THE APPLE SKIN: COLOURFUL HEALTHINESS
Developmental and environmental regulation of flavonoids and chlorogenic acid in apples

Mohamed Abdel-Ghani Awad

Promotoren: Prof. Dr. W.M.F. Jongen
       Hoogleraar in de Productontwerpen en Kwaliteitskunde

       Prof. Dr. L.H.W. van der Plas
       Hoogleraar in de Plantenfysiologie

Co-promoter: Dr. Ir. A. de Jager
       Senior Onderzoeker (PPO, Randwijk)

Committee: Prof. Dr. Habil. Dieter Treutter (Technische Universität München)
       Prof. Dr. O. van Kooten (Wageningen Universiteit)
       Prof. Dr. A. J. van Tunen (Universiteit van Amsterdam)
       Dr. C.M. Colijn-Hooymans (PPO, Wageningen)

THE APPLE SKIN: COLOURFUL HEALTHINESS
Developmental and environmental regulation of flavonoids and chlorogenic acid in apples

Mohamed Abdel-Ghani Awad

Propositions

1. The best of the apple is often thrown away.
   This thesis

2. Light is essential not only for the quantitative but also for the qualitative quality of apples.
   This thesis

3. Different flavonoid classes and chlorogenic acid are independently regulated, although they share the same biosynthetic pathway.
   This thesis

4. Different ripening events can be regulated separately which may allow retardation of undesirable and promotion of desirable processes during ripening.
   This thesis

5. Nothing is more practical than a good theory.
   Emanuel Kant (1724-1804)

6. Not all who are sitting around negotiation tables are peacemakers and not all who are carrying guns are terrorists.

7. Any civilisation reaches a stage at which it's end starts.

Propositions belonging to the Ph. D. thesis entitled:

"THE APPLE SKIN: COLOURFUL HEALTHINESS
Developmental and environmental regulation of flavonoids and chlorogenic acid in apples"

Mohamed Abdel-Ghani Awad
Wageningen, 26 June 2001

Preface

My study years passed in the Netherlands with many cloudy rainy days and only some sunny dry days. Above all, I would like to acknowledge and thank ALLAH, the merciful, for his grace and mercy which he extended to me; without his willing no body can do anything. I would like also to extend my gratitude and thanks to all people whom ALLAH made available to prepare my stay, and to support and to guide me during this period.

First of all, I would like to thank my 'daily' guide and co-promoter Dr. Ir. Anton de Jager at Applied Plant Research, Fruit Research Unit in Randwijk for all his efforts, support, encouragement and trust he gave to me during my M.Sc. and my Ph.D. work periods. I remember that day (August 26, 1991) when I arrived for the first time at Schiphol Airport near Amsterdam where I met him, with only a few English words in my mind. It was the first time I ever spoke with foreigners. He was very patient and tolerant with my "la-la" English as well as with my different way of thinking and my different cultural practices. Also I extend my thanks to his wife Mieke and their children for their kindness, hospitality and for all nice things they made for me and for my family.

I express my deep gratitude and thanks to my promoters Prof. Dr. Wim Jongen and Prof. Dr. Linus van der Plas, Wageningen University, for their interest, efforts, support and guidance during the course of the study. I acknowledge Dr. Matthijs Dekker and Dr. Alexander van der Krol, Wageningen University, for their guidance, efforts and support.

I would like to thank Lucie van Westing, Ania Meerman and Therese de Leeuw for their help and excellent support in the HPLC measurements and Kees Westerweele for his indispensable support in the storage of fruit and in the quality measurements. I thank Erik van Remortel and Jacques Withagen for their support and help in the statistical analyses of all my data. I thank Frans Roelofs and his wife Irma for friendly attitude and help. I would like to thank Dr. Bob Wertheim and Prof. Dr. Jan Tromp for their friendship and for the help in correcting the English of some early versions of the manuscripts. I thank Ir. Rob Meijer, former director of the Fruit Research Station, for his interest and support. I further express my thanks to all staff members of the Applied Plant Research, Fruit Research Unit at Randwijk and to the former workers at the 'old' site at Wilhelminadorp for all their support, help and friendship.

I would like to thank the Moslem Community in Goes and in Wageningen, especially my colleague Osama and his family, for their encouragement, support and hospitality. They all were a source of relief and contributed to make us feel at home. I thank our Dutch neighbours in Goes, Piet and Lenie de Kraker and their children Linda and Marcel, for their hospitality, friendly attitude and for all nice things they did for Abdu and Hagar.

I express my deep gratitude and thanks to my parents, my brothers and sisters for all the hard work and the efforts they made to allow me completing my study in Egypt. For them, I think no words are enough to express my thanks. I would like to thank my father and my mother in-law for their trust and encouragement. Finally, I express my gratitude and thanks to my wife and my children; with them I really enjoy life.

CONTENTS

1

General introduction

Background

The ultimate objective of the production, handling and distribution of fresh fruits and vegetables is to satisfy consumers requirements. It is generally agreed that consumer satisfaction is related to product quality. The term quality can be viewed as an absence of defects or a degree of excellence but it may mean different things to different handlers within the distribution chain (Shewfelt, 1999). In general the attractiveness of fruits and vegetables to consumers is determined both by visible and invisible quality attributes. The visible quality attributes include appearance, size, uniformity, colour and freshness, whereas the invisible quality attributes include flavour, firmness (texture), nutritional value, healthiness and toxicity. The extrinsic quality parameters, which describe how fruits or vegetables are cultivated e.g. production conditions, application of chemical sprays and fertilizers are also important aspects.

Fresh fruits and vegetables contain high levels of nutritional and healthy constituents such as minerals, vitamins, phytochemicals like folates, glucosinolates, carotenoids and phenolic compounds (e.g. flavonoids and phenolic acids) and dietary fibres. Nowadays, nutritionists, food scientists, the food industry, horticulturists and consumers are becoming more interested in the contents of these health promoting compounds in fresh or processed fruits and vegetables (Block et al., 1992; Steinmetz and Potter, 1996; Dekker et al., 2000). Flavonoids and phenolic acids are widespread in the plant kingdom, comprise a large group of naturally occurring compounds and form part of the human diet. Recent interest in these bioactive compounds has been stimulated by the potential health benefits arising from their role as antioxidants, which are scavengers of so-called reactive oxygen species (ROS) or free radicals (Bors et al., 1990; Block et al., 1992; Formica and Regelson, 1995; Steinmetz and Potter, 1996; Cook and Samman, 1996; Hollman, 1997). ROS are by-products of many of the body's normal chemical processes that can damage cell membranes and interact with genetic materials. ROS are possibly involved in the development of cancer, heart diseases and aging processes. Recent studies have shown that the majority of the antioxidant activity of a fruit or vegetable may originate from the flavonoids and other phenolic compounds (Gao et al., 1996; Wang et al., 1996).

Possible functions

It has been suggested that flavonoids act as antioxidants, primarily based on the fact that they extend the shelf life of fat-containing foodstuffs (Kuhnau, 1976; Bors et al., 1990). Flavonoids include a variety of compounds that display various functions. Koes et al. (1994) and Shirley (1996) have reviewed the possible functions of flavonoids in plants. Possible functions are protection of the photosynthetic machinery, preservation of membrane integrity, protection of DNA and proteins against the harmful effects of radiation especially UV, a crucial role in the plant sexual reproduction process especially male fertility, a visual signal in the attraction of pollinators especially for the coloured classes anthocyanins and flavonols, a signal in the symbiotic plant-microbe interaction and involvement in plant-pathogenic

microbe interactions (phytoalexins). Takahama and Oniki (1997) and Yamasaki et al. (1997) have shown evidence that the peroxidase-flavonoids/ascorbate reaction can function as a hydrogen peroxide scavenging system in plants. The flavonoid pigment anthocyanin has also been observed to function as endogenous antioxidant to reduce oxygen toxicity (Yamasaki et al., 1996) in addition to its well known role as a colouring agent for plant tissues and food additives. Like flavonoids, phenolic acids as chlorogenic acid also have antioxidant properties and act as electron donors (Takahama and Oniki, 1997). The pharmacological properties of flavonoids are well characterised and suggested to have potential as starting material in drug development programmes (Formica and Regelson, 1995). Although there is considerable evidence establishing antioxidant activity in vitro for flavonoids and phenolic acids found in the diet, there are only few studies in humans on the absorption and bioavailability of these compounds and their role in protection against cancer and heart diseases (Block et al., 1992; Hertog, 1992; Hollman, 1997; Croft, 1998).

Biosynthesis

The biosynthetic pathway of flavonoids represents one of the most intensively studied secondary metabolic pathways of higher plants (Mol et al., 1996, Holton Cornish, 1995). Genes required for flavonoids biosynthesis are regulated in a developmental and a tissue specific manner (Kubaske et al., 1992; Koes, et al., 1994). Flavonoids and phenolic acids production in plants can be induced by a variety of environmental stimuli such as high light, ozone, temperature stress, water stress, nutritional stress, pathogen attack, mechanical damage and atmospheric pollution (Kangasjarvi et al., 1994; Marten and Grimmig, 1994; Estiarte et al., 1994; Dixon and Paiva, 1995). The basic skeleton of flavonoids is a combination of products of two independent pathways, the shikimate pathway (yielding phenylalanine) and the acetate-malonate pathway (yielding malonyl-CoA) (Stafford, 1990). The Flavonoid skeleton consists of two distinct units: the C_6-C_3 moiety which contains the B-ring and the C_6 fragment (A-ring, Fig. 1) (Smith, 1972). Shikimic acid is considered the source of ring B, and a condensation of 3-acetate units form ring A. As presented in figure 1 (Chapter 6), the deamination of L-phenylalanine to trans-cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL) is the initial step in the biosynthesis of hydroxycinnamic acids, flavonoids and other phenylpropanoid polyphenols (Camm and Towers, 1973; Margna, 1977). In two subsequent reactions coumaroyl-CoA is produced. The first specific flavonoid structure naringenin chalcone arises by the condensation of 1 molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA. This reaction is carried out by the enzyme chalcone synthase (CHS). The naringenin chalcone is subsequently converted into naringenin flavanone by the enzyme chalcone isomerase (CHI). From this central step the pathway goes into several side branches producing different classes of flavonoids such as anthocyanins, flavonols, and flavanols. These compounds are present in plant tissues mostly as glycosides or methoxylated derivatives, but also as aglycones. The glycosylation reaction is carried out by the enzyme UDPG: flavonoid-3-O-glycosyltransferase, followed by transport to the vacuole and subsequent storage.
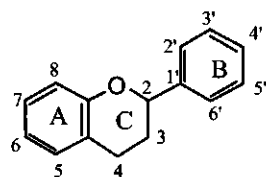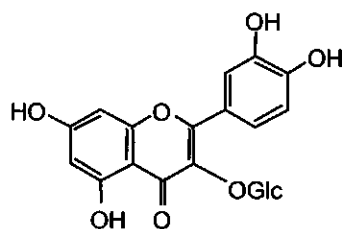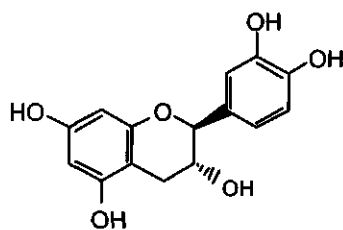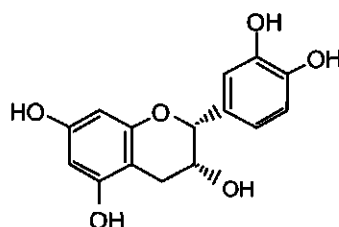
Phenylpropanoid ring
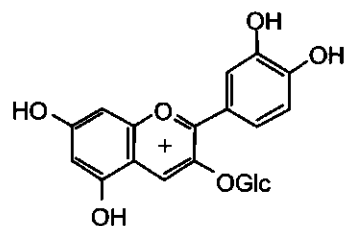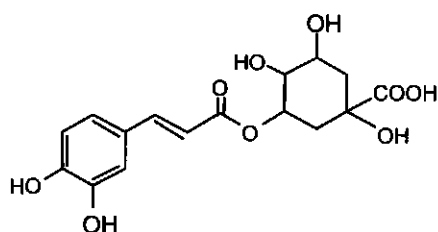
Cyanidin-3-glycoside
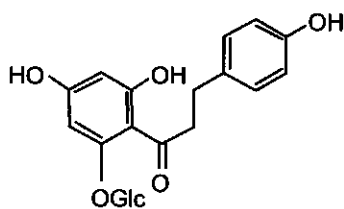
Phloridzin

Quercetin-3-glycoside

(+)-Catechin

(-)-Epicatechin

Chlorogenic acid

Fig. 1. Structure of the general phenylpropanoid ring and subclasses of flavonoids and chlorogenic acid.

Based on the degree of oxidation of the C_3 unit, flavonoids are divided into subclasses such as flavonols, flavanols, isoflavonoids and anthocyanins. Subsequent enzymatic modification of each type of flavonoids can involve hydroxylation, methylation, acylation, glycosylation, and reduction. Over 4000 different flavonoid derivatives are known in plants (Bors et al., 1990; Lancaster, 1992).

Flavonoids in apple

The main flavonoids present in apples and their products are flavonols (quercetin 3-glycosides), flavanols (catechin, epicatechin, gallocatechin, procyanidins and its polymers), dihydrochalcone glycosides (phloritin glucoside (phloridzin) and phloritin xyloglucoside), and cyanidin 3-glycoside (anthocyanins, Fig. 1). Seven quercetin glycosides have been identified so far (quercetin 3-galactoside, 3-glucoside, 3-xyloside, 3-rhamnoside, 3-rhamnoglucoside, 3-arabinoside and 3-arabinofuranoside), all glycosylated in the 3-position. Quercetin 3-galactoside is the main quercetin glycoside type (Lister et al., 1994; Nicolas et al., 1994). The main phenolic acid present in apples is chlorogenic acid (Fig. 1, Nicolas et al., 1994).

The background colour yellow/green is due to chlorophylls and carotenoids which are located in the plastids, while the red colour is primarily a consequence of the flavonoid pigments anthocyanins which are located in the vacuole. Anthocyanins are coloured glycosylated flavonoids, which are prominent in a number of plant tissues such as flower petals, leaves, and fruit peel. The chemical structure is based on an aromatic flavylium cation, and the aglycone forms are called anthocyanidins. In plants there are six groups of anthocyanidins that produce different colour shades depending on the hydroxylation/methylation status of the anthocyanidin B-ring (Stafford, 1990). These are cyanidins, pelargonidins, delphinidins, peonidins, petunidins, and malvidins. For example the cyanidins have 2 hydroxyl groups at the 3', 4' position on the B-ring and give rise to scarlet red colours, compared to pelargonidins which have 1 hydroxyl at the 4' position and give orange-red, and delphinidins, which have 3 hydroxyl groups at the 3', 4' ,5' position on the B-ring and produce blue colours. The family *Rosaceae* to which apple (Malus) belongs contains mainly cyanidin glycosides, while some peonidin and pelargonidin glycosides have been found. Delphinidin, however, and its derivatives are not found (Lancaster, 1992).

The main cyanidin glycoside in apple skin is cyanidin 3-galactoside, while cyanidin 3-arabinoside, 3-glucoside, 3-xyloside and 7-arabinoside are present in minor amounts in some red cultivars (Lancaster, 1992). The different colour shades pink, red, mauve and blue found in plants have been attributed to pigment concentration and to the phenomenon of co-pigmentation. This phenomenon has been investigated in flowers. In this process the flavonols aggregate around the anthocyanin and shield it from hydration thus stabilizing the anthocyanin as a coloured flavyllium cation. The extent of co-pigmentation depends on the concentration and the type of the anthocyanins, the molar ratio of flavonols to anthocyanins, and the chemical nature of the flavonols. In apples there are many different cultivars exhibiting a wide range of red colours. However, Lancaster et al. (1994) showed that the

process of co-pigmentation is not involved in the differences in colour intensity and that the increase in colour intensity could be attributed (1) to the increase in anthocyanin concentration, (2) to a greater proportion of darker red vacuoles, (3) to larger vacuoles and (4) to more layers of red cells. The blending of chlorophylls, carotenoids, and anthocyanins could account for the visible change in hue of the red coloration of apple skin from orange-red to bronze or to purple red.

Despite the importance of flavonoids for the intrinsic quality of apples, as it contributes to both colour and healthiness, very little is known of their biosynthetic pathways in fruit. Most published information on the biosynthesis of anthocyanin and flavonoids comes from work on flowers. Some steps in the biosynthesis pathway are assumed to be similar to those in flowers. There is some literature information on the regulation of anthocyanin biosynthesis in apples but quantitative information on the amplitude of variation and on the relationship with external factors is almost lacking. For other flavonoid substances even the variation in content due to environmental factors is poorly studied. Studies of the relationships between external factors and the formation of individual flavonoids can lead to a better understanding of the mechanisms by which flavonoid biosynthesis is regulated and thus improve the possibilities to enhance both colour and healthiness of apples for fresh consumption or for processing.

Factors influencing flavonoid formation in apples

1. Developmental factors

Apple fruit may remain growing on the tree for up to 6 months after pollination. During that time, there are changes in concentration and amount of anthocyanin and flavonoids in the apple skin related to the development and growth of the fruit (Lancaster, 1992). Apple skin accumulates anthocyanin during two phases of fruit growth. The first occurs early in the season in young fruitlets during cell division. At this time even anthocyanin accumulates in the skin of some non-red cultivars such as 'Golden Delicious' and 'Granny Smith'. Little is known about the biological significance of this early colouring. However, the nature of this anthocyanin is identical to that in ripe fruit (cyanidin 3-galactoside; Lister et al., 1994). Its biosynthesis depends on light conditions and is promoted by lower air temperature (Jaetak et al., 1998). This first phase is followed by a stage in which the anthocyanin decreases or may completely disappear. Depending on the cultivar, anthocyanin formation in the skin resumes when fruits are approaching maturation at the end of the growing season. Anthocyanin concentration at this stage is frequently used as a maturity index (Lancaster, 1992; Curry, 1997). Anthocyanin accumulation during ripening has been studied in Cox's Orange Pippin apples (Knee, 1972). The concentration of anthocyanin increased three-fold during the month of ripening. During this time, chlorophyll concentration fell fourfold and carotenoids increased fourfold.

Compared to anthocyanin, other flavonoid compounds may show different developmental patterns of accumulation. Relatively little is known about accumulation of

flavonoids and hydroxycinnamic acid derivatives in apple skin during development. Lister et al. (1994) reported that the concentration on a fresh weight basis of quercetin glycosides (seven types) and proanthocyanidins (catechin, epicatechin, gallocatechin and procyanidins B2 and B5) in the skin of a red 'Splendour' and a green 'Granny Smith' apple cultivar sharply decreased from early to mid season followed by an increase during ripening only in'Splendour'. Mayr et al. (1995) observed that, on a dry weight basis, phenolic compounds (including chlorogenic acid) in the skin and the leaves of 'Golden Delicious' apples decreased during growth without a further increase during maturation and ripening, but the individual compounds did not behave uniformly. Sannomaru et al. (1998) reported that the concentrations of total polyphenols, epicatechin and chlorogenic acid in the skin, flesh and core of 'Fuji' and 'Starking' apples decreased during the ripening process, and fell rapidly during the early stage of development. Hamauzu et al. (1999) found in the flesh of 'Tsugaru' and 'Fuji' apples that, on a fresh weight basis, the total concentration of catechins and procyanidins (total catechins units) decreased, whereas it increased on a fruit basis during fruit development in both cultivars. Oligomeric procyanidins and epicatechin content increased, on a fruit basis, during the middle stage of development and decreased thereafter, whereas polymeric procyanidin increased as fruit matured. They also found a rather stable amount of phloridzin in both cultivars during development; however, the amount of chlorogenic acid increased only in 'Fuji' apples.

Although there are some reports on the developmental changes in the concentration of flavonoids and phenolic acids in apples, a mass balance would provide more information about periods of net synthesis and/or breakdown, and also give information on the relation between the different flavonoids in the same biosynthetic route.

