Anthocyanins – more than nature's colours...

Proceedings

Edited by

Izabela Konczak
Wei Zhang

Editors Note
Texts were submitted electronically and subjected to perfunctory editorial tasks. Authors should be considered responsible for the presented concepts, data and conclusions.
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Preface

“Let food be the medicine and medicine be the food” stated the father of medicine, Hippocrates (460–377BC), over two thousand years ago. This statement is equally valid today: preventative healthcare through a well-balanced diet is the primary objective of many national and international research programs.

Among bioactive phytochemicals with a safe history of human consumption are anthocyanins: water-soluble pigments almost ubiquitous to the plant kingdom, responsible for the red to purple to blue colours of fruits, vegetables, cereal grains, and flowers. Accumulated in plants frequently in response to environmental stress, anthocyanins are responsible for photo-protection against light-induced photo-oxidation and UV-B damage and regulation of drought and cold resistance through adjustment of the osmotic pressure of vacuole content. Perhaps because of their ability to impact colour to the plant or plant product in which they are present, but more certainly because of their pharmacological properties, anthocyanins were incorporated into herbal folk medicines and food products.

Recent research data reports an array of health-beneficial effects arising from the consumption of anthocyanin-rich products and indicates new areas of their potential application in promoting good health. Anthocyanin-rich fruit and vegetable extracts evaluated in both in vitro and in vivo experimental systems demonstrated protection against oxidative damage caused by free radicals. Anthocyanins have been reported to provide protection against liver injuries and UV radiation, significantly reduce blood pressure, improve eyesight, exhibit strong anti-inflammatory and antimicrobial activities, inhibit reversed mutation caused by cooked food mutagens, and suppress proliferation of human cancer cells. Due to their wide physiological activities, anthocyanins may play a significant role in preventing life-style related diseases such as cancer, diabetes, and cardiovascular and neurological diseases. These qualities of anthocyanins have prompted researchers and the food industry towards the development of “anthocyanin-tailored” functional food products, which would deliver anthocyanins as nutraceuticals with specific health benefits.

Application of biotechnology to engineer the anthocyanin biosynthetic pathway in order to produce the desired anthocyanins as a natural food colorant with specific colour qualities, increased stability, and/or health-promotive qualities, may create new opportunities for a commercial production of selected food colorants and/or nutraceuticals in a factory setting. Biotechnology application for development of novel ornamental plants with increased decorative characteristics or with enhanced disease- or stress-resistance through manipulation of anthocyanin biosynthetic pathway is another area of anthocyanin research with a commercial focus.

We hope that scientific presentations enriched with displays of anthocyanin-containing food products by Australian and international companies as well as debates among researchers and industry managers at IWA2004 will contribute towards further understanding of the benefits that application of anthocyanins in food products can bring and their efficient utilization.

Dr Izabela Konczak
Chair, IWA2004
Workshop organizers

Cooperative Research Centre for Bioproducts

The CRC for Bioproducts, a collaborative research venture of Albright Wilson Ltd., Melbourne University, Food Science Australia, Flinders University and New Zealand Crop & Food Research Ltd., was among the first 15 CRC’s set up in Australia in 1991 under The Cooperative Research Centres (CRC) Programme.

The programme aims to bring together researchers and research users: academia, government labs and industry, to emphasise the importance of collaborative arrangements and to maximise the benefits of research through an enhanced process of utilisation, commercialisation and technology transfer. It also has a strong education component with a focus on producing graduates with skills relevant to industry needs.

The CRC for Bioproducts has been established as a centre of Biotechnological Innovation, using fundamental knowledge to support commercial application. The areas of our key capabilities are: fermentation technology (principally plant cell cultures), extraction of valuable bioproducts from agricultural wastes, commercial application of biopolymers, supported by a fundamental understanding of their structure-function relationship.

For more information about the CRC for Bioproducts visit http://www.bioproducts.org.au/.

Food Science Australia

With its 300-strong team and world-class facilities, Food Science Australia is a major long-term contributor to the prosperity of the Australian food processing industry. Our focus is to develop innovative food processes and safe, value-added food products to help make Australian food companies among the most competitive in the world.

Food Science Australia’s researchers solve today’s problems by applying existing knowledge professionally and creatively. We focus on the needs of our customers – whether in Australia or overseas – and add value to their business.

Food Science Australia’s people provide research in the areas of integrated food safety; food processing and sensing; ingredient functionality; fermentations (cheese and plant cell culture); emerging food processing technologies; and food storage, distribution and packaging.

Specialised facilities and technical advice are available from each of Food Science Australia’s three sites in Sydney, Melbourne and Brisbane. Research teams from these sites work together to provide a coordinated approach to servicing food industry businesses.

To find out more about these services or other areas of Food Science Australia visit www.foodscience.afisc.csiro.au.
Workshop sponsors

*We acknowledge the financial assistance from the following organizations:*

Sensient Technologies Australia

Sensient Technologies is the world's leading supplier of flavours, fragrances and colours used to make a diverse variety of foods and beverages, pharmaceuticals, cosmetics, home and personal care products, specialty printing and imaging products, computer imaging and industrial colours. Our name communicates what we do: Enhance SENSory experiences through specialised ingreIENTs, delivered through proprietary TECHNOLOGIES. In short, we bring life to products you enjoy using everyday.

Kingfood Australia Pty Ltd

Kingfood Australia has been established 18 years ago and is a reliable supplier of various ingredients such as natural colours, juice concentrates, natural dairy ingredients, specialty starches, vegetable extracts, seafood extracts, yeast extracts, hydrocolloids, coconut cream powder, dehydrated fruits and vegetables and others to the food manufacturing industry. The company has established systems to handle shipping, financing, storage, banking, transport. Special care is taken about the quality and regulatory issues that are important to the customers. Kingfood is able to source and supply ingredients from any part of the world. The company is able to provide customers with full data on ingredients - including specifications, GM Status, Allergens, Certificates of Analysis etc. Kingfood prides itself on its high quality standards and in ensuring ethical and confidential relationships with customers and suppliers at all times. Kingfood deals with many major international food corporations and has a comprehensive understanding of what food technologists want and provides tailored services according to their needs.

Wild is one of the world's leading suppliers of natural flavour ingredients to the food and beverage industry and the largest privately owned flavour ingredient company in the world - with production facilities both in Europe and North America. The technology used to develop and make Wild Natural Colours sets new standards for the production of natural colour extracts and food colourings. Ultra-modern technology and experience in the global sourcing of natural ingredients ensures that all products are produced to the highest standards in the industry. Under the trademark 'Colours From Nature', Wild provides natural colours which are stable, intense and highly concentrated. All colours are available as water or oil soluble liquids and powders and can be customised to meet your specific requirements.

Nutrinova (Australasia) Pty Ltd is the exclusive distribution agent for the Wild group in Australia. The company is regarded as one of the leading food ingredient companies in Australia with expert experience in all aspects of the food and beverage sectors. It has the technical resources and facilities to customise your exact flavour and colour blends as well as provide functional ingredients and technology from their international partners.
Scientific program committee

Dr Izabela Konczak, Chair, CRC for Bioproducts/Food Science Australia, Australia
Prof. Mary Ann Lila, University of Illinois, Urbana, USA
Prof. Peter Olesen, Chr. Hansen, Denmark
Prof. Kazuki Saito, Chiba University, Japan
A/Prof. De-Xing Hou, Kagoshima University, Japan
Prof. Raymond Brouillard, Université Strasbourg, France
Dr François Cormier, Colarome Inc., Canada
A/Prof. Erich Grotewold, Ohio State University, USA
Dr Wei Zhang, CRC for Bioproducts/Flinders University, Australia
A/Prof. Markus Herderich, The Australian Wine Research Institute, Australia
A/Prof. Chris Franco, CRC for Bioproducts/Flinders University, Australia
Dr Kevin Davies, Crop & Food Research Ltd., New Zealand
Dr Philip A. Franks, CRC for Bioproducts/Food Science Australia
Dr Richard Thwaities, CRC for Bioproducts/Tridian-Albright & Wilson, Australia
Dr Kathy Schwinn, Crop & Food Research Ltd., New Zealand

Local organising committee

Dr Izabela Konczak, Chair, CRC for Bioproducts/Food Science Australia
Dr Philip A. Franks, CRC for Bioproducts/Food Science Australia
Ms Shalini Jayram, CRC for Bioproducts/Food Science Australia
Ms Naomi Dittmar, CRC for Bioproducts/Food Science Australia
Dr Richard Thwaities, CRC for Bioproducts/Tridian-Albright & Wilson, Australia
Dr Michael Patane, Protech Research Pty Ltd
Ms Alyssa Hannaford, CRC for Bioproducts/Food Science Australia
Ms Kay Middleton, CRC for Bioproducts/Food Science Australia
Prof. Mary Ann Lila  
**Natural Resources & Environmental Sciences**  
**Assistant Dean for Research, College of ACES**  
**University of Illinois, USA**

Dr. Mary Ann Lila has been a Professor at the University of Illinois for nearly 20 years. She is a member of the Department of Natural Resources & Environmental Sciences, the Division of Nutritional Sciences, and the Functional Foods for Health Program. Dr. Lila’s research team is focused on the isolation and characterization of bioactive components from highly pigmented fruits and their *in vitro* cell culture systems. She uses the cell culture models to rapidly, predictably accumulate the same phytomedicinal mixtures produced in the parent plants, but without many of the interfering compounds that complicate recovery of active principles from plants. Most recently her lab team has accomplished effective 14C labelling of bioactive anthocyanins, proanthocyanidins, and other flavonoid compounds from intensively-managed berry and grape cell cultures. The radiolabeled isolated compounds are then used in animal models to track the metabolic fate of certain phytochemicals.

Dr. Lila has won several research awards including the Paul A Funk Scholarship Recognition award (the premier research award in her college), the Faculty Award for Excellence in Research, the University Scholar award, the Amoco Award for Excellence in Undergraduate Instruction, and the Lilly Teaching Fellowship. She served as the US correspondent for the International Association of Plant Tissue Culture and Biotechnology (1994-1998), the President of the Society for In Vitro Biology (2000-2002), and most recently was elected as a Fellow of the Society for In Vitro Biology (2003). Dr. Lila won a Fulbright Senior Scholar award to live and study in New Zealand in 1999, and has been back to Australasia at least once a year since then.

Prof. Taiji Adachi  
**Graduate School of Agriculture & Biological Sciences**  
**Osaka Prefecture University, Japan**

Dr. Taiji Adachi was a Professor at Miyazaki University for 30 years. Since 1999 he has been a Professor at the Osaka Prefecture University. For his outstanding contributions to the MU, he was awarded a of “Professor Emeritus” title, even though he is still not yet the age of retirement. At MU he established a Department of Applied Genetics and Biotechnology where he focused on application of biotechnology to the utilization of plant bio-resources, namely agricultural crops and its related species. One of the directions of his research team was improvement of the flower colour. Prof. Adachi has organized many international academic meeting not only in Japan, but also abroad.

A double recipient of an award from the Alexander von Humboldt Foundation: in 1973/74 and in 1880 as a senior fellow, Prof Adachi has continued ever since to work closely with German scientists. Dr. Adachi has won several research awards including The Prize of Culture, Miyazaki Prefecture and Prize for Academic Contribution of Buckwheat Research in 1998. His is a co-author of over 120 original research papers and several books in the area of plant biotechnology.

With over 30 years of experience in the area of plant biotechnology, he recently devoted himself to organizing an international colloquium on the application of plant biotechnology for further progress in productive and sustainable agriculture. It recognizes the possibility food genetic technology may have no future at all if the scientific community cannot convince the consumers of its benefits.
Key Note and Plenary Speakers

Prof. Kazuki Saito
Graduate School of Pharmaceutical Sciences
Chiba University, Japan

Dr. Kazuki Saito was born in Nagano, Japan. He graduated from the Faculty of Pharmaceutical Sciences, the University of Tokyo, Japan in 1977 and then obtained his PhD for biochemistry of pharmaceutical sciences from the University of Tokyo in 1982. After staying in Keio University in Japan and Ghent University in Belgium (Prof. Marc Van Montagu’s laboratory), he has been appointed as full professor since 1995 at the Graduate School of Pharmaceutical Sciences, Chiba University, Japan. He has also been a Special Guest Researcher in Kazusa DNA Research Institute in Kisarazu, Japan since 2002.

Prof. Kazuki has published more than 140 original papers and 50 invited reviews and book chapters. His research interests are biochemistry, molecular biology and biotechnology of primary and secondary metabolism, in particular flavonoids and alkaloids, in plants. More recently he started a research program on systems biology focused on metabolomics for global understanding of plant metabolism. He is also interested in music, movies, detective novels and Bon-Sai.

Prof. Marina Heinonen
Department of Applied Chemistry and Microbiology
University of Helsinki, Finland

Dr. Marina Heinonen was appointed professor (functional foods) in August 2002, which is a newly established chair at the University of Helsinki. She has previous experience in food chemistry and teaching at the University of Helsinki for nearly 20 years with two year-long sabbatical visits to University of California, Davis (1996) and University of Rhode Island, USA (1987-1988). Prof. Heinonen’s research team is currently focusing on the functional properties of flavonoids and other phenolic compounds in fruits, berries, oilseeds, cereals, vegetables, and other natural sources. The international research collaboration recently included an EU funded research project on “anthocyanin bioactivities” (QLRT-1999-001224) that was completed in 2003. The achievements of this project include a new insight into the significance of anthocyanins as bioactive compounds with relevance to human health.

