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ABSTRACTS

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Towards a Brighter Side of Life

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Plenary lectures

Xanthophyll-binding proteins: key mediators for delivery of the macular pigment carotenoids to the human eye

Paul S. Bernstein

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Unlike all other mammals, humans and fellow primates uniquely concentrate the xanthophyll carotenoids lutein and zeaxanthin in the retina of the eye in a yellow colored region known as the *macula lutea*. Over the past fifteen years, our laboratory has endeavored to elucidate the biochemical processes underlying the selective accumulation of these macular pigment carotenoids into the human eye. Using a combination of preparative biochemical and molecular biological approaches, we have identified the two key xanthophyll-binding proteins responsible for the uptake and stabilization of the macular carotenoids, GSTP1 (dietary zeaxanthin and non-dietary *meso*-zeaxanthin) and StARD3 (dietary lutein). In this plenary lecture, I will review how the identification of these xanthophyll-binding proteins enhances our understanding of the role of the macular pigment carotenoids in the maintenance of human ocular health, and I will place these binding proteins in the larger context of our current knowledge of carotenoid transport and deposition in diverse organisms.

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Are non-vitamin A active carotenoid cleavage products metabolically active?

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The metabolic cleavage of β-carotene has been well studied and retinoids produced from β-carotene or other pro-vitamin A carotenoids are known to be critical for night vision (retinal isomers) and for growth, reproduction and other functions (retinoic acid isomers). 9-*cis*-retinoic acid impacts a variety of other body functions as a ligand for the promiscuous nuclear receptor, RXR. An emerging area of interest are metabolic or oxidative cleavage products of other carotenoids. As many of these compounds have retinoid-like structures, it has been hypothesized that some may be agonists or antagonists in various transcription systems. For example, we have suggested that metabolic products of lycopene, the so-called lycopenoids, may influence gene expression via nuclear receptors such as RARs, RXRs, PPARs, LXRs, etc (Lindshield et al., 2007). Kopec and colleagues (Kopec et al., 2011) have identified a variety of lycopenoids in human plasma at levels near those seen for retinoids. The recent emergence of mice lacking either of the two important carotenoid cleavage enzymes, has provided models to study carotenoid cleavage products more directly. This presentation will explore studies from knock-out models and other approaches that suggest that carotenoid metabolites as well as the carotenoid cleavage enzyme null genotypes influence lipid metabolism as well as steroid hormone metabolism. In addition, the effects of tomato carotenoids on prostate carcinogenesis through a proposed anti-androgenic mechanism will be discussed.

Supported by PHS-1-RO1CA125384 and PHS-R21AT005166.

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A journey along the pathway of carotenoid biosynthesis: more enzymes and new routes of interactions with plant metabolism

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In plants, carotenoids are integral components of the photosynthetic apparatus and thus are indispensable in all green tissues. As precursors in the production of the phytohormones abscisic acid (ABA) and strigolactones, carotenoids serve central physiological and developmental functions in plant adaptation to stress conditions. In addition, they play important roles in plant reproduction by furnishing flowers and fruit with distinct pigmentation designed to attract animals.

Carotenoids are synthesized within the plastids from the methylerythritol phosphate (MEP) pathway, which accounts for the plastidial isoprenoids, though all the biosynthesis enzymes are nuclear encoded.

We are studying carotenoid biosynthesis and its regulation in flowers and fruit of tomato (*S. lycopersicum*), which has become a model system for chromoplast-containing plants. Over the years we have developed various genetic tools to decipher carotenogenesis in plants by cloning and analyzing genes that encode enzymes of the pathway. To this end, we have isolated novel mutations in tomato that alter pigmentation of flowers and fruit. Through characterization of these mutations we have identified new enzymes in the carotenoid biosynthesis pathway. Recent results demonstrated the importance of RedOx to the biosynthesis of carotenoids and revealed links between carotenoid biosynthesis and plastid biogenesis.

This work was supported by the Israel Science Foundation Grants 548/05 and 1685/09 by the EU-FP6 EU-SOL.

Excited-state dynamics of overlapped optically-allowed $1B_u^+$ and optically-forbidden $1B_u^-$ or $3A_g^-$ vibronic levels of carotenoids: possible roles in the light-harvesting function

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Pump-probe spectroscopy after selective excitation of all-*trans* Cars (n = 9-13) in nonpolar solvent identified a symmetry selection rule of diabatic electronic mixing and diabatic internal conversion, i.e., '$1B_u^+$-to-$1B_u^-$ is allowed but $1B_u^+$-to-$3A_g^-$ is forbidden'. Kerr-gate fluorescence spectroscopy showed that this selection rule breaks down, due to the symmetry degradations when the

Car molecules are being excited, and, as a result, the $1B_u^+$-to-$3A_g^-$ diabatic electronic mixing and internal conversion become allowed.

On the other hand, pump-probe spectroscopy after *coherent excitation* of the same set of Cars in polar solvent identified three stimulated-emission components (generated by the quantum-beat mechanism) consisting of the long-lived coherent cross term from the $1B_u^+ + 1B_u^-$ or $1B_u^+ + 3A_g^-$ diabatic pair and incoherent short-lived $1B_u^+$ and $1B_u^-$ or $3A_g^-$ split incoherent terms. The same type of stimulated-emission components were identified in Cars bound to LH2 complexes, their lifetimes being substantially shortened by the Car-to-BChl singlet-energy transfer. Each diabatic pair and its split components appeared with high intensities in the first component. The low-energy shifts of the $1B_u^+(0)$, $1B_u^-(0)$ and $3A_g^-(0)$ levels and efficient triplet generation were also found.

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Carotenoids and their derivatives prevent cancer by affecting the activity of diverse transcription systems

Joseph Levy

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The basis for the vivid color of carotenoids and their antioxidant activity is the multiple conjugated double bonds which are characteristic for these plant-derived micronutrients. Moreover, the cleavage of these oxidation-prone double bonds leads to the formation of apocarotenoids. An amazingly large number of different carbonyl-containing oxidation products are expected to be produced as a result of carotenoid oxidation and these can be further metabolized to the corresponding acids and alcohols. Indeed, many, but not all, of these potential products have been detected and identified in edible plants as well as in human and animal plasma and tissues. Some of these compounds were found to be biologically active as anticancer agents. In addition to the inhibition of cancer cell proliferation, several carotenoid metabolites were shown to modulate the activity of various transcription systems. These include the ligand-activated nuclear receptor family, such as the retinoic acid receptor, retinoid X receptor, peroxisome proliferator-activated receptor and estrogen receptor, as well as other transcription systems which have an important role in cancer, such as the electrophile/antioxidant response element pathway and nuclear factor-κB. Therefore, carotenoid oxidation products can be considered as natural compounds with multifunctional, rather than monofunctional, activity and, thus, can be useful in the prevention of cancer and other degenerative diseases.

Synthesis of highly ^{13}C enriched carotenoids: access to carotenoids enriched with ^{13}C at any position and combination of positions

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Carotenoids and their metabolites are essential factors for the maintenance of important life processes such as photosynthesis. Animals cannot synthesize carotenoids de novo, they must obtain them via their food. In order to make intensive animal husbandry possible and maintain human and animal health synthetic nature identical carotenoids are presently commercially available at the multi-tonnes scale per year. Synthetically accessible 13C enriched carotenoids are essential to apply isotope sensitive techniques to obtain information at the atomic level without perturbation about the role of carotenoids in photosynthesis, nutrition, vision, animal development, etc.

Simple highly ^{13}C enriched C_1, C_2 and C_3 building blocks are commercially available via 99% ^{13}CO. The synthetic routes for the preparation of the ^{13}C enriched building blocks starting from the commercially available systems are discussed first. Then, how these building blocks are used for the synthesis of the various ^{13}C enriched carotenoids and apocarotenoids are reviewed next. The synthetic schemes that resulted in ^{13}C enriched β-carotene, spheroidene, α-carotene, astaxanthin, (3*R*,3*R*')-zeaxanthin and (3*R*,3*R*',6*R*')-lutein are described. The schemes that are reviewed can also be used to synthetically access any carotenoid and apocarotenoid in any ^{13}C isotopically enriched form up to the unitarily enriched form.

This paper is written in respectful memory of Dr. Otto Isler, pioneer in the industrial production of synthetic, nature-identical carotenoids. The authors are thankful to the organizations that supplied the financial resources to carry out this work. This paper has been written with great indebtedness to the investigators in the Leiden group and researchers worldwide whose contributions have been essential to the work described in this review. The senior author is grateful for the friendship and support of the members of the carotene club.

Carotenoids – mechanisms of photoprotection in the skin

Wilhelm Stahl

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The skin is the outer barrier of the organism and protects against external stressors including UV-irradiation. Light-dependent photooxidative processes generate reactive oxygen species (ROS) which damage biologically relevant molecules and cellular structures. UV-light may further interact directly with DNA bases inducing the formation of pyridine dimers. As a result of UV damage cells respond with adaptation to stress and defense signaling. Photoprotection of the skin may be realized at different levels of defense e.g. absorption of light, scavenging of ROS, or modulation of signaling pathways.

Carotenoids have a unique structure and are essential for photoprotection in plants and likely suitable for protecting humans. Intervention studies with carotenoid supplements or diets rich in carotenoids have demonstrated that they contribute to endogenous photoprotection ameliorating UV-induced erythema (sunburn). Photoprotection through dietary components such as beta-carotene or lycopene in terms of sun protection factor is

considerably lower than that achieved with a sunscreen. However, it may contribute to basal protection and thus increase the defense against UV light-mediated damage to skin.

The mechanisms underlying this effect are not completely understood but antioxidant activity and/or interference with inflammatory, apoptotic, and adaptive signaling play a role. The solar erythema is an inflammatory reaction to overexposure to UV light and it his been discussed whether carotenoids prevent its formation or suppress a desired physiological reaction. Modulation of signaling may not only be mediated by parent carotenoids. Apocarotenals of lycopene for example address Nrf-2 dependent pathways triggering the expression of defense systems against oxidants and other electrophiles. Apo-carotenoic acids interfere with retinoic acid-sensitive signaling.

Basic research in model systems contributes to the understanding of protective mechanisms, allows to elaborate structure-activity relationship and helps to understand the properties of natural and non-natural carotenoids. Carotenylflavonoids are synthetic hybrids comprising structural elements of elements of both classes. Compared to the parent carotenoids or flavonoids these compounds exhibit improved photoprotective properties and they outperform the individual constituents with respect to antioxidant properties. Isorenieratene and 3,3'-dihydroxyisorenieratene are aromatic carotenoids carrying phenylic and phenolic residues, respectively. Both compounds are efficient antioxidants preventing lipid, protein and DNA oxidation and suppress the expression of UV-sensitive hemeoxygenase-1. In addition to other carotenoids both compounds prevent the formation of thymidine dimers which is likely due to their UV-absorbing properties related to the aromatic elements in their structure.

Xanthophyll-membrane interactions at high and low xanthophyll concentrations

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Membrane localization of some portion of carotenoids in bacteria, plants, and animals is commonly accepted. However, the function of carotenoids in membranes is unclear. To understand the basic mechanisms and effects of carotenoids, it is necessary to understand carotenoid-membrane interaction.

For systems with a high carotenoid concentration (for example, in bacteria and plants in which the local carotenoid concentration in membranes can reach a few mol%), it is most important to understand how carotenoids affect the physical properties, structure, and dynamics of the membrane. Leading conclusions drawn from previous investigations are that carotenoids (1) shift to lower temperature and broaden the main phase transition of phosphatidylcholine membranes; (2) decrease membrane fluidity and increase the order of alkyl chains; and (3) increase the hydrophobicity of the membrane interior. The effects of carotenoids are strongest for xanthophylls. These results suggest that anchoring carotenoid molecules at opposite membrane surfaces by polar hydroxyl groups is significant to enhance their effects on membrane properties.

In animals, the highest concentration of carotenoids is found in the retinas of primates. But even there, the xanthophyll concentration in the lipid-bilayer portion of the membrane is much lower than 1 mol%. For systems with a low carotenoid concentration, it is especially important to understand how the membrane itself – its composition, structure, and lateral organization – affects the organization of carotenoids in the lipid bilayer, including their orientation (transmembrane vs. parallel) and localization (distribution between membrane domains). Macular xanthophylls are not distributed uniformly in membranes containing domains. Xanthophylls are substantially excluded from domains enriched in saturated lipids that contain a high concentration of cholesterol (raft domains), and remain ~10 times more concentrated in domains that contain mainly unsaturated lipids (including highly unsaturated docosahexaenoyl acid) and much smaller amounts of cholesterol. The localization of macular xanthophylls in domains formed from unsaturated lipids is ideal if they are to act as a lipid antioxidant, which is the most accepted mechanism through which lutein and zeaxanthin protect the retina from age-related macular disease.

Supported by grant EY015526 of the NIH.

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Session 1

Nutritional Carotenoids and Their Implication in Human Health

Professor Norman I. Krinsky

in Memoriam

Professor Norman I. Krinsky
1928 – 2008

Professor Norman Irving Krinsky, a scientist who led the field of carotenoid research, passed away on November 28, 2008 after more than a half century of dedication to research with these plant pigments. Born in Michigan's Upper Peninsula, Norman began his college education at the age of 16 years at the University of Illinois at Urbana-Champaign but finished up with a B.S. and M.S. degrees in biochemistry at the University of Southern California. In 1952 he received a Ph.D. in biochemistry with a dissertation entitled "Studies of Carotenoid and Vitamin A Complexes with Protein in Plasma and Tissues". It was novel work at the time, as the biological importance of carotenoids to health was not well recognized. In 1953, Norman came to Harvard University where he worked with George Wald who received a Nobel Prize in Medicine for his work with vitamin A and vision. Their work together investigated vision in a variety of species. He was at Harvard for seven years, during the latter part of which he discovered his love of teaching while serving as an instructor and lecturer in the Department of Biology.

In 1960, Norman accepted a position as an assistant professor at Tufts University School of Medicine, where he remained for the rest of his career. It was in these early days that Norman realized that the importance of carotenoids spanned across many forms of life. His studies in algae and bacteria laid down some of the groundwork for the biochemical conversions and function of various carotenoids. Throughout these decades were studies employing the conventional laboratory rat, evaluating beta-carotene metabolism. His work with rabbits looked at the conversion of carotenoids to vitamin A and retinoic acid. The monkey model allowed for exploration into the biological control of carotenoids in the primate retina.

An additional significant contribution that Norman made to the field of carotenoids were the studies that evaluated the bioconversion of carotenoids in various food vehicles to vitamin A. Apart from his work in the field of carotenoids and antioxidants, Norman was deeply interested in the recent advances of others. In 1973, the increasing interest in the field of free radicals and related oxidants in relation to health and disease initiated a Gordon conference on "Oxy-Radicals in Biology and Medicine". Norman was a part of this initiative and was the first chair of a conference that continues today. In 1992, he chaired the first Gordon Research Conference on "Chemistry and Biology of Carotenoids" which also continues to this day.

From his early years at Harvard and throughout his career at Tufts, Norman was continually involved with students. He loved research, but was passionate about teaching and he wanted others to carry on that passion. When he retired from Tufts in 2001, he and his wife established the Norman & Susan Krinsky Excellence in Teaching Award for students in the Sackler School of Graduate Biomedical Sciences and the Medical School at Tufts. This award honors Norman's 40 years of service in the Sackler School and is meant to recognize individuals who have shown sustained teaching excellence. This award has been presented to graduate students in fields including biochemistry, genetics, immunology, microbiology and pharmacology.

Dr. Krinsky continued his dedicated interest in carotenoids, with a publication up until the year of his death. Norman passed away the day after Thanksgiving 2008 at which he enjoyed the occasion with his loving family, his wife Susan, their daughter Lisa, their son Adam, and their two grandchildren, Colin and Jenna. His wit, wisdom, and wonder will be missed by all.

INVITED LECTURES

Carotenoid and retinoid metabolism in hepatic stellate cells (HSCs) and their relationship to hepatic disease

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Approximately 70% of the retinoid (vitamin A and its metabolites) present within most healthy mammals is found in the liver. Within the liver, approximately 70-90% of retinoid is stored as retinyl ester within lipid droplets that are a distinguishing characteristic of hepatic stellate cells (HSCs). The HSC lipid droplets are also a site of hepatic β-carotene accumulation. HSCs possess all of the metabolic machinery needed for metabolizing retinoids and also express both carotene-cleaving enzymes, *Bcmo1* and *Bcmo2*. We have been interested in a number major questions regarding HSC retinoid storage and metabolism. The first concerns why evolution has selected the HSC as the site where the majority of retinoid within the body is stored. We hypothesize the retinoid storage within HSCs may protect the liver against injury. To assess this possibility, we have employed four models to induce liver injury, alcohol-induced liver disease, partial hepatectomy, carcinogen induced hepatocellular carcinoma, and CCl4-induced hepatic fibrosis in wild type mice that can store and mobilize retinoids normally, in LRAT-deficient mice which are unable to store retinoid within the liver and in RBP-deficient mice that store retinoid normally but which are unable to mobilize hepatic retinoid stores. Outcomes from these studies suggest that HSC retinoid storage and mobilization and carotenoid accumulation may be important for preventing development of liver injury. A second area of interest centers on the biochemical and cellular processes involved in the genesis and dissolution of lipid droplets present within hepatic stellate cells. Here, we are interested in understanding processes important for the formation and enlargement of these lipid droplets in times of excessive dietary vitamin A intake and in the dissolution of these droplets in times of diminished dietary vitamin A intake or upon injury to the liver. Data from these lipid droplet studies will also be presented.

Effects of carotenoids on DNA damage and repair: relevance to human disease

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Carotenoids have been considered as likely candidates for a role in protecting against disease for at least 20 years, and there is a widespread belief that antioxidants in the diet, including carotenoids, can prevent diseases such as cancer, cardiovascular disease and diabetes by counteracting the oxidative damage to biomolecules that is thought to underlie, or exacerbate, these diseases. Carotenoids are classical radical scavengers, and their ability to suppress oxidation in vitro has been demonstrated; for instance, they prevent the oxidation of low density lipoprotein particles by a free radical generating system. In cell culture, certain carotenoids have been shown to decrease the oxidation of DNA by H_2O_2.

To assess the relevance of these findings to human health, a common approach is to use DNA oxidation in lymphocytes (measured with the comet assay, single cell gel electrophoresis) as a biomarker for the effectiveness of various antioxidants and antioxidant-rich foods in vivo. An early finding was a negative correlation between plasma carotenoid concentration and DNA base oxidation in humans (though causality could not be deduced; carotenoids might simply have acted as a marker of total fruit and vegetable consumption, with another ingredient of those foods being responsible for the effect). Intervention studies with carotenoids as supplements have shown, in many but not all cases, a decrease in endogenous DNA oxidation in lymphocytes and an enhanced resistance to damage by H_2O_2 ex vivo.

However good biomarkers are, they are not a substitute fror the true endpoint, disease or death. Numerous clinical trials with β-carotene supplementation have been carried out. A recent meta-analysis (Bjelakovic et al., 2007) showed that β-carotene, vitamin A and vitamin E, taken singly or together, actually increase mortality.

Whatever the role of carotenoids in vivo, it is unlikely that antioxidant activity is the whole story. There is recent evidence from cell culture experiments that β-cryptoxanthin enhances the ability of cells to repair oxidation damage to bases in DNA (their second line of defence), and this is supported by findings of enhanced DNA base excision repair in humans given kiwifruit as a supplement, or a diet rich in fruit and vegetable products. But we are still far from understanding how the complex mix of phytochemicals in fruits and vegetables protects us from disease.

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Lutein and zeaxanthin: relationships to cognitive function in the elderly

Elizabeth J. Johnson

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Cognitive impairment affects nearly one in four community-dwelling elders and is a major risk factor for development of dementia later in life. Cognitive impairment in the elderly is receiving increased attention because of the possibility that early intervention may prevent or delay progression to dementia. Findings from our studies suggest that the xanthophylls, lutein (L) and zeaxanthin (Z), which benefit individuals with early stage age-related macular degeneration, may also be important in cognitive function in the elderly. L and Z cross the blood brain barrier and exclusively accumulate in the macular region of the retina, where they are referred to as macular pigment (MP). In a study of healthy older adults, MP was found to be significantly related to cognitive tests that assessed processing speed, accuracy and completion ability. Our work in primates showed that retinal L and Z were significantly related to brain L and Z concentrations. MP is thus a biomarker for brain L and Z concentrations. In the Georgia Centenarian Study population we found that among the serum carotenoids, L had the strongest relationships with global cognitive function, memory, recall, retention, verbal fluency, and dementia severity. In decedents of the same study we found that brain Z concentration was significantly related to antemortem measures of global cognitive function, memory, verbal fluency and dementia severity after adjusting for age, education, sex and self-reported diabetes or hypertension. Brain L concentration was related to recall and verbal fluency, but the associations were attenuated after adjustment for covariates. We have also shown that L supplementation significantly improved verbal fluency scores in healthy older women. The sum of our findings suggests that L and Z embedded in brain tissue are capable of influencing cognitive function in the elderly. Additional research will be necessary to confirm these relations.

Beta-carotene, and metabolic effect of fatty acids on the Connexin 43 expression in stressed endothelial cells.

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Cellular stressors cause changes in the inner mitochondrial membrane potential ($\Delta\psi$) leading to mitochondrial swelling and cell apoptosis, as well as induction of protective for cell survival autophagy. Connexin 43 (Cx43) is a main gap junction protein in endothelial cells. The mitochondrial redistribution of Cx43 by beta-carotene during the homocysteine/ischemia-induced cellular stress has been reported. Free fatty acids (FFA) as well as TNFα, especially in the postprandial period, are inducers of metabolic stress that lead to development of the metabolic syndrome cardio-vascular complications.

Aim of the study is to follow effects of beta-carotene, selected dietary FFA on the Cx43 expression and mitochondrial function in the endothelial cells challenged with TNFα.

Methods: HUVECs were incubated with non-toxic, physiological (10-30 μM) concentrations of beta-carotene (BC) (3-30 μM); albumin-bound palmitic (PA), oleic (OA), eicosapentaenoic (EPA) or arachidonic (AA) acids for 24 hours. 5ng/mL TNFα was added for the last 4 hours of incubation. Expression of *Cx 43* gene was analyzed by the quantitative real-time PCR (qRT-PCR) method. The Cx43 protein concentration in whole cells, as well as in isolated mitochondria was measured by western blot. Changes in the mitochondrial membrane potential ($\Delta\psi$) were measured by flow cytometry, while $\Delta\psi$ and the localization of Cx43 protein were analyzed by BD Pathway 855 Bioimager. ATP lrvel was measured by ATPlite TM Luminescence ATP Detection Assay.

Results: The significant decrease of following incubation with TNFα ($p=0.003$) as well as with PA ($p=0.042$) and OA ($p=0.002$) was observed. On the contrary, AA ($p=0.047$) as well as EPA ($p= 0.004$) led to increase of $\Delta\psi$. Initial incubation with EPA or AA also partially prevented the TNFα-induced decrease of $\Delta\psi$. Incubation with AA resulted in the up-regulation of *Cx43* gene expression. Addition of AA as well as PA significantly increased Cx43 protein cellular content. In spite of changes in the gene expression, any effect of BC on Cx43 protein and distribution was found in HUVEC.

Conclusions: The up-regulation of *Cx43* expression and Cx43 protein concentration along with normalization of the mitochondrial function ($\Delta\psi$) and increased ATP generation seems to be one of the mechanisms of EPA-mediated protective effect in the endothelial cells.

Physiological effects of single nucleotide polymorphisms in the β-carotene 15,15'-monoxygenase (BCMO1)

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Since humans are unable to synthesise vitamin A *de novo*, they must consume diets containing preformed vitamin A or provitamin A carotenoids. β-carotene, the most abundant provitamin A carotenoid in the diet, is converted to retinal by β-carotene 15,15'-monoxygenase (BCMO1). However, β-carotene absorption and/or conversion into retinal is extremely variable among individuals, with proportions of low responders to dietary β-carotene as high as 45%. Recently, two common non-synonymous single nucleotide polymorphisms (R267S; rs12934922 and A379V; rs7501331) with variant allele frequencies of 42% and 24%, respectively, were identified in the BCMO1 coding region that revealed reduced catalytic activity [1]. In addition to this, three common polymorphisms (rs6420424, rs11645428 and rs6564851) in the upstream region of BCMO1 also reduce the catalytic activity of BCMO1 by 59%, 51% and 48%, respectively. Since these data were obtained after consumption of 120 mg of β-carotene supplements, results from our on-going study using $^{13}C_{10}$ β-carotene at physiological concentrations will also be presented. In summary, a range of single nucleotide polymorphisms within and upstream of BCMO1 can influence the effectiveness of using plant based pro-vitamin A carotenoids to increase vitamin A status.

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Retinoids and carotenoids in adipose tissue biology

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Accumulated evidence indicates that, owing to genomic and extragenomic actions, vitamin A derivatives can impact body fat content in mammals through effects on energy and lipid metabolism. In adipocyte cell cultures, retinoic acid (RA), the carboxylic form of vitamin A, influences differentiation and lipid deposition, mitochondrial uncoupling, oxidative metabolism and the expression of adipokines. Moreover, treatment in vivo with RA has been shown to reduce body fat and improve insulin sensitivity in lean and obese rodents by enhancing fat mobilisation and energy utilization systemically, in tissues including brown and white adipose tissues, skeletal muscle and the liver. Recently it has been showed that β-carotene (BC)-derived RA can influence differentiation and lipid metabolism in adipocytes in culture. In adition, it has been described an adiposity reducing effect of long-term BC supplementation in intact mice. This study revealed that the effects of BC on adipocyte biology are clearly dependent on BC-15,15'-oxygenase production of retinoids, and are mediated by a reduction of PPARγ activity in adipocytes. Together, these results suggest a role of retinoids and carotenoids in adipose tissue biology, with potential implications for chronic disorders including obesity, diabetes, nonalcoholic fatty liver disease and atherosclerosis.

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Rice and maize rich in β-carotene are superior dietary sources of vitamin A

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Vitamin A is essential for the promotion of general growth, maintenance of visual function, regulation of differentiation of epithelial tissues, and embryonic development. In an effort to provide long term and sustainable prevention of vitamin A deficiency (VAD), staple foods, such as rice and maize, that biofortified with β-carotene were developed. To evaluate their vitamin A value in humans, high β-carotene rice and maize were hydroponically produced and given to human subjects (in US, Asia & Africa). To quantitatively evaluate the vitamin A equivalence of the labeled rice and yellow maize β-carotene in human subjects, a known amount of [$^{13}C_{10}$] retinyl acetate in corn oil was used as a reference dose, thus, the absorption and the conversion of β-carotene to retinol in vivo can be determined.

Scientists have engineered components of the provitamin A biosynthetic pathway into rice endosperm to produce high β-carotene "Golden Rice". The Golden Rice may contain up to 35 μg β-carotene in a gram of rice. Bioconversion studies giving cooked Golden Rice to adults and children have showed very effective conversion of rice β-carotene to vitamin A, that is, 3.8 μg of Golden Rice β-carotene provided 1 μg retinol to US adults and 2.3 μg of Golden Rice β-carotene provided 1 μg retinol to Chinese children.

Through the efforts to increase the provitamin A nutrient in yellow maize, the hybrid yellow maize was developed that contained up to 15 μg β-carotene in a gram of maize. The bioavailability and bioconversion of intrinsic deuterium labeled high β-carotene yellow maize was studied on Zimbabwe adult subjects. By using the stable isotope reference method, the study showed that vitamin A equivalent value of high β-carotene yellow maize consumed by healthy Zimbabwean adult men was 3.2 μg of maize β-carotene provided 1 μg retinol.

Thus, these staple crops that are commonly consumed in Asia (rice) and Africa (maize), when biofortified with provitamin A β-carotene, will provide good dietary source of vitamin A to these populations (funded by NIH, USAID, Nutricia Research Foundation, USDA ARS).

ORAL PRESENTATIONS

Naturally-occurring β-apocarotenoids function as retinoid receptor antagonists

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β-carotene (BC) is the major dietary source of provitamin A. β-carotene oxygenases (BCOs) are the enzymes involved in cleavage of BC. Central cleavage of BC catalyzed by BCO1 yields two molecules of retinal followed by further oxidization to retinoic acid. Eccentric cleavage of BC is catalyzed by BCO2 and perhaps by other enzymes and can also occur non-enzymatically. The polyene chain of BC is cleaved at double bonds other than the central double bond and the products of these reactions are β-apo-carotenals and β-apo-carotenones with different chain lengths. β-Apo-8'-, 10'-, 12'- and 14'-carotenals and β-apo-13-carotenone were detected in mouse and human plasma at nM concentrations and were likewise detected in BC-containing fruits and vegetables. All of the possible cleavage products of β-carotene were obtained or synthesized as the aldehydes and the corresponding carboxylic acids. Transactivation assays were employed to see whether apocarotenoids activate or antagonize RARs or RXRs. Reporter gene constructs (pRARE-Luc, pRXRE-Luc) and retinoid receptor subtypes (RARα, RARβ, RA & Rγ and RXRα) were transfected into COS-1 cells which were used to perform quantitative assays for the activation of these ligand dependent transcription factors. None of the β-apocarotenoids significantly activated RARs or RXR. However, some of the compounds (e.g., β-apo-12'-carotenoic acid, β-apo-14'-carotenal, β-apo-14'-carotenoic acid, and β-apo-13-carotenone) antagonize ATRA activation of RARs and β-apo-13-carotenone antagonizes the 9-*cis*-RA activation of RXRα. Competitive radioligand binding assays demonstrated that the putative RAR and RXR antagonists compete directly with the retinoid agonists for binding to purified receptors. Molecular modeling studies confirmed that β-apo-13-carotenone directly interacts with the ligand binding domains of the retinoid receptors. Finally, β-apo-13-carotenone inhibited ATRA-induced expression of retinoid responsive genes in HepG2 cells. These findings suggest that β-apocarotenoids function as naturally-occurring retinoid antagonists. The antagonism of retinoid signaling by these metabolites may have implications for the activities of dietary β-carotene as a provitamin A and modulator of risk for cardiovascular disease and cancer.

Provitamin A carotenoid uptake, but not retinol uptake, involves the Scavenger Receptors SR-BI and CD36

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Vitamin A, which can be found in fruits and vegetables as provitamin A carotenoids (such as β-carotene, α-carotene or β-cryptoxanthin) or in animal-based foods as retinyl esters (mainly retinyl

palmitate), is essential for normal cell growth and differentiation, immunological functions, and vision. Little is known about mechanisms involved in retinol and carotenoid uptake across the apical membrane of intestinal cells. The scavenger receptor SR-BI and CD36 have been shown to be involved in the intestinal uptake of some provitamin A carotenoids, but data are lacking regarding the uptake of the third main dietary provitamin A carotenoid: α-carotene. The objective of this study was to verify and complete cellular studies showing a role of SR-BI and CD36 on intestinal uptake of provitamin A carotenoids and retinol using human differentiated Caco-2 cell monolayers and transfected HEK cells.

We first showed that the uptake of α-carotene, β-carotene and β-cryptoxanthin was significantly diminished by BLT1 (a specific inhibitor of SR-BI) in Caco-2 cells (approximately 50% to 70%), conversely to the uptake of retinol. This result was then confirmed using transfected HEK cells. Indeed, the transfection with human SR-BI led to a significant 2- to 3-fold increase of provitamin A carotenoid uptake compared to control (cells transfected with an empty plasmid), and this increase was significantly impaired by 10 μM BLT1. Moreover, HEK cell transfection with human CD36 significantly increased carotenoid uptake from 40% to 100%, and this increase was suppressed by the addition of a chemical inhibitor of CD36: SSO (400 μM). Conversely, and according to our previous result on Caco-2 cells, the transfection of HEK cells neither with SR-BI nor with CD36 increased retinol uptake. These findings add further evidence that these scavenger receptors are involved in the absorption of β-carotene and β-cryptoxanthin. Moreover, we showed for the first time that they are also involved in absorption of the other main dietary provitamin A carotenoid α-carotene. Further research is actully under investigation to evaluate whether genetic variation in their respective genes can affect blood concentrations of provitamin A carotenoids at the population level.

Hepatic stellate cells are an important cellular site for β-carotene conversion to retinoid

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Hepatic stellate cells (HSCs) are responsible for storing 70-90% of the retinoid present in the liver. These cells have been reported in the literature also to accumulate dietary β-carotene, but the ability of HSCs to metabolize β-carotene in situ has not been explored. To gain understanding of this, we investigated whether β-carotene-15,15'-monooxygensase (*Bcmo1*) and β-carotene-9',10'-monooxygenase (*Bcmo2*) are expressed in HSCs. Using primary HSCs and hepatocytes purified from wild type and *Bcmo1*-deficient mice, we establish that *Bcmo1* is highly expressed in HSCs; whereas *Bcmo2* is expressed primarily in hepatocytes. We also confirmed that HSCs are an important cellular site within the liver for accumulation of dietary β-carotene. *Bcmo2* expression was found to be significantly elevated for livers and hepatocytes isolated from *Bcmo1*-deficient compared to wild type mice. This elevation in *Bcmo2* expression was accompanied by a statistically significant increase in hepatic apo-12'-carotenal levels of *Bcmo1*-deficient mice. Although apo-10'-carotenal, like apo-12'-carotenal, was readily detectable in livers and serum from both wild type and *Bcmo1*-deficient mice, we were unable to detect either apo-8'- or apo-14'-carotenals in livers or serum from the two strains. We further observed that hepatic triglyceride levels were significantly elevated in livers of *Bcmo1*-deficient mice fed a β-carotene-containing diet compared to mice receiving no β-carotene. Collectively, our data establish that HSCs are an important cellular site for β-carotene accumulation and metabolism within the liver.

Brain carotenoids and physical function in the very old

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As a whole, the population is aging. An implication of this is an increase in disability. Circulating carotenoids have been shown to be positively related to physical performance and muscle strength. The purpose of this study was to determine the relationship of brain carotenoids with premortem physical function measures in decedents aged >98 y. Physical function was assessed using: (1) Direct Assessment of Functional Status (DAFS) which was divided into, Basic Activities of Daily Living (BADLs) and Instrumental Activities of Daily Living (IADLs); (2) Older Adults Resource Scale (OARS_BADLs, OARS_IADLs); (3) Short Physical Performance Battery (SPPB); (4) Modified Physical Performance Mobility Examination (PPME); (5) Peak grip (GRIP); (6) Peak knee extensor or leg strength (LSTR) and (7) Georgia Centenarian Study composite scale (GCS). Carotenoid concentrations were assessed using standard lipid extraction methods and reverse phase HPLC in the cerebellum and frontal, temporal and occipital cortices of 49 decedents from the Georgia Centenarian Study who agreed to brain donation after death. Statistical analyses were performed using SPSS v19.0. Partial correlations were performed using sex, race, facility, Mini-mental state examination score and arthritis as covariates. The mean cryptoxanthin concentration in the four brain sections was significantly related to PPME ($r=0.575$, $p=0.004$) and GCS ($r=0.505$, $p=0.014$). The SPPB scores using original cut offs are population specific. Centenarians exhibited a floor effect and hence SPPB was not included in the analyses. Negative associations were observed between lutein and OARS_BADLs, and between lycopene and BADLs. No significant associations were observed between any carotenoid and DAFS, IADLs and LSTR. Zeaxanthin in the frontal ($r=0.459$, $p=0.031$) temporal ($r=0.508$, $p=0.016$) and occipital cortices ($r=0.396$, $p=0.076$) was related to GRIP, which is a measure of muscle strength. The significant relation of cryptoxanthin with PPME and GCS indicate that brain cryptoxanthin may play a role in lower extremity mobility function, while zeaxanthin may play a role in grip strength in older adults. Alternatively, those with poor physical function may not be eating carotenoid-rich foods.

POSTERS

1.1.

Lycopene induces RARE-mediated signaling in mice

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Lycopene is a lipophilic carotenoid and provides the red color to tomatoes and tomato product. It has been suggested to be one of the most potent antioxidants found in foods due to its eleven conjugated double bonds. Various studies indicate that lycopene and tomatoes / tomato products are able to positively influence various diseases associated with a chronic inflammation. The mechanism of action of lycopene to elicit these effects is largely unknown. One suggestion is that biological metabolites of lycopene may initiate nuclear hormone receptors in mammalian cells. In this study the activity of lycopene is compared to all-*trans* retinoic acid (ATRA) for the induction of the retinoic acid receptor in mice using a RARE-reporter system. The investigation included whole body scanning of the mice alongside organ specific studies. It was observed that both lycopene and ATRA induced RARE-medicated cell signaling within various organs of the mice. However the effect of ATRA was observed after 6 h of treatment whilst the effect of lycopene was not observed until 18 h post-treatment. Supplementary studies on the mice using qRT-PCR determined the potential expression of BCO1 and BCO2 the carotenoid metabolizing enzymes along with the carotenoid transporter protein CD36. The main observation form this study is that lycopene may be a precursor of a biologically active agent with potent RAR activating properties.

1.2.

Total carotenoid content in the phloem and xylem tissue of carrot root

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Carrot storage root is rich in carotenoids and is one of the most important sources of those compounds in human diet. In Europe, cultivars developing orange roots are common, but in other world regions yellow, red or purple carrots are also grown. The carrot root is composed of two main tissues e.g., a flashy secondary phloem and centrally located secondary xylem. Both tissues often differ in their colour that depends on carotenoid composition and in the case of purple carrots also on the presence of anthocyanins.

In this work we compared carrots of various origin and root colour to assess variation in carotenoid content between the phloem and xylem. For this purpose six cultivars were grown in a three-year field trial. The material comprised cultivars developing yellow, red and purple coloured roots. After harvest, the root phloem and xylem tissues were separated and the content of total carotenoids was determined in each tissue spectrophotometrically.

Carrot roots accumulated various amounts of carotenoids depending on the genotype, but on average, red roots possessed more carotenoids than purple and yellow roots. There was a distinct difference in the carotenoid content between the tissues assayed. Higher carotenoid amounts were always observed in the phloem that exceeded 2-3 times the amounts found in the xylem. This relationship was observed independent on root colour, however varied between the years. The results obtained demonstrate that the phloem is more desired for human consumption as it can provide much more of those bioactive compounds. This applies particularly to purple carrots that phloem can contain up to four times more carotenoids than the xylem. The results support also breeding efforts to ensure in modern cultivars a low share of the xylem in the whole root.

1.3.

Decreased carotenoid micellarization and Caco-2 cellular uptake in the presence of divalent minerals and trace elements

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Carotenoids are lipophilic, dietary originating antioxidants. Their regular consumption has been associated with reduced risk of developing several chronic and age-related diseases including several types of cancer and cardiovascular diseases. Prior to their availability for various physiological functions, carotenoids have to be micellarized and taken up by the intestine, both being marginally understood processes. Based on an in vitro digestion model simulating gastric and small intestinal phases, coupled to Caco-2 cells, we assessed the effect of various concentrations of abundant divalent minerals (7.5-25 mmol/L) including calcium (Ca) and magnesium (Mg), and the trace elements zinc (Zn) and iron (Fe) (range 3.8-12.5 mmol/L) on spinach-derived carotenoid micellarization and cellular uptake. Both steps were significantly inhibited by the presence of divalent minerals, with stronger effects for Fe>Zn>Ca>Mg, and for higher concentrations. Highest reduction of micellarization (87.5%, $P<0.001$) and uptake (95.0%, $P<0.001$) was found for Fe (12.5 mmol/L) while it was lowest for Mg (25 mmol/L), remaining unaltered and reduced by 30.8% ($P<0.001$), respectively. Total carotenoid cellular uptake from test meal was reduced in proportion to decreased carotenoid concentrations in the generated micelles; however, lower β-carotene and lutein micellarization was counterbalanced by improved fractional cellular uptake from the micelles. Compared to the control, fractional β-carotene uptake from the micelles was increased in samples digested in the presence of Fe, Ca and Zn, at highest ion concentrations, by up to 5-10 times ($P<0.001$, Bonferroni), while fractional lutein uptake was slightly elevated (1.4-1.6 times, $P<0.001$, Bonferroni). In

addition to the above carotenoids, we monitored the uptake of the epoxycarotenoid conversion products neochrome (from neoxanthin) and luteoxanthin/auroxanthin (from violaxanthin), showing complete epoxide-furanoid conversion under the conditions applied, but also comparable micellarization and cellular uptake with respect to lutein. The present results indicate that divalent ions may inhibit carotenoid micellarization and uptake, warranting further examination in vivo.

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1.4.

Cardiovascular related biomarkers as affected by consumption of tomato products – results from human intervention studies within LYCOCARD

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Various epidemiological studies showed prevention of cardiovascular diseases (CVD) by consumption of tomato products, being the main lycopene source in Western diets. Beside this red coloured carotenoid also other ingredients of tomatoes (e.g. ascorbic acid, folic acid, phenolic compounds) may be responsible for beneficial effects. One possible mechanism of prevention is the antioxidant potential of tomatoes and tomato products. In-vitro studies as well as animal experiments showed different mechanisms of action of lycopene. However, in-vivo studies are needed to show effects of relevance for human beings. Thus, within the European project LYCOCARD several human intervention trials were done, using different tomato products (tomato purée, tomato passata, tomato juice, tomato ketchup). Looking for primary prevention effects, volunteers were healthy people without or with CVD risk factors (smokers, postmenopausal women, obese people), ingesting between 12 and 46 mg lycopene per day out of tomato products for one week up to 18 months. Although the lycopene contents in plasma significantly increased in all studies, only small changes were observed in CVD relevant parameters. Some volunteers showed significantly increased antioxidant capacity in plasma. In addition, inflammatory markers were decreased after consumption of tomato products. In contrast, endothelial function remained unchanged (Stangl et al., 2011) as did lipid status parameters. Thus, further studies need to investigate more in detail CVD relevant anti-inflammatory effects of tomato ingredients.

Concluding all investigations within LYCOCARD, looking for primary prevention proved to be difficult as all volunteers were healthy and the intervention studies with different products were not able to improve the health status. However, these study results do not exclude preventive effects of tomato products, with CVD development often taking decades . To eat tomato products within a mixed diet is always a good and tasty choice, as lycopene is better absorbed from processed products than from raw tomatoes.

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1.5.

Carotenoid exposure of inflammational stimulated Caco-2 intestinal epithelium cells – impact on biomarkers of inflammation and the proteome

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Carotenoid dietary intake has been related to a number of health beneficial effects, including the reduction of several chronic diseases such as cancer and cardiovascular complications. This is in part due to their antioxidant potential, albeit they could act via many different pathways that may result in anticancerous or anti-inflammatory properties. However, no data is available on their action on the intestinal epithelium, being exposed to the highest concentrations of carotenoids in the human body, and where they could act in a preventive way on intestinal inflammatory diseases such as Crohn's disease and ulcerative colitis. The objective of the present study was to investigate whether lycopene and β-carotene in micelles, at concentrations that could be reached via the diet (10-25 μg/ml) could aid in the reduction of TNF-α plus IL-1 induced inflammation of human derived Caco-2 epithelium cells. The impact on biomarkers of inflammation, including IL-8, nitric oxide, prostaglandin-2α, and NF-kB and MAPK pathways of intracellular signalling cascades were evaluated vs. a control (empty micelles). Furthermore, proteomic analyses were conducted from total cellular protein extracts following 2 D-DiGE and MALID-TOF/TOF analyses. Results revealed that isolated carotenoids had no statistical significant anti-inflammatory effect on the biomarkers observed, or on the regulation of NF-kB and MAPK. Nevertheless, analyses of the proteome suggested that 15 proteins were significantly ($P<0.05$, expression ratio >1.3) differentially regulated following β-carotene exposure, participating mostly in cellular metabolism including antioxidant mechanisms, such as glutathione synthase. Only 1 protein was differentially regulated by lycopene (profilin-1). To our knowledge, this is the first attempt to investigate pathways involved in the action of carotenoids on the intestinal epithelium, including proteomic analyses.

1.6.

