Carotenoids, Tocochromanols and Chlorophylls in Sea Buckthorn Berries (*Hippophae rhamnoides*) and Rose Hips (*Rosa* sp.)

Variation during Ripening, and among Cultivars/Species and Years

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Abstract

Consumption of fruits and berries have positive effects on human health by reducing the risk of *e.g.* cardiovascular diseases, age-related macular degeneration and cataracts, and different forms of cancer. These positive effects are believed to be related to the high content of bioactive compounds such as different antioxidants in fruits and berries. Increased intake of bioactive compounds can be promoted by selecting species/cultivars with high contents and by harvesting at the optimal time.

Recent interest for cultivating sea buckthorn berries and rose hips partly depends on their more or less well documented health related statements. Products containing sea buckthorn berries or rose hips are steadily growing in number on the market and there is a need for increasing the knowledge of the content of the bioactive compounds in the fruit and berry raw material.

This thesis investigated the content of carotenoids, tocochromanols (tocopherols and tocotrienols) and chlorophylls in berries from different cultivars of sea buckhorn (*Hippophae rhamnoides*) and fruits from different species of roses (*Rosa* sp.) during the harvest period of three consecutive years. The carotenoid and tocopherol content were also investigated during processing and storage in juices with sea buckthorn berries and rose hips as ingredients. Carotenoids and tocochromanols are fat-soluble antioxidants, some of which have activity as pro-vitamin A and vitamin E. All analyses were carried out by HPLC on extracts made from lyophilised material.

The concentration of carotenoids, tocochromanols and chlorophylls generally varied depending on cultivar/species and over the ripening period in both sea buckthorn berries and rose hips. This variation was not simultaneous for different compounds of the same type, *e.g.* all carotenoids did not show similar variation. For carotenoid content, harvesting time and choice of cultivar were more important than year of harvest, and a general increase occurred over the season. The tocochromanol content was mostly influenced by cultivar/species and year of harvest, although vitamin E activity generally decreased during ripening. The chlorophyll content decreased during ripening, when the fruits/berries changed colour from green to yellow-red, and proved suitable for use as a maturity marker of optimal harvesting time.

Keywords: antioxidant, carotenes, esterified carotenoids, fruit, harvest, irradiation, pheophytin, temperature, tocopherols, tocotrienols, xanthophyll.

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Andersson S.C., Rumpunen K., Johansson E. and Olsson M.E. (2008). Tocopherols and tocotrienols in sea buckthorn (Hippophae rhamnoides L.) berries during ripening. *Journal of Agricultural and Food Chemistry 56*, 6701-6706.

II Andersson S.C., Olsson M.E., Johansson E. and Rumpunen K. 2009. Carotenoids in sea buckthorn (*Hippophae rhamnoides* L.) berries during ripening and use of pheophytin a as maturity marker. *Journal of Agricultural and Food Chemistry* 57, 250-258.

III Andersson S.C., Rumpunen K., Johansson E. and Olsson M.E. Carotenoid content and composition in rose hips (*Rosa* sp.) during ripening and use of chlorophylls for determination of harvesting time. (manuscript).

IV Andersson, S.C., Olsson M.E., Gustavsson K.-E., Johansson E. and Rumpunen K. Tocopherols in rose hips (*Rosa* sp.) during ripening. (manuscript).

Objectives

The main objective of this doctoral thesis was to study the amount and composition of carotenoids and tocochromanols, in berries of different cultivars of sea buckthorn (*Hippophae rhamnoides* L.) and in fruits of different species of roses (*Rosa sp*.), during ripening in different seasons. Secondary objectives were to evaluate how to improve levels of these bioactive compounds by choice of harvesting time and cultivar/species; to investigate how the climate interacted with the content of bioactive compounds; and to determine the effects of food processing and storage on the amounts of these bioactive compounds in fruit juice.

Introduction

Plant material

Sea buckthorn

Sea buckthorns are classified botanically as follows:

Family: Elaeagnaceae
Genus: *Hippophae*
Species: *rhamnoides*
Subspecies: *rhamnoides*
Cultivars used in the present thesis work: Ljublitelskaja and three advanced selections

Sea buckthorn grows wild in nature in temperate areas of Europe and Asia (Yang & Kallio 2006a). Domestication and plant breeding have improved the quality of the plants and berries in many different respects. For sea buckthorn berries the domestication and breeding process has been going on since the early 20th century and plants are now spread all over the world (Singh 2003; Yang & Kallio 2006a).

The genus *Hippophae* consist of seven species. One of these is the species *Hippophae rhamnoides*, which was further classified into ten subspecies by Bartish et al. (2002). The most commonly used subspecies for berry production is *H. rhamnoides*. Sea buckthorn is dioecious and about 10% males are used as pollinators in commercial production. The sea buckthorn plant grows in symbiosis with the nitrogen-fixing bacteria *Frankia* and therefore does not have any great demands for additional nitrogen (Singh et

al. 2003). However, due to the pioneer character of the plant it is sensitive to competing weeds. The berries lack an abscission layer and do not fall from the plant at maturity. Various harvesting methods have been developed to replace hand-picking. One time-saving method is cutting of whole branches, which are immediately frozen to around -20 °C and the berries are thereafter shaken off when frozen. By cutting the branches a continuous pruning action is obtained, but due to the fruit setting on second-year branches, intensive cutting may result in a biennial yield.

Rose

Roses are classified botanically as follows:

Family: Rosaceae
Genus: *Rosa*
Section used in the present thesis work: *Caninae* and *Pimpinellifoliae*
Species used in the present thesis work: *R. dumalis, R. rubiginosa* and *R. spinosissima*

Roses grow wild in nature in temperate and subtropical climates around the world. For rose hips, less domestication work has been performed than for sea buckthorn. Many of the rose hips used today for commercial food products are still collected from the wild and they are not normally commercially grown, unlike sea buckthorn berries. However, rose hips are cultivated *e.g.* for health and cosmetic-related purposes, and some domestication and plant breeding activities have been carried out for rose hips (Uggla 2004).

The genus *Rosa* has over 100 species, with a large interspecific variation in characters of importance to different quality aspects of the fruits such as taste, aroma, colour, yield, fruit density on bushes, size of fruits, ripening time, and content of specific compounds. In addition to the interspecific variation, intraspecific variation sometimes is large although in the section of *Caninae* it is restricted to the special *canina* meiosis (Nybom et al. 2004). Breeding of roses for fruit production have been made at SLU Balsgård, Sweden. In particular characters such as plant yield, plant habitus, disease resistance and content of sugar, ascorbic acid, total antioxidants and colour have been considered important for selection of commercial cultivars (Gao et al. 2000a; Uggla et al. 2003; Uggla et al. 2005).

Rose hips have a rather long ripening period and usually stay attached on the bushes long after the first night frost. During ripening the hips start to change colour on their sunny side and become fully coloured with time. Due to the fact that the majority of the rose hips used for commercial products are harvested from wild populations of plants, specific quality aspects such as content of natural vitamins and antioxidants are difficult to maintain in the raw material. After harvest the rose hips are normally cleaned, dried, exported and later processed.

Health aspects of sea buckthorn berries and rose hips

Epidemiological studies and other investigations have shown that a high intake of fruit and vegetables is correlated with positive effects on human health and decreases the incidence of diseases such as cardiovascular disease, cataracts and different cancers and other chronic diseases (Block et al. 1992; Williamson 1996; Lampe 1999; Gandini et al. 2000; Liu et al. 2000; Joshipura et al. 2001; Liu et al. 2001; Bazzano et al. 2002; Temple & Gladwin 2003; Kang et al. 2005). The positive health effects have been associated to the content of vitamins, antioxidants and other bioactive compounds in fruit and vegetables. Sea buckthorn berries and rose hips have been found to have a high content of many bioactive compounds such vitamin C, carotenoids, tocochromanols, phenolic compounds, folates and healthy fatty acids (Märki-Fischer et al. 1983; Razungles et al. 1989; Hodisan et al. 1997; Zadernowski et al. 1997; Karakaya & El Nehir 1999; Gao et al. 2000a; Hornero-Mendez & Minguez-Mosquera 2000; Hvattum 2002; Kallio et al. 2002b; Pirronen et al., 2002; Larsen et al. 2003; Rösch et al. 2003; Strålsjö et al. 2003a; Strålsjö et al. 2003b; Uggla et al. 2003; Olsson et al. 2004; Zadernowski et al. 2005; Suomela et al. 2006).

Sea buckthorn berries

Sea buckthorn berries have a long history of being used by man for health-related and food purposes. The sea buckthorn berry was described in traditional Indian ayurveda medicine 5000-500 BC and in Tibetan rGud bzi medicine 618-907 AC for use in health-related purposes (Singh et al. 2006). Sea buckthorn oil has been investigated in both human and animal studies for its skin-healing properties for burns, scalds, irradiation, wounds and atopic dermatitis (inflammatory skin disease), mucosa, and immune function, all with positive effects on symptoms to different degrees (Zadernowski et al., 1997; Yang et al., 1999; Yang & Kallio 2006b). Other studies on sea

buckthorn have investigated the anticarcinogenic effects in animals and *in vitro* and have reported positive effects (Olsson et al., 2004; Yang & Kallio 2006b). Sea buckthorn has also been shown to have positive effects on cardiovascular disease by inhibiting platelet aggregation and oxidation on low density lipoprotein (LDL) (Johansson et al., 2000; Suomela et al., 2006; Yang & Kallio 2006b). However, more investigations are needed before definite conclusions can be drawn about the effects and functions. Sea buckthorn berry juice has been under investigation for its antioxidative capacity and effects. The uptake in humans of the hydrophilic constituents, such as polyphenols and ascorbic acid, has been measured (Allmann et al. 2006).