Prof. Heinonen’s research team is experienced in investigations of the action of natural antioxidants such as phenolic compounds. Different antioxidant model systems ranging from food lipids, emulsion and meat model systems to other types of model systems such as liposomes and LDL-particles are being used to reveal the best applications in developing functional foods or other types of bioactive products such as cosmetic products. Research on functional properties also includes anthocyanin copigmentation reactions. The research team is also using the most sophisticated laboratory tools for characterization of the phenolic rich raw materials. One of the highlights of the research was the appraisal of one publication (J. Agric. Food Chem., 1999, 47, 3954-3962 “Antioxidant activity of plant extracts containing phenolic compounds”) for being the most cited paper within this area of research.
Key Note and Plenary Speakers

A/Prof. De-Xing Hou
Department of Biochemical Science and Technology
Kagoshima University, Japan

Dr. Hou is an associate professor at Department of Biochemical Science and Technology, Kagoshima University of Japan. He received a PhD degree in biochemistry from Kagoshima University of Japan in 1991, subsequently undertaking postdoctoral studies at The Institute of Physical and Chemical Research (RIKEN) until 1997. His recent research focuses on the molecular mechanism of carcinogenesis and cancer prevention by natural compounds and food factors. He is the author of several papers in the highest-ranking journals such as Nature, Nature Genetics and EMBO Journal and a Leader of international research teams including Australia, China, USA, Japan and Korea. He is also an active consultant with several companies in Japan in the area of functional food processing.

A/Prof. Kevin Gould
School of Biological Sciences, University of Auckland, New Zealand

Dr. Gould is a senior lecturer in Plant Sciences at Auckland University. He gained a PhD in Botany at Manchester University, and studied for two years as a postdoctorate fellow at the University of California, Riverside, before moving to New Zealand 16 years ago. His research focuses on the development and ecophysiology of native New Zealand plants, particularly under harsh abiotic environments. Dr Gould kindled a fascination with the anthocyanins during a sabbatical in Prof. David Lee’s laboratory at Florida International University, in a study of red- and green-leaved plants from the understory of Malaysian rainforests. His research team subsequently advanced the hypothesis that anthocyanins serve to protect plants from the effects of oxidative stress, both by reducing the quantum flux incident on chloroplasts, and by scavenging reactive oxygen species.
### Workshop Program

**Third International Workshop on Anthocyanins, January 27-29, 2004**

**Program Schedule**

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**Tuesday, January 27, 2004**

**Opening Session**

**Chair:** Izabela Konczak, CRC for Bioproducts, Food Science Australia

16.30 – 16.45  
**Welcome address:** Doug Hawley, Cooperative Research Centre for Bioproducts, Australia

16.45 – 17.30  
**Key Note Lecture:** Mary A. Lila, University of Illinois, USA  
Bioactivity of anthocyanins and importance to human health

18.00 – 20.00  
**Welcome Reception** hosted by Doug Hawley, Cooperative Research Centre for Bioproducts, Australia
Wednesday, January 28, 2004

Session I: Development of anthocyanin-based functional foods

Chair: Peter Olesen, Christian Hansen, Denmark

8.30 – 9.10  Plenary lecture: Marina Heinonen, University of Helsinki, Finland
Anthocyanin Bioactivities: Outcome of an European Collaboration (QLK1-1999-00124)

9.10 – 9.30  Kaarina Viljanen, University of Helsinki, Finland
Antioxidant activity of anthocyanins in different food models

9.30 – 9.50  Dulce M. Antunes, Universidade do Algarve, Portugal
The effect of different postharvest treatments on anthocyanin content of ‘Assaria’
pomegranate (Punica granatum L.) fruit during storage

10.00 – 10.30  Morning Tea

10.30 – 11.00  Poster session
Display of anthocyanin containing products

11.00 – 11.20  Maarit J. Eiro, University of Helsinki, Finland
Stability and enhancement of berry juice colour

11.20 – 11.40  Roland Bitsch, Friedrich-Schiller-University, Jena, Germany
Bioavailability and biokinetics of anthocyanins from red grape (Vitis Vinifera L.) juices and
red wine

11.40 – 12.00  Peter Abbott, Food Standards Australia New Zealand
Food regulations and functional foods in Australia and New Zealand

12.00 – 13.30  Lunch

Session II: Health-beneficial properties of anthocyanins

Chair: Mary A. Lila, University of Illinois, USA

13.30 – 14.10  Plenary lecture: De-Xing Hou, Kagoshima University, Japan
Do anthocyanins contribute to cancer prevention? – Introduction to molecular evidence

14.10 – 14.30  Dilip Ghosh, The Horticulture and Food Research Institute of New Zealand
Ltd., New Zealand
Anthocyanins in biology and medicine: biochemical, cellular and medicinal properties

14.30 – 14.50  Peter Clifton, CSIRO Health Sciences and Nutrition, Adelaide, Australia
Effect of grape (Vitis vinifera L.) seed extract on endothelial function.

15.00 – 15.30  Afternoon Tea

Session III: Plant cell culture and bioprocessing

Chair: Chris Franco, Flinders University, Adelaide, Australia

15.30 – 16.10  Plenary lecture: Taiji Adachi, Osaka University, Japan
Natural food colorants: anthocyanins and betalains. Biotechnological approaches in research
and commercial production

16.10 – 16.30  Izabela Konczak, CRC for Bioproducts, Food Science Australia
Natural food colorant from a high-anthocyanin accumulating sweetpotato cell line

16.30 – 16.50  Wei Zhang, CRC for Bioproducts/Flinders University, Adelaide, Australia
Toward a rational plant cell-based bioprocessing for production of anthocyanins

16.50 – 17.10  Chris Curtin, CRC for Bioproducts/Flinders University, Adelaide, Australia
Role of transcriptional regulation in variability of Vitis vinifera L. cell culture-based anthocyanin
production

18.15 Departure of buses (Murrays, Australia) for the IWA2004 banquet
19.00-22.00 IWA2004 Banquet: Captain Cook Cruise on Sydney Harbour
Thursday, January 29, 2004

Session IV: Anthocyanins in plant cell – function, biosynthesis and regulation

Chair: Simon Deroles, Crop & Food Research Institute of New Zealand

9.00 – 9.40 Plenary lecture: Kazuki Saito, Chiba University, Japan
Integration of transcriptomics and metabolomics in anthocyanin-overproducing Arabidopsis: finding of molecular networks of biosynthesis

9.40 – 10.00 Kevin Davies, Crop & Food Research Institute of New Zealand
Recent studies on some rare flower colour pigments

10.00 – 10.20 Kin-Ying To, Institute of BioAgricultural Sciences, Academia Sinica, Taiwan
Molecular engineering of flower color

10.20 – 11.00 Morning Tea
Display of anthocyanin containing products by Australian companies

11.00 – 11.40 Plenary lecture: Kevin Gould, Auckland University, New Zealand
Nature’s Swiss Army Knife. The diverse protective roles of anthocyanins in leaves.

11.40 – 12.00 Mami Yamazaki, Chiba University, Japan
Metabolite and transcript profiles in anthocyanin-overproducing form of Perilla frutescens var. crispa

12.00 – 12.20 Mandy Walker, CSIRO Plant Industry, Australia
Regulation of the flavonoid pathway in fruit

12.20 – 14.00 Lunch

Session V: From the grapevine to the glass: anthocyanins, red wine phenolics and red wine colour

Chair: Markus Herderich, Australian Wine Research Institute/University of Adelaide, Australia

14.00 – 14.20 Markus Herderich Australian Wine Research Institute/University of Adelaide, Australia
Anthocyanins, anthocyanin-derived pigments and the mysteries of red wine colour

14.20 – 14.40 Graham Jones, University of Adelaide, Australia
Incorporation of anthocyanins into complex wine pigments

14.40 – 15.00 Mark Downey, CSIRO Plant Industry, Australia
The effect of light on anthocyanin accumulation in Shiraz and Cabernet Sauvignon (Vitis vinifera L.) grapes and wine

15.00 – 15.20 Yoji Hayasaka, Australian Wine Research Institute, Australia
Confirmation of pigmented polymers present in grape (Vitis vinifera L.) skin and wine

15.20 – 15.40 Michael Schwartz, Technical University of Braunschweig, Germany
Pyrananthocyanins: potential chemical markers for the determination of the age of red wines?

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Key Note and Plenary Lectures
Bioactivity of Anthocyanins and Importance to Human Health

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Anthocyanin pigments and the flavonoid compounds naturally associated with them in nature have demonstrated ability to protect or provide therapy for myriad of human diseases. These pigments and related flavonoids have been notoriously difficult to study with regards to human health for a number of reasons. The most challenging feature of the puzzle is that anthocyanin pigments frequently interact with other flavonoids to potentiate biological effects. The complex, multicomponent structure of some of the compounds in a bioactive mixture, and the ephemeral nature of the flavonoids during harsh extraction procedures, obscures the precise assignment of bioactivity to individual pigments. In addition, these phytochemicals can be highly metabolized after ingestion, which complicates the tracking of anthocyanins and other flavonoids to assess absorption, bioavailability, and accumulation in various organs.

Anthocyanin pigments that are uniformly, predictably produced in rigorously controlled plant cell culture systems can be a great advantage for health and nutrition research, because the bioactive compounds are quickly and easily isolated, lack interferences found in whole fruits, and are amenable to biolabeling so that the metabolic fate can be investigated after ingestion. The effective use of cell culture-produced anthocyanins can now elucidate previously hidden roles of anthocyanin pigments in human health and metabolism.
Anthocyanin Bioactivities:
Outcome of an European Collaboration (QLK1-1999-00124)

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The primary objectives of an European collaboration on anthocyanin bioactivities (QLK1-1999-00124)* during Feb 2000 to Jan 2003 were to investigate i) the functional properties of anthocyanins and anthocyanin-rich food ingredients and ii) the influence of anthocyanins on parameters related to the aetiology of cardiovascular disease in humans.

The outcome of the collaboration include novel methodologies, i.e., enzymatically assisted extraction especially with pectinolytic preparations in red berry (especially black currant, *Ribes nigrum* L.) juice processing, resulting in substantial increase in the phenolic content. Chemical and physico-chemical interactions (co-pigmentation and compartmenting) between anthocyanins and other food components were found to lead increased colour stability and product quality. Of particular relevance are phenolic acids, organic acids, proteins and amino acids, hydrocolloids, sugars, sweeteners and emulsifiers. Anthocyanin-rich ingredients (extracts, juice and concentrates) as well as by-products of the fruit juice processing showed significant antioxidant potential and colouring properties.

At cellular level, the bioactivities of anthocyanins include protective effect against oxidative DNA damage. Anthocyanins are effective against cytotoxicity, DNA single strand break formation and lipid peroxidation induced by tert-butyl-hydroperoxide (TBHP).

In animal studies, dietary anthocyanins seem to be capable of sparing vitamin E in healthy, growing rats. This was not seen under increased oxidative stress induced by vitamin E deficiency. In rabbit atherosclerosis study plasma cholesterol was increased significantly in the groups dosed with purified black currant (include latin name) anthocyanin fractions (AF) and in the group dosed with vitamin C in comparison to the control group and the group consuming black currant juice. A tendency to increased atherosclerosis was observed for AF and vitamin C monitored as the amount of cholesterol in intima, the ratio intima:media and the area of intima. Black currant juice bears no harmful effects towards onset of atherosclerosis in WHHL rabbits but caution is advised regarding dietary use of purified red berry anthocyanins.

A new insight was further reached concerning the biological actions of dietary anthocyanins in relation to heart disease - no preventive role of dietary anthocyanins towards cardiovascular health could be established in a human intervention study. For the strictly controlled human intervention (*n=58*) study with ca. 30 biomarkers analyzed, the only differences between treatment effects (285-450 mg/day of anthocyanins either in black currant juice or as black currant anthocyanins) were seen in vitamin C, red blood cell GPx activity and bleeding time.

* Participants in EU-collaboration (QLK1-1999-00124)
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Do Anthocyanins Contribute to Cancer Prevention?  
- Introduction to Molecular Evidence

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Epidemiological investigations have indicated that the moderate consumption of anthocyanins through the intake of the products such as red wine or bilberry (Vaccinium myrtillus L.) extract is associated with a lower risk of coronary heart disease. Recent studies indicated that anthocyanins have strong free radical scavenging and antioxidant activities, and show inhibitory effects on the growth of some cancer cells. Animal experiment showed that oral intake of anthocyanins from purple sweet potato (Ipomea batatas L.) color and red cabbage (Brassica oleracea L.) color suppressed rat colon carcinogenesis induced by 1,2-dimethylhydrazine and 2-amino-1-methyl-6-phenylimidazo-[4,5-b] pyridine. These facts suggest that anthocyanins may play a critical role on cancer chemoprevention. However, there is little molecular evidence for their actions. Thus, the possibility and mechanisms for anthocyanin application in anticarcinogenesis need to be considered at molecular level. In more recent years, we focused on investigating the chemoprevention potential of anthocyanins by targeting the molecular mechanisms. In this workshop, we present the molecular bases for anthocyanins on several key steps involved in cancer chemoprevention: (i) suppression of anthocyanins on cell transformation by targeting AP-1 gene and MAPK signaling pathways; (ii) inhibitory effects of anthocyanins on inflammation and carcinogenesis by targeting COX-2 and iNOS genes; (iii) the effects of anthocyanins on apoptotic induction of cancer cells by targeting JNK pathway, ROS generation and caspase activation. Taking together, our data provide a detailed molecular view of anthocyanins contributing to cancer chemoprevention.