BCMO1 deficiency induces nonalcoholic steatohepatitis independent of fatty acid oxidation and synthesis

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Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver disorders worldwide. NAFLD ranges from hepatic steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. The prevalence of NAFLD is associated with obesity and insulin resistance (1). Recent evidence has shown that downstream targets of retinoic acid play a pivotal role in the aetiology of NAFLD and NASH, since transgenic mice expressing the retinoic acid receptor-alpha dominant negative form developed microvesicular steatosis and spotty focal necrosis (2). Furthermore, beta-carotene 15,15'-monoxygenase (BCMO1), responsible for the local de novo production of retinal, has shown to be involved in the development of liver steatosis (3).

Since BCMO1 knockout (BCMO1 KO) animals develop liver steatosis independently of the vitamin A status of the diet, we wanted to investigate the mechanisms contributing to this effect using a microarray approach. Here we describe that loss of retinoid signalling contributes to the production of ROS and increases portal tract inflammation. Microarray analysis indicated that both oxidative stress (21% affected genes, Z-Score 2.6) and inflammatory response (18.5% affected genes, Z-score 2.6) pathways were significantly affected, but that fatty acid oxidation and biosynthesis were not changed. Our results show that the development of NAFLD in BCMO1 KO animals does not require the progression from simple fatty liver to fibrosis (two hit hypothesis) which indicates that a different mechanism is driving the development of steatohepatitis in BCMO1 deficiency.

In addition, we screened biopsy proven NAFLD patients (n=339) for their genetic variations in the BCMO1 gene. Interestingly, carriers of 379V alleles have a higher overall risk of developing NASH with OR of 1.85 ($p<0.009$).

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1.7.

Effects of carotenoids and immunity during development on adult health and coloration in male and female mallard ducks

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In addition to providing multiple nutritional and physiological benefits, carotenoids play an important role in sexual selection by acting as pigments that create vibrant, attractive colorsinmyriad fish, lizards, and birds. Many studies have shown that adults who obtain the most carotenoid-rich foods or have a superior immune system are those that are the most colorful and attractive. However, a neglected aspect of this work on honest signaling is the role of development – that carotenoid intake and immunological history of maturing animals may have later-life, long-lasting effects on adult health and coloration. Here, we present data from a series of observational and experimental studies testing the effects of carotenoid intake and immune challenges during ontogeny on carotenoid-based bill colorationand immune function inadult mallard ducks (*Anasplatyrhynchos*). Males possess a yellow, carotenoid-pigmented beak, and those with more carotenoid-rich beaks are more immunocompetent and preferred as mates. First, we correlationally found that some morphological metrics (e.g., body condition) from early in development predicted immune function and beak coloration of adult males. We also experimentally immune-challenged individuals during different stages of development and found that age during immune challenge differentially affected adult immune responses(e.g., increased primary and secondary antibody production, decreased cutaneous immune response, no effect on nitric oxide production). Still, despite developmental perturbations, we found further support for the link betweenadult male beak color andcurrent immune function, given that there were several correlations between adult male beak color, immune response, circulating carotenoid titer, and change in carotenoid titer during the immune response. Females, which possess an orange, not yellow, carotenoid-pigmented beak for which no sexual signaling role is known, showed fewer relationships between beak color, immune response, and circulating carotenoid titers, either during development or adulthood. Thus, we find support for the hypotheses that the yellow, carotenoid-pigmented beak of males is an honest signal of immunocompetence, that early-life perturbations can affect adult immune function, and that different immune metricsare tightly linked to multiple components of carotenoid physiology. However, we found minimal support for the hypothesis that early-life perturbations affect adult beak color, suggesting that this sexually selected trait signals only current aspects of quality.

1.8.

Evaluation of lycopene bioaccessibility from tomato purée

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Nutritional benefits of carotenoids depend on their bioavailability, i.e. the proportion of the carotenoids in food which reaches blood and *in fine* the target tissue. Since most of carotenoids are lipophilic, their bioavailability strongly depends on their diffusion from the food matrix to a lipid phase inside the stomach. The present study focused on this initial step of the bioavailability called bioaccessibility. We aimed at quantifying and characterizing the lycopene diffusion from a standard tomato purée to two different lipid containing phases: oil and oil-in-water emulsion.

The partition factor (PF) and the diffusivity (D) of lycopene were defined as follows: PF is the maximum percentage of the initial lycopene which diffuses from the tomato purée to the oil, D is to the slope of the linear part of the kinetic curve. The variation of PF and D were assessed first for various oil/tomato purée ratios and then for oil-in-water emulsion/tomato purée mixtures.

In a mixture of oil/tomato purée 50/50 v/v, maximum transfer of lycopene to the oil was reached after 20 min and PF value calculated was 14.0 ± 0.09%. Varying the percentage of oil from 10 to 90%, PF changed from 7.55% to 30.03% respectively, indicating that even under non limiting conditions (i.e. with a large excess of oil), almost 70% of the lycopene remained in the tomato purée. D calculated from the model was 3.0×10^{-11} $m^2{\cdot}s^{-1}$. When oil was replaced by an oil-in-water emulsion, PF remained the same for an equivalent oil-in-water emulsion/tomato purée ratio, and maximum transfer of lycopene to the oil-in-water emulsion was reached after less than 2 minutes. This diffusivity was 2 orders of magnitude below that of sugar from melon to water [1] and one order of magnitude lower than lutein from Marigold flowers diffusion in hexane [2].

Times required for the diffusion are compatible with the residence time of the food in the stomach. The fraction of lycopene

which is transferred represents possibly all the available lycopene, which could thus exert its antioxidant activity in this compartment of digestion.

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1.9.

Physico-chemical and nutritional evaluation of novel bacterial carotenoids

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Dietary carotenoids may be submitted to oxidative degradation in the stomach. Indeed, the combination of dietary iron, dioxygen, acidity and dietary polyunsaturated lipids is a potential source of oxidative stress. Thus, investigating the susceptibility of carotenoids to iron-induced autoxidation and their antioxidant capacity (inhibition of lipid peroxidation) in model gastric conditions bears nutritional significance. On the other hand, the potential health effects of carotenoids beyond the digestive tract are largely dependent on their bioavailability, which can be pertinently studied with models of lipid digestion and intestinal barrier.

Novel spore-forming pigmented marine bacteria, *Bacillus* HU36 and GB1, were isolated. They are sources of several carotenoids with original structures displaying glycosyl moieties. These cocktails of bacterial pigments were studied in comparison with common dietary carotenoids (β-carotene, lycopene, astaxanthin, lutein) chosen as reference molecules.

Kinetic studies were conducted in aqueous systems mimicking the gastric medium. Carotenoid oxidation was initiated by Fe^{II} or Fe^{III} or by hematin (heme iron). A combination of analytical methods was developed to elucidate the chemical mechanisms involved. The antioxidant activity of the carotenoids was then evaluated by testing their ability to inhibit the iron-induced peroxidation of linoleic acid in mildly acidic micelle solutions. Finally, the bioavailability of the carotenoids was compared by assessing their bioaccessibility (in vitro digestion model) and their intestinal absorption (Caco-2 cell model). An in vivo animal study on rats fed a carotenoid-enriched diet is also being conducted.

Carotenoids can be classified as follows in terms of increasing stability: beta-carotene < lycopene < astaxanthin < HU36 and GB1 carotenoids. Bacterial carotenoids are significantly more efficient than standard carotenoids at inhibiting lipid peroxidation induced by heme iron but not by free iron, possibly because of their higher stability to iron-induced oxidation, and/or their specific location in the lipid droplets due to their polar head. The bioaccessibility of carotenoids (based on their ability to enter micelles of bile acids and lipid digestion products) can be classified as follows: lutein = HU36 and GB1 carotenoids > beta-carotene > astaxanthin > lycopene. Results of intestinal absorption (Caco-2) showed only minor differences among carotenoids.

1.10.

Effects of a fruit and vegetable concentrate on skin properties

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Microcirculation in the skin is responsible for an optimal supply with nutrients and oxygen, and can be affected by micronutrients. Antioxidants are known to be involved in antioxidant defense and photoprotection, however, less is known about their influence on skin condition, such as texture and barrier function.

In this placebo-controlled intervention study the effect of a micronutrient concentrate (Juice Plus+ ®) on skin microcirculation and skin conditions was performed. The concentrate is composed primarily of a blended fruit and vegetable pulp and juice powder concentrate, providing 7.5 mg β-carotene, 46 mg vitamin E, 200 mg vitamin C and 400 μg folic acid per day. The study was performed over a period of 12 weeks with a treatment group and a placebo group consisting of 26 healthy middle-aged women each. Skin microcirculation and blood flow of deeper vessels was evaluated with the "oxygen to see"-device, texture (skin density and thickness) was determined by ultrasound and skin hydration by corneometry. Transepidermal water loss was measured with the TEWA-meter and serum analyses for carotenoids; α-tocopherol and retinol were performed with HPLC.

Ingestion of the fruit and vegetable concentrate increased microcirculation at 12 weeks of intervention statistically significantly, but general blood flow was not affected. Skin hydration increased statistically significantly, and transepidermal water loss was slightly decreased, both parameters characterize improved skin barrier function. Skin thickness and density were increased. Serum levels of β-carotene, α-tocopherol and cryptoxanthin increased statistically significantly.

The study showed that the ingestion of a fruit and vegetable concentrate has an influence on skin microcirculation, skin hydration and texture.

1.11.

Carotenoid composition containing capsanthin and zeaxanthin from chilli extract with synergistic antioxidant property

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Chilli (Red pepper) is an important spice widely used both for its characteristic red color and pungency in various food preparations particularly in India, Korea, Spain, Mexico and other countries. The red color is essentially due to carotenoids of which capasanthin and zeaxanthin form the majorconstituents (Deli et

al., 2001). Capsanthin and zeaxanthin are present mainly in the ester form in the raw material and also in the chilliextract.

Chilli may probably be an unique dietary source providing substantial amounts of capsanthin and zeaxanthin in the diets of tropical regions.Capsanthin in free and ester form is a good radical scavenger due to conjugatedketo group and polyene chain in the molecule. In a study on both animal and human subjects , the administration of paprika juice increased the capsanthin content in plasma HDL-cholesterol significantly (Aizawa and Inakuma, 2009). Intake of capsanthin may be helpful for increasing the anti-oxidant defense in the blood stream. Capsanthin esters are known to function and possess anti-tumor promoting activity (Maoka et al., 2001). Zeaxanthin is an essential macular pigment and is available in very limited quantities from normal dietcompared to the lutein.The ratio of zeaxanthin to lutein in the macula is 2:1 and in serum 1:5. It protects the eyes from UV radiation and free radical damage to the retina.It is helpful in the prevention of age-related macular degeneration and cataract formation.In view of the above a process has been developed for preparing a carotenoid composition consistingchiefly of capsanthin and zeaxanthin from chilliextract. The method involves saponification followed by fractionation and purification. The purified material chiefly consists of *trans*-capsanthin (72%) and R,R-zeaxanthin (20%). This product can increase macular pigment concentrations, provide better vision and other health benefits.

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1.12.

A multicarotenoid beadlet for human nutrition – dissolution tests

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Since the 1980's when the predominate focus of study and use of carotenoids in human nutritional formulations was solely on beta-carotene, there has been a steady increase in research aimed to understand the role of a wide variety of carotenoids in human health. This work has increasingly demonstrated the benefits of a number of carotenoids, and there has been a corresponding increase in the number of carotenoids provided in nutritional supplements (multicarotenoids). Numerous published observations in both human and animal studies suggest significant interaction and competition between various carotenoids during absorption and metabolism, resulting in the inhibition of uptake of one over the other. This competition has the end result of denying the consumer the maximal beneficial effects of the inhibited carotenoid. To limit such competition and maximize carotenoid uptakes, a layered beadlet was designed to release a defined ratio of carotenoids sequentially. Preliminary dissolution testing will be presented showing the release profile in simulated digestive conditions of a combination of beta-carotene, alpha carotene, lutein, zeaxanthin, lycopene and astaxanthin derived from natural sources. Comparison will be made to an immediate release beadlet formulation using the same combination of carotenoids. These results will be used to guide proof of concept clinical testing for effectiveness in humans.

1.13.

Single nucleotide polymorphisms in β-carotene 15,15'-monoxygenase 1 in relation to plasma carotenoid and retinol levels

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Carotenoids may protect against diseases such as breast cancer, but associations may arise from other factors associated with fruit and vegetable intake. Genetic variation in carotenoid metabolism is one method for assessing carotenoid exposure with reduced confounding. Beta-carotene 15,15'-monooxygenase 1 (BCMO1) catalyzes the first step in converting provitamin A carotenoids to vitamin A. We assessed the association between three a priori identified single nucleotide polymorphisms (SNPs) in *BCMO1*, as well as additional SNPs within 20 kilobases (NCBI 37 chr16:81252296-81344747), with plasma carotenoid and retinol levels in 2337 women from the Nurses' Health Study prospective cohort who had been genotyped as part of previous genome-wide association studies. An additional 1737 women with genotypes for the a priori identified SNPs were also included. Plasma carotenoid levels were measured for various case-control analyses; seven endpoints are included here. All cases were diagnosed after sample collection. For women with genome-wide scans, genotypes were imputed using 1000 Genomes Project EUR data as the reference panel (August 2010 release). Imputation R^2 was ≥ 0.5 for 225/548 SNPs. P-values < 2.2E-4 (= 0.05/225) were considered region-wide significant. The rs28380273 G allele was region-wide significantly associated with 16.4% lower β-carotene (p = 6.2E-21), 16.9% higher lutein/zeaxanthin (p < 1.0E-30), and 6.5% lower α-carotene (p = 1.5E-4). When simultaneously included in the multivariate model, rs28380273 and rs12934922 remained region-wide significantly associated with β-carotene, and rs28380273 and rs12923433 remained region-wide significantly associated with α-carotene. Associations between specific SNPs and plasma α- and β-carotene levels were stronger among women with lower α- and β-carotene intakes while specific associations with plasma lutein/zeaxanthin levels were stronger

among women with higher lutein/zeaxanthin intakes. No SNPs were region-wide significantly associated with plasma β-cryptoxanthin, lycopene, or retinol. This study confirms that variants in *BCMO1* ±20 kilobases are associated with plasma carotenoid levels and may be useful markers of carotenoid exposure in future studies.

1.14.

Antioxidant capacity of crude extracts of carotenoids from the berries of various cultivars of sea buckthorn

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Comparative analysis of antioxidant capacity of crude extracts containing carotenoids obtained from fruits of sea buckthorn harvested in 2010 were conducted using FRAP (Ferric Reducing Antioxidant Power) method. The study included nine cultivars of sea buckthorn growing in the comparative cultivation at the Fruit Experiment Station in Brzezna near Nowy Sącz.

In Poland, sea buckthorn fruits and preserves obtained from them are not very well known because Sea Buckthorn are encountered occasionally as ornamental tree. However sea buckthorn is more known and cultivated at Northern Europe and Asia (Mech-Nowak et al., 2011). These fruits are regarded as a valuable for health in folk medicine. Recently, more and more fruits of sea buckthorn are the feedstock for production of many emerging on the world market drugs supporting health (Stahl and Sies, 2003). Therefore, there is a need for assessment of suitability for the cultivation of sea buckthorn in Poland, that are already known in Europe, including the evaluation of health-oriented values of obtained fruits.

Conducted analysis allowed to compare the antioxidant capacity of extracts of carotenoids using the spectrophotometric method of FRAP as well as DAD-HPLC methods. FRAP analysis showed that the highest antioxidant capacity was demonstrated by three of the sea buckthorn cultivars: Botanicheskaya, Avgustinka and Luchistaya. However HPLC methods provided the evidence that highest carotenoid content was observed in three other cultivars: Aromatnaya, Moskvichka and Arumnyj. Other antioxidants such as tocoferols might be involved.

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1.15.

Infant plasma response to lutein in formula and human milk

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Wide variations of lutein in human milk exist and are generally reflective of maternal dietary intake. Information is lacking regarding appropriate lutein supplementation levels in infant formula that result in plasma levels comparable to the breastfed infant. The objective of this study was to measure infant plasma response to lutein supplementation of infant formula. Term infants (n=28) enrolled in a controlled clinical trial, that was non-randomized and unblinded, were fed formula containing 117 μg lutein/L. The level of lutein in the formula was chosen based on plasma carotenoid responses in infants fed carotenoid-supplemented formula from phase one of this study (Mackey et al., 2008). Lutein intake and plasma response were measured at baseline and after 56 days of feeding. Statistical comparisons were made between infants fed the experimental formula and those in an exclusively breastfed group from the phase one study. Anthropometric data were collected on study days 1, 28, and 56. At baseline, mean plasma lutein levels were significantly higher in the breastfed group than in the formula-fed group. After 56 days of feeding, infants fed the lutein-containing formula had significantly greater mean plasma concentration of lutein than human milk-fed infants; however, the range of plasma lutein in the formula-fed infants was within the range of the human milk-fed infants as reported by both Mackey et al., 2008 and Bettler et al., 2010. Anthropometric data and the reported incidence of adverse events in the formula-fed group were similar to other formulas fed in phase one. Results from both phases of the clinical study indicate that the carotenoid-supplemented formulas were safe and well tolerated. This work provides evidence that 117 μg lutein/L, needed in formula to achieve the range of plasma levels corresponding to that of human milk-fed infants, is safe and well tolerated.

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1.16.

Northern berries as source of carotenoids useful for maintenance of human health

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Carotenoids are bioactive substances in foods with powerful antioxidant bioactivity. There are abundant data showing theirs preventive effects in humans for number of diseases. Due to the

limited information on carotenoids in wild berries we aimed to determine β-carotene and xanthophylls content in northern berries. Four berry species (*Rubus chamaemorus, Vaccinium myrtillus, Vaccinium vitis-idaea, Oxycoccus palustris*) were selected for this study in taiga zone of European North-East of Russia. Samples were collected in August-September 2010 and transported to laboratory, where they were quick-freezed and kept at -76°C. Carotenoids in freeze-dried samples were extracted with acetone. Individual carotenoids were separated using reversed-phase high-performance liquid chromatography (HPLC) system (Knauer, Germany), a column 4.0×250 mm Diasphere-110-C18NT. The pigments were eluted for 34 min with gradient solvent systems A (acetonitrile:methanol:water, 75:12:4, v/v) and B (methanol:ethyl acetate, 68:32, v/v) at a flow rate of 2 ml/min. Identification of carotenoids was carried out by comparing of HPLC retension times with corresponding standarts. The highest total carotenoids content among the studied berries was found in cloudberry (2840 μg/100 g DW) followed by blueberry (2140 μg/100 g). Cranberry and cowberry had the lowest carotenoid content, accordingly 210 and 150 μg/100 g DW. All berries had β-carotene but this carotenoid prevailed greatly in cloudberry (82% of total carotenoids content). Lutein was a principal carotenoid in blueberry (71%). The major contributors to the total carotenoids content of cranberry were β-carotene (28%), lutein (23%) and neoxanthin (20%). Not great quantity of violaxanthin cycle carotenoids (violaxanthin, antheraxanthin and zeaxanthin) were identified in all berries and cloudberry sepals. It was established that the carotenoid HPLC profiles of the extracts from cloudberry are complex, because the naturally occurring carotenoids (lutein, zeaxanthin, and violaxanthin) in these fruits are esterified with straight chain of fatty acids. Unidentified carotenoid constituents were presented in the unsaponified extract of cloudberry. The received results are meant a regional database of resources with increased nutritional value.

1.17.

Content of carotenoids in roots of seventeen cultivars of *Daucus carota* L.

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The aim of this study was to compare the content of carotenoids in seventeen cultivars of carrots grown in Poland. We compared conventional orange cultivars with rarely grown: white, yellow and purple with creamy core cultivars. Carrots are an important source of carotenoids (mainly β-carotene) in daily diet. In Poland, the carrot is grown on a large scale (813 000 tons) and has great economic importance. New cultivars introduced into crops are not only different in shapes and colors, but also in the content of bioactive and important for health compounds including carotenoids.

Seventeen selected cultivars of carrots were grown on plots at the Experimental Station of Agricultural University in Mydlniki. For determine the content of carotenoids, extracts made from lyophilized carrot roots were analyzed by spectrophotometric as well as HPLC methods with DAD detector. The highest content of carotenoids was found in cultivars: 'Kazan' and 'Kongo' (nearly 10 mg/100 g f.w.). We also compared the antioxidant properties of selected cultivars using the FRAP method (Benzie and Stain, 1996).

Carotenoids are needed in human diet as provitamin A. It is less known that they significantly contribute to the protection of membrane lipids as important antioxidants soluble in fats. Carotenoids as antioxidant compounds reduce the risk of chronic diseases (cardiovascular diseases, cancer ect.) (Krinsky and Yeum, 2003, Stahl and Sies, 2003). Identifying the *Daucus carota* L. cultivars with the highest carotenoid content in roots will make it possible to recommend them for cultivation, especially for farms specializing in production of preserves with high carotenoid content and contribute to further selection of new cultivars in horticultural breeding.

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1.18.

Carotenoid composition and in vitro pharmacological activity of rose hips

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The aim of the present study was to compare flower carotenoid extracts with regard to their phytochemical profiles and their in vitro anti-*Helicobacter pylori* (*H. pylori*), cytotoxic, multidrug resistance (MDR) reversal and radical scavenging activity. Carotenoid composition was investigated in the different fractionation of rose hips, using extraction methods. Six main carotenoids – epimers of neochrome, lutein, zeaxanthin, rubixanthin, lycopene, β, β-carotene – were identified from Rose hips by their chromatographic behavior and UV-visible spectra, which is in accordance with other studies on carotenoids in this plant material. The active principles in the carotenoid extract might differ, depending upon the extraction procedures.

This study, on the part of the Hungarian authors was supported by the grants Szeged Foundation for Cancer Research and OTKA K 76176, OTKA K 83898 (Hungarian Scientific Research Fund) and PTE ÁOK KA-34039-35/2009.

1.19.

Health promotion by lycopene

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Lycopene has been widely investigated as a possible cancer preventive agent, especially in prostate cancer prevention. We have already reported that lycopene is also effective for prevention against hepatitis C virus-induced liver cancer (Nishino et al., 2009). Recently, lycopene has been shown to be an useful agent for various lifestyle- related diseases, such as cardiovascular diseases, as well as cancer. We have also found that lycopene is promising as a preventive agent against osteoporosis. Therefore, lycopene seems to be an important carotenoid in preventive medicine. Health promotion by lycopene will be discussed in this presentation.

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1.20.

Dihydro-lycopenoids are lycopene-metabolites

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Lycopene has been shown to be a potent activator of RARE-mediated signaling in RARE-LUC reporter mice and therefore we are in search for lycopene metabolic pathways and bio-active lycopene-metabolites. Our initial targets were the fully conjugated and previously detected apo-lycopenoids. Various apo-lycopenoids were synthesized for this study ranging from apo-10´-, apo-12´- and apo-14´-lycopenoids. In these experiments lycopene was supplemented either orally or intra-venously for better absorption to mice and administered to cultured human adipocytes. In various organs of the lycopene-supplemented mice we could not detect any apo-lycopenoic acids using highly sensitive and specific HPLC MS-MS techniques. Fortunately, in the white adipose tissue of lycopene-supplemented animals we could identify a large peak using single ion monitoring which has a molecular weight of 2 Da higher then apo-10´-lycopenoic acid. Based on our HPLC-MS data and additional UV detection we conclude that this peak might be 7,8-dihydro-apo-10´-lycopenoic acid. We suggest that dihydro-apo-10´-lycopenoic acids might be novel lycopene-metabolites resulting in bioactive RAR and RXR activating apo-lycopenoids, which might explain potent RARE-activation induced by tomato preparations and lycopene.

1.21.

Effects of carotenoids on growth performance, mortality and carotenoid pigmentation of rainbow trout (*Onchorynchus mykiss*)

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Carotenoids are natural liposoluble pigments ranging from colorless and yellow to red and blue, and have several biological functions in plants and animals. Fish and other animals are unable to biosynthesize carotenoids de novo and must obtain them from their diet (Davies, 1985). Asataxanthin and canthaxanthin (β, β-carotene-4,4'-dione) are the most commonly used carotenoids for pigmentation of farmed salmonid fishes. In the present study the effects of dietary astaxanthin supplementation on growth performance, mortality, carotenoid pigmentation was investigated in triplicate groups each with 1000 swim up larvae of rainbow trout , derived from five groups of female broodstock fed diets with 0.07, 12.5, 33.3, 65.1 and 92.9 mg astaxanthin kg^{-1} , respectively. The first feeding fry were fed a diet not supplemented with carotenoids. Fry were initially sampled for astaxanthin content and initial weight, and in subsequent 15-day intervals to determine weights, condition factors (CF), specific growth rates (SGR) and thermal growth coefficients (TGC). Total carotenoid concentration of the larvae was highly linearly correlated to that of eggs (r^2=0.97, P=0.002). About 59-67% of fry carotenoids consisted of esterified astaxanthin, and on average 39.7% of the egg carotenoids were recovered in the fry. Overall (0-45 days) SGRs and TGCs were significantly higher ($P < 0.05$) in the offspring of the four groups of females fed supplemented diets (12.5-92.9 mg astaxanthin kg-1) than in offspring of the females fed the non-supplemented diet. TGCs (0-45 days) within groups derived from broodstock supplemented with astaxanthin were similar ($P > 0.05$), but higher than in the group derived from females fed the diet not supplemented with astaxanthin ($P < 0.05$). Mortality (average 0.76%) was not significantly affected by treatment. The study indicates that dietary supplement of astaxanthin (> 12.5 mg kg^{-1}) to maternal broodstock diets improves offspring SGR and TGC with up to 33 and 38%, respectively.

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1.22.

Antioxidants quercetin and beta carotene can modulate mitochondrial function of human preadipocytes

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One by the reason of complication of obesity, like diabetes type 2, could be mitochondrial dysfunction, increase ROS generation and decrease antioxidant activity. Quercetin and beta carotene

(BC) are nutrients which exert antioxidant activities. Quercetin, a flavonol occurring in fruit and vegetables, is one of the most potent antioxidants among polyphenols. BC derivatives such as retinoic acid (RA) of BC deeply affect mitochondrial biogenesis. The effect of these nutrients on metabolism of human preadipocytes is still not well recognized.

The aim of the study was investigated the effect of beta carotene (BC) and quercetin on mitochondrial function.

The human preadipose immortalized (Chub-S7) cells were used. Cells were incubated for 24h with BC (3 μM, 10 μM, 30 μM) or with quercetin (10 μM, 30 μM, 50 μM, 70 μM, 100 μM). Mitochondrial metabolic activity was monitored by measurements of the mitochondrial oxygen consumption rates (OROBOROS® Oxygraph-2k) and ATP generation (ATP Lite Parkin Elmer). Changes in the mitochondrial membrane potential ($\Delta\psi$) was monitored by flow cytometry (BD) and high thoroughput fluorescent microscopy in vivid cells (BD Bioimager 855).

The different effects of used compounds on mitochondrial activity was observed. Quercetin decreased mitochondrial respiration and increased mitochondrial membrane potential dependent on concentration. BC decreased mitochondrial respiration and ATP generation, especially at the low (10 μM) concentration.

In the immortalized human preadipocytes the concentration-dependent inhibitory effect of investigated nutrients on mitochondrial functions was evidenced.

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1.23.

Lutein is the predominant carotenoid in infant brain

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As lutein has increasingly been implicated to support cognitive health in adults, its role in infants merits investigation. Humans cannot synthesize lutein and breast milk typically contains higher concentrations compared to infant formula; also lutein in formula is known to be less available. Infants fed unsupplemented formula may thus be at an increased risk of low lutein status potentially resulting in increased risk of oxidative stress. Our objective was to determine carotenoid distribution in infant hippocampus, and frontal, auditory, and occipital cortices. Pre-existing voluntarily donated samples were obtained from the federally-funded Brain and Tissue Bank, which adheres to strict consent and confidentiality procedures. Subjects were otherwise healthy infants (n=30) who died during the first year of life of either SIDS (50%) or other conditions (50%). Tissue was extracted using established lipid methods and analyzed using reverse phase HPLC. All statistical analyses were performed using SPSS (v19). There was significantly greater accumulation of xanthophylls (lutein, zeaxanthin, cryptoxanthin) compared to carotenes (β-carotene, lycopene) in all four regions of the brain ($p<0.05$). The average concentration of lutein in all four brain regions (48.6±7.2 pmol/g) was significantly greater ($p<0.05$) than zeaxanthin (13.3±1.5), cryptoxanthin (6.3±1.6), β-carotene (15.9±3.8) and lycopene (1.7±1.4). Lycopene was detected in only two decedents. No differences were detected between SIDS and others for carotenoids in any brain region. Lutein concentration was positively related to cryptoxanthin (not present in infant formula) in each region indicating that decedents with higher cortex lutein levels were most likely breast fed. Carotenoid distribution in the brain regions:

Carotenoids (pmol/g)	Frontal cortex (n=29)	Hippocampus (n=24)	Auditory cortex (n=11)	Occipital cortex (n=28)
Lutein	41.33±16.94[a]	30.28±9.42[b]	53.43±18.93[b]	47.0±18.57[a]
Zeaxanthin, *trans*	11.98±3.22[a]	12.21±25.2	18.51±4.28[a,c]	12.60±3.42[c]
Cryptoxanthin	5.43±2.99	2.98± 0.68	4.44±2.47	5.56±3.07
β-carotene, *trans*	6.76±20.9[a]	4.36±1.84[a,c]	13.56±2.94[a]	8.57±2.41[c]

mean±SEM. Within a row, means sharing a common superscript are significantly different ($p<0.05$, Repeated measures ANOVA). [b]$p=0.055$.

These data demonstrate for the first time that lutein is the predominant carotenoid in the infant brain. As infant formula is not routinely supplemented with lutein, further investigation of the impact of lutein intake on neural development is warranted.

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1.24.

Apo-10'-lycopenoic acid increases both mRNA and protein levels of Sirt1 as well as decreasing the fat accumulation in the livers of ob/ob mice

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Recent studies suggest that both tomato extract and lycopene supplementation are beneficial in protecting against high fat diet induced liver injury. The underlying mechanism(s) is not well defined. In the present study, we investigate the effect of apo-10'-lycopenoic acid (ALA), a biologically active metabolite of lycopene, on the development of fatty liver in ob/ob mice. Male ob/ob mice (N=12/each group) were fed a liquid high fat diet (60% fat as energy intake) with or without ALA supplementation at a dose of 40 μg/g diet for 16 weeks. Liver histopathologic lesions including steatosis and inflammation were examined and graded. The mRNA expression of sirtuin 1 (Sirt1), PPARγ, TNFα, carnitine palmitoyltransferase (CPT), medium chain acyl CoA dehydrogenase (MCAD), acetyl co-A carboxylase 1 (ACC1) were determined by real-time PCR. Protein levels of Sirt1, acetylated FOXO1 and IκB were measured by Western blot analyses. Results showed that ALA supplementation did not affect both body and liver weight but significantly decreased the steatosis in the liver tissue, as compared to the control group without ALA supplementation ($p<0.05$). Further, ALA supplementation significantly increased both mRNA and protein levels of hepatic Sirt1, as well as the activity of Sirt1 as evidenced by the decreased levels of acetylated FOXO1 protein levels ($p<0.05$, vs. control). There were no significant differences on the hepatic levels of PPARγ, IαB, and TNFα, inflammatory foci, and expressions of CPT and MCAD between two groups. On the other hand, the expression of ACC1, an adi-

pogenesis enzyme, was significantly decreased in ALA supplemented group, as compared to controls. Since Sirt1 plays a key role in lipid homeostasis, the present study suggests that ALA protects against the development of steatosis in ob/ob mice by upregulating Sirt1 gene expression.

1.25.

Fluorescence spectroscopy of polyene antibiotic drug amphotericin B

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Amphotericin B (AmB) is an antifungal antibiotic commonly used in treating deep-seated mycosis. It has many side effects such as breath and heart rate disorder, it is nephrotoxic and hepatotoxic. That is why, it is important to discover mechanisms of its interaction with fungal and human cells. From other investigations, it is known that AmB binds to lipid membranes. The structure of energetic states of the drug in diffrent molecular forms and model of its interaction with lipid membranes are still under detailed investigations.

Auto-fluorescence of AmB may be also used to investigate the structure of energetic states of the drug in diffrent molecular forms. Auto-fluorescence of AmB incorporated to the DPPC liposomes may be used to investigate dynamic and structural properties of lipid membranes and simultaneously molecular organization of the drug with using fluorescence anisotropy.

The samples we examinated included AmB in pH7 , pH12 water buffers and DPPC liposomes with AmB in concentration 5mol% with respect to lipid.

The conclusions of our investigations are:

1. Fluorescence anisotropy spectra of sample in pH12 buffer show the presence of two molecular forms of the durg, though solution with such high pH level should containe only monomeric form. It also shows that 521 nm peak in fluorescence emission spectra is not a part of emission band of the monomeric form,
2. Comparison of the fluorescence exitation ane 1-T spectra confirms that the sample in pH12 buffer containes both the monomeric and the dimeric forms of AmB,
3. Fluorescence anisotropy spectra of AmB incorporated to DPPC liposomes suggests that dimeric form of the drug binds stronger to the membranes as compared to the monomeric form.

1.26.

Lutein supplementation fosters neuroprotection and improves associative and spatial learning and memory performance in aged mice

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Introduction: Due to changes in demographic structure, the decline of cognitive abilities with age is among the largest socio-economic problems of modern societies. Therefore, it is desirable to search for safe nutritional compounds to delay or prevent age-associated reductions in mental performance. Beneficial effects of lutein, a hydroxylated Carotenoid (Xanthophyll) which is present in several food sources including broccoli, spinach, kale and pepper, on brain functions have been previously studied in several paradigms including animal models of neurodegenerative disorders.

Methods: FloraGlo® Lutein was tested in a glutamate intoxication assay there primary cortical neurons were pretreated with Lutein. We have further tested aged animals, after chronic dietary supplementation with 9 mg/kg b.w /d. FloraGlo® Lutein in the IntelliCage System. After a 2 weeks adaptation period animals had to perform for at least 4 weeks, in four different behavioral paradigms examining exploratory-, associative-, stress-related- and spatial- learning and memory.

Results: FloraGlo® Lutein supports neuronal survival through protection against oxidative stress and against glutamate intoxication. Moreover, FloraGlo® Lutein significantly promotes associative learning and memory, learning and memory under stress and spatial- learning and memory but is ineffective in supporting other learning paradigms tested in the IntelliCage System.

Conclusion: These data suggest that supplementation with FloraGlo® Lutein may be helpful in improving specific aspects of learning and memory in daily life situations and further may help to slow the normally occurring age-associated deterioration of cognitive performance.

1.27.

Cosmetic benefits of astaxanthin oral supplementation on humans subjects

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A randomized double-blind placebo controlled study involving 36 healthy male subjects for 6 weeks was performed. Crow's feet wrinkle and elasticity; and transepidermal water loss (TEWL) improved after 6 mg of astaxanthin daily supplementation. Moisture content and sebum oil level at the cheek zone showed strong tendencies for improvement. These results suggest that astaxanthin derived from Haematococcuspluvialis may improve the skin condition in not only in women but also in men.

1.28.

Cosmetic benefits of oral supplementation combined with topical treatment of astaxanthin on humans subjects

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An open-label non-controlled study involving 30 healthy female subjects for 8 weeks was performed. Significant improvements were observed by combining 6 mg per day oral supplementation and 2 ml (350 μM solution) per day topical application of astaxanthin. Astaxanthin derived from the microalgae, *Haematococcus pluvialis* showed improvement in skin wrinkle reduction (crow's feet at week 8), age spot size (cheek at week-8), and improved elasticity (crow's feet at week-8), skin texture (cheek at week-4), moisture content of corneocyte layer (cheek in 10 dry skin subjects at week-8) and corneocyte condition (cheek at week-8). These results suggest that astaxanthin derived from *H. pluvialis* can improve skin condition in all layers such as corneocyte layer, epidermis, basal layer and dermis by combining oral supplementation and topical treatment.

Session 2

Photosynthesis, Photochemistry, and Photoprotection by Carotenoids

Professor Trevor W. Goodwin

in Memoriam

Professor Trevor W. Goodwin, CBE, FRS, 1916 – 2008

One of the all-time greats of the carotenoid world, Trevor Walworth (T. W.) Goodwin died on 7th October 2008 at the age of 92. Unusually for someone who would go on to a long and distinguished research career, he never studied for a Ph.D., though he did obtain a doctorate (D.Sc.) later. After his university training in chemistry at the University of Liverpool, and a Master degree in the then new discipline of biochemistry, he built the main foundations of his research career during World War 2, when he worked on Government projects on food and nutrition, especially vitamin A and other vitamins, interspersed with his duties as an Air Raid Warden. Particularly noteworthy is his collaboration with Prof R.A. Gregory and a goat, leading to the first experimental demonstration that vitamin A is formed from beta-carotene in vivo in the intestine. Post-war, TWG's research career blossomed, first in Liverpool, then at the University College of Wales, Aberystwyth, from where he returned to The University of Liverpool as Johnston Professor of Biochemistry and Department Chair in 1966.

Although he worked on other topics, such as riboflavin, plant sterols, Trevor Goodwin's great research love affair was with carotenoids. He built up a large research group which generated hundreds of publications, notably on the occurrence and distribution of carotenoids in a wide variety of plants and microorganisms, and then much pioneering work on carotenoid biosynthesis, making good use of newly available isotope labelling techniques. His prolific research output was recognised by a number of accolades, culminating in his election a Fellow of The Royal Society (FRS). For his research and for his major activities in the University and in various national scientific bodies and policy committees, he was granted national recognition and appointed a Commander of the Order of The British Empire (CBE).

To do justice to T.W. Goodwin's many achievements could easily fill a whole book. Here it is appropriate to select just a couple of highlights. First, he was a prolific editor of books and symposium proceedings, and was the author in 1953 of a book The Comparative Biochemistry of the Carotenoids, which he brought up to date in the 1980s as The Biochemistry of the Carotenoids, published in two Volumes, Plants (1980) and Animals (1984). These books remain valuable sources of information and insight. Second, he was an expert and experienced organiser of conferences, and served as Chair of the 6th International Symposium on Carotenoids held in Liverpool in 1981, thirty years ago this week.

Trevor Goodwin was a special person, who introduced many of us to the wonderful world of carotenoids, and remained a great influence and inspiration throughout our careers. His contribution to the carotenoid field is enormous and enduring.

INVITED LECTURES

Xanthophyll role in photoprotection and biogenesis of the photosynthetic apparatus

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The carotenoid composition of plants is one of their most conserved properties, suggesting each xanthophyll species has a specific function. The most abundant carotenoid in dark adapted plant leaves is lutein followed by beta-carotene, violaxanthin and neoxanthin. In addition, zeaxanthin is found in high light treated plants where is synthesized from violaxanthin. In order to determine the function of each xanthophyll species a combined approach has been undertaken by constructing and characterizing ko mutants in xanthophyll biosynthesis enzymes producing arabidopsis plant either lacking one specific xanthophyll species or missing all but one. In addition, individual recombinant proteins have been produced engineered in their xanthophyll content. Plants and recombinant proteins have been characterized for their capacity of quenching singlet chlorophyll excited states, producing carotenoid triplet by quenching Chl triplet excited states and for their ROS productivity upon illumination and/or capacity for scavenging exogeneously supplied ROS species. Results show that each xanthophyll species has a specific role namely: lutein is specialized in ^{3}Chl quenching, violaxanthin in $^1O_2$ scavenging and neoxanthin in superoxyde scavenging. Zeaxanthin has enhanced effect in both Chl triplet quenching and singlet oxygen scavenging. In addition it has a strong constitutive ^{1}Chl* quenching, which makes it unsuitable in limiting light conditions thus accounting for its absence in low light. Zeaxanthin synthesis is controlled by VDE (Violaxanthin de-epoxydase) a soluble enzyme in the chloroplast lumen which, at low lumenal pH, becomes membrane bound and lead to the accumulation of zeaxanthin in both the lipid phase and specific sites of monomeric Lhcb proteins CP29, CP26 and CP24 where it up-regulates NPQ (non photochemical quenching) of excess excitation energy. In addition to these direct effect in photoprotection, the xanthophyll composition also affects transcription and protein turnover in vivo thus controlling acclimation of plants to different light/temperature conditions.

Spectroscopic investigation of green plant antenna pigment-protein complexes containing specific xanthophyll pigments

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The spectroscopic properties and energy transfer dynamics of the protein-bound chlorophylls and xanthophylls in monomeric, major LHCII complexes and minor Lhcb complexes from genetically-altered *Arabidopsis thaliana* plants have been investigated using both steady-state and time-resolved spectroscopic methods. The pigment-protein complexes that were studied contain Chl *a*, Chl *b*, and variable amounts of the xanthophylls, zeaxanthin (Z), violaxanthin (V), neoxanthin (N) and lutein (L). The complexes were derived from mutants of plants denoted *npq1* (NVL), *npq2lut2* (Z), *aba4npq1lut2* (V), *aba4npq1* (VL), *npq1lut2* (NV) and *npq2* (LZ). The data reveal specific singlet energy transfer routes and excited state spectra and dynamics that depend on the xanthophyll present in the complex.

Proteins and carotenoids are bricks and mortar for constructing functional photosystem II complex architecture

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PSII first supercomplex of the electron transport chain governs the energy transfer using harvested light energy which is transformed to biochemical energy. Carotenoids can assist in assembly of photosynthetic complexes of both in higher plants and cyanobacteria. These carotenoids serve as mortar for proteins which act as bricks for construction of the acting photosynthetic machinery and they have determinative roles in oligomerization of protein subunits. X-ray crystallographic localization of carotenoids revealed that they are present at functionally and structurally important sites of both PS I and PS II reaction centres. Carotenoids are protective agents, which prevent photosynthetic complexes from degradation caused by reactive oxygen species. There are several specific carotenoids in cyanobacteria and also in higher plants with individual roles in photosynthetic reactions. They guard molecules and can protect proteins against free-radical attack generated by surplus of light exposition. A carotenoid-less *Synechocystis* PCC6803 mutant was generated in which the *crtB* gene encoding phytoene synthetase was inactivated. Consequently, not carotenoid synthesis was observed. In mutant cells the synthesis of determinative proteins involved in the assembly of PSII was blocked together a concomitant block in the assembly of PSII structure, however, PSI was assembled and it was functional.

Ultrafast excited state dynamics of carotenoids and their role in non-photochemical quenching

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The excited state relaxation and the role and character of intermediate states, in particular the so-called S*state, has been studied by ultrafast transient absorption for a variety of carotenoids of conjugation length 9-13. Our data indicate that the S* state under low intensity excitation (single photon excitation) is a vibrationally excited S1 state. Furthermore we find evidence at very short times for conical intersections of excited states in all of these carotenoids leading to coherent coupling between states which give rise to electronic quantum beats (1,2).

Non-photochemical chlorophyll quenching (NPQ) in plants protects against photochemical destruction of the photosynthetic apparatus under excess light conditions and the exact role of carotenoids, whether it is direct or indirect, in this quenching is

a matter of intense debate.While one location of the NPQ process has been shown to be centered on the major light harvesting complex II (LHCII) (Q1 type or qE quenching)(3), an additional quenching center responsible for qI type (identical to Q2 center(3)) quenching(4) has been suggested to be located on the minor light-harvesting complexes upon accumulation of zeaxanthin (Zx), in particularon CP24 and CP29 (5). We have performed femtosecond transient absorption and time-resolved fluorescence measurements of NPQ quenching in intact leaves of higher plants, in the isolated major LHC II complex in the aggregated state, and on isolated minor complexes reconstituted with various carotenoids. The major quenching mechanism(s) in these complexes in vivo and in vitro will be discussed.

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Non-linear laser spectroscopy of carotenoid-chlorophyll interactions in light-harvesting complexes

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In addition to chlorophylls (Chls) *a* and *b* plant major light-harvesting complex (LHC II) binds xanthophylls – two luteins, neoxanthin, violaxanthin (and/or its de-epoxidation products) per monomeric subunit. Xanthophylls have important functions: structure stabilization, light-harvesting and photoprotection. LHC II, in vitro as well as in vivo, can exist in various states of aggregation profoundly altering interactions between pigments. Aggregation of LHC II in vitro is thought to mimic structural changes that may be the basis of the photoprotective process assessed as the energy-dependent component (qE) of non-photochemical quenching of Chl fluorescence (NPQ). The xanthophyll cycle (light-stress induced, dark-reversible, de-epoxidation of violaxanthin via anthraxanthin to zexanthin) appears to be crucial in this regard. The molecular mechanism of xanthophyll cycle involvement in qE/NPQ is not firmly established.