2. Climatic factors

2.1. Light

The biosynthesis of anthocyanin and other flavonoids in apples, as in other plant tissues, either requires or is enhanced by light (Ju, 1998). Light-induced production of anthocyanin and other flavonoids in the epidermis and other outer tissues is considered to be a ubiquitous protection mechanism against solar radiation in higher plants (Hahlbrock and Scheel, 1989; Merzlyak and Chivkunova, 2000). Bagging the fruits about one month after full bloom until harvest resulted in an absence of anthocyanin formation and trace amounts if any of flavonoids but considerable levels of the simple phenols (chlorogenic acid). Fruit bagging also prevented the enzymes PAL, CHS, DFR and UFGalt from being induced (Ju et al., 1995a and b; Ju et al., 1997). It is observed that apples from the interior part of the tree canopy develop no or very small amounts of anthocyanin whereas exterior fruits, well exposed to light, develop much more anthocyanin and better colour (Saure, 1990; Lancaster, 1992). Anthocyanin concentration in apple skin increased with light level, up to about 50% of full sunlight (Jackson, 1980, Barritt et al., 1997). Heinicke (1966) reported that red colour development in 'Red Delicious' and 'McIntosh' apples was directly related to the amount of

light exposure, with best colour in fruit exposed to more than 70% of possible full sunlight, whereas fruit exposed to less than 40% of possible full sunlight developed inadequate colour and size. Quercetin glycosides were twice as high in peels of 'Golden Delicious' on the sunny side of fruit as compared to the shaded side. Fruits from the canopy exterior contained twice as much quercetin glycosides as the green fruit harvested from the interior part (Workman, 1963).

Thus light is generally considered a key regulatory factor in the biosynthesis of flavonoids in apple skin. However, the biosynthesis of the different flavonoid classes might have different spectral sensitivity characteristics. The biosynthesis of anthocyanin requires light of certain energy and quality. Siegelman and Hendricks (1958) reported that the formation of anthocyanin in apple skin disks is controlled by a high-energy photoreaction, with an action maximum between 650 and 670 nm. A subsidiary maximum between 430 and 480 nm has been reported by Downs et al. (1965). In this region, the energy required for appreciable anthocyanin synthesis was about three times as much as at the action maximum near 650 nm. Bishop and Klein (1975) showed that in harvested apples light of wavelength 440 nm (a blue wavelength band) was the optimum for the synthesis of anthocyanin, with an additive effect of red light. Anthocyanin synthesis in apple fruit is also stimulated by light in the UV region. Arakawa et al. (1985) observed that UV-B light (emission peak at 312 nm) is highly effective in stimulating anthocyanin synthesis and exerts a synergistic effect when applied simultaneously with white or red light. The effects of UV-B together with blue light were only additive, and UV-A (emission peak at 353 nm) was less effective than UV-B. In attached apples, UV cut-off filters prevented rapid anthocyanin formation after bag removal (Kubo et al., 1988). White plus UV-B light can also promote synthesis of different flavonoid classes in green-mature detached apples (Dong et al., 1995; Lancaster et al., 2000). Thus, as in other plant tissues, the effect of light on the biosynthesis of flavonoids in apples especially that of anthocyanin is very complex because more than one photoreceptor such as chlorophyll, phytochrome, the blue and UV-B light photoreceptor might be involved (Siegelman and Hendricks, 1958; Downs et al., 1965; Arakawa, 1988a and b; Hahlbrock and Scheel; 1989). The participation of photosynthesis is found to be necessary for full expression of the response (Downs et al., 1965; Arakawa, 1988a).

Generally, in most apple cultivars the increase in anthocyanin occurs after an initial lag phase of about 20 to 24 hours of light exposure in both attached and detached matures apples (Faragher and Chalmers, 1977; Arakawa, 1988b; Dong et al., 1995). The effectiveness of light depends on cultivar, degree of fruit maturity, and temperature. Arakawa (1988b) concluded that the responsiveness of the fruit to light, especially to white light, differed considerably between the cultivars. Under white light alone or in combination with UV-B light, 'Starking Delicious' and 'Jonathan' apples produce a maximum concentration of anthocyanin, while 'Fuji' and 'Tsugaru' apples required a higher light intensity and were more dependent on UV-B irradiation. 'Mutsu' and 'Golden Delicious' produce no anthocyanin at even a high intensity of white light but a small amount of anthocyanin is formed with the addition of UV-B light. Chalmers et al. (1973) observed that in immature fruit a reduction of light intensity resulted in a decrease in anthocyanin production, while its

accumulation continued in mature fruit. Chalmers and Faragher (1977) found that in detached immature fruit after exposure to sunlight, anthocyanin formation stopped earlier than in detached mature fruit.

In 'Reliance' grapes, the biosynthesis of peonidin 3-glucoside and malvidin 3-glucoside was much less light dependent than that of cyanidin 3-glucoside (Gao and Cahoon, 1993). Thus light might not be a regulatory factor for the biosynthesis of all different anthocyanin types. Moreover in 'Norland' red potato tuber, the red colour is due to the accumulation of anthocyanins, mainly pelargonidin and peonidin, in the periderm and peripheral cortex while potato tubers grow underground (Hung et al., 1997). This suggests the existence of a light independent mechanism for anthocyanin biosynthesis.

In conclusion, light through its energy and quality is essential for the biosynthesis of the different flavonoid classes in apples; however, its effectiveness may vary upon cultivar, stage of development, temperature and type of flavonoid.

2.2. Temperature

There is considerable literature information on the relationship between temperature and anthocyanin formation in apples (Saure, 1990); however, very little information is available on other phenolic compounds. Generally low temperatures, especially at night, during the last period of fruit development and maturation promote anthocyanin synthesis contrary to high temperatures. Uota (1952) reported that in MacIntosh apples the red colour development was significantly correlated with the low average night temperature during the last month of fruit development. He observed that at high temperature, $24.5^{\circ}C$ or above, the formation of anthocyanin was completely inhibited and suggested that a greater amount of light energy might be required to synthesise the pigment at a higher temperature. Creasy (1968) found an inverse relationship between average temperature and anthocyanin formation both under natural conditions and in artificially illuminated controlled temperature chambers. Low temperature promoted anthocyanin production whether given during the light or the dark period (Diener and Naumann, 1981). Tan (1980) showed in a post harvest experiment that, at constant light conditions, 'Red Spy' apples exposed to an alternating $6/25^{\circ}C$ (night/day) treatment had more anthocyanin than those kept constantly at $25^{\circ}C$ during the course of the experiment. Blankenship (1987) reported that in potted 'Red Chief' apple trees exposed to $26^{\circ}C$ day temperature during the period of fruit development and maturation, fruit showed more red colour development when held at $11^{\circ}C$ than at $22^{\circ}C$ night temperature. Noro et al. (1991) imposed four temperature regimes in 'Starking Delicious' apples during the last months of fruit development. He found a higher level of anthocyanin in the fruit under the low temperature regimes (constant $15^{\circ}C$, or alternating $10/20^{\circ}C$) than in those under high temperature regimes (constant $25^{\circ}C$, or alternating $20/30^{\circ}C$). There were no differences in the level of anthocyanin between constant and alternating temperature. In detached green mature 'Royal Gala' apples red colour development of skin increased to a maximum after UV-B plus white light irradiation for 2 days followed by 15 days of cold storage at $4^{\circ}C$ in dark conditions (Dong et al., 1995). They observed no further increase in

red coloration in fruit stored at 14°C or 25°C following the irradiation treatment. Low air temperature has also been found to promote red coloration of young 'Fuji' apple fruitlets (Jaetak et al., 1998).

However, applying different day/night temperatures at mid-season or late-season did not influence red colour formation and has no or very little influence on the course of the various ripening characteristics of both 'Elstar' and 'Cox's Orange Pippin' apples (Tromp, 1999). Faragher and Brohier (1984) concluded that the rise in anthocyanin with ripening appears to be more closely related to the rise in ethylene than to the fall in temperature, despite the well-known effects of low temperature in stimulating colour formation. Creasy (1968) observed that low temperature improved the efficiency of anthocyanin formation in poorly exposed skin at a low light level, but it did not reduce the obligate light requirement.

During the course of fruit maturation a shift in optimum temperature for anthocyanin formation to higher degrees has been observed in detached fruit during illumination (Diener and Naumann, 1981; Faragher, 1983; Arakawa, 1991). This suggests that each stage of development possibly has its optimal temperature regime for maximal red colour formation. Furthermore the different cultivars showed differences in temperature optimum during postharvest irradiation (Arakawa, 1991). Curry (1997) found that skin tissue disks prepared from preclimacteric fruit developed more anthocyanin than did tissues from climacteric fruit during irradiation with white light. Pre-cooling the tissue for 48 h at 2°C before incubation at 25°C increased the amount of pigment that accumulated. He also observed a different temperature optimum for each cultivar but a similar temperature optimum for a highly colouring mutant as compared to its related cultivar.

Since both flavonoids and anthocyanin are synthesised from the same biosynthetic pathway, it would be reasonable to assume that temperature affects the synthesis of both groups in the same way. However, Reay (1999) found that exposing detached 'Granny Smith' apples to a low temperature treatment followed by a warm temperature greatly enhanced the formation of anthocyanin but not that of quercetin in the skin during irradiation with UV-B plus visible light. Lancaster et al. (2000) investigated the effect of UV-B irradiation at 10°C and 20°C on the concentrations of quercetin glycosides (6 glycoside types), procyanidins (procyanidins B1 and B5, epicatechin and catechin), anthocyanin and chlorogenic acid in the skin of detached fruit of five apple cultivars. UV-B irradiation increased the quercetin glycosides concentration in the shaded side of 'Gala', 'Royal Gala' and 'Braeburn' fruit only at 20°C. Chlorogenic acid concentration increased by UV-B irradiation on both fruit sides, at both 10°C and 20°C, with greatest increases generally being found on the shaded side at 20°C in most cultivars. There was no effect of UV-B irradiation on procyanidins concentration at either temperature. UV-B irradiation increased anthocyanin concentration in both fruit sides of all studied cultivars at 20°C, and on 'Braeburn' and 'Aurora' at both 20°C and 10°C.

In conclusion, low temperature is generally considered to promote anthocyanin and red colour formation in attached apples during development whereas, in detached apples at constant illumination there is a shift in the temperature effects toward a higher optimal range depending on cultivar and degree of ripening.

3. Cultural practices

3.1. Nutrition

Fruit nutrient composition has a strong association with general external and internal quality attributes of fruits such as colour, firmness, storability and disorders resistance (Bramlage, 1993; Johnson and Ridout, 1998). Fruits high in N level tend to be larger, softer, are more likely to develop a number of physiological disorders, and develop poor red colour (Bramlage 1993). Nitrogen is considered the most important nutrient affecting red colour formation negatively (Walter, 1967; Saure, 1990). According to Johnson and Samuelson (1990) and Raese and Drake (1997) additional supply of N to fruiting trees may decrease the extent of red coloration and increase the intensity of fruit greenness. Apart from such a direct effect nitrogen may indirectly affect red colour formation by more shade caused by denser foliage (Magness et al., 1940; Saure, 1990). Faust (1965a) found that feeding skin disks with urea significantly decreased the formation of anthocyanin after illumination. Accordingly, N-containing compounds like urea and calcium nitrate have been applied to increase fruit skin greenness and to suppress the formation of anthocyanin in green cultivars where red or yellow blush is undesirable (Williams and Billingsley, 1974; Meheriuk, 1990). Boynton and Cain (1943) observed that an increase in leaf N of 1% was associated with a decrease of 30% in red fruit colour. Weeks et al. (1958) found that high N levels significantly reduced red colour formation but K appeared to offset the adverse effects of N. Awasthi et al. (1995) reported that K applied to 'Starking Delecious' apple trees either broadcast or in bands with or without 1 or 2 foliar sprays greatly increased anthocyanin concentration in fruit. Tan (1980) reported that both N and K deficiencies in the leaf clearly increased the level of anthocyanin production in 'Red Spy' apples. Larrigaudiere et al. (1996) found that pre-harvest spray with seniphos (a phosphorus-calcium mixture) significantly improved the development of anthocyanin and red colour of 'Starking Delicious' apples. Raese and Staiff (1990) reported that soil-applied calcium nitrate was associated with higher concentration of fruit Ca, lower N/Ca ratio, more red colour and lower incidence of disorders in 'Delicious' apples. In another study Raese and Drake (2000) showed that calcium spray improved fruit quality and increased red coloration of 'Red Delicious' apples. Smock (1966) showed that post harvest dipping of 'McIntosh' apples in a solution of $CaCO_3$ increased the synthesis of anthocyanin after illumination. Reay et al. (1998) reported that foliar applications (total 8 sprays at weekly intervals) of urea and magnesium sulphate increased chlorophylls and carotenoids concentrations in 'Gala' apples during maturation. But anthocyanin concentration was decreased only in urea treated fruits.

However, information on the effect of other nutrients is not consistent and therefore clear correlations with anthocyanin formation have not yet been established (Walter, 1967; Saure, 1990). Moreover the influences of nutrients on the formation of other flavonoid compounds and chlorogenic acid in apples is not yet investigated. Ciders obtained from 'Dabinett' apple trees that received NPK fertilizer were less bitter and astringent than those from control trees, which was related to an overall decrease of 17% in fruit phenolic

concentration (Lea and Beech, 1978). In tomato plants (Lycopersicon esculentum L) nitrogen deficiency increased the level of anthocyanin of leaves to 2-3-fold and total flavonoids by 14% (Bongue-Bartelsman and Phillips, 1995). Several authors observed that nutritional stresses e.g. N, P, Mn and B deficiency increased flavonoid content in different plant species (Zornoza and Esteban, 1984; Estiarte et al. 1994). A positive effect of calcium spray on apple phenolics has been also observed by Sannomaru et al. (1998). They reported in 'Starking' apples grown under conventional soil culture with or without Ca spray that epicatechin, chlorogenic acid and total polyphenols contents were significantly higher in Ca treated than in untreated fruit. Patil and Alva (1999) reported that in grapefruit, the levels of the flavonoids naringin and rutinoside and the levels of total vitamin C (ascorbic acid plus dehydroascorbic acid) decreased in the fruit with increased nitrogen application rates in the orchard. Similarly, reduced levels of vitamin C in juices of oranges, lemons, grapefruits and mandarins resulted from the application of high levels of nitrogen fertilizer to those crops, while increased potassium fertilization increased vitamin C content (Nagy, 1980). In apple shoot cultures Lux-Endrich et al. (2000) found that reducing the macronutrient content of the culture media resulted in more accumulation of phenolic acids, quercetin glycosides, catechins and procyanidins of the tissues. However, Krause and Reznik (1976) found that various P and N concentrations in buckwheat seedlings did not influence chlorogenic acid concentration, in contrast to flavonoids.

In conclusion, it is generally agreed that N is negatively affecting anthocyanin and red colour formation in apples as well as flavonoids including anthocyanin in other plants. Potassium appeared to antagonize the negative effects of N and to favour red colour formation in some apple cultivars. Calcium might positively affect anthocyanin and other flavonoids formation in apples. Information concerning the effect of other nutrients is even more conflicting, possibly due to complex interactions between many different factors which affect anthocyanin and red colour formation in apples (see Walter, 1967; Saure, 1990).

3.2. Moisture relations

Water availability is an important factor that may influence anthocyanin and flavonoids formation in fruits. Generally, the direct effect of soil moisture on anthocyanin and red colour formation per se is obscured due to interactions with nutrient uptake and the general influence on tree growth and fruit development (Walter, 1967; Sharples, 1973). However, it appears that soil moisture promotes red colour formation in apples especially in dry areas or dry seasons as long as it is adequate for normal fruit development (Saure, 1990). Unrath (1972) found that 'Red Delicious' apples picked from trees which received over-tree irrigation had a greater surface of red coloration and were riper than fruits which received under-tree or no irrigation. Furthermore, air or soil moisture may influence the effectiveness of ethephon on red colour formation (Unrath, 1973). In a drier production area, ethephon advanced maturity but did not increase red colour. The application of ethephon on trees after a 5 cm soil irrigation or on trees which received over-tree irrigation resulted in about 30% more red colour in fruits than for un-irrigated trees. On the other hand, Parchomchuk and Meheriuk

(1996) found no effect of over-tree irrigation on red colour formation in 'Jonagold' apples. These conflicting results might be due to differences in the way that cultivars respond to cooling, or to differences in climatic conditions. Excessive soil moisture may also be detrimental to red colour formation due to excess tree vigour that reduces light exposure of the fruit (Walter, 1967). No information is available on the effect of soil or air moisture on the levels of other phenolic compounds in apples.

3.3. Tree form/ Pruning/ Rootstock

Tree form, pruning and the use of rootstock or interstock may alter the canopy vigour and consequently alter the light conditions within the canopy (Walter, 1967; Wagenmakers, 1991). Root/shoot ratio, nutrient and assimilates supply to the remaining fruits and time of ripening may also be altered by pruning and rootstock types (Sharples, 1973; Saure, 1987, 1990; Autio, 1991). There are different types of pruning namely shoot, leaf and root pruning and these may be performed by several ways, at different times, and at different levels. Pruning systems are well known to influence fruit quality retention (Bramlage, 1993). Severe winter pruning stimulates vegetative growth the following season which affects negatively red colour formation and also may increase calcium-deficiency related disorders of the fruit (Sharples, 1973). In contrast, summer pruning generally improves red colour formation in most apple cultivars but tends to lower fruit size and sugar level (Morgan et al., 1984; Marini and Barden, 1987; Saure, 1987). Summer pruning decreases both leaf area index and light interception and improves fruit light exposure and red colour formation (Saure, 1987; Palmer et al., 1992). In addition to the better light exposure of the fruits, the positive effect of summer pruning may be explained by an increased Ca content, or by altered hormone levels and distribution within the tree (Saure, 1987; Bramlage, 1993).

Root pruning has generally been reported to increase red colour formation in several apple cultivars. This effect is possibly due to a restriction of the vegetative growth by which light exposure of the fruits is improved (Schupp and Ferree, 1989; Saure, 1990). Accordingly dwarfing rootstocks/interstock are generally found to stimulate the formation of anthocyanin compared to less dwarfing ones (see Walter, 1967). Rootstock can influence nutrient composition, time of ripening and storability of fruit (Sharples, 1973; Autio, 1991). To a considerable extent, this reflects tree vigour but not all fruit differences can be attributed to growth differences. Saure (1990) suggested that besides an indirect effect of root pruning or dwarfing rootstocks resulting from a better light exposure caused by less dense foliage, there is also a direct effect of the smaller supply and transfer of root-derived GAs to the fruits. To our knowledge, no literature is available about the influence of tree form, pruning and rootstock on the concentration of flavonoids and chlorogenic acid in apple fruit, except on that of anthocyanin. Since both flavonoids and anthocyanin are synthesised from the same biosynthetic pathway, it is reasonable to assume that improving light conditions within the tree canopy by using dwarfing rootstocks and proper training and pruning systems may enhance the level of other flavonoids as well.

3.4. Thinning

Fruit thinning is a very important cultural practice that affects fruit development, the availability of assimilates, the content of sugars, acids and dry matter, and the nutrients composition of apples (Poll et al., 1996; Volz and Ferguson, 1999; Wertheim, 2000). To our knowledge, no literature is available about the influence of crop load on the concentration of flavonoids and chlorogenic acid in apple fruit, except on that of anthocyanin, which is directly related to red colour and marketability (Smock, 1969; Walter, 1967; Saure, 1990). It is estimated that, under normal growth conditions, about 20% of all carbon photosynthesized by plants flows through the shikimate pathway and much of it might be used for the synthesis of the various secondary metabolites (Herrmann, 1995). According to Smith (1972), about 2% of the carbon fixed by plants is converted to flavonoids or closely related compounds. Flavonoid compounds are generally present in plants as glycosides, except flavan-3-ols (catechins) which are found in free rather than in glycosylated forms. One would expect that a treatment that causes a change in the availability of assimilate that function as flavonoid precursors might induce subsequent changes in the synthesis of flavonoids. It is found that dipping of 'McIntosh' apples in shikimic acid solution considerably increased anthocyanin formation in unripe but not in ripe fruit during irradiation (Faust, 1965b). Exogenous sucrose, galactose and glucose were effective in promoting anthocyanin synthesis in apple skin discs (Vestrheim, 1970). A pre-harvest application of 0.25M of galactose or glucose on fruit trees enhanced anthocyanin formation in 'Fuji' apples (Bae and Lee, 1995).