Rose hips

Rose hips have long been used in traditional medicine and more recently studied in relation to different human health aspects. Rose hips have been used in clinical studies for evaluation of humans suffering from osteoarthritis, rheumatoid arthritis and low back pain, with positive results, but more investigations are needed to establish evidence of their effectiveness (Warholm et al. 2003; Rein et al. 2004; Rein E 2004; Winther et al. 2005; Christensen et al. 2007; Rossnagel et al. 2007; Chrubasik et al. 2008). Different studies have investigated rose hips for their antioxidative activity and have reported higher activity compared with other fruit and berries (Costatino et al. 1994; Gao et al. 2000a; Daels-Rakotoarison et al. 2002; Halvorsen et al. 2002). Investigations on anti-inflammatory activity have shown that rose hips extracted in more fat-soluble solvents have a higher activity than water extracts (Kharazmi & Winther 1999; Larsen et al. 2003; Winther et al. 2005; Deliorman et al. 2007; Wenzig et al. 2007). Effects of rose hips on body fat, plasma and biliary lipids have been shown in various animal studies (Gonzales et al. 1989; Lutz et al. 1993; Gonzales et al. 1997; Ninomiya et al. 2007). Rose hips have also been investigated for antiulcerogenic effects in animal and human studies, with good results (Gurbuz et al. 2003; Deliorman et al. 2007). Rose hips are reported to have antimutagenic effects on *Salmonella typhimurium* and anticancerogenic effects in different *in vitro* studies on cancer cells (Karakaya & Kavas 1999; Olsson et al. 2004) and also have antimicrobial effects on different bacteria (Kumarasamy et al. 2002). Other investigations have revealed effects on blood glucose and urine excretion and composition (Can et al. 1992; Grases et al. 1992; Ninomiya et al. 2007). In addition, rose hip seed oil has been tested for different skin treatments (Shabykin & Godorazhi 1967).

Antioxidants – Free radicals

Antioxidants are compounds that can be harmful against free radicals and other reactive oxygen species (Halliwell & Gutteridge 1995). Many bioactive compounds are antioxidants, *e.g.* ascorbic acid, carotenoids, tocochromanols and phenols. Sea buckthorn berries and rose hips have both been shown to have a relatively high antioxidant capacity (Velioglu et al. 1998; Halvorsen et al. 2002; Nilsson et al. 2005).

Free radicals are often highly reactive species with one or more unpaired electrons, and can cause damage in the cell (Halliwell & Gutteridge 1995). The radicals are suggested to be involved in different processes such as ageing, cardiovascular disease, inflammation, different cancers and cataracts (Halliwell & Gutteridge 1989). The radicals are normally produced in cell metabolism, such as in the mitochondria by enzymatic activity and in the immune system as a defence against pathogens (Finkel & Holbrook 2000). Some of the radicals in biological systems involve oxygen, and are then called reactive oxygen species (ROS), including superoxide anion, hydroxyl radicals, singlet oxygen, hydrogen peroxide, peroxynitrite, nitric oxide and hypochlorous acid (Halliwell & Gutteridge 1989). Overproduction of radicals may be triggered by *e.g.* radiation, pollutants and smoke (Finkel & Holbrook 2000). In situations of surplus radicals compared with presence of antioxidants in cells, oxidative stress can lead to damage to cell membranes and lipoproteins (Finkel & Holbrook 2000; Bender & Mayes 2003). When radicals react with polyunsaturated fatty acids in cell membranes, the fatty acids in turn can become radicals, thereby starting a chain reaction that can cause great damage (Niki et al. 1982). After reacting with and neutralising the radicals, many antioxidants become radicals by themselves, although less reactive than the originals. Opportunities also exist for these new radicals to be regenerated by other antioxidants (Niki et al. 1995).

Many antioxidants may protect each other from oxidation, including tocochromanols and carotenoids (Munné-Bosch & Alegre 2002; Krinsky & Johnson 2005). The α-tocopherols and β-carotene are suggested to cooperate as antioxidants in lipid membranes, and β-carotene has been shown to work as an antioxidant at low pressure and as a pro-oxidant at high pressure (Burton & Ingold 1984; Zhang & Omaye 2000; Zhang & Omaye 2001). α-tocopherol may therefore replace β-carotene in protecting lipid membranes from oxidation at moderate partial pressures (Munné-Bosch & Alegre 2002). The synergistic effects of α-tocopherol and β-carotene are effected by the concentration with approximately equal levels showing

synergistic protection of lipid membranes (Palozza & Krinsky 1992). Lycopene has also been shown to have synergistic effects with other antioxidants (Fuhrman et al. 2000). Both tocochromanols and carotenoids may be generated by other antioxidants, such as ascorbic acid and procyanidins (Packer et al. 1979; Niki et al. 1982; Maguire et al. 1992; Mukai et al. 1992; Niki et al. 1995).

Tocochromanols

Vitamin E and tocochromanols are common names for tocopherols and tocotrienols existing in photosynthetic organisms. There are four natural tocopherols (α-, β-, γ-, δ-tocopherol) and four natural tocotrienols (α-, β-, γ-, δ-tocotrienols), but a total of 14 theoretical forms are possible (Bramley et al. 2000). Vitamin E was first discovered as a necessary nutritional factor in pregnant rats for foetus viability (Evans & Bishop 1922). All tocopherols and tocotrienols are considered to have vitamin E activity, with α-tocopherol being the most active. Amongst the tocotrienols, only α-tocotrienol has been proven to have significant vitamin E activity (Ingold et al. 1990; Bender & Mayes 2003). The vitamin E activity for the different tocochromanols is calculated using the equation (Bramley et al. 2000):

$$C_E = C_\alpha + C_\beta \times 0.5 + C_\gamma \times 0.1 + C_\delta \times 0.03 + C_{\alpha_3} \times 0.3 \qquad (1)$$

where C_α = α-tocopherol content; C_β = β-tocopherol content; C_γ = γ-tocopherol; C_δ = δ-tocopherol; and C_{α_3} = α-tocotrienol content. All tocochromanols are antioxidants but tocotrienols are suggested to suppress reactive oxygen species more efficiently than tocopherols (Schaffer et al. 2005). The most prominent function of tocopherols is protection of polyunsaturated fatty acids from lipid peroxidation by quenching and scavenging various reactive oxygen species (Munné-Bosch & Alegre 2002). Tocopherols are lipid-soluble and are normally found in seeds and other fatty parts of the plant (Collakova & DellaPenna 2003). Therefore vitamin E deficiency is a rare phenomenon in the Western world due to high intake of different staple foods containing tocochromanols (Bramley et al. 2000). The essential Vitamin E is required for muscle, immune and neural functions (Pryor 2000; Brigelius-Flohe et al. 2002). The tocochromanols are also suggested to be involved in reducing the risk of cardiovascular disease, cancer and cataracts (Pryor 2000; Brigelius-Flohe et al. 2002). The functions of tocopherols in plants are suggested to be protection of the chloroplast membranes from photooxidation and creating a supportive environment for

the photosynthetic functions (Fryer 1992; Munné-Bosch & Alegre 2002). Many of the proposed functions of tocochromanols are generally related to their antioxidant properties (Fukuzawa & Gebicky 1983; Munné-Bosch & Alegre 2002).

The synthesis of tocopherols and tocotrienols takes place in the chloroplasts. In general, the tocopherol consists of a polar chromanol ring and a 15-carbon lipophilic prenyl chain derived from homogentisic acid and phytyl diphosphate. The last part of the biosynthesis of tocopherols starts from 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ), which can later be methylated to 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) (Figure 1) (Collakova & DellaPenna 2003). The MPBQ and DMPBQ can be synthesised to δ- and γ-tocopherol, respectively, and δ- and α-tocopherol can be synthesised to β- and α-tocopherol, respectively (Collakova & DellaPenna 2003). The different steps in the synthesis involve enzymatic activities (Collakova & DellaPenna 2003).

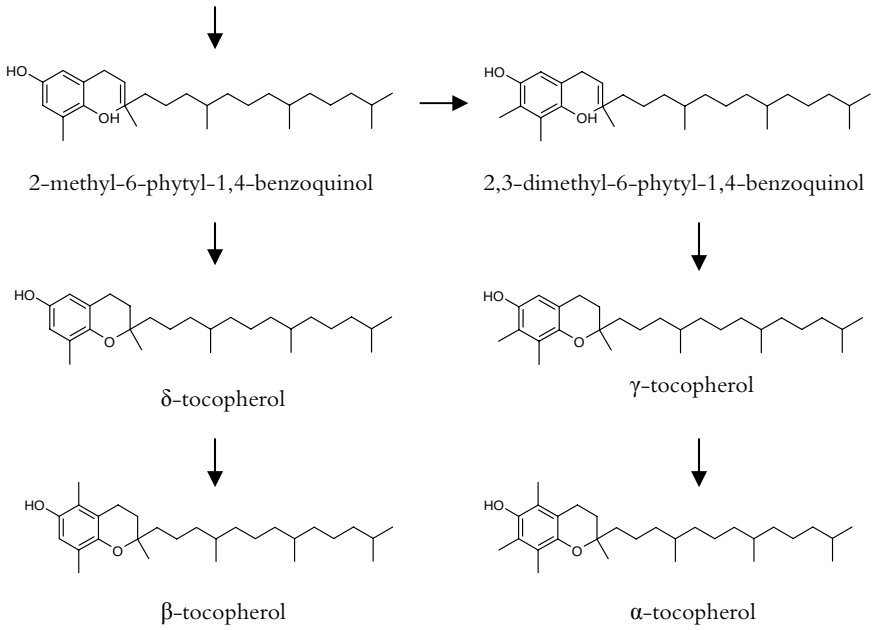

Figure 1. Later stages of tocopherol biosynthesis with structures of tocopherol compounds.

The synthesis of tocopherols is affected by different stress factors, such as drought, low temperature and high light intensity (Shigeoka et al. 1986; Tanaka et al. 1990; Moran et al. 1994; Leipner et al. 1999). Low stress levels might induce increased levels of antioxidants and with higher stress levels the antioxidant content increases, until the stress induce damages where no production of antioxidants are possible (Munné-Bosch & Alegre 2002). Annual variations in tocopherols have e.g. been found in olive oil (Failla et al. 2002). Seasonal variations have been found e.g. in drought-stressed leaves of *Salvia officinalis*, with the highest levels during the period with the lowest degree of drought stress and the lowest levels of α-tocopherol found at midday (Munne-Bosch et al. 2001).

Health effects of tocopherols

The lipid-soluble vitamin E is necessary in animal cells for different antioxidant functions, especially in cell membranes and plasma lipoproteins. It participates in preventing the proliferation of oxidative chain reactions, and it plays possible roles in atherosclerosis and cancer prevention (Lopaczynski & Zeisel 2001; Bender & Mayes 2003). However, investigations on humans have shown that supplements of vitamin E may result in negative health effects for heavy smokers, enhancing carcinogenesis in the lung (Heinonen et al. 1994; Albanes et al. 1996). A lower incidence of prostate and colorectal cancer but higher cancer rates in the stomach are also reported with vitamin E supplements (Albanes et al. 1995). The reasons for these conflicting results are still unclear. A number of epidemiological investigations related to correlations of cardiovascular events and tocopherol intake show a positive relationship between intake of tocopherol and reduction of disease (Asplund 2002). Investigations in different *in vitro* and *in vivo* studies show a correlation between tocochromanols and lower cholesterol levels (Schaffer et al. 2005). The tocochromanols have been suggested to be an essential part of the diet in preventing Alzheimer's disease (Morris et al. 2005).