Two-photon excitation with tunable 100 fs-laser pulses was performed in the presumed xanthophyll $1^1A_g^- \rightarrow 2^1A_g^-$ (S1) transition region with LHC II samples containing different xanthophyll complements. For comparison, two-photon excitation spectra of Chls *a* and *b* in solution were recorded in the same spectral region.

Nonlinear polarization spectroscopy in the frequency domain (NLPF) was used to investigate the changes in the interactions between xanthophylls and Chls in LHC II upon alteration of its aggregation state by incubation at different detergent concentrations (Voigt et al., 2008; Lokstein et al., 2011). NLPF spectra of slightly aggregated and trimeric LHC II were measured – pumping in the Chl-Q_y region and probing in the xanthophyll $2^1B_u^+$-region. NLPF spectra of trimeric LHC II are dominated by a peak at about 652 nm (due to Chl *b*) – with virtually no contribution from Chl *a*. For LHC II aggregates the spectra peak in the Chl *a* Q_y region (at about 682 nm). These changes are discussed with regard to alterations in the interactions of xanthophylls and Chls. Implications of the results for recently proposed mechanism(s) of qE/NPQ will be discussed.

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Significance of lipids in molecular mechanism of the xanthophyll cycle

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Xanthophyll cycle is the main photoprotective mechanism operating in plants. It regulates and optimises the amount of light used by the photosynthetic apparatus for photochemical reactions. There are several types of xanthophyll cycle described in plants and algae. One of them is violaxanthin (Vx) cycle which involves interconversion between Vx, antheraxanthin and zeaxanthin. In the another type, the diadinoxanthin (Ddx) cycle, interconversion between Ddx and diatoxanthin occurs. It is known since long time that lipids are involved in the operation of the xanthophyll cycle. Further studies revealed, that they play a crucial role in the molecular mechanism of de-epoxidation of the epoxyxanthophylls participating both in the Vx cycle and the Ddx cycle. Enzymatic activity of Vx de-epoxidase and Ddx de-epoxidase in the presence of various lipid types have been systematically studied. We determined several properties and parameters of lipids which influence the enzymatic activity of the investigated de-epoxidases. The basic requirement is the shape of the molecule, allowing the lipid to form inverted hexagonal phases in water surrounding. Other important factors are the physical properties of the structures formed by lipids such as size of the inverted micelles, thickness, fluidity and molecular dynamics of their hydrophobic fraction and also solubility of substrates (Vx and Ddx) in various kind of lipids. The data obtained in the model systems are in agreement with our studies on pigment protein complexes present in the thylakoid membranes. Analysis of different LHCII preparations showed that the amount of Vx in the LHCII well correlated with the concentration of the main thylakoid lipid monogalactosyldiacylglycerol (MGDG) associated with this complex. A model of de-epoxitation of Vx present in the LHCII and the role of MGDG associated with this complex will be presented.

ORAL PRESENTATIONS

Photoprotection by carotenoids of *Plantago media* photosynthetic apparatus in natural conditions

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Carotenoids, apart of their antenna function in photosynthesis, play an important role in protection of photosynthetic apparatus. Role of protection mechanisms increases with enhancement of climate instability and under environmental stresses. Daily pattern of photosynthesis and carotenoids were studied in the sun and shade plantain plants in the field (62°45 N, 55°49 E) in July 2007-2011 (Golovko et al., 2011). The sun plants growing on the open site differed from shade plants growing in dense herbage in terms of CO_2 exchange rate and photosynthetic pigments content. HPLC-analysis revealed the presence of β-carotene (β-Car) and xanthophylls in carotenods pool. The total pool of xanthophylls [violaxanthin (V) + antheraxanthin (A) + zeaxanthin (Z)] and the conversion state (Z+0.5A)/(V+A+Z) increased from morning to midday in sun plants. The percentage of V in VAZ pool was the greatest (85-90%) at midnight and decreased to 10% at noon. An increase in Z content occurred concomitantly with the V decrease. Maximum part of Z in VAZ pool was 60%. The conversion state of violaxanthin cycle pigments (VCP) was significantly lower in shade plants than in sun plants, especially in the morning. With using of epoxidase inhibitor we showed that V which was not involved in conversion was about 20% of total V pool. The photosynthesis of sun leaves was depressed strongly at midday, but changes of maximum quantum yield of PS2 (Fv/Fm) were not apparent at that time. The highest qP (photochemical quenching) was found at early morning and afternoon. qN (non-photochemical quenching) in the sun leaves increased sharply at midday. The direct relation between heat dissipating and the conversion state of VCP in plantain leaves was revealed. Apart from VCP, other carotenoids (lutein, neoxanthin, and β-carotene) can also take part in protection of PA. The results presented here clearly demonstrate that the plantain leaves resistance to excess solar radiation is determined by activation of qN mechanisms associated with the VCP de-epoxidation.

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Orange Carotenoid Protein related photoprotective mechanism in cyanobacteria

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Photosynthetic organisms have developed mechanisms protecting themselves from high light by thermal dissipation of excess absorbed energy. In cyanobacteria the photoactivation of the Orange Carotenoid Protein (OCP) is required for triggering one of such photoprotective mechanisms. The OCP is a soluble 35 kDa protein carrying a ketocarotenoid, the 3'-hydroxyechinenone (hECN) and composed of two domains: an all -helical N-terminal domain and an α/β C-terminal domain. The inactive orange form of OCP under high light illumination undergoes structural changes in the carotenoid and in the protein leading to the formation of the red active form of OCP. The active red form induces an increase of thermal dissipation of the energy absorbed by the phycobilisome, the cyanobacterial extra-membrane antenna of the photosystem II. This diminishes the effective size of the antenna, decreasing the energy arriving to the reaction center and it is accompanied by a decrease of phycobilisome fluorescence. We have identified a novel protein that mediates the recovery of the full antenna capacity when irradiance decreases: the Fluorescence Recovery Protein (FRP). In *Synechocystis* PCC 6803, this protein is encoded by the slr1964 gene, downstream the OCP encoding gene. Homologous of the *slr1964* gene are present in all the OCP – containing strains. The FRP is a 13 kDa protein that interacts with the active red form of the OCP and accelerates its conversion into the orange inactive form. Recently we have reconstituted the photoprotective mechanism in vitro using isolated phycobilisomes, OCP and FRP in order to further understand the interaction of these three elements. This characterization is a new essential approach in the understanding of the OCP-related photoprotective mechanism in cyanobacteria.

Electric field-induced Fano effect in UV absorption spectra of carotenoids

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The order and symmetry properties of electronic excited states of carotenoids are still a matter of debate, especially concerning the UV-excited states higher in energy than the 1^1B_u state attainable by excitation with visible light. A general difficulty is associated with the occurence of "dark" states of A_g symmetry, which are not observed in absorption spectra. In principle, the perturbation of the electronic states should cause a state mixing which can be observed as an electric field-induced light absorption. Apparently no such effect was observed in electronic transitions in carotenoids examined with the Stark effect (i.e. electroabsorption) spectroscopy in the spectral range covering the lowest 2^1A_g and 1^1B_u states, and in the higher energy "cis" band of carotenoid isomers (Krawczyk et al., 2006). However, we have observed significant and characteristic electric field-induced change in absorption, covering the next UV band resulting from a *g-u* transition. The electroabsorption signal consists of a dispersion-like, wide and apparently structureless biphasic band which adds to the typical derivative-like shape characteristic for carotenoids. This effect was observed in three carotenoids examined – lycopene, violaxanthin and zeta-carotene, and some of their isomers. The shape of this additional signal suggests an electric field-induced mixing of the discrete-energy excited state with the continuum of vibronic states of some lower-energy electronic state, and seems to be the case of the Fano effect (Fano 1961), well known in the exciton spectra of solids. This effect depends on the symmetry of electronic states involved which are mixed by the electric field. It seems that the analysis of this effect by its quantitative modeling based on vibronic coupling theory in linear polyenes can supply good premise for the complete classification and symmetry assignment to the higher excited states of carotenoids, and indirectly also to lower states, and to explain the apparent lack of such effects in lower-energy spectra of carotenoids.

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Solvatochromism and ultrafast laser spectroscopic investigations of carbonyl carotenoids in neat ionic liquids and their binary mixtures with organic solvents

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Femtosecond Pump-SuperContinuum Probe (PSCP) spectroscopy has been used to investigate the photoinduced dynamics of carbonyl-substituted apocarotenoids, such as 12'-apo-β-caroten-12'-al (12'C) and 12'-apo-β-carotenoic-12'-acid (12'CA), in a range of neat ionic liquids (ILs).[1][2] The results have been compared with those in heterogeneous solvation environments of IL/organic solvent mixtures as a function of composition. These probe molecules possess a unique electronically excited state (S_1/ICT) with intramolecular charge transfer character. Its spectral evolution provides details of the solvation dynamics in neat ILs, which consist of two components: a fast sub-picosecond contribution, probably due to the inertial translation of ions, and a slower response, which likely involves cage deformation and reformation processes in the IL. The latter one is conveniently described by a stretched-exponential function. Lifetimes and spectral properties of the S_1/ICT state suggest that the polarity of ILs is comparable to that of short-chain alcohols. We also find experimental indications that specific interactions between the cations of the IL and the negatively charged carbonyl end of the apocarotenoid probe influence the spectral dynamics. Comparative studies of the ultrafast solvation dynamics in a series of imidazolium-based ILs, such as $[C_x\text{mim}]^+[Tf_2N]^-$ (x = 2,4,6) and $[C_4\text{mmim}]^+[Tf_2N]^-$, highlight specific issues such as the role of hydrogen-bonding and the influence of the alkyl chain length.

Solvatochromic studies in binary mixtures of $[C_6\text{mim}]^+[Tf2N]^-$ / acetonitrile show no indications for preferential solvation around the carotenoid probe by the IL. Results from time-resolved laser spectroscopy indicate, that the lifetime of 12'CA in the lowest S_1/ICT electronic state in these mixtures linearly correlates with composition. This suggests similar local and bulk environments. Our current results will be compared with complementary studies of solvent relaxation times from dielectric and other spectroscopies.

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POSTERS

2.1.

Light induced switch between carotenoid excited states in the Orange Carotenoid Protein

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To cope with the deleterious effects of excess light illumination photosynthetic organisms developed photoprotective strategies, where the absorbed excess energy is dissipated in the form of heat within or from the antenna system, in a process known as nonphotochemical quenching. A mechanism of nonphotochemical quenching in cyanobacteria is activated by blue-green light and allows the harmless dissipation of energy from the main antenna system, the phycobilisome [1]. A crucial step in the process is the activation of a small monocarotenoid protein, known as Orange Carotenoid Protein (OCP) which binds 3'-Hydroxyechinenone. While the spectroscopic properties of the inactive form of OCP have been elucidated [2], the nature of the excited states in the active form still awaits elucidation.

We applied transient absorption spectroscopy to the dark form of OCP upon 480, 495, 540 and 550 nm excitation. The sample was subsequently illuminated by a LED emitting in the 450-500 nm region to induce OCP activation and the experiment repeated to study the excited state dynamics of the active form. We show that activation of OCP leads to the population of new carotenoid excited states. More specifically shortly after excitation a state characterized by a very pronounced charge transfer character and a lifetime of about 500 fs is populated. This state decays to the S_1 state which in turn relaxes to the ground state in about 2 ps. A third experiment performed on the pre-illuminated sample after a dark (non-illuminated) relaxation period shows that the new excited states can be created in a completely reversible manner.

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2.2.

Two-photon fluorescence excitation (TPF) spectroscopy of pigment-protein complexes and photosynthetic pigments

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Chlorophylls and carotenoids are light-harvesting pigments and essential structural components of photosynthetic pigment-protein complexes. Due to the optically forbidden character of the lowest excited singlet state ($S_1/2^1Ag^-$) of relevant carotenoids for one-photon excitation from the electronic ground state ($S_0/1^1Ag^-$) the relative energy position of the carotenoid S_1 state cannot be readily investigated by conventional spectroscopic techniques. This state, however, is generally assumed to be involved in excitation energy transfer to adjacent chlorophyll molecules, based on its supposed close energetic proximity to the chlorophyll S_1 (Q_y) state.

The carotenoid S_0- to S_1-transition is assumed to be two-photon allowed and consequently spectral peaks in the TPF spectra (detected by chlorophyll fluorescence) of light-harvesting complexes are usually ascribed to this transition. We conducted TPF studies with the plant major light-harvesting complex and chlorophylls in solution. From direct comparison to TPF spectra of relevant chlorophylls in solution we infer that there is no effective energy transfer from the carotenoid 2^1Ag^- state onto chlorophyll Q_y and/or only direct two-photon excitation of chlorophyll excited states. Consequences of these findings for recent models of carotenoid-to-chlorophyll energy transfer and non-photochemical quenching of chlorophyll fluorescence will be discussed.

2.3.

The light and dark paths of the excited state deactivation of all-*trans* and 15-*cis*β-carotene in ionic liquids

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β-Carotene in ionic liquids mimics well spectroscopic data of this pigment in situ. The light and dark paths of excited state deactivation of all-*trans* and 15-*cis*β-carotene isomers, very important in photosynthesis, in ionic liquids: $[C_8\text{-}C_1Im][BF_4]$, $[C_7H_{15}OCH_2\text{-}C_8Im][BF_4]$, $[C_8H_{17}OCH_2\text{-}C_1Im][BF_4]$were study by fluorescence and photoacoustic spectroscopy.

Comparison of absorption spectra of designed by us ionic liquids and literature data has shown that our ionic liquid purification technique is very efficient. The $[BF_4]^-$ based imidazolium ionic liquids are not as transparent as commonly has been believed and have non-negligible absorption in the UV-VIS region with the absorption red tail extending far into the visible region.

Inner-filter effect is responsible for distorted fluorescence emission spectra and nonlinear calibration curves between fluorescence intensity and fluorophore concentration. According to our study, in a case of samples like ionic liquids, in order to minimize inner-filter effect the micro-cell ought to be used. Other methods for example dilution may cause changes in the nanostructure of ions and generate artifacts. Also an excitation wavelength dependent fluorescence effect (different for various ionic liquids) known as 'red edge effect' is observed. The energetically different associated species in ionic liquids are responsible for this 'red tail'. The maxima of the fluorescence emission spectra of β-carotene in the investigated ionic liquids are at around 530 nm. Quenching of the fluorescence intensity of ionic liquids by β-carotene is not linear with path length of the cell and the values of fluorescence intensities of ionic liquid spectra differ considerably with their structures.

The dark (thermal) deactivation of both β-carotene isomers in investigated ionic liquids takes place in the similar way. Comparison of thermal deactivation of both isomers shows additional path of deactivation of excited state 1^1Ag^+ (*cis* peak).

The excited state deactivation of all-*trans* and 15-*cis*β-carotene isomers are very sensitive to the ionic liquid structure in a case of light path in contrast to the dark path-almost no sensitive to the kind of ionic liquid.

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2.4.

Seasonal changes of violaxanthin cycle pigments de-epoxidation in wintergreen and evergreen plants

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Information on the functioning of carotenoid protective mechanism in various species and ecological groups are important for understanding plant adaptation processes. We studied the seasonal changes of the carotenoids (Car) in the underwood of evergreen conifers (*Abies sibirica, Juniperus communis, Picea obovata*), and in the understory evergreen shrub (*Vaccinium vitis-idaea*), and wintergreen herbaceous plants (*Ajuga reptans, Pyrola rotundifolia*) growing in the middle taiga subzone of the European North-East. Mature green leaves and two-year-old needles were used in the experiments. Separations and quantifications of Car were carried out by reserved-phase high-performance liquid chromatography (HPLC). The total carotenoid content varied in range 500-2800 μg/g DW in dependence on plant species. Car pool was presented mainly by xanthophylls (up to 90% of the total Car) independently of species and season. Among the xanthophylls, the part of lutein was 70-75%, neoxanthin – near 10%, and violaxanthin – up to 15%. Maximum of the violaxanthin cycle pigments de-epoxidation (DEPS) was revealed in December – March, minimum was noted in May-September in all plant species. The understory herbs were characterized by the twice lower values of DEPS as compared to woody plants in which the DEPS level was up to 60% in the winter and early spring period. We found out that the *Ajuga* plant grown in sunny habitats increased in the pool of VXC pigments up to 60% (Dymova et al., 2010). A strong positive correlation between thermal dissipation of light energy and VXC pigment de-epoxidation (DEPS) was shown for the evergreen conifers (Yatsko et al., 2011). The role of Car protection in adaptation evergreen and wintergreen life forms of plants to northern environments will be discussed.

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2.5.

Antioxidant properties of carotenoids in model pigment-protein systems

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Carotenoids are structurally and functionally a very diverse group of isoprenoid pigments. They occur commonly in all photosynthetic organisms carrying out diverse functions. Apart from light harvesting and structural function (photo)protection is considered as one of their most important role, however still not fully understood. Carotenoids are well known physical quenchers of chlorophyll excited states and reactive oxygen species. They also act as efficient chemical quenchers of reactive species undergoing irreversible modifications (Fiedor et al., 2005).

In the present study we investigated the protective role of carotenoids toward chemically oxidised (bacterio)chlorophylls in model pigment-protein complexes. In this approach a series of model LH1 complexes was prepared with carotenoids that significantly differed in structure (i.e. length of their C=C bonds, presence of additional side groups), following a recently developed method (Fiedor et al., 2004). For oxidation reactions various concentrations of either potassium ferricyanide or hydrogen peroxide was used. The progress of oxidation reaction of model LH1 complexes was monitored by following the changes in their UV-VIS absorption, fluorescence and thermoluminescence spectra. Oxidation of model pigment-protein complexes with either potassium ferricyanide or hydrogen peroxide leads to irreversible changes in their absorption spectra indicating progressive decomposition of the pigments. The changes in absorption spectra are paralleled with the decrease of emission intensity in fluorescence spectra and a temperature shift observed in thermoluminescence spectra. Our results indicate that carotenoid presence do affect the stability of a complex exposed to chemical oxidation. The most stable are complexes reconstituted with long-chain carotenoids.

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2.6.

The influence of carotenoid population on the structure and fluorescence emission properties of the LH2 light-complex from *Rhodopseudomonas palustris*

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It is known that varying the growth conditions of purple photosynthetic bacteria different carotenoids may be incorporated into the light-harvesting (LH) complexes. This can be achieved by either culturing the cells under different light regimes, or in the in the presence of diphenylamine (DPA) which inhibits the activity of phytoene desaturase. We will present our recent work on the influence of carotenoid type on the structure and fluorescence properties of isolated LH2 molecules.

2.7.

Excitation energy dependence of intramolecular charge transfer dynamics of fucoxanthin

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Carotenoids containing a carbonyl group in conjugation with their polyene backbone are naturally-occurring pigments in marine organisms and are essential to the photosynthetic light-harvesting function in aquatic algae. These carotenoids exhibit spectral characteristics attributed to an intramolecular charge transfer (ICT) state that arise in polar solvents due to the presence of the carbonyl group. In this study, we report the spectroscopic properties of the carbonyl containing carotenoid fucoxanthin in polar (methanol) and nonpolar (cyclohexane) solvents investigated by steady-state absorption and femtosecond pump-probe measurements. Transient absorption associated with the optically forbidden S_1 (2^1Ag^-) state and/or the ICT state were observed following one-photon excitation to the optically allowed S_2 ($1^1B_u^+$) state in methanol. The transient absorption measurements carried out in methanol showed that the ratio of the ICT-to-S_1 state formation increased with decreasing excitation energy. We also showed that the ICT character was clearly visible in the steady-state absorption in methanol based on a Franck-Condon analysis. The results suggest that two spectroscopic forms of fucoxanthin, blue and red, exist in the polar environment.

2.8.

COP1 regulates biosynthesis and degradation of xanthophylls in *Arabidopsis* seedlings

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COP1 (*constitutive photomorphogenesis 1*) is an E3 ubiquitin-protein ligase involved in signaling pathways mediating gene expression in both plant and animal cells. In plants, COP1 is crucial in the developmental switch to autotrophy during the first exposure of emerging seedlings to light (deetiolation). During deetiolation, the light-triggered photoreduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide), catalyzed by light-dependent protochlorophyllide oxidoreductase (LPOR), has a key role in the regulation of Chl biosynthesis. Carotenoids are photoprotective and antioxidant pigments synthesized in plants. In particular, xanthophylls were found to be essential for the formation of the prollamellar body (PLB) [1]. Violaxanthin was demonstrated to be bound in photoactive complexes of NADPH:LPOR [2]. In this study, using Reversed Phase-HPLC and fluorescence spectroscopy, we estimated pigment composition in 5 days old etiolated seedlings of *cop1* mutant, previously shown to accumulate high amounts of Pchlide not complexed in PLB's. In darkness and under moderate light conditions (100 μE $m^{-2}s^{-1}$) the concentrations of lutein and violaxanthin in *cop1* seedlings were 2-3 times higher in comparison to wild type (WT). In contrast, the irradiation with an excessive light intensity (400 μE $m^{-2}s^{-1}$), resulted in the decrease of violaxanthin concentration in *cop1* mutant, whereas lutein concentration was not altered under that conditions. Preferential degradation of violaxanthin was neither accompanied with the formation of antheraxanthin/zeaxanthin nor observed in WT seedlings. This effect correlated with a rapid photodegradation of Pchlide observed in *cop1* seedlings in strong light. Our results indicate that COP1 is a negative regulator of xanthophyll biosynthesis in darkness. Additionally, under light conditions, COP1 may regulate processes that maintain physiological levels of photoprotectants and/or anitioxidants during seedling deetiolation.

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2.9.

Electrochromism of carotenoid photosensitizers adsorbed on TiO_2

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The development of new organic sensitizers for the purposes of dye sensitized solar cells and photocatalysis requires a better understanding of the complexity of their electronic states on the surface of TiO_2 – the oxide semiconductor widely used in research and prototype constructions. This communication presents a study of carboxylated derivatives of all-*trans* carotenoids retinoic acid (RA) and bixin by Stark effect (electroabsorption) spectroscopy in glassy ethanol at low temperature. They were studied both as free monomeric species and when adsorbed to nanoparticles of titanium dioxide in the presence of variable concentration of acetic acid. Both pigments adsorbed completely at the lowest acid contents (<0.2%), but become partially desorbed at the higher acid concentration (2%). Adsorption of pigments on TiO_2 was accompanied by an increase of difference dipole moment between the ground and excited state of RA from 14.5 D for free pigment to 22.5 D for pigment adsorbed on TiO_2. A smaller change in dipole moment was estimated for bixin. The remarkable spectral inhomogeneity exhibited by significant fractions of both pigments with larger changes in dipole moment is ascribed to the differences in binding sites on the surface of TiO_2. We observed a gradual shift of absorption and electroabsorption spectra due to the charging of nanoparticles induced by the increasing acid concentration. This spectral shift and electrooptical parameters of adsorbed pigments were used to estimate the change in surface electric field of charged nanoparticles, which is approximately (2±0.7) MV/cm.

2.10.

Effect of cold acclimation and the *pgp1* mutation on the expression of GLK1 and GLK2 and chloroplast development in *Arabidopsis thaliana*

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The *pgp1* mutation in *Arabidopsis thaliana* results in a 30% decrease in phosphatidylglycerol (PG) content compared to WT. Although growth and development of the mutant at 20°C exhibited a phenotype indistinguishable from that of WT, growth of *pgp1* at 5°C resulted in leaves with a distinct yellow-white phenotype compared to either the WT or other lipid mutants (*mgd1* and *dgd1*) grown under identical conditions. The appearance of this low-temperature phenotype was accompanied by a reduction in total leaf chlorophyll (Chl) and carotenoids content and an increase in the Chl *a*/*b* ratio compare to WT. Immunoblot analysis indicated a lower abundance of Lhcb1 and Lhcb2 polypeptides with minimal changes in the content of the PSII reaction center polypeptide, D1. In contrast, growth of *pgp1* at 5°C resulted in a significant decrease in abundance of the PSI-associated polypeptides, PsaA, PsaB and PsaD. TEM revealed a reduced number of thylakoids per grana stack in *pgp1* grown at 20°C compared to WT, while the number of stromal thylakoids remained the same. However, the formation of granal stacks was completely inhibited and the number of stromal thylakoids per chloroplast was significantly reduced during growth at 5°C in *pgp1* compared to WT. These cold induced ultrastructure features in *pgp1* mutant are associated with reduced expression of GLK1 and GLK2 transcription factors, implicated in regulation of photosynthesis related genes and chloroplast development. Since the transcription of GLK1 and GLK2 is controlled by chloroplast to nucleus retrograde signaling, the role of PG in the regulation of chloroplast biogenesis and functioning of thylakoid membranes at low temperature will be discussed.

2.11.

The S* state of carotenes is the vibrationally hot ground electronic state S_0*

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Once a carotenoid is promoted to the S_2 state by one-photon excitation, the simple electronic relaxation pathway $S_2 \rightarrow S_1 \rightarrow S_0$ is frequently assumed for interpreting the dynamics. Additional states have been proposed as potentially important intermediates, e.g. the so-called S* state, yet its relevance in the excited-state network is currently hotly debated. [1] Several interpretations regarding the nature of the S* state have been put forward by various groups, describing it either as an electronically excited state, the vibrationally hot ground electronic state (S_0*) or the vibrationally hot S_1 state. By performing ultrafast Pump-SuperContinuum Probe (PSCP) spectroscopy over a particularly wide spectral range in conjunction with temperature-dependent steady-state absorption experiments we demonstrate that the characteristic S* spectral signatures are due to highly vibrationally excited carotene molecules in the ground electronic state (S_0*), which are formed by internal conversion from the S_1 state. [2,3] The spectral features are observed for a range of structurally different carotenoids and decay with a time constant in the 10 ps range which is characteristic for collisional deactivation of the vibrationally hot S_0* molecules by the solvent. We also demonstrate that previous experiments reported in the literature, which originally assigned the S* spectral features to an excited electronic state, are actually in agreement with the findings of the current investigations.

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2.12.

Effects of hypersaline stress in the xanthophyll cycle and antioxidant metabolism of the Mediterranean seagrass *Posidonia oceanica*

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Chronic hypersaline stress induces partial inhibition of photosynthetic carbon assimilation of the Mediterranean seagrass *Posidonia oceanica*. The aim of the present study was to determine if under hypersaline stress the species is able to activate protective mechanisms through thermical light excitation dispersion (xanthophyll cycle) and/or antioxidant protection. To this end *P. oceanica* was exposed to control (37 psu) and increased salinity (43 psu) during 11 weeks in a mesocosm system. Subsequently, the photochemical (Fv/Fm-Fv'/Fm') and non-photochemical quenching (NPQ) were determined with a PAM fluorometer, together with the analysis of chlorophylls and the carotenoids involved in the xanthophyll cycle under two approximations: 1) along a daily cycle under ambient irradiance in the mesocosm and 2) exposing the plant leaves to high irradiance levels to induce NPQ. Antioxidant enzyme activities (catalase and GST) and lipid peroxidation (MDA) were also analyzed in plant leaves but just in the first approximation. During the daily light cycle simulated in the mesocosm system the following results were observed: a) no accumulated damage on photosystem II, b) low NPQ values in hypersaline and control treatments (0.2-0.3), c) no de-epoxidation of violaxanthin was detected in both treatments, d) catalase activity increased in hypersaline-stressed plants and e) no sings of lipid membrane degradation (lipid peroxidation, MDA) was found in any case. These results suggest that under ambient conditions hypersaline stress induce the activation of effective antioxidant mechanisms, but not of NPQ maybe because leaves were not under excessive light levels. In fact, when leaves were exposed to high light, NPQ drastically increased to 7.1 in control plants and reached significantly ($p < 0.01$) higher values (9.0) in the hypersaline treatment. Unexpectedly, these differences in NPQ were not accounted by the de-epoxidation state of xanthophylls, which was also reflected by the similar fast recovery of NPQ between both treatments. The slow NPQ recovery phase, associated to the D1 protein turnover, indicated a higher accumulated photodamage in leaves of the hypersaline condition. In conclusion, our results suggest that hypersaline stress induce the antioxidant capacity in *P. oceanica* leaf tissues but not the xanthophyll-dependent thermal dissipation mechanisms.

2.13.

Different colors of canthaxanthin in feathers of *Eudocimus ruber*, *Iodopleura isabellae* and *Piranga rubra*

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Feather coloration can be pigmentary, structural, or a combination of the two. Pigmentary color can be due to melanin and also to carotenoids, representing an interesting illustration of the contribution of carotenoids to natural colors.

Several carotenoids have been reported in bird feathers and novel ketocarotenoids were recently identified in burgundy feathers. Feathers containing the same carotenoid can have different colors as a consequence of the binding between the carotenoid and the feather structural protein keratin. The aim of the present study is to use resonance Raman spectroscopy to investigate carotenoid-keratin interactions responsible for the different coloration of *Eudocimus ruber, Iodopleura isabellae* and *Piranga rubra*. These feathers all contain the same carotenoid, canthaxanthin, but are red, purple and red-orange respectively.

Chromatography and resonance Raman spectroscopy data will be presented and discussed. The understanding of such phenomenon may help in the elucidation of color variation and changes due to carotenoid-protein interactions in other living organisms.

2.14.

The role of carotenoid isomerase in photoprotection in rice

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In this work, we isolated a yellowish-leaf mutant from *Oryza sativa* (rice). Map-based cloning approach revealed that the gene encodes a predicted carotenoid isomerase named as *OsCRTISO*. Comparison of wild type, the zeaxanthin (Z) was hardly detectable and lutein was only 30%, while violaxanthin (V) and antheraxanthin (A) were increased nearly 3-fold and neoxanthin and -carotene increased evidently. In addition, the xanthophylls cycle (A+Z)/(A+Z+V) decreased by about 45% under growth light condition (400 μmol photons $m^{-2}s^{-1}$) and 53% under high light (1000 μmol photons $m^{-2}s^{-1}$) in the mutant. These results indicate that both the de-epoxidation of antheraxanthin to zeaxanthin and conversion of lycopen to lutein were suppressed in the mutant. Further analysis indicates that the mutation caused decrease in (1) LHCII trimers and supercomplex of photosystem II (PS II), (2) the capacity of PS II; (3) other thermal dissipation pathways, such as nonphotochemical quenching and state transitions; (4) accumulation of hydrogen peroxide in the leaves. The suppression of PS II capacity was much more significantly under the high light condition. Further investigation indicates that the amount of the core protein CP43 and CP47 especially in the supercomplexes of PS II and their expression in the transcript or translation level was suppressed in the mutant. Based on those results, we suggested that the low level of lutein and suppression of xanthophyll cycle increase the sensitivity of rice plant to light. The possible role of caroteinoid isomerase in photoprotection of PS II is discussed.

2.15.

DFT studies of open chain carotenoid radicals: dependence on conjugation length

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Previous EPR (electron paramagnetic resonance) and DFT (density functional theory) studies on carotenoids containing terminal rings like zeaxanthin and violaxanthin (Focsan et al., 2008), lutein (Lawrence et al., 2008), 9'-*cis* neoxanthin (Focsan et al., 2009) and astaxanthin (Polyakov et al., 2010) have shown that carotenoid radical cations deprotonate to form neutral radicals. Proton loss occurs preferentially from the methylene and methyl groups of the terminal rings, while the presence of functional groups like epoxy, carbonyl and allene on the terminal rings prevents this loss. As a consequence of deprotonation, a change in the unpaired electron spin distribution produces larger hyperfine coupling constants for the neutral radicals than for the radical cations and thus permits their identification in a mixture analyzed by using Mims ENDOR techniques.

Here we describe open chain carotenoids, either symmetric or asymmetric, with varying conjugation length (n=9-15 carbon atoms) and different functional groups. The addition of protons and functional groups like methoxy or carbonyl to a fully conjugated open chain carotenoid causes a preferential proton loss from the radical cation formed. Formation of carotenoid radicals of dehydrolycopene, lycopene, neurosporene, spheroidene, spheroidenone, anhydrorhodovibrin and spirilloxanthin has been investigated using DFT calculations which revealed the most favorable neutral radicals, their unpaired spin density distribution, the relative energies and the hyperfine coupling constants.

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2.16.

Carotenoids content in etiolated seedlings of three *A. thaliana* ecotypes

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Angiosperms require light for their morphogenesis. While growing in the dark, seedlings become etiolated, which means, among others, that they are yellowish, contain no chlorophyll, and develop etioplasts instead of chloroplasts. Biosynthesis of chlorophyll is stopped at the level of protochlorophyllide (Pchlide) formation, that accumulates in etioplasts. The light-triggered reduction of Pchlide to chlorophyllide, catalysed by a light-dependent protochlorophyllide oxidoreductase (EC 1.3.1.33), induces the deetiolation process. Furthermore, light regulates also biosynthesis of carotenoids, indirectly via phytochrome-controlled level of phytoene synthase, the first enzyme of the pathway. Etiolated seedlings accumulate

carotenoids, however, their significance for the deetiolation process has not yet been revealed.

The presented study was aimed at revealing differences in carotenoid composition of three wild ecotypes of *Arabidopsis thaliana: Columbia (Col-0), Landsberg erecta (Ler)* and *Wassiliewska (WS)*. It is a part of the research to investigate the role of carotenoids in the deetiolation process and correlations between carotenoids and chlorophyll biosynthesis pathways.

Carotenoid composition was determined in etiolated seedlings by reversed-phase high-performance liquid chromatography (HPLC). The developed method enabled us to separate up to 16 compounds both from chlorophyll as well as carotenoid biosynthesis pathways. Lutein was the most abundant in all three ecotypes and significant changes were observed in the level of polar carotenoids with respect to that of Pchlide between the ecotypes. We have also measured the carotenoid content in seedlings during their deetiolation in different conditions (light intensity and duration). The importance of changes in the carotenoid content in relation to chlorophylls will be discussed.

2.17.

Simulation of two-color photon echo signals of all-*trans* lycopene and spheroidene observed by transient four-wave-mixing spectroscopy: evidence for the presence of the $3A_g^-$ and $1B_u^-$ states below the $1B_u^+$ state in energy

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Recently, Davis et al. have identified quantum-beat signals in all-*trans* lycopene and spheroidene by a two-color three-pulse photon echo experiment (Davis JA et al., 2010). The pulse duration was 100 fs and the signals were observed in the phase-matched direction $k_4=-k_1+k_2+k_3$ when ω_1 = 19 880 cm^{-1} and $\omega_2=\omega_3$=18 800 cm^{-1} for lycopene and and ω_1 = 20 700 cm^{-1} and $\omega_2=\omega_3$=19 740 cm^{-1} for spheroidene. The first pulse is resonant with the $1A_g^-(0)\rightarrow 1B_u^+(0)$ bright transition (numbers in parentheses denote a vibrational quantum number) and the energy difference between ω_1 and ω_2 is nearly equal to that of the C-C stretching vibrational mode (~1000 cm^{-1}) for both molecules.

We performed numerical simulations of the photon echo signals for cases when the first and second pulses hit the sample simultaneously by using the third-order nonlinear response function. The result of simulations showed that there must be a vibronic state such that it is ~1000 cm^{-1} below the $1B_u^+(0)$ state in energy and has a large value of Frank-Condon factor as well as an appreciable value of electronic transition dipole between it and the electronic ground state, suggesting the presence of a new electronic state just below the $1B_u^+(0)$ state for each molecule. The measurement of resonance-Raman excitation profiles and fluorescence spectroscopy for carotenoids has predicted the $3A_g^-(0)$ state for lycopene and the $1B_u^+(1)$ state for spheroidene located ~1000 cm^{-1} below the $1B_u^+(0)$ state (Koyama Y et al., 2010). Therefore, the observed photon echo signals by Davis et al. confirm the presence of the $1B_u^-$ and $3A_g^-$ states below the $1B_u^+$ state in carotenoids. Possible origins of the quantum best will be discussed.

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2.18.

Ultrafast excited state dynamics and spectroscopy of 13,13'-diphenyl-β-carotene

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The excited state dynamics of the newly-synthesized artificial β-carotene derivative 13,13'-diphenyl-β-carotene has been investigated in several solvents by ultrafast transient broadband absorption spectroscopy based on the Pump-Supercontinuum Probe (PSCP) technique. Time-resolved spectra were recorded in the wavelength range 340 770 nm with ca. 60 fs cross-correlation time after excitation to the S_2 state [1]. The dynamics of the internal conversion (IC) processes $S_2\rightarrow S_1$ and $S_1\rightarrow S_0$* have been compared with results for β-carotene to elucidate the influence of phenyl substitution at the polyene backbone. The transient spectra were analyzed globally to extract IC time constants and time-dependent spectra for the electronic species involved. Transient changes in the S_1 spectrum indicate intramolecular vibrational relaxation (IVR), which is considerably accelerated by phenyl substitution and also solvent-dependent. We also performed DFT and TDDFT-TDA calculations showing that the phenyl substituents are oriented almost perpendicularly with respect to the plane of the carotene backbone. They are therefore largely electronically decoupled from the conjugated double bond system of the polyene. The findings are in agreement with observations based on other experimental techniques: For instance, an up-field chemical shift is found in the ^{1}H NMR spectrum which arises from a ring-current effect for the adjacent hydrogen atoms. Also, the red-shift of the $S_0\rightarrow S_2(0\text{-}0)$ transition energy in the steady-state absorption spectrum relative to β-carotene is small. Moreover, almost the same $S_1\rightarrow S_0$* IC time constant as in β-carotene is extracted from the kinetic analysis, which suggests a similar S_1-S_0 energy gap. The oscillator strength of the $S_0\rightarrow S_2$ transition of the diphenyl derivative is reduced by ca. 20%, which is possibly due to absorption contributions from configurations with more strongly tilted phenyl rings due to the thermal motion at room temperature.

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2.19.

Comparison of the de-epoxidation of violaxanthin associated with the light-harvesting complexes of spinach and the algae *Mantoniella squamata* (Prasinophyceae)

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In plants and algae the xanthophyll cycle (XC) plays an important role for the adaptation to different light conditions. Under low light conditions the major part of the light-harvesting pigment violaxanthin (Vx) is associated with the proteins of the antenna system. Under saturating light conditions Vx is released from its binding site into the surrounding lipid phase, where it is converted to the energy-dissipating pigment zeaxanthin (Zx) via the intermediate antheraxanthin (Ax). This reaction is catalyzed by the enzyme Vx de-epoxidase (VDE). In contrast to higher plants where the main de-epoxidation product is Zx, the prasinophyceaen alga *Mantoniella squamata* shows in vivo an incomplete XC, leading to a strong accumulation of Ax. Another exceptional feature of *M. squamata* is the presence of a unique type of light-harvesting complex (LHCp) which shows a different structure and a more complex pigment composition in comparison to other antenna proteins, including the main PSII light-harvesting complex (LHCII) of higher plants. In the present study we investigated the de-epoxidation of Vx which was still associated with the light-harvesting complexes (LHC) of spinach or *M. squamata*. The LHC were isolated with different concentrations of natively bound lipids and Vx by sucrose gradient centrifugation or successive cation precipitation. In both LHC types the decrease of the concentration of LHC-associated Vx was accompanied by a diminished content of the main thylakoid lipid monogalactosyldiacylglycerol (MGDG), with the difference that the LHCp contained much lower Vx concentrations than the LHCII. Furthermore, the convertibility of LHC-associated Vx was studied by addition of isolated VDE. The de-epoxidation of LHCII-associated Vx depended on the Vx/MGDG ratio, i.e. a reduced Vx de-epoxidation was observed below and above an optimal Vx/MGDG ratio where nearly all Vx was efficiently converted. In contrast, the de-epoxidation of Vx associated with LHCp was more strongly influenced by the Vx concentration or the MGDG content than the Vx/MGDG ratio.

2.20.

Intramolecular charge-transfer state of carbonyl carotenoids in LH1-RC complexes of purple bacteria

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Intramolecular charge-transfer (ICT) state is an excited state specific of carbonyl carotenoids. ICT state is strongly coupled to the S_1 state, forming new electronic state usually denoted as S_1/ICT. It is identified by its characteristic ICT-like transition, which becomes pronounced in polar environment and it is red-shifted from the well-known S_1-S_n transition (Frank et al., 2000).

Using femtosecond time-resolved spectroscopy, we performed experiments on LH1-RC complexes of 1) wild-type *Rhodobacter sphaeroides* with carbonyl carotenoid spheroidenone 2) *Rhodobacter sphaeroides* G1C strain containing a non-carbonyl carotenoid neurosporene.

While LH1-RC complexes with neurosporene exhibited typical S_1-S_n transition observed in other LH complexes having non-carbonyl carotenoids, transient absorption spectrum of LH1-RC complex with spheroidenone is dominated by a new spectral band centred at 750 nm that we identified as due to the ICT state of spheroidenone. The S_1/ICT lifetime of spheroidenone in LH1-RC complex is significantly shorter than the S_1 lifetime of neurosporene in LH1-RC, suggesting more efficient S_1-mediated energy transfer from spheroidenone. This could be caused by the increased $S_0 \rightarrow S_1$/ICT transition dipole moment and more favourable spectral overlap of S_1/ICT and bacteriochlorophyll Q_y (Polívka and Frank, 2010).

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2.21.

Interconversion of the xanthophyll pigments under heat stress in etiolated and green seedlings of triticale

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The effect of heat stress (HS, 42-44 °C) on carotenoid composition was studied in the leaves of short- and long-stem triticale cul-

tivars. In the leaves of etiolated seedlings, the ratio of lutein, β-carotene, and xanthophylls (violaxanthin + antheraxanthin + zeaxanthin) was evaluated as 55, 5, and 34 – 40 % of the total carotenoid content, respectively. Upon HS quite distinct changes in the ratio of xanthophyll pigments were observed: the violaxanthin content decreased and the zeaxanthin one increased. The efficiency of conversion violaxanthin-zeaxanthin increased with the time the seedlings were heated (3-5 h). The HS affected the relative content of violaxanthin and zeaxanthin to a greater extent than antheraxanthin as its ratio in the xanthophyll composition (39-49 %) decreased to no more than 20 % by heating. Generally, carotenoids associated with the violaxanthin cycle in both triticale cultivars demonstrated no significant distinction in their HS response. Thus, after heating for 5 h, degree of de-epoxidation [zeaxanthin/(zeaxanthin+violaxanthin)] attained 0.9 in both varieties, with higher relative content of violaxanthin in the leaves of a short-stem triticale.

In green seedlings of both varieties, lutein (about 47%) and β-carotene (about 22%) predominated in the carotenoid composition. Total content of pigments associated with the violaxanthin cycle did not exceed 18% of all carotenoids, with violaxanthin comprising 97% of total xanthophylls content. Heating of triticale green seedlings under illumination (64 μmol m^{-2} c^{-1}) even over a period of 3-5 h at 44°C did not result in the xanthophyll cycle activity as well as in alterations of the content of other carotenoids.

Our data give evidence that HS-promoted interconversion of carotenoids from the xanthopyll cycle is activated exclusively in etiolated seedlings of triticale. Furthermore, lack of hyperthermia-induced transformations of xanthophyll pigments in green triticale plants under moderate illumination suggests that systems other than xanthophyll cycle act as HS-protectors. Additionally, our data indicate that the composition of carotenoids under HS was independent from the level of endogenous abscisic acid (1.4-times higher content in a short-stem cultivar), with a part of violaxanthin pool presumably serving as a substrate.

2.22.

Application of resonance Raman microscopy to in vivo carotenoid

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The high antioxidant activity of astaxanthin has been attracted considerable attention in these days. One of the major antioxidant activities of this carotenoid is anti-photoaging. We have been focusing our attention on this particular issue. The anti-photoaging activity should be functioning in inner skin. In this study we tried to find out the fact that astaxanthin that has been swabbed on the outer surface of the skin has really passed through and reached to the inner skin. For this purpose resonance Raman microscopy was applied to the rat skin sample on which astaxanthin was swabbed on its outer surface. Astaxanthin gives rise to a unique Raman spectrum that is characteristic of its molecular structure. Therefore, we can easily identify the presence or absence of astaxanthin in the area of the rat skin that is subjected to this spectroscopic measurement.