Thus, the fruit to leaf ratio is expected to have a clear effect on anthocyanin and other flavonoids formation. In earlier literature Magness (1928a) showed the importance of providing adequate leaf area for obtaining acceptable fruit quality and colour. He found for 'Delicious' and 'Winesap' apple that fruit with 10 leaves per fruit and an almost perfect light exposure developed only 23% of its surface red colour compared to 58% red colour in fruits with a leaf to fruit ratio of 75. Magness (1928b) concluded that 40 leaves per fruit were minimally necessary to produce acceptable fruit quality and colour in both 'Delicious' and 'Winesap' apples, while 20 to 25 leaves were enough for 'Jonathan'. Similarly, Fletcher (1932) found in 'Jonathan' and 'York Imperial' apples that 50 leaves per fruit were enough to produce good sized and well coloured apples and increasing the number of leaves to 100 per fruit resulted in a further increase in red colour. Wertheim (1987) observed that in 'Jonagold' more leaf area per fruit and per tree resulted in more anthocyanin formation. He pointed out that 45 leaves per fruit were optimal for anthocyanin formation while fruit size showed a further increase with increasing number of leaves up to 220 leaves per fruit. Saure (1990) concluded in his review that the beneficial effect of thinning on fruit size and colour is known, but the amount of thinning necessary for maximum colour formation is still disputed and depends on many factors inside and outside the fruit. On the other hand, several studies did not show any relation between sugar content and anthocyanin formation in apples (Uota, 1952; Blankenship, 1987; Noro et al., 1988). Moreover, summer pruning improves light conditions within the canopy, increases anthocyanin formation but reduces assimilate supply and sugar level in fruit (Saure, 1987).

3.5. Fruit bagging

Covering apple fruit with paper bags during development starting about one month after full bloom and removing bags a few weeks before maturation is still widely applied in Japan as a practical method to increase anthocyanin formation and to protect fruit against insects and diseases (Mink, 1973). This treatment may even induce some anthocyanin formation in cultivars that usually do not show any red colour upon maturation like 'Mutsu' and 'Golden Delicious', after bag removal (Proctor and Lougheed, 1976; Noro, 2000). Temporary fruit bagging with foil bags was more effective in enhancing red colour formation than supplementary illumination in the orchard (Proctor and Lougheed, 1976). They also observed an optimum time for bag removal (20-30 days before harvest) for obtaining maximum red colour formation probably related to a certain stage of fruit growth and maturity. Arakawa (1988b) found that, in a number of apple cultivars, fruit bagging increased light sensitivity of fruit and stimulated anthocyanin formation when fruits were re-exposed to post-harvest white or UV-B light at both immature and mature stages. However, this capacity of anthocyanin synthesis greatly decreased during ripening in previously bagged fruit while it continued to increase in previously unbagged fruit.

The effect of fruit bagging on the synthesis of other flavonoids has been studied by Ju et al. (1995a). They found that apples that were kept bagged until harvest developed no anthocyanin but trace amounts of flavonoids (quercetin glycosides and procyanidins). In apples harvested 20 days after bag removal, both anthocyanin and flavonoids formation increased rapidly but the final level of anthocyanin was similar and that of flavonoids was lower than those in the unbagged control fruit. In another experiment the concentration of simple phenols (mainly phenolic acids) was not affected by a temporary bagging treatment (Ju et al., 1995b). Bagging treatments did not affect fruit maturation (Ju, 1998) and did not show any consistent effect on ethylene production (Kubo et al., 1988). However, it may reduce soluble solids and chlorophyll concentration (Proctor and Lougheed, 1976). Bagging might temporarily raise the level of phytochrome thus increasing the capacity of anthocyanin formation shortly after bag removal (Saure, 1990).

3.6. Growth regulators and other chemicals

Fruit growers have since long used chemical sprays for improving red colour in apples. Most widely used substances have been NAA and the herbicides 2,4,5-TP or 2,4,5-T. Some other chemicals like sodium thiocyanate, calcium carbonate, Diuron (3(3,4-dichlorophenyl)-1, 1-dimethylurea) but also fungicides such as Captan and Tuzet have been used (Walter, 1967; Saure, 1990). These substances were used as a pre-harvest foliar spray to partly improve red colour formation in addition to their main effects. During the last decades many compounds have been tested to increase red colour formation but most of these chemicals were not practically used because of inconsistent or insufficient effects, or because of negative side effects as enhancing fruit ripening (Walter, 1967; Saure, 1990). The growth ripening retardant diaminocide (Alar, SADH) has been used also as a colour stimulating agent (Faust, 1973), but

its use is prohibited since 1985 because of potential health hazards. Currently ethephon (an ethylene-releasing compound) is most widely used both as growth regulator and to improve fruit coloration. This compound has the undesirable side effect of accelerating fruit ripening and thus of decreasing the storability of the fruit which limits its use. However, Blanpied et al. (1975) showed that under certain restrictions ethephon promoted colour formation without substantially hastening ripening of 'McIntosh' apples when applied at a reduced concentration in combination with a preceding application of Alar. Murphey and Dilley (1988) concluded also that enhancement of anthocyanin biosynthesis may require only a brief exposure to ethylene which may be insufficient to negatively affect other fruit ripening characteristics. However, in another study a low concentration of ethephon (25 ppm) advanced ripening of 'McIntosh' apples by about one week (Greene et al., 1974). Therefore it is possible that the effect of ethephon on fruit maturation may depend upon cultivar and environmental conditions (Saure, 1990). Seniphos (a phosphorus-calcium mixture) has been reported to decrease internal ethylene and to improve colour without affecting maturation of 'Starking Delicious' apples (Larrigaudiere et al., 1996). According to Schmitz and Noga (1998) repeated application of a vitamin E formulation (containing 25% alpha-tocopherol) during growing improved resistance against scab infection and increased red colour formation of 'Elstar' and 'Jonagold' apples.

Little information, however, is available on the influence of such chemicals on other flavonoid classes and chlorogenic acid in apples. Ju et al. (1995a) found that ethephon application (250 ppm) at 20 days before maturation increased anthocyanin accumulation and UDPGal:flavonoid-3-o-glycosyltransferase (UFGalT) activity during maturation, but had no effect on flavonoids concentration and chalcone synthase (CHS) activity in both 'Delicious' and 'Ralls' apples. However, ethephon applied at 60 days before maturation induced high UFGalT activity and increased the accumulation of flavonoids but did not induce anthocyanin formation when the fruits were picked at 40 days before maturation. They also found that application of an ethylene inhibitor, aminoethoxyvenylglycine (AVG) (500 ppm) alone did not affect activity of both CHS and UFGalT, or the accumulation of anthocyanin and flavonoids. But when applied with ethephon, AVG partially counteracted the effect of ethephon. Prohexadione-Ca, an inhibitor of gibberellin metabolism, which is currently being developed as growth retardant in apple favoured the accumulation of eriodictyol and luteoliflavan, which does not normally occur in apple tissue (Roemmelt et al., 1999). In grapefruit, Berhow and Vandercook (1992) applied four different growth regulators, separately in a lanolin paste after fruit set and found that GA_3 remarkably decreased and benzyladenine increased the concentration of naringin in both peel and juice whereas naphthaleneacetic acid (NAA) and abscisic acid (ABA) had no significant effect.

4. Post-harvest factors

4.1. Changes during storage

Generally, in apples, the concentrations of phenolic compounds decrease during fruit development to reach a low, more or less steady, level during maturation and ripening (Burda et al., 1990; Mayr et al., 1995). However, the available literature concerning changes in phenolics during storage and shelf life is much more contradictory. Enzymatic browning of apple fruit tissues and its processed products during storage is generally associated with polyphenol oxidase (PPO) which is able to oxidise phenolic compounds in the presence of oxygen (Nicolas et al., 1994). Mosel and Herrmann, (1974) reported that in 'Boskoop' apples the concentrations of catechin, epicatechin and phenolic acids significantly decreased during regular storage. Piretti et al. (1994) found that the most important phenolics in 'Granny Smith' apple skin, epicatechin, quercetin glycosides, procyanidins and other, unknown phenolic compounds generally decreased from day 100 to the end of storage at day 205, both at regular and low oxygen storage (1.0% O_2 $+2.0\%$ CO_2). Further decreases were also found during one week of shelf life at $20^{\circ}C$. Apple juice pressed from 'Granny Smith' apples stored for 9 months at $1^{\circ}C$ contained a much lower concentration of phenolic compounds, especially procyanidins and catechins, than juice pressed from fruit stored for 3 months. Storing the juice concentrates for 9 month at $25^{\circ}C$ caused loss of about 36% of cinnamic acids, loss of 50-60% of quercetin and phloretin derivatives, and a complete loss of procyanidin (Spanos et al., 1990). Ju et al. (1996) reported for 'Delicious' and 'Ralls' apples stored for 4 to 5 months at regular storage that no changes occurred in the concentrations of simple phenols (mainly chlorogenic acid), flavonoids and anthocyanin. However, during 7 days at $20^{\circ}C$ following storage simple phenols and flavonoids rapidly decreased. They also found that anthocyanin decreased during shelf life but only in early picked fruit.

Kolesnik et al. (1977) reported that the concentration of anthocyanins and flavonols increased during regular storage while catechins and leucoanthocyanins decreased indicating a different behaviour of individual phenolic compounds. Lin et al. (1989) studied the stability of three types of anthocyanins (i.e. cyanidin3-galactoside, cyanidin3-arabinoside and an unidentified cyanidin arabinoside) in the skin of 'Starkrimson' apples stored unpackaged, under in-package modified atmosphere ($5\%O_2$, $10\%CO_2$, and 85% N_2) and in heat shrinkable wrap at $2^{\circ}C$ and 73% RH for up to 30 weeks. They found that CO_2 levels of higher than 73% in in-package modified atmosphere severely destabilised the three anthocyanins. Cyanidin 3-galactoside was more stable in the skin of unpacked apples than for shrink-wrapped apples. On the other hand, Burda et al. (1990) reported that the concentration of epicatechin, procyanidin B2 and phloridzin as major phenolics of three apple cultivars 'Golden Delicious', 'Empire' and 'RI Greening' in both the flesh and the skin remained at a relatively constant concentration during 6 months of regular storage. Other reports have also shown that the total phenol concentration in apples is relatively stable during storage (Coseteng and Lee, 1987; Kang and Seung, 1987). In 'Conference' pears stored in CA with various concentrations of O_2 and CO_2, the concentration of total phenols was not affected by storage conditions (Veltman et al., 1999). Zhang et al. (2000) reported for litchi fruit that flavan-3-ols monomers and dimers in addition to cyanidin-3-glucoside were major phenolics that declined with storage or browning and these phenolics were apparently the major substrates for the enzymatic oxidation.

However, in other fruits the biosynthesis of different anthocyanin types continues after harvest and during regular storage even at low storage temperature in dark conditions as found in blueberry (Kalt and McDonald, 1996), pomegranates (Holcroft et al., 1998) and strawberry (Kalt et al., 1993; Holcroft and Kader, 1999; Tomas-Barberan et al., 2000). Increasing the CO_2 concentration around fruit inhibits the post-harvest increase in anthocyanin, by affecting biosynthesis, degradation or both (Holcroft et al., 1998; Holcroft and Kader, 1999). In strawberry fruit, the concentrations of other phenolics as ellagic acid, catechin, quercetin and kaempferol derivatives were also increased during storage but these were not affected by CO_2 concentration in the storage atmosphere (Holcroft et al., 1998). Tomas-Barberan et al. (2000) observed an increase in anthocyanin concentration in nectarine, cherry, grape and strawberry fruits during regular storage whereas flavonoids and hydroxycinnamic acid derivatives remained constant with the exception of resveratrol in grapes and ellagic acid in strawberry that were also increased.

In conclusion, the complexity of results from investigations of phenolics changes during storage might be due to differences in the way that cultivars or species respond to storage conditions and/or possibly to differences in preharvest conditions.

4.2. Post-harvest irradiation

Apples exposed to sunlight after picking often blush rapidly compared to well illuminated apples still hanging on the tree and this has long been used as a commercial practice for improving red colour formation (Walter, 1967; Saure, 1990). Arakawa (1985) showed that anthocyanin formation in detached apples was greatest with UV-B irradiation and white light combined. Simultaneous irradiation with red and UV-B light had also a synergistic effect on anthocyanin formation. Arakawa (1988b) observed that the responsiveness of the fruit to light especially to white light, differed considerably between the cultivars. Under white light alone or in combination with UV-B light, 'Starking Delicious' and 'Jonathan' apples produce a maximum concentration of anthocyanin. 'Fuji' and 'Tsugaru' apples produced smaller amounts of anthocyanin and required a higher light intensity and were more dependent on UV-B irradiation. However, 'Mutsu' and 'Golden Delicious' produce no anthocyanin at even a high intensity of white light but a small amount of anthocyanin formation occurs with the addition of UV-B light. They observed that under white plus UV-B light, bagged fruit which had been covered with paper bags since about one month after flowering produced much higher anthocyanin amounts at both immature and mature stages than non-bagged ones, regardless of cultivar. Post-harvest UV-B irradiation has also been reported to increase the concentration of anthocyanin and other phenolics as quercetin glycosides and catechins and chlorogenic acid in several apple cultivars (Dong et al., 1995; Reay, 1999; Lancaster et al., 2000). However, the response to this treatment may vary upon cultivar, previous light exposure, temperature and the type of flavonoids examined (Lancaster et al., 2000). Tomas-Barberan et al. (2000) reported that post harvest UV-B and UV-C irradiation did not induce any increase in anthocyanin or flavonoids concentration of nectarine, cherry, grape and strawberry fruits, except that of resveratrol in grapes. The system

of anthocyanin biosynthesis may remain intact and active for several months during regular and controlled atmosphere storage in darkness. Bishop and Klein (1975) found that in contrast to fruit stored at regular cold storage, 'McIntosh' apples stored for about 8 months under controlled atmosphere conditions showed no diminution in synthetic capacity over time.

5. Genetic factors

As in other plants, flavonoids contents in apples are shown to be specific for particular organs or tissues and, genetically, developmentally, and environmentally determined in a very complex and yet unclear way (Lancaster, 1992). Although the genetics and biochemistry of anthocyanin biosynthesis in apples has been studied by several researchers, the conclusions are sometimes contradictory (Cheng et al., 1996; Ju et al., 1999a). According to Ju et al. (1999a), anthocyanin biosynthesis in apples is controlled by a family of structural genes. These structural genes are tightly linked and their hereditary behaviour follows the same pattern as one gene with red as dominant (Cheng et al., 1996). They exist in all apple cultivars, whether the cultivar bears red or non-red fruit and their expression in different cultivars may be controlled by regulatory genes. Under field conditions, environmental factors might affect anthocyanin biosynthesis through these regulatory genes. Significant genotypic variation has been observed for the concentration of flavonoids, especially that of anthocyanin. Thus classical breeding would be a possible tool for improving the levels of flavonoids including anthocyanin in apples. In flowers, manipulation of flavonoid biosynthesis has become feasible after characterization of several enzymes and cloning their corresponding structural and regulatory genes. Recent advances in molecular biology as gene isolation, manipulation and transfer between species have made it possible to alter flower colour or plant properties in a commercially meaningful way (Mol et al., 1989; Bevan, 1993; Holton and Cornish, 1995). De Vos et al. (2000), introduced and over-expressed two genes encoding the maize Lc and C1 transcription factors into tomato. This resulted in transformed plants with up to 60-fold higher levels of kaempferol in their fruit, mainly in the flesh, compared to their parent plants. Quercetin levels did not change by ectopic expression of the Lc/C1 genes. However, transformation of the petunia gene encoding CHI resulted in transgenic tomato lines that contain 70-fold higher levels of quercetin in their fruit peel compared to their parent plants. Like in tomato fruits, expression of the Lc/C1 genes in potato tubers resulted in a marked accumulation of kaempferol. It was concluded that flavonoid pathway engineering is a powerful tool to improve potentially healthy flavonoid compounds in crop plants. It appears that genetic engineering provides opportunities that will eventually influence flavonoids contents in fruit. However, the time lag of 10 to 15 years to deliver a commercial transgenic cultivar and the question of how this will be accepted by growers, handlers and consumers must be considered. The reader is referred to the review articles of e.g. Mol et al. (1989 and 1996) and Holton and Cornish (1995) for genetics and biochemistry of flower pigmentation in different species and of Lancaster (1992) for potential research areas in molecular regulation of apple skin colour.

Aim of the thesis

Since flavonoids and hydroxycinnamic acid derivatives most possibly possess health benefits for consumers, information on important dietary sources of these compounds and how to increase their content in fruit would be useful. Studies of the relationships between environmental and developmental factors and the formation of individual flavonoids and chlorogenic acid during fruit growth can lead to a better understanding of the mechanism of overall flavonoids accumulation and thus potentially improve the possibilities to enhance both fruit colour and healthiness. The aim of the work described in this thesis was therefore, to obtain knowledge on the extent to which the contents of flavonoids and chlorogenic acid in the skin of apples varies, how they develop during fruit growth phase, ripening phase and post harvest phase and how they can be manipulated.

Outline of the thesis

In Chapter 2 the natural variation in flavonoids and chlorogenic acid concentrations due to within fruit, within tree, between orchards, between cultivars and among mutants of 'Elstar' and 'Jonagold' apples is characterised. In Chapter 3, the natural distribution of light within the tree canopy and its relation with the existing variation in flavonoids and chlorogenic acid concentrations in fruit is analysed. The relationships between fruit nutrients and the concentrations of flavonoids and chlorogenic acid were studied in Chapter 4. The hypothesis that, assimilate availability would influence the synthesis of secondary metabolites as flavonoids and chlorogenic acid was evaluated in Chapter 5. Chapter 6 describes the changes that occur during fruit development and the interrelation between different flavonoid classes and chlorogenic acid. In Chapter 7, the influence of some plant growth regulators and fruit maturation on the accumulation of flavonoids and chlorogenic acid were investigated. The changes in these compounds during postharvest storage at regular and controlled atmosphere storage conditions and during shelf life were determined in Chapter 8. In Chapter 9 an attempt was made to integrate the results of the previous chapters in a general discussion.

2

Flavonoid and chlorogenic acid concentrations in apple fruit: characterisation of variation

Mohamed A. Awad, Anton de Jager, Lucie M. van Westing

Abstract

Variations in flavonoid and chlorogenic acid concentrations within fruit, within tree, between orchards, between cultivars and among mutants were characterised and quantified in 'Elstar' and 'Jonagold' apples by reversed-phase high performance liquid chromatography (RP-HPLC). The sun-exposed skin of individual fruit had much higher cyanidin 3-galactoside (anthocyanin) and quercetin 3-glycoside concentrations than the shaded skin, while phloridzin, catechins and chlorogenic acid were similar in the skin of both sides. Individual flavonoid and chlorogenic acid concentrations were not equally distributed within the fruit. Quercetin 3-glycosides and anthocyanin were almost exclusively found in the skin. Catechins were mostly found in the skin but some were present in the flesh. Phloridzin was most abundant in the seeds, with intermediate concentrations in both the core area and the skin, and the lowest concentration in the flesh. Chlorogenic acid was mainly present in the core area and the seeds with an intermediate concentration in the flesh and a low concentration in the skin. The concentrations of anthocyanin, quercetin 3-glycosides and total flavonoids were highest in fruit borne in the top of the tree followed by fruit from the outer tree parts, whereas the lowest concentrations were found in fruit from the inner tree. Terminal fruit contained the highest concentrations of these compounds, including catechins, compared to lateral and spur fruit. Phloridzin and chlorogenic acid were not affected by the position of the fruit in the tree nor by the bearing wood type. There were large differences in flavonoid and chlorogenic acid concentrations in 'Elstar' fruit between two normally productive orchards differing mainly in growth vigour. 'Jonagold' and its mutants had considerably higher concentrations of flavonoid and chlorogenic acid than 'Elstar' and its mutants. The most blushed mutants of both cultivars had higher concentrations of anthocyanin but not of flavonoids or chlorogenic acid compared to the standard cultivar and to the less blushed mutants. The most blushed mutants had a higher number of red cells per cell layer and more cell layers containing red cells than the standard cultivar and the less blushed mutants.