References
Natural Food Colorants: Anthocyanins and Betalains; Biotechnological Approaches in Research and Commercial Production

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The application of plant biotechnology for modification of flower color began in 1970s. At that time information on biosynthesis of plant pigments in flower petals, including anthocyanins, was limited. Genetic engineering of flower color seemed to be a very attractive model for the application of plant biotechnology: anticipated color changes were easy to detect through the application of microscopic and chemical analyses. Modification of flower color became a vast growing area of research, contributing not only towards understanding of the biosynthetic pathways of pigments and methods of their regulation, but also resulted in commercial outcomes such as the development of novel decorative plants – petunias, lisianthus or carnations. At present this knowledge can be utilized to modify plant pigments not only for flower color, but also in the development of novel natural colorants used in the food industry. Both, anthocyanins and betalains have been a target for biotechnological studies. The approaches used to modify these groups of pigments are similar and sharing the outcome of this research may bring benefits to the scientific community and industry. After reviewing the historical account on biotechnological approaches, I would like to describe our own research focusing on the application of biotechnology for modification of pigments by the means of plant tissue culture.
Integration of Transcriptomics and Metabolomics in Anthocyanin-overproducing Arabidopsis: Finding of Molecular Networks of Biosynthesis

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Systems biology approach including metabolome analysis may provide precise information on cellular molecular networks under particular genetic alteration. To integrate transcriptomics and metabolomics under a particular-gene expression in Arabidopsis thaliana, we analyzed global gene expression profile by DNA microarray and metabolite profile by combination of mass spectrometry. The metabolite profiles of pap1-D mutant and pap1 cDNA transgenic lines over-expressing a Myb gene by LC-MS indicated that ~10 different anthocyanins were over-accumulated. In addition, the level of quercetin-type flavonols increased, whereas that of kaempferol-type metabolites decreased. However, the global metabolomes analyzed by FT-MS were dominantly governed by growth conditions and organs of plants more than the mutation. These results indicate that pap1 gene is specifically involved in regulation of anthocyanin and quercetin biosynthesis. Thus, we could assign the function of particular genes whose expression with DNA microarray was enhanced in the pap1 overexpressing lines to particular steps of anthocyanin production.
Nature’s Swiss Army Knife. The Diverse Protective Role of Anthocyanins in Leaves

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Anthocyanin pigments, once considered as waste products in leaves and other vegetative organs, are now believed to play pivotal roles in plant protection. Red leaves are especially common on shoots growing under harsh environments, and they can often be induced in the laboratory by subjecting plants to a variety of biotic and abiotic stressors. A growing body of experimental evidence indicates that anthocyanins in leaf cell vacuoles serve as important moderators of the effects of environmental stress.

Two properties of anthocyanins are considered to be particularly important in stress tolerance: their physical properties of light absorption, and their chemical properties as antioxidants. The absorption of visible and ultraviolet light has been postulated to: (i) increase leaf temperature; (ii) protect cellular organelles from the effects of UV-B; (iii) deter herbivory by causing leaves to look unpalatable; (iv) prevent the degradation of photolabile defensive chemicals; (v) reduce the onset and extent of photoinhibitory damage; (vi) minimise the production of free-radicals by photooxidative processes; and (vii) facilitate nitrogen re-assimilation from senescing foliage. Both the red vacuolar forms and the colourless cytosolic forms of anthocyanins have been shown capable of scavenging most species of reactive oxygen and reactive nitrogen. The anthocyanins are significant components of the antioxidant pool in some species, and could supplement the enzymatic antioxidants under conditions of severe stress.

Anthocyanins, therefore, offer a versatile, possibly unique assemblage of protective roles for shoots under stress. Recent research indicates that this protective potential translates to an advantage in the field.
Oral Presentations
Antioxidant Activity of Anthocyanins in Different Food Models

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Anthocyanins inhibit both lipid and protein oxidation in different food model systems. The antioxidant activity of anthocyanins towards lipid oxidation is well known, but their effect on protein oxidation needs further clarifications. In foods, proteins interact with other food constituents including phenolic compounds such as anthocyanins and lipid oxidation products. Polyphenol interactions with proteins occurring through complex formation are postulated to inhibit protein oxidation thus leading to improved quality of food.

The objective of this research was to study the effect of anthocyanins (cyanidin, delphinidin, pelargonidin and their sugars) on both lipid and protein oxidation in liposome and emulsion model systems. Liposome oxidation was studied with incorporation of lactalbumin (0.16 %) into the membrane system and the oxidation of the oil-in-water emulsion with a model system consisting of whey protein concentrate (2 %) and purified rapeseed oil (10 %). Both food models were oxidized in the dark at + 37 °C with copper. Protein oxidation was measured by following loss of natural tryptophan fluorescence and formation of protein-lipid carbonyl compounds. Lipid oxidation was measured by following both formation of conjugated diene hydroperoxides and hexanal. Cyanidin, delphinidin, pelargonidin and their aglycons were tested at concentrations of 10 and 20 µM in liposome model, and at 50 and 200 µM in emulsion model. The antioxidant activity of different anthocyanins in different food models was calculated after 6 days of oxidation.

The antioxidant activity of anthocyanins was different in different food models. In liposome model all tested anthocyanins acted as antioxidants, while in emulsion model some anthocyanins acted as prooxidants towards the loss of tryptophan fluorescence. It is well known in theory that the antioxidant activity of phenolic compounds usually enhance when the amount of hydroxyl groups in the B-ring increase. However, in this study, delphinidin having three hydroxyl groups in the B-ring inhibited lipid oxidation less than cyanidin and pelargonidin as measured by the formation of conjugated diene hydroperoxides both in liposome and emulsion models. Delphinidin was better antioxidant than cyanidin and pelargonidin in inhibiting protein oxidation at 20 µM and pelargonidin was best antioxidant at 10 µM in liposome model. Glycosylation altered the antioxidant activity order so that cyanidin glucosides were the most effective toward protein oxidation in liposome model at both tested concentrations, while there were no significant differences between pelargonidin- and delphinidin-3-glucoside at 20 µM. In emulsion model cyanidin and its glucosides inhibited more loss of tryptophan fluorescence than delphinidin, pelargonidin and their glucosides at 50 µM, while at 200 µM delphinidin-3-glucoside was the best.

In conclusion, oxidative deterioration of liposomes and emulsions due to protein-lipid interaction is inhibited by anthocyanins and their glycosides present for example in berries. Further studies have shown that anthocyanins isolated from berries such as bilberries, lingonberries, raspberries and black currants exhibit antioxidant activity towards both protein and lipid oxidation.
The Effect of Different Postharvest Treatments on Anthocyanin Content of ‘Assaria’ Pomegranate (Punica granatum L.) Fruit During Storage

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“Assaria” pomegranate is a sweet Portuguese cultivar typically grown in Algarve, not yet studied. The effect of different storage conditions of “Assaria” pomegranate fruits on anthocyanins concentration was studied. The anthocyanins are quantified either by comparison with an external standard of cyanidin 3-glucoside or using cyanidin 3-rutinoside. It was observed that using cyanidin 3-rutinoside as external standard the total anthocyanin content was significantly higher. Considering the individual anthocyanins, only delphinidin 3-glucoside content was indifferent to the methodology used. The concentrations ranged from 26 to 75 mg/l when cyanidin 3-rutinoside was used as external standard, and 28 to 67 mg/l when delphinidin 3-glucoside was used. Delphinidin 3,5-diglucoside was the main anthocyanin in the juice when cyanidin 3-rutinoside was used as standard whereas delphinidin 3-glucoside was the main one when the other quantification method was used.

The storage time as well as the fruit treatment performed prior to storage influenced total anthocyanins content. The highest level was observed after one month of storage in almost all treatments (141-211 mg/l). The storage of fruits in boxes covered with low density polyethylene film showed the best results.

The present work reveals the importance of the quantification methodology of the anthocyanins as well as the storage conditions of the “Assaria” pomegranate on the accumulation of anthocyanins in the fruit.
Stability and Enhancement of Berry Juice Colour

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Attractive colour is one of the main sensory characteristics of fruit and berry products and this important quality parameter strongly affects consumer behaviour. Unfortunately the colour of red juices is unstable and easily susceptible to degradation, leading to a dull and weak juice colour. The colour stability of anthocyanins is influenced by pH, storage temperature, light, structure and concentration of the anthocyanins, and the presence of other compounds such as other flavonoids and phenolics (1). The consumption of berry products has been encouraged world widely because of their possible health benefits. The promotion of high consumption of berry juices would benefit from colour enhancement by more stabilized colour and prolonged shelf life of the juices.

This study was designed to investigate the colour stability and copigmentation of four different berry juices enhanced by phenolic acids. The berry juices used were lingonberry (Vaccinium vitis-idaea, L.), cranberry (Vaccinium oxycoccus L.), strawberry (Fragaria ananassa L.) and raspberry (Rubus idaeus, L.) juices. The phenolic acids used as copigments were ferulic, sinapic and rosmarinic acids. Phenolic acid enrichment improved and stabilized the colour of the berry juices during 100 days of storage. The colour enhancement was intensive in strawberry and raspberry juices and effective in lingonberry and cranberry juices. Sinapic acid induced the strongest colour in strawberry juice, ferulic and sinapic acids improved raspberry juice colour equally and rosmarinic acid enhanced the colour of lingonberry and cranberry juices the most.

The total anthocyanin content of all the juices decreased during storage. Strong copigmentation reactions took place in the juices since the juice colours did not fade in the same ratio as the anthocyanin content decreased. The use of phenolic acids produced new peaks to the end of HPLC chromatogram, indicating a formation of new compounds. Sinapic acid produced the most abundant peaks with strawberry juice. With raspberry juice ferulic acid induced the biggest peaks. Rosmarinic acid stabilized lingonberry and cranberry juice anthocyanins but did not induce a formation of new compounds.

The four juices can be classified into two different groups by their composition but also by their colour behaviour during storage. Strawberry and raspberry juices contain less phenolics and anthocyanins than cranberry and lingonberry juices. Also the pH is significantly higher in the two former juices compared to the latter ones. The copigmentation reactions and colour enhancement occurred similarly within one group and differently between the two groups. Also the used phenolic acids can be categorized into two groups, which differ in their behaviour as copigments. It can be assumed that the non-conjugated sinapic and ferulic acids formed new intramolecular copigmentation compounds with berry anthocyanins whereas the conjugated rosmarinic acid stabilized anthocyanins intermolecularly.

References
Bioavailability and Biokinetics of Anthocyanins from Red Grape (Vitis vinifera L.) Juices and Red Wine

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In a comparative study the bioavailability and biokinetics of anthocyanin glucosides from red grape juice and red wine was tested in cross over in 9 healthy volunteers, 5 females and 4 males. After ingestion of a single oral dose of 400 ml red grape juice or red wine either with dose adjusted anthocyanin content (279.6 – 283.5 mg total anthocyanins) the anthocyanins were detected in plasma as well as in the urinary excretion (Netzel et al. 2001, Frank et al. 2003). Additionally, the plasmatic antioxidative activity was assessed after intake. From the analytical parameters biokinetic criteria were calculated, such as AUC, c max, t max, and the elimination half life time t½ from plasma (Frank et al. 2003). The relative availability of the analysed glucosides of cyanidin, delphinidin, malvidin, peonidin and petunidin from red wine, estimated from dose normalized urinary excretion, was calculated to 65.7, 61.3, 61.9, 291.5 and 57.1 % compared to the red grape juice. The urinary excretion of total anthocyanins amounted to 0.18 % (red wine) and 0.23 % (red grape juice) of the administered dose. The different excretion rate was also reflected in the plasma level. In addition, the plasmatic antioxidant activity increased to higher levels after juice ingestion compared to red wine. The anthocyanins of red grape juice seem to be better available from the gastrointestinal tract than those of red wine, suggesting a possible synergistic effect of the glucose content of the juice. The improved absorption resulted simultaneously in an enhanced bioactivity in plasma.

References

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Food Regulations and Functional Foods in Australia and New Zealand

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Food which might be considered to have ‘functional’ properties comprises a small but increasing proportion of the food supply in Australia and New Zealand. There is currently no specific framework for regulating these foods, however, some types of functional foods are addressed by current regulations. Functional foods are not currently defined in food legislation but are generally considered to be foods that have been modified to have physiological roles beyond the provision of simple nutrient requirements. A general requirement of food legislation is that food entering the food supply must be ‘safe’ and ‘suitable’ while the Food Standards Code specifies in greater detail regulatory requirements. Functional foods raise issues in relation to both safety and efficacy.

In relation to safety, there are issues because of the nature of the functional ingredient itself and/or the increased level of exposure. In 2001, regulations were introduced which require so-called ‘novel’ foods to undergo a pre-market safety assessment. This is a broadly based regulation, the purpose of which is to ensure that non-traditional foods entering the market are safe.

In relation to the efficacy of functional foods, claims in relation to the function properties of a food can range from content claims, enhanced functional to risk-reduction claims. The latter two are generally considered to be health claims, which currently are not permitted in Australia and New Zealand. Following a recent decision of the Health Ministers, however, Food Standards Australia New Zealand will be developing over the next 12-months a regulatory framework to allow limited health claims on foods.
Anthocyanins (Greek *anthos*, flower and Greek *kyanose*, blue), the terminology used originally to describe the blue pigment of the cornflower (*Centaurea cyanus*, Marquant, 1835), are the most important group of water-soluble plant pigments visible to the human eye. They belong to the most widespread class of phenolic compounds, collectively named flavonoids with more than 4000 structures reported as at year 2000. During the past two decades an increasing number of studies have investigated the diverse protective effects elicited by polyphenolics present in various fruits and vegetables, against cancer, ischemic heart disease, anti-tumorigenic, anti-microbial, anti-inflammatory and allergic, anti-mutagenic and other physiological effects. Polyphenolic research has recently intensified due to our increasing understanding and awareness of the potential beneficial human health effects. Until today, anthocyanins have received less attention than other flavonoids, despite their far-reaching effects. The aim of the present article is to summarise the known bio-medicinal power of anthocyanins and to help bridge the gap in our understanding of their functional mechanisms.
Effect of Grape (*Vitis vinifera* L.) Seed Extract on Endothelial Function

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Aim: To demonstrate that 2g/day of grape seed extract (GSE) [containing 1g of polyphenols] alone, or with 1g/day of added quercetin in yoghurt, favourably alters vascular function, endothelial function and degree of oxidative damage in comparison to a control yoghurt.

