000), Wolter *et al.* (2001)) and Col2 (Nam *et al.* (2001)).

No one has previously reported cell volume increase following exposure to resveratrol or other chemopreventive substances. Whether the change in cell morphology is cell type specific still needs to be investigated. It is clear though that reorganization of the cytoskeleton takes place during cell volume changes, but the exact mechanism is not known. Clustering of the integrins are likely one of the factors as well as involvement of G-proteins (such as rho, rac and cdc42) (Pedersen *et al.* (2001)).

Increased cell adhesion to artificial extracellular matrix is a possible marker for chemopreventive activity (Meng *et al.* (2000)). In contrast to the present study, previous experiments have shown that resveratrol inhibits the adhesion of endothelial cells and inhibit the expression of both intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (Ahn *et al.* (2000), Ferrero *et al.* (1998)).

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## **EFFECT OF STORAGE ON PHENOLIC PROFILES AND ANTIOXIDANT ACTIVITY OF CHERRIES (*PRUNUS AVIUM* L.)**

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### **Abstract**

Samples of four different sweet cherry varieties, Burlat, de Saco, Summit, and Van, were harvested and stored at 2 °C and 15 °C for 30 and 6 days, respectively. Phenolics were extracted before and after storage and the phenols were quantified by the Folin procedure and profiled by HPLC. Total phenols in the extracts of the freshly harvested cherry samples varied from ~1000 - 2300 mg/L and generally increased during storage at 15 °C, mostly attributable to increases in anthocyanins. Cool storage induced no changes or decreased phenols levels. There was a dose-response relation between addition level of phenols and antioxidant activity to inhibit human low density lipoproteins (LDL) oxidation *in vitro* when phenolic extracts of cherry samples were evaluated at 10 – 20 µM levels of total phenols on 0.05 mg/mL LDL protein. Freshly harvested Summit cherries exhibited the highest antioxidant activity compared to the other varieties. The data obtained show that the mode and length of storage influence significantly the phenolic profiles of cherries, and that this in turn apparently affect their antioxidant properties.

Key words: Sweet cherries, antioxidant phenolics.

### **Introduction**

Fresh, sweet cherries represent an important, but fragile, commodity in the Portuguese agricultural export market. Since the harvesting season is very short, storage is used to balance the supply-demand in the season. However, knowledge on the effects of different storage conditions on cherry quality is not well developed. Sweet cherries can be considered good sources of both flavonoids and phenolic acids, and extracts of sweet cherries were previously shown to exert antioxidant activity on human low-density lipoproteins (LDL) oxidation *in vitro* (Heinonen et al., 1998). However, very little is known about the effect of storage on the phenolic profiles of different cherry varieties, and about how any possible changes in the phenols during storage affects the antioxidant properties of cherries. We

evaluated the evolution of phenols in four different cherry varieties Burlat, de Saco, Summit, and Van, when stored at 2 °C and 15 °C for 30 and 6 days, respectively. In addition we tested the antioxidant activities of extracts of fresh and stored cherry samples on human LDL oxidation *in vitro*.

## Experimental

### *Cherry Samples*

The *Prunus avium* cherry cultivars, 'Burlat', 'Summit', 'Van' and 'De Saco' were harvested according to a randomised scheme from an orchard in Vila Real, Portugal in summer 2001. Cold storage was at 2 °C and 90 % RH for 30 days; ambient storage was at 15 °C for 6 days.

### *Extraction and analyses of phenolics*

At fixed sampling times the cherries were pitted and freeze dried, and phenols were then extracted three times from the freeze dried cherry samples in 60 % aqueous methanol (10 min. solvent contact time, filtration through Whatman No. 1 filter paper). Total phenols were determined by the Folin-Ciocalteu procedure and expressed as mg/L gallic acid equivalents (GAE) (Singleton and Rossi, 1965). HPLC analysis was carried out as described by Lamuela-Raventos and Waterhouse (1994) with identification and quantification of compounds based on spectral identification (diode array detection) and comparison of retention times and peak areas with authentic standards.

### *Inhibition of human LDL oxidation*

Antioxidant activities of cherry extracts were assessed as inhibition of copper-catalysed oxidation of human LDL (0.05 mg/mL LDL protein, 37 °C, 5 µM CuSO<sub>4</sub>) evaluated by monitoring conjugated diene hydroperoxides at 234 nm (Esterbauer et al., 1989).

## Results and discussion

As expected, the phenols extracted decreased markedly from the 1<sup>st</sup> to the 3<sup>rd</sup> extraction. In total, the levels of phenols in the extracts of the freshly harvested cherry samples varied from ~ 770 – 1220 mg/L GAE, with highest levels in de Saco (data not shown). The absolute and relative levels of hydroxycinnamates and anthocyanins varied among the different varieties, where the dark red Burlat berries contained highest levels of anthocyanins, and de Saco was particularly rich in hydroxycinnamates as dominated by neo-chlorogenic acid (Table 1). Epicatechin, catechin, and quercetin-rutinoside were also detected; these compounds each constituted 3 – 9 % by weight of the total phenols in the four cherry varieties (data not shown). The results agreed well with the available data on phenolics in cherries (Gao and Mazza, 1995)

**Table 1.** Anthocyanins and hydroxycinnamates in extracts of freshly harvested cherries (mg/L as quantified by HPLC).

	cya-3-glu	cya-3-rut	peo-3-rut	neo-chloro	<i>p</i> -cou-quinic	chlorog
Burlat	79	156	9	119	144	20
Saco	9	113	ND <sup>§</sup>	878	71	56
Summit	6	109	0.4	218	183	53
Van	5	101	ND	469	40	35

<sup>§</sup> ND: Not detected

The levels of total phenols increased during storage at 15 °C, where the most significant increases were in the principal anthocyanins cyanidin-3-rutinoside and -3-glucoside. In contrast, cold storage for 30 days at 2 °C, gave decreased or unchanged phenols levels as compared to the fresh cherries. There was a dose-response relation between addition level of phenols and antioxidant activity to retard copper-catalysed human LDL oxidation *in vitro* when phenolic extracts of cherry samples were evaluated at 10 – 20 µM total phenols. At equal micromolar concentrations of phenols, the extracts of freshly harvested cherry samples exhibited higher antioxidant activities than extracts of stored samples (irrespective of the storage temperature of the samples). At 20 µM addition level of total phenols, the extracts of freshly harvested Burlat and Summit cherries blocked the LDL oxidation. Differences in the antioxidant potencies of the different cherry varieties were correlated to differences in their phenolic compositions.

## Conclusions

The phenolic profiles of the four cherry varieties varied. The storage temperature impacted the evolution of phenolics during storage, where storage at 15 °C increased the phenols levels and changed the phenolic composition, and cold storage led to decreased phenols levels or had no significant effect on the concentration and profile of phenols. Since antioxidant potency of cherry samples were related to their phenolic composition, the storage induced changes in phenolic profiles can be concluded to affect the antioxidant properties of cherries.

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## **PHYTOCHEMICALS IN ORGANICALLY GROWN CARROTS (*DAUCUS CAROTA*. L).**

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### **Abstract**

The content of different polyacetylenes, carotenoids and isocoumarins in two organically grown genotypes of carrots subjected to various processing and/or storage conditions was investigated in order to evaluate the nutritional value and potential beneficial effect of carrots on the human health. The content of polyacetylenes, carotenoids and the isocoumarin, 6-methoxymellein, were determined in organically grown genotypes of carrots subjected to 1) freezing after blanching, 2) cold storage for 5 months at 1°C after which the samples were blanched and frozen or 3) frozen raw.

Keywords: carrot, polyacetylenes, carotenoids, isocoumarins, secondary metabolites.