We used 532 nm laser light for probing the resonance Raman scattering of astaxanthin. Astaxanthin shows three strong Raman lines at 1508, 1145, and 993 cm^{-1}. These three lines are ascribable to the C=C stretching, C-C stretching, and C-CH_3 in-plane rocking vibrational modes, respectively. We have constructed confocal Raman microscope that has the spatial resolution of 1 μm. 3-Dimensinal mapping of the Raman spectrum has been performed in order to determine the distribution of astaxanthin in the rat skin.

2.23.

Dark excited states of carotenoid in photosynthetic light harvesting complex studied by multi-pump spectroscopy

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In photosynthesis, carotenoids play important roles in light harvesting (LH) and photoprotective functions. The S_2 and S_1 excited states in carotenoids are important in the LH function. Recently, another dark state, S*, has been identified in LH complexes (Polívka and Sundström, 2004). Here, we have applied multi-pump spectroscopies to the reconstituted LH1 complex from *Rhodospirillum rubrum* S1. The prepump-pump-probe spectroscopy shows that the pre-excitation of bacteriochlorophyll (BChl) increases the formation of the S* state in carotenoid (Car) (Nakamura et al., 2011). The vibrational dynamics of the S_1 and S* states of Car in LH1 have been observed by femtosecond stimulated Raman spectroscopy. The relaxation of the ν_1 vibrational mode (C=C stretch.) of the S_1 state has a time constant of 0.4 ps. Since the BChl Q_y state has the similar formation time constant, the hot S_1 state is important in the light harvesting function. The Raman signal of the S* state is different from the S_1 state but has similar structure with the triplet state of Car. Our findings provide an explanation for observed spectroscopic features, including the excitation-intensity dependences of the S* state debated so far and offer new insights into energy transfer and deactivation mechanisms inherent in the LH antenna.

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2.24.

Light-induced isomerization of neoxanthin in LHCII

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The largest pigment-protein antenna complex of the Photosystem II, LHCII, is the structure responsible for a collecting and transfer of excitation energy to the reaction center. A rate of excitation transfer needs to be adjusted to a capacity of the photochemical reactions, in order to protect the photosynthetic apparatus against oxidative damage. Green plants have developed different photoprotection mechanisms operating at all the organization levels. Besides reorientation of leaves and translocation of chloroplasts, high-light induced reactions in the photosynthetic apparatus at the level of single photosynthetic pigment-protein complexes are observed. The role of LHCII-pound xanthophylls in photoprotection at that level is a subject of intensive research of many laboratories worldwide.

Our analyses reveal that illumination of the isolated LHCII leads to isomerization of the protein-bound neoxanthin from the conformation 9'-*cis* to 9',13- and 9',13'-*dicis* forms. At the same time decreasing in chlorophyll a fluorescence intensity and shortening in fluorescence lifetimes is observed as a light-driven excitation quenching result. Both processes, the neoxanthin isomerization and the chlorophyll excitation quenching, are reversible in dim light. The results of the 77 K florescence measurements of LHCII show that illumination is associated with appearance of the low energy states, which can act as energy traps in antenna complex subjected to excess excitation.

We propose the model of excitation quenching in LHCII, based on neoxanthin photo-isomerization.

Session 3

Chemistry, Analysis, Chemical Synthesis, and Industrial Production of Carotenoids

Professor Dr. Hans-Dieter Martin
and
Dr. Rodney Lee Ausich
in Memoriam

Professor Dr. Hans-Dieter Martin
1939 – 2009

Hans-Dieter Martin was born on January 18, 1939 in Berlin and later moved with his parents to Singen where his father managed a pharmacy. In 1958, he started to study chemistry at the Albert-Ludwigs University in Freiburg where he finally received his PhD in 1969 with a thesis on the topic "Thermolysis of Cyclopropane Compounds". Working in the group of Professor Prinzbach, Hans-Dieter Martin became interested in small, carbon composed ring systems some of unusual, aesthetic structure. He was studying their thermal, photochemical, and catalyzed behavior and considered these small tensed ring systems as "a playground for an organic chemist". During post doc visits abroad he studied Photoelectron Spectroscopy in Basel (Prof. Heilbronner) and Gas Phase Kinetics in Reading (Prof. Frey).

In 1975, Hans-Dieter Martin habilitated at the Albert-Ludwigs University Freiburg and became Professor for Organic Chemistry at the Julius-Maximilian-University Wuerzburg. His research interests extended to natural and synthetic dyes, pigments, heterocyclic compounds, their synthesis, reaction mechanisms, spectroscopic properties and photochemistry. Among these polyenic compounds were the carotenoids to which he further dedicated a large part of his scientific activity. In 1980 he received the Carl-Duisberg-Award of the German Chemical Society and became Professor of Organic Chemistry at the Institute for Organic Chemistry and Macromolecular Chemistry at the Heinrich-Heine University, Duesseldorf; this was a position that he held until his retirement in 2006. Although suffering from cancer, he was heading a research group on "Micronutrients-Photoprotection and Photodynamic" in a collaborative research center, afterwards. Professor Martin died on March 8, 2009 in Wuerzburg.

Hans-Dieter Martin was an outstanding scientist with over 150 publications covering organic, physical and biochemistry and was cooperating with major industrial companies such as BASF or Henkel. However, he also was an engaged academic teacher most popular with students. Following his initiative, the University of Duesseldorf installed a new, integrated course on economic chemistry. He considered carotenoid research as "his most colourful activity" and his interest covered the entire field from chemical synthesis and basic research to application of carotenoids in industry and human health. Hans-Dieter Martin established numerous synthetic routes for natural carotenoids and non-natural analogs. Among them are natural aromatic carotenoids like dihydroxyisorenieratene or artificial hybrids composed of polyenic carotenoids and aromatic flavonoids or porphyrins. Spectroscopic and physicochemical properties, absorption dynamics or aggregate formation related to structural features were in his focus as well as the biochemical activities related to quenching of excited state molecules, radical scavenging, light absorption, impact on gap junctional communication, retinoid signaling or gene expression. He could oversee great parts of the research area and was always willing to assist with advice on various laboratory projects.

Hans-Dieter Martin was a highly considered colleague and well respected not only in the carotenoid community but in the scientific community at large and his death was a huge loss not only for his wife and family but also for all other who knew him. Professor Martin is survived by his wife Marianne Martin, and two daughters, Stefanie and Barbara. Hans-Dieter loved nature and his hobbies were photography and diving.

Dr. Rodney Lee Ausich
1953 – 2010

Dr. Rodney Lee Ausich fought a heroic battle against urothelial cancer. On June 25, 2010 at age 56 Rod lost his battle at the Mercy Hospital in Des Moines surrounded by his wife, children, father and cousin. Rod was a dad, husband, son, brother, cousin, nephew and brother-in-law. Rod gave it all. He gave it all to the family he and Rebecca loved together. He gave it all to his dear parents, John and Leda. He gave it all to Kemin.

Rod was born on October 28, 1953 to John and Leda Ausich in Casper Wyoming. Growing up in Casper, he attended McKinley Elementary School, Dean Morgan Junior High School, and Natrona County High School graduating in 1972. He subsequently graduated from the University of Wyoming in 1976, earning a BS degree in Biology/Botany. He was a member of both Phi Beta Kappa and Phi Kappa Phi. He received his master's and Ph.D. degrees in plant sciences from Indiana University in 1978 and 1980 respectively. He also received a Master of Business Administration from Lake Forest Graduate School of Management in 1991. He began his career working as a research scientist in 1980 for Amoco Corporation where he researched plant tissues identifying genes that would increase the production of valuable chemicals.

Rod joined Kemin in 1994 where he engaged in groundbreaking research and development, ultimately becoming the founding father of Kemin Health after realizing the benefits of lutein for eye health in combating age-related macular degeneration (AMD). Rod authored 7 published and 5 pending patents while at Kemin. Kemin Health is the world's leading supplier of lutein. Kemin Health grew from 2 employees (Rod and Chuck) to 150 employees, separate from Kemin Industries under the leadership of Rod as the President, Kemin Health.

Rod was an active member of the Des Moines community serving on the Board of Directors of Mercy College, and ultimately Chairman of that Board for 4 years. Rod is survived by his wife, Rebecca, and two children, Evan and Brandyn. Rod will be remembered by those who knew him as a man of quiet presence, but passionate about his family, his work, and our natural environment, which he deeply enjoyed and championed. Growing up in Casper, he had a passion for sports, participating in Little League, Babe Ruth League, and American Legion baseball. As an adult, he was an accomplished golfer, he fostered a joy of skiing in Colorado with his children, and enjoyed boating whenever possible. Cooking and playing with family and friends were his real interests. He loved the big blue skies of Wyoming and had a passion for the west. He was a voracious reader. He was never without a book and had an entire library of books that he was planning to immerse himself in "some day".

At Kemin Rod was well known for the unusual hobby of collecting unique beer glasses and mugs from all over the world. Rod always believed in education. Several students have benefited from his scholarship fund including a sponsored child living in Peru and students from Roosevelt High School and Natrona County High School.

INVITED LECTURES

Dietary carotenoids – impacts of technological processing

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Since carotenoids are not synthesized by humans their ingestion with the diet is a prerequisite. Although carotenes and xanthophylls are accumulated in human plasma and tissues equally well, only about 20 carotenoids have been detected in human beings (Britton et al., 1998) The major carotenoids in human plasma are β-carotene, lycopene, β-cryptoxanthin, lutein, α-carotene and zeaxanthin. Carotenoids predominantly occur in their *all-trans* configuration. Owing to the highly unsaturated system of double bonds, they are susceptible to degradation and to isomerization to their *cis* forms thus, resulting in alterations of physicochemical and biological properties, i.e. reduced provitamin A activity and bioavailability. Apart from naturally occuring in plants, *cis*-isomers have been demonstrated to be formed during technological processing such as drying, microvaving, canning, cooking and heat preservation. Furthermore, storage time an conditions such as temperature and light may affect *cis*-isomerization (Carle and Schieber, 2008). This review summarizes our recent investigations on the effects of processing on carotenoid stability in vegetables, fruits, functional foods, and dietary supplements. Particular attention was given to methods for the determination of carotenoid stereoisomers and to the role of the physical state of carotenoids and the plant matrix for their stability.

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Carotenoids with kappa end-group: retrospect and recent progress

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Capsanthin and capsorubin, the first carotenoids containing κ-end group were isolated from red paprika (*Capsicum annuum*) (Deli and Molnár, 2002). These red pigments are biosynthetised from carotenoid-5,6-epoxides by means of specific enzymes, the capsanthin-capsorubin synthases (Bouvier et al., 1994). Capsanthin is the main carotenoid of paprika but also occurs in other plants in much lower amount. Capsorubin and cryptocapsin are minor components accompanying capsanthin. Capsanthin 5,6-, 5,8- and 3,6-epoxides had also been isolated from red paprika.

Some carotenoids with an unique cyclopentyl enolic β-diketone group conjugated to a polyene chain in its structure were isolated as major or minor carotenoid from marine invertebrate animals (Hertzberg et al., 1988).

Rare carotenoids like capsorubin and cryptocapsin were also found in high amounts in some wild tropical fruits. A new special family of carotenoids bearing 3-hydroxy- and 3-dehydroxy-κ-end groups were found in tropical fruits of extremely high pigment content. Some of them, for example sapotexanthin, contains β-end group, too, which means they have potential vitamin A activity (Murillo et al., 2011). We describe the isolation, structure elucidation and reaction of these carotenoids and a proposed biosynthetic pathway of the κ-carotenoids is suggested as well.

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Directed synthesis of oxygenated carotenoid derived compounds

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Chemical oxidation of carotenoids affords a complex mixture of oxygenated carotenoid cleavage products. However this method is unsuitable for the preparation of pure compounds on a preparative scale [1]. Only a few examples of directed syntheses of these structures have been published so far [2].

One of the basic tools of polyene chemistry in BASF is a construction set made up of bifunctional C_5- and C_{10}-intermediates as well as C_{15}-phosphonium salts. By smart combination of these building blocks among themselves and with commercially available C_2- and C_3-units a plethora of carotenoid oxidation products are efficiently accessible via directed synthesis, using Wittig- and Wittig-Horner olefinations as key steps. This strategy is demonstrated for a bouquet of apo- and diapocarotenoids, e.g. lycopene-12´-, 10´- and 8´-derivatives, 6,10´-, 8,12´-, 6,14´- and 10,12´-diapolycopenedial as well as some apocarotenoids with cyclic end groups.

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Partial and total synthesis of serum carotenoids and their metabolites

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Human serum and tissues contain in excess of 12 dietary carotenoids and several metabolites that originate from con-

sumption of fruits and vegetables (Khachik et al., 2006). Among these are hydroxycarotenoids: (*3R,3'R,6'R*)-lutein {1}, (*3R,3'R*)-zeaxanthin {2}, (*3R,6'R*)-α-cryptoxanthin {3}, and (*3R*)-β-cryptoxanthin {4}. In addition, several dehydration products of {1} have also been identified in human serum, these are: (*3R,6'R*)-3-hydroxy-3',4'-didehydro-β,γ-carotene {5} (*3R,6'R*)-3-hydroxy-2',3'-didehydro-β,ε-carotene {6}, and (*3R*)-3-hydroxy-3',4'-didehydro-β, β-carotene {7}. Hydroxycarotenoids {1} and {2} appear to undergo extensive metabolism to several ketocarotenoids by a series of oxidation-reduction and double bond isomerization reactions. For example, (*3R,3'S,6'R*)-lutein (3'-epilutein, {8}) and (*3R,3'S;meso*)-zeaxanthin {9} are among the metabolites of {1} and/or {2} in human serum and ocular tissues that are formed by such reactions. The lack of commercial availability of some of these non-vitamin A active dietary carotenoids has limited the investigation of their metabolism and their biological activity. While the total synthesis of {1} and four of its stereoisomers has been reported (Khachik and Chang, 2009), the isolation of this carotenoid from marigold flowers (*Tagetes erecta*) on industrial scale has proven to be the most viable and inexpensive route to this carotenoid. In addition, 1 with stereocenters at 3,3',6'-positions serves as an excellent precursor in the partial synthesis of hydroxycarotenoids with ε–and β-end groups. Therefore, several semi-synthetic processes have been developed that separately transform {1} into {4} *via* {7} as well as {1} into {8}. While {8} is converted into {2} by base-catalyzed isomerization, {7} is transformed into {2} and its (*3R,3'S;meso*)-stereoisomer {9} by regioselective hydroboration followed by enzyme-mediated acylation that allows the separation of these carotenoids (Khachik et al., 2007). In another process, regioselective allylic deoxygenation of {1} afforded {3} that has been successfully transformed into (*6'R*)-α-cryptoxanthin {10}. The preparation and resolution of (*3R*)-3-hydroxy-β-ionone and its (*3S*)-stereoisomer that are important precursors in the total synthesis of {2} and {4} and their stereoisomers will be described.

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Isomers and apo-carotenoids in biological matrices

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Conversion of carotenoids to geometrical (E, Z) isomers and more recently to apo-carotenoids and apo-lycopenoids has been the subject of various investigations. Analysis of carotenoids has revealed the formation of several geometrical isomers created during thermal processing of carotenoid rich plant foods. However, the susceptibility of carotenoids to isomerization appears to be matrix dependent and favored when carotenoids are associated with lipid. Previous reports have demonstrated that *cis* isomers of beta-carotene are not readily absorbed in humans. In contrast, lycopene isomers are absorbed and in-vivo isomerization reactions proceed readily following uptake. Data from clinical trials in humans show that relatively high percentage of lycopene isomers (approx. 60-80%) are found circulating in blood plasma and deposited in tissues. However, the mechanism for in-vivo conversion remains to be elucidated but appears to proceed to equilibrium during absorption and circulation within the bloodstream at physiological temperatures. Although oxidative eccentric cleavage mechanisms of carotenoids have been well documented, few reports have unequivocally identified the in-vivo apo-carotenoid and apo-lycopenoid metabolic products. Our laboratory has developed highly selective and sensitive LC MS/MS methodology to analyze these compounds in foods and biological tissues. In collaboration with other researchers at the Ohio State University several eccentric cleavage products have been identified by comparison to synthesized authentic compounds and their potential biological activity demonstrated.

Superlative carotenoids: the shortest, the longest, the cleanest, the fattiest, the most precious, the most water-soluble, the ultimate antireductant, the smallest aggregated and the best DNA-carrier carotenoid

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Syntheses to carotenoids have long been the ultimate goal for carotenoid chemists. Syntheses with carotenoids are still hampered by the popular opinion that carotenoids are too sensitive for chemical manipulations. However, some of the commercially available carotenoids bear reactive functional groups such as -COOR, -CHO, -CO, -OH. A carotenoid with COOH can, therefore, be considered a highly unsaturated fatty acid, which can form highly unsaturated fatty acid alkali salts or inherently colored soaps. Highly unsaturated carotenoic acids may also replace saturated fatty acids in glycerolipids and phospholipids, which result in the formation of highly unsaturated fats. Carotenoid acids and carotenols can be esterified with hydrophilic substituents inducing water-solubility or water-dispersibility to the otherwise hydrophobic carotenoids. The C=O group in ketocarotenoids can easily be replaced with C=S, which is highly absorptive on Ag or Au for monolayer assembling. The CHO group invites to carotenoid prolongation in Wittig reactions. Other reactions with carotenoids can soon reach the reasonable limit in yield and stability. Still, low yields in a one-step synthesis may counterbalance higher yields in multistep syntheses.

One positive effect of carotenoids cannot be overestimated: carotenoids introduce color to otherwise sallow compounds providing instant visual confirmation of product formation; the carotenoid color facilitates work-up procedures and allows chiroptical detection of typically "invisible" molecules, e.g. carotenoid glycerolipid enantiomers give CD spectra.

The presentation illustrates the use of carotenoids in synthesis, highlights some unique results and exemplifies the use of carotenoid-modified compounds in spectroscopy, as pharmaphores, and in the study of aggregation and surface properties. Right and proper acknowledgements will be given to the many collaborators participating in this work.

ORAL PRESENTATIONS

Synthesis of water soluble PEG-carotenoid conjugates

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Natural carotenoids are hydrophobic antioxidants usually occur esterified with long chain fatty acid which make the even more lipophil. For therapeutic uses and also for food additives derivatives or formulations are needed which are water soluble to some extent. In the literature there are only a few examples for such derivatives: they are the sodium salts of lutein and astaxanthin disuccinate and diphosphate and the dilysinate of astaxanthin.

The polyethyleneglycol (PEG) is widely used for the enchancement of pharmacological properties of bioactive compounds. In theory, there are several posibilities to couple a partially or fully functionalized PEG derivative to a carotenoid: esterification, etherification, cycloaddition (eg. click reaction). We have synthetized various PEG-carotenoid succinate diesters with hydroxy carotenoids (eg. *capsanthin, cryptoxanthin, zeaxantin*, 8'-β-*apocarotenol*) and carotenoid dimers with PEG spacer. Water solubility of the products varied from moderate to good depending the PEG content of the molecules. Recently, we successfully coupled carotenoid pentinoates with PEG azides via click reaction. Some of the new compounds were tested for their antioxidant activity on human liver cells and showed considerable improvement over native carotenoids.

Some star-shaped trimers were also synthesized in which there is a PEG spacer between the carotenoid "arms" and the aromatic core.

This study was supported by OTKA PD 77467 (Hungarian National Research Foundation).

Identification of carotenoid pigments and their esterified forms in avian integuments: preliminary differences between wild and captive red-legged partridge

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Yellow-orange-red ornaments present in the integuments (feathers, bare parts) of birds are often produced by carotenoid pigments, serving to signal the quality of the bearer. Although carotenoid esterification in tissues is a common phenomenon, most of the work on avian carotenoids has been focused on the identification of free forms or have been done after sample saponification. In the present work, we determined free and esterified carotenoid composition in a bird species with red ornaments: the red-legged partridge (*Alectoris rufa*). Carotenoids from integuments were analyzed combining chromatographic techniques, and the result of this combination has been the identification of two major carotenoids and their esterified forms present in the integuments. The main carotenoid (λ_{max} 478 nm and $[M+H]^+$ at m/z 597.2) was identified as astaxanthin by comparison with standards. A second carotenoid (λ_{max} between 440 and 480 nm and $[M+H]^+$ at m/z 581.3) was not identified among any of the commercially available carotenoid standards. Both the unidentified carotenoid and astaxanthin formed monoesters with fatty acids, but only astaxanthin was in its diesterified form. Monoesters were mainly formed with palmitic, stearic, oleic and linoleic acids. Differences were detected between wild and captive birds and different integuments (among others). These discrepancies will be discussed taking into account the biology of the species. This is the first chromatographic analysis of the carotenoid composition of integuments in this bird, and the first HPLC analysis of esterified carotenoids in any avian species.

Structural changes of astaxanthin in a unicellular algae upon thermal stress: in situ Raman and DFT study

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Haematococcus pluvialis is the biomanufacture of astaxanthin (AXT), a superpotent antioxidant. Biosynthesis of AXT in an algae is stimulated by environmental stress among others high temperature. (Boussiba, 2000) Temperature is also a very important factor causing of degradation or isomerization of carotenoids in general. (Schieber and Carle, 2005) Therefore, heat-promoted structural changes of AXT in *Haematococcus* were investigated in situ by means of Raman spectroscopy and DFT computations (B3LYP/6-31+G(d,p)). No visual, but discernible spectral changes in algae cells were observed upon increase of temperature from -100°C systematically up to 150°C. The exponential increase of the Raman shift of the ν C=C band at *ca*. 1520 cm^{-1} along with the change of the intensity ratio of bands at 1190 and 1160 cm^{-1} was observed, that correlates with the changes predicted by calculations for astaxanthin conformers ordered by decreasing energy.

It is assumed that AXT molecules, initially in the form of H-aggregates with the *trans* conformations of the end-rings, interconvert toward more stable *gauche* forms upon thermal stress of the algae. It was confirmed that the results, obtained for a single algae cell, can be generalized for their statistical number.

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Synthesis of stabilized carotenoids as molecular wires

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Carotenoids are adopted by Nature as electron shuttles in various biological processes and are the most potent organic molecular wires (Frank et al., 2004). The inherent instability of carotenoids in vitro, however, does not allow full investigation of the potentials of these biological molecular wires. We devised the conceptually new and seemingly stable artificial carotenoids containing the phenyl groups with a *para*-substituent X (OMe, Me, H, and Br) of diverse electronic natures at C-13 and C-13' positions, which provided the carotenoids with various conductance as well as stability (Meang et al., 2010). The stability of carotenoids by the phenyl groups might be expected from the repulsive steric interactions with attacking nucleophiles and/or the reversible trapping of incoming radicals (e.g. reactive oxygen species) that would cause fragmentations of the conjugate polyene chain of carotenoids.

An electric circuit has been assembled by embedding carotenoids in an insulating and supporting monolayer of methyl(octadecyl)sulfane on a gold surface. Adsorptions of methyl(octadecyl)sulfane and carotenoids on a gold substrate were confirmed by XPS. The self-assembly of 2 nm-size gold particles on top of the other methylthio group of the embedded carotene wires completed the electric circuit. A gold-coated AFM probe was used as an electrode to contact the carotenoid molecule through a gold nanoparticle. Reproducible measurement of molecular conductance has been reported by through-bond contacts between molecules and gold particles (Cui et al., 2001).

The orthogonal phenyl substituents (*p*-X-C_6H_4-) to the polyene chain increase the conductance according to the electron-donating capability of X. The carotenoid containing two electron-releasing *para*-anisyl groups (X = OMe) gives the highest conductance of 33.46 nS. An electron-withdrawing *para*-bromophenyl group (X = Br) provides more resistance rather than conductance. The most resistive carotenoid among the series is the wire containing two para-bromophenyl groups, and its conductance value is 3.37 nS. It is believed that electron transport of a molecular wire proceeds through the frontier orbital of the molecule, which is closest to the Fermi levels of the electrode, and that the electronic effect of the substituents may shift the frontier molecular orbital and alter the electron transport efficiency through the molecule. The electron-donating substituent (X) places the LUMO of the carotenoid closer to the Fermi level of gold electrode than the electron-withdrawing substituent does, and thereby facilitates the electron transport process.

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A HPLC method for the simultaneous determination of geometrical isomers of major carotenoids in human plasma

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The development of the HPLC C30 stationary phase can facilitate the study of the occurrence of geometrical isomers of carotenoids in human blood from different sources. The study of geometrical isomers is interesting as they are known to have a different metabolism and function in terms of susceptibility to oxidation, bioavailability, vitamin A activity, etc. In the present study we developed a new HPLC method for the simultaneous determination of the geometrical isomers of major carotenoids found in human plasma and tissues. For this purpose, appropriate standards including lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, lycopene, phytoene and phytofluene were dissolved in ethanol and stereomutated by heating at temperatures around 80°C for some 30 minutes. The combination of aliquots of such extracts was used for the optimization of the separation of the isomers on a YMC C30 column (3 μm, 4.6 × 150 mm) by using methanol and methyl-tert-butyl ether in the mobile phase. Up to 46 peaks were separated in 61 minutes by this method. The analysis of the posprandial plasma carotenoid responses of a human volunteer of Asian ethnicity 24 hours after the consumption of a vegetable mix (carrot, celery, beets, parlsey, lettuce, watercress, spinach) and tangerine juices revealed the presence of (all-*E*)-β-carotene, (all-*E*)-β-cryptoxanthin, (all-*E*)-lutein, (all-*E*)-lycopene and several (*Z*)-isomers of the two latter carotenoids. The analysis of that fraction in a human volunteer of Caucasian ethnicity 4 hours after the intake of thermally-processed carrots and orange peppers, and juices containing mixed vegetables and fruits (mango, peach and orange) revealed the presence of (all-*E*)-α-carotene, (all-*E*)-β-carotene, (all-*E*)-β-cryptoxanthin, (all-*E*)-zeaxanthin, (all-*E*)-lutein, (all-*E*)-lycopene and several (*Z*)-isomers of the three latter carotenoids. No detectable levels of either phytoene or phytofluene were found, although the method proved suitable for their determination. However we detected, in addition to β-carotene and lycopene isomers, two geometrical isomers of phytoene and 5 of phytofluene in lung tissue of ferrets receiving a diet containing tomato extract supplement. To the best of our knowledge, this is the report of the highest number of carotenoid geometrical isomers separated with a HPLC method.

Controlling aggregation – carotenoids aggregates with predefined size

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Nearly all carotenoids are hydrophobic. Their inherent hydrophobicity limits carotenoid application in water-based environment. Carotenoids research is therefore often restricted to organic solvents. Recently, carotenoids were modified with hydrophilic groups making them water-soluble and water-dispersible [1,2].

These hydrophilic carotenoids easily self-aggregate in water. However, aggregation cannot be predicted: aggregates from few nm to m size are obtained. Hydrophilicity plays an essential role in absorption and distribution; the permeability of the cellular membrane depends on the size of the particles.

Control on aggregate size was now achieved by preparing self-assembled monolayers from carotenoid selenium compounds on gold nanoparticles. By selecting carotenoid phospholipids with different chain lengths and by choosing gold nanoparticles with various diameters the size of the aggregates can be tailored allowing a high degree of control. We present the synthesis of the modified carotenoids, the preparation and characterization of the carotenoid particles.

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Synthesis of longest chain carotenoids with 27 double bonds

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Most carotenoids have 11 C=C bonds; the natural maxima is reached with 14 C=C bonds. Notwithstanding extending the π-system by increasing the number of double bonds beyond the natural boundary is a goal for theoretical and practical consideration. The λ_{max} of polyenes increase asymptotically to the limiting value λ_{∞}; in spite of many calculations λ_{∞} is still unknown. Polymer polyenes only show a partial conjugation of double bonds. Synthetic chain elongation is therefore the only way to sustain molecular calculations.

The synthesis of long-chain carotenoids under classical Wittig conditions is always accompanied with substantial decomposition. The purity of previously synthesized C60:19 can be questioned, (Anderson et al., 1995) a supposed C70:23 carotenoid decomposed at low temperatures under argon (Broszeit et al., 1997) It is therefore likely that the limit for the classical carotenoid syntheses is reached with C60 or C70.

We have now found that the synthesis of long chain carotenoids can substantially be improved, when the Wittig reaction is performed under microwave irradiation. The microwave variant of the Wittig reaction allowed us to synthesize the longest carotenoid ever synthesized, the zeaxanthin derivate C80 with 27 double bonds. The synthesis, purity and spectroscopic properties of the zeaxanthin series will be presented from natural C40 to C50, C60, C70 and ultimate C80.

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POSTERS

3.1.

In situ detection of a single carotenoid crystal in a plant cell using Raman microspectroscopy

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It is the first report of direct, in situ detection of carotenoids at the subcellular level by using Raman microspectroscopy. Single crystals sequestered in a carrot cell were measured using FT-Raman spectrometer equipped with a microscope and 40×objective. The observed characteristic bands centered at 1518 cm^{-1} and 1156 cm^{-1} proved the crystals contained carotenoids. They were predominantly composed of beta-carotene, but a complex structure of carotenoid signal indicates that crystals were not homogenous and might contain also alfa carotene or lutein. The obtained results show the potential of Raman microspectroscopy for identification and analysis of compounds localized in cytoplasm by taking measurements directly from a single plant cell.

The research was supported by the Polish Ministry of Science and Higher Education (grant N204013635, 2008-2011).

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3.2.

β-carotene as antioxidant during cholesterol thermal oxidation

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Cholesterol, like all unsaturated lipid, can be attacked by reactive oxygen species and the products formed are most commonly termed cholesterol oxidation products (COP). These compounds are related to several negative biological effects which can culminate on the development of chronic and/or degenerative diseases. β-carotene was added to model systems containing solid cholesterol heated at 140, 180 and 220°C in the presence of oxygen in order to prevent or reduce COP formation due to its ability to scavenge free radicals. The efficiency of β-carotene as antioxidant was evaluated, by HPLC, considering the amounts of COP formed during heating in comparison to systems with only cholesterol. Heating resulted in the formation of the 5 major COP usually found in foods, 7-ketocholesterol (7k), 7α- and 7β-hydroxycholesterol (7α- and 7β-OH), α- and β-epoxycholesterol (α- and β-EP). 7k was the most abundantly COP, probably because it can be generated by two ways, directly from 7-OOH dehydration or by the 7-OH decomposition. The amounts of 7-hydroperoxycholesterol (7-OOH), which is an intermediate compound of the COP formation, were also determined. Results showed that β-carotene was able to reduce COP formation at all studied temperatures, in spite

of its own thermal degradation. At 140 and 220°C, the reduction of 7-OOH formation was 26 and 56%, respectively. However, at 180°C, 7-OOH content doubled in comparison to pure cholesterol model system. Moreover, systems containing β-carotene also delayed the time to achieve the maximum COP formation. A decrease in 7k amounts occurred at all temperatures; however, the reduction of the other COP was only noticed at 220°C. At 180°C only the levels of β-EP and 7β-OH reduced, while at 140°C, only the contents of α-and β-EP decreased. Those differences could be explained by the action of β-carotene at the distinct pathways of cholesterol oxidation mechanisms as well as cholesterol conformation during heating.

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3.3.

Effect of annatto powder on lipid oxidation in pork patties during frozen storage

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The antioxidant activity of annatto seeds is mainly attributed to its major carotenoid bixin. The effects of annatto powder on lipid oxidation in pork meat were evaluated and compared to sodium erythorbate, a synthetic antioxidant, usually added to meat products. Annatto powder alone or combined with sodium erythorbate was added to minced pork loin. Four types of patties were formulated, (1) the control without addition of annatto or sodium erythorbate, (2) addition of 0.05% annatto, (3) 0.1% sodium erythorbate and (4) 0.05% annatto plus 0.1% sodium erythorbate. The patties were packed in polyvinyl chloride film, stored at -18°C for 120 days and grilled at 165°C, for approximately 4 minutes per side, before analysis. Measurements of thiobarbituric acid reactive substances (TBARS), levels of conjugated dienes, cholesterol oxides contents and fatty acids composition were carried out in order to follow the lipid oxidation. Simultaneously, the stability of bixin in the pork patties during storage time was evaluated. In general, the levels of conjugated dienes showed no significant differences during 120 days. Patties containing annatto, sodium erythorbate and annatto plus sodium erythorbate showed significantly lower TBARS levels than the control patties during storage. Annatto showed the same protection levels as sodium erythorbate on cholesterol oxidation. Bixin contents in pork patties with annatto were always higher than those in the patties with annatto plus sodium erythorbate. These values decreased until day 75, remaining constant until the end of storage. The pork patties with sodium erythorbate and sodium erythorbate plus annatto showed low losses of polyunsaturated and monounsaturated fatty acids contents during storage. The overall results showed that annatto is an efficient antioxidant and can be used as an alternative for food industry to protect the lipids and cholesterol from oxidation in pork meat.

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3.4.

Screening of antioxidant activity of extracts from husks of Chinese lantern (*Physalis alkekengi* L.)

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Compounds as carotenoids, flavonoids and triterpenoids belong to plant secondary metabolites. Some of the secondary metabolites have beneficial role in healthy functioning of animal and human organisms. For example among carotenoids, zeaxanthin and lutein are reported (Ma and Lin, 2009) to have important role in eye health.

Chinese lanterns (*Physalis alkekengi* L.) is a well-known ornamental plant. It was found to be a good source of carotenoids zeaxanthin and β-cryptoxanthin (Weller and Breithaupt, 2003; Pintea et al., 2005). Other components of different parts of the plant are less known and no in vitro antioxidant activity profile has been published yet.

The aim of this study was to find the optimal extraction conditions for acquiring different groups of compounds present in the dried orange husks of *Physalis alkekengi* and measuring antioxidant activity of the extracts in vitro by different published tests. Consecutive extraction was made with n-hexane, ethyl acetate, methanol and 70% methanol$_{(aq)}$ in this order. Screening of different groups of compounds in the extracts was done by thin-layer chromatography.

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MA L and LIN XM. 2009. Effects of lutein and zeaxanthin on aspects of eye health. *Journal of the Science of Food and Agriculture* 90: 2-12.

3.5.

Separation of carotenoids using UHPLC and predictive mapping

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Carotenoids have been measured using HPLC with pressures up to 6000 psi for nearly 35 years. Recently, UHPLC, offering pressures up to 18,000 psi, has become readily available. These high pressures permit the use of <2 μm particle packings which produce very high efficiencies and rapid separations. Alternatively, UHPLC can be used to produce much higher resolution separations of carotenoids. At this time there are limited sub-2 μm column packings available.

Objective: To compare several commercial columns and mobile phase combinations to determine the separation efficiency and selectivity using UHPLC.

Results: A good HPLC separation with a 3 μm column produces about 20,000 plates. Multiple UHPLC columns were used in series to achieve unique selectivity and nearly 50,000 plates. The data from carotenoid standards was used to predict elution of other carotenoids and choose conditions for high resolution separations. Selected conditions were used to separate serum and food carotenoids.

Conclusions: UHPLC was successfully used in high-resolution mode to achieve separations previously unobtainable with conventional HPLC. Mapping the retention of known standards permitted the prediction of unidentified components.

3.6.

Total synthesis of an oxidation product of γ-carotene, a pro-vitamin A food carotenoid

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Human serum carotenoids and their metabolites are known to function as antioxidants and inflammation mediators. In 1992, an oxidative metabolite of lycopene was isolated from human serum and tomato-based food products which was later prepared by partial synthesis from lycopene and characterized as 2',6'-cyclolycopene-1',5'-diol {1} (Khachik et al., 1998). Results of in vitro studies demonstrated that {1} was more effective at inhibiting the growth of solid human tumor cells than lycopene (Monks et al., 1991). While the metabolism of prominent hydrocarbon carotenoids such as lycopene and β-carotene have been extensively studied, the functional role of γ-carotene remains unexplored. Because the chemical structure of γ-carotene is a hybrid of lycopene and β-carotene, the total synthesis of 2',6'-cyclo-γ-carotene-1',5'-diol {2} which is a structural analog of {1} was targeted. This was accomplished in six steps from commercially available citral using a $C_{15}+C_{10}+C_{15}$ Wittig coupling strategy in an overall yield of 15%. In another semi-synthetic approach, 12'-apo-Ψ-caroten-12'-al was transformed into {2} in a modest yield by epoxidation at the 5,6-position followed by rearrangement and elongation. The present methodology provides novel access to an oxidation product of γ-carotene that could be potentially formed in humans or biological systems. Further, supplementation studies with γ-carotene from food sources or with dietary supplements would be expected to provide an insight into possible metabolic oxidation of this carotenoid to 2. In view of the well-established health benefits of lycopene and β-carotene in the prevention of chronic diseases, investigation of the metabolism of γ-carotene that has structural features of both of these carotenoids is essential.

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3.7.

Carotenoid composition of mamey fruit (*Pouteria sapota*)

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A survey of local plants in Panama has revealed the presence of ketocarotenoids in a range of species. Examples with high concentrations of ketocarotenoids were found among fruits, e.g. 'Mamey' (*Pouteria sapota*), 'Maracuya Chino' (*Cionicisсyos macrunthus*) and 'Jipijapa' (*Carludovica palmata*), and in young brown leaves and red seeds of *Zamia dressleri*.

This paper focuses on the HPLC investigation of the carotenoid composition of Mamey fruit.

Three 'Mamey rojo' and one 'Mamey naranja' varieties were investigated. By using HPLC-DAD and HPLC-MS systems 50 compounds were detected. The parallel use of HPLC and classic column chromatography (CC) allowed the identification of numerous minor carotenoids. Based on their UV-VIS and mass spectrum as well as co-chromatography with authentic samples, the following carotenoids were identified: neoxanthin, violaxanthin, luteoxanthin, capsanthin 5,6-epoxide, β-cryptoxanthin-5,6,5',6'-diepoxide, (9*Z*)-violaxanthin, β-cryptoxanthin-5,6,5',8'-diepoxide, β-cryptoxanthin-5,8,5'8'-diepoxide, cryptocapsin 5,6-epoxide, cryptocapsin, 3'-dehidroxy-capsanthin, 3-dehydroxy-capsorubin, β-carotene, β-carotene-5,6-epoxide, β-carotene-5,8-epoxide and β-carotene-5,8,5',8'-diepoxide. Some carotenoids with κ-end group but with no oxygen substituents on the cyclopentane ring were tentatively identified by their UV-VIS and MS spectra and their adsorption properties. The isolation and structure elucidation of these compounds are in progress.

This study was supported by the grant OTKA K 83898 (Hungarian National Research Foundation) and SENACYT (Secretaria Nacional de Ciencia Tecnologia de Panamà).

3.8.

Novel microalgae as potential source of lutein: optimization of solvent extraction conditions

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Microalgae have been one of the most important natural source of products. Among these, carotenoids play a protective role for many diseases. Within this group, lutein has received increasing attention due to its potential health benefits, particularly in the prevention of age-related macular degenaration. It has also been claimed that lutein displays cancer-preventing properties. Nowadays, microalgae have been proposed as potantial source of lutein. For this reason, new studies must be directed towards its accurate identification and quantification. This study aims to find out the optimum solvent extraction conditions for lutein in order

to develop new systems for its efficient production from novel microalgae *Scenedesmus protuberans* and *Oocystis sp.* at industrial scale. For this purpose, the effect of extraction solvent, extraction time, extraction number, saponification time, biomass amount and temperature on lutein content were investigated. The experiments were performed using ultrasonic bath. All extraction and work-up procedures were conducted under yellow light to prevent photo-isomerization and degradation of carotenoids.

Internal standard (β-apo-12'-carotenal) calibration method was applied for the determination of lutein content in samples. Analyses were performed by using HPLC-DAD at 446 nm with a flow rate of 1.0 mL/min. Separation was achieved on a YMC Carotenoid (5 μm 4.6×250 mm) column at 25.0°C with gradient elution of methanol, methyl tert-butyl ether and water, each containing 0.1% TEA. Additional identification was carried out comparing the spectral data obtained with diode array detector with the reported values. The results showed that THF:DCM extraction gives the highest lutein yield within 10 minutes. According to the experimental evidence both of the microalgae high lutein content. Therefore, it is concluded that *Scenedesmus protuberans* and *Oocystis sp.* have a great potential for large-scale extraction of lutein. In conclusion, the results of this study can be a reference for the extraction of lutein from other microalgae.

3.9.

Carotenoids determination in raw eggs of bluefin tuna (*Thunnus thynnus*) and in their salted product "bottarga"

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This contribution presents the first investigation on the carotenoids composition in raw eggs and in their salted product, known as "bottarga" in Italian and "karasumi" in Japanese, of wild Atlantic bluefin tuna (ABT) *Thunnus thynnus*. Tuna eggs, roe from maturing ovaries, and bottarga are both considered a delicacy by the consumers and the study of their carotenoids composition become relevant considering the importance of these micronutrients in our diet and of a precise knowledge of the food composition and quality.

Astaxanthin was the main carotenoid detected in the samples investigated with an average content of 7.4 μg/g, w/w; β-cryptoxanthin and β-carotene were also detected in small amounts. Astaxanthin was determined in "bottarga" at a concentration of 0.91 μg/g. Astaxanthin chiral isomers were also directly separated on a chiral column and a distribution of respectively (3*S*,3'*S*) 13.1%, (3*R*,3'*S*) 47.8%, (3*R*,3'*R*) 8.4% astaxanthin isomers in the eggs of wild tuna samples was determined for the first time.

This study could also be useful in the formulation of technologically appropriate functional diets for the aquaculture of this species which is seriously endangered by global massive overfishing.

3.10.

Determination of carotenoids and their esters in chilli red pepper (*Capsicum annuum* L.) by comprehensive (LC x LC) liquid chromatography

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The great difficulties that are found when analyzing complex carotenoid samples, due to the high natural variability of these compounds as well as to the presence of carotenoid esters are well documented. Due to the significant higher separation power of comprehensive LC when compared to its 1D counterpart, the technique has experienced large interest in diverse fields, and the number of applications is continuously increasing. More recent trends are towards the implementation of UPLC methods for high efficiency and reduced analysis time, as well as miniaturization of the system to reach higher sensitivity and reduce solvent consumption. In this contribution, new developments in the comprehensive LC instrumentation will be presented, with applications to the analysis of natural products. A dedicated software was developed to directly transform raw LCxLC data into 2D and 3D chromatograms. Retention time values and spectroscopic data were available directly from the 2D chromatogram, making peak identification easier. In particular, UP-LCxLC has been applied to the separation of the native carotenoids present in chilli red peppers. Thirty-five compounds were detected and separated into ten different chemical classes in the 2D plane. The applicability of UP-LCxLC to the separation of xanthophylls bearing keto groups has been shown. The UP-LCxLC separations were used with two different set ups for the second dimension separation; the first set up having two serially connected columns showed a better resolving power compared to the set up having a single column in the second dimension, especially for the separation of mono-esters and free carotenoids.

3.11.

Cryptocapsin epoxides, new carotenoids isolated from mamey (*Pouteria sapota*)

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The well known carotenoids containing κ-end group such as capsanthin, capsorubin and cryptocapsin occur mainly in red papri-

ka (*Capsicum annuum*) (Deli and Molnár, 2002). Capsanthin has also been found in the pollen anthers of *Lilium tigrinum* and in the fruit of *Berberis* spp. and *Asparagus officinalis*. Capsorubin has also been isolated from the integument of *Encephalartos altensteinil*, the petals of *Cajophora lateritia*, and the fruits of *A. officinalis* (Deli et al., 2000).

In this year, the isolation of β,κ-carotene-6'-one from a panamian fruit mamey (*Pouteria sapota*) was published (Murillo et al., 2011). The structure elucidation was performed by MS and NMR. It was also established that the mamey fruit contains some carotenoids with κ-end group and the main carotenoid is cryptocapsin. In this study, we describe the isolation and structure elucidation of natural cryptocapsin 5,6-epoxide, cryptocapsin 5,8-epoxides and 3'-deoxy-capsanthin 5,6-epoxide, new carotenoids from the fruits of mamey. The cryptocapsin 5,6-epoxide was also prepared by the epoxidation of cryptocapsin. The structures of the natural and semisynthetic compounds were compared.