Method. Fully randomised, crossover trial in 36 men and women with above average vascular risk from high cholesterol, smoking or high blood pressure with three 4 week treatment periods.

Measurements. Weight, blood pressure, vascular compliance as assessed by an HDI pulse wave analyser, flow mediated dilatation assessed ultrasonically, fasting lipids, CRP, oxidized LDL, endothelial adhesion molecules (ICAM1, VCAM1), Von Willebrand factor (VWF), nitrates, tissue type plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) and urinary F2 isoprostane.

Results. GSE alone improved flow mediated dilatation by an absolute 1.1% compared with control. There was no effect of the combination of GSE with quercetin. No other blood or urine measure was altered.

Conclusions. Sufficient GSE appears to be absorbed to influence endothelial nitric oxide production, although the level of polyphenols did not appear to be high enough to influence other endothelial functions. GSE has the potential to favourably influence vascular function.
Natural Food Colorant from a High–anthocyanin Accumulating Sweetpotato Cell Line

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Application of biotechnology for a commercial production of selected phytochemicals, including pigments, flavour compounds, novel food ingredients and drugs, offers numerous advantages over the conventional agriculture. Plant cell/tissue culture presents an opportunity for a continuous production of large quantities of plant biomass in a factory setting independent of environmental conditions. Skilful application of cell culture techniques could ensure uniformity of the raw products never observed with field grown material.

One of the novel sources for a commercial production of anthocyanins as natural food colorants is a Japanese sweetpotato (*Ipomoea batatas* L.), cv. Ayamurasaki. Due to the outstanding stability of the sweetpotato anthocyanins they are applied as natural food colorants in multiple food products such as confectionary, cakes, juices, yogurts, vinegar, ice-creams or soft drinks both, in Japan and USA. We have generated a high-anthocyanin accumulating cell line (PL) from the storage root of the Ayamurasaki cultivar. The cell line displays unique characteristics such as i) ability to accumulate anthocyanin pigment in the dark ii) morphological differentiation towards formation of small aggregates with an average size of 3-5 mm which facilitate resistance to shear forces and successful growth in a bioreactor iii) 3 to 4-fold higher level of pigment accumulation than that in the field-grown plant.

Both medium optimization and cell line selection have resulted in a 4 to 6-fold increase of the anthocyanin level: the Colour Value (CV) of the crude pigment extract from the *in vitro* grown tissue increased from 6.0 to 20±2 in multiplication medium (MM) and 35±2 in production medium (PM). The composition of *in vitro* produced pigment consists of cyanidin 3-sophoroside-5-glucoside (YGM-0a) and it’s mono- and di-acylated and/or methylated derivatives. Beside anthocyanins identical with those accumulating in the field-grown plant we have detected also cell line-specific pigments. Accumulation of anthocyanins with highly evolved molecular structures (increased number of sugar moiety attached, mono- and di-acylation) enhances the stability of pigment extract and therefore is of a significant importance from the commercial point of view. Several methods have been identified to regulate the biosynthesis of anthocyanins in our system. Decreasing the level of NH$_4^+$ ions from 20 to 2.5 µM in the culture medium or decreasing the temperature from 25 to 15 ºC lead to accumulation of acylated pigments. Feeding a precursor p-coumaric acid at the level of 2 mM resulted in a complete conversion of non-acylated anthocyanins into their mono- and di-acylated derivatives. These changes were concomitant with an approximately 2-fold increase the total amount of accumulated pigments. The addition of the elicitor methyl jasmonate (MeJ) in concentrations 4.5 – 44.5 µM also promoted biosynthesis of di-acylated anthocyanins.

An extract from a purple-fleshed sweetpotato has been reported to possess health-promoting qualities. Therefore cell line aqueous extracts produced under MM and PM medium conditions were evaluated for antioxidative, antimutagenic and antiproliferative activities. The assays indicated that both aqueous extracts of the PL cell line exhibited significantly stronger antioxidative, antimutagenic and anticancer activities than the field-grown storage root extract with the superior effect of MM extract. These results suggest that the PL cell line may serve as an alternative source of natural food colorants and valuable physiologically active components of functional food products.
Plants are probably the best cell factories on this planet from which a diversity of >100,000 low molecular secondary metabolites was discovered with an estimate of total number in plants exceeding 500,000. Plant cell-based bioprocessing is to use the biosynthetic pathways of plant cells/tissues for the production of these metabolites and biotransformation. Despite many years' efforts, this technology still remains as a potential yet with limited commercial success. To advance our knowledge and tools in translating “potential” into “commercial success”, the present report intends to establish strategies and technical framework for a rational molecular plant cell-based bioprocessing approach. Three main tasks of this rational approach are (i) characterization of all genes involved, their protein products and their metabolic products in a given biosynthetic pathway; (ii) characterization of their respective regulatory functions and roles; (iii) manipulation of the biosynthetic pathways for a given application via engineering metabolism. To illustrate this approach, we presented some data on the functional analysis of metabolic pathways for biosynthesis of anthocyanins from profiling of gene expression and protein expression to metabolic profiling in *Vitis vinifera* cell culture as a model system. Emphasis was placed on a global correlation at three molecular levels, as well as on the interactions between biosynthetic pathway and post-biosynthetic events.

Using techniques such as precursor feeding, elicitation, metabolic inhibitors, redirected transport and analysis of strains, the dynamic profiles of mRNA expression, enzyme activities and anthocyanin metabolites of the biosynthetic pathways in *Vitis vinifera* cell culture were characterized. One example was the functional analysis of *V. vinifera* cell cultures that were elicited with jasmonic acid, light, and sucrose alone and in combination. All these single conditions enhanced anthocyanin production and exhibited a additive improvement when combined. Transcriptional studies by quantitative RT-PCR indicated a strong correlation between transcriptional expression and improved anthocyanin biosynthesis and a role of light irradiation in up-regulating UFGT. Metabolic profiling implicated the competition between anthocyanin and stilbene pathways, and the importance of methylated and acylated anthocyanin species in enhanced production. In recognition that the post-biosynthetic steps may play equally crucial roles in its yield improvement, we have been investigating the characteristics and roles of glutathione S-transferases (GSTs) and anthocyanin vacuolar inclusions (AVIs) in anthocyanin transport and storage, respectively in grape cells. The AVIs were isolated and are being characterized using an integrated post-genomic approach. Initial results indicated AVIs may compose of several protein species and have the selectivity for acylated anthocyanins. It is expected that these studies will provide new targets for rational metabolic engineering.
Role of Transcriptional Regulation in Variability of *Vitis vinifera* L. Cell Culture-based Anthocyanin Production

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One of the prevailing challenges to commercialization of plant cell culture based bioprocesses for secondary metabolite production is the heterogeneity they exhibit in terms of productivity. It is not uncommon over the course of repeated subculturing for a plant cell culture line to lose capacity to produce the target secondary metabolite. A better understanding of the factors contributing to this variability is needed and to this purpose we have investigated heterogeneity in terms of anthocyanin and stilbene production in *Vitis vinifera* L., v. Gamay Fréaux cell culture.

Long-term subculturing of FU-01 cell line illustrated metabolic instability, where over 130 weekly subcultures average biomass was 10.7±2.5g/l dry cell weight (DCW), while anthocyanin content was 27.8±16.9 CV (colour value)/g-DCW. Another cell line, FU-02, which was highly selected for increased anthocyanin production, exhibited the long-term subculture effect of loss of metabolic capacity. Initially characterised by an anthocyanin content of >200 CV/g-DCW and a predominance for accumulation of malvidin derivatives, within 18 months the line reverted to being peonidin and cyanidin dominant (as for FU-01) and had a pigment level around 20 CV/g-DCW. In both cell lines content of piceid, the major stilbene present, was less variable than that of anthocyanins, and to some degree there was a competitive relationship between the two.

In order to investigate the mechanism for this alteration in metabolic output, a non-pigmented cell line (FU-03) and a new highly-pigmented cell line (FU-04) were selected by microcalli and clonal procedures from original FU-01 material. There was little difference in piceid production by the cell lines. Elicitation experiments were conducted with FU-03 and FU-04 alongside FU-01, to gain insight into how these subpopulations of cell types respond at a pathway and molecular level.

The three cell lines exhibited a similar gene expression response to elicitation, particularly in terms of which elicitation conditions induced the maximal transcript levels for key anthocyanin pathway genes PAL (Phenylalanine ammonia-lyase), CHS (Chalcone synthase), DFR (Dihydroflavonol 4-reductase), UFGT (UDP-glucose flavonoid glucosyltransferase), and the stilbene pathway gene StSy (Stilbene synthase). Differences in the anthocyanin content were only reflected by expression of UFGT, early pathway genes PAL, and CHS were present at similar levels in FU-01 and FU-03 after elicitation despite a 10-fold difference in anthocyanin content.

Malvidin, particularly the acylated form, is the most stable *Vitis vinifera* L. anthocyanin and its presence correlates with the highest anthocyanin yields within our cell cultures. Both FU-02 and FU-04, selected for high pigment level over 2-years apart, were characterised by a higher proportion of malvidin derivatives than FU-01. The key pathway steps critical to synthesis of this metabolite are F3’5’OH (Flavonoid 3’5’-hydroxylase) and subsequent methylation of these hydroxyl groups. Analysis of F3’5’OH expression indicates that FU-04 has an increased level of transcript for this particular gene.

In conclusion, metabolic differences observed between three cell lines can be attributed to differential regulation at the transcript level of F3’5’OH and UFGT. Future research could focus on strategies to achieve stable expression of these key genes and potentially reduce the variability in both anthocyanin production and composition observed for *Vitis vinifera* L. cell culture.
Recent Studies on Some Rare Flower Colour Pigments

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We have a programme aimed at understanding the basis of colour variation in nature, so that strategies for modifying production of pigments, both in planta and in cell culture, can be developed. Chlorophyll, carotenoids and flavonoids are the most common pigments in plants. In particular the common 3-hydroxyanthocyanins are widespread in plant tissues, and anthocyanin biosynthesis is the most amenable pigment pathway from a biotechnology point of view. However, there are many plant pigments of rarer distribution that also feature as key pigments in flowers or vegetative organs of some species. Some of these are in widespread use as food pigments, while others offer attractive routes for introduction of novel colours into target species via genetic modification approaches. Our current research is investigating the biosynthetic mechanisms of three of these rare pigment types; aurones, 3-deoxyanthocyanins, and betalains.

We have previously isolated cDNAs that may encode enzymes involved in the biosynthesis of 3-deoxyanthocyanins in Sinningia cardinalis, including cDNAs for flavanone 4-reductase (FNR), anthocyanidin synthase and a range of putative flavonoid glycosyltransferases. We have now expressed the FNR and GTs in E. coli, to study their substrate preference, and their role in 3-deoxyanthocyanin production. We have also developed transient protein expression (using Agrobacterium infiltration) and RNAi gene inhibition techniques for petals that can be used for rapid assays of gene function, and it is planned to use these in future research to confirm in vitro results.

Aurones are bright yellow flavonoid pigments synthesized from chalcones by a polyphenol oxidase (PPO) variant, termed the aureusidin synthase. The identification of this activity in Antirrhinum majus was one of the first examples of a specific biosynthetic role for plant PPOs. Using plant transgenic analysis we have further defined the function of the aureusidin synthase, and the transgenic phenotypes suggest that it can carry out the ring-closure to form flavanones from chalcones that is normally carried out by chalcone isomerase.

The betalains are nitrogenous pigments that are the most taxonomically restricted of the major plant pigment groups, being found only in a few families of the order Caryophyllales and some fungi. Curiously, their occurrence is mutually exclusive to that of the anthocyanins. We have been testing the application of the known betalain biosynthetic genes for introduction of betalain biosynthesis into novel species. By expressing the DOPA-dioxygenase in A. majus petals we were able to show the formation of yellow pigments, presumably betaxanthins, was possible if sufficient L-DOPA was present in the tissue. We are now confirming the identity of the novel pigments using stably transformed transgenic plants, and investigating approaches for increasing L-DOPA levels in cells.
Chalcone synthase (CHS) is a key enzyme in the anthocyanin biosynthesis pathway. We have cloned a full-length cDNA (1170 bp) encoding CHS from a Petunia cDNA library of violet flower. DNA sequence analysis showed 85-92% identity with other chs sequences from higher plants including tobacco (91%). The accumulation of the cloned chs mRNA was found in flowers but not in leaves or roots of Petunia plants. As a first step in engineering of flower color, the gus reporter gene in the binary vector pBI121 was replaced by the Petunia chs cDNA, and a sense chs construct (pCHS) was obtained. The expression vector pCHS containing Petunia chs cDNA under the control of CaMV 35S promoter was then transformed into tobacco (Nicotiana tabaccum) via Agrobacterium-mediated method. Southern blot analysis and T1 progeny assay suggested the presence of one or two copies of transgene in 7 transgenic tobacco plants obtained. Among them, 4 transformants carrying white flowers and 3 transformants carrying pale red flowers were observed as compared to red flowers of wild-type tobacco. Thin layer chromatography analysis revealed the absence of cyanidin in all white-flowered transformants. Northern blot analysis showed that total chs mRNA levels was significantly decreased in the white-flowered transformants, suggesting that both transgenic and endogenous chs transcripts were under suppressed; by contrast, a significant increase of chs mRNA was observed in those pale red-flowered transformants. It is consistent that overexpression of chimeric sense or antisense chs constructs suppresses chs message accumulation in white-flowered transformants from other plants; however, significant increase of chs mRNA in transformants carrying evenly reduced color of flower has not been reported yet. Species-specific reverse transcription-polymerase chain reaction (RT-PCR) analysis was further designed to dissect that the relative level of endogenous tobacco chs mRNA was obviously less than that of transgenic Petunia chs mRNA in each white-flowered line. In addition, plant/T-DNA junction sequence analysis was conducted to exclude the possibility of losing flower pigmentation in those white-flowered transformants by T-DNA inactivation of pigment-synthesizing genes in tobacco genome. These results provide extensive data for the hypothesis that cosuppression occurs in transgenic plants with white flowers so that there is not enough CHS enzyme activity to make pigments.
Metabolite and Transcript Profiles in Anthocyanin-overproducing Form of *Perilla frutescens* var. *crispa*

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The metabolite profiles and gene expression in two chemo-varietal forms, red and green forms, of *Perilla frutescens* var. *crispa* were investigated. Striking difference in anthocyanin content was observed in leaves. Anthocyanin, mainly malonylshisonin, was highly accumulated in the leaves of the red form but not in the green form. Less obvious differences were also observed in the stems. However, there was no remarkable difference in the contents and patterns of flavones and primary metabolites such as inorganic anions, organic anions and amino acids. These results suggest that only the regulation of anthocyanin production, but not that of other metabolites, differs in red and green forms. The red form specific genes were profiled by differential display and PCR-select subtraction. The genes involved in anthocyanin production/accumulation and the several genes related to light response were expressed specifically in red form. The possible regulatory network leading to differential anthocyanin accumulation in a form-specific manner will be discussed.
Flavonoids, especially anthocyanin and proanthocyanidin (tannin), contribute colour and mouthfeel to fruit and vegetables. Colour of fruit and vegetables is one of the major determinants of quality which influences the consumer when purchasing a product. Anthocyanins contribute the dominant red colour to apple fruit and the patterns and hue are important to the purchaser. Anthocyanin and tannin, extracted from grape berries, are vital components of wine, and appear to have significant health benefits when wine is consumed in moderation.