### **Introduction**

Several studies indicate that a high daily intake of fruits and vegetables have a significant protective effect against certain types of cancer, cardiovascular diseases and diabetes (Block et. al, 1992, Trolle et al., 1998). In most fruits and vegetables including carrots, a wide range of phytochemicals may contribute to this protection. Many phytochemicals are produced by the plants as defence compounds, and thus possess biological activity towards a wide range of organisms including humans and therefore may provide health benefits (Dillard and German, 2000).

Carrot is one of the main vegetables in Denmark with an annual intake of more than 10 kg per person. Some of the known phytochemicals in carrots are the polyacetylenes, e.g. falcarinol, falcarindiol and falcarindiol-3-acetate, the carotenoids,  $\alpha$ - and  $\beta$ -carotene being the most abundant and the isocoumarins, including 6-methoxymellein. As carrots are consumed throughout the year either raw or processed, it is important to elucidate the loss of these compounds in relation to factors such as genotype, processing and storage conditions.

### **Aim**

The aim of the present study was to determine the content of the polyacetylenes falcarinol, falcarindiol, falcarindiol-3-acetate, the carotenoids  $\alpha$ - and  $\beta$ -carotene and the isocoumarin 6-methoxymellein in relation to genotype, processing (blanching before freezing) and storage conditions (cold storage or frozen storage), in order to evaluate the health benefits of the

phytochemicals in carrots. The aim was also to investigate if the stability of these compounds is similar in order to recommend procedures for processing and storage of carrots.

### **Results**

The content of the polyacetylenes falcarinol, falcarindiol and falcarindiol-3-acetate, the carotenoids  $\alpha$ - and  $\beta$ -carotene and the isocoumarin 6-methoxymellein in carrot genotypes that have been processed and frozen after harvest or have been stored at 1°C for 5 months and then processed and frozen were determined. Furthermore, the content of these compounds in carrot genotypes subjected to blanching prior to freezing were compared to samples that were not blanched prior to freezing.

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## **HOME PROCESSING AND DISTRIBUTION OF ANTIOXIDANTS AND PESTICIDES IN APPLES**

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### **Abstract**

Two varieties of Danish apples, Discovery and Jonagold, were sprayed with pesticides, and after harvest the apples were peeled in order to diminish pesticide levels.

Contents of pesticides and the natural antioxidants, flavonoids and vitamin C, were investigated in peel, flesh and whole apples. Pesticides were determined by GC with ECD and NPD, flavonoids by HPLC with PDA and by LC-MS, and vitamin C by HPLC as ascorbic acid and dehydroascorbic acid.

Peeling was an effective method for reducing pesticide residues. Likewise, contents of the presumed health promoting flavonoids and vitamin C were also significantly reduced by peeling off the skin of the apples.

Keywords: Pesticides, flavonoids, vitamin C, home processing, peeling, apples.

### **Introduction**

Although residue levels found in fruit on the Danish market are generally low and without any health risk, many consumers wish to avoid intake of pesticides. Flavonoids are natural antioxidants and among the polyphenolic compounds often associated with the health promoting effects of a high intake of fruit and vegetables. Processing of fruit and vegetables has a potential of affecting pesticide residues as well as natural components, like antioxidants and vitamins.

In this study the effects of home processings on residues were investigated. As both flavonoids and pesticides are mostly found in the outer layers of fruit after harvest, the effect of peeling was investigated on these compounds. In addition the antioxidant vitamin C was measured.

### **Materials and methods**

Two varieties of field grown apples, Discovery and Jonagold, were sprayed twice (approximately 30 and 14 days before harvest) with 13 pesticide formulations at double the

dose recommended by the manufacturer. The different pesticides and metabolites were selected to represent various physical and chemical properties, among pesticides commonly observed in fruit and vegetables in the Danish monitoring programme (Danish Veterinary and Food Administration 2001).

Peeling of the apples was done by hand with a peeler removing 2 mm of peel. Stalk and blossom were removed and put aside for analysis together with peel. The weight of peel, stalk and blossom represented 18% and 16% of the whole apple of Discovery and Jonagold, respectively.

**Pesticides analysis:** 25 g of homogenised apple was extracted with acetone, cyclohexane, ethyl acetate and anhydrous sodium sulphate. The extract was cleaned by GPC, the pesticide fraction concentrated to nearly dryness, and resolved in ethyl acetate and cyclohexane (1:1).

Extracts were quantified by GC-ECD and GC-NPD. For further details see Poulsen and Granby (2000).

**Flavonoid analysis:** Apple flesh and peel were lyophilized (peel twice to compensate for the large variation within apple peel.). 1 g of homogenised sample was extracted with water repeatedly, centrifuged, and filtered (5 µm). The extract was cleaned by SPE on a C18 Bond-Elut column and flavonoids eluted by methanol. The eluate was evaporated to dryness and resolved in methanol. Flavonoids were quantified by HPLC using a C18 column, as solvent a gradient of formic acid, methanol, acetonitrile and water, and PDA detection. The identity of the individual components was verified by LC-MS.

**Vitamin C analysis:** Jonagold was the only variety investigated for vitamin C. Whole apples, peel and flesh were analysed by HPLC with UV-detection of ascorbic acid and postcolumn fluorometric detection of dehydroascorbic acid after derivatisation. The method is further described by Kall and Andersen (1999).

## **Results and discussion**

Pesticide levels of the unprocessed apples were within the normal range of fruit (European Commission, 2002), thus setting a realistic basis for the study, and not constituting any health risk. The results show that peeling significantly reduced the contents of all pesticides in both apple varieties. This indicates that the residues were mostly kept in the peel and that only smaller amounts had penetrated into the flesh of the apples, as earlier reported by Holland et al (1994) for fruits in general.

Flavonoids were mainly found in the peel of both apple varieties. Six glycosides of quercetin were found and only in the peel, while phloridzin was identified in peel as well as in flesh. Intake of the potential healthy flavonoid components are thus significantly reduced by peeling the apples before consumption. Contents of flavonoids varied rather much within the apple variety and even within the same apple, probably partly due to small differences in growing conditions, like position towards the sun.

Vitamin C was also mainly found in the peel of the apples, with ascorbic acid giving a major and dehydroascorbic acid only a modest contribution to the total vitamin C content..

## **Conclusion**

Peeling could effectively reduce pesticides in apples. Flavonoids and vitamin C were mainly found in the peel of the apple varieties, thus peeling apples before consumption would significantly reduce the intake.

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## **A RAT MODEL ON COLORECTAL CANCER SUITABLE FOR MEASURING THE EFFECTS OF NUTRITIONAL INTERVENTIONS**

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### **Abstract**

A rat model for colorectal cancer (CRC) has been fully developed and characterised at the Biomedical Laboratory, University of Southern Denmark. By giving 4 subcutaneous injections with Azoxymethane to rats of the BDIX inbred rat strain, a reproducibly high incidence of colon cancer occurred after a latency period of about 28 weeks. This model has been characterised histopathology, immunohistochemically, and genetically. This model will be used in the near future for examining the effects of preventive and therapeutic treatments.

Key words: Colorectal cancer, rat, model, prevention.

### **Background**

Colorectal cancer (CRC) remains one of the most common cancer forms in industrialised countries, where it accounts for up to 15% of all cancers. The prognosis for this disease is very poor, only 40-50% survive the following five years after the surgical removal of the tumour. Many efforts focus on finding better cures for this disease, and only a minor part of the studies are concerning studies on the prevention of this disorder. Studies of the human population provide insufficient information about carcinogenesis, pathogenesis and treatment of CRC. Therefore animal models are needed for understanding the different steps in the process under well-controlled conditions. The use of a well-standardised dietary composition and other experimental factors, the examination of putative intermediate biomarkers, studies on possible mechanisms of carcinogenesis, and quantitative development of tumours in a relatively short time are –among others- the advantages of using animal models in colorectal cancer research.