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3.12.

Synthesis of carotenoid dimers and trimers

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In the present poster we describe the synthesis of some carotenoid dimers, trimers and their precursors which can serve as a starting point for further studies with other hydroxy-carotenoids and form a basis for the synthesis of carotenoid dendrimers. The apocarotenoids retinol and 8'-β-apocarotenol were chosen as model compound for other carotenoids, being not too expensive, easily accesible and bearing a reactive primary hydroxy group. These dentritic structures can have enchanced or altered biological activity and can be used in aggregation studies of carotenoids.

Our first aim was to make dicarotenoid diesters of bivalent acids in which secondary interactions are possible between the two polyene chains. To achieve this, retinol and some other carotenoids were esterified with several bivalent acid anhydrides (maleic, succinic and phtalic). These intramolecular anhydrides were chosen after several unsuccessful trials with the corresponding acyl dichlorides [1,2]. This way dimers of retinol with other carotenoids (heterodimers) and dimers of the same carotenoids (homodimers) were synthetized with satisfactory yields [3].

Some podand-like structures bearing a central aromatic moiety were also synthesized from aromatic di- and triacids and hydroxy-carotenoids with DCC coupling of the acids and the carotenoid alcohols [4]. Di- and tribenzylic alcohols were coupled by the same manner to carotenoid succinates to give dimers and trimers with acceptable yields. A synthesis of star-like carotenoid trimers with PEG spacers in the arms was elaborated as well to enchance water solubility of the products. Some of the new compounds were tested for their antioxidant activity on human liver cells and showed considerable improvement over native carotenoids.

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3.13.

Investigation of carotenoid composition in flowers of *Chelidonium majus* L. with CLC and HPLC techniques

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Chelidonium majus L. (greater celandine) is a member of the family Papaveraceae. The flowers comprise four yellow petals and two sepals. The whole plant is toxic as it contains a range of isoquinoline alkaloids, but there are numerous therapeutic uses when applied at the correct dosage (Szabó, 2005). The current research project aimed at the carotenoid analysis of total extract of the flower of *Chelidonium majus* L. To the best of our knowledge, we report for the first time the carotenoid composition of this medicinal plant.

The fresh flowers were extracted three times with MeOH and once with Et_2O. The methanolic extracts were combined and transferred into the mixture of toluene and hexane in a separation funnel. After evaporation of this solution the residue was dissolved in Et_2O. The ethereal solutions were combined and this total extract was saponified in heterogeneous phase (30% KOH/MeOH) overnight. The saponified extract was distributed between MeOH : H_2O (9:1) and hexane (Schiedt and Liaaen-Jensen, 1995). The partition resulted in a hypophasic and an epiphasic fraction, which were analyzed separately by CLC and HPLC.

The carotenoid content of the flowers: 1.1 mg/g wet plant material; 13.6 mg/g dry plant material. In the total extract the identified carotenoids were violaxanthin (13.2%), lutein-5,6-epoxide (69.7%), flavoxanthin + crysanthemaxanthin (5.9%), (9*Z*)-lutein-5,6-epoxide (6.9%), (13*Z*) + (13'*Z*)-lutein-5,6-epoxide (4.3%) and lutein (<1%). The separation of the total extract by classic column liquid chromatography (CLC) resulted in 5 fractions. The carotenoid composition of these fractions was also determined by HPLC. From the fourth fraction lutein-5,6-epoxide was isolated in crystalline form.

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3.14.

Pharmacological activity of lutein-RAMEB complex on sensory neurones in vitro and in vivo

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Transient receptor potential (TRP) ion channels, such as TRP vanilloid 1 and ankyrin repeat domain 1 (TRPV1 and TRPA1), are expressed on primary sensory neurons. Lutein, a natural carotenoid, can be incorporated into membranes and might modulate TRP channels. Therefore, the effects of the water-soluble randomly methylated-β-cyclodextrin (RAMEB) complex of lutein were investigated on TRPV1 and TRPA1 activation.

Lutein-RAMEB (100 μM) significantly diminished Ca^{2+} influx to cultured rat trigeminal neurons induced by TRPA1 activation with mustard oil, but not by TRPV1 stimulation with capsaicin, as determined with microfluorimetry. Calcitonin gene-related peptide release from afferents of isolated tracheae evoked by mustard oil, but not by capsaicin, was inhibited by lutein-RAMEB. Mustard oil-induced neurogenic mouse ear swelling was also significantly decreased by 100 μg/ml s.c. lutein-RAMEB pretreatment, while capsaicin-evoked edema was not altered. Myeloperoxidase (MPO) activity indicating non-neurogenic granulocyte accumulation in the ear was not influenced by lutein-RAMEB in either case.

It is concluded that lutein inhibits TRPA1, but not TRPV1 stimulation-induced responses on cell bodies and peripheral terminals of sensory neurons in vitro and in vivo. Based on these distinct actions and the carotenoid structure, the ability of lutein to modulate lipid rafts in the membrane around TRP channels can be suggested.

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3.15.

Determination of carotenoid contents in banana varieties cultivated in Thailand

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In Thailand, banana (*Musa* spp.) is grown widely throughout the country and is a widespread food and fruit for Thai people and worldwide. Some varieties of banana cultivars are good source of β-carotene known as vitamin A precursor. Therefore, the aim of this work was to evaluate the carotenoid content in banana varieties collected in many part of Thailand. The ripped bananas of thirty cultivars were analyzed for carotenoids content using HPLC. In most banana cultivars, β-carotene and lutein were two major carotenoid constituents. The β-carotene content ranged from 30.15 μg/100g fresh-weight in Kluai Sae-Law to- 1680.07 μg/100g fresh-weight in Kluai Saw-Kra-Toup-Hor. The lutein content ranged from 10.60 μg/100g fresh-weight in Kluai Chom-Jan to 60.87 μg/100g fresh-weigh in Kluai Hom-Tong. The results highlight the potential of banana variation in pro-vitamin A composition, and the data is also necessary for encouragement for consumption in order to prevention of vitamin A deficiency and chronic disease in Thailand.

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3.16.

New trends in the use of visible spectroscopic data for the analysis of carotenoids towards the rapid screening of large sample sets

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The availability of objective instrumental methods to assess the colour of foodstuffs is very important for the food industry due to the relationship of this attribute with their acceptability. These methods have many advantages, like rapidity, possibility of automation, non-destructiveness, among others. Since the colour of foods is due to pigments like carotenoids that are known to provide some health benefits, studies on the applicability of objective colour parameters in the assessment of pigment levels appear interesting for different purposes, like the rapid screening of large populations of samples, monitoring quantitative/qualitative changes as a result of different practices, the objective determination of maturity stages, the grading of foods according to their organolpeptic of nutritional quality, among others. In this review we discuss new tendencies in the application of colour and visible spectroscopic data in the analysis of carotenoid pigments beyond the traditional quantification of extracts based on absorbance readings at a single wavelength. More specifically, we focus on studies dealing with the study of the applicability of these data to estimate total and individual carotenoid contents, the weight of individual carotenoids in the colour of foods the relationships between the structure and the colour of these pigments and the effect of changes in them in the colour of foods.

3.17.

Carotenoid composition from unexploited Amazonian fruits

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Brazil has a wide variety of native, wild and not well-known fruits, such as the Amazonian fruits, and few information about their constituents are available. Maná-cubiu (*Solanum sessiflorum*) has 5-6 cm in diameter and the comestible fraction represents approximately 91% of total fresh weight (9% of peel). Piquiá (*Caryocar villosum* (Aubl.) Pers) presents 7-9 cm in diameter and the pulp represents approximately 12% of the total fresh weight (24% of seed and 64% of shell). Both fruits are irregularly oblong-globose with a yellowish pulp. Since the carotenoid profiles of maná-cubiu and piquiá were not reported yet in the literature, the main purpose of this work was to identify and quantify by HPLC-DAD-MS/MS (APCI – positive ion mode) the carotenoids from these Amazonian fruits. The carotenoids were separated on a C30 column and identified according to the UV/Vis and MS spectra features as compared to standards (lutein, zeaxanthin, antheraxanthin, violaxanthin, neoxanthin and β-carotene) and carotenoid extracts from other sources (kale and mango with or without HCl acidification), all analyzed in the same conditions. The contents of total carotenoids in the freeze-dried pulp of maná-cubiu was 12.43 μg/g and the main carotenoids found were all-*trans*-lutein (2.74 μg/g), all-*trans*-β-carotene (2.66 μg/g), all-*trans*-violaxanthin (2.59 μg/g), 9-*cis*-luteoxanthin (2.01 μg/g), 9-*cis*-violaxanthin (1.29 μg/g) and all-*trans*-neoxanthin (1.13 μg/g). The total carotenoid level in the freeze-dried pulp of piquiá was 16.96 μg/g and the main carotenoids found were all-*trans*-antheraxanthin (3.44 μg/g), all-*trans*-zeaxanthin (2.88 μg/g), all-*trans*-neoxanthin (2.26 μg/g), all-*trans*-violaxanthin (1.10 μg/g) and all-*trans*-β-carotene (0.73 μg/g). A non-identified carotenoid was also found in piquiá (2.84 μg/g), presenting λ_{max} at 445 nm in methanol/MTBE (%III/II = 55 and no *cis* peak) and an in-source fragment at 583 *m/z* ($[M+H-18]^+$). Since the major carotenoids identified in both fruits presented epoxide groups, all analysis were performed in both fresh and freeze-dried pulps, with and without addition of $NaHCO_3$ during extraction step, and the carotenoid profiles did not show any difference. Considering that the essential role of biodiversity for its sustainable use in food security and nutrition is world-wide recognized, these natural resources from Amazonia should be commercially exploited.

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3.18.

Chemical and colour changes associated to the epoxidation and oxidative cleavage of β-carotene

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The oxidation of carotenoids leads to important changes in their colour, biological and other properties. The objective measurement of colour could therefore be harnessed to monitor the oxidation of these compounds and for the rapid estimation of oxidation products, some of which are attracting much interest as they seem to be involved in the modulation of intracellular signalling pathways. In this sense, the objectives of this study were two: (1) to identify by HPLC-DAD-MS/MS the oxidation products derived from β-carotene after epoxidation with meta-chloroperbenzoic acid (MCPBA) and oxidative cleavage with $KMnO_4$; and (2) to assess the related colour changes. In the epoxidation reaction, the major carotenoids identified were 5,6-epoxy-β-carotene, 5,6,5',6'-diepoxy-β-carotene, 5,6,5',8'-diepoxy-β-carotene and 5,8-epoxy-β-carotene, while β-apo-8'-carotenal, semi-β-carotenone, 10'-apo-β-carotenal, 12'-apo-β-carotenal, 14'-apo-β-carotenal and 15-apo-β-carotenal were the major products identified in the oxidative cleavage reaction. The (13*Z*)- and (9*Z*)- isomers of β-carotene were also detected in both reactions. More than 95% of (all-*Z*)-β-carotene oxidized after 60 minutes; however, the amounts of (all-Z)-β-carotene lost were not compensated by those of the new compounds formed. As a result of both reactions, most of the compounds formed had shorter chromophore and therefore shorter maxima wavelengths as compared to those of β-carotene (450 nm). This led to important changes in the values of colour parameter b* and colour differences (ΔE^*). Thus, the values of ΔE^* relative to the original β-carotene solution after 1 hour of reaction were 16.2 (epoxidation) and 48.1 (oxidative cleavage), indicating that colour changes could be visually noticed. The values of b* ($r>0.99$) and C^*_{ab} ($r>0.99$) highly correlated with the absorbance at 450 nm, thus these parameters seem promising for the rapid assessment of the formation of oxidative derivatives. Since C^*_{ab} is the quantitative attribute of colourfulness, visual changes in colour vividness can be used to monitor de oxidation of β-carotene.

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3.19.

The influence of the spreading solvent on the Langmuir monolayer characteristics of β-carotene

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Our work was aimed at finding appropriate solvent for spreading β-carotene onto the air/water interface for Langmuir monolayer formation. We have thus chosen a number of organic solvents and their mixtures that dissolve β-carotene, namely: benzene, cyclohexane, chloroform, isooctane, petroleum ether, benzene/dichloromethane, chloroform/hexane/methanol. In each of the tested solvent we observed the increase of surface pressure upon film compression, which was monotonous, without typical collapse at the end of compression. However, a surface pressure increase alone is an insufficient criterion to evidence a true monolayer formation. Therefore, in addition to the surface pressure monitoring, we engaged an optical method – Brewster angle microscope (BAM), for a direct visualization of a film structure. We observed that β-carotene deposited from all the tested solvents onto the water surface, form crystalline domains instead of a homogenous film. Therefore, an increase in surface pressure observed for β-carotene can thus be accounted for the decrease of available total surface area upon compression that gives rise to the domains compacting, and not monomolecular layer formation. Therefore so called "isotherm" recorded for β-carotene is

misleading and cannot be regarded as a monomolecular film. In the literature, however, β-carotene was applied for Langmuir monolayer formation [1,2]. In the above mentioned papers, the film-forming abilities of β-carotene were inferred only from surface pressure-area isotherm, and none of the other complementary method was used to probe the structure of layers formed at the interface. Since our BAM experiments indicated the domain formation of β-carotene dropped from a wide variety of organic solvents, we tried to improve its spreading behaviour by adding a small amount of surface active substance (isopropanol or n-pentanol) into the solution. This method of adding different additives to the spreading solution to improve the stability and monolayer structure has long been known [3] and was further applied by many authors (see e.g. [4]). Unfortunately, all these experiments failed in obtaining a homogeneous monolayer from β-carotene, indicating lack of film-forming properties.

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3.20.

Degrading of carotenoids by the DyP peroxidase MsP2 from *Marasmius scorodonius*

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From the perspective of human physiology and nutritional sciences, the symmetric cleavage of carotenoids yielding retinoids has attracted much attention. Retinoids act as regulating and signalling molecules and as visual pigments. The excentric cleavage of the carotenoids' polyene chain yields a wealth of apocarotenoids, with the plant hormone abscisic acid being the most prominent example. Many apocarotenoids, such as α- and β-ionone, geraniol and β-damascenone act as potent flavour compounds (Winterhalter and Rouseff, 2002). Apart from the generation of retinoids, plant hormones or flavour compounds, there is a strong interest of the detergent and food industries in carotenoid degradation for bleaching fabrics, whey and other substrates. Few data are available on microbial carotenoid degradation pathways and the enzymes involved.

Basidiomycete fungi are a potent source of unique lignin decomposing enzymes. In a screening more than 100 basidiomycetes in an agar plate based assay, 37 strains also showed a strong carotenoid degrading activity. Some β-carotene cleaving enzyme activities were described previously (Zorn et al., 2003). In particular, the DyP peroxidase MsP2 of the "garlic-mushroom" *Marasmius scorodonius* was characterized on the biochemical and molecular level (Scheibner et al., 2008). In the current work, the gene encoding MsP2 was cloned from the cDNA and functionally expressed in different *Escherichia coli* strains using pCold (a cold shock induced expression) system. Fusing the MsP2 sequence with the N-terminal His tag was effective for the purification of the target protein. Biologically active peroxidase was obtained as proven by β-carotene destaining. Aside from degrading β-carotene, the stable MsP2 enzyme was able to effectively decolorize lycopene, lutein and capsanthin as well.

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3.21.

Isolation and characterization of two novel capsorubin like carotenoids in the red mamey (*Pouteria sapota*)

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The most common C_{40} carotenoids have six-membered rings as end-groups (β or ε rings), carotenoids with five-membered rings (κ-rings) are rare. Capsorubin and capsanthin isolated from paprika are the most well-known carotenoids with κ-end group. The κ-end group of these carotenoids always bear a hydroxyl group. Mamey is a fruit native in Panama, Central America and Mexico, much appreciated for its pleasant taste and attractive red-orange colour of flesh. Recently, we reported the complete isolation and characterization of the sapotexanthin (β,κ-caroten-6'-one) – which bears non-hydroxylated κ-end group- from red mamey pulp. In our poster we present evidence showing the existence of two new and unknown di-keto-di-κ carotenoids which are structurally related to capsorubin. The carotenoids were isolated and characterized by combining the techniques of open column chromatography, TLC and HPLC-DAD, HPLC-MS and qualitative tests of reduction and acetylation. Three carotenoids (A, B and C) were isolated from the pulp of red mamey that have two keto groups conjugated to the polyene chain. UV/Vis spectra of the three carotenoids and their reduction products are the same as those of capsorubin which suggests that A, B and C have the same chromophore as capsorubin. Carotenoid A was identified as capsorubin (3,3'-dihydroxy-κ,κ-caroten-6,6'-dione) by co-chromatography (TLC and HPLC), MS and qualitative tests. The carotenoid B has one hydroxyl group and its molar mass is 584, while C has no hydroxyl group and its molar mass is 568.

These data allow us to suggest that B corresponds to 3-hydroxy-κ,κ-caroten-6,6'-dione and C is κ,κ-caroten-6,6'-dione. The carotenoids B and C were also characterized by their ^{1}H NMR and CD spectra. The presence of the direct metabolic precursor of B and C in red mamey supports this proposal, as well.

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3.22.

New sources of κ-ring carotenoids in Panama's biodiversity

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There are few sources of carotenoids with κ-ring end groups. Capsanthin, capsorubin, capsanthin 5,6 epoxide and cryptocapsin are the most known carotenoids with κ-ring end groups. The presence of a carbonyl group conjugated with the polyenes chain, is responsible of the red color of these carotenoids and vegetables that contain them. Panama is a humid tropical country with a large native biodiversity, where there are many plants with red parts (fruit, leaves, flowers and seeds), which have not been investigated. Our group has studied the carotenoids of several of these sources, had been isolated and identified the main carotenoids ?, combining open column chromatography, TLC, HPLC-DAD, HPLC-MS and qualitative tests, comparing with standards. We found that red-brownish leaves of Zamia (*skinneri, dressleri* and *neurophyllidia*), plants considered living fossils, containing capsorubin, capsanthin and capsanthin 5,6-epoxy, where the main carotenoid is capsorubin. This is the first report on the presence of carotenoids with κ-ring in leaves. The Chinese passion fruit (*Cionosicyos machranthus*) contains cryptocapsin, capsanthin and cryptocapsin 5,6-epoxy. The red mamey fruit (*Pouteria sapota*), appreciated for its pleasant taste, contains cryptocapsin, sapotexanthin, cryptoxanthin 5,6-epoxy, capsanthin 5,6 epoxy and others. This is the first report of edible fruits, with high content in cryptocapsin. This carotenoid has been reported in the paprika, but in trace amounts. The inflorescence of Jipijapa (*Carludovica palmata*) contains capsorubin, capsanthin and capsanthin 5,6-epoxy. The seeds of Zamias (*skinneri, neurophyllidia, nesófila, acuminata, fairchildiana* and *oblikua*) contain capsorubin, capsanthin and capsanthin 5,6-epoxy. Probably the striking color of these parts of the plant, helps its spread by animals. The fruit of niguito (*Cordia collococca*) contains capsorubin and capsanthin. This is a wild fruit not consumed by humans very often, but greatly appreciated by birds. The results show that the κ-ring carotenoids are found in all of the plant part (fruit, leaf, seeds and flowers).

3.23.

Carotenoid-cysteine conjugates

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The hydrophobic natural carotenoids are efficient antioxidants, however, chemical synthesis of their water-soluble derivatives is reasonable to gain better bioavailability. Isozeaxanthin gives allylic cation on acidic treatment which reacts readily with thiol nucleophiles (Nagy et al., 2010). Using N-acetylcysteine as nucleophile the obtained products are carotenoid-cysteine conjugates in which the amin oacid moiety connects to the carotenoid through sulphur in position 4. The water solublity of the product can be increased by deprotection of the amino group.

The antioxidant activity of the products were examined on human liver cells in hydrogenperoxide induced oxidative stress. The intake of the synthesized products by the cells was facilitated by preparing lyposomes (C. Socaciu et al., 1999).

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3.24.

Spectroscopy analysis for simultaneous determination of lycopene and β-Carotene in fungal biomass of *Blakeslea trispora*

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Blakeslea trispora is a good source for producing lycopene and β-carotene. The objective of this research was to elaborate a method for the simultaneous determination of lycopene and β-carotene using UV-vis spectrophotometer.

The standard solutions of the mixture of different concentrations of β-carotene and lycopene were measured with the UV-vis method and a correlation formula for the extinction coefficients of 1% standard solution of lycopene in the solvent (hexane) and the ratios of the optical densities at the character peaks of 470 and 502 nm was defined:

$$A_{lyc}^{1\%} = \left(-1.79 + 11.306 \cdot \frac{D_{470}}{D_{502}} - 0.061 \cdot \left(\frac{D_{470}}{D_{502}}\right)^2\right) \cdot e^{\left(5.696 - 0.09 \cdot \left(\frac{D_{470}}{D_{502}}\right) + 0.147 \cdot \left(\frac{D_{470}}{D_{502}}\right)^2\right)}$$

in which $A^{1\%}_{lyc}$ is the extinction coefficient of a 1% lycopene solution; D_{470} is the optical density of the spectrum at λ=470 nm; D_{502} is the optical density of the spectrum at λ=502 nm.

Substituting the result into the classic formula, gives a possibility to calculate the concentrations of lycopene in the mixture:

$$C_{lyc} = \frac{D_{470} \cdot V}{m \cdot l \cdot A^{1\%}_{lyc-tab}}$$

in which C_{lyc} is the percentage of lycopene in %; D_{470} is the optical density of the investigated solution at the wavelength of 470 nm; V is the solvent (hexane) volume, being spent for the preparation of the investigated solution in ml; m is the weight of the sample in g; l is the thickness of the cuvette for the optical density measurements in cm; $A^{1\%}_{lyc\text{-}tab}$ is the table extinction coefficient of the 1% solution of pure lycopene, equals to 3450.

Then the optical density for β-carotene ($D_{\beta\text{-car}}$) is calculated as:

$$D_{\beta\text{-car}} = D_{470} - D_{lyc}$$

in which $D_{\beta\text{-car}}$ is the optical density for β-carotene at the wavelength of 470 nm; D_{470} is the optical density of the mixed

carotenoids in the mixture at the wavelength of 470 nm; D_{lyc} is the optical density of pure lycopene at the wavelength of 470 nm.

Knowing the extinction coefficient of the 1% solution pure β-carotene in hexane ($A^{1\%}_{\beta car}$=2116) at the wavelength of 470 nm, it is possible to determine the actual content of β-carotene in the carotenoid mixture. The prediction quality of the UV-vis method was sufficient and the obtained results were very close to the ones, being measured with the HPLC technique.

The proposed method can be used for both routine industrial work and academic research, providing the rapid analysis for simultaneous measurements of lycopene and β-carotene.

3.25.

Agro-food wastes as substrates for biotechnological carotenoids production

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Carotenoids are one of the most abundant groups of natural pigments, with broad range of biological effects, serving mainly as lipophilic antioxidants and some of them present provitamin A activity. The all-*trans*-β-carotene has the higher provitamin A activity, and is produced chemically and biotechnologically. Nowadays, there are a lot of efforts towards sustainable development of chemicals with less environmental impact, meaning production of 'all-natural' chemicals under mild conditions including biotechnological approaches. The microorganism used in industrial scale for β-carotene production is the mated cultures of a heterothallic fungus *Blakeslea trispora*. Despite the intense research for β-carotene production by *B. trispora*, natural substrate utilization has not been extensively studied (Dholakia and Modi, 1982). The utilization of solid agro-food wastes such as cabbage, watermelon rind and peach peels from northern Greece as main carbon source into submerged *B. trispora* cultures for carotenoids production, is examined here. There was used 100 g/L agro-food waste in each case, with an estimated water content 88-95%, giving a biomass accumulation about 10-13 g/L, comparable to that from synthetic substrate where pure glucose 50 g/L was used (Papaioannou and Liakopoulou, 2010). All these natural substrates gave a β-carotene percentage 80-90%, estimated by HPLC analysis, in respect to total main carotenoids obtained: namely γ-carotene, β-carotene and lycopene. This β-carotene percentage is higher from the synthetic substrates used previously. In addition total carotenoids volumetric production was quite high (~200-250 mg/L), with cabbage exhibiting the best performance. Though, optimization studies are necessary with each substrate in order to maximize the yield. The above results further support that *B. trispora* may utilize also different origins agro-food wastes for carotenoids production.

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3.26.

Tomato peel lycopene recovery under mild conditions assisted by enzymatic pre-treatment and non-ionic surfactants

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Tomato peel residues, a by-product of the tomato processing industry, are annually generated in large amounts usually creating environmental problems. These residues contain significant quantities of lycopene, a lipophilic carotenoid pigment that possesses higher antioxidant activity compared to other carotenoids. The enzymatic pretreatment of agro-wastes is an already established approach with many applications for recovering compounds of biological significance and other high added value products (Lavacchia and Zuorro, 2008). However, the use of surfactants to facilitate the recovery of such compounds from their natural matrices is another alternative (Chatzilazarou et al., 2010). In this study, the enzymatic pretreatment of tomato peels, using two commercially available pectinolytic enzyme preparations is examined with regard to pretreatment time, enzyme amount and pH. One enzyme preparation (Citrozym® CEO) appears to perform better with an optimum concentration 250 U/mL for 1 h, while the pH effect is not significant. Lycopene surfactant – assisted extraction was further investigated, showing that the most suitable surfactant was "Span 20" (among eight different surfactants used) with an optimum stoichiometry >5 molecules of surfactant/ lycopene. The sequential utilization of the enzymatic pretreatment and surfactant-assisted extraction (30 min for each step) was evaluated, showing that it can lead to an improvement in lycopene extraction yield, with lower surfactant stoichiometry (i.e. 4 to 5). In the latter case, the yield of lycopene recovery almost doubled compared to simple 1 hr enzymatic pretreatment, and was approximately ten times higher with reference to recovery from untreated peels. Furthermore, in this case, the recovered lycopene is in an aqueous emulsion formulation readily usable for applications in the food and cosmetics industries; this is of paramount importance for such a lipophilic compound recovery, avoiding the use of organic solvents and thus being environmental friendly.

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3.27.

Determination of all-*trans*-lutein in spinach

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The aim of our work was to find the optimum conditions for determination of lutein in lyophilised spinach sample using non-

toxic solvents and miniaturised and simple preparation of the test solution. We were also interested in influence of addition of antioxidants during the preparation of test solution and extraction in air or in nitrogen atmosphere.

Extraction of 10 mg of lyophilized and powdered plant material with 10 ml of ethanol with magnetic stirring for one hour at room temperature in tubes gave a satisfactory test solution after centrifugation. Two replicates of sample test solutions, two replicates of standard solutions and two replicates of spiked test solution with the same concentration of standard were simultaneously prepared. The same procedure was repeated with addition of 10 mg of antioxidants butylated hydroxytoluene (BHT) or *tert*-butylhydroquinone (TBHQ) to all standard solutions, extracts and spiked extracts. The whole experiment with magnetic stirring of solutions of standards, extracts of spinach and spiked extracts was performed in air and under nitrogen. Absorbances of standard solutions at 445 nm before and after the stirring were measured and served for the determination of the concentration of lutein in standard solutions, taking the published value 2550 as specific absorption coefficient at 445 nm. HPLC determination of all-*trans*-lutein was performed on Prontosil C30 column, 250×4.6 mm, with 5 μm particles. Gradient of acetone-water was used for the separation of compounds. The eluted compounds were detected by PDA. For the calculation of the content of lutein in lyophilised spinach sample and determination of % recovery, standard treated in the same way as samples was taken. Extraction in nitrogen gave significantly higher values for the content of lutein in spinach compared to the extraction in air (about 10%), but the influence of antioxidants was not significant. Recovery was close to 100% at extraction in nitrogen, but lower in air, which means that lutein in the mixture with other compounds in the spinach extract was more susceptible to degradation than in standard solution during stirring. The results were verified on the certified material for the determination of carotenoids.

3.28.

Metabolomic characterization of sea buckthorn products based on carotenoid fingerprint

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Sea buckthorn (*Hippophaë rhamnoides* L.) is a deciduous tree-like shrub with yellow or orange berries, rich in bioactive compounds i.e. vitamins, phenolics, lipids, tocopherols, carotenoids and phytosterols with benefitial effect on human health and nutrition (Sabir et al., 2005). Among the biologically active substances, carotenoids have essential functions in photosynthesis, photoprotection, and also possess various activities such as anti-oxidant, antimutagenic and anti-tumour activity (Pintea et al., 2001). Using a metabolomic approach, untargeted fingerprinting of carotenoids was performed in order to characterize sea buckthorn products like fruits, juice, seed oil and seeds. The major carotenoids found in sea buckthorn products were lutein, zeaxanthin, lycopene, β carotene and esters of β cryptoxanthin, zeaxanthin and lutein, mainly esterified with saturated fatty acids like palmitic and myristic. Among sea buckthorn based products, sea buckthorn berries had the highest total carotenoid content (151 mg/100g DW) compared to 3.74 mg/100g FW, 43 and 22 mg/100g DW found in sea buckthorn juice, seed oil and seeds respectively. The esterified carotenoids were the predominant fraction in all carotenoid extracts, corresponding to aprox. 62 % of the total carotenoids in sea buckthorn berries, 73% in sea buckthorn juice, 90% in sea buckthorn seed oil and 60% in sea buckthorn seeds. Among the free forms of investigated carotenoids, ? carotene was detected in the highest percentages. The results obtained suggested that carotenoids can be used as important biomarkers for metabolomic studies, having also important application in food science to evaluate quality, authenticity/adulteration and traceability of sea buckthorn products.

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3.29.

Study of surface properties of highly unsaturated conjugated soaps

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The surface activity of ionic sodium and potassium salts of C12 – C18 saturated fatty acids is known since ancient times [1]. Only at an almost recent time soaps found scientific consideration [2]. The special properties of soaps led to the concept of surface tension, of micelles, critical micelle concentration, surface molecular area and to the designation "surfactant" (surface active agents).

In nutrition saturated fatty acid gained lately a bad reputation in contrast to the numinous properties of unsaturated fatty acids. However, cleansing with highly unsaturated soaps is a subject that never came up; saturated soaps stand all times strong and maintain the bathroom monopoly.

There are both reasons for alikeness or disparity of saturated and polyunsaturated soaps. In consequence, the surface properties of saturated and conjugated unsaturated fatty acids were systematically compared. Any such investigation is severely hampered by the inherent instability of unsaturated fatty acids with extended conjugation. Therefore, it was evident right from the outset of our study that we should make a draft on stable unsaturated conjugated acids. These acids are known as apo-carotenoid acids. C20- (retinoic acid) and C30-carotenoid acid (food color E 160f) are commercially available. Other acids were synthesized. When we compared the critical micelle concentration of the Na and K salts of C4 – C22 saturated acids with the K, Na and Cs salts of C10 – C22 carotenoid acids the results were largely in agreement. However, the K, Na and Cs salts of the longer chain unsaturated acids deviate considerably from the short and medium unsaturated carotenoid salts.

We present our preliminary data demonstrating the commonalities for shorter chain saturated and unsaturated soaps and point to the unexpected discord in surface properties for the long chain unsaturated soaps.

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3.30.

Carotenoid as antireductants

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The electron-rich carotenoids are prime examples for their electron donor property to reactive radicals. The reverse reaction, the uptake of electrons by Car, has not yet been observed in nature and is difficult to perform in the laboratory. Cyclic polyenes easily take up electrons (Birch reduction), but chain polyenes resist in electron transfer reactions. Carotenoids were forced to take up electrons by difficult chemical procedures (Na in high vacuum) or with methods requiring special instruments (electrochemistry, flash photolysis, pulse radiolysis). Nevertheless, it has been predicted theoretically that carotenoids could act favorably as electron acceptors. Recently, crocetindial has been reacted in a simple procedure with the electron donator alkaline DMSO = $H_3C(S{=}O)CH_2^-$ = $DMSO^-$ (Øpstad et al., 2010). An immediate two-electron uptake reaction to crocetin dienolate was observed by a color change to blue.

We present now the synthesis of a series of dialdehydes (C10:3 to C50:19) and describe their electron uptake properties with regard to the chain length. Carbonyl carotenoids, like any other carotenoid, may act as antioxidants by releasing electrons. We demonstrate that carbonyl carotenoids also easily accept electrons; therefore, these carotenoids react as antireductants.

This project is financially supported by the Higher Education Commission Pakistan (HEC) and the Norwegian Centre for International Cooperation in Higher Education (SIU).

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Session 4

Carotenoids in the Prevention of Cancer and Cardiovascular Disease

Invited Lectures

Role of Nrf2 and heme oxygenase-1 in tumor growth

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Antioxidant genes can protect the tumor cells against the effect of therapies, but due to their pleiotropic activities may be also involved in tumor initiation, growth and progression. The nuclear factor erythroid-2 related factor-2 (Nrf2) is a transcription factor mediating the cellular response to oxidative stress and is involved in regulation of the expression of both phase II and antioxidant genes, including heme oxygenase-1 (HO-1). Overexpression of Nrf2 and its target genes can protect the cells against tumor transformation and hence Nrf2 upregulation is considered as the sort of chemopreventive strategies. However, both Nrf2 and HO-1 expression are very often boosted in tumor tissues and could be further elevated in response to radio- or chemotherapy.

Interestingly, our data indicate that the influence of HO-1 can be tumor dependent: in melanoma it accelerates tumor cells proliferation, increases their angiogenic potential and promotes metastasis (Was et al., 2006). Similar effects have been observed in pancreatic cancer, Kaposi sarcoma, chronic myelogenous leukemia and some other types of cancer. On the other hand HO-1 can protect against skin cancer development, but in the already growing tumors it seems to favor their progression toward more malignant forms (Was et al., in revision).

Interestingly, our recent studies indicate that in human non-small cell lung cancer (NSCLC) HO-1 overexpression attenuates tumor growth, angiogenesis and metastasis. The growth of HO-1 overexpressing NSCLC in NOD-SCID mice was attenuated in comparison to wild type cells. However, NSCL cells overexpressing HO-1 seem to be more protected against some chemotherapeutics. The microarray analysis revealed that HO-1 affects the expression of numerous genes involved in cells proliferation, migration and metastasis. Those effects can be mediated by the microRNAs, as suggested by changes in expression of several microRNAs in NSCLC overexpressing HO-1.

In sum, both Nrf2 and HO-1 can be upregulated in tumors and apparently are playing a role in the their growth and metastasis and can affect the anti-cancer therapies. The specific influence seems to be, however, cell-type dependent. MicroRNAs, acting in a cell-specific context may be responsible for such effects.

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Is the effect of β-carotene on prostate cancer cells dependent on their androgen sensitivity?

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The androgen receptor (AR) is involved in the development and maintenance of the normal prostate and the development and progression of prostate cancer. The classical role of AR is modulation of gene transcription by binding specific DNA sequences called androgen response elements in the promoter regions of target genes. To carry out this role, AR interacts with many coregulator proteins which either enhance or inhibit its activity. Altered expression or misregulated activation of a co-regulator protein may significantly alter AR activity and the basal transcription rate of androgen responsive genes. Results of epidemiological studies indicate that the overwhelming majority of prostate cancers originate from environmental factors and dietary errors.

β-carotene (BC) for many years is known as importing factor modulating the cell growth. However no studies have directly investigated a possible influence of BC on the sensitivity of hormone and the possibly sequence on tumor cell growth. In this study, we addressed by assessing the observed role of β-carotene on cell growth in prostate cancer cell lines. We showed that β-carotene acted as a potent growth-inhibitory more in PC-3 than hormone dependent LNCaP cell line. The carotenoid downregulated in a dose- and time-dependent manner the expression of β-catenin and c-myc at mRNA and protein levels and inhibited AKT phosphorylation which, in turn, stimulated apoptosis by increasing the activity of caspases, including caspases-3, -8 and -9. The results clearly indicated that any of factors used alone led to different response of both cell lines. Analysis of microarray data (Affymetrix HG-U133A; 22 000 genes) of untreated and variously treated LNCaP cells showed that many of genes involved in replication, transcription and translation processes, steroid, cholesterol and eicosanoid metabolism were affected. The changes in expression of the genes which control G1/S checkpoint – cyclins and CDK, Rb, p107 and E2F were also noticeable. The results indicate links between BC and steroid signaling. In the view of the recent interest in the prognostic significance of lipogenic enzymes and their potential role as targets for antineoplastic therapy, our findings on the regulation of these enzymes by BC may provide a novel insight into the complex mechanisms by which androgens affect prostate cancer cells.

Retinoids and myoblast differentiation: the role of oxidative stress

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Ligands of retinoic acid receptors (RAR) can regulate cell differentiation. They also can cause an oxidative stress, leading to induction of Nrf-2 transcription factor and upregulation of cytoprotective genes such as heme oxygenase-1 (HO-1). RAR are expressed in the muscle cells and regulate both expression and transcriptional activity of myoD. Depending on their concentration the RAR ligands can activate or repress myogenesis. Here we investigated the role of HO-1 in muscle maturation.

We found that differentiation of satellite cells into myotubes was inhibited in cells isolated from HO-1-deficient mice, as evidenced by cell morphology and reduced expression of myogenin, MyoD, and myosin. Accordingly, 10-fold upregulation of HO-1 activity in C2C12 myoblast line stably overexpressing HO-1, improved the cell proliferation and survival under oxidative stress, while inhibited the differentiation, as indicated by reduced formation of myotubes, diminished activity of creatine phosphokinase, and decreased expression of myogenin, MyoD and myosin. These effects were fully reversed by pharmacological or genetic HO-1 inhibition. Importantly, effects of HO-1 overexpression were mimicked by HO-1 inducer or by carbon monoxide, one of HO-1 products. Upregulation of HO-1 reduced the total pool of cellular pre-miRNAs and miRNAs, and specifically inhibited the myomirs, miR-1, miR-133a, miR-133b, and miR-206, while increased production of SDF-1. Effects of HO-1 were mocked by treatment of cells with SDF-1 protein and reversed by overex-

pression of miR133a, miR133b and miR206. Role of HO-1 was also shown in vivo: after injection of control C2C12 myoblasts to the injured murine gastrocnemius muscle their numbers remained stable for at least three weeks. In contrast, HO-1 overexpressing cells proliferated continuously, and formed big, hyperplastic, undifferentiated tumors.

Thus, HO-1 can improve survival of myoblasts under oxidative stress. It is also a potent regulator of muscle differentiation, acting independently of anti-oxidative activity.

Molecular mechanisms by which retinoids inhibit tumor angiogenesis

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Tumor angiogenesis is a good target for treatment of solid cancers. Tumor cells induce angiogenesis via producing angiogenic factors, such as vascular endothelial growth factor (VEGF), and angiopoietins (Ang). The VEGF/VEGF receptor (VEGFR) signaling pathway is essential for drawing endothelial cells (ECs) from preexisting blood vessels and stimulating their growth, whereas the Ang/Tie2 signaling pathway is important for sustaining interaction between ECs and mural cells and stabilizing the vasculature.

Retinoids (vitamin A and its derivatives) exerts anti-tumor activity by modification of transactivation of growth regulation genes including $p21^{CIP1}$, IFN receptor, STAT and transglutaminase. All-*trans* retinoic acid (atRA) inhibits angiogenesis on chorioallantoic membrane (CAM) through disruption of vascular remodeling via inducing Ang2 expression and suppressing Ang/Tie2 signaling. Acyclic retinoid (ACR), an agent under clinical trials to suppress recurrence of hepatocellular carcinoma (HCC) through its activity to induce apoptosis in premature HCC cells, has anti-cancer effect in vivo, although it shows weak apoptosis-inducing activity against mature HCC cells. ACR inhibits angiogenesis within CAM. Whereas suppression of angiogenesis by atRA was partially rescued by Ang1, suppression of angiogenesis by ACR was not rescued under the same condition at all. On the other hand, whereas suppression of angiogenesis by ACR was partially inverted by VEGF, suppression of angiogenesis by atRA was not affected under the same condition. ACR selectively inhibited phosphorylation of VEGFR2 as well as ERK without changing their protein expression levels, and inhibited EC growth, migration, and tube formation. The inhibition in phosphorylation of ERK, endothelial growth, migration, tube formation and angiogenesis by ACR was rescued by overexpression of constitutively active MAPK kinase. Finally, ACR but not atRA inhibited HCC-induced angiogenesis in a xenografted CAM model. These results suggest that ACR will be clinically useful also as an anti-angiogenic agent in addition to current usage as a chemopreventive agent. Studies are underway to clarify mechanism underlying CAR's anti-phosphorylation activity.

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Carotenoids inversely modulate estrogenic and glucocorticoid activity in cancer and bone cells

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Exposure to estrogens is a major risk factor for breast and endometrial cancer. Carotenoids and other phyto-nutrients were found by us to inhibit estrogen signaling, in breast and endometrial cancer cells. In addition, we and others have shown that these phyto-nutrients induce the antioxidant response element (ARE) and the Nrf2 transcription factor. Using overexpression of Nrf2 and siRNA for this gene, we demonstrated that Nrf2 is involved in the phytonutrient-induced inhibition of the estrogenic activity. Although the effect of estrogens in breast and endometrial cancer is harmful, it is beneficial for bone formation. Thus, we investigated the effect of carotenoids and their oxidized derivatives as well as other phyto-nutrients on estrogenic activity in osteoblasts. We found that these compounds, which inhibit estrogenic activity in cancer cells, did not inhibit and even stimulated the expression of estrogen-induced genes in osteoblast-like cells. The effect of glucocorticoids in bone is opposite to that of estrogens and glucocorticoid treatment leads to bone resorption and osteoporosis. Thus, we determined whether phytonutrients inhibits glucocorticoid activity in bone. We found that the expression of glucocorticoid-dependent bone-destroying gene (RANKL) and the glucocorticoid inhibition of bone-supporting genes (osteocalcin, osteoprotegerin) were both reversed by the carotenoid derivatives. As discussed above, Nrf2 was found to be involved in the inhibition of estrogenic activity in breast cancer cells. In contrast, in bone cells, over-expression of Nrf2 enhanced estrogen-induced transcription but reduced glucocorticoid-induced transcription, similar to the effect of the carotenois. In addition, reduction of Nrf2 level, by siRNA, leads to a decrease in caotenoid supported activity of estradiol in bone cells. In addition to their positive effect on osteoblasts which can lead to increased bone formation, the carotenoids were found to interfere with RANK-Ligand dependent osteoclastic differentiation, which can lead to reduction in bone resorption. In conclusions, carotenoids and their derivatives, which inhibit estrogenic activity in cancer cells, do not inhibit and even stimulate estrogen signaling in osteoblastic bone cells but inhibit the deleterious effects of glucocorticoids in these cells. The results suggest that Nrf2 is partially involved in these activities.

Carotenoid metabolism and biological functions: its significance in lung cancer prevention

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Many epidemiological studies show the benefit of carotenoid-rich fruits and vegetables on the risk of lung cancer; however, clinical beta-carotene supplementation trials have returned null findings, or evidence of harm in certain populations. Based on these results carotenoid supplementation is not recommended for the general population, and smokers and consumers of alcohol are warned to avoid high-dose carotenoid supplements. However, the

metabolism and molecular biological properties of many carotenoids remain to be determined. Recent studies, including ours, indicated that carotenoids other than beta-carotene may be active in several important cellular signaling pathways in lung carcinogenesis, and carotenoid metabolites could have greater biological roles than their parent compounds in several molecular targets. This information is critically needed for future human studies involving carotenoids for prevention of lung cancer. In particular, studies from seven large well-implemented cohorts consistently show that among all of the specific carotenoids examined, increased dietary intake or elevated blood levels of beta-cryptoxanthin is strongly associated with a reduced risk of lung cancer. In our previous in vitro study, we provided experimental evidence that beta-cryptoxanthin inhibits the growth of both pre-malignant and malignant lung cell lines, and show the anti-proliferation activity of beta-cryptoxanthin is associated with both the induction of retinoic acid receptor-alpha tumor suppressor gene and the increase of retinoic acid response element-dependent promoter activity in cells co-transfected with retinoic acid receptor expression vector. In our recent in vivo studies, we demonstrated that beta-cryptoxanthin supplementation decreased dose-dependently the tobacco smoke-induced lung inflammation, TNF-alpha levels and squamous metaplasia in ferrets. We further demonstrated that beta-cryptoxanthin supplementation reduced significantly the multiplicity of lung tumors, and cyclin D1 proteins in the lungs of A/J mice treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. In addition, we showed that dietary beta-cryptoxanthin is effective in targeting the nicotinic acetylcholine receptor alpha7 subunit (alpha7-nAChR) and phospho-AKT pathway which mediates the tumor growth signaling, inflammation and angiogenesis. In summary, these studies indicate that beta-cryptoxanthin has certain unique beneficial effects against cigarette smoke-induced lung lesions and that β-cryptoxanthin is a potentially effective chemopreventive agent against the development of lung cancer.