We are studying the regulation of the flavonoid pathway in apple and grape by examining the developmental and light-induced expression of the pathway genes. In grapes, synthesis of flavonols requires exposure to light whereas synthesis of anthocyanins and tannins can occur in the dark. Cripps Red apples, in contrast to grapes, make no anthocyanin if kept covered to exclude light. When the green apples are exposed to light two weeks before harvest, anthocyanins accumulate and the flavonoid pathway genes generally show a large increase in expression, as determined by Real Time PCR. This induction of gene expression must be due to the increase in the expression or activity of one or more regulators and this provides a good system to isolate and characterise transcription factors that regulate the pathway genes.

Our research is focused on the understanding of the regulation of the flavonoid pathway in a number of species, including model plant systems and important crops to provide tools for the modification of flavonoids in horticultural products.
Anthocyanins, Anthocyanin-derived Pigments and the Mysteries of Red Wine Colour

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The importance of grape anthocyanins to grape quality and red wine colour is well established. During winemaking and ageing, however, anthocyanin concentrations decrease rapidly and only small amounts of grape anthocyanins can be detected in aged red wines. Together with the anthocyanins, two types of pigments are essential for the colour of red wines: pigmented polymers, formed by the condensation of anthocyanins with other grape-derived polyphenols such as tannins, and stable 'small' pigments (such as the vitisins) that originate from the modification of grape anthocyanins with pyruvate and other fermentation by-products. This presentation will give an overview on concepts and compounds that have been utilized to explain aspects of red wine colour, and we will report outcomes from our production-scale winemaking trials and model experiments with focus on the formation of anthocyanin-derived pigments. While the commonly accepted paradigm (Somers, Phytochemistry, 1971: 10, 2175-2186) predicted that "a gradual transition from monomeric anthocyanins through oligomers to polymeric pigments" occurs during ageing, our data clearly identify the importance of yeast-mediated reactions during the few days of alcoholic fermentation and confirm the contribution of non-coloured condensed grape tannins with regard to pigment formation and red wine colour.
The anthocyanins extracted from grape skins during vinification and are responsible for the colouration of red wines. During winemaking anthocyanins undergo a range of reactions to form more colour-stable, complex wine pigments that are key components in maintaining red wine colour during ageing. Important in these reactions is the formation of ethyl-linked dimers and multimers of malvidin-3-glucose (and other grape-derived anthocyanins) and flavan-3-ols through a Baeyer reaction involving acetaldehyde. These compounds are formed quickly and early in fermentation to the extent that during a 9 day fermentation 75% of the available anthocyanin pigments are present as the ethyl-linked compounds. They are more stable to pH-dependent hydration and bisulfite bleaching than the parent anthocyanins, phenomena explained by steric hindrance to attack by nucleophiles, but they are temporally unstable and decrease to low concentrations in red wines a few weeks after the completion of fermentation. The loss of the ethyl-linked pigments is linked to the formation of active intermediates which go on to form long-lived pyranoanthocyanin pigments which are also resistant to bisulfite bleaching and oxidation. Furthermore, because of their resistance to hydration at wine pH as measured by their ionization and hydration constants, these new pigments show significantly greater colour expression than the parent anthocyanins. A knowledge of the factors affecting the formation of the various forms of wine pigments, particularly the ethyl-linked pigments, is important in maximizing the colour expression and potential mouthfeel of red wines, since between 50 and 80% of all anthocyanins present in the grape berry before winemaking remain in the grape marc after winemaking.

References
The effect of light on Anthocyanin Accumulation in Shiraz and Cabernet Sauvignon (*Vitis vinifera* L.) Grapes and Wine

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Anthocyanins in grapes contribute to wine quality and are one of the flavonoid compounds in wine thought responsible for the health benefit known as the “French paradox”. Thus, vignerons seek management practices that will increase the anthocyanin content of grapes and therefore wine. In a range of plant species, including mustard, pea and apple, light is required for anthocyanin biosynthesis. In grapes, light is also considered important for colour development, however while some research supports this, other work shows no effect or the opposite effect, being a decrease in colour with exposure to light. Here we sought to clarify the effect of light on anthocyanin accumulation in winegrapes by excluding light from developing bunches of Shiraz grapes (*Vitis vinifera* L.) over three seasons. Bunches were shaded prior to flowering with polypropylene boxes designed to minimise changes in temperature and humidity while excluding light. There was no significant effect of shading on berry development, however chlorophyll concentration was much lower in the shaded fruit, which appeared pale yellow until veraison. In two of the three seasons studied there was no significant change in anthocyanin content. However, anthocyanin composition was consistently altered in the shaded fruit, which had a greater proportion of cyanidin and peonidin derived anthocyanins, whereas the exposed fruit had a greater proportion of delphinidin, petunidin and malvidin derivatives. In the third season, wine was made from the shaded fruit. There was no difference in grape colour between the shaded and exposed fruit, however wines made from the shaded fruit showed lower total anthocyanins and lower colour density.

A further trial was conducted on Shiraz and Cabernet Sauvignon grapes that increased bunch exposure by vertically positioning the canopy and leaf plucking in the fruit zone. Colour in the highly exposed Shiraz fruit was around 50% higher than in the control. While total anthocyanins in the wine made from the highly exposed fruit were not significantly different (α=0.05, n=5) from the control, ionised anthocyanins (anthocyanins in the coloured flavylium form) were around 30% higher and colour density 20% higher. In Cabernet Sauvignon, there was no difference in the fruit colour at harvest, however there was a 10-20% increase in total wine anthocyanins and colour density. These results suggest bunch exposure to light does play a role in anthocyanin biosynthesis. However, the different outcomes in the wine and changes in anthocyanin composition with different levels of exposure suggest a complex relationship between grape colour and wine colour, which may also be varietal dependant.
Confirmation of Pigmented Polymers Present in Grape (Vitis vinifera L.) Skin and Wine

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Since Somers' initial report (Phytochemistry, 1971, 10: 2175-2186) in which he claimed that pigmented polymers are likely to be responsible for the major portion of colour in aged red wine, the importance and contribution of pigmented polymers to wine colour has been the subject of many studies. Since pigmented polymers in aged wine are thought to be a complex mixture of anthocyanin and proanthocyanidins and individual pigments are present in very low quantities, the characterisation of pigmented polymers remains an analytical challenge. In addition, the presence of pigmented polymeric materials in red grape skins has remained unexplored.

Recent studies on the characterisation and confirmation of pigmented polymers present in grape skin and wine using chromatographic and mass spectrometric techniques will be discussed.

\textit{Pigmented materials isolated from Shiraz grape skins.} Pigmented materials remained in the aqueous stationary phase after the progressive extraction from the grape skins with organic solvent by means of multilayer countercurrent chromatography. The majority of the pigmented materials were neither genuine anthocyanins nor their monomeric derivatives but appeared to be direct condensation products of anthocyanins with an extension to trimers.

\textit{Pigmented materials isolated from a three-year old Pinot noir wine.} Pigmented materials from the wine were isolated using size exclusion chromatography and confirmed to be polymeric by gel permeation chromatography. The pigmented isolates were confirmed to be direct condensation products of anthocyanin with proanthocyanidins with an extension to octamers.
The pyranoanthocyanins form a group of pigments that bear an additional pyran ring between C-4 and the hydroxyl group attached to C-5 of the anthocyanidin core. So far, they have only been detected in aged red wines and black carrot juice stored for a longer period of time, but not in fresh grapes or other fruits.

Vitisin A, isolated from red wine, was one of the first members of this new group of "aged" anthocyanins. At first, two slightly different structures were proposed, but today the structure suggested by Fulcrand et al. (1998) bearing a carboxyl substituent at the new pyran ring has been confirmed by several researchers. It was found that vitisin A evolves from the reaction between malvidin 3-glucoside and pyruvic acid, a yeast metabolite excreted into the wine during fermentation. In HPLC chromatograms of aged red wines, vitisin A was sometimes the only visible discrete peak, while genuine monomeric anthocyanins had already completely vanished. For this reason it was discussed that vitisin A, generated by the slow reaction between malvidin 3-glucoside and pyruvic acid during wine ageing, could be a suitable marker to determine the age of red wines. However, our analysis of a vertical row (vintages 1987-2002) of Chilean Cabernet Sauvignon wines from the same winery and vineyard showed that the highest content of vitisin A was present in the youngest wines. Afterwards, a somewhat fluctuating – attributable to e.g. yearly varying initial concentrations of malvidin C-glucoside and course of fermentation - but in the end steady decrease set in. Vitisin A is therefore clearly not suited as an ageing indicator for red wines [1].

A second class of pyranoanthocyanins bears a variably substituted 4-vinylphenol substituent instead of the carboxyl group on the newly formed pyran ring. Especially in Pinotage red wines, we observed high concentrations of pinotin A, formally the 4-vinylcatechol adduct of malvidin 3-glucoside. A total of 50 Pinotage wines (between 0.5 and 6.5 years old) were analyzed for the content of pinotin A, malvidin 3-glucoside, caffeic acid, and caftaric acid and statistical analyses were performed in order to determine the factors that influence pinotin A formation during wine ageing. With prolonged storage time we observed an exponential increase of the concentration of pinotin A. Significant differences in pinotin A levels were found in wines of different ages. Pinotin A formation depended to a larger extent on the concentration of free caffeic acid than on malvidin 3-glucoside. A statistical model was developed that allows prediction of the pinotin A concentration by taking into account age of the wine and caffeic acid concentration. Pinotin A synthesis in the wines proceeded as long as a certain level of malvidin 3-glucoside was present in the wines. Only in wines >5-6 years old degradation or polymerization of pinotin A finally exceeded the rate of its de novo synthesis [2].

We found that pinotin A is a suitable chemical marker for the age of Pinotage red wines. Importantly, formation of pinotin A and similar pyranoanthocyanins is not limited to this cultivar and will proceed in each wine containing free hydroxycinnamic acids.

References
Poster Presentations
Pharmacokinetics of Dietary Anthocyanins Following Consumption of Elderberry (Sambucus nigra L.) Extract and Blackcurrant (Ribes nigrum L.) Juice

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Pharmacokinetic parameters and the bioavailability of several dietary anthocyanins following consumption of blackcurrant juice and elderberry extract were compared exploratory in six healthy volunteers.

Three females and three males were given a single oral dose of either 137 ml of blackcurrant juice (144.8 mg total anthocyanins [delphinidin- and cyanidin-3-glycosides]) or 137 ml of diluted elderberry extract (147.3 mg total anthocyanins [cyanidin-3-glycosides]). Blood (plasma) was sampled over a period of 3 h after intake. Urine was collected over a period of 7 h after intake. Plasma and urine concentrations of anthocyanidin glycosides were assayed by HPLC-DAD [Miyazawa et al. 1999; Netzel et al. 2001; Frank et al. 2003].

Within 7 hours the urinary excretion of unchanged anthocyanins was 0.04% and 0.37% of the administered dose following blackcurrant juice and elderberry extract ingestion, respectively. After ingestion of elderberry extract, a statistically significant 8.7 fold higher estimate of the maximum excretion rate of total anthocyanins was calculated as compared with blackcurrant juice. Pharmacokinetic parameters derived by noncompartmental models from plasma and urine concentrations exhibited higher variability after ingestion of elderberry extract. Anthocyanin absorption was unambiguously enhanced following drinking of elderberry extract compared with blackcurrant juice as shown by 5.3 and 6.2 fold higher estimates of dose-normalised Cmax and AUC(0-t z) of total anthocyanins, respectively. The geometric means of t 1/2 were not significantly different following elderberry extract (1.74 h) or blackcurrant juice ingestion (1.73 h, p>0.05).

On a low level, urinary excretion of intact anthocyanins was fast and the excretion rates seem to exhibit monoexponential characteristics over time both after ingestion of blackcurrant juice and elderberry extract. However, to evaluate the impact of anthocyanins on health protecting properties of blackcurrant juice and elderberry extract, further studies will be needed tracing both the unchanged glycosides and their in vivo metabolites in human plasma and urine.