Carotenoids

The carotenoids consist of over 600 fat-soluble pigments, providing yellow to red colours in nature (Krinsky & Johnson 2005). The carotenoids in plants are normally C_{40} tetraterpenoids which consist of 5-carbon isoprenoid units (Rodriguez-Amaya 2001) (Figure 2). The carotenoids can be categorised into xanthophylls and carotenes, but some carotenoids can also become esterified. The xanthophylls are oxygenated and therefore normally

more polar and visible at lower wavelengths than many of the carotenes (Krinsky & Johnson 2005). Examples of xanthophylls are neochrome, neoxanthin, violaxanthin, rubixanthin, lutein, zeaxanthin and β-cryptoxanthin (Figure 3). The carotenes are normally less polar and therefore visible at higher wavelengths. Examples of the carotenes are α-, β-, γ-, ζ-carotene and lycopene.

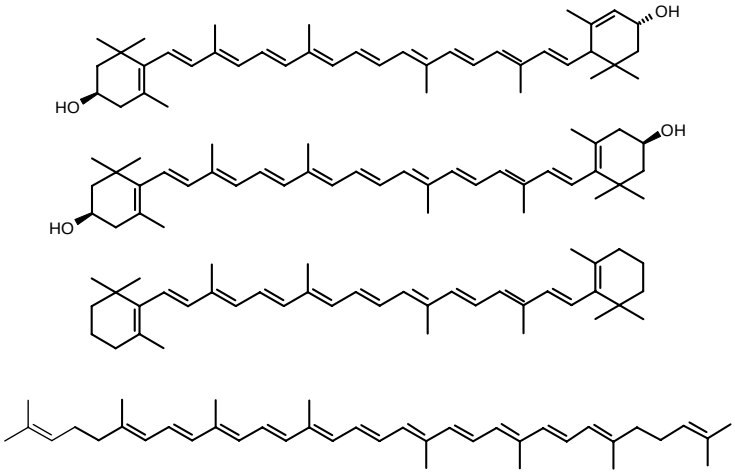

Figure 2. Structure of lutein (top), zeaxanthin (second from top), β-carotene (third from top), and lycopene (bottom).

About 10% of the carotenoids found in fruits and berries are pro-vitamin A and can be converted into retinol (Martin et al. 1999). The basic requirement for a carotenoid to have vitamin A activity is an unsubstituted β ring with a C_{11} polyene chain (Rodriguez-Amaya 2001). β-carotene has 100% pro-vitamin A activity, while carotenoids with one unsubstituted β ring, *e.g.* α-, γ-, δ-carotene and β-cryptoxanthin, have about 50% activity (Rodriguez-Amaya 2001). Other carotenoids missing β rings or containing a β ring with hydroxy, epoxy and carbonyl substitutes are not pro-vitamin A, with some exceptions (Rodriguez-Amaya 2001). Examples of such carotenoids are lycopene, ζ-carotene, rubixanthin, lutein and zeaxanthin. In ripe fruits most carotenols (hydroxycarotenoids) are esterified with fatty acids (Rodriguez-Amaya 2001), *e.g.* lutein, zeaxanthin, β-cryptoxanthin and rubixanthin.

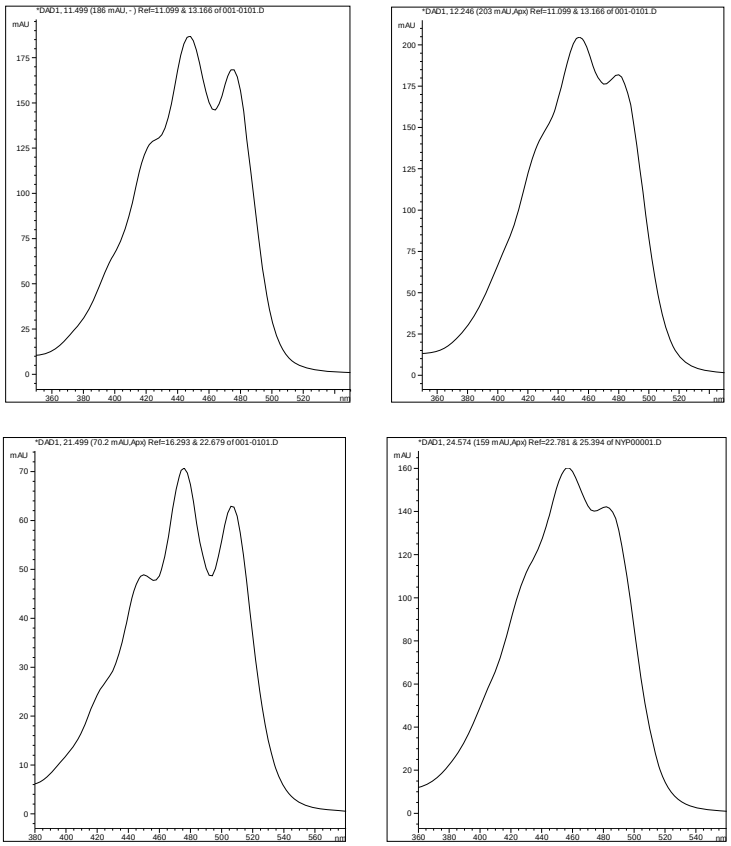

Figure 3. Spectra of standards of lutein (top left) at 11.5 min (λ_{max} 448, 474 nm), zeaxanthin (top right) at 12.2 min (λ_{max} 454, 480 nm), lycopene (bottom left) at 21.5 min (λ_{max} 450, 476, 506 nm), and β-carotene (bottom right) at 24. 6 min (λ_{max} 458, 482 nm).

The carotenoids such as β-carotene and lutein are involved in photosynthesis by harvesting light and act as co-pigments to the chlorophylls. (Demmig-Adams et al. 1996). Other carotenoids protect the photosynthetic system through the xanthophyll cycle, and eliminate singlet oxygen formed during photosynthesis (Halliwell & Gutteridge 1989; Demmig-Adams et al. 1996). Different carotenoids differ in strength of antioxidant activity (Terao 1989).

In the early stages of the synthesis of carotenoids, 5-carbon isoprenoid units are built and these later form the first compound of the C_{40} tetraterpenoids, the phytoene (Rodriguez-Amaya 2001). The later stages of

carotenoid biosynthesis are schematically drawn in Figure 4 (Rodriguez-Amaya 2001). The different steps in the biosynthesis involve various reactions, *i.e.* desaturation, cyclisation, hydroxylation, epoxidation and epoxidefuranoxide rearrangement (Rodriguez-Amaya 2001).

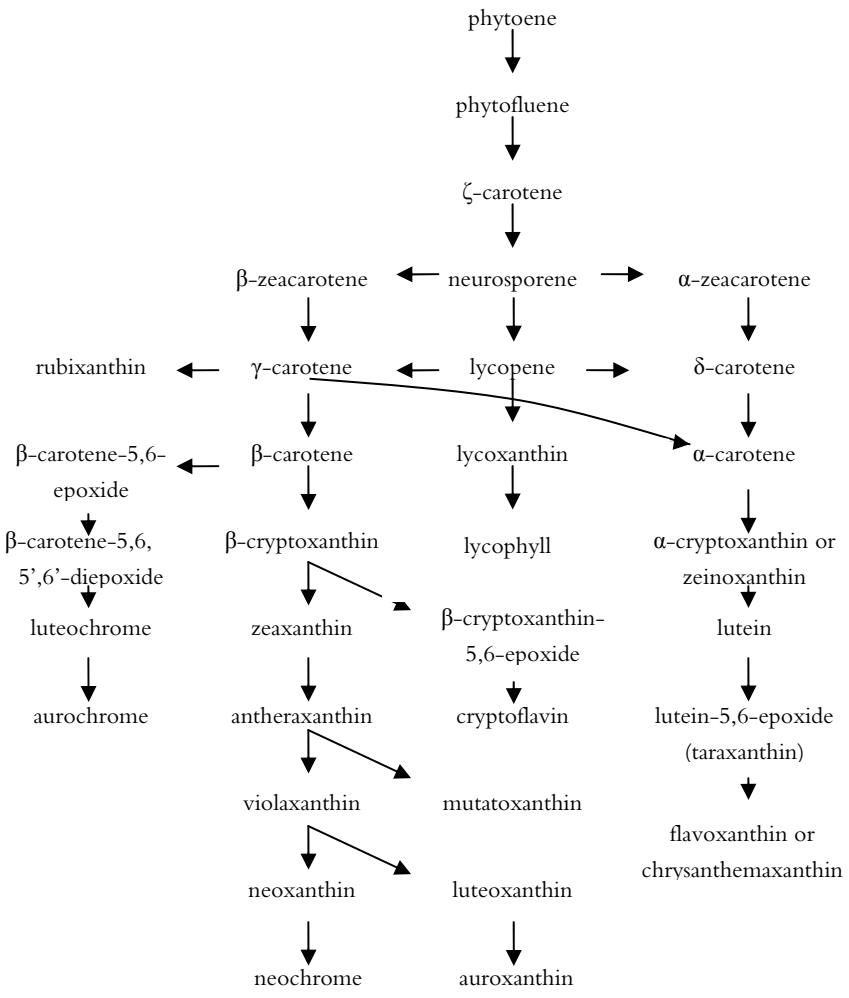

Figure 4. Later stages of carotenoid biosynthesis and possible transformations of carotenoids.

Investigations have shown that the biosynthesis of carotenoids is influenced by temperature and irradiation, but light is not essential for carotenogenesis (Goodwin 1980). β-carotene, lutein, and zeaxanthin have been shown to increase in sun-exposed leaves compared with shaded leaves (Demmig-Adams et al. 1996), while capsorubin in fruit of *Capsicum annuum* is enhanced by shade (Lopez et al. 1986). Temperature has been shown to affect lycopene biosynthesis in tomatoes, with highest production at 12-21 °C and inhibition above 30 °C (Tomes 1963). For β-carotene, no temperature effect for production has been found (Goodwin 1980). For optimal accumulation of carotenoids in sea buckthorn berries, warm and sunny weather with moderate precipitation has been suggested (Bekker & Glushenkova 2001).