1. Introduction

The attractiveness of apples to consumers is determined both by appearance and by internal attributes of firmness, taste, and health benefits. Flavonoids, phenolic secondary plant metabolites, contribute to both fruit colour and human health. Flavonoids are widely believed to possess anti-oxidative, anti-microbial, anti-mutagenic and anti-carcinogenic properties (Koes et al. 1994; Formica and Regelson, 1995; Shirly, 1996; Robards and Antolovich, 1997). Epidemiological studies have shown an inverse relationship between the intake of fruits, vegetables and beverages rich in flavonoids and the incidence of coronary heart disease, but the relationship with cancer is not clear (Hollman, 1997). Apple is one of the main sources for flavonoid intake in the European diet, after onion and tea (Hertog et al., 1993). The major

flavonoid classes occurring in apple fruit are flavonols such as quercetin 3-glycosides, monomeric and oligomeric flavan-3-ols such as catechin, epicatechin and procyanidins, dihydrochalcones such as phloridzin and, in red-coloured cultivars, anthocyanins such as cyanidin 3-glycosides. Apple fruit also contain considerable amounts of hydroxycinnamic acid derivatives which are mainly represented by chlorogenic acid (Lancaster, 1992; Nicolas, 1994). Flavonoids and chlorogenic acid also contribute to the quality aspects of apples. Their red colour is primarily due to the flavonoid cyanidin-3-galactoside located in the vacuoles of skin cells (Sun and Francis, 1967; Lancaster, 1994), and the browning occurring in processed apple such as juices and ciders is mainly due to oxidation of chlorogenic acid by oxidative enzymes (Nicolas, 1994).

The biosynthesis of flavonoids in apple, as in other plant tissues, includes precursors from both the shikimate and the acetate-malonate pathways via several enzymatic steps (Stafford, 1990; Lancaster, 1992; Van der Meer et al., 1993). Flavonoids are generally present in plant tissues as glycosides. In apple, the predominant sugar involved in glycosylation is galactose. Other sugars involved are glucose, rhamnose, xylose, arabinose and the disaccharide rutinose. Contrary to other flavonoids, flavan-3-ols are generally found in free rather than in glycosylated forms. The different flavonoid classes are predominantly located in the skin (McRae et al. 1990; Guyot et al., 1998).

McRae et al. (1990) concluded that culture and growing conditions have limited effects on the polyphenol profiles of the cortex and peel of apple fruits but did not discuss effects on actual concentrations. There is considerable qualitative information on the developmental and environmental regulation of anthocyanin biosynthesis in apples (Saure, 1990; Lancaster, 1992), but quantitative information on the amplitude of variation and hence the potential for control is almost lacking. For other flavonoid substances even the variation in content due to varietal and environmental factors is poorly studied.

Plant health substances in fruit contribute to fruit quality as precepted by consumers and more quantitative knowledge is needed of natural variation in order to increase or optimise their concentration in fruits. The purpose of the present research was, therefore, to measure variation in concentrations of major flavonoid compounds and chlorogenic acid in apples due to location within the fruit, within the tree, between orchards and between cultivars and their mutants.

2. Materials and Methods

2.1. Plant material

Mature apple fruit of the cvs Elstar, Jonagold and some of their mutants were collected at commercial harvest from trees grafted on M 9 rootstock, trained as slender spindles and grown in commercial orchards in The Netherlands. Samples generally consisting of 15 fruit were peeled

with a hand peeler, frozen in liquid nitrogen and freeze-dried. The freeze-dried skin was ground and sieved to separate it from adhering fleshy parts. The dry skin samples were kept at -20 °C for later flavonoid and chlorogenic acid analysis.

To characterise within-fruit variation, in 1996 a sample of fruit was collected from the periphery of 15 trees (one fruit per tree) of 'Jonagold', 'Elstar' and two related mutants, 'Elshof' and 'Red Elstar', at commercial harvest. The peel was divided into two parts: (1) shaded (no or very little red colour) and (2) sun-exposed (nearly full red colour). In 1998, samples of fruit from the periphery of trees of 'Jonagold' and of 'Elstar' were collected from two replicates each of 15 trees at commercial harvest. Fruit was divided into skin, flesh, core area and seeds and then frozen in liquid nitrogen and prepared as described above for the skin.

To characterise within-tree variation, the position of fruit in the tree and the type of bearing wood were defined as sources of variation. Positions were top, inner, and outer, and for outer position the sectors north (N), east (E), south (S) and west (W) were distinguished. At each position fruits were taken from one year terminals (shoots >10 cm), one year laterals (lateral position on one-year-old shoots) and spurs. In 1996 two 'Elstar' orchards were involved: Orchard A had a North-South orientation, a planting system of four-row beds, a light soil and vigorous growth. Orchard B had an East-West orientation, with a planting system of three-row beds, rather heavy soil and moderate growth. Both orchards were normally productive and received the standard commercial cultural practices. In each orchard, 24 trees were randomly selected. At commercial harvest one box of fruit (about 12 Kg) was picked for each bearing wood type at each position in each orchard. In the laboratory, a sample was randomly taken from each box, and peeled. The skin was prepared as described earlier for analysis.

Cultivar and mutant variation were studied in 1998 by collecting, at commercial harvest, fruit samples from the periphery of trees of standard 'Elstar' and 'Jonagold' and some of their corresponding mutants. Standard 'Elstar' was compared with the mutants 'Elshof', 'Red Elstar', 'Elstar Roelse' and 'Bel Elstar' using samples from two replicates of five trees (3 fruits per tree) for each cultivar. Standard 'Jonagold' was compared with the mutants 'Jonagored', 'Red Jonaprince', 'Jonaveld', 'Crowngold' and 'Decosta' sampled as described above. Both 'Jonagold' and 'Elstar' and their corresponding mutants were growing in the same orchard. Fruit of all cultivars and mutants were peeled and the skin prepared as described earlier for analysis.

2.2. Microscopic study of anthocyanin-containing cells

Cross-sections were prepared with a slide microtome from the red skin of mature 'Jonagold' and its dark-red mutant 'Red Jonaprince' shortly after picking. Each section was placed on a glass slide and covered with droplet of glycerin-gelatin and a cover slip. Micrographs were made using a light microscope (350 x).

2.3. Extraction and quantification of flavonoids and chlorogenic acid

Flavonoids and chlorogenic acid were extracted and quantified by adaptation of the method of Lister et al., (1994). Freeze dried apple skin (0.5 g) was extracted in 20 ml of methanol/10% acetic acid, 85/15 for 30 min in an ultrasonic bath. The extract was concentrated almost to dryness under vacuum at 35 °C and dissolved in 1 ml methanol plus 1 ml of 10% acetic acid in an ultrasonic bath for 1 min. The extract was transferred into a 25 ml volumetric flask and the volume brought to 25 ml with 10% acetic acid and filtered through a 0.2 μm chromafil filter before injecting onto the RP-HPLC.

The RP-HPLC system consists of a Merck Hitachi (L 6200A) pump with a Marathon automatic sample injector, a UV Perkin-Elmer (LC-85B) detector and a visible Spectra-Physics (SP 8480 XR) detector. The RP-18 column (Hypersil, C18 (ODS), 5 μm, 250 mm x 4.6 mm) was fitted with a direct connect prefillable guard column (Alltech). Chromatographic traces were recorded using the Maestro (Chrompack) computer programme. The HPLC eluate structure and wavelength used for monitoring the individual flavonoids and chlorogenic acid were as follows: (1) 10% acetic acid/acetonitrile (70/30) monitored at 530 nm for cyanidin 3-galactoside and at 280 nm for phloridzin; (2) 10% acetic acid/acetonitrile (91/9) monitored at 366 nm for quercetin 3-glycosides and at 313 nm for chlorogenic acid; and (3) 10% acetic acid monitored at 280 nm for catechin and epicatechin; in the latter the eluent was switched to 10% acetic acid/acetonitrile (70/30) every 10 min for about 5 min in order to clean up the column before the next sample injection. The eluate was de-aerated by vacuum filtration through a 0.2 μm filter. Samples of 20 μl were injected onto the column which was maintained at 30 °C using a Marathon column heater. The flow rate was maintained at 1 ml/min. The chromatogram peaks of individual compounds were identified by comparing retention times with those of authentic compounds. Integrated peaks were calculated by comparison with standard solutions of known concentration. Standards used to quantify the HPLC data were cyanidin 3-galactoside, quercetin 3-galactoside, quercetin 3-rhamnoglucoside, quercetin 3-glucoside and quercetin 3-arabinoside (Routh). Quercetin 3-xyloside was purchased from Plantech, Reading U.K. and Quercetin 3-rhamnoside from Sigma. (+)-Catechin and (-)-epicatechin were obtained from Aldrich. Phloridzin and chlorogenic acid were obtained from Fluka. Analytic quality control was performed by including control samples with a known amount of flavonoids and chlorogenic acid in every series of analysis. All determinations were carried out in duplicate. When duplicates differed more than 10%, sample extraction and measurement was repeated. Data were statistically analysed by analysis of variance using the statistical package Genstat 5, release 4.1 (Rothamstead, UK).

3. Results

3.1. Difference between sun-exposed and shaded skin of the same fruit

Sun-exposed skin parts clearly contained higher concentrations of cyanidin 3-galactoside (anthocyanin) and quercetin 3-glycosides than shaded skin parts of the same fruit (Table 1). This held both for standard cultivars and their coloured mutants. There were no significant differences in the concentrations of phloridzin, catechins and chlorogenic acid between the sun-exposed and shaded skin. In both exposed and shaded skin, quercetin 3-glycosides were the dominant phenolics followed by catechins.

3.2. Distribution within individual fruit

Since there were no significant interactions between tissue zone and cultivar in flavonoid concentrations, the data for the two cultivars were combined to determine the distribution of flavonoids in the fruit. Table 2 shows that cyanidin 3-galactoside was mainly located in the skin with traces in other parts, whereas quercetin 3-glycosides were exclusively found in the skin. Except for the flesh, the concentration of phloridzin increased from the skin to the seeds. Phloridzin was the principle flavonoid in the seeds, where it contributed 98% of total flavonoids. Catechins concentration increased in the direction from the seeds to the skin. For distribution of chlorogenic acid, interaction was found between tissue and cultivar. In 'Jonagold' the chlorogenic acid concentration was maximal in the core area and in 'Elstar' in the seed.

3.3. Variation within the tree and between orchards

Position of the fruit in the tree, type of bearing wood and orchard all significantly affected flavonoid and chlorogenic acid concentrations in 'Elstar' fruit skin (Tables 3 and 4). The concentrations of cyanidin 3-galactoside, quercetin 3-glycosides, and total flavonoids were significantly higher in fruit from the top than in fruit from other positions and in fruit from the outer position compared to fruit from the inner position. There were no significant differences in flavonoid and chlorogenic acid concentrations among the outer sectors (N, E, S and W). The concentration of phloridzin, catechins and chlorogenic acid did not significantly differ among all positions. Terminal fruit contained significantly higher concentrations of cyanidin 3-galactoside, quercetin 3-glycosides, catechins and total flavonoids than lateral and spur fruit.

Table 1. Flavonoids and chlorogenic acid concentrations in sun-exposed and shaded skin of the same fruit in mature 'Jonagold', 'Elstar' and two 'Elstar' mutants, 'Elshof' and 'Red Elstar' (1996)[a]

Fruit side	Cultivar/Mutant	Flavonoids and chlorogenic acid (mg g⁻¹ dw)					
		Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Sun side	Jonagold	0.81	0.81	0.90	12.14	14.66	0.20
	Elstar	0.63	0.52	1.83	6.38	9.36	0.04
	Elshof	1.04	0.54	2.27	7.00	10.85	0.06
	Red Elstar	1.41	0.44	1.87	5.29	9.01	0.05
Shaded side	Jonagold	0.01	0.71	0.80	3.27	4.79	0.24
	Elstar	0.05	0.46	1.88	2.03	4.42	0.03
	Elshof	0.11	0.48	1.84	2.11	4.54	0.04
	Red Elstar	0.11	0.53	1.74	1.95	4.33	0.00
Mean							
Sun side		0.97	0.58	1.72	7.7	10.97	0.09
Shade side		0.07	0.54	1.57	2.34	4.52	0.08
F-test (for the mean)		**	NS	NS	**	**	NS
$LSD_{0.05}$		0.48	–	–	3.86	3.80	–

[a] Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhaminoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3-glycosides; NS: not significant and (**) significant at level $P = 0.01$, respectively; (–) not calculated.

Chlorogenic acid concentration was higher in terminal fruit than spur fruit. Lateral and spur fruit did not differ significantly in flavonoid and chlorogenic acid concentrations. Phloridzin concentration was not influenced by bearing wood type. Fruit from orchard B had significant higher concentrations of cyanidin 3-galactoside, catechins, total flavonoids and chlorogenic acid than fruit from orchard A. There were significant interactions among position, bearing wood type and orchard on the concentration of cyanidin 3-galactoside (Tables 3 and 4).

3.4. Variation between cultivars and their corresponding mutants

The HPLC chromatographic profiles of flavonoids were qualitatively similar for all cultivars and their corresponding mutants, yet quantitatively different (Table 5). On the whole, 'Jonagold' and its mutants had a significantly higher concentration of total flavonoids, especially quercetin 3-glycosides, than 'Elstar' and its mutants. The largest differences occurred in some individual quercetin 3-glycosides and in chlorogenic acid. 'Elstar' fruit contained a significantly higher concentration of quercetin 3-rhamnoglucoside and significantly lower concentrations of both quercetin 3-rhamnoside and chlorogenic acid compared to 'Jonagold' fruits. The visually more blushed mutants (e.g. 'Red Jonaprince' and 'Bel-Elstar') also had a higher concentration of cyanidin 3-galactoside than the standard or the less blushed mutants. Except for cyanidin 3-galactoside, there were no significant differences in the concentration of flavonoid compounds between a cultivar and its related mutants.

Table 2. Distribution of flavonoids and chlorogenic acid in different tissues of mature 'Jonagold' and 'Elstar' apples (1998)[a]

Tissue zone	Flavonoids and chlorogenic acid (mg g dw)					
	Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Skin	1.27	0.79	1.94	5.41	9.41	0.20
Flesh	0.03	0.08	0.37	0.00	0.48	0.48
Core	0.06	1.94	0.43	0.00	2.43	2.10
Seed	0.05	7.41	0.10	0.00	7.56	1.13
$LSD_{0.05}$	0.18	1.06	0.21	–	1.25	0.14
F-test						
Cultivar (C)	NS	**	***	–	NS	*
Zone (Z)	***	***	***	–	***	***
C x Z	NS	NS	NS	–	NS	***

[a] Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhaminoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3-glycosides; NS: not significant; and *, ** and *** , significant at levels $P = 0.05$, 0.01 and 0.001, respectively; (–) not calculated.

Table 3. Flavonoids and chlorogenic acid concentrations of 'Elstar' fruit skin at commercial harvest as affected by position of fruit on tree, bearing wood and orchard type (1996)[a]

Variable		Flavonoids and chlorogenic acid (mg g dw)					
		Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Position	Top	0.26	0.64	1.83	4.21	6.93	0.043
	Inner	0.04	0.56	1.78	1.52	3.90	0.047
	North	0.14	0.69	1.90	2.85	5.58	0.042
	South	0.16	0.62	1.72	3.18	5.67	0.048
	East	0.15	0.61	1.76	2.94	5.47	0.043
	West	0.15	0.72	1.72	2.97	5.56	0.048
$LSD_{0.05}$		0.05	–	–	0.91	1.20	–
Wood type	1YT	0.20	0.68	1.91	3.60	6.39	0.049
	1YL	0.13	0.62	1.72	2.75	5.22	0.044
	>2Y	0.12	0.61	1.73	2.50	4.95	0.043
$LSD_{0.05}$		0.04	–	0.13	0.64	0.82	0.006
Orchard	A	0.09	0.62	1.61	2.78	5.10	0.041
	B	0.21	0.66	1.97	3.12	6.00	0.049
$LSD_{0.05}$		0.08	–	0.11	–	0.67	0.005
F-test							
Position (P)		***	NS	NS	**	**	NS
Wood type (W)		***	NS	*	**	**	*
Orchard (O)		***	NS	***	NS	*	**
P x W		*	NS	NS	NS	NS	NS
P x O		*	NS	NS	NS	NS	NS
W x O		**	NS	NS	NS	NS	NS

[a] 1YT, 1YL, and >2Y, 1 year terminal, 1 year lateral, and more than 2 years, respectively. Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamninoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3-glycosides; NS: not significant; and *, ** and ***, significant at levels $P = 0.05$, 0.01 and 0.001, respectively; (–) not calculated.

The microscopic study (Fig. 1) showed that even in the reddest part of the skin many cells have no anthocyanin in their vacuoles. The cellular distribution of red colour was different for the standard cultivar and its derived mutants. In standard 'Jonagold', red cells occurred in the epidermis and in the outer hypodermal layer, whereas in 'Red Jonaprince' anthocyanin was found in the epidermis and in three hypodermal layers. The pigmented hypodermal cells usually contained larger and darker red vacuoles than the pigmented epidermis cells. The mutant 'Red Jonaprince' had a higher proportion of dark red cells than standard 'Jonagold'.

Table 4. The interaction effect of position, bearing wood type and orchard type on cyanidin 3-galactoside concentration in mature 'Elstar' fruit skin[a]

		Cyanidin 3-galactoside (mg g dw)							
		Position (P)						Orchard (O)	
		Top	Inner	North	South	East	West	A	B
Wood type (W)	1YT	0.41	0.05	0.19	0.20	0.21	0.16	0.11	0.29
	1YL	0.17	0.03	0.15	0.12	0.13	0.18	0.07	0.19
	>2Y	0.20	0.05	0.07	0.16	0.13	0.11	0.10	0.14
Orchard (O)	A	0.18	0.04	0.07	0.10	0.11	0.05		
	B	0.33	0.04	0.20	0.22	0.19	0.25		

[a] $LSD_{0.05}$ for P x W, P x O, and W x O = 0.093, 0.076 and 0.054, respectively.

4. Discussion

Very low concentrations of anthocyanin, moderate concentrations of quercetin 3-glycosides and relatively high concentrations of phloridzin, catechins and chlorogenic acid were found in the shaded skin of an individual fruit (Table 1) and also in the skin from fruit borne in the inside of the canopy (Tables 3 and 4), indicating that anthocyanin synthesis is a light dependent process, while the synthesis of other phenolic metabolites is slightly if at all light dependent. These results support the suggestion of Ju (1998) that the genes controlling the synthesis of different phenolic compounds might have a different sensitivity to light. Dong et al. (1995) found that exposing green 'Royal Gala' apple fruit (picked from the inside of the tree) to white plus UV light increased anthocyanin and flavonoids to a similar level as naturally coloured fruits. Our data confirm those of Workman (1963) who found for 'Golden Delicious' a double amount of quercetin glycosides in exposed fruit compared to ones from shaded tree parts and a 2 fold higher concentration of quercetin glycosides in sun-exposed peel than in shaded peel of the same fruit. In contrast, Ju et al. (1996) found for 'Delicious' and 'Ralls' a similar concentration of flavonoids (flavonols and procyanidins) in both sides although in sun-exposed peel twice as much anthocyanin was found than in shaded peel.