References

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Urinary Excretion of Cyanidin–glycosides and –glucuronides in Healthy Humans after Elderberry (Sambucus nigra L.) Juice Ingestion

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Much attention has been paid to the antioxidant activity of anthocyanins, since epidemiological surveys and animal studies showed a close relationship between the consumption of flavonoids and a reduced incidence of cancer and heart diseases. Anthocyanins, a subgroup of the flavonoids are important components of human nutrition, but their metabolism, however, is still not fully understood. In the present study, the urinary excretion of anthocyanins and anthocyanin-glucuronides of seven healthy subjects was measured, after ingestion of a concentrated elderberry juice, rich in anthocyanins.

After an overnight fasting, seven healthy non-smoking volunteers (6 women and 1 man with a mean body mass index of 21.5 kg/m²) consumed a bolus quantity of 150 ml of a concentrated elderberry juice (containing 0.21 g cyanidin-3,5-diglycosides, 2.24 g cyanidin-3-sambubioside and 1.10 g cyanidin-3-glucoside), together with white rolls and cheese. HPLC-DAD analyses of urinary samples (0-5 h post-ingestion) were performed before and after hydrolysis of glucuronid-conjugates by ß-glucuronidase (Netzel et al. 2001; Bub et al. 2001; Wu et al. 2002; Andlauer et al. 2003).

Within 5 hours, the urinary excretion of total anthocyanins (unchanged cyanidin-glycosides and their glucuronide-conjugates) was 0.053 ± 0.030% of the administered dose. Only 6.9 ± 2.2% of this amount accounted for the glucuronide-conjugates. Based on this recovery, the percentage of anthocyanin-glucuronides eliminated in the volunteers urine was 0.003 ± 0.001% (calculated as the ratio of anthocyanin-glucuronides excreted to anthocyanin-glycosides ingested).

In further studies the exact binding-sites of conjugation have to be revealed and the contribution of these in vivo metabolites to the increasing antioxidant potential of the organism after drinking elderberry juice shall be determined.

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Investigation of Anthocyanic Vacuolar Inclusions (AVIs) in Anthocyanin Accumulation and Storage in *Vitis vinifera* L. Suspension Culture

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The low level of anthocyanin accumulation in plant cells is the first hurdle to overcome in the development of an industrial scale bioprocess. Much effort has focused on augmenting anthocyanin biosynthetic pathways, with little success. Furthermore, evidence is emerging that the post-biosynthetic pathways (anthocyanin modification, transport, storage and degradation) may be crucial in augmenting production of these labile compounds. The purpose of this research was to investigate and characterize the anthocyanin storage site(s) towards determining their ultimate role in anthocyanin accumulation and storage.

Anthocyanic vacuolar inclusions (AVIs) are protein matrices that bind and concentrate anthocyanins in plant cells. They have been documented in over 70 plant species and their appearance correlates with an intensified color. This is clearly visible in Lisianthus petals that possess lighter and darker regions due to the absence and presence of AVIs, respectively. We have purified AVIs from various *Vitis vinifera* L. cell-suspension cultures differing in their anthocyanin profile and level of accumulation and found that they selectively bind acylated (p-coumaroylated) anthocyanins over the monoglucosides. The profile of anthocyanins bound to the AVIs directly correlates with the expression of anthocyanins in the whole cell in different cell lines, but retains a 4-5 fold increased specificity for the acylated species.

Preliminary two-dimensional gel analysis reveals that the AVIs are comprised of a small number of high molecular weight proteins, with mass spectrometry and amino-terminal sequencing being utilized to identify these.

These results suggest the AVIs as ancestral enzymes that have lost their catalytic function, yet still retain substrate specificity and adopted a storage role. Further results will be discussed at the workshop.
Anthocyanins are becoming favoured alternatives to synthetic food colours due to their documented nutraceutical benefits. Therefore, much research effort has focused on the design of an industrial-scale bioprocess for their production, primarily by upregulating the enzymes in the biosynthetic pathway. However, the influence of the anthocyanin post-biosynthetic pathway (including anthocyanin transport, storage and degradation) has been largely overlooked. Bronze-2 and An9 are glutathione S-transferases (GSTs) that have been shown to be critical in the transport of anthocyanins to the vacuole in maize and petunia, respectively, by transposon tagging. The current model of anthocyanin transport has the GST acting as a ligandin (escort protein) for the anthocyanin and depositing it into the vacuole without glutathionation of the molecule. The purpose of this research was to characterize anthocyanin transport in Vitis vinifera L. cell-suspension culture and determine whether augmenting transport could lead to enhanced production.

We have purified and characterized 4 GSTs from Vitis vinifera L. (grapevine) cell-suspension cultures by affinity chromatography and 2-dimensional gel electrophoresis. These species are deemed VvGSTI (25kDa), VvGSTII (22.5kDa), VvGSTIII (21.5kDa), VvGSTIV (25.5kDa), all with pIs ranging from 5-7 and have been confirmed as similar to other known plant GSTs by mass spectrometry. The amino-terminal sequencing is being completed on these proteins to clone the genes. Biolistic transformation of maize kernels deficient in Bronze-2 activity with the cloned GSTs will be utilized to identify candidate transport proteins. This analysis uses Bronze-2 knockout corn seeds that cannot transport anthocyanins to the vacuole and so appears bronze in colour. When transformed with a GST gene that phenotypically complements for the knockout, the region of transformed cells turns red from anthocyanin being transported to the vacuole. Further to this, quantitative reverse transcriptase PCR data will be presented on all genes for cell lines under various elicitation conditions.

This is the first evidence of multiple GSTs identified in grape. This is the first example of a cloning strategy family of GSTs approach can be universally applied to plant cells where knockout phenotypes are available and complementation can be easily scored, as with anthocyanin transport. Furthermore, the manipulation of the transport mechanism may also simplify the purification of anthocyanins from cell-suspension cultures.
Molecular and Biochemical Pathway Analysis in Optimisation of *Vitis vinifera* L. Cell Culture–based Anthocyanin Production

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Despite the great potential of plant cell culture for secondary metabolite production, few commercial successes have been achieved via traditional optimisation approaches. It is thought that incorporation of advanced knowledge on regulation of secondary metabolite pathways into bioprocess design may provide another avenue for productivity enhancement. The purpose of this research was to demonstrate applicability of such an approach in optimisation of anthocyanin production using a *Vitis vinifera* L. var. Gamay Fréaux cell culture.

Regulation of anthocyanin and stilbene biosynthesis were investigated under conditions that represent typical bioprocess improvements – light irradiation, medium carbohydrate level, elicitation with jasmonic acid (JA). Each of these factors alone increased anthocyanin content by 2-3 fold, when combined they provided an additive enhancement of 6-7 fold over controls. In contrast, less than a 2-fold increase in content of Piceid, the major stilbene to accumulate in grape cell culture, was observed for any of the experimental conditions, alone or combined.

Gene expression data gathered by Real-time RT-PCR implicated four major groupings of differentially regulated genes, based on transcript induction kinetics and magnitude of induction for the different culture conditions. PAL (Phenylalanine ammonia-lyase) and StSy (Stilbene Synthase), CHS (chalcone synthase) and DFR (Dihydroflavonol 4-reductase), and F’3’5OH (Flavonoid 3’5’-hydroxylase) were all genes that did not reflect anthocyanin production in terms of which condition induced maximum transcript. They differed in terms of induction kinetics, with PAL and StSy reaching maximum at 12 hours, CHS and DFR at 24 hours, and F3’5’OH 96 hours after elicitation. Maximum UFGT (UDP-Flavonoid Glucosyl Transferase) transcript was reached by 24 hours, but was differentially regulated by the culture conditions and its expression level reflected anthocyanin accumulation.

Enzyme assay data for UFGT and HPLC analysis of 3’5’OH anthocyanins supported gene expression data, and suggested that the post-biosynthetic events of methylation and acylation were similarly regulated as F3’5’OH.

Experiments based on pathway regulation data were carried out to optimise anthocyanin production, with a further enhancement of 20% achieved by a multiple time-point elicitation. Attempts were also made to improve anthocyanin composition to the more stable structures (methylated at the 3’ or 5’ position and/or acylated at C6 of the glucosyl moiety) by staggering induction with the different inducers, and there was some success in altering the rate of methylation. Results of experiments currently underway to further improve anthocyanin production and compositional quality will be discussed.
Purification of an acyltransferase from *Thunbergia grandiflora*

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The objective of the research was to isolate an acyltransferase from *Thunbergia grandiflora*, catalyzing the transfer of ferulic acid to an anthocyanin, malvidin 3, 5 diglucoside. The enzyme catalyzes 5-acyl transfer resulting in the formation of Malvidin 3-glucoside, 5-feruloylglucoside. The enzyme was purified by a two-step purification involving ultrafiltration through a 50 KDa polysulphone membrane and gel filtration using Sephadex G 100. 32-fold purity was obtained after gel filtration and a single band was obtained upon SDS PAGE of the purified protein. Molecular weight of the enzyme was determined to be 60,700 Daltons and the Km value was determined using the Hanes plot to be 0.28 µmoles/ml.
Multiple Allelism in Flavonoid Hydroxylation in Lisianthus (Eustoma grandiflorum Raf. Shinn.) Flowers and Colors

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The inheritance of three major anthocyanidins, pelargonidin (Pg), cyanidin (Cy) and delphinidin (Dp), was studied by self- and reciprocal cross-pollinations in lisianthus flowers. A clear phenetic segregation from progenies by the pollination was observed in which the B-ring hydroxylation of flavonoid synthesis was under regulation completely complementing with each other between Pg- and Dp-syntheses. The PgDp-phenotype does not exist and may accompany the Cy-synthesis. Based on progenies observation, it is suggested that multiple alleles control the flavonoid B-ring hydroxylation. Segregation patterns could be explained by five alleles designated $H_T$, $H_F$, $H_D$, $H_Z$ and $H_O$ in lisianthus flowers, controlling the 3'-; 5'-; 3',5'-; 3',5'-; and 3'- and 3',5'-hydroxylation, respectively. The Pg segregation ratio fit an expected one gene ($Pg$) model (3:1), concluding the presence of locus $Pg/pg$ in the dihydroflavonol reductase and/or leucopelargonidin oxidase levels (Figure). By numeric analysis of flower coloration using the CIELab color coordinate ($L^*$, $C^*$, $h$) in conjunction with phenotypes and genotypes of petal anthocyanidins, a new methodology has been proposed for breeding flower colors (Table).

Figure: Proposed multiple alleles in the B-ring hydroxylation system for flavonoid precursors involved in anthocyanin synthesis in lisianthus flowers. The $H$ refers the the flavonoid 3'- and 3',5'-hydroxylation.

Table. Example of cross-pollination between S4 and S3 lines to produce the objective F1 color ($L^*$- lightness, $C^*$- chromaticity, $H$ - hue angle).

<table>
<thead>
<tr>
<th>Lines</th>
<th>Phenotype</th>
<th>Genotype</th>
<th>L*</th>
<th>C*</th>
<th>h</th>
<th>Pg (%)</th>
<th>Cy (%)</th>
<th>Dp (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mellow Lavender S4</td>
<td>CyDp</td>
<td>$H_OH_Opgpg$</td>
<td>54.5</td>
<td>53.8</td>
<td>331.8</td>
<td>-</td>
<td>63.9</td>
<td>36.1</td>
</tr>
<tr>
<td>Asuka no Maihime S3</td>
<td>PgCy</td>
<td>$H_TH_TPg$</td>
<td>35.8</td>
<td>65.1</td>
<td>358.7</td>
<td>95.3</td>
<td>4.7</td>
<td>-</td>
</tr>
<tr>
<td>F1 hybrid (S4 x S3)</td>
<td>PgCyDp</td>
<td>$H_OH_TPpg$</td>
<td>44.0</td>
<td>58.8</td>
<td>345.0</td>
<td>36.5</td>
<td>60.9</td>
<td>2.6</td>
</tr>
</tbody>
</table>
Identification of Stilbenes from *Vitis vinifera* L.
Suspension Culture

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The interesting biological activities recently found in stilbenes such as induction of apoptosis in colon cancer and blood sugar reduction revealed the importance of plants containing stilbenoids as resources for the development of new drugs/functional foods. The *Vitis vinifera* L. is known to contain stilbenes; however, the low content levels of those compounds remain as a problem to be solved. The accumulation of stilbenes in *Vitis vinifera* L. cell culture, especially resveratrol, was reported to be significantly stimulated by adding elicitors, such as jasmonic acid.

One of the aims of our research is to develop an efficient method for obtaining the purified resveratrol from high-resveratrol producing *Vitis vinifera* L. suspension culture system. Effort is also directed to isolate and identify rare stilbenes, which are unable to purchase as commercial products at present, for analyzing their biological activities.

The material from the cell suspension culture was extracted by 100% MeOH, and then separated to hydrophilic and hydrophobic phases using EtOAc and H₂O. Rapid chromatographic estimation by TLC showed that EtOAc extract contained stilbenes like resveratrol and piceid. Resveratrol was further purified from the EtOAc extract by silica chromatography eluted using stepwise method (CHCl₃-MeOH-H₂O). A couple of unknown compounds that were purified by a combination of silica and Sephadex LH-20 column chromatography are being analyzed for chemical structures by NMR and LC-MS.
Large-scale Recovery of Anthocyanin Pigments from Red Wine by Low Speed Rotary Countercurrent Chromatography (LSRCCC)

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The separation capability of a “low speed rotary countercurrent chromatograph” (LSRCCC) was compared to a “high speed countercurrent” system (HSCCC) [1] for preparative isolation of pure anthocyanins from a Californian red wine. The tested LRCCC apparatus is a prototype system consisting of a single 2.75 liter teflon tube (8.2 mm ID). In comparison to HSCCC, scale-up of column capacity is much easier to accomplish due to a decreased rotational speed (60-80 rpm). For good retention of the stationary phase and mixing effects of the phase system a convoluted tube system is required. Interestingly, the solvent system used for separation of anthocyanins by HSCCC (tert-butyl methyl ether/ n-butanol/ acetonitrile/ water 2:2:1:5) showed excellent retention on the LSRCCC apparatus, even at higher flow rates of the mobile phase [2]. Separation of 3 g (HSCCC) and 10 g (LRSCC) of purified XAD-7 anthocyanin extract from the red wine yielded a baseline separation of anthocyanins. Order of elution was the same for both techniques. Structure elucidation of the separated fractions was done by HPLC-DAD, HPLC-ESI-MSn, 1H-, and 13C-NMR spectroscopy.