Health effects of carotenoids

Fruit and vegetables rich in carotenoids have in epidemiological studies been associated with health, specifically with a decreasd risk of various diseases such as different cancers, cardiovascular and eye diseases (Krinsky & Johnson 2005). Only about 25 carotenoids have been found in human blood and tissues, and the most investigated carotenoids are β-carotene, lycopene, lutein and zeaxanthin (Krinsky & Johnson 2005). A number of epidemiological investigations comparing the risk of various cancer forms and intake of different carotenoids have shown an inverse relationship between intake of carotenoids and risk of cancer (Krinsky & Johnson 2005). Some of these studies also showed a positive correlation between increased intake of β-carotenoid supplement and risk of cancer and smoking, alcohol intake and people with asbestos lung (Heinonen et al. 1994; Omenn et al. 1996). However, another study comparing intake from fruit and vegetables, and supplements of β-carotene, did not find any increased risk for lung cancer among smokers and asbestos exposed people for fruits and vegetables, while supplements effected negatively on the uptake of protective compounds from the food (Neuhouser et al. 2003). Results from epidemiological studies on intake of different carotenoids and the risk of coronary vascular disease have shown both a decreased risk and no significant relationship (Asplund 2002; Krinsky & Johnson 2005). One study showed that extra intake of β-carotene and vitamin A supplements gave a non-significant increase in the risk of mortality in a group of smokers and asbestos-exposed people (Evans et al. 1998). The protective mechanism of the carotenoids is suggested to be as antioxidants to protect the low-density lipoproteins from being oxidised. Low-density lipoproteins have also been

shown to be the major transporter of lycopene and β-carotene in the blood system (Krinsky & Johnson 2005).

Different carotenoids have been investigated for their protective effects against sun irradiation. Investigations examining whether different carotenoid supplements function as sun block on the skin have shown a positive correlation between carotenoid application and protection against irradiation (Offord et al. 2002; Aust et al. 2005; Darvin et al. 2007). Lutein and zeaxanthin are the only carotenoids found in the retina and lens of the eye, causing the yellow colour. Therefore, these carotenoids are suggested to protect the eyes from harmful irradiation and to prevent light-initiated oxidative damage, thus protecting against age-related deterioration and cataracts (Snodderly 1995; Yeum et al. 1995; Bernstein et al. 2001; Krinsky & Johnson 2005). Lutein and zeaxanthin in the eyes are suggested to be protective in two different ways, by functioning as antioxidants and thereby to protect against the specific oxidative stress created by metabolism and light (Ham 1983; Khachik et al. 1997), and as a filter, filtering out the blue light, which can be harmful to photoreceptors and retinal pigment epithelium (Ham 1983; Ham et al. 1984).

Chlorophylls

The chlorophylls are the photosynthetic pigments present in thylakoid membranes, contributing to the green colour of plants. Two different chlorophylls are found in higher plants: chlorophyll a and b, together with other chlorophyll metabolites, presented as spectra (Figure 5**)**. Chlorophyll consists of a tetrapyrrole ring structure with a magnesium atom in the middle and a phytol tail (Salisbury & Ross 1992). Pheophytin is a chlorophyll molecule with two hydrogen atoms instead of the magnesium atom (Salisbury & Ross 1992), and if the phytol tail is removed from the pheophytin it becomes a pheophorbide molecule (Johnson-Flanagan & Thiagarajah 1990). Investigations have shown that the content of chlorophyll declines during ripening in many fruits and berries. Chlorophyll content is used for indicating ripeness in different fruits, such as apple, banana and mango (Knee 1980; Li et al. 1997; Bron et al. 2004).

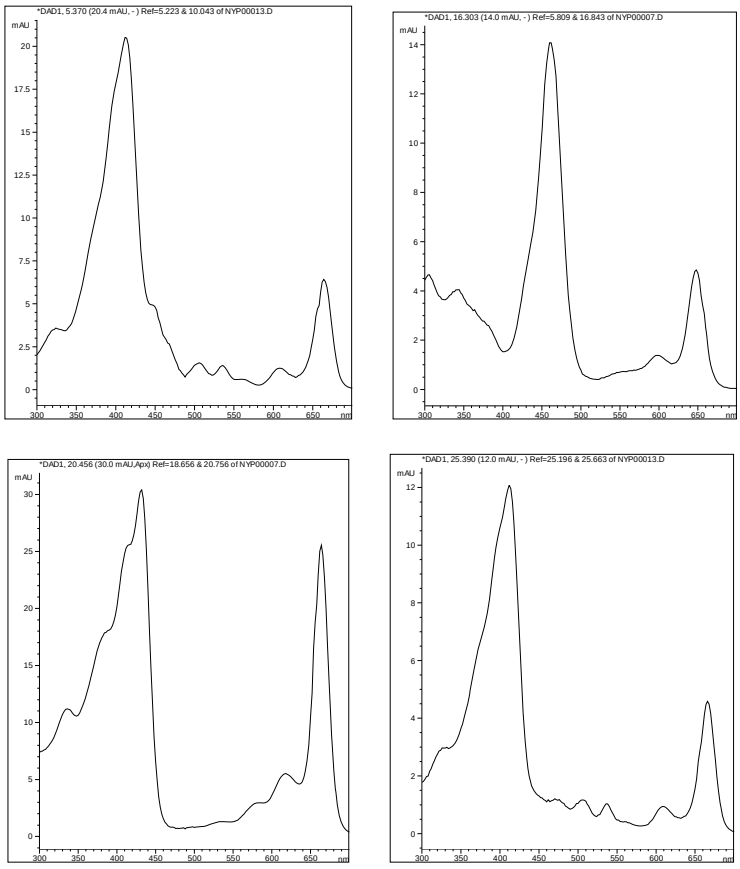

Figure 5. Spectra of pheophorbide a (top left) at 5.4 min (λ_{max} 412, 664 nm), chlorophyll b (top right) at 16.3 min (λ_{max} 462, 648 nm), chlorophyll a (bottom left) at 20.5 min (λ_{max} 432, 664 nm), and pheophytin a (bottom right) at 25.4 min (λ_{max} 412, 666 nm) (from analyses of *R. rubiginosa*).

Variations in content of tocochromanols, carotenoids and chlorophylls

The bioactive compounds in plants can differ in content and composition between families, genera, species, cultivars and genotypes, but also between years, climate zones and ripening and harvesting time. Other pre- and post-harvest factors can affect the content and it can differ in different parts of the plant.

Genetic diversification

The bioactive compounds in plants can differ in both content and composition between different families, genera, species and genotypes. Sea buckthorn berries exhibit differences in carotenoids and tocopherols between different cultivars/genotypes (Gao et al. 2000b; Kallio et al. 2002b; Ranjith et al. 2006). Rose hips from different species have differences in carotenoid content (Gao et al. 2000a). Many rose hips contain the carotenoid rubixanthin, which is found in *Rosa* sp. and in a few other plants (Rodriguez-Amaya 2001), but also contain common carotenoids, such as β-carotene, lycopene, lutein and zeaxanthin commonly to many other plants.

Annual variation/environmental effect

Annual variations are mainly dependent on the climate, but differences in management, soil quality and other possible specific within-year activities, such as pathogens, may also affect the content of bioactive compounds. Climate factors have been shown to have a large influence on different bioactive compounds (Weston & Barth 1997). Temperature, irradiation and water availability during the annual growing season has been shown to affect the content of carotenoids and tocochromanols in fruit and vegetables to various extents (Weston & Barth 1997; Munné-Bosch & Alegre 2002).

Chlorophylls constitute an essential part of the light harvesting system that is used to capture light energy in the plants. The content of chlorophylls in photosynthetic plant parts is related to the amount of light the particular plant part receives, *i.e.* sun leaves contain more chlorophyll than shade leaves. In many fruits and berries there is a continual decrease in the content of chlorophylls during ripening, and this is sometimes used as a ripening indicator or marker.

Harvesting and ripening time

Harvesting time can largely influence the content of bioactive compounds in different plant parts. For α-tocopherol and other antioxidants a diurnal rhythm has been revealed (Franzen et al. 1991; Wildi & Lutz 1996; Munne-Bosch et al. 2001). Reasons for the variation during the day can be light and temperature, but may also be water-related, especially if results are based on fresh weight. There is also a risk of mechanical injury at harvest affecting the content of bioactive compounds. Further more, the degree of ripeness and methods of analyses may affect the content of various bioactive compounds. Access to a ripening indicator, external or internal, facilitates sampling at

proper time and thus makes comparisons among different samples more valid.

Storage

Storage conditions may affect the content of bioactive compounds depending on product, packaging, duration of storage, light, temperature, humidity and other environmental factors in the storage room. Both tocopherols and carotenoids are relatively stable compared with the water-soluble ascorbic acid, which decreases more quickly (Kalt 2005). Temperature is crucial for maintaining the bioactive compounds in a product (Wills et al. 2007). Lower temperature decreases metabolic activity, although if too low the product may be damaged (Wills et al. 2007). If light is used during storage, it may affect compounds involved in photosynthetic activity, such as carotenoids, tocopherols and chlorophyll, also non-photosynthetic activities involving enzymes may affect the content of bioactive compounds (Wills et al. 2007).

Processing and bioavailability

Processing methods involve heating, pressure, extractions and possible exposure to oxygen, as well as inclusion of antioxidants, and all these various treatments may affect the content of certain bioactive compounds. Different investigations of sea buckthorn berries and rose hips have been studied methods of processing and the effects on bioactive compounds (Beveridge et al. 1999a; Singh et al. 2003; Gao et al. 2005; Nybom & Rumpunen 2005). Investigations have shown that the bioavailability differs between different tocopherols, tocotrienols and carotenoids, and that it is also affected by processing (Bramley et al. 2000; Krinsky & Johnson 2005). Furthermore, bioavailability of tocopherols and carotenoids is dependent on product, handling, additives to the food product, different processing methods such as cooking, chopping and addition of fat have been shown to increase the bioavailability for compounds such as lycopene ((Bramley et al. 2000; Krinsky & Johnson 2005). The carotenoids and tocochromanols are fat-soluble and have the same intestinal adsorption route as dietary fat.

Methodological aspects

This section gives an overview of the methodological aspects of Papers I-IV. For methodological details in specific studies, see the materials and methods section in the individual papers.

Field aspects

Sea buckthorn berries

All sea buckthorn berry material was collected from plants growing in the experimental fields at SLU, Balsgård, during the period 2004-2006. The layout of the plants in the field did not follow any statistical design, with plants of the same cultivar being grown together in rows aligned in the south-north direction (Figure 6). The spacing between sea buckthorn plants within rows was 1.0 m, and the distance between different cultivars was at most 30 m.

Rose hips

All rose hip material was collected from plants growing in the fields at SLU, Balsgård, during the period 2004-2006. The layout of the plants in the field did not follow any statistical design, with plants of the same species being grown together in rows aligned in the south-north direction, except for *R. dumalis* hybrid plants, which were grown together in a border next to the fields (Figure 7). The spacing between rose plants was 1.0 m and the distance between different cultivars/species was at most 40 m, except for the *R. dumalis* hybrid which were planted approximately 200 m apart from the other accessions.