Oral Presentations

Structural and formulation factors influencing carotenoid lipid-based DNA delivery

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The success of nucleic acid delivery requires the development of novel delivery vectors and formulations that overcome cellular barriers for effective transport. This presentation will describe our ongoing efforts towards the synthesis and nucleic acid carrying potential of novel cationic, carotenoid-based lipids. The series of novel lipids described within contain a hydrophobic C30-carotenoyl component, and differ in the length of the second (saturated) alkyl chain. Our aim in synthesizing the cationic carotenoid lipids was to determine if a structure-function relationship would emerge based on the balance of rigid and flexible lipid in the lipid series by investigating physicochemical behaviors, as well as pDNA delivery efficiency. Lipid-DNA particles were generated using these carotenoid-based lipids employing two distinct co-lipids, namely 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cholesterol. The performance of these carotenoid lipid vectors and the associated co-lipid formulations were compared to two established non-viral delivery vectors, 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (EPC). To determine the relative gene transfer efficiency, lipoplexes were formed using a plasmid that contained a gene encoding the enzyme, beta-galactosidase, driven by a cytomegalovirus early/intermediate promoter, designed to give strong expression of beta-galactosidase should the gene become localized in the nucleus of target cells. Chinese hamster ovary (CHO) cells were chosen based on their common use as a target for transfection. Beta-galactosidase activity and sample protein concentration were determined 48 hr after transfection. We found that in general, the vector formulations employing cholesterol as co-lipid were more efficient (greater β-gal expression) and less toxic than those with DOPE as co-lipid. The carotenoid C30-14 / cholesterol formulation out-performed all other lipids at a (+/-) molar charge ratio of 1.5, whereas the C30-20 / cholesterol formulation revealed the greatest overall performance at a (+/-) molar charge ratio of 3.0, out-performing the commercial lipids EPC and DC-Chol. At the (+/-) molar charge ratio of 3.0, with the exception of the C30-16 lipid, the increasing contribution of flexibility in the alkyl chain appears to balance the rigid properties of the carotenoid chain leading to more efficient gene transfer. These results establish the novel carotenoid lipids as promising new nucleic acid transfer agents.

Preventive effects of marine carotenoids on inflammatory bowel disease

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Carotenoids are a family of natural pigments with at least 700 members. Fruits and vegetables are the major sources of carotenoids in diets. Marine organisms also contain many kinds of carotenoids such as astaxanthin (AX) and fucoxanthin (FX).

Ulcerative colitis and Crohn's disease are major forms of inflammatory bowel diseases (IBD), which are chronic uncontrolled inflammation of intestinal mucosa. The incidence of ulcerative colitis has risen sharply in the developed country in recent decades. In this study, we focused on carotenoids as good candidates to be used for the prevention and/or therapy for IBD. The effects of AX and FX on the experimental colitis induced by dextran sulfate sodium (DSS) in mice were investigated. AX and FX at 200 ppm in the diet significantly suppressed a DSS-induced mucosal colitis determined by histopathology score. Both carotenoids alsoinhibited the expression of pro-inflammatory cytokines such as interleukin-6 (IL-6) and IL-1 mRNA in the intestinal tissue. In addition, COX-2 and iNOS mRNA expressions were also suppressed by AX and FX. As nuclear factor-kappaB (NF-kB) activity plays a critical role in the inflammation, we further examined the NF-kB expression in the inflamed intestinal tissue. Immunohistopathology score showed reduction of NF-kB expression in mice fed AX and FX. The suppression of NF-kB-DNA binding was also observed in murine macrophage-like cells, RAW 264.7 treated with AX. Further, AX significantly inhibited the occurrence of colonic mucosal ulcers, dysplastic crypts, and colonic adenocarcinoma on colitis-associated colon carcinogenesis induced by azoxymethane (AOM) / DSS in mice at 20 weeks. These results indicate that AX and FX are potential preventive compounds for IBD.

POSTERS

4.1.

Effect of lycopene on the progression of atherosclerosis development in New Zealand White Rabbits

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In the frame of the EU-funded project "LYCOCARD" (www.lycocard.com) the role of tomato products, in particular lycopene, in reduction of progression of cardiovascular diseases was studied in an animal model. Lycopene is the main carotenoid found in high concentrations in tomatoes. Strong epidemiological evidence suggests that lycopene may provide important protection against cardiovascular diseases. We aimed to investigate cardiovascular protective effects of lycopene on the progression of high fat-induced atherosclerosis in New Zealand White Rabbits. For that purpose, animals were divided into four groups with 9 animals each, that were fed either a standard diet, a high-cholesterol diet containing 0.5% cholesterol, a high-cholesterol diet containing placebo beadlets, or a high-cholesterol diet plus 5 mg/kg body weight/day of lycopene (in the form of lycopene beadlets) for a time period of 4 weeks.

We found significantly elevated lycopene plasma levels in the lycopene treated group. This was associated with a 50% reduction in total cholesterol and LDL cholesterol serum levels in the lycopene group, compared to the high-cholesterol and the placebo group. However, we could not observe a significant decrease in the extent of atherosclerotic lesions in aortae of the lycopene group. In addition, no differences in the intima-media thickness between the groups were found. Endothelial-dependent and endothelial-independent vasodilation in isolated rabbit aortic and carotid rings was not different between all treated groups.

In summary, lycopene supplementation for 4 weeks increased lycopene plasma levels in the animals. Although we found strongly reduced total and LDL cholesterol serum levels in the lycopene treated group, no significant differences in atherosclerotic lesions and morphological changes in the aortae could be detected.

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4.2.

Beta-Cryptoxanthin inhibits phosphoinositide 3-kinase (PI3K) signaling and lung cancer cell motility

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Recent epidemiologic studies show that increased dietary intake or higher blood levels of beta-Cryptoxanthin (BCX) relates to a reduced

risk of cigarette smoking-induced lung carcinogenesis. Here we show that BCX is effective at inhibiting migration and invasion of alpha7-nicotinic acetylcholine receptor (alpha7-nAChR) positive lung cancer cells by suppressing actin remodeling, ruffling/lamellipodia formation, but not in alpha7-nAChR negative cells. We establish that inhibition of alpha7-nAChR expression and its mediated PI3K signaling pathways potentially contribute to these activities of BCX. The PI3K downstream molecules such as Rac1 and ARF6 contribute to inhibition of lamellipodia formation and cell motility by BCX. BCX-induced cell migration inhibition is attenuated by overexpression of constitutively active PI3K or mutant of PTEN, activators of alpha7-nAChR/PI3K signaling, constitutively active Rac1 or ARF6. Overall, our studies show that BCX is efficient at suppressing PI3K signaling and lung cancer cell motility, which might be one of the potential mechanisms for the chemopreventive effect of BCX against lung cancers.

4.3.

Quenching effect on singlet oxygen, suppression against generation of reactive oxygen species and melanin synthesis in skin, and inhibition of multidrug resistance in cancer cells by capsorubin and capsanthin

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Capsorubin and capsanthin showed strong quenching activity for singlet oxygen. These activities were about 32 times (for capsorubin) and 25 times (for capsanthin) higher than that of β-carotene. These carotenoids also effectively suppressed the generation of reactive oxygen species (ROS) in mouse skin by irradiation of UVA. UVB-exposure increased significantly melanin concentration in the human skin equivalent model (HSEM) after 17 days culture. Topical application of carotenoid decreased significantly melanin production in comparison with the UVB-exposed group, suggesting that capsanthin and capsorubin had potent suppressive effects against UVB-induced melanogenesis in the HSEM. Capsorubin and capasanthin enhanced the Rhodamine 123 accumulation in human MDR1-gen transfected mouse lymphoma cells and reduced the expression of multidrug resistance efflux pump in xenografted PZX-40/46 human pancreatic adenocarcinoma cells. Apoptosis was induced by these carotenoids in cancer cells. The presence of vitamin C might influence the biological actions of these carotenoids differently.

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4.4.

The effect of 9-*cis* β-carotene on plasma lipid profile and atherosclerosis in LDL receptor-deficient mice

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Introduction: Atherosclerosis, a major risk factor of cardiovascular disease, is a complex disease caused by interactions between oxidized lipoproteins, invading inflammatory cells and vascular wall cells. The pro-vitamin A, β-carotene is a precursor for retinol, retinal and retinoic-acid. The alga *Dunaliella bardawil* is a rich source for β-carotene which under specific conditions accumulates β-carotene, reaching 10% of its dry weight, composed of approximately 50% all-*trans* and 50% 9-*cis* β-carotene isomers. Previous studies in our laboratory showed that β-carotene rich *Dunaliella* powder reduced plasma cholesterol levels and atherosclerosis development in mouse models. Importantly, by using *Dunaliella* powder composed of different ratios of 9-*cis* to all-*trans* isomers, we found that the protective effect is 9-*cis* β-carotene dependent.

Aim: In the current work we aimed to study the effect of isolated 9-*cis* β-carotene compared to 9-*cis* β-carotene-rich *Dunaliella* powder on plasma cholesterol levels and atherogenesis in female and male LDL Receptor knockout (LDL R-/-) mice fed high-fat Western diet.

Results: In female mice, 9-*cis* β-carotene inhibited atherosclerosis development significantly, along with a trend of decreased plasma cholesterol levels compared to the control group; however, 9-*cis* β-carotene-rich *Dunaliella* was more effective. In male mice, 9-*cis* β-carotene increased plasma cholesterol levels and had no effect on atherogenesis. In contrast, 9-*cis* β-carotene-rich *Dunaliella* powder inhibited atherogenesis and lowered plasma cholesterol.

Conclusions: The results show that 9-*cis* β-carotene rich *Dunaliella* powder is more effective in inhibition of atherogenesis than isolated 9-cis β-carotene. The difference in the effect of 9-*cis* β-carotene rich *Dunaliella* powder and isolated 9-*cis* β-carotene on atherogenesis suggests that the alga *Dunaliella* contains additional active ingredients that may play a role in inhibition of atherosclerosis.

4.5.

Photoprotective effects of aromatic carotenoids in human dermal fibroblasts (hdF)

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Dihydroxyisorenieratene (DHIR) and isorenieratene (IR) are naturally occurring aromatic carotenoids. DHIR is produced by the *Brevibacterium linens* which is used for the production of red smear cheeses. It is an unsual carotenoid comprising phenolic and polyenic structural elements. To evaluate those structure-related properties of DHIR, luteine and IR were studied for comparison.

Due to this unique structure and based on previous studies, which have shown a high antioxidative capacity (Martin et al., 2009), it was suggested that DHIR provides protection against UVB-induced cell damage.

Among other parameters we analyzed the formation of thymine dimers, as a measure of direct DNA damage, in hdF exposed to UVB-light in the presence and absence of the carotenoids mentioned above. In cells preincubated with DHIR and IR a significant decrease in formation of thymine dimers was found, whereas the treatment with luteine had no effect. In the alkaline Comet assay similar effects were determined for the aromatic carotenoids. In this assay also luteine showed some protection but less pronounced than DHIR and IR. Additionly, the formation of reactive oxygen species (ROS) and intracellular zinc release were determined. Both parameters were decreased in cells preincubated with DHIR in comparison to untreated cells or to those treated with luteine, which had no effect (Lutter et al., 2010). The data provide further evidence for the high antioxidative capacity of DHIR and confirm the hypothesis that DHIR is protective against UVB-induced damage. As expected from the structure DHIR acts as a multifunctional antioxidant with radical scavenging and UVB absorbing properties.

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4.6.

Beta-cryptoxanthin inhibits lung tumor by suppressing α7 nicotinic receptor expression and AKT activation in A/J mouse model

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The efficacy of beta-cryptoxanthin (BCX) for lung carcinogenesis has not been studied despite its association with a reduced risk of lung cancer in epidemiological studies. The activation of nicotinic acetylcholine receptor α7 subunit (α7-nAChR) and its downstream pathway can promote lung tumor growth. We hypothesize that BCX prevents the tobacco carcinogen-induced lung tumorigenesis by targeting α7-nAChR signaling. Male A/J mice were randomly assigned to six groups (n=16): the experimental arm received the NNK (4-[N-nitrosomethylamino]-l-[3-pyridyl]-l-butanone) injection with or without supplementation with BCX at two doses (0.2 and 2 mg/kg/day); and the control arm without NNK injection received the same BCX supplementation. The incidence and multiplicity of lung tumor and α7-nAChR expression and AKT activation were examined after 16 weeks post the carcinogen injection. There were 52% and 63% reductions of lung tumor multiplicity in mice supplemented with BCX at two doses, respectively ($P < 0.001$), as compared to the NNK alone group. The reduced lung tumor multiplicity by BCX was associated with the suppressions of the NNK-induced α7-nAChR expression and AKT activation measured by real-time polymerase chain reaction and immunoblotting. By high-performance liquid chromatography analysis, a significant increase was observed for hepatic retinyl palmitate, hepatic BCX, and for serum BCX concentrations of the mice supplemented with BCX. These results suggest that BCX is an effective chemopreventive agent for prevention of tobacco smoke components-induced lung carcinogenesis.

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4.7.

β-Cryptoxanthin supplementation prevents cigarette smoke-induced lung inflammation, oxidative damage and squamous metaplasia in ferrets

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In epidemiologic studies, high intake of β-cryptoxanthin has been associated with a decreased risk of lung cancer, particularly among current smokers (Mannisto et al., 2004; Gallicchio et al., 2008). However, data are not available from well-controlled animal studies to examine the effects of β-cryptoxanthin on cigarette smoke-induced lung lesions, and the biological mechanisms by which β-cryptoxanthin might affect lung carcinogenesis. We evaluated the effects of β-cryptoxanthin supplementation on cigarette smoke-induced squamous metaplasia, inflammation, and changes in protein levels of pro-inflammatory cytokine [tumor necrosis factor alpha (TNFα)] and transcription factors [nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1)], as well as on smoke-induced oxidative DNA damage [8-hydroxy-2'-deoxyguanosine (8-OHdG)] in the lung tissue of ferrets. Thirty six male ferrets were assigned to cigarette smoke exposure or no exposure and to low-dose, or high-dose β-cryptoxanthin, or no dose (2 x 3 factorial design) for 3 months. β-Cryptoxanthin supplementation dose-dependently increased plasma and lung β-cryptoxanthin levels in ferrets, whereas cigarette smoke exposure lowered plasma and lung β-cryptoxanthin levels. β-Cryptoxanthin at both doses significantly decreased smoke-induced lung squamous metaplasia and inflammation. β-Cryptoxanthin also substantially reduced smoke-elevated TNFα levels in alveolar, bronchial, bronchiolar and bronchial serous/mucous gland epithelial cells and in lung macrophages. Moreover, β-cryptoxanthin decreased smoke-induced activation of NF- B, expression of AP-1 and levels of 8-OHdG. The beneficial effects of β-cryptoxanthin were stronger for high-dose β-cryptoxanthin than for low-dose β-cryptoxanthin. Data from this study indicate that β-cryptoxanthin provides a beneficial effect against cigarette smoke-induced inflammation, oxidative DNA damage and squamous metaplasia in the lungs.

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Session 5

Interaction of Carotenoids with Reactive Oxygen Species

INVITED LECTURES

The quenching effect of singlet oxygen by carotenoids and vegetables. Development of singlet oxygen absorption capacity (SOAC) assay method

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Lipid peroxyl radical ($LOO^{\bullet}$) and singlet oxygen ($^1O_2$) are two well-known representative reactive oxygen species (ROS) generated in biological systems. In recent years, the method to assess the total oxygen radical absorption capacity (ORAC) of foods and plants has been developed. On the other hand, the method to assess the $^1O_2$-quenching activity of foods and plants which include carotenoids and phenolic antioxidants has not been developed. Thus, we developed the new, versatile method to assess singlet oxygen absorption capacity. A kinetic study of the quenching reaction of $^1O_2$ with 8 kinds of carotenoids and some kinds of vegetable extracts was performed in ethanol:chloroform:D_2O (50:50:1, v/v/v) solution at 35°C. The overall rate constants, k_Q (= $k_q + k^r$, physical quenching + chemical reaction), for the reaction of carotenoids with $^1O_2$ were measured, using the competition reaction method, where 3-(1,4-epidioxy-4-methyl-1,4-dihydro-1-naphthyl) propionic acid (endoperoxide) was used as a singlet oxygen generator, 2,5-diphenyl-3,4-benzofuran (DPBF) as an UV-Vis absorption prove, and α-tocopherol as a standard compound. The rate constants, k_Q (S) and k_Q ($t_{1/2}$), were determined by analyzing the first-order rate constant (S) and the half-life ($t_{1/2}$) of the decay curve of DPBF with carotenoids, respectively, showing good accordance with each other. Similar measurements were performed for some extracts of vegetables rich in carotenoids. Based on the results, a new assay method that can quantify the singlet oxygen absorption capacity (SOAC) of foods and plants including carotenoids and phenolic antioxidants was proposed.

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Reactivity of carotenoids and new putative lycopene metabolites in a biomimetic experimental model of oxidative stress

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Carotenoids, among which lycopene, are supposed to participate in the prevention of degenerative diseases such as cancers and cardiovascular diseases. Mechanisms underlying these effects are not well known. Regulation of gene expression has been demonstrated in in vitro studies. Antioxidant mechanisms could be especially relevant prior to intestinal absorption, i.e. in the gastro-intestinal (GI) tract where dietary antioxidants can accumulate in large concentrations after a meal rich in plant products and where different forms of oxidative stress can take place. Carotenoids metabolites are of interest for their possible biological activity which could be different from or even higher than those of the parent carotenoid. Only few lycopene metabolites were identified in vivo: 2,6-cyclolycopene-1,5-diol in humans [1], apo-12'- and apo-8'-lycopenals in rats [2] and apo-10'-lycopenal in ferrets [3]. Recently Kopec et al. [4] found for the first time a serie of apo-lycopenals in the plasma of human subjects having consumed tomato products. The same apo-lycopenals being present in the tomato products, it is not known whether they were formed in humans or if they were ingested and further absorbed.

We have synthesised three series of potential lycopene metabolites, the apo-11-, apo-10'- and apo-14'-lycopenoids, each with four different terminal chemical functions: aldehyde, carboxylic acid, ester and alcohol. Our synthetic strategy was based on chemical reactions classically used in carotenoid synthesis: the Horner-Wadsworth-Emmons and Wittig condensations, together with reduction, oxidation and saponification reactions for functional group modifications. We determined the antioxidant activity of carotenoids and putative metabolites of lycopene using a home-made lipid peroxidation test mimicking oxidative stress in the GI tract [5]. A mathematical treatment yielding useful kinetic parameters was developed to achieve a quantitative comparison between the investigated antioxidants and to establish structure-activity relationships. The antioxidant activity of the apo-lycopenals increased with increasing length of the conjugated double bond system, i.e. in the order: apo-11-, apo-14'-, apo-12'-, apo-10'-, apo-8'-, apo-6'-lycopenal. For the apo-10'- and 14'-lycopenoids, the order of increasing reactivity in relation with the terminal function was: OH < COOEt ≤ CHO < COOH. Ionisation potentials for all investigated apo-lycopenoids and energies of peroxyl radical addition were estimated for data interpretation.

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Novel functionality and molecular mechanism of fucoxanthin

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We have found that brown seaweed carotenoid, fucoxanthin, is regarded as playing an important role in the prevention of human

disease and maintaining good health. This unique carotenoid improved obesity-induced disorders mainly on the basis of specific molecular mechanism. Fucoxanthin showed anti-obesity effect through the induction of uncoupling protein 1 (UCP1) expression in visceral white adipose tissue (WAT) mitochondria leading to oxidation of fatty acids and heat production in the WAT. Fucoxanthin improved insulin resistance and decreased blood glucose level through the down-regulation of adipokines such as TNFα, MCP-1, IL-6, and PAI-1 in WAT and promotion of translocation of glucose transporter 4 (GLUT4) to the cell membrane of skeletal muscle from the intracellular compartments. The down-regulation of mRNA of above adipokines was partly due to the inhibitory effect of fucoxanthin on the infiltration of macrophages into adipose tissue. Obese adipose tissue is characterized by an increased infiltration of macrophages, which results in a paracrine loop involving synergistic increases in TNFα and free fatty acids between infiltrated macrophages and adipocytes. Fucoxanthin effectively stops this adverse circulation between adipose cells and macrophages, and then inhibits excess secretion of adipokines from visceral WAT. In addition, fucoxanthinol, a main fucoxanthin metabolite, decreased nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA expression in RAW264.7 cells stimulated by palmitic acid. The down-regulation of COX-2 and iNOS mRNAs found in macrophages with fucoxanthinol may contribute to suppress or ameliorate the increased inflammation in WAT and insulin resistance. Fucoxanthin metabolites accumulated in animal tissues down-regulated NADPH oxidase expression and up-regulated antioxidant enzyme expressions. These effects of fucoxanthin and its ability to scavenge free radicals resulted in the less oxidative stress of the viscera WAT and the liver of animals, which will be another reason to explain its physiological effect. Physiological effects of fucoxanthin are likely linked to its structural characteristic – an allene bond and an additional hydroxyl substituent on the side group of the fucoxanthin metabolites, fucoxanthinol and amarouciaxanthin A. Present study demonstrates that characteristic physiological activity of fucoxanthin is mainly based on the mechanisms of action that are independent of the antioxidant activity, although the antioxidant properties of fucoxanthin will be effective to prevent the biological tissues against oxidative damage.

Carotenoids: from guardians of oxidative damage to redox regulators

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It has been suggested that carotenoids, one of the major groups of antioxidants found in fruits and vegetables, exhibit health-beneficial effects, including prevention of chronic diseases, such as cancer, cardiovascular, inflammatory, and metabolic diseases, by virtue of their antioxidant activity. In fact, their polyene chain is the structural feature that determines the chemical reactivity toward free radicals, and hence, their ability to inhibit oxidative processes to lipids, DNA and proteins. However, recent literature suggests that carotenoid molecules can actually "perform" activities and roles independent of such capacity and involving a modulation of redox signalling. Electrophiles, reactive oxygen species, and reactive nitrogen species are known to act as second messengers in the modulation of many cellular signalling pathways leading to gene expression changes and pharmacological responses. Recent studies from our laboratory show that carotenoids may control redox-sensitive molecular targets at different levels, affecting enzyme activities and expressions, binding to membrane or nuclear receptors, modulating the activation of MAPKs and transcription factors, such as NF-kB and AP-1, Nrf2. Inductive or signalling effects may occur at concentrations much lower than those required for effective antioxidant activity and may be responsible for a regulation of ROS-mediated cellular functions in both physiological and pathophysiological conditions.

Beta-carotene degradation products – formation, toxicity and prevention of toxicity

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Introduction: After β-carotene (BC) failed in clinical trials, there is evidence that BC might have harmful effects at high dosages. Hypothesis: Negative side effects might be mediated by BC breakdown products (BCBP). Within the BCBP there is a multitude of aldehydiccoumpounds, which exert similar effects compared with HNE. BC is cleaved non-enzymatically by liberated oxidants of PML [1]. Supplementation of BC seems to be important in various diseases. Therefore, it is necessary to think on safe conditions expecially for high-dosage supplementation.

Materials and Methods: BC degradation was investigated under various conditions. BC was rapidly degraded by PML, too. Hepatocytic mitochondrial respiration and genotoxicity were evaluated in presence of BCBP. In parallel experiments antioxidants such as alpha-tocopherol, ascorbic acid, uric acid etc. were used to avoid negative side effects.

Results: BCBP modify the activities of enzymes and transport proteins such as Na-K-ATPase, NADPH oxidase, and adenine nucleotide translocator. At nanomolar concentrations BCBP exert genotoxic effects [2,3,4]. BCBP impair mitochondrial functions at low mikromolar concentrations [5,6]. During incubation of mitochondria with BCBP GSH and protein SH decreased, GSSG and MDA increased. PML mediated BCBP formation was reduced in presence of antioxidants. The inhibition of ADP-stimulated respiration was also mitigated in presence of antioxidants. Even genotoxic effects could be avoided if antioxidants were present [7]. Best protective effects were exerted by vitamin E.

Discussion Conclusions: The data indicate a basic pro-oxidative mechanism of high concentrated BC at heavy oxidative stress leading to rapid formation of BCBP. Antioxidants mitigate potential toxic effects [7].

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Interactions of dietary carotenoids with activated (singlet) oxygen and free radicals: potential effects for human health

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Singlet oxygen is important in the skin and eye and carotenoids are efficient singlet oxygen quenchers with corresponding rate constants near diffusion controlled (typically $\approx 10^{10}\ M^{-1}s^{-1}$) with lycopene exhibiting the most efficient quenching in organic solvents. However, in membrane environments there is little or no difference in the quenching efficiency between the dietary carotenoids. Furthermore, aggregation of carotenoids markedly reduces singlet oxygen quenching efficiency. Free radical interactions with carotenoids leads to at least three processes, electron and hydrogen atom transfer and adduct formation. The most studied is electron transfer where the carotenoid loses an electron to become a radical cation. The reactivity/lifetime of such carotenoid radicals may lead to a switch from anti- to pro-oxidant behaviour of carotenoids. These reactions are related to carotenoid redox potentials with lycopene being the lowest (most easily oxidised) allowing lycopene to reduce/repair all other carotenoid radical cations and lycopene 'sacrificed' where mixtures of carotenoids are present in oxidative environments. Such redox-controlled reactions may lead to deleterious as well as beneficial health effects.

Carotenoids, mitochondria and oxidative stress

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Carotenoids are the precursors for vitamin A and are proposed to prevent oxidative damage to cells. Mammalian genomes encode a family of structurally related non-heme iron oxygenases that modify double bonds of these compounds by oxidative cleavage and *cis*-to-*trans* isomerization. The roles of the family members BCMO1 and RPE65 for vitamin A production and vision have been well established. Surprisingly, we found that the third family member BCDO2 is a mitochondrial carotenoid-oxygenase. BCDO2 converts both carotenes and xanthophylls by oxidative cleavage at position 9,10 and 9',10. In BCDO2-deficient mice, carotenoid homeostasis was abrogated and carotenoids accumulated in several tissues. In hepatic mitochondria, accumulated carotenoids induced key markers of mitochondrial dysfunction such manganese superoxide dismutase and reduced rates of ADP-dependent respirationby 30%. This impairment was associated with an 8- to 9-fold induction of phosphor-MAP kinase and phosphor-AKT, markers of cell signalling pathways related to oxidative stress and disease. Administration of carotenoids to human HepG2 cells depolarized mitochondrial membranes and resulted in the production of reactive oxygen species. Expression of recombinant BCDO2 prevented oxidative stress in this cell line. Thus, our studies in BCDO2-deficient mice and human cell culture indicate that carotenoids can impair respiration and induce oxidative stress. Mammalian cells thus express a mitochondrial carotenoid-oxygenase that degrades carotenoids to protect these vital organelles.

Oral Presentations

Direct observation of natural antioxidants β-carotene and resveratrol reaction with hydroxyl radical

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Hydroxyl radical is widely accepted as the most oxidizing oxygen reactive species (ROS). Natural antioxidants β-Carotene and resveratrol are found reacting readily with hydroxyl radical following submicrosecond laser photolysis of N-hydroxypyridine-2(1H)-thione (N-HPT) as a "photo-Fenton" reagent generating hydroxyl and thiyl radicals in acetonitrile:tetrahydrofuran (4:1, v/v) solution. Based on time-resolved spectral evidences, and supported by kinetic considerations and thermodynamic calculations, a short-lived transient species with an absorption maximum at ~750 nm and τ~150 ns for β-carotene, is suggested to be the long-sought neutral β-carotene radical formed by hydrogen-atom abstraction (Chen C.-H. et al., 2011 and Galano, A. et al., 2009). Whereas, a relatively stable radical with an absorption maximum at ~360 nm and τ~3 μs for resveratrol observed, is suggested to be an adduct product by hydroxyl radical addition to resveratrol. Different behaviours of the reaction with hydroxyl radical for β-carotene and resveratrol are discussed based on theoretical quantum calculations. The mechanistic studies in present study may contribute to a better understanding of natural antioxidants under extreme oxidative stress.

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Elevated ambient temperatures impair antioxidant status and carotenoid-based sexual ornamentation

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Recent warming of Earth's climate has had many impacts on organism function and fitness, but few have considered effects on some of the traits – such as condition-dependent signals (e.g. songs, colors) – that are most likely, strongly, and rapidly to be affected. Even fewer experimental approaches have been taken in this area, which would permit isolation of causal influences of thermal conditions per se from other concurrent environmental changes. Here I manipulated ambient temperature in captive male zebra finches (Taeniopygia guttata) using climate-controlled environmental chambers and examined effects on their antioxidant system (e.g. circulating carotenoids and vitamins), body mass, and expression of a sexually selected trait (carotenoid-based beak colouration). Exposure to an elevated ambient temperature cycle for one month significantly lowered levels of circulating carotenoids and a lipid-soluble vitamin (tocopherol) as well as beak redness, without altering body mass. These experimental results in a native warm-climate (Australian grassland) species implicate carotenoid-derived sexual advertisements as reliable and sensitive indictors of climate change.

POSTERS

5.1.

Synthetic growth regulator cytodef influence on the carotenoids level in cucumber plants affected by heavy metals

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Environment contamination with toxic heavy metals (HM) is hazardous to plants. HM excess inhibits the seeds germination, seedlings growth, breaking of metabolic processes in plants. HMs intensifies generation of activated oxygen species (AOS) and promotes oxidative stress in plants. Antioxidative system promote the level of AOS and contain both enzymatic and low molecular antioxidants. Carotenoids as part of this system strongly inhibit the singlet oxygen, and can neutralize others AOS too. Biologically active substances are capable to modify plants reaction to stresses. However there are a few researches about plant growth regulators effects on plants at HM stress. In this work the influence of synthetic cytokinin-like growth regulator cytodef and HM ions on the carotenoids level in cucumber (*Cucumis sativus* L., cv. "Graceful") leaves was investigated.

Cucumber seeds were germinated in plastic pots in liquid medium supplemented with 10 μM (suboptimal doses) or 1 μM (sublethal doses) Pb^{2+}, Sr^{2+}, Ni^{2+} or Zn^{2+} under following conditions: 21-23°C, 14 h photoperiod, illumination about 200 μM photons·m^{-2}·s^{-1}. Some of seeds were exposed by 0.1 μM cytodef, control plants grown on distilled water. In 7-days seedlings we detected carotenoids level in cotyledonous leaves spectrophotometrically, as well as superoxide generation.

Carotenoids level varied in cucumber cotyledonous leaves affected by HM. So, Sr^{2+} and Pb^{2+} practically didn't influence the carotenoids level, whereas Zn^{2+} or Ni^{2+} treatment decrease carotenoids level. Cytodef didn't influence carotenoids in water, however in HM treated plants carotenoids level raised in comparison with variants without growth regulator. The exception were marked at 1 mM Pb^{2+}, Ni^{2+} and Zn^{2+}. As a rule, protective action of cytodef dropped among $Ni^{2+} > Zn^{2+} \geq Sr^{2+} > Pb^{2+}$.

HMs enhance superoxide generation in cucumber plants, but cytodef application reduce this enhancement, most effectively in case of Ni^{2+} and Zn^{2+} in low concentrations. So, protective effects of cytodef on cucumber plants affected by HMs can occur through increase of antioxidative activity, including carotenoids participation.

This work was supported by the Russian Ministry of Education and Science under the Analytical Departmental Target Program "Development of Scientist Potential of Higher Scool", Project no. 2.1.1/624.

5.2.

Preliminary study on the effect of intraperitoneal injections of astaxanthin in gilthead seabream (*Sparus aurata*) broodstock performance

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Dietary astaxanthin plays a key role in fish reproductive response. A carotenoid-depleted diet in aquaculture fish species results in pale gonads and poor broodstock performance. Due to the low apparent digestibility of dietary astaxanthin in fish, alternative administration routes of astaxanthin are required. The objective of this work is to evaluate the effect of administering astaxanthin through the intraperitoneal cavity on gilthead sea bream reproductive performance, astaxanthin deposition, oxidative stress and fatty acid profile.

Duplicate groups of gilthead sea bream were injected with preparations of astaxanthin made from Carophyll Pink (DSM). Two treatment groups were injected once with doses of 10 (IP10) and 50 (IP50) mg of astaxanthin. The control was injected with phosphate buffered saline, without astaxanthin. The experiment lasted 3 weeks. A ratio of males to females of 2:1 was maintained in each group. After injection of astaxanthin, the fish were fed a commercial feed, 1.5% of body weight. The feed used contained 25 mg astaxanthin kg^{-1}. Egg batches were collected daily for evaluation of egg production, egg quality and hatchability. Samples for biochemical analyses were taken every week.

During the first two weeks of the experiment, no differences ($P \geq 0.05$) were found in total egg production. On week three, the IP50 treatment group presented higher production ($P \leq 0.05$) than the other treatment groups. Regarding percentage of viable eggs, both IP10 and IP50 presented higher values ($P \leq 0.05$) than the control on week two; however, on week three only IP50 differed from the control group. Hatchability of IP10 and IP50 treatment groups were higher ($P \leq 0.05$) than control only in week three. Astaxanthin deposition in eggs increased with time, and the amount of astaxanthin injected. The amount of malondialdehyde (MDA) in eggs was lower ($P \leq 0.05$) in IP10 and IP50 on week three. Egg fatty acids such as eicosapentaenoic acid (EPA) and arachidonic acid (AA) were both significantly higher in IP10 and IP50. These results could indicate a protective role of astaxanthin or a promotion of the biosynthesis of n-3 and n-6 PUFA. Also, total monosaturated fatty acids, an important energy source in fish, were significantly reduced significantly in both IP10 and IP50, suggesting a stimulation of lipid utilization. Results clearly demonstrate the significant benefis of astaxanthin administered through the intraperitoneal cavity in terms of improved egg quality and production, astaxanthin egg deposition, response to oxidative stress, and egg fatty acid profile.

5.3.

Cigarette smoking and lycopene isomerisation

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It is often assumed that smokers have lower plasma carotenoids such as β-carotene and lycopene than non-smokers. Cigarette smoke is a very complex mixture and it has been demonstrated that one of its constituents, peroxynitrite may cross the lung alveolae. It has been postulated that it may interact with components of the blood including plasma Low Density Lipoproteins (LDL) which comprises of a protein apolipoprotein B100 and lipids. LDL particles are also largely responsible for transport of lycopene and β-carotene in the blood. In this study we recruited 25 smokers and non-smokers from the Merseyside area, to assess oxidation of LDL particles and its lycopene content. Blood was taken from the volunteers and full lipid profiles, oxidation of LDL and HPLC analysis of lycopene and its isomers along with -carotene was undertaken. The first observation was that the smokers had a pro-atherogenic profile (higher total cholesterol and lower HDL cholesterol). It was also observed that smokers had a greater concentration of oxidised LDL, indicating they were under a greater oxidative stress. β-Carotene concentrations were lower in the smokers ($p<0.05$) but there was no significant difference in total lycopene. Upon further analysis it was noticed that smokers had an altered all (E):Z lycopene ratio compared to non-smokers; this ratio was more apparent in the heavier smokers (>15 cigarettes per day). This study indicates that -carotene concentrations were lower in smokers and that cigarette smoke may influence the lycopene isomerisation within human plasma.

5.4.

Enhancement of in vivo renal reducing ability by dietary lycopene, the main carotenoid in tomato (*Lycopersicon esculentum*): an estimation by a radiofrequency electron paramagnetic resonance method

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The kidney is the organ that excretes and reabsorbs various substances in blood. If relatively greater amounts of oxidative substances pass through the kidney, the increased oxidative stress, such as reactive oxygen species (ROS) production, will cause nephropathy. Thus, prevention of renal oxidative stress by antioxidants is effective in suppressing nephrotoxicity. Lycopene is the main carotenoid in tomato and tomato-based products and is well known to be a potent antioxidant in various in vivo and in vitro models. Although it has been reported that dietary lycopene, improved drug-induced nephropathy, there are no reports on the effect of orally administered lycopene on the in vivo renal reducing ability. The radiofrequency electron paramagnetic resonance (EPR) method is a unique technique by which the in vivo reducing ability of an experimental animal can be studied. In this study, the in vivo changes in the renal reducing ability of rats orally administered lycopene were investigated using a 700 MHz EPR spectrometer equipped with a surface-coil-type resonator. Rats were fed either a control diet or a diet containing 0.25% lycopene. After 2 weeks, in vivo EPR measurements were conducted. The renal reducing ability, which was estimated based on the half-life of the EPR signal of nitroxide radical (TEMPOL), in rats administered lycopene was significantly greater than that of the control. On the other hand, direct chemical reaction between lycopene and TEMPOL was not observed. This is the first verification of in vivo antioxidant enhancement via dietary lycopene administration.

5.5.

Ferric reducing and peroxyl radical scavenging activity of lycopene compounds

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Due to their chemical structure of a highly unsaturated hydrocarbon chain, carotenoids provide potential antioxidant properties. Of the >750 carotenoids, which were identified from natural sources, only six represent >90% of the total content of carotenoids in human blood of Western populations, with lycopene as the most important one (Rao et al., 2006). Carotenoids can react by certain mechanisms to inhibit oxidation of a substrate (Krinsky and Johnson, 2005). Therefore, the activities of the main dietary carotenoids (using (*all-E*)-isomers) in hydrogen atom transfer (HAT)-reactions were evaluated by determining the peroxyl radical scavenging capacity (PSC). Furthermore, firstly the ferric reducing activities of the carotenoids were analyzed, which illustrates the reactivities against transition metals underlying single electron tranfer (SET)-based reactions.

Lycopene as (*all-E*)-isomer is abundant in raw fruits and vegetables. No differences were observed in the ferric reducing activity of (*all-E*)-lycopene compared to the (5*Z*)-, (9*Z*)-, and (13*Z*)-isomers, which are (*Z*)-isomers of lycopene that account for more than 50% of the total lycopene content in human tissues and are found in substantial amounts in processed foods such as tomato products (Rao et al., 2010). Furthermore, the antioxidant activity of prolycopene, abundant in *Tangerine* tomatoes, was analyzed.

Moreover, the antioxidant activity of lycopene is possibly associated with short-chain lycopene metabolites. Analyzing various apo-lycopenoids, we observed a strong influence of length of the polyene chain and type of terminal function on the ferric reducing activity and on PSC.

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5.6.

Development of singlet oxygen absorption capacity (SOAC) assay method and the quenching activity of singlet oxygen by carotenoids and vegetables

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Lipid peroxyl radical ($LOO^{\bullet}$) and singlet oxygen ($^1O_2$) are two well-known representative reactive oxygen species (ROS) generated in biological systems. In recent years, the method to assess the total oxygen radical absorption capacity (ORAC) of foods and plants has been developed. On the other hand, the method to assess the $^1O_2$-quenching activity of foods and plants which include carotenoids and phenolic antioxidants has not been developed. In the present work, measurements of the quenching rate of $^1O_2$ (k_Q) for eight carotenoids were performed by using the competition reaction method. The k_Q values of carotenoids obtained were 50-100 times greater than that of α-tocopherol which was used as a standard compound. Furthermore, we measured the k_Q values of three kinds of carotenoid-rich vegetable extracts and the concentrations of seven kinds of carotenoids included in vegetable extracts, using a HPLC technique. From the results, it has been clarified that the total $^1O_2$-quenching activity for vegetable extracts may be explained as the sum of the product $\{\sum k_Q^{\text{Car-i}} [\text{Car-i}]_i\}$ of the rate constant ($k_Q^{\text{Car-i}}$) and the concentration ([Car (i)]) of carotenoids included in vegetable extracts. Based on the results, a new assay method that can quantify the singlet oxygen absorption capacity (SOAC) of foods and plants including carotenoids and phenolic antioxidants was proposed.

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Session 6

Biosynthesis, Genetics, and Metabolism of Carotenoids

INVITED LECTURES

New insights into strigolactones biosynthesis

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Strigolactones (SLs) are isoprenoids with a typical C_{19}-structure consisting of a tricyclic lactone (ABC-rings) connected via an enol ether bridge to a further lactone, a butenolide group (D-ring). SLs act as phytohormones regulating plant architecture, in addition to their role as chemical signals involved in plant-plant and plant-fungi interactions. It was proposed that the synthesis of SLs is initiated through a carotenoid cleavage step yielding a C_{15}-aldehyde subjected to a series of reactions leading to the ABC C_{14}-skeleton, which is then coupled to a butenolide group of unknown origin. Plant branching mutants indicated the involvement of the carotenoid cleavage dioxygenases (CCD) 7 and 8 in the SLs biosynthesis, and it was supposed that these enzymes catalyze a sequential cleavage leading to β-apo-13-carotenone (C_{18}) which structure has little in common with that of SLs. Our study provides evidence that the activities of CCD7 and 8 are sufficient surpass the gap between carotenoids and SLs. Using in vitro assays performed with enzymes from different plant species, we show that combined CCD7/8 catalysis leads to a product similar to SLs in its structure and biological activity. The formation of the SL-like compound indicates that CCD8 catalyzes a series of reactions including isomerization, Baeyer-Villiger-like rearrangement and repeated dioxygenation.

Regulation of carotenoid biosynthesis: impacts on plant development

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In plants, carotenoids are required for photosynthesis, photoprotection and the biosynthesis of at least two hormones, namely abscisic acid and strigolactones. The carotenoid biosynthetic pathway bifurcates after lycopene to produce lutein or beta-carotenes and its derivatives. Thus the branch point modulates which carotenoids accumulate [1]. We have shown how the branch point can be regulated by a chromatin-modifying histone methyltransferase, Set Domain Group 8, (SDG8), targeting the carotenoid isomerase (*CRTISO*) [2]. SDG8 controls the permissive expression of a small number of genes by histone methylation of lysine 4 and/or 36 of chromatin surrounding key gene targets such as *CRTISO* [3]. Regions within the *CRTISO* promoter are required for SDG8 recruitment as well as function, and tissue specific expression of *CRTISO* is similar to that of *SDG8* [4]. We are exploring the molecular nature by which SDG8 regulates *CRTISO* and how modulating carotenoid flux through the pathway may perturb the production of carotenoid-derived signaling molecules. The chromatin modifying nature of SDG8 and novel functions for CRTISO in regulating plant development have opened a new door to improve our understanding of epigenetic processes.

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Carotenoid biosynthesis in tomato at the crossroad between genomics and metabolic engineering

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Tomato (*S. lycopersicum*) is the second most important horticultural crop worldwide, a model system for fleshy fruit development, and the genetically best-characterized plant after Arabidopsis and maize. Together with *S. pimpinellifolium*, its closest wild progenitor, accumulates the unusual carotene, lycopene, in its fruits. A large number of mutants, affected specifically in fruit carotenoid biosynthesis, are available, thanks to widespread gene duplication in the biosynthetic pathway. My lab has been interested, since the 1990's, in metabolic engineering in tomato and potato and, more recently, in the genomics of the pathway in the two plants. The topics touched upon in my talk will be:

a) the regulatory relations between carotenoid genes and metabolites in tomato fruits, as revealed by metabolically engineered plants and mutants
b) the structure and regulation of the carotenoid pathway genes in tomato and its wild progenitor species
c) the likely molecular events that resulted in the emergence of red-fruited, wild species in the tomato clade, and the effects of man-madedomestication.

Pathway engineering for efficient production of astaxanthin in lettuce plants

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Among ketocarotenoids (carotenoids including 4-ketolated β-ring), astaxanthin and canthaxanthin (specifically the former), are commercially important pigments as nutraceuticals and cosmetics that have anti-oxidation and anti-aging effects, while other ketocarotenoids are likely to have industrial potentials. Pathway engineering is to engineer biosynthetic pathways for compounds of interests in heterologous organisms such as microbes and higher plants. Pathway engineering researches for production of astaxanthin have been performed in higher plants, by using carotenoid β-ring 4(4')-ketolase (4(4')-oxygenase) genes, which contain *crtW, bkt1, bkt2*, and *crtO* genes, as reviewed (Misawa, 2009). Recently, the marine bacterial (*Brevundimonas* sp.