Table 5. Flavonoids and chlorogenic acid concentrations in fruit skin at commercial harvest of 'Jonagold' and 'Elstar' and some of their corresponding mutants (1998)[a]

Cultivar/Mutant	Flavonoids and chlorogenic acid (mg g dw)											
	Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides							Total Flavonoids	Chlorogenic acid
				Q.gal.	Q.rha.	Q.glu.	Q.xyl.	Q.rhgl.	Q.ara.	Total		
Jonagold St	0.63	0.78	2.41	3.4	2.5	0.61	0.83	0.21	0.13	7.69	11.49	0.38
Red Jonaprince	4.12	0.83	2.39	3.5	2.8	0.47	0.88	0.17	0.13	7.96	15.29	0.31
Jonagored	1.62	0.92	2.10	3.2	2.8	0.53	0.90	0.17	0.14	7.71	12.33	0.34
Decosta	1.35	0.77	2.25	2.9	2.5	0.48	0.79	0.16	0.12	6.94	11.31	0.35
Jonaveld	1.12	0.86	2.20	3.3	2.7	0.56	0.90	0.19	0.14	7.83	12.00	0.26
Crowngold	0.58	0.92	2.12	3.0	2.7	0.53	0.86	0.16	0.12	7.40	11.01	0.29
Elstar St	0.38	0.70	2.00	3.1	0.64	0.83	0.63	0.48	0.12	5.81	8.88	0.05
Bel-Elstar	1.92	0.74	2.17	2.9	0.53	0.66	0.56	0.41	0.11	5.11	9.93	0.15
Elstar roelse	1.64	0.71	2.21	2.4	0.45	0.57	0.51	0.36	0.10	4.41	8.97	0.15
Elshof	0.75	0.92	2.53	3.3	0.70	0.77	0.67	0.28	0.12	5.83	10.02	0.07
Red Elstar	0.70	0.68	2.00	2.7	0.57	0.68	0.56	0.42	0.12	5.10	8.43	0.04
LSD$_{0.01}$	0.65	0.14	0.39	–	0.55	0.24	0.17	0.12	0.03	1.90	2.55	0.13
F-test	***	***	*	NS	***	**	***	***	*	***	***	***
Mean												
Jonagold type	1.57	0.84	2.24	3.2	2.66	0.53	0.86	0.17	0.13	7.59	12.24	0.32
Elstar type	1.10	0.75	2.18	2.9	0.58	0.70	0.58	0.39	0.11	5.25	9.25	0.09
F-test	***	***	NS	*	***	***	***	***	**	***	***	***

[a] Date of picking was 8 and 29 of September for Elstar and Jonagold types respectively. Catechins, the sum of catechin and epicatechin; Q.gal., quercetin 3-galactoside; Q.rha., quercetin 3-rhaminoside; Q.glu., quercetin 3-glucoside; Q.xyl., quercetin 3-xyloside; Q.rhgl., quercetin 3-rhamnoglucoside; Q.ara., quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3-glycosides; NS: not significant; *, ** and ***, significant at level $P = 0.05$, 0.01 and 0.001, respectively; (–) not calculated.
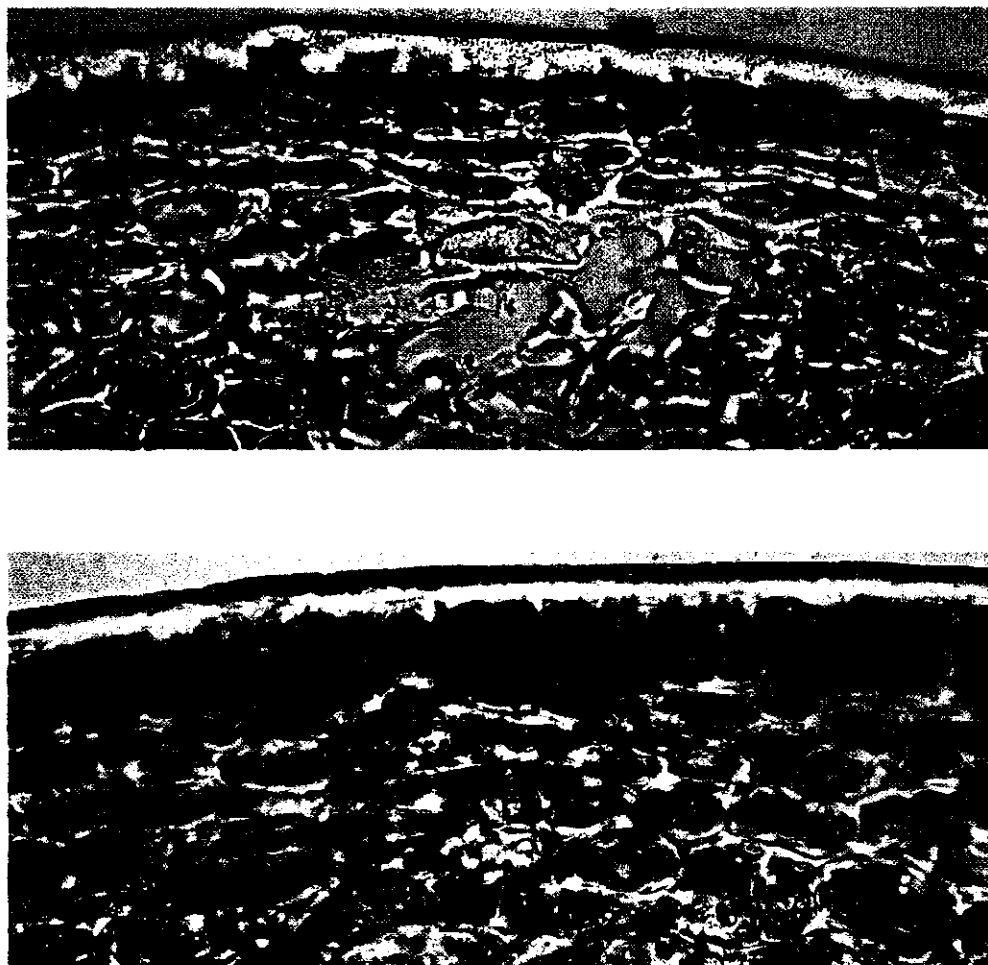
Fig. 1. Anthocyanin distribution in cross-sections of apple skin of (a) standard 'Jonagold' and (b) its mutant 'Red Jonaprince'. Magnification: 350x.

Moreover, in contrast to our data, the concentration of simple phenols (mainly chlorogenic acid) was significantly higher in the shaded than in the exposed peel.

With regard to the distribution of flavonoid and chlorogenic acid within the apple fruit our results (Table 2) confirm those of Guyot et al. (1998), indicating that the synthesis and the accumulation of phenolic compounds is tissue specific. The higher concentrations of phloridzin, catechins and chlorogenic acid in deeper tissue zones may indicate that the genes controlling their synthesis are not light dependent. However, it is not certain whether flavonoid biosynthetic genes are active in these unpigmented tissues. Alternatively, an intercellular transport of flavonoids,

precursors and/ or enzymes in the fruit might be postulated as assumed for petunia flower by Koes et al. (1990). The genetic activity of the various parts of the apple fruit might be different as it is a pome fruit (not a true fruit). The core and seeds originate from the ovary and the fleshy part from the floral tube, the fused bases of sepals, petals and stamens (Pratt, 1988). Yao et al. (1999) found that MADS-box genes (involved in development) in apple fruit are preferentially expressed in the different apple parts. Strict separation in red coloration between adjacing cells subject to the same illumination (Fig. 1) has also been observed by Dayton (1959) and Lancaster et al. (1994). The mechanism behind this striking on/off phenomenon is unclear.

Much of the within-tree variation in fruit quality can be attributed to two factors: position of the fruit in the canopy, which determines the light microclimate under which fruit develop, and bearing wood type (Volz et al., 1994). It is known that the intensity and the composition of the light are different in the exterior and interior regions of the tree canopy. Fruit in the top of the tree may receive a higher light intensity with relatively more UV and red light, and relatively less far-red light than in other positions (Looney, 1968; Proctor, 1975). Such conditions would stimulate the synthesis of anthocyanin and other flavonoids in fruit at the top and outer position (Kubo et al., 1988; Lister et al., 1994). The higher concentrations of flavonoids especially cyanidin-3-galactoside and quercetin 3-glycosides in terminal fruit compared to lateral and spur fruits (Tables 3 and 4) may be explained partly by better light conditions and by a better availability of metabolites and minerals. Volz et al. (1994) found that terminal flower buds on one-year-old wood flower earlier, form a larger leaf area, and produce larger fruit with higher Ca and Mg concentrations than buds on other wood types. It is interesting to note that differences in ripeness (as determined by starch stage, firmness, sugar and acid levels) between various positions in the tree and wood types were only slight (data not reported). This may be an indication that the formation of cyanidin 3-galactoside and quercetin 3-glycosides in apple fruit is mainly dependent on a specific spectral distribution, as also suggested for colour development by Proctor et al. (1975).

The higher concentration of flavonoids in orchard B (Tables 3 and 4) might be due to its lower external and internal shading and also to higher P and Ca and lower N concentrations (paper in preparation). Both lower external and internal shading results from lower growth vigour giving more free space between trees and less room occupied by leafy shoots within trees. Higher P and Ca concentrations have been indicated as possible potential factors by Larrigaudiere et al. (1996) who showed that in 'Starking Delicious' apples anthocyanin concentration was positively influenced by a treatment with seniphos, a compound containing mainly P and Ca. By this treatment ripening and ethylene production were not affected. Tan (1980) suggested that low N and K promote the accumulation of the enzyme PAL, and thus stimulate anthocyanin formation in apple. Considering all of the fruit characteristics (but especially starch and firmness) fruit from orchard B did not appear to be more advanced in ripeness.

Cultivar variation in flavonoids and chlorogenic acid concentrations has also been reported

by McRae et al. (1990) and Perez-Ilzarbe et al. (1991). Fruit response to light in anthocyanin synthesis varies considerably among apple cultivars (Arakawa, 1988b). The differences between the standard cultivars and their mutants show potential anthocyanin accumulation may increase several fold in more blushed mutants without influencing the concentrations of other flavonoids (Table 5). If potential maximum concentrations of flavonoid compounds in apple is mainly genetically determined, this could be an important consideration in apple breeding programmes.

In conclusion, these results show that there is much room for optimising the concentration of healthy substances in fruit through cultivation, selection of varieties and sorting. Since the skin is such an important source of phenolics, any promotion of apple consumption should imply the skin and this, in turn, should be facilitated by safe, e.g. organic, ways of growing.

Acknowledgements

3

Effects of light on flavonoid and chlorogenic acid concentrations in the skin of 'Jonagold' apples

Mohamed A. Awad, Patricia S. Wagenmakers, Anton de Jager

Abstract

The objective of our work was to determine how fruit position on the tree affects flavonoid and chlorogenic acid contents. Light was measured at different positions within the canopy of 10-year-old 'Jonagold' apple trees on M.9 rootstock raised as slender spindles. Fruit from the top of the canopy contained the highest percentage of blush and the highest concentrations of cyanidin 3-galactoside (anthocyanin) and quercetin 3-glycosides, followed by fruit from the outside of the canopy, and then those from the canopy interior. There were no significant differences in the concentrations of catechins, phloridzin and chlorogenic acid among fruit from the different canopy positions. Light level was directly correlated with the concentrations of cyanidin 3-galactoside and quercetin 3-glycosides and with the percentage of blush in the fruit skin. Light in the interior of the canopy was poorer in UV-A, blue, green and red but richer in far-red light than at all other positions. Consequently, the FR/R ratio was much larger at the interior of the canopy than at all other positions. Both anthocyanin and quercetin 3-glycoside concentrations were clearly related to light level, and there was a critical FR/R ratio of about 1 above which no anthocyanin and only minimal quercetin 3-glycosides were formed.

1. Introduction

Flavonoids, plant-derived phenylpropanoid compounds, can serve as free radical scavengers and metal chelators (Stafford, 1990; Rice-Evans et al., 1997). Recently, they have been attributed possible inhibitory action against certain cancers and coronary heart disease (Formica and Regelson, 1995; Steinmetz and Potter, 1996; Hollman, 1997). Apple fruits are known to be rich in flavonoid compounds such as anthocyanins, dihydrochalcones, quercetin 3-glycosides, catechin, and epicatechin and its polymers, which are mainly located in the skin (Lancaster, 1992; Awad et al., 2000). Their red colour is due to anthocyanin pigments, mainly cyanidin 3-galactoside, which can scavenge superoxide radicals in an in-vitro system (Yamasaki et al., 1996). Apple fruits also contain considerable amounts of hydroxycinnamic acid derivatives, mainly represented by chlorogenic acid (Lancaster, 1992; Nicolas, 1994), which showed higher antioxidant capacities than vitamins C and E in in-vitro studies (Rice-Evans et al., 1997). Flavonoid and chlorogenic acid concentrations in fruits vary greatly among cultivars, orchards, positions within the tree and within individual fruit (Awad et al., 2000). Effects of environmental factors and cultural practices on the formation of anthocyanin have been well documented (Walter, 1967; Saure, 1990; Lancaster, 1992). Two environmental factors are especially important: temperature and light. Light conditions within the canopy can be improved by using dwarfing rootstocks, suitable planting systems, optimum row orientation and proper training and pruning systems (Wertheim et al., 1986; Wagenmakers and Callesen, 1995). Anthocyanin concentration in apple skin increased with light level, up to about 50% of full sunlight (Jackson, 1980, Barritt et al., 1997). According to Siegelman and Hendricks (1958), the

formation of anthocyanin in apple skin is controlled by a high-energy photoreaction, with an action maximum between 650 and 670 nm. A subsidiary one at 430 to 480 nm has been reported by Downs et al. (1965). In post-harvest, white plus UV-B light at 312 nm was optimal for anthocyanin synthesis, with red and blue light additive (Arakawa et al., 1985; Arakawa, 1988a). In attached apples, UV cut-off filters prevented rapid anthocyanin formation after bag removal (Kubo et al., 1988). White plus UV light can also promote synthesis of different flavonoid classes (Dong et al., 1995).

The objectives of this study were (1) to characterise the natural light level and its spectral distribution within a 'Jonagold' apple tree canopy grown in a humid climate and (2) to determine any relationship between the measured characteristics of light and the concentrations of flavonoid and chlorogenic acid in the fruit skin.

2. Materials and methods

2.1. Plant material

In 1997, four 10-year-old commercially grown 'Jonagold' apple trees (replicates) on M.9 rootstock and trained as slender spindle in The Netherlands were used. The planting system was a single row, oriented north to south with spacing of 1.25 m by 3.0 m.

2.2. Experimental procedure

Radiation within a tree canopy was measured between 330 and 1000 nm at 5 nm intervals during a cloudy and a sunny day using a spectroradiometer (LI-COR, Model LI-1800) equipped with a remote light collector held horizontally during all measurements. Readings were taken at four zones within the canopy of each tree. Zone 1 was the canopy top about 2 m from the ground; zone 2 was the inner position of the canopy at 1 m; zones 3 and 4 were the outer east and west sides about 20 cm inside the canopy at 1.5 m. In these zones the averages of 3, 4, 3 and 3 reading points 25 cm apart were used, respectively. A reference spectrum was measured above the tree immediately before measuring within each tree. Spectral irradiances at 735 and 645 nm were used to calculate the FR/R ratios (Kasperbauer 1987). The measurements under cloudy condition were done on August 27 between 14.00 and 15.00 h. Sunny conditions were measured on September 2 between 11.00 and 12.00 h. and repeated between 13.00 and 14.00 h.

Daily global radiation data during the period of anthocyanin formation (about 5 weeks before harvest, August 27 to October 2) were collected from a weather station located about 30 km from the orchard. The global radiation was 10.7 MJ/m^2 on August 27, 15.0 MJ/m^2 on September 2 and the daily average from August 27 to October 2 was about 12.0 MJ/m^2.

At commercial harvest (October 2) 10 fruit were picked from each zone for each tree. The percentage of blushed area of each fruit was measured with the colour measuring vision system

'Keurmeester' (AWETA fruit grading company, Nootdorp, The Netherlands) adjusted to a specific setting for 'Jonagold' apples. Fruit were then peeled and the peel was immediately frozen in liquid nitrogen and then vacuum dried. The freeze-dried peel was ground and sieved to separate the skin from adhering fleshy parts. The dry skin samples were then kept at –20 °C for later flavonoid and chlorogenic acid analyses.

2.3. Flavonoid and chlorogenic acid analyses

The extraction and reversed-phase high performance liquid chromatography (RP-HPLC) quantification of flavonoids and chlorogenic acid were carried out as previously described by Awad et al. (2000).

2.4. Statistical analysis

All data were subjected to analysis of variance and the means separated by F-test and by the least significant difference (LSD) test at the 5% level using the statistical package Genstat 5, release 4.1 (Rothamstead, UK). Regression analysis was used to relate light level and the concentration of cyanidin 3-galactoside and quercetin 3-glycosides and blush percentage in the fruit skin.

3. Results

3.1. Light level

Under cloudy and sunny conditions, the inner tree received significantly less light than the other positions (Table 1). Under cloudy conditions, differences between the top and the outer east and west positions were not significant, but under sunny conditions the top and outer east positions received more radiation than the west ones. Within each position far-red light penetrated better than light of shorter wavelengths.

3.2. Spectral composition

Under both cloudy and sunny conditions, the inner position received a significantly lower proportion of UV-A, blue, green and red but a higher proportion of far-red light than all other positions (Table 2). There were no significant differences between the top and the outer (east and west) positions. The relative light composition under cloudy and sunny conditions was similar. The FR/R ratio was significantly larger at the inner position than at all other positions.

3.3. Flavonoid and chlorogenic acid concentrations in fruit skin and percentage of blush

The cyanidin 3-galactoside (anthocyanin) concentration in fruits from the top of the tree was higher than in fruit from all other positions (Table 3). Quercetin 3-glycosides and total flavonoids were highest in fruit from the top position. Cyanidin 3-galactoside, quercetin 3-glycosides and total flavonoids were significantly lower in inner fruit than in those from all other positions. Phloridzin, catechins and chlorogenic acid concentrations were not significantly affected by fruit position. Percent of blush on fruit followed the same trend as found for cyanidin 3-galactoside.

3.4. Relationship between light level and the concentrations of cyanidin 3-galactoside, quercetin 3-glycosides and percentage of blush

Significant correlations were found between light level and the concentrations of cyanidin 3-galactoside ($r = 0.66$, $P<0.05$), quercetin 3-glycosides ($r = 0.86$, $P<0.05$), and percentage of blush ($r = 0.61$, $P<0.05$) in the fruit skin. It is evident that at low light level and above a critical FR/R ratio (about 1) there was virtually no anthocyanin formation (Fig. 1a and b). Quercetin 3-glycosides concentration was probably zero at zero light level (Fig. 2a) but above a FR/R ratio of 1 there was still a minimum level of about 2.5 mg/g dw (Fig. 2b).

Table 1. Percent of incoming light at different positions in a 'Jonagold' apple tree canopy measured under cloudy and sunny conditions[a]

Position on tree	Light level (percentage of above tree)					
	UV-A	Blue	Green	Red	Far-red	Total radiation
Cloudy conditions						
Top	59	59	60	60	69	61
Inner	8	8	8	9	17	10
Outer east	55	54	54	55	64	56
Outer west	57	58	58	58	63	53
F-test	***	***	***	***	***	***
LSD $_{0.05}$	25	26	25	24	20	16
Sunny conditions						
Top	65	67	68	69	80	70
Inner	7	7	7	6	15	8
Outer east	75	75	81	84	95	82
Outer west	50	49	49	49	56	50
F-test	***	***	***	***	***	***
LSD $_{0.05}$	16	19	18	21	22	19

[a] Values are the means of four trees. UV-A, 330-400 nm; blue 400-450 nm; green, 450-530 nm; red, 600-700 nm; far red, 700-750 nm; total radiation, 330-750 nm. The measurements under cloudy conditions were between 14.00 and 15.00 h on August 27; those under sunny conditions were on September 2 between 11.00-12.00 h and were repeated between 13.00 and 14.00 h. ***, significant at $P = 0.001$.

Table 2. Spectral composition and FR/R ratio (735/645 nm) at different positions in a 'Jonagold' apple tree canopy measured under cloudy and sunny conditions[a]

Position on tree	Spectra composition (percentage of total available light)					
	UV-A	Blue	Green	Red	Far-red	FR/R
Cloudy conditions						
Above tree	7.2	9.0	18.0	18.8	10.5	0.83
Top	6.5	8.3	16.5	17.5	11.5	0.97
Inner	4.0	5.3	10.7	11.4	14.8	2.00
Outer east	6.6	7.8	16.1	17.1	11.7	1.10
Outer west	6.4	8.3	16.6	17.5	11.6	1.00
F-test	***	***	***	***	***	***
LSD $_{0.05}$	0.5	0.6	0.8	1.1	0.8	0.25
Sunny conditions						
Above tree	6.1	8.7	17.7	20.4	10.7	0.82
Top	5.6	7.9	16.3	18.7	11.6	0.99
Inner	2.9	4.1	9.5	7.9	15.4	3.20
Outer east	5.2	7.4	16.0	19.0	11.7	0.98
Outer west	5.9	8.0	15.9	17.8	11.4	1.03
F-test	***	***	**	***	***	***
LSD $_{0.05}$	0.7	0.9	2.8	1.4	0.9	0.73

[a] Values are the means of four trees. UV-A, 330-400 nm; blue 400-450 nm; green, 450-530 nm; red, 600-700 nm; far red, 700-750 nm. The total of available light used was in the range of 330-1000 nm. The measurements under cloudy conditions were between 14.00 and 15.00 h on August 27; and under sunny conditions were on September 2 between 11.00 and 12.00 h and were repeated between 13.00 and 14.00 h. **, ***, significant at $P = 0.01$ and 0.001, respectively.