Figure 6. Sea buckthorn plant about 2 m in height.

Figure 7. Rosa dumalis plant about 2 m in height.

Plant material

Sea buckthorn berries

The sea buckthorn cultivars used in the experiments were cv. Ljublitelskaja, originating from Russia, and three advanced selections, BHi72587, BHi72588 and BHi727102, originating from SLU Balsgård. The criteria for selection of different cultivars to be included in the experiment were high yielding bushes with good-sized berries, and a nice taste, besides sufficient harvesting material and proper growth habitus. Sea buckthorn berries were collected for use from five ramets of each species, which all were planted in the field year 2000 at the age of 2 years.

Rose hips

The rose hip species used in the experiments were *R. rubiginosa*, *R. dumalis*, *R. dumalis* hybrid and *R. spinosissima*. The criteria for selection of plant material to be included in the study were high yield, uniform ripening and availability of rose hips. For *R. spinosissima,* the change to ripe colour of the rose hips was observed to be about a month earlier than in the other rose hips investigated. Rose hips were collected from five ramets of *R. dumalis* and *R. rubiginosa*, three ramets of *R. dumalis* hybrid, and two ramets of *R. spinosissima*. All Roses were planted in the field as one-year-old plants in 2000 except for *R. dumalis* hybrid, which was planted in 1995.

Harvesting

Sea buckthorn berries

Harvesting of sea buckthorn berries began when the colour changed from green to the characteristic yellow to red colour, and ended when over-ripeness was apparent as rancid aroma and desiccation (Figure 8). Harvesting was carried out during daytime and branches with berries were cut and regularly moved from the field into -20 °C for short time storage. When the full harvest was carried out for the whole season, the berries were loosened gently from twigs and leaves and small twigs were removed in a berry rinse at 8 °C. The berries were then transferred to long-term storage at -80 °C. Sea buckthorn berries from the same cultivar were pooled on each harvesting occasion to one sample, to give a representative and homogenous sample with sufficient material throughout the harvesting season.

Figure 8. Hippophae rhamnoides berries (BHi727102) harvested on different dates in 2005. The berries are frozen.

Rose hips

Harvesting of rose hips began when the rose hips changed from green to red, and ended when over-ripeness was apparent as general fruit desiccation on the plants (Figure 9). Harvesting was carried out by hand during daytime and fruits were regularly moved from the field into -20 °C. When the full harvest was carried out, the fruits were transferred to long-term storage at -80 °C. All rose hips from the same species were pooled on each harvesting occasion to one sample to give a representative and homogenous sample as well sufficient material throughout the harvesting season.

Figure 9. Rose hips of *R. rubiginosa* harvested on different dates in 2005. The hips are frozen.

Sample preparation

For sample preparation, frozen sea buckthorn berries (40-80 g) were crushed in a mortar and then lyophilised for four days. The frozen rose hip samples (about 40 g) were cut in half and lyophilised for four days. Seeds were removed from the lyophilised fruits and berries and the soft parts were ground to a powder. For each of the triplicate samples, 1 g milled material was extracted with 20 mL ethanol:n-hexane (4:3 v/v) in sealed tubes in darkness for 20 hours at 4°C for carotenoid, chlorophyll and tocopherol analyses. Extraction trials were carried out with ethanol:n-hexane in different concentrations for carotenoids and tocochromanols and with 80% acetone for chlorophylls (its commonly used extractant). For identifying the esterified carotenoids, saponification trials were performed according to (Granado et al. 2001). However, the ordinary samples were not saponified

due to difficulties in obtaining uniform replicates with a number of samples. This problem has been recognised previously and is attributed to the fact that hydroxy- and epoxycarotenoids (lutein, violaxanthin, neoxanthin, rubixanthin) can be reduced during saponification (Khachik et al. 1986; Rodriguez-Amaya 2001). However, the pro-vitamin A carotenoids (α-, β-, γ-carotene and β-cryptoxanthin) can resist saponification (Rodriguez-Amaya 2001). The samples were centrifuged and thereafter placed in vials for direct analysis of carotenoids. Back-up samples and samples for tocochromanol analyses were stored at -80 °C.

Chemical analyses

For both sea buckthorn berries and rose hips, the carotenoids and chlorophylls were analysed by reversed phase high-performance liquid chromatography (HPLC) with a binary gradient, following a method modified from Kachik *et al.* (1991). The modifications involved evaluating by different columns, different concentrations of chemicals and different speeds of flow and binary gradients. A diode array detector was used for quantification, while identification was made by comparisons with standards of lutein, zeaxanthin, β-cryptoxanthin, lycopene and β-carotene (Extrasynthese, Genay, France) and with literature data (Khachik et al. 1989; Yamauchi & Watada 1993; Britton et al. 1995; Rodriguez-Amaya 2001). For carotenoid quantification the standard used was *all-trans*-β-carotene, which was quantified in a spectrophotometer before each HPLC analysis due to possible breakdown (Chen & Yang 1992). For the tocochromanol analysis, methods were modified from Panfili *et al.* (2003). The tocopherols were analysed by an isocratic HPLC method with a gradient. Different gradients for separation were evaluated before the method was finalised. However, no esterified tocopherols could be found and therefore no saponification trials were carried out for the tocopherols. A fluorescent detector was used for quantification. The tocopherols were identified using standards for all four tocopherols (Calbiochem, Merck, Darmstadt, Germany), while the tocotrienols were identified by comparison with literature data (Abidi 2000; Kamal-Eldin et al. 2000; Panfili et al. 2003). All tocopherols and tocotrienols were quantified with a standard of α-tocopherol (DL-R-tocopherol 97%; Alfa Aesar, Karlsruhe, Germany). Chlorophylls were identified by comparisons with literature data (Johnson-Flanagan & Thiagarajah 1990). All results are presented in terms of dry matter content.

Statistics

GLM

Statistical analyses of HPLC results were performed on three replicate samples of each cultivar and harvesting date. General linear model (GLM) analyses were used, followed by *t*-test for comparisons of means for each parameter, harvest, date, year and cultivar. The significance level was set at $P<0.05$ (SAS Institute Inc., Cary, NC).

Correlations

Pearson correlation coefficients were calculated for specific compounds and totals (*i.e.* total amount of carotenes, carotenoids, *etc.*) for the different harvesting dates, species/cultivars and years, using Minitab 15 (Minitab Inc.). Correlations were also calculated for climate data and specific compounds/totals for different harvesting dates, species/cultivars and years. Climate data (Figure 10) were obtained from the Swedish Meteorological and Hydrological Institute (SMHI) at Kristianstad airport (station 1651).

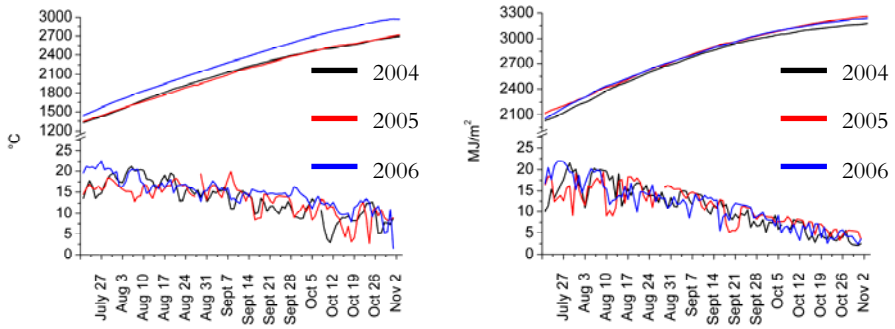

Figure 10. Daily means and sums (from metrological spring) of temperature (left) and global irradiation (right) during the period 2004-2006.

Conversion of harvesting time into ripening time

Analyses of total amounts of carotenoids and chlorophylls revealed a strong correlation between the total amount of chlorophylls including derivates in the various species/cultivars and years over the whole harvesting period (*i.e.* the total amount of chlorophylls varied similarly between years and cultivars). The general decrease in the amount of total chlorophylls including derivates was used to convert harvesting date into ripening time for each

year, in order to investigate the usefulness of the chlorophylls and derivates as a measurable marker for berry ripening. The whole annual harvest for each species was converted into an estimated ripening time by fitting the cultivars by the whole sequence of weekly harvesting dates to the content of total chlorophylls including derivates (Figure 11). The best fit was obtained by comparing the coefficient of variation for chlorophyll at all ripening times for all cultivars and years (only comparable data), with the smallest value as selection criterion (see example CV in Figure 11). Ripening time was evaluated by comparing the coefficient of variation for all compounds investigated at all ripening times for all cultivars and years with the smallest value as positive criterion. The ripening time was plotted against the mean content of total chlorophylls including derivates, a trend line was added, and an equation was derived (Excel 2003, Microsoft).

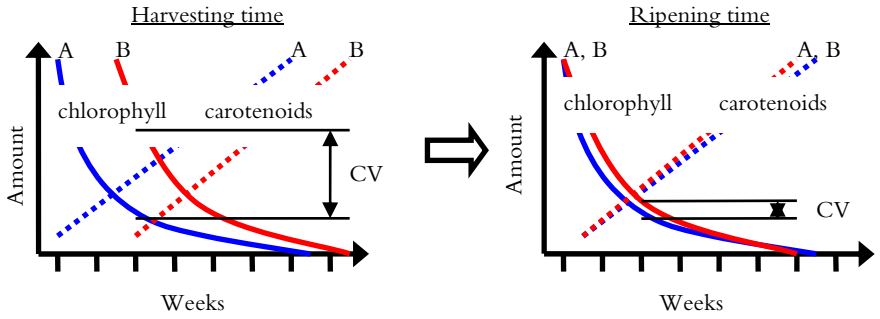

Figure 11. Schematic conversion of harvesting time into ripening time. A (blue) and B (red) represent different species/cultivars. Filled lines represent chlorophyll and dotted lines represent carotenoids. CV is an example of the values compared for calculating the coefficient of variation to estimate the best fit.