## Methods

On the basis of a review of the literature (Kobaek-Larsen et al., 2000) and studies using three different inbred rat strains, four subcutaneous azoxymethane (AOM) injections (15 mg/kg body weight per wk) in the BDIX rat strain was chosen as the most optimal model for the induction of colorectal cancer (Kobaek-Larsen et al., 2002). Furthermore, the model was characterised with respect to histopathology, anti-tumour immune response by immunohistochemical analysis, and genetical parameters. The microsatellite status and K-ras mutations were analysed by molecular biological methods, whereas mutations of *apc* and *p53* were analysed by immunohistochemical analysis. On the basis of this characterisation, a comparison to the human counterpart was made.

## Results

The BDIX rat strain developed a relatively high tumour incidence (>75%) in both sexes, which turned out to be highly reproducible. The BDIX rat had a relatively short latency period, as compared to the other inbred rat strains tested. The time between the first AOM injection and first diagnosis of colon cancer was about 28 weeks.

Histopathological examination revealed that the characteristics of the disease in this strain resemble those in the human (Kobaek-Larsen et al, 2002). The tumours were mainly located in the final part of the large intestine. The appearance of the tumours was both flat and polypoid. Formation of different stages of the colon carcinogenesis was observed, as aberrant crypt foci (ACF), adenomas and adenocarcinomas were present in this model. Most of the tumours represent the early stages of the carcinogenesis (ACF and adenomas) and the invasion of the adenocarcinomas was restricted to the submucosa (referred to as Dukes' A stage in the human process). No metastases were observed in the animals under our experimental conditions.

The anti-tumour immune response in our model was characterised (Kobaek-Larsen et al. submitted). The results from this study show that the model resembles the human situation in which the tumours are infiltrated mainly by cells from the non-innate immune system and only to a minor degree by mature cells or activated lymphocytes from the innate immune system.

The primary goal of the genetical studies of the model was to evaluate the microsatellite status of the model. This was done using molecular biological methods. It was shown that under our experimental conditions, all tumours that had developed were characterised as microsatellite stable. The *Apc*, *K-ras* and *p53* of the tumours were analysed for mutations, since these genes are highly affected in humans. No mutations of these genes were observed (Soerensen et al, in preparation).

## Conclusions

Histopathological examination revealed that the characteristics of the disease in the BDIX rat strain resembles colorectal cancer in humans. The latency time of about 28 weeks needed for the development of tumours is considered a suitable period for examining the possible preventive effects of nutritional interventions. The fact that mainly early stages of CRC are developed in this model makes it suitable for prevention studies of this disease. This model can be considered as an immunocompetent model, and the anti-tumour immune response is similar to that seen in humans. This makes the model useable for immune therapeutic studies. Analyses focussing on the genetical changes revealed the absence of mutations in *apc*, *ras* and *p53* genes in the rat model; these mutations are usually found in human colorectal cancer patients.

Finally, the genetical characterisation showed that the model represents the majority of the human group of CRC patients having microsatellite stable tumours. In humans this is the case in 85% of the patients.

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## ENZYMATIC ENHANCEMENT OF ANTHOCYANINS AND OTHER PHENOLICS IN BLACK CURRANT JUICE

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### Abstract

This paper summarises part of our work on enzymatic pre-press maceration treatments of black currants with the purpose of increasing the levels of anthocyanins and other phenolic phytochemicals in the expelled juice. Ten different plant cell wall degrading enzyme preparations were evaluated in a statistically designed experimental plan encompassing variations in enzyme addition level, degree of berry crushing, treatment time and reaction temperature. The levels of total phenols and anthocyanins in the resulting juice samples varied significantly in response to the different enzymatic maceration conditions. Increased maceration time, enzyme dose, and increased maceration temperature influenced positively the levels of phytochemicals released into the juice. More finely crushed black currants gave higher levels of anthocyanins in the juice. The data obtained demonstrated that it is possible to increase the levels of phenols, including anthocyanins, in black currant juice by modifying the enzymatic maceration of black currants prior to juice pressing.

Key words: Black currant juice, enzymatic treatment, anthocyanins, antioxidant phenols.

### Introduction

The cardioprotective effects of moderate wine consumption have been linked to the ability of red wine phenolics to retard lipid oxidation in low-density lipoproteins as demonstrated *in vitro* (Frankel et al., 1993). Consumption of American Concord grape juice has also been reported to improve a number of cardioprotective markers in humans (Keevil et al., 2000). However, even though grapes constitute a major source of several, potentially bioactive phenolic substances, where e.g. Concord grape juice is particularly rich in anthocyanin monoglucosides, many of the same type of compounds are present in other fruits and berries. Black currants are among those berries that contain high amounts of phenolic compounds, notably anthocyanins, that may constitute up to 2% by weight of the black currant skins (Koeppen and Herrmann, 1977). Other principal phenolics present in black currants include flavonols, procyanidins, and various phenolic acids, particularly hydroxycinnamates. Only a very small portion of black currants are consumed fresh, while the major part is processed for

juice concentrate. We previously demonstrated enhanced extraction of antioxidant phenols from black currant juice press residues via enzymatic polysaccharide hydrolysis (Landbo and Meyer, 2001). The purpose of the present work was to explore if the concentration of phenols in black currant juice could be enhanced - without compromising juice yields - by a forced enzymatic maceration treatment of the black currants prior to pressing. We evaluated the phenols, anthocyanins, juice clarity, and juice yields obtained under different enzyme reaction conditions with 10 plant cell wall degrading enzyme preparations in a statistical design comprising 25 different experimental maceration conditions.

## Experimental

### *Black currants, enzymes*

Frozen black currants (*Ribes nigrum*) cv. Ben Lomond were supplied by Vallø Saft A/S (Køge, Denmark). The enzyme preparations were obtained directly from the different enzyme manufacturers: Pectinex Superpress, Pectinex BE, Pectinex Ultra SP-L, vinozyme G (Novozymes A/S, Denmark), Rapidase BE Super, Rapidase EX Color, Rapidase Vino Super, Klerzyme Color (DSM Gist Brocades, NL), Macer 8™ [FJ] (Biocatalysts Ltd., UK), Rohapect B5L (Röhm, D).

### *Juice production and chemical analyses*

Maceration with each of the individual enzyme preparations were tested in a central composite circumscribed experimental frame with the factors enzyme dose, maceration time, reaction temperature, and degree of berry crushing as test parameters. Juice was extracted by pressing in a stainless steel hydraulic press (HST Tinkturen Press, Germany). The concentration of total phenols in juice samples was determined by the Folin-Ciocalteu procedure with total phenols expressed as mg/L gallic acid equivalents (GAE) (Singleton and Rossi, 1965). Turbidity in FNU (formazan nephelometric units) was measured by nephelometry at 90° light scattering, 860 nm, with a Nephla reader (Dr. Lange, Düsseldorf, Germany). 1 FNU equals 2.5 mg/L SiO<sub>2</sub>. Prior to measurement samples were diluted to 3.0 °Brix. Total anthocyanins were assessed by the pH differential method and calculated as cyanidin-3-rutinoside equivalents (mg/L) (Wrolstad, 1976).

## Results and discussion

The different enzyme preparations induced different responses in the phenols, anthocyanins, juice yield and clarity levels. Multivariate statistical analyses of the data showed that for all the enzyme preparations high enzyme dose, increased maceration time, and increased maceration temperature influenced positively the levels of total phenols released into the juice (Table 1).

**Table 1.** Summary of data on the effect of the four test parameters on increased total phenols, anthocyanins, yield, and clarity of black currant juice. Data given as number of enzyme preparations responding positively out of the 10 enzymes tested.