References
Anthocyanin Contents in Mulberry Fruits and their Purification with Macro-porous Resins

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Mulberry (\textit{Morus} sp), a tree of the Moraceae family, is widely used for its foliage to feed the silkworm, \textit{Bombyx mori} L. in many countries, especially in China. Like many other forage crops, breeding in mulberry mainly aimed at enhancing the foliage production through heterosis breeding. Owing to its small size, relatively low output, short storage-life, etc., mulberry fruit was not paid enough attentions to for a long time. The application of new technologies as breeding, processing, etc. made it possible to get good commercial profit in developing mulberry fruit. Mature mulberry fruit is rich in red pigments, and is a good source for production of natural mulberry red pigment. Anthocyanins are the major pigments responsible for the colour of mulberry fruits and the major compounds identified were cyandin-3-glucoside and cyanidin-3-rutinoside. The objective of this study was to check the anthocyanin contents in different cultivars of mulberry, and study the industrial process of mulberry anthocyanins as a natural food pigment. In the 105 cultivars of mulberry, the monomeric anthocyanin contents were between 147.68~2725.46 mg/L juice calculated as cyandin-3-glucoside. Extraction and purification using macro-porous resin seemed an efficient way to produce mulberry pigments on an industrial scale.
New Family of Bluish Pyranoanthocyanins

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Anthocyanins are the most important group of water-soluble plant pigments visible to the human eye. Their use has been investigated for the preparation of foods and beverages natural colorants as they seem to have non-toxic effects. In this context, vinylpyranoanthocyanins characterized by the general structure (Figure 1) were recently found to naturally occur in ageing red wine [1]. This new family of anthocyanin-derived pigments may be obtained directly through the reaction between genuine anthocyanins or their derivatives. Some of these newly formed pigments have been found to exhibit a bluish color at acidic pH. The formation of bluish pigment was obtained through reaction between anthocyanidin monoglucosides and vinylflavanol adducts. The formation of similar bluish pigments was attempted using different other vinyl precursors. The pigments were fully characterized by mass spectrometry and several NMR techniques.

The chromatic features of this kind of pigments bring promising expectations concerning the use of these naturally occurring blue pigments in the Food Industry.

Figure 1. General structure of vinylpyranoanthocyanin pigments wherein R₁, R₂, R₃ and R₅ independently of each other is H, OH, alkoxy, an –O-glycosyl group, an –O-glycosyl group which is substituted with one or more acyl groups, R₄ is H or OH, R₆ is an alkyl or aryl.

References
Photo-stability and Antioxidant Mechanism of Red Radish 
(*Raphanus sativus* L.) Anthocyanins

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The photo-stability and antioxidant mechanism of pelargonidin 3-sophoroside-5-glucoside acylated with different cinnamic acids (caffeoyl, feruloyl, or p-coumaroyl units) from red radish were examined. The photo-irradiation (fluorescence light, 5,000 lx; at 30 ºC) indicated that the red radish anthocyanins were more stable at lower pH than at higher pH. HPLC analyses of the red radish anthocyanins revealed that di-acylated anthocyanins were stabler at low pH (pH 3) than mono-acylated anthocyanins. Meanwhile, the number of the intramolecular acyl units would influence the photo-stability of acylated anthocyanins. At high pH (> pH 5), the species and binding site of the acyl units would influence the photo-stability, rather than the number of the acyl units. On the other hand, the antioxidant activity of acylated anthocyanins varied in the characteristics of the acyl units. The identification and formation behaviors of the reaction products of acylated anthocyanins with peroxyl radicals suggest that acylated anthocyanins would have radical scavenging abilities on some common sites even if the characteristics of the acyl units are different, and that the reaction rate of acylated anthocyanins are affected by the characteristics of the acyl units.
Delphinidin-3-rutinoside (D3R) is the major anthocyanin component isolated from blackcurrant (*Ribes nigrum* L.) fruits. Although anthocyanin is well known to have an ophthalmic activity, little is known about the detailed pharmacological properties of D3R. Present experiments were designed to analyze the relaxation mechanism of bovine ciliary smooth muscle (CM) with D3R. D3R produced a sustained and progressive relaxation during the contraction caused by endothelin (ET)-1. Change in phosphorylation ratio of myosin light chain in CM preparation was closely related to the D3R-induced relaxation. The relaxation in response to D3R was significantly attenuated by NOARG as NOS inhibitor, carboxy-PTIO as NO scavenger, ODQ as guanylyl cyclase inhibitor or BQ788 as ETB receptor antagonist and accompanied by the increased cGMP production. The inhibition with NOARG was reversed by excess L-arginine supplementation. However, iberiotoxin as Kca channel inhibitor, propranolol and indomethacin failed to modify the D3R-induced relaxation. Although ETA and ETB receptors were detectable by [*125*I]-ET-1 binding study, ETB receptor was predominant in both ciliary endothelium (EC) and CM, and kinetics of the binding was different in two preparations. These results suggest that D3R possibly stimulates ETB receptors localized in EC to produce/release NO, thereby causing relaxation of the bovine CM.
The Effect of Two Methods of Pomegranate (*Punica granatum*, L.) Juice Extraction on Quality during Storage at 4°C

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The pomegranate juice is not yet commercialised in Portugal, but it is an interesting alternative sell usage for fruits with bad appearance. In the present work two ways to obtain the pomegranate juice were compared (centrifugation of seeds and squeezing of half fruits with an electric lemon squeezer). Some quality parameters were evaluated (acidicity, pH, °Brix, sugars, organic acids and anthocyanins), along during 72 hours at 4°C. The results showed no significant differences at P<0.05, between the two treatments in the parameters studied. The exception was the titratable acidity, in which an important decrease was detected after 72 hours of storage in juice obtained by seed centrifugation, but not by fruit squeezing. The pH values ranged from 2.9, at the beginning of the experiment, to 3.2, at the end. The °Brix readings decreased over the time (14.6 to 13.7). Glucose and sucrose constituted the most important sugars of pomegranate juice, with concentrations that not exceeded 2 g/l. Oxalic and tartaric acids were predominant in fruit juice; pyruvic, malic, ascorbic, maleic, citric and fumaric acids could also be detected. The major acid levels decreased during the first storage hours (5-15 hours) and suddenly increased reaching the maximal values after 32 hours of cold storage (293 mg/l for oxalic acid and 229 mg/l for tartaric acid). The most important anthocyanin present in the pomegranate juice was delphinidin 3-glucoside (45-69 mg/l), followed by delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside and cyanidin 3-glucoside. Pelargonidin 3,5-diglucoside and pelargonidin 3-glucoside were present in the smallest amounts (0.1-6 mg/l). The content of almost all anthocyanins decreased over the time, independent on the treatment, with the exception of delphinidin 3,5-diglucoside, that showed no alterations.

It seems that juice characteristics were not significant affected by the way juice was obtained, within the time period tested. Other assays must be done in order to prior introduction it into the market.
Cultures conditions influencing anthocyanin production in *in vitro* cultures of *Daucus carota* L.

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*Daucus carota* L. cells cultured in a MS medium with indole-3-acetic acid and kinetin developed a red pigmentation. Maximum yield of anthocyanin at the end of three weeks was 5.4% on dry weight basis. The cultures subjected to phosphate and nitrate stress produced 7.2 and 8.5% anthocyanin respectively. Feeding of sucrose at 7.5% level resulted in production of 15% anthocyanin. Mannitol as osmoticum had positive influence on anthocyanin production. A 2-fold increase in anthocyanins was obtained by elicitation with elicitors derived from the extract of the yeast *Rhodotorula rubra*. Treatment with calcium ionophore A 23187 resulted in the enhancement of both growth and anthocyanin production. Ionophore treatment also resulted in higher Ca$^{2+}$ ATPase activity. Calcium-channel blockers, verapamil and chlorpromazine, resulted in lowering of growth and anthocyanin production, suggesting the involvement of calcium in anthocyanin production. The cell aggregate size had influence on anthocyanin content in suspension cultures. Over 92% of biomass was present in the aggregates of 500 – 1500 µm with maximum anthocyanin content in the cell aggregate diameter up to 500 – 850 µm. Anthocyanin was found to be potent antioxidant compared to classical antioxidant such as BHA, BHT and α-tocopherol. High yield of anthocyanin in cell cultures holds promise for scale up in bioreactors.
Bunch Exposure and Accumulation of Anthocyanins in Different Grape Cultivars (*Vitis vinifera* L.)

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Recent viticultural research has focused on the impact of bunch exposure on berry quality. Previous work in this field has been limited to a small number of cultivars without a clear trend emerging. Here we investigated the effect of shading on the anthocyanin concentration in the skins of 13 table and 14 winegrape cultivars. A shading treatment that enclosed the bunches and excluded light without otherwise affecting microclimate (temperature and humidity) was applied before the onset of ripening, in warm irrigated vineyards around Mildura in northwest Victoria, Australia. The grapes were harvested at a level of ripeness consistent with industry standards (table grapes 18-20°Brix; winegrapes 23.5-24.5°Brix). Skins were removed from the harvested grapes and anthocyanins extracted and analysed by reversed-phase HPLC. Anthocyanins were quantified against a commercial standard of malvidin-3-glucoside and the anthocyanin concentration was compared between the shaded and exposed fruit. In table grapes, the anthocyanin concentration was reduced by shading in 9 of the 13 cultivars examined. In two cultivars there was no significant difference in total colour and in two cultivars total colour increased with shading. In the winegrape cultivars, total anthocyanins decreased in six of the 14 cultivars with shading. There was no significant difference in colour in six cultivars and in two cultivars colour increased with shading. The anthocyanin composition of table grapes and winegrapes was also altered by shading. However, there was no discernible pattern to these changes. These results show that anthocyanin accumulation in some grape cultivars appears to be light sensitive. However, other cultivars showed no response to shading. Based on these data, over a single season, the general trend is a decrease in anthocyanin concentration with shading.
Genistein, found in soy beans, and resveratrol, found in grapes, are phenolic antioxidants. They may have effects on human cells manifest as changes in acute survival and chronic population growth potential. In addition, their antioxidant properties confer free radical (reactive oxygen species, ROS) scavenging abilities which may confer anticarcinogenic properties, although their mechanism of action is not fully understood. ROS are generated in cells as by-products of oxygen metabolism, as well as being induced by radiation and some xenobiotic compounds. ROS can be generated in an experimental setting using hydrogen peroxide.

The assays used in the experiments to be reported at the workshop are the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay which measures the number of viable human cells (WIL2NS lymphoblastoid cell line) with active mitochondria as an indicator of acute toxicity. Also, population expansion in flasks is measured by monitoring cells over a 7 day period and counting total viable cell number. In addition, the cytokinesis block micronucleus (CBMN) assay detects damage to chromosomes, including chromosome breakage, rearrangement or loss from the nucleus. Cells are prepared as slide smears, stained and score microscopically for the presence of chromosome fragments manifest as micronuclei.

The initial MTT assays indicate that resveratrol is not toxic to human cells at doses below 100 µM. Genistein showed lower toxicity when acute survival was measured using the MTT assay where survival of cells was almost the same as the control for 100 and 1000 µM. When monitoring population growth in flasks over a 7 day period, genistein showed only a small decrease in survival at doses of 100 µM and showed inhibition of population growth in flasks of approximately 50% at doses of 1000 µM. For the CBMN assay, doses of 1, 10 and 100 µM of Genistein, the frequencies of micronuclei were equivalent to the untreated control, whereas a dose of 1000µM showed an increased frequency. This toxicity, population growth and CBMN data needs to be replicated and followed up for different exposure times. This initial data are all from a 1 hour exposure. Additional data are being conducted on longer incubation times and will be presented at the workshop. Experiments are also being conducted on the protective properties of genistein and resveratrol against hydrogen peroxide induced damage. These results will be discussed at the workshop.
New Pyranoanthocyanins from *Vitis vinifera* L. cv. Pinotage Wines: Isolation, Identification, and Mechanism of Formation

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For the first time, we were able to isolate previously unknown anthocyanin-derived ageing products from Pinotage red wines (*Vitis vinifera* L.) by countercurrent chromatography and semi-preparative HPLC. The structures were fully elucidated by one- and two-dimensional NMR analyses and the pigments identified as the 4-vinylcatechol adducts of malvidin 3-glucoside, malvidin 3-(6''-acetylglucoside), and malvidin 3-(6''-coumaroylglucoside) [1].

So far, the formation of these so-called pyranoanthocyanins has been explained by the reaction of anthocyanins with substituted vinylphenols, which in turn should be generated during alcoholic fermentation of the must by decarboxylation of the corresponding hydroxycinnamic acids through a decarboxylase side activity of the wine yeasts. However, in model experiments we could show that such a reaction would be completed in a short time following fermentation and cannot explain the steady increase in the concentration of “aged” pigments observed in Pinotage red wines during years of storage.

After further model reactions we were able to prove that the predominant ageing product pinotin A 1 is formed by a direct reaction of malvidin 3-glucoside 2 with caffeic acid 3 (cf. figure). Other *p*-hydroxyphenyl-substituted pyranoanthocyanins are generated by reaction between anthocyanins and coumaric acid, ferulic acid, or sinapic acid. Substitution in *para*-position with an electron-donating group is essential for the reaction as it stabilizes the intermediate carbenium ion 4 [2].