Processing and storability

Tocopherols and carotenoids were investigated for their stability during processing, using methods such as pasteurisation and homogenisation of juice containing sea buckthorn berries and rose hips. The juice mixtures of rose hip (*R. dumalis*) and sea buckthorn berry (cv. Sunny) were also evaluated for taste, aroma, mouth feel, colour, sweetness, *etc.* (results not shown). The juice included ingredients such as purée of sea buckthorn berries and fresh rose hips or dry rose hip powder, with or without oil. The

amounts of the respective ingredient and size of sample (fresh weight) varied (results not shown). However, the total amounts of ingredients in the juice samples were of equal volume in all samples, except for juices without oil and/or rose hip purée, which were complemented with water and milled rose hips to an equal total amount. In one batch, the juices were mixed, homogenised and pasteurised, and samples were taken before and after pasteurisation. In another batch, juices were mixed, pasteurised, packed in Tetrapaks and stored for up to 35 days at 4 °C or 20 °C. Samples were taken on days 0, 5, 10, 21 and 35. All the samples were lyophilised and analysed as triplicates according to the methods described in Papers I-IV. All results are presented in dry weight

Results and discussion

Content of tocochromanols, carotenoids and chlorophylls

Sea buckthorn berries

The tocochromanols identified in sea buckthorn berries were α-, β-, γ-, δ-tocopherol and α-, γ-, δ-tocotrienol (Paper I), confirming results in other studies (Kallio et al. 2002a; Kallio et al. 2002b; Zadernowski et al. 2003; Cenkowski et al. 2006; Ranjith et al. 2006). The total tocochromanol content was found to be in the range 316.6-1250.9 μg/g dry weight (DW), which corresponds to 4.6-12.4 mg/100g fresh weight (FW). This range has also been reported in previous investigations (Beveridge et al. 1999a; Kallio et al. 2002b). Compared with other fruits and berries, the total tocochromanol content is relatively high in sea buckthorn berries (Pirronen et al., 2002; USDA 2009), due mainly to the fact that tocochromanols are primarily found in seeds of other products. The mean relative amounts of different tocochromanols found were 65% α-tocopherol, 3% γ-tocopherol, 18% δ-tocopherol, 5% α-tocotrienol, and 8% δ-tocotrienol.

The major carotenoids found in the sea buckthorn berries were lutein, zeaxanthin, β-cryptoxanthin, lycopene, γ-carotene, β-carotene and esterified carotenoids (Paper II**)**. This is in agreement with earlier findings (Kudritskaya et al. 1990; Beveridge et al. 1999b; Gao et al. 2000b; Yang & Kallio 2002; Raffo et al. 2004; Pintea et al. 2005). The total content of carotenoids was in the range 119.9-1424.9 μg/g DW, which corresponds to 1.5-18.5 mg/100g FW, also in the same range as other findings (Gao et al. 2000b; Raffo et al. 2004). In comparison with other fruits and berries, sea buckthorn berries have a relatively high content of carotenoids (Olsson et al.

2004). The relative mean amounts of different carotenoids in sea buckthorn berries were 1% lutein, 8% zeaxanthin, 0.3% β-cryptoxanthin, 8% lycopene, 4% γ-carotene, 14% β-carotene and 10 % minor carotenoids. The esterified carotenoids corresponded to 55% of the total carotenoids.

In the sea buckthorn berries, the only chlorophyll compound found was pheophytin a (Paper II), in concentrations in the range 264.2 μg/g DW to below detectable limits, which corresponds to 3.3 mg/g FW. Pheophytin a is a metabolite of chlorophyll a and therefore it may be possible to find chlorophyll a in unripe berries earlier in the season.

Rose hips

In the rose hips investigated, only α- and γ-tocopherol were found and the total tocopherol content was in the range 99.3-240.2 μg/g DW, corresponding to 2.7-5.2 mg/g FW (Paper IV). α-tocopherol made up 90% of the total tocopherols found. Earlier investigations of tocopherols in rose hips pulp are few (Piironen et al. 2002; Yoruk et al. 2008; Kazaz et al. 2009), and the amount of α- and γ-tocopherol are in the same range as Piironen *et al.*, (2002). In relation to other fruits and berries, the content of tocopherols in rose hips is relatively high (Piironen et al. 2002; USDA 2009). However, sea buckthorn berries has twice the amount of tocopherols in comparison to rose hips.

The carotenoids found in rose hips differed to some extent between the species investigated (Paper III). The carotenoids identified in all species were lutein & zeaxanthin and β-carotene. In *R. spinosissima*, the other carotenoids found were neochrome, neoxanthin, violaxanthin and one unknown xanthophyll and three unknown carotenes. In the other three species, besides the common carotenoids, rubixanthin, lycopene, prolycopene, γ- and ζ-carotene were also found. Esterified carotenoids were found in all species. All carotenoids identified have been reported in earlier investigations but some carotenoids reported previously were not identified, such as α-carotene, gazanixanthin, β-cryptoxanthin, antheraxanthin and auroxanthin (Valadon et al. 1975; Hornero-Mendez & Minguez-Mosquera 2000; Novruzov 2005). Thus no esterified carotenoids were identified in this study. The total carotenoid content was in the range 40.9-1590.4 μg/g DW (0.8-42.5 mg/100G FW), and this is in agreement with other reports (Jacoby & Wokes 1944; Valadon & Mummery 1969; Toth & Szabolcs 1970; Gao et al. 2000a; Olsson et al. 2005). In comparison with other fruits and

berries, rose hips have a high content of carotenoids (Olsson et al. 2004). Of the total carotenoids found in the rose hips investigated here, lutein & zeaxanthin made up 2%, β-carotene 22%, xanthophylls 5%, carotenes 48% and esterified carotenoids 47%. Lycopene corresponded to 25% of total carotenoids in the red-coloured rose hips.

In the rose hips, the chlorophyll compounds found was chlorophyll a, chlorophyll b, pheophytin a and pheophorbide a (Paper III), The total chlorophyll a content was in the range 360.9 μg/g DW to below detectable limits, which corresponds to 6.8 mg/g FW.

Genetic diversification

Sea buckthorn berries

There were large differences among the sea buckthorn cultivars investigated. For example, the total tocopherol content was almost two-fold higher between the highest and the lowest cultivar (Paper I). The mean content of tocochromanols in the different cultivars was 800.7 μg/g DW (9.4 mg/100g FW) in Ljublitelskaja, 552.6 μg/g DW (7.2 mg/100g FW) in BHi72587, 450.9 μg/g DW (5.5 mg/100g FW) in BHi72588 and 405.8 μg/g DW (5.7 mg/100g FW) in BHi727102 (Paper I).

The carotenoids in the sea buckthorn berries investigated here showed large differences between the species, with two of the species having about double the content in the other two cultivars (Paper II). The mean content of total carotenoids was 499.0 μg/g DW (5.9 mg/100g FW) in Ljublitelskaja, 1161.5 μg/g DW (15.1 mg/100g FW) in BHi72587, 1130.3 μg/g DW (13.8 mg/100g FW) in BHi72588 and 663.7 μg/g DW (9.4 mg/100g FW) in BHi727102.

Rose hips

The tocopherols found in the rose hips showed differences between the species investigated (Paper IV). The mean content of total tocopherols was 164.0 μg/g DW (4.1 mg/100g FW) in *R. rubiginosa*, 145.3 μg/g DW (3.9 mg/100g FW) in *R. dumalis*, 160.4 μg/g DW (4.3 mg/100g FW) *R. dumalis* hybrid, and 195.9 μg/g DW (4.4 mg/100g FW) in *R. spinosissima*. The species with higher content of α-tocopherol showed a lower content of γ-tocopherol and *vice versa*.

In the rose species investigated there were large differences between the total carotenoid content in the rose hips, with more than three-fold higher content in *R. dumalis* hybrid compared with *R. spinosissima* (Paper III). The mean content of total carotenoids was 726.0 µg/g DW (18.1 mg/100g FW) in *R. rubiginosa*, 629.3 µg/g DW (17.0 mg/100g FW) in *R. dumalis*, 1020.8 µg/g DW (27.5 mg/100g FW) in *R. dumalis* hybrid and 297.1 µg/g DW (6.7 mg/100g FW) in *R. spinosissima*.

Harvesting date

Sea buckthorn berries

During the harvesting period, the sea buckthorn berries showed decreasing tendency for amounts of total tocopherols, however the tendency was not regular for consecutive harvesting dates (Paper I). The predominant α-tocopherol, which contributes about 96% of the vitamin E activity, showed a general decrease during ripening (Figure 12). The largest decrease in α-tocopherol during the whole harvesting period for sea buckthorn berries was observed for cv. Ljublitelskaja in 2005, with a decrease of about 27% (486.1 to 355.4 µg/g DW) during the seven weeks of harvesting. This is in the same range as the differences between the species in terms of mean values (Paper I; Table 2). Differences between years had a smaller impact on the content of α-tocopherol.

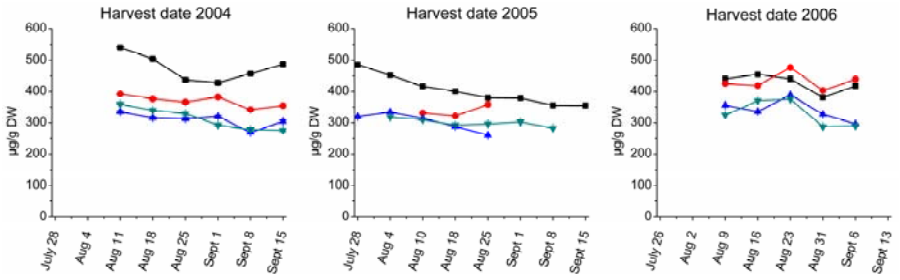

Figure 12. Content of α-tocopherol ± standard deviation (µg/g DW) in berries from four cultivars of sea buckthorn sampled on various harvesting dates over three years. Cultivars: Ljublitelskaja (■); BHi 72587 (●); BHi 72588 (▲); BHi 727102 (▼).

The total carotenoid content in sea buckthorn berries increased throughout the harvesting period (Figure 13), (Paper II). The largest increase

in total carotenoids during the whole harvesting period was observed for cultivar BHi727102 in 2005, with a five-fold increase (147.2 to 883.0 μg/g DW) during five weeks, which also shows the relatively fast increase between the harvesting times. However, the rate of increase in compounds is of less significance due to the length of the measuring period and the concentration of the compound on the first harvesting occasion. For example, if first harvest occasion has a relatively low content as 0.1 or 1.0 μg/g DW and the final harvest occasion has content of 100.0 μg/g DW, there is a 10-fold difference in the increase depending on the content of first harvest occasion.