SD212) *crtW* and *crtZ* (carotenoid β-ring 3(3')-hydroxylase) genes, whose nucleotide sequence is modified to codon usage of higher plants, were successfully overexpressed in the chloroplasts of tobacco plants (*Nicotiana tabacum*), and astaxanthin level produced there reached 5.44 $mg \cdot g^{-1}$ dry weight (74% of total carotenoids) (Hasunuma et al., 2008). This finding means that direct expression of the foreign genes in plant chloroplasts is a promising approach for efficient production of astaxanthin. We performed chloroplast transformation of an edible plant lettuce (*Lactuca sativa*), and expressed three key genes for ketocarotenoid biosynthesis in lettuce leaves, which were the *crtW* and *crtZ* genes, and the *idi* gene originated from marine bacterium *Paracoccus* sp. N81106. Subsequent transplastomic lettuce plants produced astaxanthin predominantly, along with other ketocarotenoids such as fritshiellaxanthin (4-ketolutein), 4-ketoantheraxanthin and canthaxanthin, and another commercially important carotenoid lutein.

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Transcriptional regulation of phytoene synthase levels in *Arabidopsis*

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Carotenoids are isoprenoid pigments synthesized by all photosynthetic organisms and some non-photosynthetic bacteria and fungi. In plants, they play central roles in photosynthesis and photoprotection and provide color to non-photosynthetic roots, flowers and fruits. Additionally, their oxidative cleavage generates apocarotenoids such as the hormones abscisic acid (ABA) and strigolactones that regulate plant development and responses to external stimuli. However, the molecular mechanisms controlling plant carotenogenesis are not well understood yet.

The metabolic precursors for plant carotenoid biosynthesis derive from the methylerythritol 4-phosphate (MEP) pathway and are shared by other plastidial pathways leading to the production of different isoprenoid-end products such as gibberellins and the side chain of tocopherols and chlorophylls. Such MEP-derived precursors are specifically channelled to the carotenoid pathway by the enzyme phytoene synthase (PSY). Environmental factors such as light and salt stress are important regulators of PSY accumulation at the gene expression level. We have shown that the only gene encoding PSY in *Arabidopsis thaliana* is repressed in dark-grown seedlings by direct binding of the phytochrome-interacting transcription factor PIF1 to specific motifs in the *PSY* promoter. Degradation of PIF1 upon interaction with photoactivated phytochromes during deetiolation results in a rapid derepression of *PSY* gene expression and a burst in the production of carotenoids in coordination with chlorophyll biosynthesis and chloroplast development for an optimal transition to photosynthetic metabolism. Our data on the regulation of *PSY* expression by PIFs beyond deetiolation and by other transcripcion factors in response to salt stress will also be presented.

Carotenogenesis in the potato tuber

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Despite extensive studies characterising the biosynthetic genes involved in the carotenoid pathway little is known about the mechanisms regulating carotenoid accumulation in non-green tissues. Early transgenic studies have demonstrated that tubers have the capacity to accumulate nutritionally significant levels of a range of carotenoids, either by manipulation of biosynthesis using single or combinations of transgenes, or by altering the sink capacity for storage of carotenoids. We have used a range of techniques in an attempt to discover the genes that underpin natural variation in potato tuber carotenoid content. Two diploid populations that segregate for tuber carotenoid content were developed and dense genetic maps were constructed. Two major QTL, affecting overall tuber carotenoid content were identified on chromosome 3 and 9. Whereas a known biosynthetic gene was shown to underpin the QTL on chromosome 3 (*crtR-b2*), no known biosynthetic gene maps to the chromosome 9 QTL. A genetical genomics approach was used to identify candidate genes for this QTL. Further QTL associated with different aspects of tuber carotenoid content, are being studied.

Additional candidate genes for tuber carotenoid content have been identified in a series of microarray experiments, analysing samples bulked according to individual carotenoid traits. Transgenic potato plants have been developed silencing one of these candidate genes encoding a carotenoid cleavage dioxygenase (*CCD4*), resulting in increased levels of tuber carotenoids and unexpected effects on tuber morphology that mimic a heat sprouting phenotype (Campbell et al., 2010). Another carotenoid cleavage dioxygenases (*CCD8*) was also silenced resulting in major effects on stolon and tuber development.

A key issue in understanding tuber carotenoid accumulation is the nature of the organelle in which carotenoids accumulate. To address this, transgenic lines in which carotenoid synthesis related enzymes have been tagged with RFP have been developed. The localisation of the carotenoid biosynthetic enzymes was revealed by this analysis, with different locations for phytoene synthase and β-carotene hydroxylase.

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The revised carotenoid biosynthetic pathway in plants and discoveries for obtaining sustainable agricultural solutions to global vitamin A deficiency

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Development of cereal crops with increased provitamin A carotenoids could provide a sustainable solution to eliminating the global health problem of vitamin A deficiency. Maize is a major staple carbohydrate source and an important model for

studying carotenogenesis in cereals. Using the maize carotenoid mutant collection, our laboratory discovered 15-*cis* zeta-carotene isomerase (Z-ISO), a new carotenoid biosynthetic pathway enzyme needed in all plants [1]. Z-ISO is especially needed in absence of light, such as in roots and endosperm, the target tissue for provitamin A biofortification. For other pathway enzymes, we used bioinformatics and cloning to identify genes encoding enzymes for biosynthesis and degradation of carotenoids in maize and related grasses. We found extensive gene duplication that is conserved in the grasses. We have been investigating the role of gene family members in contributing to carotenogenesis. To assess roles in endosperm, we took advantage of the extensive maize germplasm collection. We compared endosperm transcript profiles with carotenoid content and composition. Thus we elucidated pathway control points and identified natural alleles for breeding higher levels of endosperm provitamin A carotenoids. Building on earlier collaborative studies, we discovered maize *HYD3* encoding a non heme di-iron beta-carotene hydroxylase, as the partner locus needed for controlling endosperm provitamin A content [2]. We also identified new targets for controlling pathway flux that can be used in future efforts to enhance carotenoid levels in endosperm [3,4]. Our ongoing research focuses on regulatory and structural aspects of carotenoid biosynthesis to facilitate predictive metabolic engineering for a sustainable solution to vitamin A deficiency.

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ORAL PRESENTATIONS

An effective in vitro system for the functional characterization of carotenogenic genes in rice (*Oryza sativa*)

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Carotenoids play fundamental roles in human nutrition. The mechanisms that control carotenoid accumulation in plants are complex and only partly understood. The amount of carotenoids in plant tissues and organs do not appear to depend solely on carotenogenic enzyme activities responsible for their synthesis. The upstream precursor (MEP derived IPP and GGPP) pathways may also positively influence their accumulation, while downstream degradation pathways that metabolize carotenoids may deplete the carotenoid pool. Recent studies in our laboratory and elsewhere indicate that an effective strategy to enhance carotenoid accumulation in staple crops requires the simultaneous introduction and coordinated expression of multiple transgenes. A rapid functional expression assay would be very advantageous in assessing the nature and specific combinations of particular transgenes to be used in any given experiment aiming towards maximizing carotenoid accumulation reliable, inexpensive and conclusive. We present and discuss recent results from experiments utilizing combinations of transgenes involved in carotenogenesis in stably engineered rice cell lines which exemplify the utility of such in vitro cell assays to validate the function of multiple carotenogenic genes.

Microarray analysis of gene expression changes associated with the accumulation of carotenoid pigments in the storage root of carrot (*Daucus carota*)

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Carrot (*Daucus carota* var. *sativus* L.) provides more than 30% of the pro-vitamin A carotenoid pigments the US alone, making research into the accumulation of these pigments in carrot important. The storage root of orange carrot contains α-and β-carotene, but available carrot germplasm also accumulates high concentrations of other carotenoid pigments such as lycopene and lutein,

making carrot an ideal model for the study of carotenoid biosynthesis. With whole genome sequence available for many plant species, the study of gene expression has moved to large scale whole genome analyses to compliment a gene by gene approach. This project followed that trend by evaluating whole genome changes in expression using the Affymetrix Medicago GeneChip microarray. This project used sibling inbred lines derived from the well characterized mapping population B493 X QAL, a cross between an USDA inbred line and Queen Anne's Lace, or wild carrot. These materials represent the extremes of the carotenoid accumulating phenotypes, in a common genetic background. This method identified specific changes in gene expression during the accumulation of carotenoid pigments, and more importantly identifies genes that have not been previously researched using candidate gene approaches. Real time quantitative PCR (RT-qPCR) was be used to verify the genes determined to be differentially expressed by microarray analysis.

Carotenoid accumulation in roots: same target – different principles

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Phytoene synthase has frequently been reported as the rate-limiting step governing the flux through the carotenoid biosynthetic pathway in higher plants. Accordingly, increased carotenoid accumulation often correlates with high PSY transcript levels or can be achieved by PSY overexpression. However, we have found cases where this correlation did not hold suggesting the presence of different mechanisms.

For instance, naturally occurring root color variants of cassava are not caused by high PSY protein or transcript levels. Instead, a single nucleotide polymorphism within a PSY gene resulting in a nonsynonymous amino acid exchange within a highly conserved region was decisive (Welsch et al., 2010). By in vivo and in vitro approaches we confirmed the activity-enhancing impact of this modification thus explaining higher carotenoid levels by increased enzymatic effectiveness. This finding provides means for the generation of provitamin A-enriched cassava roots both through transgenic as well as through breeding approaches.

Orange carrot roots contain high PSY levels in contrast to white (wild-type) genotypes. However, this difference is not reflected by equivalently different PSY transcript levels (Maass et al., 2009) suggesting the involvement of posttranscriptional mechanisms. A novel feedback loop impacting PSY protein but not RNA levels is proposed that originates from carotenoids downstream of β-carotene.

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Regulation of carotenoid metabolism and colour diversity in *Citrus* fruits

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Citrus is the first fruit-tree crop in the world and the massive world consumption of both fresh fruit and processed juice made of this crop an important source of carotenoids for human nutrition and health. Fruits of the different *Citrus* species and varieties exhibit a high diversity of colours, ranging from the yellow of grapefruit and lemon, to the typical colour of orange and mandarins, with some red and pink coloured grapefruit and orange mutants either in the peel or pulp. Colour singularities in citrus fruits are due to a complex accumulation of carotenoids but the mechanisms involved in their biosynthesis are starting to be elucidated. Therefore, understanding the regulation of carotenoid biosynthesis and catabolism are important challenges to decipher colour singularities in *Citrus* fruit and also for future biotechnological applications to improve fruit colour and nutritional status. In this communication we will summarise recent breakthroughs into molecular mechanisms regulating carotenoid metabolism in fruits of different *Citrus* species and mutants and how they may be related to the specific carotenoid complement. Gene expression analysis of carotenoid biosynthetic genes in the peel and pulp of orange-coloured fruits revealed that phytoene synthase (*PSY*), a chromoplast-specific β-lycopene-cyclase (*βLYC*) and β-carotene hydroxylase (*βCHX*) appears to be key regulatory steps of the pathway with good correlation with carotenoid content and composition. However, the relative balance between the expression of upstream genes in the pathway and that of *βCHX* is also an important factor determining accumulation of intermediate xanthophylls, such as β-cryptoxanthin. We have also investigated the mechanism of lycopene accumulation in orange and grapefruit mutants and the overall results indicate that even *βLYC* is a critical step in the pathway additional processes may be affected to lead lycopene accumulation in such mutants. Moreover, accumulation of C30 apocarotenoids, such as β-citraurin or 8-β-apocarotenal which impart an attractive reddish colour to the fruit peel, is a specific feature of citrus fruit. Progress in the isolation and characterization of novel carotenoid-cleavage dioxygenases (*CCD*s) genes involved in the synthesis of these apocarotenoids as well as potential regulatory mechanisms will be presented and discussed.

POSTERS

6.1.

β-carotene cleavage in *Blakeslea trispora* and *Phycomyces blakesleanus*

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Mixed cultures of strains of opposite sex ("mated cultures") of *Blakeslea trispora* and *Phycomyces blakesleeanus* (Mucoromycotina, Mucorales) may be used as industrial sources of β-carotene. These cultures produce apocarotenoids that induce an increased β-carotene content and the early morphogenetic processes of the sexual cycle.

A new family of apocarotenoids with 7 carbons has been identified in *Phycomyces* (2 compounds) (Polaino et al., 2010) and *Blakeslea* (the same and three others). These new molecules represent the missing link that proves that β-carotene is split into fragments of 18, 15 and 7 carbons, heads of three separate families of apocarotenoids.

The cocktail of apocarotenoids in Mucorales varies not only between species, but between strains. Thus, the mated cultures of *Blakeslea*, wild-type strains F921 and F986, contain eleven apocarotenoids: two C_{18}, two C_{15}, a C_{13} and five C7 compounds. Six of them are new natural products and two are new for *Blakeslea*, while 14 other apocarotenoids reported from other strains of *Blakeslea* have not been found now.

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6.2.

β-carotene enhancement in orange fruits

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Citrus is a very important fruit crop throughout the world not only because of its economic significance but also for its great value for human nutrition and well-being. Complex and heterogeneous accumulation of carotenoids accounts for the typical colouration of the peel and pulp of most citrus fruits, thus influencing the commercial and visual quality of the fruits. Orange fruit is an excellent natural dietary source providing health-promoting compounds such as vitamin C, flavonoids and folic acid in a combination and concentration unique among fruits and vegetables, but it has low provitamin A content. The main carotenoid with provitamin A activity is β-carotene, a compound hardly detectable in the pulp of most oranges. The most abundant/important provitamin-A compound in oranges is -cryptoxanthin, which has half provitamin A activity than β-carotene. However, this carotenoid is usual in mandarins but relatively scarce or absent in most orange cultivars.

With the aim of increasing orange fruit nutritional value, we have attempted to enhance its content in β-carotene (provitamin A) by metabolic engineering of carotenoid biosynthesis. Recent advances in the identification and isolation of the genes responsible for the carotenogenesis in citrus fruits combined with the availability of genetic engineering tools have made feasible a metabolic engineering approach to improve the content and composition of certain carotenoids in oranges. In this work, we blocked the expression of the endogenous β-carotene hydroxylase gene (*β-CHX*), involved in the conversion of β-carotene into xanthophylls, using RNA interference (RNAi) technology. Transgenic plants were obtained that showed important changes in carotenoid content and composition in both fruit peel and pulp, with β-carotene increases accompanied by a general decrease in the accumulation of downstream xanthophylls and enhanced production of flavor-related apocarotenals. The implications of these changes in nutritional value and volatile composition of transgenic oranges will be discussed.

6.3.

Studies on carotenoids and their volatile apocarotenoid products in flowers of *Osmanthus fragrans*

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The unique scent of flowers of *Osmanthus* fragrans is dominated by carotenoid-derived constituents. In essential oil nearly 100 flavor compounds originated from carotenoids have been identified including β-ionone, α-ionone, dihydro-β-ionone, oxo-β-ionone, and hydroxy-β-ionone (Kaiser, 2002). The essential oil contains up to 19.5% of β-ionone and 11.7% β-ionone (Wang et al., 2009).

We report the contribution of *Osmanthus* carotenoid cleavage enzymes in the bio-generation of β-ionone and α-ionone. cDNAs encoding carotenoid cleavage dioxygenases (CCDs) were identified based on conserved CCD sequences. To elucidate whether the sequences encode functional CCDs cDNAs were transferred into gluthathione fusion vectors for expression in *E-coli* and the recombinant enzymes purified prior functional analysis. The relation of carotenoids, volatile emission, and CCD transcripts was investigated by determination of their changes over the floral development and photo-rhythmic periods. Our results indicate that OfCCD1 is likely involved in the C_{13}-apocarotenoid biogenesis in flowers of Osmanthus fragrans. The volatile reaction products, β-ionone and α-ionone, have very low odor perception thresholds for humans and exhibit a strong impact on floral scents. By sensory evaluation we confirmed the impact of both C_{13}-apocarotenoids on the changes in scent perception of flowers of *Osmanthus fragrans* in the course of the day (Baldermann et al., 2010).

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6.4.

Diversity of carotenoid composition in carrot gene bank collection

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The subset of carrot (*Daucus carota* L.) gene bank collection comprising 85 accessions and including commercial cultivars, old varieties, landraces and two wild carrot relatives was used for the assessment of biodiversity related to carotenoid content. The whole marketable roots of field grown plants were sampled and homogenized, and then carotenoids were extracted in acetone containing 0.1% *t*-butylated hydroxytoluene. Detection of carotenoids was done after HPLC separation in a Nucleosil 100 C18 column using a diode array detector. Luteine, lycopene, α- and β-carotenes were detected at 454 nm, whereas phytoene was detected at 296 nm.

The total carotenoid content calculated as the sum of the four main carrot compounds lycopene, lutein, α- and β-carotenes varied among the accessions and corresponded to their root colour. Carrots developing white roots and two wild carrot relatives were usually free of carotenoids. The remaining accessions accumulated carotenoids up to 42 mg/100 g FW, but such high level was observed in orange roots only. High carotenoid content was mainly caused by the presence of β-carotene followed by α-carotene. Lutein was important component of total carotenoids in roots that were rather poor in those compounds; lycopene was detected in only two accessions. Some accessions developing purple roots also contained considerable amounts of carotenoids. The carotenoid precursor phytoene was not found in 31 accessions, which roots were white. Only one accession developing white roots and with a low carotenoid content possessed high phytoene level. This indicates that in carrot roots the carotenoid biosynthetic pathway may be blocked at different steps.

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6.5.

Identification of gene *carD*, encoding the aldehyde dehydrogenase responsible for neurosporaxanthin biosynthesis in *Fusarium fujikuroi*

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The fungus *Fusarium fujikuroi*, well known for its ability to produce gibberellins, is also a model for genetic and biochemical analysis of carotenoid biosynthesis. Its major carotenoid product is neurosporaxanthin (NX), an acidic apocarotenoid formerly discovered in *N. crassa*. NX is produced in *F. fujikuroi* through the activity of the enzymes encoded by genes carRA (cyclase and phytoene synthase), *carB* (phytoene desaturase), and *carT* (torulene cleaving oxygenase). The enzyme responsible for the last reaction of the pathway, the oxidation of the aldehyde group of beta apo 4´ carotenal to yield NX, has not been described in this fungus. Based on our former results with *ylo-1* in *N. crassa*, we have identified the F. fujikuroi gene carD, coding for an aldehyde dehydrogenase putatively responsible of this enzymatic reaction. In contrast to other *car* genes of *F. fujikuroi*, RT-PCR analyses of carD mRNA levels showed a light-independent expression. However, the mRNA levels were increased in carotenoid overproducing mutants. Crude protein extracts from an *E. coli* strain expressing a *carD* cDNA version were able to convert beta apo 4´ carotenal into the corresponding apocarotenoic acid, confirming the expected enzymatic activity. CarD was also active on shorter carotenoids, including acyclic ones, such as 8'-lycopenal, indicating the irrelevance of the cycled end of the molecule for substrate recognition. Also, we expressed the enzyme in a beta-apo-4'-carotenal producing *E. coli* strain and we got NX production in vivo. Finally, targeted *ΔcarD* mutants, obtained by gene replacement with a hygromycin resistant cassette, accumulated beta-4'-apo-carotenal instead of NX. Unexpectedly, beta-4'-apo-carotenal was reduced into beta-4'-apo-carotenol along incubation time, conferring a yellowish pigmentation to aged *ΔcarD* colonies.

6.6.

The effects of formulated carotenoid diets on New Zealand sea urchin (*Evechinus chloroticus*) roe pigmentation

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Sea urchin roe is a highly valued product in the international marketplace. New Zealand's coastal zones have significant stocks of sea urchin (*Evechinus chloroticus*; local name Kina), but quality issues including colour variation and limited export opportunities.

Kina roe varies from a desirable (bright yellow to orange red) to an undesirable (brown or black) coloration. Understanding Kina carotenoid metabolism and developing a modified

carotenoid diet to control roe pigmentation will help establish a Kina roe export industry for New Zealand.

The current belief is that carotenoid compounds are responsible for sea urchin roe pigmentation. Sea urchins cannot synthesise carotenoids *de novo*, and are therefore dependent upon dietary carotenoid intake, which may be assimilated selectively or transformed to other forms.

Comparison of the carotenoid profiles extracted from Kina tissues revealed that echinenone was the predominant carotenoid in both light and dark pigmented roe. There were also increased amounts fucoxanthin, astaxanthin and β-carotene being detected in dark brown roe compared to light coloured roe. The predominant carotenoids present in the gut wall were fucoxanthin, violaxanthin and echinenone.

The source of echinenone in Kina roe is uncertain, as it is not present in the natural diet of Kina, *Macrocystis pyrifera* (kelp), which instead contains mainly fucoxanthin with minor amounts of β-carotene and violaxanthin. We suggest here that echinenone is generated from available β-carotene found at low levels in kelp. We also suggest that the gut is therefore directly involved in the metabolism of carotenoid compounds and that separate carotenoid modification events are occurring in different tissues.

A four-month Kina diet trial was carried out with specially formulated diets to evaluate the effect of increasing or decreasing beta-carotene dietary levels on Kina roe colour. We found that by modifying the carotenoid content in the diet, it was possible to manipulate roe colouration and potentially assist in the development of a Kina export market for New Zealand.

6.7.

Rapid positive selection of carotenoids-overproducing mutants of the yeast *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) using mixture of heavy metals as a selective agent

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The yeast *Phaffia rhodozyma* is the producer of carotenoids, such as carotenes and xanthophylls. Biomass of this yeast can be strategic source of compounds with a high biological activity. The wild strains produce carotenoids in the level of about 0.3 mg/g, among them 80% is related with antioxidant astaxanthin (Goswami G. et al., 2010). The mutant strains were isolated with genetic blocks in synthesis of some astaxanthin precursors (Gural et al., 2011). We propose to select the mutants with higher levels of carotenogenesis using a simple and rapid procedure based on positive selection on a medium containing heavy metals. For mutagenesis, ultraviolet irradiations, as well as nitrosoguanidine were used. As the selective factors, compositions of mixtures of heavy metals salts (Cu^{2+}, Zn^{2+}, Co^{2+}, Pb^{2+}) and (Cu^{2+}, Zn^{2+}, Co^{2+}, Pb^{2+} and Cd^{2+}) were used in different concentrations (Iutynska et al., 2000, Bhosale P., 2004). The survival rate as a function of the dose and metals concentration has been estimated. Carotenoids' content in the most productive mutants has been analyzed.

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6.8.

Carotenoid composition in native and commercial potato (*Solanum* sp.) cultivars

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The genus *Solanum* comprises more than 2000 species, being the potatoes (*S. tuberosum* L.) the most important, with more than 4000 potato varieties world while, most of them developed through man selection. Potato is, after cereals, the most important staple food for the human population, being considered an important source of carbohydrates and other nutrients such as vitamin C. In the last ten years, an increasing interest has been raised on the study of potatoes as a good source for phytonutrients such as carotenoids and anthocyanins. For this reason, several breeding programmes are being conducted with the aim of increasing the carotenoid and/or anthocyanin contents in potatoes, and as a result a particular attention has been paid to the characterization of the old and native cultivars as a source of genetic variability for increasing these traits, as well as to help in the understanding of the genetic and molecular basis governing their biosynthesis. In the present study we have characterised the carotenoid composition of 60 potato cultivars (commercial, bred, old and native cultivars) belonging most of them to the species *S. tuberosum* subsp. *tuberosum* and *S. tuberosum* subsp. *andigena*, and a few to the species *S. goniocalix* and *S. stenotonum*. The analysis of the carotenoid profiles allowed the cultivars to be segregated into three groups according to the presence of violaxanthin, lutein and neoxanthin as main pigment, suggesting a different control of the biosynthetic pathway in the three groups. In addition, a direct correlation was found between the total carotenoid content and the concentration of the main pigment (namely violaxanthin, lutein and neoxanthin), corresponding the varieties with higher carotenoid content to those having violaxanthin as main pigment. The presence of xanthophyll esters has also been investigated, with a direct correlation being observed for the carotenoid content and the fraction of carotenoids being esterified, which is in accordance with the idea that the esterification process allows the plants to accumulate these lipophyllic compounds within the plastids. Therefore, the presence of xanthophyll esters should be phenotypic character to be included in the breeding studies, and more efforts should be dedicated to the understanding of the biochemical process leading to this structural modification of carotenoids in plants.

6.9.

Modification of carotenoid pigments in *Iris germanica* L. by overexpression of phytoene synthase gene (*crtB*) from *Pantoea agglomerans*

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Iris germanica L. is one of the most horticulturally important, tall, bearded irises. Flower color in irises is determined by two distinct biochemical pathways. The carotenoid pathway imparts yellow, orange and pink while anthocyanin pathway produces blue, violet and purple flowers. Stoppage in one of the early steps in either of two biosynthetic pathways is probably responsible for white flowers. Naturally, there are no truly red iris flowers and conventional breeding methods have so far failed to generate them. Genetic engineering provides a means of introducing new traits into iris by expanding the gene pool beyond what has been available in iris genome. With a goal of developing red iris flowers, we transformed the pink iris cultivar 'Fire Bride' with a bacterial phytoene synthase gene (*crtB*) from *Pantoea agglomerans*, fused to Rubisco small subunit transit peptide from tobacco, and under the control of several different promoters (pCaMV35S, E35S-PchsA, AP3 and Llccs). This approach aimed to increase the flow of metabolites into the carotenoid pathway that would ultimately lead to the elevated levels of lycopene and a "deeper" red color. Overexpression of the *crtB* in iris cv 'Fire Bride' produced a color change in callus tissue from yellow to orange-red and red. Carotenoid analysis by HPLC confirmed that the major red pigment in the transgenic callus was lycopene. Transgenic plants were regenerated successfully from calli transformed with *crtB* driven by *AP3* and *Llccs* promoters and grown to maturity. In both cases, the most prominent color changes occurred in ovaries and anthers. Naturally green ovaries turned deep orange and snow-white anthers turned pink. Unexpectedly however, the intensity of pink color in flowers of both types of transgenic plants decreased compared to wild type. Future studies will focus on determining tissue specific expression of *crtB* transgene under *AP3* and *Llccs* promoters by RT-PCR and correlating expression levels to qualitative and quantitative changes of carotenoid pigments.

6.10.

Structural and biochemical analyses of the carotenoid biosynthesis enzymes CRTI and CRTISO

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The plant enzyme carotene isomerase (CRTISO) and the bacterial phytoene desaturase (CRTI) have structural similarities in size and amino acid sequence. CRTI catalyzes four desaturation steps along with *cis* to *trans* isomerization of the polyene chain, while converting phytoene into all-*trans* lycopene. In contrast, CRTISO only catalyses the *cis* to *trans* isomerization of *cis*-neurosporene and *cis*-lycopene. Our study is aiming at discovering the structural features in these enzymes that determine their different functions. In the absence of crystallographic three-dimensional structures, we have introduced specific mutations in these enzymes in order to unravel amino acid residues that are associated with specific catalytic functions. Chimeric polypeptides of CRTISO and CRTI were tested in *E. coli* for desaturation and isomerization activities. Our results indicate specific domains in each of the enzymes that are imperative for their activities. A domain of 44 amino acids near the FAD-binding motif in CRTI was tested with specific amino acid substitutions. Several amino acids within this domain were demonstrated crucial for the catalytic activity of the enzyme. Substitution of these residues with those that occur in the same position in CRTISO abolished or reduced enzyme activity and exposed cis-ζ-carotene and trans-neurosporene as intermediates in phytoene desaturation by the mutated CRTI.

6.11.

An EPR study of thylakoid membrane dynamics in mutants of the carotenoid biosynthesis pathway of *Synechocystis* sp. PCC6803

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Thylakoid membranes are the site of light-dependent reactions of photosynthesis in plants and cyanobacteria.The photosynthetic activity has been found to be significantly altered in *Synechocystis* mutants blocked at different steps of carotenoid biosynthesis.

The rigidity of thylakoid membranes isolated both from wild type Synechocystis and from mutants in genes encoding acyl-lipid desaturases and/or selected enzymes of carotenoid biosynthesis pathway, was studied by EPR spectroscopy using 5-SASL and 16-SASL spin probes. Cyanobacteria were cultivated at 25°C and 35°C at different light regimes: photoautotrophically (PAG) and/or in light-activated heterotrophic conditions (LAHG).

RO mutant is not able to synthesize xanthophylls because of disruption of genes encoding β-carotene hydroxylase CrtR and β-carotene ketolase CrtO. ROAD mutant has the same characteristic with additional disruption of genes encoding acyl-lipid desaturases A and D responsible for introducing double bonds in Δ12 and Δ6 positions of C_{18} fatty acids [1]. ΔcrtH mutant is not able to synthesize *cis* to *trans* carotene isomerase. This mutant cultivated under LAHG conditions produces *cis*-carotenes and small amounts of *trans*-carotenes but no xanthophylls, whereas under PAG conditions is not distinguishable from the wild type [2]. ΔcrtH/B mutant is carotenoidless mutant with a disruption in crtB gene encoding phytoene synthase, in ΔcrtH background [3].

EPR spectra of spin labeled thylakoid membranes were recorded at temperature range from 5°C to 65°C. Order parameters and rotational correlation times were calculated from the spectra.

The values of the measured parameters obtained for membranes from all analyzed mutants were not significantly different in comparison to the wild type *Synechocystis*. These results suggest that the changes both in carotenoid composition and in the content of unsaturated glycerolipids have not a substantial effect on the rigidity of thylakoid membranes in cyanobacteria under both LAHG and PAG culture conditions.

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6.12.

Activation by light of carotenoid biosynthesis in the fungus *Neurospora crassa*

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The ascomycete fungus *Neurospora crassa* accumulates the carotenoid neurosporaxathin in vegetative mycelia after exposure to blue light, presumably to protect the cell from excessive light. Blue light is perceived by the White Collar Complex (WCC), a photoreceptor and transcription factor complex that binds the promoters of light-regulated genes to activate transcription. The WCC is composed of the blue-light photoreceptor WC-1 and its partner WC-2. Additional photoreceptors have been characterized in *Neurospora crassa*. These include two red-light photoreceptors (the phytochromes PHY-1 and PHY-2), a blue-light photoreceptor (the cryptochrome CRY-1), and the rhodopsin NOP-1. The WCC is the main photoreceptor in *Neurospora* as wc mutants are defective in all the responses to light. The other photoreceptors should play secondary roles in *Neurospora* photoreception as deletion of the corresponding genes does not impair fungal vision.

Mycelia of the wild-type strain of *N. crassa* accumulates 143 μg/g dry mass of carotenoids after one day of light exposure, compared to 3 μg/g dry mass of carotenoids in mycelia kept in the dark. The accumulation of carotenoids after one day of light in the photoreceptor mutants was similar to that in the wild-type strain, with the exception of *wc-1* or *wc-2* mutants that failed to accumulate any carotenoids, as expected. A clear reduction in the amount of carotenoids accumulated after light exposure was observed in a strain with a mutation in the *ve-1* gene, a homolog of a repressor of photomorphogenesis in the fungus *Aspergillus nidulans*. Our results confirmed the secondary role for the *Neurospora* photoreceptors in the activation by light of carotenoid accumulation.

The activation of carotenoid accumulation by light is a complex response. Carotenoid accumulation is observed with light intensities higher than 0.1 J/m^2 but the response saturates and increases again after exposing mycelia to light of 100 J/m^2. The presence of two components in photocarotenogenesis suggested the action of additional photoreceptors optimized to operate at different light intensities. We have assayed the presence of the two components in the photocarotenogenesis of the photoreceptor mutants of *N. crassa*. Our results show that the secondary photoreceptors play a key role in the reception of low-intensity light in combination with the WCC.

6.13.

Purification of biologically active violaxanthin de-epoxidase from *Escherichia coli*

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Violaxanthin de-epoxidase (VDE) is one of two enzymes of the xanthophyll cycle, an important and widely distributed photoprotective mechanism in plants. VDE catalyzes de-epoxidation of violaxanthin (Vx) to zeaxanthin (Zx) via the intermediate antheraxanthin (Ax).

Traditionally, VDE is isolated mainly from plants thylakoids. However, plants have low contents of this enzyme and therefore a lot of plant materials is needed for VDE isolation.

Moreover, the existing isolation procedures are not satisfactory since the amount of the obtained enzyme and the level of its purity are low.

In the presented studies isolation and purification of VDE from transgenic *E coli* strain C43 have been developed. *E. coli* was transformed by the gene of VDE from *Arabidopsis thaliana* tagged with 6xHis. After induction, VDE gene expression resulted in high concentration of the enzyme as evidenced by SDS-PAGE and Western blot analysis.

Purification of VDE has been performed using Ni-NTA Affinity Resin. Analysis by SDS-PAGE indicated presence of VDE both in washed and eluted fractions. In the eluted fraction concentration of VDE was lower, but purity of sample was the highest.

Purified enzyme has been used to catalyze conversion of Vx to Zx in the in vitro system. Biological activity VDE was tested by HPLC-method. The de-epoxidation of Vx and Ax, catalyzed by obtained enzyme was observed both for enzyme with 6xHis tag and after its removal by thrombin digestion.

6.14.

A search for carotenoid-binding proteins in the New Zealand sea urchin *Evechinus chloroticus* (Kina)

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The sea urchin *Evechinus chloroticus*, locally known as Kina, is endemic to costal New Zealand. Kina are harvested commercially as their edible roe (gonad) is considered a delicacy on both local and international markets. The revenue attained by roe on the market is in proportion to the desirability of pigmentation, which appears to be due to varying quantities of carotenoids chemically modified in and transported from the viscera via protein interactions [1].

Carotenoid-binding proteins are likely to have an important role in the pigmentation of sea urchin roe. In addition to acting as metabolic enzymes, carotenoid-binding proteins may have a variety of important roles, including; cell surface receptors, transmembrane transporters and stabilisation of carotenoids deposit-

ed in tissue [2]. Carotenoid-binding proteins have been described extensively in plants and bacteria, however much less is known about their roles in invertebrates and vertebrates.

A method is being developed for the purification of carotenoid-binding proteins from the roe of *E. chloroticus*. Density-adjusted ultra-centrifugation is used to separate the roe homogenate into a low-density lipid-rich fraction and a clarified solution. In addition to large quantities of protein, both fractions contain significant amounts of the major roe carotenoid echinenone and are being investigated for the presence of carotenoid-binding proteins. It is hypothesised that the lipid fraction may contain lipoproteins that bind non-specifically to large numbers of carotenoid molecules. The solution fraction is more likely to contain small soluble carotenoid-binding proteins of the lipocalin type, in which the interaction between protein and carotenoid is both specific and stoichiometric.

The techniques of solution phase iso-electric focusing and ion exchange chromatography have been investigated as purification procedures and have proved promising in co-purifying carotenoid and protein. The next phase of the procedure will utilise size-exclusion chromatography to increase the purity of carotenoid-binding proteins. As a final purification step, an immobilised carotenoid affinity chromatography column [3] will be developed, to selectively purify carotenoid-binding proteins. It is then hoped that the isolated proteins will be analysed and identified by MALDI TOF/TOF mass spectrometry and structures will be elucidated by crystallographic techniques.

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6.15.

Geranylgeranyl diphosphate synthase isoforms involved in carotenoid biosynthesis in *Arabidopsis thaliana*

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Geranylgeranyl diphosphate (GGPP) is the precursor for the biosynthesis of carotenoids and other important plant isoprenoids such as gibberellins and the side chain of chlorophylls, tocopherols, and isoprenoid quinones. GGPP is produced by the enzyme GGPP synthase (GGDS), encoded by ten genes in *Arabidopsis thaliana*. We hypothesize that different GGDS isozymes might be involved in the production of specific isoprenoids, including carotenoids, by channeling GGPP to their corresponding biosynthetic pathways. Carotenoids are synthesized in plastids and they preferentially accumulate in photosynthetic tissues of *Arabidopsis*. Based on these features and on the role of carotenoids as photoprotectants, GGDS isoforms involved in their production are expected to be targeted to plastids, highly expressed in green tissues, and light-induced. Among the seven *Arabidopsis* genes encoding true GGDS enzymes with a predicted plastid-targeting peptide, only At4g36810 (herein referred to as GGDS1) met the expression criteria. The total loss of GGDS1 function is lethal, but we have identified a mutant (*ggds1-1*) in which a 40% decrease in the expression of *GGDS1* results in smaller plants with a significant reduction in carotenoid levels. These results suggested that GGDS1 is the main (or only) isoform involved in carotenoid biosynthesis. Alternatively, the expression patterns of other GGDS isoforms might not be appropriate to rescue the loss of GGDS1 in the mutants. To test this latter possibility we have generated constructs for expression of several plastid-targeted active GGDS isoforms under the control of the *GGDS1* promoter or a constitutive promoter (*35S*). GFP fusions will also be expressed for complementation experiments. Lines complementing the *ggds1-1* mutant with *GGDS1:GGDS1*, *35S:GGDS1* and *35S:GGDS1-GFP* constructs are already available, and immunoprecipitation experiments using those overexpressing GGDS1-GFP are in progress to identify proteins that interact with GGDS1 in in vivo complexes. It is expected that we will find enzymes of the carotenoid pathway among them. Similar experiments using lines overexpressing other GFP-tagged GGDS isoforms should provide useful information on whether protein partners are shared among different GGDS-containing complexes. Together, these experiments should serve to verify what GGPP isoforms are specifically involved in the production of carotenoids, which would optimize future biotechnological strategies aimed at obtaining new plant varieties rich in these compounds. Our latest progress along these lines will be presented.

6.16.

α-Carotene and its derivatives have a sole chirality in phototrophic organisms?

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Eukaryotic phototrophic organisms necessarily synthesize not only chlorophylls but also some carotenoids, which can be divided into two groups; β-carotene and its derivatives (zeaxanthin, violaxanthin, neoxanthin, fucoxanthin, peridinin, diadinoxanthin, etc.), and α-carotene and its derivatives (lutein, loroxanthin, siphonaxanthin, prasinoxanthin, etc.). Distribution of α-carotene and its derivatives is reported to be limited in some taxonomic groups of phototrophic organisms. In addition, only (6'*R*)-type of α-carotene and its derivatives have been reported from algae and land plants, although C-6' in α-carotene, between ε-end group and conjugated double bonds, is chiral, (6'*R*)- and (6'*S*)-types. To confirm the reliability of chirality, we re-examined distribution of α-carotene and its derivatives, and analyzed their C-6' chirality using CD or NMR after purification of the carotenoids.

We found α-carotene and/or its derivatives from Rhodophyceae (macrophytic group), Cryptophyceae, Euglenophyceae, Chlorarachniophyceae, Prasinophyceae, Chlorophyceae, Ulvophyceae, Trevouxiophyceae, Charophyceae, and land plants, while we could not detected them from Glaucophyceae, Rhodophyceae (unicellular group), Chryosophyceae, Raphidophyceae, Bacillariophyceae, Phaeophyceae, Xanthophyceae, Eustigmatophyceae, Haptophyceae, and Dinophyceae. In addition, loroxanthin and siphonaxanthin, which are synthesized from lutein, were found from Euglenophyceae, Chlorarachniophyceae, Prasinophyceae, Chlorophyceae, and Ulvophyceae. We analyzed chirality of α-carotene and/or its derivatives from around 40 species described above, and found they had only (6'*R*)-type.

In biosynthesis of α-carotene in land plants, both lycopene β-cyclase and lycopene ε-cyclase are needed to produce α-carotene from lycopene. They have high homology with each other, and therefore lycopene ε-cyclase gene might be produced by duplication of lycopene β-cyclase gene. In enzymatic reaction of cyclization, the mechanisms of lycopene β-cyclase, lycopene (6'*R*)-ε-cyclase, and lycopene (6'S)-ε-cyclase are almost the same; the products are depending on the carbon number to eliminate H^+ and on the direction of elimination. Therefore, both lycopene ε-cyclases could be exist, but only lycopene (6'*R*)-ε-cyclase was found based on the presence of only (6'*R*)-type. Since the stereochemistry of (6'*R*)- and (6'S)-α-carotenes are different for the direction of ε-end groups, the binding site on the protein should not be identical. Consequently, the protein moiety might restrict to one chirality of α-carotene, (6'*R*)-α-carotene.

6.17.

Specific non-natural C_{50} carotenoid pathways constructed by the combinatorial expression of laboratory-evolved enzymes

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Rapid expansion of available carotenogenic genes has enabled to produce various carotenoids in heterologous systems. In addition, non-natural carotenoids can be also produced using combinatorial expression of laboratory-evolved carotenogenic enzymes in heterologous hosts of *Escherichia coli* (Lee et al., 2003; Umeno et al., 2005). However, carotenogenic enzymes are in general promiscuous, and it is especially so for the laboratory-evolved ones. Thus, mere combination of these enzymes results in the complex mixture of carotenoids containing only a minor amount of the target. Here, using our C_{50} and C_{35} carotenogenic pathways as a model system, we explored how pathway engineers can systematically 'evolve' the specificity of the artificial carotenogenic pathways. It includes: (1) creation of geranylfarnesyl diphosphate (C_{25}PP) synthase from geranylgeranyl diphosphate (C_{20}PP) synthase CrtE, (2) development of the size mutant of diapophytoene synthase CrtM, (3) combinatorial expression of above mentioned mutants for efficient and selective production of C_{50} backbone carotenoids, and (4) desaturation of C_{50} carotenoids with the mutant of phytoene desaturase CrtI. Our current effort to further diversifying this C_{50} pathway, together with extensive analytical effort, will be also presented.

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6.18.

Unique carotenoid lactoside, P457, in *Symbiodinium* sp. of dinoflagellate

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The dinoflagellates are a large group of unicellular protists common mostly in marine and also in fresh water environments. They are characterized by possession of two types of flagellum with a different direction. About half of dinoflagellates are photosynthetic, and some of them are known as an endosymbiont of various kinds of marine animals, such as scleractinian corals, sea cucumbers, and sea anemones. Symbiotic dinoflagellates are called zooxanthellae. Another feature of photosynthetic dinoflagellates is that they have peridinin, a light-harvesting carotenoid in photosynthesis. In addition to peridinin, an unique carotenoid, P457, was first descrived by Jeffery in 1968as highlypolar pink-orange carotenoid from *Amphinidium*. P457 was also found in photosynthetic dinoflagellates including zooxanthellae. Its structure from *Amphinidium* was determined by Liaaen-Jense group (1993); neoxanthin-like with an aldehyde group and a lactoside.

Presence of P457 in *Symbiodinium* derived from marine animals was not reported. In this study, we reconfirmed the molecular structure of P457 from *Symbiodinium* sp. NBRC104787, isolated from sea anemone, using spectroscopic methods including CD, FD-MS, and ^{1}H-NMR. In addition, we investigated the distribution of P457 in various *Symbiodinium* sp. and scleractinian coral species, and possible biosynthetic pathways of carotenoids including P457 are proposed.

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6.19.

Engineering ketocarotenoid biosynthesis in maize endosperm

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Astaxanthin is a highly valued ketocarotenoid with applications in the nutraceutical, cosmetic, food, and animal feed industries. It is not a typical plant carotenoid although its precursors β-carotene and zeaxanthin are abundant. It is possible to genetically engineer

astaxanthin biosynthesis by engineering plants and plant cells with microbial β-carotene ketolase genes. We had generated previously a diverse population of maize plants in terms of carotenoid profiles. A specific plant lineage was shown to accumulate astaxanthin and other ketocarotenoids; however, astaxanthin accumulation in this specific line was shown to be rather low compared to β-carotene. It has been demonstrated experimentally that the limited astaxanthin accumulation in plants is due to a bottleneck in the conversion of adonixanthin to astaxanthin. This is in agreement with the hypothesis that the low efficiency of β-carotene ketolases in ketolating zeaxanthin to astaxanthin is the major limitation for astaxanthin accumulation in engineered plants.