<i>Factor level</i>	<i>Total phenols (GAE mg/L)</i>	<i>Anthocyanins (mg/L)</i>	<i>Juice yield (g/g fw)</i>	<i>Clarity (FNU)</i>
<i>High enzyme dose</i>	<i>All enzymes</i>	<i>5 of the enzymes</i>	<i>All enzymes</i>	<i>All enzymes</i>
<i>Long maceration time</i>	<i>9 of the enzymes</i>	<i>2 of the enzymes</i>	<i>8 of the enzymes</i>	<i>All enzymes</i>
<i>High temperature</i>	<i>All enzymes</i>	<i>7 of the enzymes</i>	<i>5 of the enzymes</i>	<i>None<sup>1</sup></i>
<i>More crushed berries</i>	<i>5 of the enzymes</i>	<i>All enzymes</i>	<i>None<sup>2</sup></i>	<i>None<sup>3</sup></i>

<sup>1</sup> 4 of the enzymes gave increased clarity with low temperature maceration. <sup>2</sup> 2 of the enzymes gave increased yields with less berry crushing. <sup>3</sup> 3 of the enzymes gave better clarity with less berry crushing.

More finely crushed berries gave higher levels of anthocyanins in the juice with all the enzymes tested (Table 1). Some of the enzymes preparations increased the levels of anthocyanins in the juice with shorter maceration time. One preparation gave both better clarity (lower FNU), and higher anthocyanins concentration at low maceration temperature. From the analysis of the data, an optimal treatment for black currant mash could be defined with respect to obtaining a high content of anthocyanins balanced with as high juice yield as possible.

## Conclusions

The data confirmed our hypothesis that it is possible to increase total phenols and anthocyanin levels in black currant juice without compromising the juice yields by adjusting the pre-press enzymatic maceration treatment. On the assumption that the phenolic phytochemicals in black currants can elicit cardioprotective effects, the enzymatic pre-press treatment may provide a starting point for producing healthier black currant fruit juices.

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## THE EFFECT OF RESVERATROL ON EXPRESSION OF NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN (NGAL)

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### Abstract

During a cDNA array screening, the most highly induced gene following exposure to resveratrol was found to be neutrophil gelatinase-associated lipocalin (NGAL). This severe response was further confirmed and found to be time- and dose-dependent. The molecular mechanism is unclear as well as the cellular effect of the enhancement of NGAL.

The aim of this study is to identify the molecular mechanism for the resveratrol mediated induction of NGAL

Keywords: *Resveratrol, neutrophil gelatinase associated lipocalin, chemoprevention, NF- $\kappa$ B*

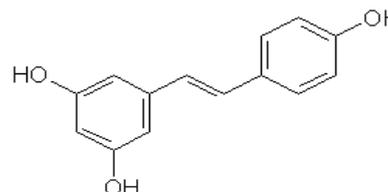
### Background

During the analysis of the molecular effects of resveratrol on human colon cancer cells using a cDNA-array, we identify a severe up-regulation of the NGAL expression following exposure to 50  $\mu$ M resveratrol for 24 h. This result was surprising, as NGAL is not known directly to be related to cell growth, changed cell morphology or adhesion that we have observed when treated with resveratrol.

Because of the significant up regulation of NGAL, we have speculated that NGAL could be an important signaling factor in the effects of treatment with resveratrol.

#### *Resveratrol*

Resveratrol (3,4',5-trihydroxystilbene) (figure 1) is a phytoalexin found in some plants, including grapes, peanuts and in wine. It is produced in the grapes in response to injury or fungal attack. Resveratrol is an interesting naturally occurring compounds, as it has been shown to provide cardiovascular and cancer protection properties.



**Figure 1:** Structure of resveratrol  
(3,4',5-trihydroxystilbene)

## NGAL

Neutrophil Gelatinase-Associated Lipocalin is also known as lipocalin 2, Human Neutrophil Lipocalin (HNL) and is homologue to mouse oncogene 24p3 and rat  $\alpha$ -2 microglobulin related protein. NGAL is a 25-kDa protein originally found associated with purified human neutrophil 92-kDa gelatinase/MMP9. It has been suggested that NGAL may exert modulatory actions on gelatinase (Kjeldsen *et al.* (1993)), and NGAL also forms a complex with the proenzyme form of matrix metalloproteinase-9 (pro-MMP-9, or progelatinase B) via an intermolecular disulphide bridge. This protein belongs to the family of the lipocalins or low molecular weight proteins that are able to bind and transport small hydrophobic molecules. The exact cellular function of NGAL remains to be determined.

### Aim of the study

The aim of the study is to describe the expression of NGAL in human colon cells (DLD-1) following exposure to resveratrol, and to identify the molecular mechanism by which resveratrol modulate NGAL expression.

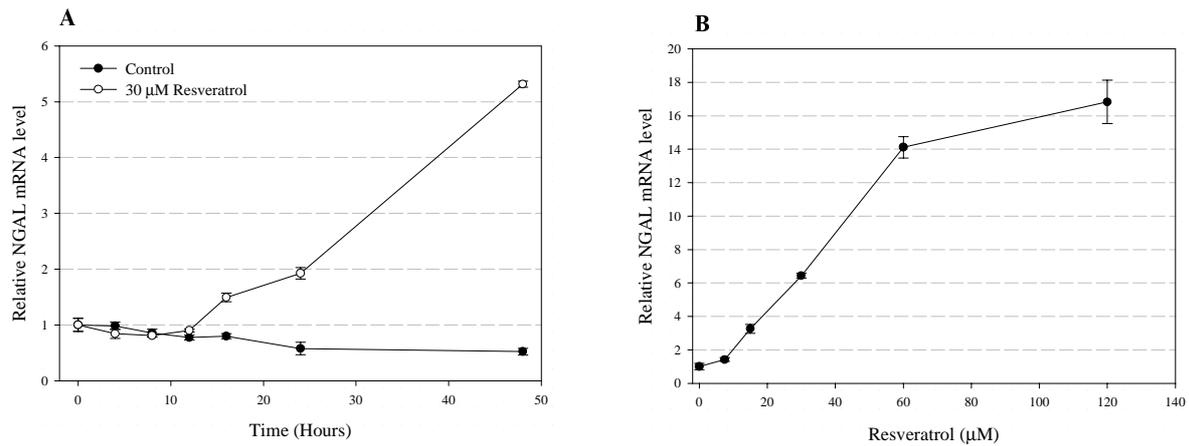
### Results

#### *The effect of resveratrol on NGAL expression*

The effect of resveratrol on the NGAL expression was investigated by exposure of human colon cells (DLD-1) to 30  $\mu$ M resveratrol for 0- 96 h. In a dose-dependent experiment DLD-1 cells were exposed for up to 120  $\mu$ M resveratrol for 48 h. Thereafter, mRNA and total proteins were purified. Detection of NGAL expression was done using Northern blot whereas NGAL proteins was detected using western blot.

When DLD-1 cells were exposed to resveratrol, the NGAL expression increase significantly beyond 8 h, and the expression continue to increase up to 48 h where the expression was 13 times higher than the control (Figure 1A). Furthermore, we observed that resveratrol induce NGAL expression dose-dependently (Figure 1B).

As the phorbol ester TPA is shown to be an inducer of both NGAL and MMP-9 (Kjeldsen *et al.* (1993)), we want to test the effect of resveratrol on TPA-treated cells. In experiments where the growth of DLD-1 cells were stimulated with 10  $\mu$ M TPA, the increased cell growth were blocked by a simultaneously treatment with 60  $\mu$ M resveratrol.



**Figure 2:** Resveratrol induce NGAL expression time- (A) and time-dependently (B). DLD-1 cells were exposed to DMSO or 60 μM resveratrol for up to 48 (A) or increasing concentrations of resveratrol in 48 h (B).