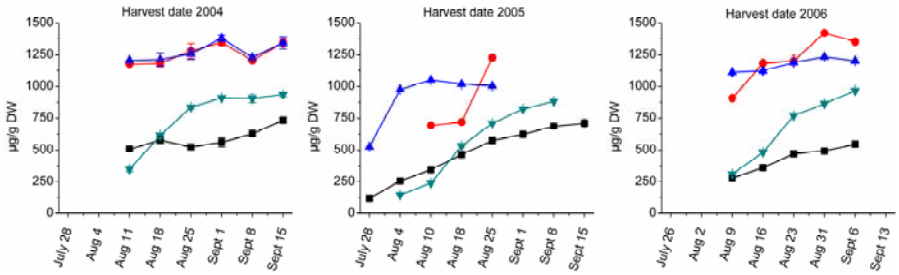

Figure 13. Total carotenoid content ± standard deviation (μg/g DW) in berries from four cultivars of sea buckthorn sampled on various harvesting dates over three years. Cultivars: Ljublitelskaja (■); BHi 72587 (●); BHi 72588 (▲); BHi 727102 (▼).

Rose hips

Due to the longer ripening period in rose hips, harvesting was investigated for a longer period of time than in sea buckthorn berries except in 2004. Amount of total tocopherols, γ- and α-tocopherol (Figure 14) showed irregularities over the harvesting period, although a general decreasing trend was noted (Paper III). For α-tocopherol the largest difference was observed in *R. spinosissima* in 2006, with a 1.5 fold increase (154.4 to 232.1 μg/g DW).

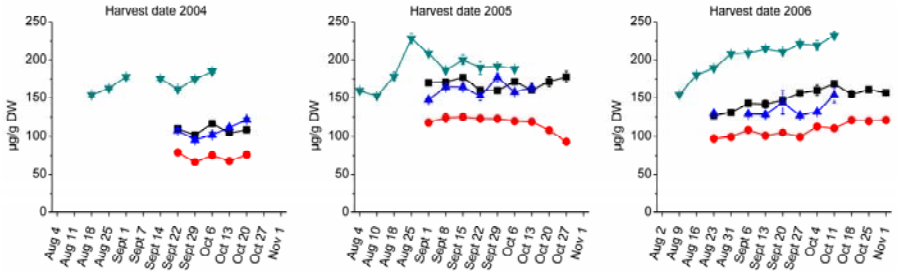

Figure 14. Content of α-tocopherol ± standard deviation (µg/g DW) in hips from four species of rose sampled on various harvesting dates over three years. Species: *R. rubiginosa* (■), *R. dumalis* (●), *R. dumalis* hybrid (▲) and *R. spinosissima* (▼).R. rubiginosa.

Amount of total carotenoids as well as other groups of carotenoids in the rose hips increased from the first to later harvesting dates (Oct 12 and Oct 19) (Figure 15), (Paper IV). The largest increase in total carotenoids was observed for *R. rubiginosa* in 2006, with about 16-fold increase (61.7 to 1050.6 µg/g DW). This increase tailed off at later harvesting dates for different carotenoids and groups of carotenoids in different species and years.

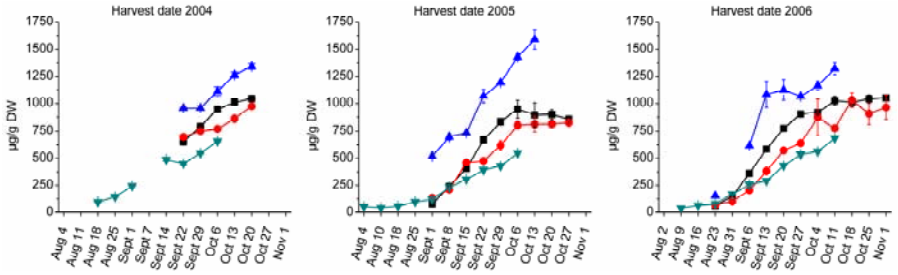

Figure 15. Total carotenoids ± standard deviation (µg/g DW) in hips from four species of rose sampled on various harvesting dates over three years. Species: *R. rubiginosa* (■), *R. dumalis* (●), *R. dumalis* hybrid (▲) and *R. spinosissima* (▼).

Ripening time

When comparing amounts of various compounds in different plant materials, accurate comparisons may be difficult due to different degree of ripening of the plants and associated accumulation of compounds. Use of an internal ripening scale with a valid marker can be practical when comparing plant materials. However, it is important that the compound used for the

marker has a similar pattern of development within all plant materials being compared. Thus, comparisons of the coefficient of variation of the harvesting and ripening time are problematic in terms of choosing the compounds to be included in the evaluation. In these studies the carotenoids showed more significant correlations between years and species than the tocochromanols, which did not show any consistent regular variation between the years investigated. The tocochromanols were therefore not included in evaluation of compounds for conversion to ripening time. The carotenoids impart most of the colour to maturing fruits, but can be visually masked by the chlorophylls, which mainly cause the green colour of immature fruits. When the fruit/berry ripens and changes to characteristic colour, a genetically controlled conversion of the chloroplasts into the carotenoid-accumulating chromoplasts takes place (Gillaspy et al. 1993; Bonora et al. 2000).

For sea buckthorn berries, conversion from harvesting time into ripening time resulted in a lower coefficient of variation for 71% of the comparisons made (60 out of 84) (Paper II) For rose hips, similar conversion resulted in a lower coefficient of variation for 57% of comparisons (115 out of 203) (Paper IV).

Annual variation – climate effect

The variation between years can be seen as a climate effect if the management practices remain consistent. There are other parameters besides annual fluctuations that affect the content of bioactive compounds, such as soil quality and pathogens. During this investigation no extraordinary events were observed that could explain variations between years, with the exception of excessive harvesting in one of the sea buckthorn cultivars (BHi72588) during 2004, where cutting of the plants limited the berries in the 2005 harvest. Differences in daily means and sums of solar irradiation and temperature during different years within the harvest period can be seen in Figure 10.

Sea buckthorn berries

In terms of variations between years in content of tocochromanols, the highest content of mean total tocopherols was found in 2004, 36% higher than in 2005 and about 10% higher than in 2006 (Paper I). The mean α-

tocopherol content was highest in 2006, about 4% higher than in 2004 and about 11% higher than in 2005.

The sea buckthorn berries also showed differences in content of different carotenoids and groups of carotenoids between different years (Paper II). The mean content of total carotenoids was 49% higher in 2004 than in 2005 and 11% higher in 2006 than in 2005.

Rose hips

Rose hips had a 37% higher content of mean total tocopherols in 2005 than in 2004 and 9% higher in 2006 than in 2004 (Paper IV). The mean a-tocopherol content in 2005 was about 33% higher than in 2004 and about 7% higher than in 2006.

The amounts of different carotenoids and groups of carotenoids varied between years (Paper III). The mean content of total carotenoids in 2004 was about 29% higher than in 2005 and about 24% higher than in 2006.

Correlations with climate data

For both sea buckthorn berries and rose hips, there were no consistent significant correlations between climate data and tocopherols for all three years investigated (Paper I and Paper IV). For sea buckthorn berries, the most consistent significant correlations were found between pheophytin a and esterified carotenoids, and between cumulative irradiation and cumulative temperature for most years and cultivars (Paper II), which reflects the continuous decreasing/increasing trends of the compounds pheophytin a and esterified carotenoids. For rose hips, the most significant correlations were found between total carotenoids, total carotenes, lycopene, prolycopene and total a chlorophylls, and different climate data including cumulative sums (Paper III). There were also many significant correlations between amounts of certain compounds in different species during different years.

Dry weight

When presenting data on bioactive compounds, *e.g.* carotenoids, tocopherols and chlorophylls, it is common to present the results based on either dry or fresh weight. However, the results in Papers I-IV are presented in terms of dry weight, partly to limit the influence of possible short-term

weather fluctuations resulting in change in the fresh weight. The dry matter content of sea buckthorn berries, all years (Table 1) was 9.9-12.7% for Ljublitelskaja, 11.9-14.7% for BHi72587, 10.9-13.6% for BHi72588 and 12.2-15.5% for BHi727102 (Papers I-II). The dry matter content of rose hips, all years (Table 2) was 16.5-28.3% for *R. rubiginosa*, 21.8-30.0% for *R. dumalis*, 21.1-31.2% for *R. dumalis* hybrid and 16.7-27.7% for *R. spinosissima* (Papers III-IV).

Table 1. *Dry matter content (%) of berries from four sea buckthorn cultivars at different harvesting times and in different years*

	2004				2005				2006			
AHD	Ljub	587	588	7102	Ljub	587	588	7102	Ljub	587	588	7102
July 28					12.6		12.3					
Aug 4					11.7		12.4	13.8				
Aug 10	10.9	13.2	11.7	13.5	12.7	12.5	13.6	14.8	12.7	14.7	13.5	15.5
Aug 17	11.2	12.8	11.6	13.7	12.2	12.5	12.5	15.0	12.0	13.5	13.1	14.1
Aug 24	10.6	12.4	11.4	14.6	12.5	13.6	11.9	15.2	12.0	13.7	12.5	13.9
Sept 1	11.1	11.9	10.9	13.8	12.1			14.7	11.9	13.0	12.2	12.2
Sept 7	9.9	12.1	11.4	13.8	12.5			14.5	11.9	13.2	12.4	13.4
Sept 14	10.1	12.3	11.2	13.7	12.1							

AHD = average harvesting date. Ljub = Ljublitelskaja, 587 = BHi72587, 588 = BHi72588, 7102 = BHi727102.

Table 2. *Dry matter content (%) of hips from four species of rose at different harvesting times and in different years*

	2004				2005				2006			
AHD	rub	dum	hyb	spin	rub	dum	hyb	spin	rub	dum	hyb	spin
Aug 4								16.7				
Aug 10								18.5				18.9
Aug 17				20.1				21.3				18.9
Aug 24				22.3				21.7	16.5	21.8	21.1	19.1
Sept 1				19.9	18.6	23.8	26.2	22.3	20.4	22.6	-	21.8
Sept 7				-	20.4	24.1	28.3	23.6	22.6	23.6	26.6	22.9
Sept 14				25.8	22.7	26.5	26.7	23.3	24.8	26.6	28.1	24.1
Sept 21	25.9	26.9	24.6	27.2	24.3	26.3	27.9	24.4	26.5	27.0	27.3	24.8
Sept 28	26.2	27.3	25.5	27.7	26.0	27.5	27.4	24.6	26.4	28.3	31.2	24.0
Oct 5	26.1	27.6	25.6	27.7	25.8	28.5	27.3	24.3	25.9	27.4	31.2	22.7
Oct 12	28.3	28.3	28.4		27.0	28.7	26.7		26.8	28.2	28.0	23.9
Oct 19	25.4	26.7	24.1		28.1	29.9			28.1	29.0		
Oct 26					27.9	30.0			26.5	29.4		
Nov 1									25.3	29.9		

AHD = average harvesting date. rub = *R. rubiginosa*, dum = *R. dumalis*, hyb = *R. dumalis* hybrid, spin = *R. spinosissima*.