Our strategy to overcome this bottleneck is to screen for bacterial ketolase genes encoding particular enzymes with preferential substrate specificity for zeaxanthin and iontroduce these into plants. We have identified two promising candidates, the ketolase genes from *Brevundimonas* SD 212 and *Chlamydomonas reinhardtii*, which mediate the formation of substantial amounts of astaxanthin in engineered *E. coli* and transgenic rice callus. A further elements of our strategy to enhance astaxanthin accumulation in maize endosperm is to enhance ketolase versus hydroxylase expression levels in the maize transformants by maize-specific codon modifications of these two transgenes, use of strong endosperm-specific promoters and of a 5'-UTR which is a translational enhancer in monocotyledonous plants.

Session 7

Carotenoids and Vision

INVITED LECTURES

Disturbed accumulation and abnormal distribution of macular pigment in retinal disorders

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Macular pigment (MP) consists of different xanthophylls, lutein and zeaxanthin and meso- zeaxanthin. It is highly accumulated along the axons of the cone photoreceptors in the central retina. The concentration and spatial deposition of MP, which are entirely of dietary origin, vary substantially between normal subjects. In general, MP shows a peak at the foveal centre, that rapidly decreases with eccentricity. However, recently it has been shown that in macular telangiectasia type 2, the MP is reduced within the central retina with a surrounding ring-like structure of preserved MP at about 6 degrees eccentricity. Further, the Sjögren-Larsson syndrome appears to be a disease with a genetically caused deficiency of MP. The mechanism of deposition of MP in the central retina is still unknown. The latter diseases, with their inability to accumulate MP in the central retina, might serve as a model to elucidate the mechanisms of MP deposition in the retina.

Light distributions on the retina: relevance to macular pigment photoprotection

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Purpose: Light exposure has been implicated in age-related macular degeneration (AMD). This study was to measures the cumulative light distribution on the retina over extended periods. Since AMD damage is most pronounced in the macula, we hypothesize that this is where light distributions would peak. If this is correct, macular carotenoids would be ideally located to reduce photooxidative damage.

Methods: An eye-tracker recorded a video of the subject's field of view, superimposed the gaze position, and recorded pupil size. Fifteen na?ve subjects,divided into 3 age groups formed a test group; five subjects formed a control group. In phase 1, subjects viewed photographic images projected on a screen; in phase 2, they observed a PowerPoint consisting of 78 images; in phase 3, they performed arbitrary computer tasks while viewing a monitor; in phase 4, they viewed a projected video; in phase 5, they moved freely around the building. The control group was specifically instructed to gaze at bright features in the field of view and, in a second test, at dark features. All participants in the test group were allowed to gaze freely. Using the subject's gaze coordinates and the corresponding pupil diameter, we calculated the cumulative light distribution over 5 minute periods on a central ~20°(H)×14°(V) area of the retina.

Results: Retinal light distributions were obtained for all 20 subjects. As expected for the control group, cumulative retinal light distributions peaked and dipped in the fovea when the subjects gazed at bright or dark features respectively in the field of view. The distribution maps obtained from the test group showed a consistent tendency to peak in the macula in phase 3, a variable tendency in phases 4 and 5 but no tendency in phases 1 and 2. Age was not a factor.

Conclusions: A tendency for the cumulative light distribution on the retina to peak where macular pigment is most pronounced appears to be a characteristic of some individuals and of certain specific tasks, such as viewing a computer monitor. However at this stage, we have not observed a potential relationship between light distribution on the retina under general viewing conditions and the spatial occurrence of AMD.

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Effects of macular pigment on static and dynamic visual function

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Introduction: It is likely that the mechanisms involved in accumulating retinal lutein (L) and zeaxanthin(Z) (termed macular pigment, MP) evolved outdoors. This talk will present data on the effects of macular pigment on two ecological measures of vision: Visibility: The ability to see distant objects is obscured due to the preferential scatter of short-wave (blue) light in the atmosphere. MP could extend visual range by selective filtering of *blue haze* (the Visibility hypothesis) Visual reaction time (RT): The ability to make a motor response coincide to an object in time and space is an important skill (particularly for athletes). L and Z, in the retina and brain, could improve neural efficiency and speed of processing (the Neural Efficiency hypothesis) thereby improving dynamic visual function.

Methods: 72 young healthy subjects were evaluated. MP optical density (OD) was measured using customized HFP. Visibility was assessed by measuring contrast sensitivity thresholds at 8 cycles/deg using an optical system that passed xenon-light through the sine-wave grating. Blue haze was simulated using an ecologically valid broad-spectrum filter. Coincidence anticipation timing (CAT) was measured using individual white LEDs along a linear 120 LED track that are lit in sequence, creating the appearance of a small, moving light bar. Subjects pressed a button to stop the light bar at a specified point along the track. Bar speed was randomly varied between 5, 10, 15, and 20 MPH. Fixed reaction time was measured by a button press in response to one of the LEDs, repeatedly presented at the same position on the track. Variable reaction time required a button press in response to one of the LEDs presented at a random location along the 120 LED track.

Results: MP was significantly related to contrast sensitivity under blue haze conditions ($p<0.01$). MP was also significantly related to fixed ($p<0.02$) and variable RT ($p<0.01$) and CAT assessed at multiple speeds.

Discussion: Recent published data from our laboratory suggests that the MP carotenoids reduce glare discomfort and disability, shorten photostress recovery times, and enhance chromatic contrast. These data show that MP may increase visual range (how far one can see in the distance). Our past data has also shown that L and Z (likely within the brain) increase temporal processing speeds. These data show that MP is related to psychomotor responses to dynamic visual stimuli. Taken together, these visual function improvements suggest that MP is particularly significant when ecologically valid stimuli are used. Increasing L and Z levels within the retina may be particularly significant to athletes (e.g., baseball players).

Macular pigment changes after cataract surgery with intraocular lens implantation

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Purpose: Macular pigment optical density (MPOD) levels after cataract surgery was compared between eyes with clear intraocular lenses (IOLs) and yellow-tinted IOLs implantation.

Design: Prospective, comparative case series.

Patients and Methods:The data from 259 eyes (clear IOL group, 121 eyes; yellow-tinted IOL group, 138 eyes) of 259 Japanese patients who met the inclusion criteria, i.e., a postoperative visual acuity (VA) of 0.8 and better and no fundus diseases were analyzed. Patients provided informed consent to participate in this study based on the approval of the institutional review board.MPOD levels were measured using resonance Raman spectroscopy on day 1 (baseline value), months 1, 3, and 6, and years 1 and 2 postoperatively. The following parameters were analyzed by multiple regression analysis: age, gender, body mass index, smoking history, glaucoma, diabetes, preoperative VA, preoperative refractive error, and IOL power and type.

Results: We found no significant differences in the baseline characteristics between the two groups. Until 6 months postoperatively, the MPOD levelsdid not differ significantly between the groups. However, from 1 year onward, the levels were significantly higher in the yellow-tinted IOL group compared with the clear IOL group. By multiple regression analysis, 1 day postoperatively, older age and diabetes were correlated with lower MPOD levels; 1 year postoperatively and thereafter, however, lower MPOD levelswere correlated with clear IOLs.

Conclusions: Cataract surgery with clear IOLs induced a greater decrease in MPOD compared with yellow-tinted IOLs during a longer follow-up period. The reason of the deterioration has not been unclear, but excessive light exposure through clear IOLs might beone reason.

Antioxidant properties of macular carotenoids and their susceptibility to degradation: protective and deleterious effects on cultured retinal pigment epithelium

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Lutein and zeaxanthin and their metabolites accumulate in the human retina reaching submilimolar concentrations in the area responsible for acute vision, the macula. These macular carotenoids exhibit potent antioxidant properties in free radical scavenging and particularly in quenching of singlet oxygen. The retina requires efficient antioxidant protection because it is inherently at risk of oxidative stress due to high oxygen tension, extremely active mitochondrial metabolism, accumulation of weakly chelated iron, exposure to visible light and abundant polyunsaturated lipids, such as docosahexaenoate (DHA). Some epidemiological studies indicate that people with dietary rich in macular carotenoids are at lower risk of developing age-related macular degeneration (AMD) – the major cause of blindness in the elderly. AMD is associated with an increased oxidative stress in the retina.

As a result of exposure to reactive oxygen species, carotenoids are susceptible to degradation. Degradation products of lutein and zeaxanthin obtained by exposure to autooxidized DHA, iron ions, light or combinations thereof exhibit potent photosensitizing properties (quantum yields of singlet oxygen generation of ~30%) and cytotoxicity to cultured human retinal pigment epithelial cell line, ARPE-19. Vitamins C and E can inhibit macular carotenoid degradation and therefore provide synergistic protection against oxidative damage, even under conditions where carotenoid alone exacerbates oxidative damage to ARPE-19 cells and increases cytotoxicity. In turn, macular carotenoid can partly protect from deleterious effects of vitamin C in photosensitized damage. Our results demonstrate that there is a need for a very intricate balance between different antioxidants to provide antioxidant protection in vitro. Increasing concentration of a single antioxidant, such as macular carotenoid, is inefficient as a way of increasing antioxidant protection, and may even lead to deleterious effects. Our results indicate that caution may be needed with supplementation of the elderly with lutein and zeaxanthin, and development of optimized antioxidant combination is required.

Lutein and zeaxanthin in the monkey retina – results of a successful research collaboration between academia and industry

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(for the Research Group listed in the acknowledgement)

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This lecture reviews results of a lutein and zeaxanthin supplementation experiment in 18 monkeys that had been fed a xanthophyll-free diet since conception. Consequently, these monkeys did not have any lutein or zeaxanthin in plasma nor was macular pigment optical density (MPOD) detectable in their retinas.

12 of these monkeys were supplemented with pure (i.e. zeaxanthin-free, see acknowledgement) lutein or zeaxanthin at 2.2. mg per kg body weight and day for 56 weeks, while the remaining 6 monkeys were left on their original feed. A group of monkeys on usual diet with normal levels of xanthophylls in plasma and macula served as further control. During supplementation, plasma xanthophylls as well as MPOD were monitored at regular intervals and raised substantially, but without reaching levels observed in monkeys on normal diets, indicating that the livelong absence of xanthophylls may have impaired the ability to accumulate xanthophylls.

At the end of the experiment, retinas were excised and analyzed for various parameters. Morphologically, irregularities in the cellular distribution profile in the RPE (Retinal Pigment Epithelium) were found, which further indicated a possible con-

sequence of the lifelong xanthophyll deprivation, particularly because supplementation partly corrected these irregularities. Chiral analyses of the xanthophylls in the retina by HPLC unequivocally revealed lutein as the source of meso-zeaxanthin, which could not be detected in monkeys fed zeaxanthin.

In order to evaluate the role of xanthophylls in blue light protection, retinas of xanthophyll-free, supplemented and normal control monkeys were exposed to blue laser light and consecutively analyzed regarding the size of the resulting photochemical lesions in dependence of the radiant exposure energy. Exposure was within fovea (MPOD present) or parafovea (MPOD absent). Xanthophyll-free animals exhibited identical vulnerability to blue light in fovea and parafovea, whereas in supplemented animals the vulnerability of the fovea was substantially reduced, actually to vulnerability levels found in normal control animals, demonstrating that supplementation has provided the retina with substantial protection from blue light.

Acknowledgement: Felix Barker – Salus University, Philadelphia; Joachim Gerss, Wolfgang Köpcke – University of Münster; Alfred Giger (with colleagues) – Chemical Research Centre DSM, for the preparation of 50 kg of zeaxanthin-free lutein; Elizabeth Johnson – Tufts University, Boston; Ivan Leung – Singapore Polytechnic; Martha Neuringer – Oregon National Primate Research Centre; Max Snodderly – University of Texas, Austin.

Genes and nutrition are related to age-related macular degeneration

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Objective: The hepatic lipase (*LIPC*) and cholesterol ester transfer protein (*CETP*) genes in the high-density lipoprotein cholesterol (HDL) pathway are significantly related to advanced age-related macular degeneration (AMD). HDL is the major lipoprotein transporter of lutein and zeaxanthin in the body. Therefore, we evaluated the association and interaction between these genes and dietary lutein and AMD.

Methods: Participants with advanced AMD or no AMD were evaluated. AMD status was determined using fundus photography. Covariates included cigarette smoking, body mass index (BMI), antioxidant intake and dietary lutein. Individuals were genotyped for snps in the *LIPC* and *CETP* genes as well as seven previously identified AMD genetic loci. Unconditional logistic regression analyses were performed.

Results: The TT genotype of the *LIPC* variant was associated with a reduced risk of AMD controlling for age, gender, smoking, body mass index (BMI), nutritional factors and other genes. The magnitude of the effect was similar for both atrophic and neovascular forms of AMD. Cigarette smoking and higher BMI increased risk, while higher dietary lutein reduced risk of advanced AMD, adjusting for genetic variants.

Conclusions: *LIPC* and *CETP* genes are associated with advanced AMD and higher dietary lutein reduces risk, independent of demographic, environmental and other genetic variables. Behavioral, lifestyle, and genetic factors predict risk of AMD.

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POSTERS

7.1.

Comparison of dietary supplementation with lutein diacetate and lutein: effects on macular pigment and serum

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Purpose: A study was conducted to compare a new dietary lutein formulation – a diacetate ester – with crystalline lutein, the endpoints being serum lutein and macular pigment optical density (MPOD) changes. The lutein diacetate formulation, "Micro-Mic™ Lutein," was manufactured in the form of micelles.

Methods: Ten subjects were assigned to each of three groups and given a 24 week supply of gel caps containing lutein diacetate (group 1), lutein (group 2), and a placebo (group 3). The amount of lutein for groups 1 and 2 was 20 mg per day. Serum samples were collected at baseline, and at weeks 6, 12, 18, and 24, and analyzed by HPLC. On the same occasions, MPOD was obtained by heterochromatic flicker photometry.

Results: The average serum lutein for weeks 6 to 24 expressed as a ratio to the baseline value (±SD) was 5.52±2.88 for group 1, 4.43±1.61 for group 2, and 1.03±0.25 for group 3. Macular pigment response for each subject was expressed as the rate of change of MPOD (milli-absorbance units/week). For groups 1, 2, and 3, these were 1.92±1.43, 1.69±1.75, and -0.75 ±3.03 mAU/wk, respectively. For both serum lutein and MPOD responses, the differences were significant between groups 1 and 3, and 2 and 3 (one-tail t-test), but not between 1 and 2 (two-tail t-test).

Conclusions: The average serum lutein response was ~ 25% higher for group 1 (lutein diacetate) compared with group 2 (lutein) and, correspondingly, the average MPOD response was 14% higher for group 1 compared with group 2. While these differences did not reach a level of statistical significance, they justify a forthcoming larger study of this new lutein formulation to determine whether its higher bioavailability than crystalline lutein can be substantiated.

Support: Industrial Orgánica SA de CV.

7.2.

Comparison of serum and macular pigment responses between subjects taking two formulations of a lutein supplement

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Introduction. A double-blind, placebo controlled study of the responses of subjects to lutein in two different, chewable commercial supplement formulations was undertaken with the goal of assessing the extent to which the products produce both serum and macular pigment optical density (MPOD) responses.

Methods. A placebo (n=9) was compared to two different lutein formulations, L1 and L2; each provided a dose of 9 mg/day but they differed in that L1 contained a suite of additional nutrients (n=10, in each group). Measurements of MPOD, and serum lutein levels were obtained at 0, 6, 12, 18 and 24 weeks of supplementation. The relative increase in these measures was used to assess the response for each group.

Results. At the outset of the study there was no significant difference between the baseline serum levels of the lutein groups when compared to the placebo group. The average baseline for lutein concentration at day 0 for all subjects was 0.12±0.038 μg/mL. Average serum levels, computed for each subject from weeks 6 and 12 of the supplementation period, showed that serum lutein levels increased for both supplements when compared to baseline and placebo. The increase in the average lutein concentration (weeks 6-12) for subjects taking L1 was highly significant compared to the placebo group, p=0.005 (one-tailed t-test), reaching 5.6 times that at baseline. Among subjects taking formulation L2 the average increase in serum lutein concentration (weeks 6-12) was 2.4 times baseline with a p value of 0.19. For subjects who consumed L1, a positive average rate of the MPOD increases of 2.4±0.2 milli-absorbance units per week (mAU/wk) was observed versus 0.19±0.3 mAU/wk for the placebo group. For the L2 group the average rate of MPOD increases was 1.1±0.2 mAU/wk.

Conclusion. The serum lutein response and rate of MPOD increase observed for the L1 chewable formulation is consistent with similar doses of lutein in oil-solublized formulations [1]. The rates of MPOD increases observed for the two formulations also correlate with serum lutein responses. The differences between the results for these two similar products are illustrative of the influence that formulations can exert on bioavailability of carotenoids.

Support: Four Leaf Japan Ltd.

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7.3.

Position statement on lutein and its role in cognition and eye health

The Science and Nutrition Advisory Board on the Macular Xanthophylls and DHA consists of: Paul S. Bernstein[1], Gary M. Chan[1], Anne B. Fulton[2], Elizabeth J. Johnson[3], John T. Landrum[4], and Lewis P. Rubin[5]; they were retained to assess the extent to which evidence supports the hypothesis that the xanthophylls function to maintain optimal health of the eye and brain throughout all stages of life.

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Lutein (L) and zeaxanthin (Z), known as the macular pigment (MP), are selectivelytaken up by the retina and bind to specific

binding proteins [1,2]. Humans cannot *de novo* synthesize L or Z; they must be obtained from dietary sources. L and Z protect the retina by absorbing damaging blue light and a putative role providing antioxidant protection to photoreceptors and retinal pigment epithelium (RPE) [3]. L and Z are present in the serum and the retina of neonates, evidence that they are transported across the placenta [4]. L and Z are also present in colostrum and breast milk [5,6]. L levels are greater than those of Z in neonates and during early retinal development. L supplementation improves neonates' retinal sensitivity and plasma L concentration is correlated with robustness of neonates' retinal responses [7]. Retinal Z increases during the first 3 years of life, in part by the conversion of L into meso-zeaxanthin within the macula [4]. This evidence supports the hypothesis that these xanthophylls may be critical to normal retinal development. New data indicate that the relatively low serum levels of L and Z in preterm infants increases their risk for progressive retinopathy of prematurity (ROP). Xanthophyll supplementation may provide protection against ROP. In older adults, higher MP densities correlate positively with reduced risk of age-related maculardegeneration and better cognitive function. Evidence indicates that L and Z play a critical role in retinal health throughout life, and may also play a role in cognitive function [8]. Older women (60-80 yrs) taking a dietary supplement containing L and DHA showed improved cognitive function as measured by verbal fluency. The actions of xanthophylls and DHA in maintaining optimal ocular health are well supported by data for adults. Their role in development during early life is potentially important. Their mechanisms of action will require extensive additional research.

Support: Abbott Nutrition.

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7.4.

Transgenic expression of human macular zeaxanthin-binding protein in mouse retina

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Age-related macular degeneration (AMD) is a leading cause of blindness in the developed world. Abundant clinical data indicate that the macular pigment (MP) carotenoids, lutein and zeaxanthin may help to prevent or reduce the risk of AMD; however, the physiological roles of MP carotenoids and their protective mechanisms are incompletely understood. Among mammals, only primates concentrate carotenoids specifically in the central retina region known as the *macula lutea*. Other mammals, such as mice, have only trace amounts of carotenoids in their retinas even when supplemented at very high doses. This fact has hindered studies of carotenoid effects on mouse models of AMD. We therefore endeavored to engineer mice with enhanced uptake of lutein and/or zeaxanthin in their retinas by overexpressing each specific binding protein in photoreceptor cells. Here, we report initial results of a transgenic mouse line produced by overexpression of human *GSTP1* (the primate retina zeaxanthin-binding protein previously identified in our laboratory) under the control of a mouse rhodopsin gene promoter. Expression of human *GSTP1* mRNA was detected by RT-PCR in transgenic mouse retinas, and western blots demonstrated that the human GSTP1 protein was strongly expressed in the retinas of one-month-old mice. Immunohistochemistry results showed that human GSTP1 is distributed specifically in mouse rod photoreceptors, from the spherule to the outer segment. Feeding studies to determine if these mice have enhanced zeaxanthin uptake into the retina are in progress. These transgenic "macular-pigment mice" could be valuable tools to investigate the protective mechanisms, metabolism, and physiological functions of carotenoids in the retina.

7.5.

Lutein and zeaxanthin protect against induced photosensitization in retinal pigment epithelial cells

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Lutein and zeaxanthin are dietary carotenoids which accumulate in the anatomic structures of human retina, including the retinal pigmented epithelium (RPE). The RPE is essential for photoreceptor survival and the loss of RPE cells is related to several eye diseases, including age related macular degeneration (AMD). Beside phagocytosis, the high level of irradiation and the presence of photosensitizers are important causes of generation of reactive oxygen species in the RPE. The aim of this study was to investigate the effect of lutein and zeaxanthin on the oxidative status of photosensitized RPE cells.

RPE cells (line D407) were cultivated in standard conditions. Control cells and cells pre-treated with lutein and zeaxanthin (10 ?M in culture medium for 24 h) were incubated with medium containing 500 nM Rose Bengal, for 1 h. The culture medium was replaced with HBSS buffer and cells were exposed to light (580 nm filter) for 30 min. The cells viability was estimated by the MTT assay, the cytotoxicity was determined by LDH leakage assay, and the level of lipid peroxidation – by a fluorimetric method. The generation of intracellular ROS was determined by using a fluorescent probe – DCF-DA, reduced glutathione by DTNB assay, and antioxidant enzymes glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) by classic methods.

Our study showed that both lutein and zeaxanthin protect RPE cells against photooxidative damage by improving cell viability, by reducing intracellular ROS and lipid peroxidation levels. Carotenoids have had little effect on the activity of antioxidant enzymes and on the reduced glutathione level in this experimental model. This suggests the involvement of carotenoids in the antioxidant protection of the RPE by direct absorption of light and by quenching singlet oxygen, rather than by their effect on the antioxidant enzymes. The results are consistent with studies, some very recent, which demonstrate in different experimental models and by different techniques that lutein, zeaxanthin and

mezozeaxanthin are effective quenchers of singlet oxygen (Wrona et al., 2004, Kim et al., 2006, Li et al., 2010).

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Session 8

Model Systems, Computational and in silico Studies of Carotenoids

INVITED LECTURES

Exploring the nature of the intramolecular charge transfer state (ICT) of peridinin analogues

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Peridinin is a highly substituted carotenoid with C37 carbon skeleton present in the light harvesting complex PCP from dinoflagellates, a group of marine algae, where plays a role of a dominant photosynthetic pigment along with chlorophyll *a*. The spectroscopic properties of peridinin are significantly different from majority of carotenoids and are affected by presence of a carbonyl group in the structure. Lifetime of the lowest excited singlet (S_1) state has been reported to depend on the solvent polarity and attributed to the presence of an intramolecular charge transfer (ICT). The nature of ICT state is still under debate. Up-to-date proposals include, a separate electronic state from S_1 [1], a state being quantum mechanically mixed with S_1 [2], or a S_1 state with large intrinsic dipole moment caused by strong coupling with S_2 [3].

To explore the nature of the ICT state, steady-state and ultrafast optical spectroscopic techniques, including femtosecond transient absorption and picosecond time-resolved fluorescence have been performed on peridinin and its three synthetic analogues, C_{33}-, C_{35}-, and C_{39}-peridinin at ambient temperature and at 77 K. The molecules are structurally similar on the way that they possess the same functional groups however have different numbers of conjugated carbon-carbon double bonds. Such systematic change in the pigments group allows study trends in the spectral features of S_1, S_2, and ICT states and behavior of their dynamics upon solvent polarity and temperature changes.

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Orientation of lutein in a lipid bilayer – revisited

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Lutein and zeaxanthin comprise the macular pigment of the human retina. They are located in rod outer segment (ROS) membranes and their highest concentration is in perifoveal ROS membranes. They also intercalate into model membranes.

Lutein is a C40 xanthophyll with hydroxyl groups located at the 3 and 3' positions. It has both the β- and ε-ionone ring, that are not symmetric. In the ε-ionone ring, the covalent bond between C6' and C5' is single, whereas in the β-ionone ring, the C6-C5 bond is double. As C6', that is attached to the conjugated polyene chain, has sp3 hybridization, the entire ε-ring has some rotational freedom to rotate about the C6'-C7' bond, which is not the case for the β-ring. Moreover, the hydroxyl group of the ε- and β-ring are oppositely directed.

For the reasons stated above, there is a chance that in the model membrane, and possibly in biomembranes, lutein is orientated not only parallel but also partially perpendicular to the bilayer normal with both hydroxyl groups located at the same bilayer surface. These two, mutually perpendicular orientations of lutein in the model membrane were observed experimentally [1]. As the membrane-spanning orientation of lutein in the phospholipid bilayer should be favored due to geometrical and energetic reasons, we have repeated the experiment performed in [1] using molecular modeling approach to get detailed information about the preferential orientation of lutein molecules in the bilayer, its stability as well as basic lutein-phospholipid interactions that stabilize this orientation. In the experimental study, xanthophyll was incorporated into egg yolk phosphatidylcholine (EYPC) liposomes. In the computer modeling study, palmitoyloleoylphosphatidylcholine (POPC) was used as a EYPC matching phospholipid.

The OPLS-AA force filed was used to parameterize lipid and lutein molecules. The parameters for the C8'-C7'-C6'-C5' torsional potential, missing in the OPLS-AA, were obtained by calculating the energy profiles for rotation about the C6'-C7' bond using the procedure described in [2]. Molecular dynamics simulations of two bilayer systems were carried out for 200 ns each. One POPC bilayer contained six lutein molecules in the perpendicular orientation and the other POPC bilayer contained six lutein molecules in the parallel orientation. Each bilayer was hydrated with 6000 water molecules.

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Carotenoid aggregates: self-assembly, spectroscopy and prospective utilization in solar cells

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It is well known that carotenoids form aggregates when dissolved in hydrated polar solvents, and that aggregation is characterized by changes in their absorption spectra. Depending on water content, initial carotenoid concentration and solvent, two types of carotenoid aggregates are usually observed: H-aggregate with blue-shifted absorption maximum, and J-aggregate whose absorption band occurs at wavelengths longer than 500 nm (REF). Carotenoids also tend to aggregate when deposited on surfaces, which is important for their prospective use in devices for solar energy conversion. Here we present aggregation study of synthetic carotenoid 8'-apo-β-carotenoic acid (ACOA). Monomeric ACOA has absorption maximum at 440 nm in ethanol. In hydrat-

ed ethanol, it forms predominantly H-aggregates with absorption peak at 390 nm, but J-aggregates coexist in the sample as evidenced by weak absorption band around 510 nm. Excited-state dynamics are significantly changed upon aggregation. Excitation of H-aggregates at 400 nm reveals prolongation of the S_1 lifetime as compared with monomer (38 vs. 24 ps). In addition, contrary to monomers, transient signal of ACOA aggregates does not decay within 100 ps, indicating presence of long-lived species, most likely a triplet state generated by singlet-singlet homofission. ACOA also forms H-aggregates when attached to TiO_2 film. As observed earlier for monomeric ACOA-TiO_2 system in colloidal solution (Pan et al., 2002), excitation of ACOA aggregate on TiO_2 film generates ACOA radical on sub-picosecond time scale due to electron injection from the S_2 state. Contrary to monomeric ACOA, however, the ACOA aggregate also exhibits a slower phase of electron injection that may occur from the S_1 state of the ACOA aggregate.

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Exceptional molecular organization of canthaxanthin in lipid membranes

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Canthaxanthin (β, β-carotene 4, 4' dione) is a carotenoid pigment widely distributed in nature. It is found in green algae, bacteria, crustaceans, and fish. In the last 30 years it has been a popular E161g food additive and cosmetic colorant due to a very attractive color.

There are many reports on undesirable health effects caused mainly by the formation of canthaxanthin crystals in the *macula lutea* membranes of the retina, called canthaxanthin retinopathy.

Our experiments done on model systems indicate an exceptional molecular organization of canthaxanthin in lipid membranes as well as a very strong effect of this carotenoid on the physical properties of the lipid membranes.

It has been found that the canthaxanthin localization and orientation in the model lipid membranes depends strongly on its concentration. The mean angle between the dipole transition moment and the axis normal to the plane of the DPPC membrane was determined as 20°- at 0.5 mol% which confirms vertical orientation of the axis connecting opposite keto-groups of the xanthophyll at the 4 and 4' positions and 47°- at 2 mol% of canthaxanthin, which implies the possibility that canthaxanthin incorporated into lipid membranes can be distributed in such a way that its small fraction can be oriented parallel to the plane of the lipid membrane.

Strong interactions between canthaxanthin and lipids were found under experimental conditions. For model DPPC lipid membranes and canthaxanthin concentration below 1 mol% significant changes in the membrane properties were observed, in some cases at the pigment concentration as low as 0.05 mol% with respect to lipid. Based on experimental data on phosphatidylcholines several molecular mechanisms of canthaxanthin action can be listed such as strong van der Waals interactions between polyene chain of canthaxanthin and the lipid alkyl chains, modifications of the lipid properties in its polar zone, introduction of a new thermotropic phases upon the incorporation of canthaxanthin, formation of the hydrogen bonds between canthaxanthin keto- groups and the C=O group of lipid or hydrogen bonds between the canthaxanthin polyene chain and water. Two last mechanisms can have a crucial significance in formation of molecular aggregates of canthaxanthin leading to further development of canthaxanthin- induced retinopathy.

The *cis*-carotenoid-membrane interactions

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The heterogeneity of carotenoids is greatly increased by the existence of their geometrical isomers. Carotenoid geometry is a factor that determines their solubility and orientation in the lipid memebrane as well as antioxidant capacities and bioavailability. The polyene-chain double-bonds present in carotenoids can exist in mono-*cis*, poly-*cis*, or all-*trans* configurations; however the vast majority of naturally occurring carotenoids exist in the thermodynamically more stable all-*trans* conformation rather than in *cis* configuration. *Trans-cis* conversion occurs at elevated temperature and/or in the presence of intensive light and triplet sensitizers.

The role of the all-*trans* carotenoids in the modulation of the physical properties of lipid membranes has been a subject of research for the last three decades. Effects of *trans*-carotenoids on the structural and dynamic properties of lipid bilayers have been studied with application of various techniques, such as differential scanning calorimetry, fluorescence, electron spin resonance and nuclear magnetic resonance spectroscopy, diffractometry, and others. However, the effect of the *cis*-isomers of carotenoids on the membrane properties is less investigated.

Our results indicate that the *cis*-isomers of xanthophylls, similarly to the *trans*-isomer adopt a transmembrane orientation and do not show a tendency to organize into high aggregates as *trans* configuration. Molecules of carotene in form *trans* and *cis* are distributed homogeneously without any well-defined orientation within the membrane. The influence of this nonpolar carotenoid on the memebrane properties is negligible and such effects can be related to the low solubility of carotene in the lipid bilayers.

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Structural aspects of antioxidant activity of lutein in models of photoreceptor membranes

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It was shown that in membranes containing domains, such as raft-forming mixtures (composed of dioleoylphosphatidylcholine

[DOPC], sphingomyelin [ESM], and cholesterol, mixed at a molar ratio of 1:1:1), and in models of photoreceptor outer-segment membranes (containing palmitoyl-docosahexaenoyl-PC [PDHAPC], distearoyl-PC [DSPC], and cholesterol mixed at a molar ratio of 1:1:1), macular xanthophylls, lutein, and zeaxanthin are not distributed uniformly. They are substantially excluded from raft domains rich in saturated lipids and cholesterol and remain ~10 times concentrated in bulk domains, which are enriched in unsaturated lipids (DOPC or PDHAPC) (Wisniewska and Subczynski, 2006; 2006a). The selective accumulation of lutein and zeaxanthin in direct proximity of unsaturated lipids, which are especially susceptible to lipid peroxidation, should be very important as far as the antioxidant activity of these xanthophylls is concerned. Therefore, the protective role of lutein against lipid peroxidation was investigated in raft-forming mixtures and photoreceptor model membranes and compared with their antioxidant activity in homogenous membranes composed of unsaturated lipids. Lipid peroxidation was induced by photosensitized reaction using rose bengal and monitored by an MDA-TBARS test, iodometric assay, and oxygen consumption (using EPR spectroscopy oximetry and the mHCTPO spin label as an oxygen probe). The results show that lutein protects unsaturated lipids more effectively in raft-forming mixtures than in homogenous membranes. This suggests that the selective accumulation of macular xanthophylls in the most vulnerable regions of photoreceptor membranes may play an important role in enhancing their antioxidant properties and their ability to prevent age-related macular diseases (such as age-related macular degeneration [AMD]).

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ORAL PRESENTATIONS

Importance of stability tests of lycopene in experimental animal feed

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The EU-funded project "LYCOCARD" (www.lycocard.com) investigated the role of lycopene, tomatoes/tomato products in reducing the risk of cardiovascular diseases. An animal study with NZW-rabbits (Charité Berlin, Germany) examined the influence of lycopene on atherogenesis induced by high-cholesterol diet.

Lycopene is sensitive to degradation and isomerization. Therefore, an important part of the animal study was to investigate the lycopene stability in lycopene-enriched experimental feed according to diverse processing methods, formulations and storage conditions. These experiments were mandatory to ensure the correct daily dose of lycopene in animal diets. Therefore, storage stability of carotenoids should always be monitored in chow used for animal studies.

Lycopene beadlets, provided by DSM Nutritional Products Ltd. (Basel, Switzerland), were used for production of different experimental diets (ssniff Spezialdiäten GmbH, Soest, Germany or Altromin Spezialfutter GmbH & Co. KG, Lage, Germany). To test the stability of lycopene in rabbit chow enriched with lycopene beadlets, lycopene content as well as the isomer pattern were measured in a variety of chows (different processing conditions: e.g. temperature, pressure, added oxygen absorber) over the course of time (8 days, 3 months) under various storage conditions (different temperatures, with and without exposure to light and oxygen) by using C30-HPLC (Fröhlich et al., 2007).

In contrast to the high stability of unprocessed lycopene beadlets (6 months at room temperature – no significant change), strong decrease of lycopene contents in chows enriched with beadlets was observed. After 8 days of storage, the lycopene contents were reduced significantly by about half of the basal level. Exposure to light showed no significant effects on lycopene stability. During the long-term stability tests (3 months), reduction of lycopene was significant and depending on the storage temperature. Surprisingly, no significant changes in the isomer ratio (all-E:Z) of lycopene were observed during all storage experiments.

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Stark spectroscopy of carotenoids bound to LH3 antenna pigment-protein complexes from *Rhodopseudomonas molischianum*

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Light-harvesting (LH) complexes of purple photosynthetic bacteria absorb sunlight and efficiently transfer captured energy to reaction centers. It is well known that there are two types of LH complexes; one is peripheral LH2 complex and the other is LH1 core antenna complex that is strongly conjugated with the reaction center. There is another type of peripheral LH complex called LH3. The LH3 complexes are generated under stressed growth condition (e.g. low light illumination at low temperature) in some species of purple photosynthetic bacteria. Marked feature of the LH3 complex is the shift of the bacteriochlorophyll (Bchl) *a* Q_y absorption band from 850 nm in LH2 to 820 nm in LH3. X-ray crystal structure analysis of both the LH2 and LH3 complexes from *Rhodopseudomonas (Rps.) acidophila* has been reported [1,2]. Both the LH2 and LH3 complexes have nonameric ring structures and only a slight distortion of pigment molecules when they bound to the LH3 complex have been reported. On the other hand, although the X-ray crystal structure analysis of the LH2 complex from *Rps. molischianum* has been reported [3], the structure of the LH3 complex from this bacterium has yet to be clarified. The LH2 complex from *Rps. molischianum* has octameric ring structure and it is obviously different from that of *Rps. acidophila*. In this study we have applied Stark absorption spectroscopy at cryogenic temperature to the LH3 complexes isolated from *Rps. molishianum* strain DSM-120 to make a good access to the structural information of carotenoids bound to these complexes. The Stark absorption spectroscopy is one of promising spectroscopic methods to exploit the molecular structure as well as electrostatic environment surrounding the photosynthetic pigments [4].

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Femtosecond stimulated Raman spectroscopy on photosynthetic carotenoids

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Femtosecond Stimulated Raman Spectroscopy (FSRS) is a new promising spectroscopic tool complementary to femtosecond IR spectroscopy. Apart from all benefits associated with Raman spectroscopy, such as the possibility of resonance enhancement or working at experimentally convenient wavelengths, it also yields an orders of magnitude higher signal in comparison with spontaneous Raman scattering. FSRS not only allows one to investigate the molecular vibrations with femtosecond time resolution but due to the changes in the selection rules of the excited molecules it allows examination of their electronic state dynamics through their Raman signatures that often display more specific fingerprints than broad electronic absorption bands. The price to pay for all these benefits originates in the coherent nature of the process which is associated with disadvantages not known to steady state Raman spectroscopy. Though FSRS in principle breaks the standard time-bandwidth limitation of ultrafast spectroscopy it is associated with complex coherent artifacts. Both their theoretical and experimental investigation is just in its beginnings. If the method should bring all the power it is capable to supply a deeper understanding of the process is required and new experimental approaches have to be developed. I will demonstrate our first FSRS on carotenoids, I will discuss some of the problems and I will indicate some potential solutions.

Zeaxanthin epoxidation – an in vitro approach

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Zeaxanthin epoxidase (ZE) is an enzyme operating in the violaxanthin cycle, one of the six existing types of the xanthophyll cycles, which protect plants against overexcitation. Under strong light condition violaxanthin (Vx) is converted to zeaxanthin (Zx) and this reaction is catalyzed by another xanthophyll cycle enzyme, violaxanthin de-epoxidase (VDE). The reverse reaction of Zx to Vx epoxidation is catalyzed by ZE. The intermediate product in both reactions is antheraxanthin. There exist model systems to study Vx de-epoxidation with the use of isolated enzyme. Such approach in the case Zx epoxidation is difficult because the respective enzyme has not been isolated and purified by now. This study deals with development of a model system of Zx epoxidation. Three assay systems are presented in which reduction in the Zx amount was observed. In these assays two mutants of *Arabidopsis thaliana* which have active only one of the two xan-

thophyll cycle enzymes were used. Npq1 mutant produces ZE and it may convert Zx to Vx but VDE is inactive. The other mutant, npq2, produces VDE and converts Vx to Zx under strong light condition but reverse reaction is not possible. The first assay containing thylakoids from npq1 and npq2 mutants of *Arabidopsis thaliana* gave positive results but with low efficiency of epoxidation reaction. Similar results were obtained in the second type of assay where thylakoids of both mutants were supplemented with MGDG. The third kind of assay contained thylakoids npq1 mutant of *A. thaliana* and exogenous Zx with MGDG. In this system, due to epoxidation, the amount of Zx was reduced to 33% of its initial level. To optimize high efficiency of epoxidation reaction additional factors facilitating both fusion of the two types of thylakoids and incorporation of Zx to their membranes were also studied. This in vitro system of Zx epoxidation enables analysis some properties of the enzyme without necessity of its isolation.

Posters

8.1.

FTIR studies of carotenoid-containing lipid membranes

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Carotenoid pigments are commonly present in living cells and are recognized to play several vital physiological functions. Carotenoids also stabilize the structure of proteins and cell membranes. Typically, carotenoids are present in the cell membrane bound to proteins or remaindirectly in the lipid phase, where they can create molecular aggregates. The molecular structures and possible physiological effects of carotenoid aggregates is not fully known. The process of incorporation and molecular organization of carotenoid pigments in model lipid membranes as well as their influence on the membrane properties is the main subject of this work. Multibilayer lipid membranes formed with dipalmitoylphosphatidylcholine (DPPC) and containing carotenoid pigments: β-carotene, zeaxanthin (3,3'-dihydroxy-β-carotene) and lutein (3,3'-dihydroxy-β,ε-carotene) was studied by infrared absorption spectroscopy FTIRwith applicationof the ATR (Attenuated Total Reflectance) technique and LD (linear dichroism). The analysis of the IR absorption spectra let conclude that:

1. Polar xanthophylls incorporate to the membranes and adopt an orientation in which the average angle between the axis normal to the membrane plane and the transition dipole of the C=C bonds is 40-45 deg., in the case of zeaxanthin, and 41-46 deg., in the case of lutein.
2. The presence of zeaxanthin in the lipid phase, in the concentration range 1-5 mol%, with respect to lipid, increases the order parameter of alkyl chains, in contrast to lutein which causes a decrease in the order parameter.

8.2.

Adhesion properties of DPPC vesicles containing β-carotene: an atomic force microscopy study

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Carotenoids play a role as modulators of physical properties of biological membranes, which was shown both in model systems and in vivo (Gruszecki and Strzalka, 2005). The influence of carotenoids on thermotropic phase transition of lipids has been a subject of intensive studies in the past few years. In particular, DSC measurements revealed the shift of pretransition towards lower temperature of about 2.3°C in DPPC vesicles containing 1% β-carotene (Kostecka-Gugala et al., 2003). β-carotene may also affect molecular dynamics of membrane causing the change of adhesion properties of vesicles. Direct force measurements using

atomic force microscopy (AFM) enabled us to characterize adhesion interactions between unruptured vesicles and the AFM tip. Studies revealed a significant change in the dependence of adhesion force on temperature caused by the presence of β-carotene in the lipid bilayer.

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8.3.

Spectroscopic properties of astaxanthin aggregates in hydrated solvents

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It is well known that carotenoids form aggregates when dissolved in hydrated polar solvents, and that aggregation is characterized by dramatic changes in their absorption spectra. In this study, aggregates of the carotenoid astaxanthin were studied. Depending on water content and organic solvent used for preparations of primary stock solution, two types of aggregates were produced: H-aggregates with absorption maximum around 390 nm, and J-aggregates with red-shifted absorption band peaking at wavelengths >550 nm. The large shifts in respect of absortion maximum of monomeric astaxanthin (470-495 nm depending on solvent) are caused by excitonic interaction between aggregated molecules as demonstrated also by CD spectra. While in hydrated acetone and methanol only H-aggregates were generated, ambivalent behavior was observed in hydrated dimethyl sulfoxide (DMSO) in which both types of aggregates are observed and their ratio could be controlled by varying the water content. We applied molecular modeling simulations to elucidate structure of astaxanthin dimer in water, and resulting structure was used as a basis for calculations of absorption spectra. Absorption spectra of astaxanthin aggregates in hydrated DMSO were calculated for various structures, including structural disorder, using excitonic model. The resonance interaction energy between astaxanthin monomers was determined by quantum chemical calculation, and both underdamped intramolecular vibrational modes and the overdamped solvent modes were included to fit the absorption lineshape. For selected astaxanthin aggregates, excited-state dynamics were studied by femtosecond transient absorption spectroscopy.

8.4.

Violaxanthin and diadinoxanthin de-epoxidation in various model lipid systems

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The xanthophyll cycle is an important photoprotective process functioning in plants. One of its forms, the violaxanthin (Vx) cycle, involves interconversion between: Vx, antheraxanthin (Ax) and zeaxanthin (Zx). Another kind of the xanthophyll cycle is the diadinoxanthin (Ddx) cycle in which interconversion between Ddx and diatoxanthin (Dtx) occurs. In this study an information on molecular mechanism and regulation of these two types of the xanthophyll cycle is presented. The influence of lipids on the de-epoxidation of the xanthophyll cycle pigments was investigated, with special focus put on the significance of physical properties of the aggregates formed by inverted lipid micelles, which are necessary for activity of tha xanthophyll cycle enzymes. In particular, thickness of the hydrophobic fraction of the aggregates, size of the inverted micelles, suggested by mathematical description of the structures and solubility of Vx and Ddx in various kind of lipids were studied. Obtained results show that the rate of de-epoxidation is strongly dependent on the physicochemical properties of the lipids used. The key role for enzyme activation play non-bilayer lipids and the parameters of inverted micelles created by them, such as thickness, molecular dynamics of hydrophobic core and their diameter. The presented results show that MGDG and other non-lamellar lipids like different forms of phosphatidylethanolamine are necessary for the Vx and Ddx de-epoxidation because they provide the three-dimensional structures, which are needed for the binding of de-epoxidases and for the accessibility of Vx and Ddx to these enzymes.

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INSTRUCTIONS TO AUTHORS

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