#### *The molecular mechanism of the modulation by resveratrol*

The expression of NGAL is known to be regulated via the NF-κB pathway, where IκB prevents the activation of NF-κB. When IκB is phosphorylated it is degraded and NF-κB is activated. Several papers have shown that NF-κB activation is inhibited by resveratrol (Manna *et al.* (2000)). To verify the role of NF-κB in the resveratrol induced NGAL induction, DLD-1 cells have been transfected with plasmids encoding either normal IκB or mutated IκB that cannot be phosphorylated and thereby not activate NF-κB. If resveratrol act via prevention of IκB inactivation, resveratrol will induce NGAL expression in cells transfected with wild type IκB but not in cells transfected with mutated IκB.

The results will be presented.

#### **Discussion**

Neutrophil Gelatinase-Associated Lipocalin is induced dose- and time-dependently in human colon cancer cells by resveratrol. NGAL is induced as an inflammatory response in colon cells but not detected in normal colon cells (Nielsen *et al.* (1996)). No one have previously reported that resveratrol regulate the expression of NGAL. Other inducers of NGAL are bacterial derived formylpeptides and lipopolysaccharides (Cowland and Borregaard (1997)) and phorbol ester (Kjeldsen *et al.* (1993)).

The underlying mechanism is unknown. A recent paper indicate that in a lymphocytic cell line, cytokine withdrawal (IL-3) cause an induction of NGAL, and that the secreted NGAL induce apoptosis (Devireddy *et al.* (2001)). The apoptotic effect in these cells was caused by protection of Bad protein to be phosphorylated. On the other hand, NGAL has been found to stimulate the growth of estrogen dependent mammary epithelial cells (Seth *et al.* (2002)).

In various reports it have been shown that resveratrol inhibit several cellular responses induced by phorbol ester, i.e. cyclooxygenase activation (Subbaramaiah *et al.* (1998)), inhibition of intercellular communication (Nielsen *et al.* (2000)) and the phorbol ester induced cell proliferation in the present experiment. It is not clear how resveratrol and phorbol ester interact with each other, as phorbol ester induces NGAL expression in several other cellular systems whereas resveratrol induce NGAL in the present model.

The role of NGAL in the observed cell growth retardation, changed cell morphology and increased cell adhesion is still unclear, but NGAL is believed to be a secreted protein, in some cells, that could act as a paracrine modulator that can affect cell proliferation and survival (Seth *et al.* (2002)). Further studies are needed to answer this.

### Future perspectives

The receptor protein(s) that resveratrol interact with is not known. The proteins which resveratrol binds to will be identified by using Isothermal Titration Calorimetry (ITC). The method may measure the small changes in heat when a ligand interacts with a receptor in a cellular extract. Using this method, the relevant proteins may be purified from DLD-1 cells.

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## CHARACTERISATION OF THE N-METHOXYINDOLE-3-HYDROXYMETHYL (NI3C) INDUCED CELL CYCLE ARREST IN HUMAN BREAST AND COLON CANCER CELL LINES

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### Abstract

Two gram N-methoxyindole-3-hydroxymethyl (NI3C) has been synthesized and for the first times it has been shown that NI3C inhibits cellular growth of human colon cancer cells (DLD-1 and HCT-116). Furthermore, NI3C was a more potent inhibitor of cell growth compared to indole-3-hydroxymethyl (I3C), which has recently been shown to inhibit DNA synthesis and cell division of human breast cancer cells (MCF-7). Concurrent to the inhibition of cellular growth, NI3C caused an accumulation of cells in G<sub>2</sub>/M phase, in contrast to I3C, which lead to accumulation of the colon cells in G<sub>0</sub>/G<sub>1</sub> phase. These results together with expression studies indicate that NI3C and I3C inhibit the cell growth by different mechanisms.

Keywords: N-methoxyindole-3-hydroxymethyl, human colon cells, cell cycle arrest, chemoprevention.

### Introduction

I3C and NI3C are degradation products of glucobrassicin and neoglucobrassicin, respectively. The glucosinolates are primarily found in *Brassica* vegetables and may in part explain the cancer preventive responses of cruciferous vegetables. In contrast to I3C, very little is known about the biological effects of NI3C. As previously found for I3C, NI3C induces cytochrome P-450 1A1 *in vitro* (Stephensen *et al.* (2000)) and *in vivo* in rats (Bradfield and Bjeldanes (1987)), probably via activation of the Ah-receptor (Stephensen *et al.* (2000)) as has been shown with I3C.

Recently, I3C was shown to inhibits DNA synthesis and cell division of human breast cancer cells, MCF-7 cells, by a G<sub>1</sub> cell cycle arrest, via a specific down-regulation of cyclin-dependent kinase 6 (CDK6) (Cover *et al.* (1998), Cover *et al.* (1999), Cram *et al.* (2001)), and preliminary experiments in our laboratory have shown that NI3C may be even more active to inhibit MCF-7 cell growth. I3C has an identical growth inhibitory effect in prostate cells

(Chinni *et al.* (2001)). Beside these effects, I3C significantly inhibits the cell adhesion, spreading, and invasion associated with an upregulation of PTEN (a tumor suppressor gene) and E-cadherin (a regulator of cell-cell adhesion) expression in human breast cancer cells (Meng *et al.* (2000)).

N-methoxyindole-3-hydroxymethyl is not commercially available, but we have now synthesized more than 2 gram of pure NI3C (characterized by NMR and HPLC).

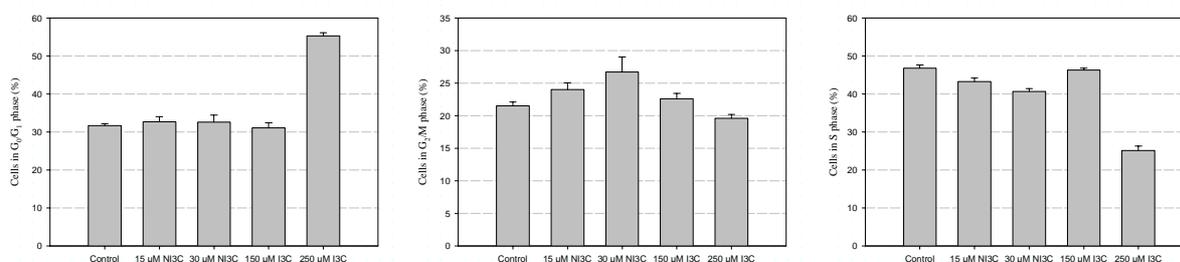
## Results

### *Inhibition of cell growth*

The growth inhibitory effect of NI3C was tested in two human colon cancer cells, DLD-1 and HCT-116, and the effect compared with the effect of I3C. The cell number was determined after a 48-hr treatment period with indoles or vehicle. In HCT-116 cells, the mean 50% inhibitory concentration (IC<sub>50</sub>) for NI3C and I3C was 35.3  $\mu$ M ( $\pm$  4.8  $\mu$ M) and 250  $\mu$ M ( $\pm$  67  $\mu$ M), respectively. In the DLD-1 cells, the mean IC<sub>50</sub> values for NI3C and I3C were 25.9  $\mu$ M ( $\pm$  3.8  $\mu$ M) and 270  $\mu$ M ( $\pm$  38  $\mu$ M), respectively. These data show that NI3C is a 7 to 10 fold more potent growth inhibitor compared with I3C.