Processing and storage

Processing

The results from the processing and storage studies are summarised in terms of total carotenoid and α-tocopherol content (μg/g DW). The total carotenoids in different juices showed statistical differences before and after pasteurisation and homogenisation in the four juice mixtures (Figure 16, left). The decrease in total carotenoids due to pasteurisation and homogenisation was 11.1% for juice including purée of rose hips, 5.5% for juice including oil and purée of rose hips, 40.2% for juice including dried powder of rose hips, and 16.8% for juice including oil and dried powder of rose hips. The juices containing oil retained carotenoids better than the juices without oil. The oil used in the juices contained minor amounts of carotenoids compared with the fruit/berry raw material, especially in relation to the amounts of the respective ingredients used (< 2% of total). However, the oil is rich in α-tocopherol (<13% of total) and the smaller decrease of carotenoids in juices with oil can be due to antioxidative protection of carotenoids by the α-tocopherol (Figure 16, right). Previous

investigations have shown that tocopherols are involved in antioxidative relationships with carotenoids (Palozza & Krinsky 1992). The juices with dried and milled rose hips showed a larger relatively decrease in carotenoids compared with the juices with rose hip purée. This may have been due to the purée containing more antioxidants than the dry rose hips, since the drying process can harm antioxidants.

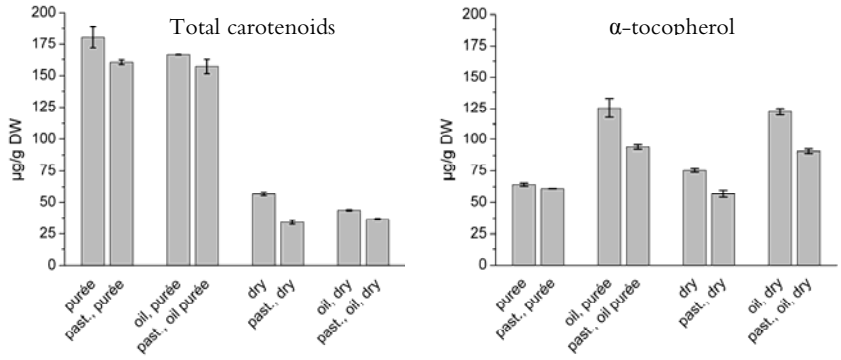

Figure 16. Content (µg/g DW) of total carotenoids (left) and α-tocopherol (right) before and after pasteurisation and homogenisation (past.) of juices containing ingredients such as purée of sea buckthorn berries, purée or dried powder (dry) of rose hips, without or without oil (oil). Results are means of triplicates ± standard deviation.

The α-tocopherol is the main form of tocochromanols in both rose hips and sea buckthorn berries (Paper I and Paper IV), as well in the oil used in some of the juices. The α-tocopherol in different juices showed statistical differences before and after pasteurisation (Figure 16, right). The decrease in α-tocopherol due to pasteurisation and homogenisation was 5.1% for juice including purée of rose hips, 25.2% for juice including oil and purée of rose hips, 24.4% for juice including dried powder of rose hips, and 25.9% for juice including oil and dried powder of rose hips. The juices containing oil showed a larger decrease in α-tocopherol compared with the juices without oil. As mentioned above, the reason may be the preventive effects of α-tocopherol for the carotenoids. The juice with rose hip purée without oil also showed a smaller decrease in amount of α-tocopherol compared with the other three juices, but a higher decrease in total carotenoids compared with the juice with purée and oil.

Storage

The total carotenoid content in juices with and without oil stored for up to 35 days at different temperatures was significantly different on day 0 compared with days 5, 10, 21 and 35 days (Figure 17, left). In terms of storage, the first days after pasteurisation and homogenisation proved to be critical for the decrease in carotenoids. After day 5, small or no changes were seen for total carotenoids in any juice. However, for storage at 20 °C until day 21 and day 35, the amounts of carotenoids were different for juices with and without oil, although the reasons for this are unclear. The largest decrease occurred between day 0 and day 5 for all juices, especially those containing oil. The decrease of total carotenoids was 43.6% for juice with oil stored at 20 °C; 32.4% for juice with oil stored at 4 °C; 29.1% for juice without oil stored at 20 °C and 5.5% for juice without oil stored at 4°C. The large decrease in carotenoids in the juices containing oil may depend on the carotenoid-friendly environment of the oil and possibly increased oxidation of the carotenoids.

The juice without oil stored at 4 °C showed the highest carotenoid content from day 5, while the juices stored at 20 °C showed the lowest mean content. Temperature had a large influence on the content of total carotenoids in juices during the first days of storage.

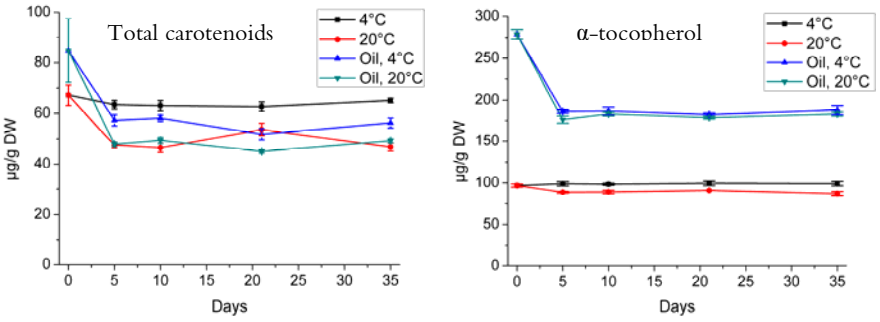

Figure 17. Content (µg/g DW) of total carotenoids (left) and α-tocopherol (right) during storage at 4 °C and 20 °C of pasteurised juice with ingredients such as purée of sea buckthorn berries, purée of rose hips, without or without oil (oil). Results are means of triplicates ± standard deviation.

The α-tocopherol content in pasteurised juices with oil stored for up to 35 days at different temperatures was significantly different on day 0 compared with days 5, 10, 21 and 35 (Figure 17, right). The decrease in α-tocopherol between day 0 and day 5 was 36.8% for juice with oil stored at 20 °C and 33.2% for juice with oil stored at 4°C, while after day 5 minor or no changes occurred. In juices without oil, the decrease in α-tocopherol was 8.5% for storage at 20 °C, while for storage at 4 °C no significant differences were found. The juices in the storage study were not homogenised, unlike the juices in the processing study. The impact of storage temperature on α-tocopherol content in the juices was of minor importance, and no or minor differences were found from day 5 to day 35.

Concluding remarks and future perspectives

This study investigated fat-soluble tocochromanols and carotenoids in different cultivars/species of sea buckthorn berries and rose hips during the harvesting period in different years. The different bioactive compounds were found to vary in content and composition as follows:

- The content of tocopherols was relatively high in both sea buckthorn berries and rose hips compared with other fruits and berries. α-tocopherol was the dominant tocochromanol, with the highest vitamin E activity.

- The content of carotenoids in sea buckthorn berries and rose hips was relatively high compared with other fruits and berries. The dominant carotenoids were generally lycopene and β-carotene, with β-carotene having pro-vitamin A effect.

- There were large variations in the contents of tocochromanols and carotenoids between the different species/cultivars of sea buckthorn and rose hips. Choice of species/cultivars is thus very important in improving the content of these bioactive compounds.

- Harvesting should be carried out earlier if a higher vitamin E activity is desired in sea buckthorn berries and rose hips. However, the differences between harvesting dates are relatively small and later harvest does not severely impair the vitamin activity. Harvesting time showed small decreasing trends for α-tocopherol and vitamin E activity in both sea buckthorn berries and rose hips. However, the total tocopherol content

increased slightly in sea buckthorn berries, while it decreased in rose hips.

- Late harvesting is recommended if a high content of carotenoids is desired in sea buckthorn berries and rose hips. Harvesting time caused major variations in carotenoid content, with an increasing trend for the major carotenoids and for pro-vitamin A activity.

- The decrease in chlorophyll content in both sea buckthorn berries and rose hips can be used for predicting harvesting time or for comparing plant material at earlier stages of harvesting. However, in rose hips there was a variation between the different species in terms of the decrease in chlorophylls in relation to the increase in carotenoids. In the red-coloured rose hips the consistent increase of prolycopne, lycopene, total carotenes and total carotenoids may be used as ripening indicators.

- Variations between years had an impact on the content of tocochromanols and carotenoids in both sea buckthorn berries and rose hips. When producing sea buckthorn berry and rose hip products for their tocochromanols and carotenoids, such annual variations should be taken into consideration when planning the final amounts.

- In pasteurised and homogenised juices with rose hips and sea buckthorn berries as raw materials, the content of carotenoids and α-tocopherol is dependent on the ingredients used in addition to the rose hip and sea buckthorn berries. Storage conditions in the first few days are critical, with the temperature affecting the carotenoid content in the juices, while the content of α-tocopherol is more affected by the ingredients used.

Future perspectives

In future investigations of tocochromanols or carotenoids in sea buckthorn berries, rose hips, some interesting areas include:

- Screening large quantities of genetic materials of different cultivars/species of sea buckthorn berries and rose hips to document the actual content and variation in tocochromanols and carotenoids. A useful measurement would be the ripening scales presented in this thesis.

- Identifying all the carotenoids in *R. spinosissima* and investigating whether other *Rosa* species have different carotenoids.

- Studying how the tocochromanols and carotenoids vary for different time spans, for example by harvesting fruits and berries at different states of ripeness every hour for 24 hours.

- Studying how the tocochromanols and carotenoids vary in different cultivars/species in different climates, by planting ramets in different geographical locations. However, this would be similar in some respects to our investigations in different years. Such a project would have to be planned some years in advance to get sufficient plant material for harvesting.

- Investigating different health aspects of rose hips and sea buckthorn berries, especially the fatty phase of the fruits and berries, including the tocochromanols and carotenoids. For example, having groups of human subjects (including a placebo group) drink different concentrations (or amounts) of juice with known amounts of bioactive compounds and investigating any differences between the groups, *e.g.* eyesight and cholesterol levels. External application of oil from sea buckthorn berries and rose hips could also be investigated for its effects on skin problems. Different *in vitro* studies on cancer cells, bacteria and artificial skin could investigate different aspects of sea buckthorn berries and rose hips. Studies comparing supplemental tocopherols and carotenoids with their natural equivalents would also be interesting, since existing data suggest avoiding excessive intake of supplement tocopherols and carotenoids.

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