Table 3. Flavonoid and chlorogenic acid concentrations (mg g dw) and % of blush at commercial harvest in 'Jonagold' apple skin as affected by fruit position in the tree[a]

Position on tree	Percentege of blush	Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Top	38.0	0.55	1.2	3.0	8.8	13.5	0.17
Inner	0.0	0.02	1.1	3.6	2.5	7.2	0.20
Outer east	14.2	0.23	1.2	3.7	7.0	12.2	0.20
Outer west	20.5	0.25	1.2	3.5	6.8	11.8	0.21
F-test	***	***	NS	NS	***	**	NS
LSD$_{0.05}$	7.8	0.12	–	–	2.0	2.6	–

[a] Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside, quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3-glycosides. NS, not significant; **, ***, significant at level $P = 0.01$ and 0.001 respectively; (–), not calculated.
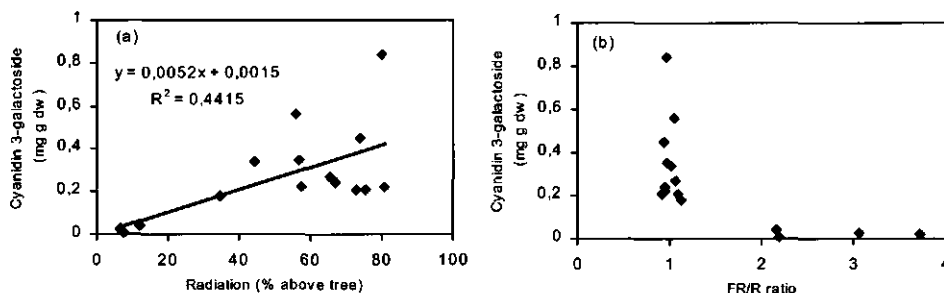
Fig. 1. Relationship between the concentration of cyanidin 3-galactoside in Jonagold apple skin and (a) percentage of radiation and (b) FR/R ratio.
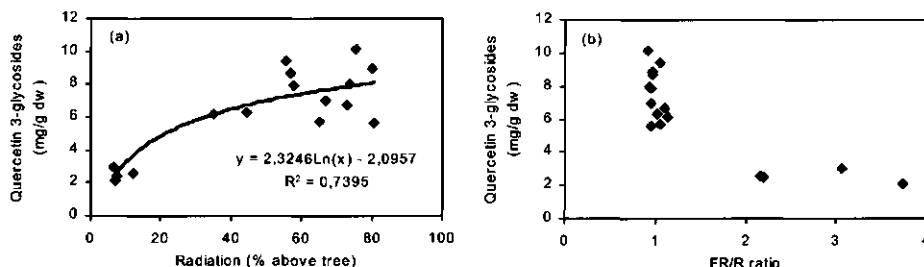

Fig. 2. Relationship between the concentration of quercetin 3-glycosides in Jonagold apple skin and (a) percentage of radiation and (b) FR/R ratio.

4. Discussion

Canopy shading clearly decreased the concentration of cyanidin 3-galactoside and quercetin 3-glycosides in 'Jonagold' apple skin. The concentrations of catechins, phloridzin and chlorogenic acid were, however, largely independent of light/canopy position (Table 3). Though the light dependence of anthocyanin formation is well known, that of quercetin 3-glycosides has not been reported before. Our data also conflict with the conclusion of Ju et al. (1999b) that the total concentration of flavonoids is not influenced by light quantity or canopy positions. They, however, only measured total flavonoids and did not discriminate between quercetin glycosides and other phenolic classes. Catechins, phloridzin and chlorogenic acid are part of the same biosynthesis pathway as quercetin 3-glycosides and cyanidin 3-galactoside (Lancaster, 1992) and one would expect competition for the same precursor molecules. However, accumulation of catechins, phloridzin and chlorogenic acid was apparently not affected by the synthesis of cyanidin 3-galactoside and quercetin 3-glycosides, even in fruit grown on the top of the tree where there is a substantial accumulation of both of these two compounds (Table 3). This

suggests that the biosynthesis of the catechins, phloridzin and chlorogenic acid on the one hand and quercetin glycosides and cyanidin 3-galactoside on the other, occur independently of each other. Perhaps synthesis of different flavonoid classes occurs in different cell types of the apple skin. The observation that in the reddest area of apple skin anthocyanin only accumulates in some cells may support this suggestion (Awad et al., 2000). Alternatively, different independently operating multi-enzyme complexes may channel substrate conversion towards either catechins, phloridzin and chlorogenic acid or quercetin 3-glycosides or cyanidin 3-galactoside (Burbulis and Shirley, 1999).

The lower level of UV-A, blue, green and red light, the higher level of far-red light and the higher FR/R ratio in the interior of the canopy (Tables 1 and 2) may suppress the synthesis of both cyanidin 3-galactoside and quercetin 3-glycosides in the inner position (Table 3). Both quercetin glycoside and cyanidin 3-galactoside concentrations were related to light level; the regression lines (linear for cyanidin 3-galactoside, logarithmic for quercetin 3-glycosides, Figs. 1a and 2a) most likely passed through zero. This corresponds to the results of Ju et al. (1997) with bagged fruit, where there was no synthesis of anthocyanin and quercetin glycosides before bag removal. Furthermore, there appeared to be a requirement for a FR/R ratio of about 1 for significant cyanidin 3-galactoside and quercetin 3-glycosides formation (Figs. 1b and 2b) which did not occur in the tree interior. The higher concentrations of quercetin glycosides, cyanidin 3-galactoside, and percentage of blush in fruit from the top than in ones from the outside of the canopy (Table 3) might be due to a longer time of light exposure of fruit at the top than fruit at the outer positions.

Light is considered one of the most important factors in the control of flavonoid synthesis acting upon a complex system involving several photoreceptors: UV, blue, phytochrome and the photosynthetic system (Tobin and Silverthorne, 1985; Arakawa, 1988a). Phytochrome activity is controlled by the FR/R ratio and is believed to regulate flavonoid gene expression in many plant systems (Mohr and Herrel, 1983; Tobin and Silverthorne, 1985). The differences in fruit maturity between fruit from the different canopy positions were generally slight and are unlikely to explain the stimulatory effects of light/canopy position on cyanidin 3-galactoside and especially on quercetin 3-glycosides formation (Awad et al., 2000; Ju, 1998).

A large portion of the tree canopy received less than 20% of the available light. This means that even in trees trained as a slender spindle, further action is needed to increase light penetration within their canopy for increasing cyanidin 3-galactoside and quercetin 3-glycoside concentrations, leading to improved fruit colour and healthiness. Mutants with more blush, which are able to accumulate anthocyanin even in fruit at the inner canopy position, did not show higher quercetin 3-glycoside concentrations than their standard cultivars (Awad et al., 2000). Therefore, improving light conditions within the canopy, especially at the final stage of fruit development, by some cultural measure e.g. summer pruning, repositioning branches or covering the orchard floor with reflecting films, may improve both healthfulness and colour of the fruit.

Acknowledgements

Abstract

The relationships between fruit N, P, K, Mg and Ca concentrations during the season and flavonoid and chlorogenic acid concentrations in skin of 'Elstar' apples at maturity have been studied during three seasons in a nutrition experiment (with the mutant 'Elshof'), and in a separate experiment with standard 'Elstar' using within-tree variation in nutrient concentration due to fruit position in the tree. Negative correlations were frequently found between the concentration of N and Mg and the N/Ca ratio, and occasionally with that of K, in fruit during growth and at maturity, and anthocyanin and total flavonoids concentration at maturity. Calcium concentration showed occasionally positive correlations with anthocyanin and total flavonoids concentration. Chlorogenic acid concentration showed only in the second experiment a correlation with some of the studied nutrients (positive with P and Ca and negative with N, K, Mg and N/Ca). Regression models accounted for up to 40% and 30% of the variance in anthocyanin and total flavonoids concentration of 'Elshof' mutant apples, and up to 70% and 65% of the variance in anthocyanin and total flavonoids concentration of standard 'Elstar' apples, respectively. The most important variable in predictive models for the anthocyanin and total flavonoids concentration was N concentration in the fruit. The results suggest that the concentration of flavonoids in fruit skin could be increased by optimising fertilization, especially that of N.

1. Introduction

Plant flavonoids constitute one of the largest group of naturally occurring phenolics, possessing ideal chemical structural as antioxidants, free radical scavengers and metal chelators (Rice-Evans et al., 1997). Hertog et al. (1993) estimated the daily Dutch intake of flavonols as 23 mg/day (expressed as aglycones). However, reliable quantitative data on the intake of other flavonoids such as anthocyanin, catechins, phloridzin and phenolic acids are not yet available. Apple fruits are rich in quercetin glycosides, catechin, epicatechin, procyanidins, dihydrochalcones (such as phloridzin) and in blushed cultivars in anthocyanin (such as cyanidin glycosides), all of which are concentrated mostly in the skin (Lancaster, 1992; Awad et al., 2000). Hydroxy cinnamic acids, such as chlorogenic acid, which are also reported to have in-vitro antioxidant properties (Rice-Evans et al., 1997) are present predominantly in the flesh (Awad et al., 2000).

Plant phenolics are considered as stress metabolites and can be induced by a wide range of environmental stimuli such as light, ozone, temperature, water stress and wounding (Tan, 1980; Lancaster, 1992; Kangasjarvi et al., 1994; Matern and Grimmig, 1994; Estiarte et al., 1994). A number of pre and post harvest studies have been mainly conducted to increase anthocyanin concentration because of its direct relation with fruit colour and marketability (Smock, 1966; Faust, 1973; Walter, 1967; Saure, 1990). Fruit nutrient composition has a strong association with general external and internal quality attributes of fruits such as colour, firmness, storability and disorder resistance (Bramlage, 1993; Johnson and Ridout, 1998). Walter (1967) concluded from his review that, in general, nitrogen has a negative effect on

apple fruit red colour. However, information on the effect of other nutrients on fruit colour is inconsistent and clear correlations with anthocyanin formation have not yet been established (Walter, 1967; Saure, 1990). Nitrogen, potassium and phosphorus deficiency has been reported to increase anthocyanin formation in maize (Lawanson et al., 1972) and in buckwheat seedlings (Krause and Reznik, 1976). In tomato plants N deficiency increased the level of anthocyanin of leaves 2-3-fold and that of total flavonoids by 14% (Bongue-Bartelsman and Phillips, 1995). Patil and Alva (1999) reported that in grapefruit, the levels of the flavonoids naringin and rutinoside and the levels of total vitamin C (ascorbic acid plus dehydroascorbic acid) decreased in the fruit with increased N application levels. In apple shoot cultures Lux-Endrich et al. (2000) found that, increasing the sucrose content and reducing the macronutrient content of the culture media, resulted in a higher accumulation of phenolic acids, quercetin glycosides, catechins and procyanidins of the tissues.

There appears to be, however, no published work on the effect of nutrients on flavonoids and chlorogenic acid concentration in apples. Therefore, we report here on the relations between fruit nutrient concentration during growth and the concentration of several flavonoid classes and chlorogenic acid of 'Elstar' apple skin at commercial harvest. Possibilities for optimising the level of flavonoids in apples via nutrient fertilization are discussed.

2. Materials and methods

2.1. Experimental

Expt. 1.

Trees of 'Elstar' apple ('Elshof' mutant) were planted in 1993 at the experimental orchards in Wilhelminadorp to study the relationship between fruit and leaf nutrient concentration of N, P, K, Mg and Ca and post-harvest quality development. Fruit samples were taken in the 1996, 1997 and 1998 seasons to study the effect of these nutrients on the concentrations of flavonoids and chlorogenic acid in fruit skin. Trees were planted in a single row system at a density of 6000/ha and trained as slender spindles. To increase the range of nutrient concentrations, half of the rows were planted in former grass strips and the other half in former grass-free strips. Half of the trees were grown directly on M.9 rootstock and half with interstock 'Dubbele Zoete Aagt', both types being evenly distributed over replications. 'Golden Delicious' trees were planted as pollinizer at a 1:10 ratio. Treatments were 5 nutrients (N, P, K, Mg and Ca) each at 5 levels (see table 1) with 4 replicates (11 trees for each) in a randomised complete block design. For each treatment two replications were allotted at random to former grass strips and two to former bare strips. For each treatment/block, one nutrient was given at different levels, while the remaining four nutrient fertilizers were administered at normal level (level 2). Nitrogen was applied to the soil in two doses (April and June) as NH_4NO_3 mixed with $CaCO_3$ (a commercial product containing 26% N). Potassium was applied once to the soil in March as K_2SO_4 (42% K). Phosphor (Ca

$(H_2PO_4)_2)$ (25% P), calcium $(CaCl_2)$ (29% Ca) and magnesium (Mg_2SO_4) (10% Mg) were applied as foliar sprays in 0.1% 'Citowett' (BASF) wetting agent using a portable high pressure sprayer. Trees were sprayed until run-off at two weeks intervals starting from full bloom until one week before commercial harvest. Fruit samples (25 fruits of each) were randomly collected from the tree periphery at monthly interval from June to August and at commercial harvest for N, P, K, Mg and Ca analysis. At the same date leaf samples were randomly taken from the extension shoots (two full grown leaves per tree). At commercial harvest (17, 8 and 2 September for the season 1996, 1997 and 1998, respectively) samples of 15 fruits of each replicate were randomly taken from the tree periphery for flavonoids and chlorogenic acid analysis. In 1996 and 1998, 30 objects out of 100 and in 1997, 60 objects were sampled representing a wide range of nutrient concentrations.

Table 1. Levels of applied fertilizer (Kg/ha) for 'Elstar' apple trees ('Elshof' mutant), either broadcast to the soil (br) or sprayed on tree (sp)

	Application level				
	0	1	2	3	4
Type of fertilizer					
N (br)	0	35	70	140	210
P (sp)	0	1.15	2.3	4.6	101*
K (br)	0	25	50	100	150
Mg (sp)	0	2.5	5.0	10.0	20.0
Ca (sp)	0	2.75	5.5	11	16.5

* Applied broadcast as triple super-phosphate.

Data of daily mean temperature and precipitation were obtained from a weather station located in the same orchard where the experiment was performed. In August, during the later stages in fruit development, the mean temperature was 18°C, 21.5°C and 18°C for the seasons 1996, 1997 and 1998, respectively, whereas the precipitation was 65, 34 and 65 mm, respectively, showing that this month in 1997 was exceptionally warm and dry.

Expt. 2.

Within-tree and between-orchards variations in flavonoids and chlorogenic acid concentration of standard 'Elstar' apples have been reported elsewhere (Awad et al., 2000). Using the same fruit samples in 1996, we determined the relationships between concentration of N, P, K, Mg and Ca in the fruit at commercial maturity and the concentration of flavonoids and chlorogenic acid in fruit skin with simple and multiple regression analyses.

2.2. Nutrient analysis of fruits and leaves

For each fruit sample, one sector (free of seeds and stalks) of about 30 g fresh weight was taken from each fruit. For the fruitlets sampled in June the whole fruitlet was used after removing seeds and stalk. Fruit and leaf samples were dried at 70°C, ground, and analysed by standard methods. After digestion in H_2SO_4, the concentrations of N and P were determined

colorimetrically by an autoanalyzer (ALPKEM, RFA-300). The concentration of K, Mg and Ca was measured by atomic absorption spectrophotometry (PERKIN-ELMER, 2380). The concentrations of all nutrients were expressed as mg/100g fresh weight (FW).

2.3. Determination of flavonoids and chlorogenic acid in fruit skin

Blush percentage of harvested fruits were measured, the fruit peeled, and the combined peel of each sample immediately frozen in liquid N and then vacuum dried. The freeze-dried peel was ground and sieved to separate the skin from adhering fleshy parts and kept at -20 °C for flavonoids and chlorogenic acid extraction. The extraction and the RP-HPLC quantification of flavonoids and chlorogenic acid were done as previously described by Awad et al. (2000) with some modifications (Awad et al., 2001b).

2.4. Estimation of blush percentage

At commercial harvest fruit samples from both Expt. 1 and 2 were used to estimate the percentage of blushed area of each individual fruit of each sample by the colour measuring vision system 'Keurmeester' (AWETA Fruit Grading Company, Nootdorp, The Netherlands) adjusted to a specific setting for 'Elstar' apples.

2.5. Statistical analysis of data

Data were subjected to simple and multiple linear regression analyses or to analysis of variance (ANOVA) and the means were separated by F-test or the least significant difference (LSD) test using the statistical package Genstat 5, release 4.1 (Rothamstead, UK).

3. Results

In experiment 1, increasing the amounts of N and Ca fertilizers increased the concentrations of each nutrient in fruit (Table 2a). Increasing N fertilization decreased the concentration of P and Ca in fruits. Increasing the amount of N fertilizer also caused a significant increase in the N/Ca ratio (5.9, 7.8 and 10.8 at rates of 0, 2, and 4 of N fertilizer, respectively; $P<0.05$) in fruits. Increasing the amount of Ca fertilizer decreased the N/Ca ratio (8.8, 7.5 and 7.2 at rates 0, 2 and 4 of Ca fertilizer, respectively; $P<0.05$) in fruits. Application of P, K and Mg had no clear effect on their respective concentration in fruits. In leaves, the concentration of N, P, K, Mg and Ca were generally increased by the applications of the respective fertilizers (data not shown).

The concentrations of cyanidin 3-galactoside (anthocyanin), catechins and total flavonoids (Table 2b) and % of blush (Table 2c) were generally decreased by increasing the amount of N fertilizer. Phloridzin concentration responded positively to Mg fertilizer. Catechins concentration increased by increasing the amount of P and decreased by increasing the amount of Ca fertilizer.

A simple regression analysis was performed to calculate the correlation among the concentration of different flavonoid classes and fruit nutrient concentrations in 'Elshof' mutant and in standard 'Elstar' apples (Table 3). For 'Elshof' the concentrations of N and Mg and the N/Ca ratio in fruit of both early (June) and late (September) analysis were negatively correlated with cyanidin 3-galactoside (anthocyanin) and total flavonoids concentration of fruit skin at commercial harvest in the three seasons 1996, 1997 and 1998 (r of 0.18 to 0.84), except for some of the data on Mg in 1997. In 1997, P and K showed negative, though rather poor, correlations with cyanidin3-galactoside and total flavonoids concentration, especially in the September analysis. The concentration of Ca was not related to the concentration of anthocyanin and total flavonoids in the three seasons. Similar results were also observed for the relations between fruit nutrient in the July and August analyses and the concentration of anthocyanin and total flavonoids in fruit skin at commercial harvest (data not shown). Chlorogenic acid concentration was not related to any of the studied nutrients in this experiment (data not shown). Leaf mineral concentrations were, generally, much less related to the concentration of flavonoid compounds in fruit than fruit mineral concentrations (data not shown).

For standard 'Elstar' the concentrations of N and K and the N/Ca ratio in fruit at commercial harvest were negatively and of Ca was positively correlated with cyanidin 3-galactoside and total flavonoids concentration in fruit skin (Table 3). Magnesium concentration was negatively correlated with cyanidin 3-galactoside concentration, but not with total flavonoids. The concentration of P was not related to cyanidin 3-galactoside and total flavonoids concentration in fruit skin. Chlorogenic acid concentration was negatively correlated with the concentration of N, K and Mg and N/Ca ratio, and positively correlated with P and Ca concentration in fruit (r of 0.33 to 0.42).