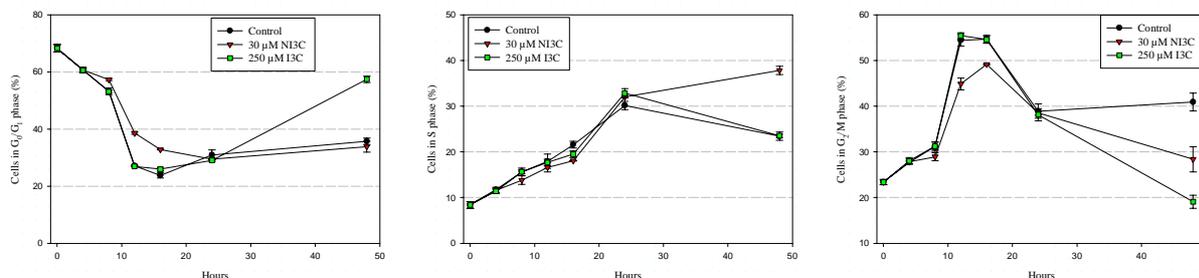
### *Effects on cell cycle*

The effect of NI3C on cell cycle distribution was analyzed using cell flow cytometry. Two different experimental set-up's have been used: i) Using asynchronous HCT-116 cells, we have determined how the exposure to the indoles for 48 h change the cell cycle phase distribution. ii) When HCT-116 cells were grown with low serum concentration for 72 h, 70% of the cells were accumulated in G<sub>0</sub>/G<sub>1</sub> phase (synchronized). Addition of serum initiates the cells to run through the cell cycle phases (G<sub>1</sub>  $\rightarrow$  S  $\rightarrow$  G<sub>2</sub>  $\rightarrow$  M  $\rightarrow$  G<sub>1</sub>) as one wave. The indoles were added simultaneous with the serum and a possible retardation may be observed. Following 48 hours exposure of asynchronous HCT-116 cells to 30  $\mu$ M NI3C, a significant increase in the number of cells was observed in the G<sub>2</sub>/M phase (27 % in the NI3C treated cells compared to 22 % in the control cells, Figure 1). In comparison, 250  $\mu$ M I3C significantly accumulate the cells in G<sub>0</sub>/G<sub>1</sub> phase (55 % compared with 32 % in control cells).



**Figure 1:** NI3C and I3C accumulate cells in G<sub>2</sub>/M and G<sub>0</sub>/G<sub>1</sub> phase, respectively. HCT 116 cells were exposed to NI3C (15 or 30  $\mu$ M) or I3C (150 or 250  $\mu$ M) for 48 h. The cell cycle phase distribution was determined using flow cytometry.

Synchronized HCT-116 cells were exposed to 0, 30, or 250  $\mu\text{M}$  I3C for up to 48 hr. Figure 2 shows that 30  $\mu\text{M}$  NI3C retards the cells in  $G_0/G_1$  phase compared to cells treated with the vehicle or 250  $\mu\text{M}$  I3C, which immediately move into the S phase after addition of serum. On the other hand, 250  $\mu\text{M}$  I3C causes an accumulation in the  $G_0/G_1$  phase after 48 hr of exposure (57 % compared to 36 % of control cells), whereas 30  $\mu\text{M}$  NI3C causes an accumulation of cells in  $G_2/M$  phase (37 % compared with 23 % in control cells).



**Figure 2:** NI3C delay the  $G_1 \rightarrow S$  transversion, and NI3C and I3C accumulate cells in  $G_2/M$  and  $G_0/G_1$  phase, respectively. HCT 116 cells were synchronized by growing the cells with low serum for 72 h. Then the cells were changed to normal serum and exposed to 30  $\mu\text{M}$  NI3C or 250  $\mu\text{M}$  I3C for 48 h. The cell cycle phase distribution was determined using flow cytometry.

### *Expression of genes regulating the cell cycle*

To understand the mechanism by which the indoles regulate cell division, the expression of several cell cycle related genes were analyzed during (synchronized cells) or after a 48 h (asynchronous cells) exposure to NI3C or I3C. The genes analyzed were: i) cyclins (Cyclin A2, B1, D1 and E1), ii) CDK's (CDK1, CDK2, CDK4 and CDK6) and iii) CDK inhibitors (p21 and p27). Total RNA was isolated from HCT-116 cells treated with 0, 15, or 30  $\mu\text{M}$  NI3C, or 150 or 250  $\mu\text{M}$  I3C for 48 hr, and the expression levels were investigated using northern blotting, normalized to the 18S rRNA levels.

In general, exposure to NI3C or I3C for 48 hr reduced the levels of cyclins, in contrast to cyclin E1, which was induced. The level of CDK mRNA's were in general reduced, whereas CDK6 mRNA levels were unchanged. The p27 expression was reduced by I3C and NI3C.

The short-term effects of the indoles on the expression of 4 genes (p21, CDK4, cyclin E1 and CDK1) were determined in synchronous HCT-116 cells. The p21 and CDK4 levels were increased beyond 8h and 16h, respectively, in cells exposed to NI3C. I3C induced the p21 expression after 24 h of exposure, but reduced the CDK1 level at 24 h and cyclin E1 at 8 h.

### **Discussion**

In contrast to the numerous papers showing different biological responses of I3C, only very few papers have focused on the biological activities of NI3C. This paper is the first one, showing that NI3C inhibits cellular growth, and that in human colon cells NI3C is a more

potent inhibitor than I3C. Preliminary data indicate that this may also be the case in MCF-7 cells.

The mean  $IC_{50}$  for I3C was 250-270  $\mu$ M in the human colon cells analyzed (HCT-116 and DLD-1 cells). These levels are somewhat higher than observed for other cell types. In MCF-7 cells, the thymidine incorporation was reduced to 50% by 30  $\mu$ M I3C (Cover *et al.* (1998)) and only 30  $\mu$ M I3C was required to reduce the cell numbers of human prostate cells to 80% at 48 hr (Chinni *et al.* (2001)). It is still unclear, whether I3C or products of I3C formed in the medium during the incubation are the active compounds. LTr-1 is one of the products formed from I3C in an acid environment, and 20  $\mu$ M and 25  $\mu$ M LTr-1 inhibit the cell number by 50 %, of MDA and MCF-7 cells, respectively (Chang *et al.* (1999)).

In the present experiment an accumulation of cells in G<sub>2</sub>/M phase was observed, when exposed to NI3C. This is in contrast to the observed accumulation in G<sub>0</sub>/G<sub>1</sub>, when human colon cells were exposed to I3C. A similar accumulation in G<sub>0</sub>/G<sub>1</sub> phase was previously observed in MCF-7 cells (Cover *et al.* (1998)), and human prostate cells (Chinni *et al.* (2001)). The delayed transition from G<sub>1</sub> to the S phase by NI3C indicate a transient inhibition of the G<sub>1</sub>→S transition.

Both NI3C and I3C enhance the expression of cyclin E1, which are involved in the G<sub>1</sub>→S transition, and reduce the cyclin A2 expression, maybe because of the reduced level of CDK2 and 4. IN contrast, a previous report showed a significant reduced CDK 6 protein following exposure to I3C was observed (Cover *et al.* (1999)). Of the CDK inhibitors, we have observed maximal p21 expression shortly after addition of NI3C. The different effect of NI3C and I3C on cell cycle distribution and the various genes related to cell cycle indicate that the growth inhibition by NI3C and I3C run via different mechanisms.

The anticarcinogenic response of I3C has previously been linked to the modulation of cytochrome P-450 1A enzymes via the Ah receptor or inhibition of the enzyme activity, but the growth inhibition by I3C is Ah receptor independent (Cover *et al.* (1999)). NI3C induce CYP1A activity (Stephensen *et al.* (2000)), but the growth inhibition by NI3C is not caused by the Ah receptor, as only DLD-1 but not HCT-116 cells do show CYP1A inducible activity.

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## **MODEL SYSTEM OF DIFFERENTIATED MUSCLE-CELLS (MYOTUBE CULTURES) FOR STUDYING QUENCHING OF RADICAL OXYGEN SPECIES**

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### **Abstract**

Human studies on various effects of naturally occurring health promoting compounds e.g. antioxidants often result in limited effects because of limitations in both dosage, duration and severity of the stress imposed on the subjects. We present a model-system of myotube cultures (primitive muscle fibres) established from a mouse C2C12 myoblast line, for studying quenching of radical oxygen species (ROS). Stress is induced on the myotubes by anoxia or hypotonic shock which is accompanied by cell swelling and a concomitant activation of osmolyte-release, determined by [ $^{14}\text{C}$ ] taurin efflux. Simultaneously an increase in ROS is observed, which may be reduced in the presence of unspecific antioxidants. In this model system we found a decreased stress induced [ $^{14}\text{C}$ ] taurin efflux and ROS production in the presence of green tea extract and the pure compound epigallo-catechin-gallat (EGCG) which is the quantitatively most predominant catechin in tea.