References
Isolation and Identification of New Pyranoanthocyanins from Black Carrot (*Daucus carota* L.) Juice

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Black carrots contain diverse glycosylated and acylated pigments with cyanidin as common aglycon [1, 2]. In case of the three predominant pigments, cyanidin is substituted with a 6-O-acyl-β-D-glucopyranosyl-(1→6)-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranosyl moiety in position 3. The acyl group is mostly ferulic acid; the pigments esterified with coumaric acid or sinapic acid are usually present in lower concentrations. Two minor components are lacking the 6-O-acyl group or the 6-O-acyl-β-D-glucopyranosyl moiety, respectively. Because of the high pH-stability of these pigments, extracts from black carrot are commonly applied as natural food colorants.

For a short time, pure black carrot juices are available in health food stores in Germany and their consumption is being promoted with the high content of health-promoting anthocyanins. In this juice, additional pigments with a higher retention time and a hypsochromically shifted absorbance maximum compared to the known anthocyanins were detected by HPLC-DAD. The relatively high concentrations of caffeic acid (30 mg/l) and ferulic acid (20 mg/l) gave rise to the suspicion that substituted pyranoanthocyanins have been formed in the juice during storage, following the same reaction pathway as recently described for red wines [3]. The occurrence of such pigments was evidenced by HPLC-ESI-MS^3^ analyses of the unknown anthocyanin derivatives isolated by countercurrent chromatography. Five new pyranoanthocyanins were identified for the first time, namely the 4-vinylcatechol and 4-vinylguaiaicol adducts of 6-O-feruloyl-β-D-glucopyranosyl-(1→6)-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranosyl-(1→O^3^)-cyanidin (1, 2), and β-D-xylopyranosyl-(1→2)-β-D-galactopyranosyl-(1→O^3^)-cyanidin (3, 4), respectively. The 4-vinylphenol adduct of the latter (5) was also detected, but only in trace amounts.

References

Effect of Processing and Packaging Material on Anthocyanins Retention in Pomegranate (*Punica granatum* L.)

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Pomegranate (*Punica granatum* L.) belonging to the *Punicacea* family, also called Chinese apple, has been cultivated extensively in Mediterranean countries. In pomegranate fruit cracking at maturity is major problem leading to huge economic loss to farmers. The traditional utilization of the fruit lies in drying the seeds of these cracked fruits to yield a value added bi-product known as *anardana* used as acidulent in Indian curries also used in Ayurvedic and Unani medicines. One of main feature of quality grade anardana is the bright red colour of the seeds and this red colour is due to Anthocyanins, which are naturally occurring compounds. This colour depends on the anthocyanins concentration and change in amount of anthocyanins directly affect this quality factor. The major anthocyanins present in pomegranate are cyanidin-3, glucoside, delphinidin 3 glycoside and 3,5-diglucoside. Anthocyanins belong to a group of plant compounds called flavonoids that have antioxidant abilities and anticancer potential, prevents blood clotting, and defend cells against dangerous carcinogens. Therefore, attempts were made to study the effect of processing and packaging materials on retention of anthocyanins in anardana under different storage conditions.

Physico-chemical and organoleptic analyses of seven genotypes were carried out for processing into value added bi-product *anardana*. The results indicated Bassein Seedless having anthocyanin content 77.85 mg/100 g to be the best genotype for processing. Total anthocyanins were estimated for grains of various genotypes that ranged from 10.38-to 77.85-mg/100 g. In general most of the inter-genotype differences were quite high and significant. Similarly wide variation was observed for anthocyanin values in different *anardana* samples. It differed significantly for most of the samples prepared from different genotypes. Genotype Bassein Seedless showed highest value (305.06). Further studies were carried to standardize the best drying condition as well as the best pre-treatment for preparation of anardana. Steam blanching for 5 minutes before drying in a cabinet drier was considered essential for preparation of quality grade anardana. Sulphuring prior to drying as pre-treatment was rejected on nutritional aspect as it bleaches the anthocyanins of the fruits. Anthocyanin values were statistically significant with regard to interaction of packaging material, storage temperature and period. During six months of storage at ambient temperature anthocyanins (as OD) were maximum (0.582) in Plastic bottles (50% transparent) package and minimum (0.416) in High Molecular Milky package. At low temperature their value ranged from 0.602 (Low density polyethylene) to 0.810 (Plastic bottles (50% transparent)). The interaction effect of storage temperature and storage period on anthocyanins of *anardana* was also found to be statistically significant.
Photoprotective Role of Anthocyanin in *Commiphora wightii* (Guggul)

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*Commiphora wightii* (*Burseraceae*) is a highly valued endangered medicinal plant of arid zones of India and Pakistan. The contents of anthocyanins in the plants growing *in vivo* and *in vitro* were analyzed with UV-Spectrophotometer and High Performance Liquid Chromatography1. Present study shows the photoprotective role of anthocyanin in this plant. This plant has evolved a number of protective strategies, which minimize the impact of high temperature and UV-B radiation by synthesizing anthocyanin. In this plant anthocyanin is synthesized at the apical part of young stems under natural condition to protect meristematic cells, but when growing under shade, the apical part of young stems of the plants turns green. The UV-B radiation enhanced the anthocyanin biosynthesis by several fold.

References

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Sustainable Harvest and Trade of Medicinal and Ornamental Plants

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Sustainable harvesting of non-timber products (NTFPs) of which medicinal and aromatic plants (MAP) is a part, is yet to be followed in Nepal. High anthocyanin accumulating plants are among the many MAP plants growing in the Humla region, one of the country’s remote places. Since sustainability is linked, among many other factors with trade of MAP especially in areas such as Humla, where a majority of population is below poverty line, a mechanism must be developed which would allow the local people to gain maximum benefits out of “their” natural resources. We have identified 51 species of MAP, in this paper we discuss the mechanism for developing sustainable harvesting methods and to expedite marketing of MAP products.
Anthocyanins are widespread pigments of the plant kingdom. Previously, we have reported that dietary cyanidin 3-glucoside (C3G)-rich purple corn color significantly suppressed the development of obesity and ameliorated hyperglycemia induced by high-fat diet feeding in mice. In this study, we examined gene expression profile in adipocytes treated with anthocyanins and identified altered genes including lipid metabolism. Rat adipocytes isolated from epididymal adipose tissues were suspended in Dulbecco's modified Eagle's medium containing 0.5 % of bovine serum albumin and treated with 100 µM of C3G, its aglycon (cyanidin; Cy) or vehicle (0.1 % dimethyl sulfoxide) for 24 h. cRNA preparation and scanning of rat genome U34A array (Affymetrix) were performed according to the manufacturer's protocol. After normalization using control samples and performing quality control of gene intensity, 3962 genes were analyzed, and increased more than 1.5-fold genes compared to controls are listed and examined. One hundred and twenty eight genes were up-regulated in both C3G and Cy groups compared to the control group. The up-regulated genes include lipid metabolism and signal transduction related genes, however, the altered genes were different between C3G and Cy group. These results suggest that the chemical structure (aglycon or glucoside) can affect different response of gene expression in adipocytes.

Green tea is an effective chemopreventive agent in animal tumor bioassays and some human cancers. Much of its cancer preventive effects appear to be mediated by its major polyphenolic constituent (-) epigallocatechin-3-gallate (EGCG). In order to better understand the molecular regulation underlying the anti-proliferative activity of EGCG in prostate cancer, we have utilized cDNA microarray to elucidate how EGCG alters program of gene expression in prostate carcinoma LNCaP cells. Fluorophore-labeled cDNA probes synthesized from the untreated LNCaP cells or the cells treated for 12 h with EGCG (12 µM), a physiologically achievable dose, were competitively hybridized to the microarray that contained a total of 250 kinases and phosphatases genes. Such high-throughput screening has identified a number of EGCG-responsive gene candidates. Of these, we found that EGCG induced a subset of genes that functionally could exhibit inhibitory effects on cell growth. The genes repressed by EGCG mostly belonged to the G-protein signaling network. Interestingly, the protein kinase C-alpha (PKC-alpha) form, whose inhibition of expression has been shown to inhibit cell growth in some cancer cells, was selectively repressed by EGCG while the expression of six other PKC isoforms (beta, delta, epsilon, micro, eta and zeta) was unaffected. These EGCG-responsive genes may provide key insights into the mechanisms of action of other polyphenolic compounds in prostate cancer chemoprevention.
Advances in Genetic Engineering of Ornamental Plant

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The cloning and applications of the plant anthocyanin biosynthetic genes flower organ identity genes and flower senescence genes were reviewed. Plant anthocyanin biosynthetic pathways and involved enzymes have been characterized. Many structural and regulatory genes controlling flower color were cloned using direct gene introduction anti-sense RNA and co-suppression techniques. Some flower plant cultivars with different colors were obtained. Flower pattern can be changed from modifying the genes controlling flower organ identity. The cloned genes opened up a wide range of application in plant science research, such as the creation of cultivars with new flower color, leaf color, flower form, and postponed flower cutting period.
Overexpression of Anthocyanin Regulatory Genes in Wheat Endosperm and Its Implication for Functional Genomics

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Functional genomics is the major issue of biological and agronomic sciences in this era. With genomics progress, more and more genes are sequenced. Nevertheless, the function of most genes, which can be invaluable both academically and potential commercially, remains unclear. Gene tagging via transposon insertion mutagenesis is an important strategy for new gene isolation and characterisation. Future genetic improvement of wheat, one of the major cereal crops, will increasingly be dependent on knowledge of the roles of individual genes rather than large genetic loci.

In Arabidopsis, gene disruption has been used to great effect to generate knockout mutants that permit functional analysis of the affected gene. Where the disruption is coupled to the use of insertion sequences such as T-DNA or transposable elements this can lead to the very rapid linkage of phenotype and gene.

It is our intention to construct a similar system in cereals; however, a number of issues arise. Arabidopsis produces a large quantity of small seeds that are easy to sow at high density on to selection plates and identify the relatively rare individuals in which transposition has occurred. This selection system may not then be feasible with wheat, which produces much fewer, as well as much larger seed than Arabidopsis. We wish therefore to set up a system based on visual markers. The visual system we have been testing is based on the production of the red pigment anthocyanin. Several regulatory genes have been isolated from maize that when over-expressed cause the over-production of anthocyanin in different tissues. In wheat, however, it has been reported that excess anthocyanin can inhibit normal development. We therefore wished to confine its expression to the endosperm. We engineered two constructs containing either the regulatory gene C1 or Lc driven by the endosperm specific promoter pHMW-glutenin. Results indicated that both C1 and Lc genes could be essential for anthocyanin production in the immature embryo of wheat seed tissue.

Both genes, when driven by the CaMV35S promoter, were required to be co-transformed into wheat embryos via particle bombardment in order to generate any anthocyanin production. More recently, we used the endosperm specific promoter driven C1 vector combined with CaMV35S::Lc to transform wheat endosperm. In the early stages of young endosperm development (< 7 days past anthesis), large sharp sectors of anthocyanin were formed.

This data suggests a visible section system based on anthocyanin could be used for large scale of wheat transformation studies, in particular in the generation of a transposon tagged population. Incorporation of the C1 and Lc genes into our Ac/Ds constructs should coincide with the demonstration of stably transformed wheat and these will be among the first constructs to be transferred. The constructs are also available for transfer into rice.
Regulation of Anthocyanin and Stilbene Biosynthesis in
_Vitis vinifera_ L. Suspension Culture by Elicitation

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Growing interest in the use of anthocyanins and stilbenes as chemo-preventative agents for cancer and cardiovascular diseases in humans inspired us to investigate the production of these metabolites in plants and plant cell cultures. Elicitors, the substances of biotic or abiotic origin, have been widely used to increase the production of secondary metabolites in plant cell and tissue cultures. The aim of this study is to investigate the regulatory mechanism of several potential elicitors on anthocyanin and stilbene biosynthesis in _Vitis vinifera_ L. v. Gamay Fréaux suspension culture toward the metabolic optimization of anthocyanin and/or stilbene production.

To differentiate the regulation patterns of various elicitors, seven single elicitors (methyl-β-cyclodextrin (MC), betaine, 3-methyl-salicylic acid (MSA), salicylic acid (SA), chitosan, jasmonic acid (JA) and β-glucan) and three elicitor combinations (SA and JA, β-glucan and JA, chitosan and JA) were examined. The elicitors were added on day 4 of the culture and the culture kinetics were analysed on day 7 and 10. Among the elicitors examined, JA was the only elicitor, which enhanced anthocyanin production. Anthocyanin production decreased with β-glucan treatment, while SA and chitosan did not have any effect. The combination of JA with SA and β-glucan decreased anthocyanin production compared with JA added as a single treatment. The production of _trans_-resveratrol was unchanged in response to MC, betaine, MSA, JA, SA, β-glucan and chitosan were found to increase the production of intracellular _trans_-resveratrol to reach a maximum level of 10 to 15 mg/L, which was approximately 2 to 3-fold higher than that of the control. The combination of SA and JA had a negative effect; and β-glucan and JA did not have any additive effect on the production of intracellular resveratrol.

The inhibition of anthocyanin production by β-glucan was observed even though the overall production of phenolics was increased. However β-glucan increased the production of intracellular resveratrol. It is known that stilbene synthase (STS) and chalcone synthase (CHS) - the branch-point enzymes of the biosynthetic pathways of stilbenes and anthocyanins that use the same substrates _p_-coumaroyl CoA and malonyl CoA. Therefore, the decrease in the level of anthocyanins is possibly explained by competition for common substrates between STS and CHS. In contrast to the effect of β-glucan, JA stimulated the accumulation of both anthocyanins and stilbenes (piceid, and intracellular and extracellular resveratrol).

These results demonstrate the specific regulatory role of elicitors on different metabolic pathways and could be applied to redirecting the metabolic flux toward the desired bioproducts.
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