Table 4 presents selected multiple regression models for the relation between cyanidin 3-galactoside and total flavonoids concentrations in fruit skin at commercial harvest and the nutrient concentration in fruit at an early stage of development (June) and at commercial harvest (September). For 'Elshof' mutant, fruit N concentration was particularly important for predicting the concentration of cyanidin 3-galactoside and total flavonoids. In 1996 fruit N concentration in June and September accounted for 46% and 41% of the variation in cyanidin 3-galactoside, respectively. Fruit N concentration in June accounted for 27% and the N/Ca ratio in September accounted for 23% of the variation in total flavonoids. In 1998 fruit N concentration in June and September accounted for 70% and 41% of the variation in cyanidin 3-galactoside, respectively and for 53% and 25% for total flavonoids, respectively. However, in 1997 the relations between nutrients and flavonoids were very poor especially between the June nutrient concentrations and flavonoid concentrations at commercial harvest. In general,

Table 2a. Nutrient concentration of 'Elshof' mutant apples at harvest as affected by application level of nutrient fertilizers[a]

| | Nutrient concentration (mg.100g fw) | | | | | | | | | | | | | | |
| | N | | | P | | | K | | | Mg | | | Ca | | |
Application level	0	2	4	0	2	4	0	2	4	0	2	4	0	2	4
Type of fertilizer															
N	32.0	41.1	54.1	13.2	13.4	12.1	145	146	146	6.1	6.3	6.4	5.6	5.4	5.0
P	40.6	41.4	41.5	13.1	14.2	13.9	148	153	152	6.5	6.4	6.5	5.5	5.4	4.9
K	40.0	40.1	37.5	13.8	13.7	13.4	148	150	149	6.4	6.4	6.3	5.1	5.4	5.4
Mg	40.2	39.7	39.9	12.9	13.5	13.7	145	146	146	6.1	6.2	5.5	5.5	5.4	5.5
Ca	40.0	40.1	45.9	13.0	12.9	13.8	146	142	153	6.2	6.1	6.5	4.7	5.4	6.5
LSD 0.05		3.4			0.9			7.7			0.3			0.6	

[a] Data are the mean of the 1997 and 1998. LSD value for the comparison within and between columns for each nutrient.

Table 2b. Flavonoids and chlorogenic acid concentration in the skin of 'Elshof' mutant apples at harvest as affected by application level of nutrient fertilizers[a]

| | Flavonoids and chlorogenic acid (mg g dw) | | | | | | | | | | | | | | | | | |
| | Cyanidin 3-galactoside | | | Phloridzin | | | Catechins | | | Quercetin glycosides | | | Total flavonoids | | | Chlorogenic acid | | |
Application level	0	2	4	0	2	4	0	2	4	0	2	4	0	2	4	0	2	4
Type of fertilizer																		
N	1.10	0.86	0.64	0.86	0.95	0.75	3.0	3.0	2.5	4.9	4.8	4.3	9.8	9.5	8.2	0.047	0.053	0.045
P	0.73	0.63	0.84	0.76	0.82	0.80	2.5	2.6	3.0	4.4	4.6	4.9	8.4	8.7	9.5	0.047	0.048	0.048
K	0.80	0.78	0.81	0.79	0.67	0.79	2.8	2.7	2.9	5.0	4.7	5.1	9.5	8.8	9.6	0.042	0.072	0.042
Mg	0.74	0.75	0.59	0.62	0.73	0.80	2.5	2.7	2.7	4.7	4.5	3.8	8.6	8.6	7.9	0.052	0.052	0.058
Ca	0.91	0.83	0.73	0.77	0.77	0.88	3.0	2.7	2.5	5.3	5.3	4.7	9.9	9.8	8.7	0.047	0.060	0.052
LSD 0.05		0.26			0.12			0.4			1.2			1.6			0.019	

[a] Data are the mean of the 1997 and 1998 seasons. Catechins, the sum of catechin and epicatechin; quercetin glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3- glycosides. LSD value for the comparison within and between columns for each compound.

Table 4. Multiple regression models and their coefficient of determination (R^2) for prediction of cyanidin 3-galactoside and total flavonoids concentration in the skin of 'Elshof' mutant and standard 'Elstar' apples at maturity by fruit nutrient concentrations in June and September for the seasons 1996, 1997 and 1998[a]

June analysis		September analysis	
'Elshof' mutant			
Season 1996			
Cyanidin 3-galactoside	R^2	*Cyanidin 3-galactoside*	R^2
(1) 2.0 − 0.0061 * N	46	(1) 1.55 − 0.02 * N	41
(2) 1.16 − 0.01 * N + 0.04 * P	52	(2) 0.50 − 0.02 * N + 0.01 * K	44
Total flavonoids		*Total flavonoids*	
(1) 11.5 − 0.02 * N	27	(1) 9.0 − 0.23 * N/Ca	23
(2) 7.5 − 0.03 * N + 0.2 * P	35	(2) 1.74 − 0.24 * N/Ca + 0.04 * K	31
Season 1997			
Cyanidin 3-galactoside		*Cyanidin 3-galactoside*	
(1) 1.51 − 0.05 * P	3	(1) 2.1 − 0.23 * Mg	9
(2) 1.9 − 0.05 * P − 0.003 * N	5	(2) 2.2 − 0.21 * Mg − 0.3 * N/Ca	11
Total flavonoids		*Total flavonoids*	
(1) 11.6 − 0.33 * N	5	(1) 18.8 − 0.07 * K	10
(2) 18.4 − 0.032 * N − 0.04 * K	9	(2) 20.8 − 0.06 * K − 0.08 * N	14
Season 1998			
Cyanidin 3-galactoside		*Cyanidin 3-galactoside*	
(1) 3.52 − 0.02 * N	70	(1) 2.2 − 0.03 * N	41
(2) 3.0 − 0.21 * N + 0.03 * P	71	(2) 1.13 − 0.03 * N + 0.01 * K	43
Total flavonoids		*Total flavonoids*	
(1) 21.2 − 0.1 * N	53	(1) 15.2 − 0.1 * N	25
(2) 15.3 − 0.1 * N + 0.33 * K	55	(2) 12.8 − 0.11 * N + 0.13 * N/Ca	28
Standard 'Elstar'			
		Cyanidin 3-galactoside	
		(1) 0.82 − 0.01 * N	66
		(2) 0.54 − 0.002 * N + 0.04 * Ca	70
		Total flavonoids	
		(1) 13.42 − 0.14 * N	57
		(2) 8.73 − 0.2 * N + 0.07 * K	65

[a] Total flavonoids, the sum of cyanidin-3-galactoside, phloridzin, catechins and 6 different types of quercetin-3- glycoside.

cultures higher quercetin glycosides, catechins and procyanidins accumulation by reducing the macronutrient content of the culture media in general. Similarly, ciders obtained from 'Dabinett' apple trees that received NPK fertilizer were less bitter and astringent than those from control tress, which was related to an overall decrease of 17% in fruit phenolic concentration (Lea and Beech, 1978). Our results also agree with those of Patil and Alva (1999) who found in grapefruit that naringin levels and the levels of total vitamin C in fruit decreased with increased nitrogen application rate in the orchard. Several authors observed that nutritional stresses e.g. N, P, Mn and B deficiency increased flavonoid content in a wide variety of plants (Zornoza and Esteban, 1984; Bongue-Bartelsman and Phillips, 1995; Estiarte et al. 1994). A positive effect of calcium spray on apple phenolics has been observed by Sannomaru et al. (1998), who found that epicatechin, chlorogenic acid and total polyphenols contents of 'Starking' apples were higher in Ca treated than in untreated fruit. Raese and Staiff (1990) reported that soil-applied calcium nitrate was associated with higher concentration of fruit Ca, lower N/Ca ratio, more red colour and lower incidence of disorders in 'Delicious' apples as compared to control.

Regression models combining up to two variables accounted for up to 40% and 30% of the variance of anthocyanin and total flavonoids concentration in 'Elshof' mutant apples, respectively and up to 70% and 65% of the variance of anthocyanin and total flavonoids concentration in standard 'Elstar' apples, respectively (Table 4). The most important variable that showed repeatedly significant contribution to the predictive models of anthocyanin and total flavonoids concentration was N concentration in the fruit. Due to the high level of inter-correlation between the different explanatory variables, addition of more variables did not further improve the models (data not shown).

The mode of action of different nutrients on phenolics formation in plant tissues is still a matter of speculation, though less for N. According to the generally accepted concept of competition between phenolics and proteins for their common precursor, L-phenylalanine, nitrogen may inhibit flavonoid synthesis via enhancing the channeling of L-phenylalanine towards proteins (Faust, 1965a; Phillips and Henshaw, 1977; Margna et al., 1989). Alternatively, N might negatively influence the enzyme system involved in the biosynthesis of phenolics (Krause and Reznik, 1976; Tan, 1980). Furthermore, there are some indications that N might influence flavonoid synthesis at the gene level. Bongue-Bartelsman and Phillips (1995) found that N deficiency increased flavonoid in tomato leaves and produced changes in the steady-state mRNA levels of chalcone synthase, chalcone isomerase and dihydroflavonol-4-reductase enzymes. We generally found that fruit maturity characteristics were only slightly influenced by nutrient fertilizers (data not shown). Thus, the effects of nutrients on flavonoid concentrations cannot be explained by any influences on fruit ripening.

The relationships between nutrition and chlorogenic acid concentration in apples are not consistent and further study is required. For standard 'Elstar' apples the concentration of chlorogenic acid was negatively related to the concentration of N, K and Mg and the N/Ca ratio but positively related to both P and Ca. For 'Elshof' mutant, on the contrary, we found that chlorogenic acid was not related to the studied nutrients. The same confusion exists in literature. Lux-Endrich et al. (2000) found in apple shoot culture more accumulation of

chlorogenic acid and other hydroxy cinnamic acids by reducing the macronutrient content of the culture media in general, whereas Krause and Reznik (1976) found that varying P and N concentration in buckwheat seedlings did not influence chlorogenic acid concentration, in contrast to flavonoids. In conclusion, our results show evidence that, in addition to improving light conditions, the concentration of flavonoids and chlorogenic acid in fruit skin could be further increased by optimising fertilization especially that of N.

Acknowledgements

5

Formation of flavonoids and chlorogenic acid in apples as affected by crop load

Mohamed A. Awad, Anton de Jager, Matthijs Dekker, Wim M.F. Jongen

Abstract

The effects of crop load on the concentrations of flavonoid and chlorogenic acid in skin and on quality characteristics of mature apple fruit has been studied. Three crop load levels of about 50, 100 and 200 fruits/tree (about 35, 60 and 100 t/ha) representing low, moderate and high loads, respectively, were established on 'Jonagold' apple tress by fruit thinning 8 weeks after full bloom (FB). Also three crop load levels of about 60, 100 and 140 fruits/tree (about 35, 50 and 70 t/ha), representing low, moderate and high loads, respectively, were established by thinning 'Red Elstar' apple trees at: FB, and 4 and 8 weeks after FB. Similar results were found for both cultivars. At a low crop load, fruit weight, soluble solids, acidity and firmness were significantly higher than at high and moderate loads. However, the concentrations of flavonoid and chlorogenic acid were not affected by crop load. For 'Red Elstar', the time of thinning did not affect the concentrations of flavonoid and chlorogenic acid in fruit skin or fruit weight, soluble solids, acidity and firmness. Removal of only the interior fruits (about one-third of total fruit) at about 4 weeks before expected commercial harvest did not influence the concentrations of flavonoid and chlorogenic acid or quality of the remaining exterior fruits of either 'Jonagold' or 'Elstar'. The results indicate that, assimilate availability is not a major regulatory factor in flavonoids and chlorogenic acid formation in apples.

1. Introduction

Phenolic compounds occur naturally in a large number of edible plants. Flavonoids, one of the most important phenolic classes, are common constituents of the human diet and contribute largely to the antioxidant potential of the diet. Tea, onions and apples are considered the most important sources of Dutch dietary flavonols (Hertog et al., 1993). Apple fruits are rich in flavonoids (e.g. flavonols, catechins, phloridzin and anthocyanins) and contain considerable amounts of hydroxycinnamic acid derivatives, mainly represented by chlorogenic acid (Lancaster, 1992; Nicolas et al., 1994; Awad et al., 2000). Flavonoids are a product of a combination of precursors of two independent pathways, the shikimate pathway (yielding phenylalanine) and the acetate-malonate pathway (yielding malonyl-CoA) via several enzymatic steps (Stafford, 1990). Generally these compounds are present in plant tissues as glycosides, except flavan-3-ols (catechins) which are found in free rather than in glycosylated forms.

A treatment that causes a change in the availability of the flavonoid precursors, shikimic acid, acetate and sugar, might also induce subsequent changes in the synthesis of flavonoids. Exogenous sucrose, galactose and glucose promoted anthocyanin synthesis in apple skin discs (Smock, 1969; Faust, 1965b; Vestrheim, 1970). A pre-harvest application of 0.25M of galactose or glucose on fruit trees enhanced anthocyanin formation in 'Fuji' apples (Bae and Lee, 1995). On the other hand, several studies did not show any relation between sugar content and anthocyanin formation in apples (Uota, 1952; Blankenship, 1987; Noro et al., 1988). Lux-Endrich et al. (2000) reported that increasing the sucrose content and reducing the

macronutrient content of the culture media enhanced the accumulation of phenolic acids, quercetin glycosides, catechins and procyanidins in cultured apple shoots.

Little research has been conducted to enhance the concentration of phenolics in field grown apples. Fruit thinning is a very important cultural practice that affects fruit development, the availability of assimilates, the sugars, acids and dry matter contents, and mineral composition of apples (Poll et al., 1996; Volz and Ferguson, 1999; Wertheim, 2000). To our knowledge, no literature is available about the influence of crop load on the concentration of flavonoids and chlorogenic acid in apple fruit, except for anthocyanins (Smock, 1969; Walter, 1967; Saure, 1990). In the present study, we hypothesised that under conditions of high carbohydrate availability, plants may increase the formation of their secondary metabolites, such as phenolic compounds, as a method for storing surplus fixed carbon. Crop load was manipulated by applying fruit or flower thinning at different stages of development and at different severities. The concentrations of several flavonoids and chlorogenic acid in apples were measured at harvest and fruit quality characteristics were recorded.

2. Materials and methods

2.1. Experimental

Experiments were conducted in the 1997 and 1998 seasons using 'Elstar' and 'Jonagold' apple trees. The trees were trained as slender spindle on M.9 rootstock and planted in a single row system with spacing of 3.0 x 1.25 m for standard 'Jonagold' and 'Elstar' and 3.0 x 1.0 for 'Red Elstar' mutant in commercial orchards.

In 1997, 12 'Jonagold' trees of 10-year-old were selected for uniformity. On June 30, after the natural drop had ceased, different levels of crop load of about 50, 100 and 200 fruits/tree (about 35, 60 and 100 t/ha) were established by hand-thinning and are referred to as a low, moderate and high crop load, respectively. Thinning involved the removal of all but the king flower or fruit of each cluster. The experimental design was a randomised complete block design with 4 replicates of one tree each. At commercial harvest (Oct. 2), 15-fruit samples from the outer tree canopy were collected for measurements.

In 1998, an experiment was conducted with 7-year-old trees of 'Red Elstar'. Thinning treatments were applied at: full bloom (FB), 4 and 8 weeks after FB. On each occasion, different levels of crop load of about 60, 100, and 140 fruits/tree (about 35, 50 and 70 t/ha) were established by hand-thinning and are referred to as a low, moderate and high crop load, respectively. An unthinned control (about 220 fruits/tree) was also included. Thinning involved the removal of all but the king flower or fruit of each cluster. The experimental design was a factorial randomised complete block design with 4 replicates of 3 trees each. At commercial harvest (Sept. 15) 15-fruit samples, from only 2 trees per replicate, were collected from the outer tree canopy for later analysis.

An additional experiment with 'Elstar' and 'Jonagold' was conducted during the 1998 season to study the effect of removing only the interior fruits (about one-third of total fruit) at

about 4 weeks before expected commercial harvest on the concentration of flavonoids and chlorogenic acid and on the quality characteristics of the remaining exterior fruits. For each cultivar, 12 uniform 11-year-old trees having a normal crop level were selected. The interior fruits of 6 randomly selected trees (replicates) were removed by hand and the remaining 6 trees served as controls (without thinning). At commercial harvest (Sept. 8 and 29 for 'Elstar' and 'Jonagold', respectively) 15-fruit samples were collected from the outer tree canopy for later analysis.

2.2. Determination of fruit quality characteristics

At commercial harvest, weight (g/fruit), flesh firmness of the background side (kg, Instron, 11 mm probe, 8 mm depth in 2 seconds), soluble solids (% brix) and titratable acidity (% malic acid in juice) were measured (De Jager and Roelofs, 1996). Starch conversion stage was assessed after dipping a transverse fruit section into a solution of 1% (w/v) iodine and 4% (w/v) potassium iodide; the image was scored on a 1-10 scale (1 = completely black, 10 = completely white). The streif maturity index (firmness/(starch * % brix)) was also calculated (Streif, 1996). The percentage of blush in 'Red Elstar' apples was estimated in all fruits (including fruits from the inner and top positions of tree) picked from one tree per replicate by the colour measuring vision system 'Keurmeester' (AWETA Fruit Grading Company, Nootdorp, The Netherlands). Fruit diameter (mm) was also recorded by this machine. The background colour of the skin of each individual fruit of each sample was measured spectrometrically (Minolta CR 200) using the 'a-value' (ca –20 hard green and +5 yellow) in 'Jonagold' and 'Red Elstar'.

2.3. Determination of flavonoids and chlorogenic acid in fruit skin

After measuring weight and colour, the fruits were peeled and the combined peel of each sample was immediately frozen in liquid nitrogen and vacuum dried. The freeze-dried peel was ground and sieved to separate the skin from adhering fleshy parts. The dry skin samples were then kept at –20 °C for later flavonoids and chlorogenic acid extraction. The extraction and the RP-HPLC quantification of flavonoids and chlorogenic acid were done as described by Awad et al. (2001b).

2.4. Statistical analysis of data

All data were statistically analysed by analysis of variance (ANOVA) and the means were separated by F-test or the least significant difference (LSD) at 5% level using the statistical package Genstat 5, release 4.1 (Rothamstead, UK).

3. Results

At the same harvest date the concentrations of flavonoid and chlorogenic acid were not significantly different at different levels of crop load in 'Jonagold' apples (Table 1a). However, cyanidin 3-galactoside (anthocyanin) tended to increase and phloridzin, catechins, quercetin 3-glycoside and total flavonoids tended to decrease at a low crop load.

Table 1a. Flavonoid and chlorogenic acid concentrations (mg g dw) in skin of 'Jonagold' apples as affected by crop load; season 1997 [a]

	Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Crop load						
High	0.26	1.04	2.54	6.1	9.9	0.26
Moderate	0.22	0.90	2.36	5.1	8.5	0.23
Low	0.44	0.80	2.31	5.0	8.6	0.28
F-test	NS	NS	NS	NS	NS	NS

[a] High, moderate and low crop loads were about 200, 100 and 50 fruits/tree, respectively. Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3-glycosides. NS, not significant.

Fruit weight, % of brix, acidity and firmness were higher at a low crop load than at high and moderate loads (Table 1b). Fruit maturity, as characterised by both the streif and starch indexes, was not similar at different crop loads though there was a tendency to a higher streif index and a lower starch index at a low crop load. The ground colour of fruit ('a-value') was less green at a low crop load than at high and moderate loads.

Table 1b. Fruit quality characteristics of 'Jonagold' apples as affected by crop load; season 1997 [a]

	Fruit weight (g)	% Brix	Acidity % malic acid	Firmness (Kg/cm^2)	a-value	Streif index	Starch index
Crop load							
High	198.5a	13.3a	0.57a	7.4a	-14.7a	0.075	7.6
Moderate	232.6b	13.6a	0.63b	7.2b	-14.3a	0.087	6.5
Low	255.5c	14.5b	0.70c	7.6c	-10.8b	0.092	5.8
F-test	***	*	**	*	**	NS	NS

[a] A high, moderate and low crop load about 200, 100 and 50 fruits/tree, respectively. NS, not significant; *; * *; ***, significant at $P = 0.05, 0.01, 0.001$, respectively.