It is suggested that this myotube culture system may be used as a model for screening various compounds/mixtures capable of quenching radical oxygen species within muscle tissue.

Keywords: Myotubes, antioxidants, tea, screening.

### **Introduction**

Epidemiological studies indicate a number of beneficial health effects of increased intake of fruit and vegetables, and a major group of antioxidative compounds in fruit and vegetables, the polyphenols, has been suggested to contribute to these beneficial effects (Hertog *et al.*, 1995). Studies on various effects of naturally occurring antioxidants often result in limited effects (Cherubini *et al.*, 1999) because of limitations in both dosage, duration and severity of the stress imposed on the subjects. Humans at rest with no nutritional deprivation has a very well balanced antioxidative system, and in order to increase the chances of revealing effects of naturally occurring compounds several studies have either depleted the antioxidant intake before intervention and throughout the experimental period (Young *et al.*, 2002) or tried to increase the stress e.g. using smokers (Princen *et al.*, 1998; Young *et al.*, 2002). Also long-duration or intense physical exercise is assumed to increase the stress on the muscle (Alessio

*et al.*, 1993), and increased levels of radicals and lipid peroxidation products (Ashton *et al.*, 1998) has been reported in human experiments. The exercise-induced stress could be either due to increased oxygen intake (Alessio *et al.*, 1993), or due to local anoxia within the muscle cells. The aim of the present study was to set up a model system of myotube cultures suitable for screening a range of compounds capable of quenching radical oxygen species. Stress was induced on the myotubes by anoxia or hypotonic shock resulting in ROS production and [ $^{14}\text{C}$ ] taurin efflux.

## Materials and Methods

Myotube cultures were established from a mouse myoblast line (C2C12) from the American type culture collection, as described by (Oksbjerg *et al.*, 2000). For measuring taurin efflux cultures were loaded with [ $^{14}\text{C}$ ] taurin (10 nCi/ml) for 24 h at 37 °C and for measuring reactive oxygen species cultures were loaded with 2',7', dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) (10 μM) for 2 h at 37 °C. Cells were washed with isotonic Krebs-Hepes buffer and incubated with either hypotonic Krebs-Hepes buffer (2½ h for ROS determination and 30 min for taurin release) or isotonic Krebs-Hepes buffer in an argon atmosphere (2 h for determination of taurin release) in the presence of antioxidants. [ $^{14}\text{C}$ ] Taurin efflux was determined as disintegrations per minute (dpm) and intracellular ROS was determined by fluorescence from H<sub>2</sub>DCFDA (excitation: 490 nm, emission: 515 nm).

## Results and Discussion

Experiments on rats have shown that pure catechins are absorbed and transported to the tissues, and in human subjects catechins have been found in plasma (Nakagawa *et al.*, 1997), indicating absorption of the compounds. Catechins are thus present in the blood vessels and possibly in the interstitial fluid after ingestion of catechin rich foods or drinks. The myotube culture model presented in this work simulate the effect of antioxidants in the interstitial fluid. Addition of BHT, α-tocopherol, green tea extract, the pure compound epigallo-catechin-gallat (EGCG) which is the quantitatively most predominant catechin in tea decreased hypotonic stress induced ROS production in the cells (Figure, A). It has been suggested that anoxia itself, cell swelling, and free radicals activates phospholipase A<sub>2</sub> which in turn increases the production of reactive oxygen species (ROS) and 5-lipoxygenase products causing osmolyte-release (Lambert *et al.*, 2002). Osmolyte release was determined by efflux of [ $^{14}\text{C}$ ] taurin, and was reduced in the presence of both green tea extract and EGCG upon hypotonic induced stress (Figure, B). The more lipid-soluble antioxidants BHT and α-tocopherol reduced ROS over a 2 h time-interval, but reduced the taurin release to a lesser degree when exposed to the cells for only 30 min (Figure, B). However, when the antioxidants were pre-incubated with the cells for 24 h both BHT and α-tocopherol reduced taurin release (data not shown). Anoxia induced taurin release was also reduced by both tea extract and EGCG, as

well as BHT, whereas  $\alpha$ -tocopherol only had a minor effect. At the highest concentrations used of BHT and  $\alpha$ -tocopherol the protective effect against ROS production was reduced probably due to decreased solubility or a biphasic action (A). Both tea extract and EGCG seemed to be toxic at very high concentrations over prolonged exposure (2h) (C) since taurin efflux increased. The experiments presented on C2C12 myotubes are at present being carried out on primary cells isolated from *m. semi membranosus* of ca. 7 week old pigs.

## Conclusion

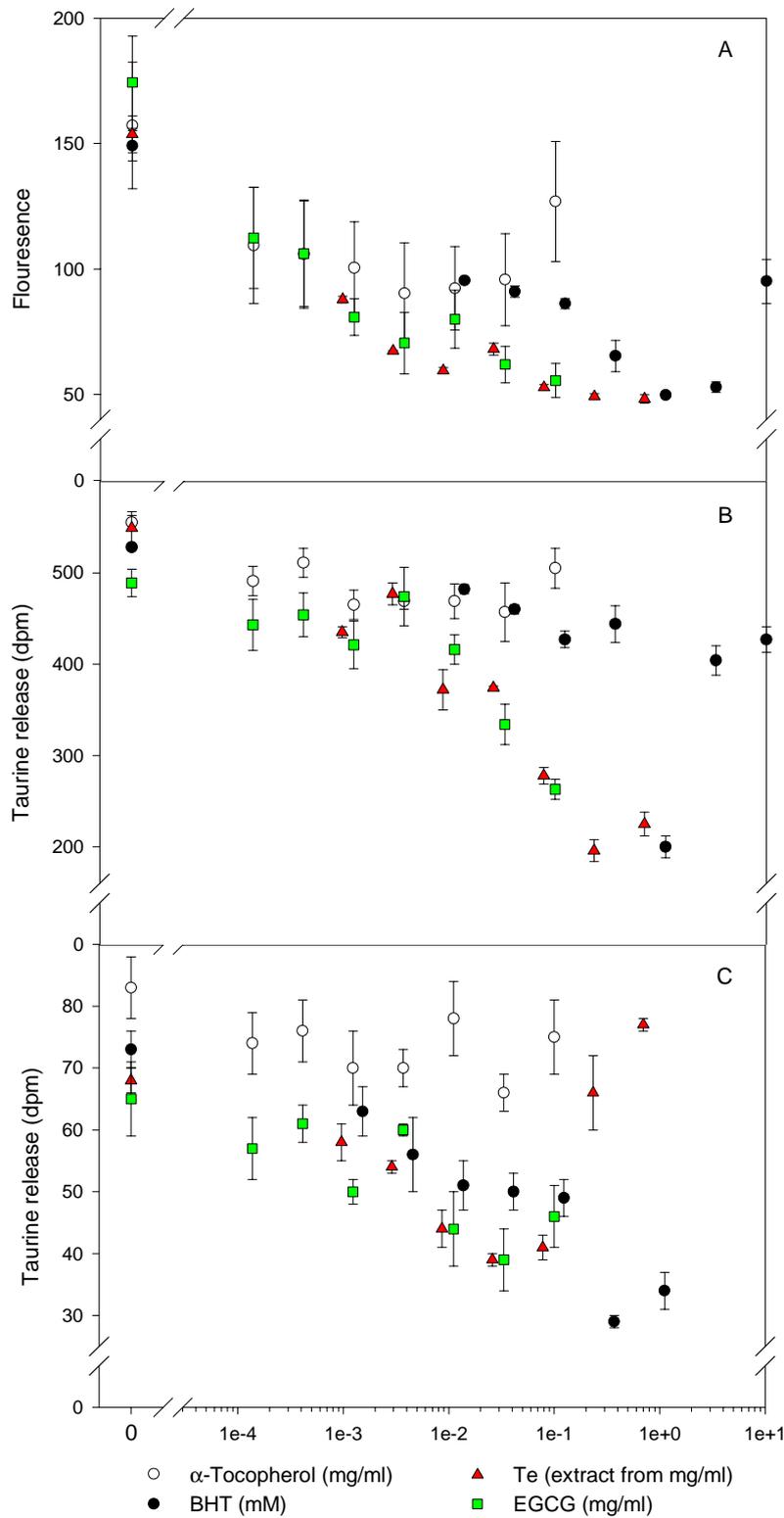
In a myotube culture system we found a decreased stress induced [ $^{14}$ C] taurin efflux and ROS production in the presence of green tea extract and the pure compound epigallo-catechin-gallat which is the quantitatively most predominant catechin in tea. It is suggested that this myotube culture system may be used as a model for screening various compounds/mixtures separately or in combination (antagonism or synergism) capable of quenching radical oxygen species within muscle tissue.

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**Figure.** Intracellular ROS from myotubes exposed to hypotonic shock for 2½ h(A), determined as fluorescence from H<sub>2</sub>DCFDA (excitation: 490 nm, emission: 515 nm) and [<sup>14</sup>C] Taurin efflux from myotubes exposed to hypotonic shock for 30 min (B) and anoxia for 2 h (C) determined as disintegrations per minute (dpm) (B). Effects of  $\alpha$ -tocopherol, BHT, tea extract and EGCG.

## QUANTIFICATION OF POLYACETYLENES BY LC-MS IN HUMAN PLASMA AFTER INTAKE OF FRESH CARROT JUICE (*DAUCUS CAROTA* L.)

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Epidemiological investigations indicate that a high intake of fruits and vegetables protect against cancer and cardiovascular diseases [1, 2]. High intake of vegetables is associated with decreased risk of certain cancers but  $\beta$ -carotene, the orange pigment in carrots, which is a potent antioxidant, has failed to reduce incidence of cancer in a human intervention study [3]. However, carrots also contain other bioactive components such as polyacetylenes. Falcarinol, the most bioactive of the carrot polyacetylenes, has been shown to have a pronounced cytotoxic activity against several human tumour cell lines *in vitro* [4, 5] and preliminary *in vivo* evaluation of the cytotoxic activity of falcarinol have also demonstrated its potential for *in vivo* antitumour activity [5]. Hence, falcarinol could be one of the active components that may explain the health promoting properties of vegetables.

The bioavailability of falcarinol in human subjects was investigated in a pilot study. A breakfast meal was ingested consisting of 800 ml carrot juice containing 28 mg falcarinol and standardized amounts of bread and butter. Subjects excluded all food items containing falcarinol from their diets from one week before the test-day until the last blood samples were drawn 18 days later. Blood samples were drawn just before the test meal (after a 12 h fast) and regularly thereafter. Blood samples were centrifuged at 4 °C and plasma-aliquots were stored in dark tubes under nitrogen at -80 °C until analysis by LC-MS.

For the quantification of falcarinol and related polyacetylenes in plasma samples a sensitive LC-MS method was developed. Plasma proteins were precipitated by addition of 2 parts of acetonitril to one part plasma. The extracted polyacetylenes were analysed on a Quattro LC LC/MS system using a reversed phase C18 column (50 mm  $\times$  2.6 mm ID) and a binary gradient solvent system consisting of 0.1% formic acid in water and acetonitril. The MS system was operated in the electro-spray positive mode. Falcarinol and falcarindiol were detected using an MRM method with argon as collision gas. The detection limit was 0.5 ng/ml plasma for both compounds. The concentration of falcarinol after intake of carrot juice was in the range of 6–15 ng/ml plasma. This is the first investigation to show the bioavailability of highly bioactive polyacetylenes.

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## **COMPANY PROFILE OF DANISH TOXICOLOGY CENTRE (DTC)**

### **Documented Safety for novel foods and food supplements**

In the European Union, novel foods include completely new foods and food components as well as conventional foods produced by new processes. New foods are e.g. plants or plant constituents that have never been consumed in a significant amount before May 1997 in the EU. EU legislation stipulates that the nutritional, microbiological, and toxicological properties of the food or food component must be evaluated, and a formalised risk analysis of the effects of the food on human health must also be included in the product dossier.

DTC has specialists in molecular biology, nutrition, food toxicology, medical microbiology, and microbial ecology, as well as in food legislation - all working together on novel foods and under one roof. In addition, these specialists are all familiar with the food industry. All this ensures the preparation of stringent and uniform scientific documents - carried out by a part independent of the food company.

DTC has routine in interpreting the EU law complex that regulates novel foods, and is acquainted with the present attitudes and administrative practices of the various national and international authorities, such as the EU and Codex Alimentarius. DTC is well worth consulting at the early stages of the development of novel foods. The DTC-staff is experienced both in food-grade R&D strategies and in the safety evaluation of bioindustrial processes. These consultations can support industry at critical decision steps in development strategies. Early consultations can secure that unfortunate consequences in the finished product do not appear, e.g. ill-conceived cloning strategies or the presence of undesirable secondary fermentation metabolites.

### **The DTC in general**

The Danish Toxicology Centre (DTC) is a self-governing, non-profit, independent technological service institute founded in 1982 and affiliated to the Danish Academy of Technical Sciences (ATV). Since 1986, DTC has been approved by the Danish Minister of Business and Industry as a technological service institute (GTS-institute).

The basis of DTC's activities is to provide independent toxicological expertise to industry, trade and society. The prime objective is to generate, assess, gather, evaluate and communicate information regarding the effects of substances and materials on humans and the environment with the purpose of eliminating or reducing undesired effects.

DTC's hallmark and strength is the toxicological expertise combined with a knowledge of the needs of the industrial sector, the legislative requirements related to chemical substances and a working relationship with the competent authorities.

DTC provides technological service in the following main fields:

- Risk assessment including evaluation of exposure to substances, products and materials.
- Risk assessment of pollution in relation to humans and the environment.
- Preparation of documentation, such as toxicological assessments, criteria documents or expert reports on substances and materials, *e.g.* pharmaceuticals and pesticides. Registration or notification of substances, pharmaceuticals and pesticides.
- Planning, monitoring and interpretation of toxicological and physical-chemical laboratory tests and animal studies.
- Chemical management. Classification, labelling and preparation of safety data sheets, issued in the relevant languages in accordance with the legislative requirements of the European Union and EFTA countries.

Because of many international customers, DTC carries out monitoring of the legislation, in particular on chemicals in the European Union and EFTA-countries.

The question of maintaining confidentiality of information received from clients from the private sector as well as from the authorities is a very important issue for DTC. A wide range of technical, administrative and personnel security routines are established and enforced on a daily basis. On request, DTC provides a confidentiality statement to its clients.

The DTC staff comprises more than 50 highly qualified persons with long time experience in the field. All academic staff members have obtained Masters or Ph.D. degrees in subjects relevant to toxicology, inter alia biology, food science, chemistry, engineering, pharmacy and veterinary science. The working languages of DTC are Danish and English. Furthermore, a considerable number of the staff members master German, French, Spanish, Swedish, Russian, Polish and Chinese. DTC has a proven ability in submitting reports in English.

As it is DTC's main task to obtain and assess scientific information, it was necessary for DTC to build up a library specialised in toxicology and related sciences. The library is continuously kept up-to-date by acquisition of books and publications. A full-time research librarian, proving the given high priority of the task, manages the library.

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