The concentrations of phloridzin and quercetin 3-glycosides were decreased by fruit thinning in 'Red Elstar' apples, while those of other flavonoid classes, total flavonoids and chlorogenic acid remained the same (Table 2a). Time or degree of thinning did not affect the concentrations of flavonoid and chlorogenic acid. At a low crop load, fruit weight, % of brix, acidity and firmness of 'Red Elstar' apples were higher and fruit size was larger than at moderate and high loads (Table 2b). The ground colour of fruit ('a-value') was less green and the % of blush was higher at a low crop load than at high and moderate loads. However, fruit maturity, as characterised by both the streif and starch indexes, was similar at the different crop levels or times of thinning.

Table 2a. Flavonoid and chlorogenic acid concentrations (mg g dw) in skin of 'Red Elstar' apples as affected by fruit thinning treatments; season 1998 [a]

	Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Treatment						
Control	0.71	1.14	2.18	5.1	9.2	0.083
Thinning	0.83	0.93	2.25	4.0	8.0	0.082
F-test	NS	**	NS	**	NS	NS
Time (T)						
FB	0.77	0.92	2.21	3.7	7.6	0.089
4 WAFB	0.86	1.00	2.25	4.4	8.5	0.079
8 WAFB	0.85	0.87	2.28	4.0	8.0	0.078
F-test	NS	NS	NS	NS	NS	NS
Crop Load (C)						
High	0.83	0.97	2.16	4.2	8.2	0.079
Moderate	0.81	0.90	2.32	4.1	8.1	0.088
Low	0.84	0.92	2.28	3.8	7.8	0.079
F-test	NS	NS	NS	NS	NS	NS
T x C	NS	NS	NS	NS	NS	NS

[a] FB, at full bloom; 4 and 8 WAFB, 4 and 8 weeks after full bloom, respectively. High, moderate and low crop loads were about 140, 100 and 60 fruits/tree, respectively. Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3- glycosides. NS, not significant; * *, significant at $P = 0.01$.

Table 2b. Fruit quality characteristics of 'Red Elstar' apples as affected by fruit thinning treatments; season 1998[a]

	Fruit weight (g)	Fruit size (mm)	% Brix	Acidity % malic acid	Firmness (Kg/cm2)	a-value	% Blush	Streif-index	Starch-index
Treatment									
Control	112.4	70.4	12.8	0.76	6.4	-15.7	37.3	0.18	2.9
Thinning	155.2	75.5	13.7	0.93	6.9	-12.5	43.1	0.17	3.2
F-test	***	***	***	***	***	**	NS	NS	NS
Time (T)									
FB	151.2	76.7	13.7	0.94	6.9	-12.8	39.7	0.17	3.1
4 WAFB	161.4	74.7	13.9	0.93	6.8	-12.1	44.5	0.15	3.4
8 WAFB	153.1	75.0	13.6	0.92	6.8	-12.7	45.1	0.17	3.1
F-test	NS	NS	NS	NS	NS	NS	NS	NS	NS
Crop Load (C)									
High	149.5a	73.5a	13.5a	0.91a	6.8a	-13.1a	36.4a	0.18	3.0
Moderate	153.1a	75.1a	13.6a	0.92a	6.8a	-13.5a	39.6a	0.16	3.3
Low	163.0b	77.8b	14.2b	0.97b	7.0b	-11.0b	53.3b	0.16	3.3
F-test	**	**	**	***	**	**	***	NS	NS
T x C	NS	NS	**	***	NS	NS	**	NS	NS

[a] FB, at full bloom; 4 and 8 WAFB, 4 and 8 weeks after full bloom, respectively. High, moderate and low crop loads were about 140, 100 and 60 fruits/tree, respectively. NS, not significant; ** and *** significant at $P = 0.01$ and 0.001, respectively. Within each column, values with the same letters are not significant.

Removal of only the interior fruits at about 4 weeks before commercial maturity had no influence on the concentrations of flavonoid and chlorogenic acid of the remaining exterior fruits of either 'Elstar' or 'Jonagold' apples (Table 3a). Also, % of brix, acidity and firmness

of fruit were not influenced by this treatment in either cultivar (Table 3b). However, this treatment retarded fruit maturity as measured by the streif and starch indexes in 'Elstar' but not in 'Jonagold' apples.

Table 3a. Flavonoid and chlorogenic acid concentrations (mg g dw) in skin of 'Elstar' and 'Jonagold' apples as affected by late removal of the interior fruits; season 1998[a]

	Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Elstar						
Control	0.35	0.87	2.42	3.9	7.5	0.055
Treatment	0.35	0.88	2.37	3.8	7.4	0.045
F-test	NS	NS	NS	NS	NS	NS
Jonagold						
Control	0.63	0.95	2.54	6.7	10.8	0.27
Treatment	0.60	1.02	2.47	6.0	10.1	0.23
F-test	NS	NS	NS	NS	NS	NS

[a] Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3- glycosides. NS, not significant.

Table 3b. Fruit quality characteristics of 'Elstar' and 'Jonagold' apples as affected by late removal of the interior fruits; season 1998[a]

	% Brix	Acidity % malic acid	Firmness (Kg/cm^2)	Streif index	Starch index
Elstar					
Control	12.6	0.88	6.9	0.37	1.8
Treatment	12.5	0.89	7.1	0.43	1.3
F-test	NS	NS	NS	**	**
Jonagold					
Control	14.4	0.66	7.5	0.081	6.9
Treatment	14.3	0.64	7.4	0.082	6.6
F-test	NS	NS	NS	NS	NS

[a] NS, not significant; * *, significant at $P = 0.01$.

4. Discussion

Our results clearly show that, under normal field conditions, crop load had little or no effect on flavonoids and chlorogenic acid concentration in apples despite the larger pools of sugars and acids that were established at a low crop load (Tables 1b and 2b). It was expected that enhancing the availability of assimilates would favour the formation of the secondary metabolites flavonoids and chlorogenic acid. Poll et al. (1996) found that in 'Jonagored' apples grown in pots, at a low fruit/ leaf ratio, there were higher concentrations of total dry matter, soluble solids and acids, greater firmness and higher production of fatty acids and aroma compounds than at a higher fruit/leaf ratio. They suggested that the greater availability of assimilates relieved internal competition at a low fruit/leaf ratio, which increased formation of aroma compounds in the fruit. In apple shoot cultures Lux-Endrich et al. (2000) found that

increasing the sucrose content and reducing the macronutrient content of the culture media resulted in more accumulation of phenolic acids, quercetin glycosides, catechins and procyanidins. However, osmotic stress might also stimulate phenolic formation in the shoots by increasing the sucrose content of the culture media (D. Treutter, personal communication). The different secondary metabolites may also internally compete for assimilates. Miller et al. (1996) observed in 'Red Delicious' apples that the effect of canopy position on acetate esters production (important aroma compounds of apples) was generally opposite to that of anthocyanin, suggesting a direct competition between the formation of aroma esters and anthocyanin through the common precursor, acetate.

The lack of influence of crop load on flavonoid formation could be explained by the fact that even at high crop load (low free assimilate level) carbon supply is not a limiting regulatory factor for secondary metabolite synthesis; a further increase in metabolite level does not take place. This does not correspond to results with pot-grown apples (Poll et al., 1996) or in-vitro grown shoots (Lux-Endrich et al., 2000).

Lakso et al. (1998) compared the seasonal supply/demand of apple fruit and concluded that during the season two periods of potential carbon limitation for fruit growth occur: about 2-4 weeks after bloom and in the last weeks before harvest. However, Awad et al. (2001b) found that, generally, the total amount of flavonoids rapidly increase after bloom in fruitlets while anthocyanin only accumulates during the first month after bloom and during maturation. Moreover, it has been reported that summer pruning increases average irradiance within the canopy, increases anthocyanin formation but reduces assimilate supply and sugar level in fruit (Saure, 1987).

The lack of influence of removing interior fruits one month before harvest on both flavonoid and chlorogenic acid concentration and ripening characteristics of the remaining exterior fruits (Tables 3a and b) are partly in agreement with those of Youljae and Hanchan (1999). They found in 'Fuji' apples that, manipulating fruit/leaf ratio by artificial defoliation at 20, 30 or 40 days before harvest did not affect soluble solids, acidity and firmness of the fruits. However they found that, at the different defoliation times, anthocyanin contents of fruit skin significantly increased as the extent of defoliation increased due to increased light intensity around the fruits.

The general conclusion to be drawn from these results is that under normal field conditions sufficient numbers of well exposed leaves per fruit may promote primarily fruit characteristics such as weight, size, the content of sugar and acid, whereas flavonoid formation, especially that of anthocyanin, may be primarily a direct effect of light that actually strikes the fruit as suggested by Heinecke (1966).

Acknowledgements

6

Flavonoid and chlorogenic acid changes in skin of 'Elstar' and 'Jonagold' apples during development and ripening

Mohamed A. Awad, Anton de Jager, Linus H.W. van der Plas, Alexander R. van der Krol

Abstract

Apple fruits are important dietary sources of potentially healthy phenolics. In two successive seasons, changes in the concentration and amount of individual flavonoids and chlorogenic acid during development and ripening were investigated by reversed-phase high performance liquid chromatography (RP-HPLC), in 'Elstar' and 'Jonagold' apples from the outside and the inside of the tree canopy. 'Jonagold' had a higher concentration and amount of flavonoids and chlorogenic acid than 'Elstar' during fruit development and ripening. In both cultivars, the concentration on a dry weight basis of quercetin glycosides, phloridzin and chlorogenic acid was highest early in the season but decreased at different rates during fruit development to reach a steady level during maturation and ripening. Catechins (catechin plus epicatechin) concentration showed a similar pattern, but a temporary increase was observed in an early stage of development. The concentration of cyanidin 3-galactoside (anthocyanin) was relatively high early in the season, gradually decreased to a very low steady level during growth, but started to increase near maturation, especially in the outer fruit. On a fruit basis the amount of quercetin glycosides increased during development and was about two times higher in 'Jonagold' compared to 'Elstar', both in outer and inner fruit. These compounds were the most abundant flavonoids in the skin of both cultivars and their accumulation showed a strong dependency on fruit position on tree. In contrast, the amount of the second most abundant flavonoid type, catechins, increased during development to a maximum and then showed some decrease by mid season which was independent of fruit position on tree. The amount of phloridzin increased only early in the season reaching a steady level during development and ripening, and was independent of fruit position on tree. The amount of chlorogenic acid in both cultivars initially increased, but subsequently decreased to reach a low, steady level and was slightly higher in outer than in inner fruit. Although anthocyanin concentration was relatively high at early stages of development, significant accumulation on a fruit basis only occurred during maturation and ripening. The accumulation of anthocyanin, similar to that of quercetin glycosides, showed a strong dependency on fruit position on tree. Remarkably, the difference in accumulation of anthocyanin and quercetin glycosides in outer and in inner fruits had no effect on the accumulation of catechins, phloridzin and chlorogenic acid in these fruits. The results indicate that, in general, the overall production of total flavonoids and chlorogenic acid in apple skin is completed during fruit development before the onset of maturation.

1. Introduction

A protective role against diseases from fruit and vegetable consumption is generally attributed to their vitamins C and E, flavonoids, carotenoids, lycopene, selenium and dietary fiber constituents (Steinmetz and Potter, 1996). Flavonoids and phenolic acids show a powerful antioxidant capacity both in in vitro and in vivo systems (Formica and Regelson, 1995; Cook and Samman, 1996; Rice-Evans et al., 1997; Yamasaki et al., 1997). Many dietary phenolic compounds derived from plants have a stronger in vitro antioxidant activity

Phenylalanine ◄——————— Shikimic acid pathway

⬇ PAL

Cinnamic acid

⬇ C4H

p-Coumaric acid ——————▶ Phenolic acids (e.g. Chlorogenic acid)

⬇ 4CL

p-Coumaroyl-COA

⬇ CHS

3x Malonyl-CoA ◄——————— Krebs Cycle

Naringenin chalcone ——————▶ Phloridzin

⬇ CHI

Naringenin flavanone

⬇ F3H

Dihydroquercetin ——FS——▶ Quercetin ——3GT——▶ Quercetin 3-glycosides

⬇ DFR

Leucoanthocyanidins ——FR——▶ Catechins

⬇ ANS

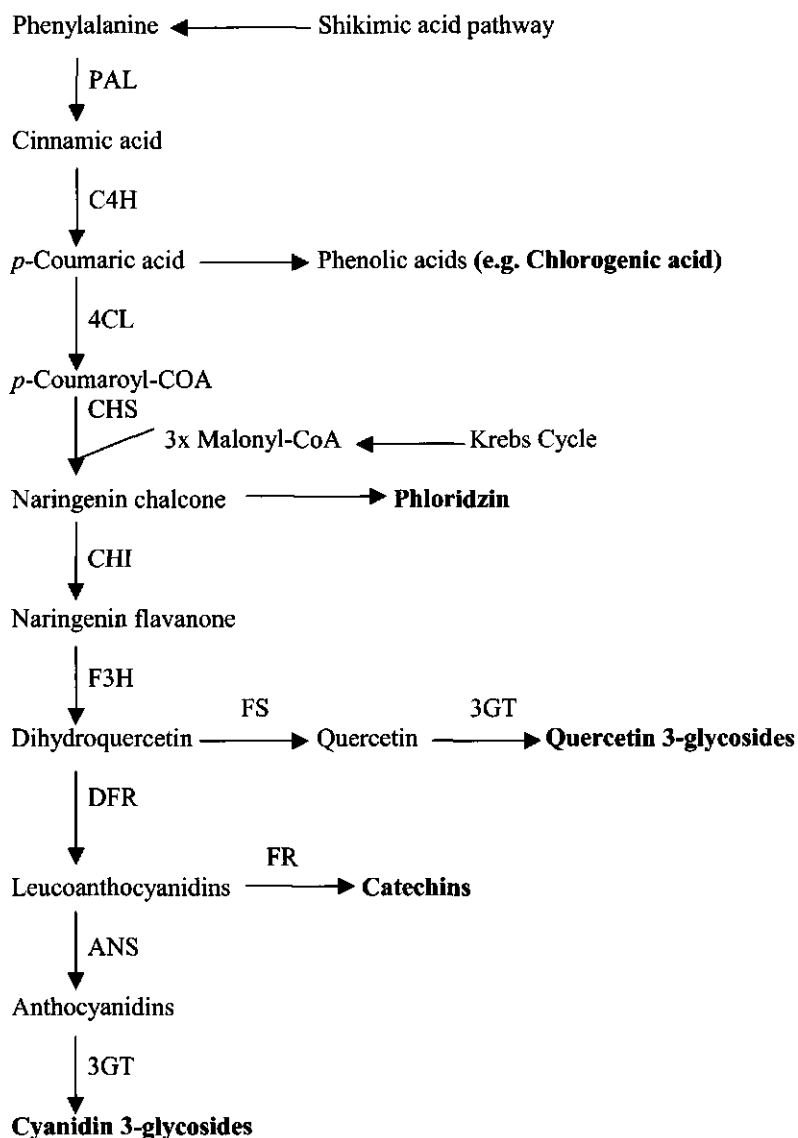Anthocyanidins

⬇ 3GT

Cyanidin 3-glycosides

Fig. 1. Putative biosynthetic route of chlorogenic acid, phloridzin, quercetin glycosides, catechins and cyanidin glycosides in apple skin as proposed by Lancaster (1992) with some modifications. PAL, phenylalanine ammonia-lyase; C4H, cinnamate hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone flavanone isomerase; F3H, flavanone-3-hydroxylase; FS, flavonol synthase; DFR, dihydroflavonol-4-reductase; FR, flavan-3-, 4-cis-diol 4-reductase; ANS, anthocyanidin synthase; 3GT, cyanidin/flavonol 3-O-glucosyl transferase. The highlighted compounds are those measured here.

on a molar basis than the classic antioxidant vitamins such as vitamins C and E (Rice-Evans et al., 1997). Flavonoids and phenolic acids are also involved in the quality characteristics of fresh fruits and its processed products, like texture, colour and taste e.g bitterness and astringency (Lidster et al., 1986; Lea and Timberlake, 1974; Lancaster, 1992). Apple fruits,

especially the skin, are rich in flavonoids (e.g. flavonols, catechins, phloridzin and anthocyanins) and contain considerable amounts of hydroxycinnamic acid derivatives, mainly represented by chlorogenic acid (Lancaster, 1992; Nicolas et al., 1994; Awad et al., 2000). Figure 1 shows a diagrammatic representation of the biosynthesis pathway of the phenolic compounds that were measured in this study. The deamination of L-phenylalanine to *trans*-cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL) is the initial step in the biosynthesis of hydroxycinnamic acids, flavonoids and other phenylpropanoid polyphenols (Margna, 1977; Jones, 1984). Phenylpropanoids are a product of a combination of precursors from both the shikimate (phenylalanine) and the acetate-malonate (malonyl-CoA) pathways via several enzymatic steps (Stafford, 1990).

Relatively little is known about accumulation of flavonoids and hydroxycinnamic acids derivatives in apple skin. The role of environmental and developmental factors in the regulation of accumulation of flavonoids other than anthocyanin is poorly documented. Generally, the concentration of anthocyanin shows two peaks: the first occurs in young fruitlets during cell division and the second one in fully developed apples during maturity (Saure, 1990). Lister et al. (1994) reported that the concentration on a fresh weight basis of quercetin glycosides and proanthocyanidins in the skin of 'Splendour' apples decreased by 50% from early to mid season followed by an increase during ripening. In contrast, Mayr et al. (1995) observed that on a dry weight basis phenolics in the skin and the leaves of 'Golden Delicious' apples decreased during growth without further increase during maturation and ripening, but the individual compounds did not behave uniformly.

Although there are some reports on the developmental changes in the concentration of flavonoids and phenolic acids in apples, a mass balance would provide more information. It would enable the identification of periods of net synthesis and/or breakdown, and also give information on the relation between the different flavonoids in the same biosynthetic route. In this paper, we report the changes that take place in the concentration and the amount of individual flavonoids and chlorogenic acid in the skin of the two main apple cultivars grown in the Netherlands, Elstar and Jonagold, during their development and ripening.

2. Materials and methods

2.1. Plant material

In 1997 and 1998, samples of 15-25 fruits each from 'Elstar' and 'Jonagold' (planted in a single row system with spacing of 3.0 x 1.0 and raised as slender spindle on M.9 rootstock) were collected from a commercial orchard at several dates throughout the season starting at 3 weeks after full bloom, from both the outer and the inner side of the tree canopy. At each date of sampling, different trees within the same rows in the orchard were used to avoid a thinning effect due to repeated sampling.

In 1997, one sample for each cultivar was picked on 29 May, 12 and 26 June, 10 and 24 July, 7 and 21 August, 4, 11 and 18 September. Extra samples for 'Jonagold' were taken at 25 September and 2, 9 and 23 October.

In 1998, two samples (replicates) for each cultivar were picked on each date. 'Jonagold' samples were collected on 26 May, 8 and 23 June, 7 and 21 July, 4 and 18 August, 1, 15, 23 and 29 September, 6, 13, 20 and 27 October and 3 November. 'Elstar' samples were picked on 26 May, 8 and 23 June, 7 and 21 July, 4, 18 and 25 August, 1, 8, 15, 23 and 29 September and 6 and 13 October. In 1998, additional samples of 'Elstar', only from the outside of the tree canopy, were collected from another orchard at the same dates as for the previous orchard.

Immediately after picking, the fruits were peeled, and the combined peel of each sample was immediately frozen in liquid nitrogen and then vacuum dried. The freeze-dried peel was ground and sieved to separate the skin from adhering fleshy parts. The dry skin samples were weighed and then kept at -20 °C for later flavonoids and chlorogenic acid analysis. The total amount (mg per apple) of individual phenolic compounds were calculated from the measured concentration and the weight of the dry skin.

2.2. Determination of fruit maturity indices

A few weeks before commercial maturity, additional samples of 15 fruits were collected at each week to measure fruit maturity indices as firmness (kg, Instron, 11 mm probe, 8 mm depth in 2 s), soluble solids (% brix), titratable acidity (% malic acid in juice) and starch conversion stage by the starch iodine test (scale 1-10) using the standard protocol. The streif maturity index (firmness/(starch x TSS)) was also calculated (Streif, 1996).

2.3. Determination of flavonoids and chlorogenic acid in apple skin

The extraction and the RP-HPLC quantification of flavonoids and chlorogenic acid were done as previously described by Awad et al. (2000) with some modifications. Samples (20 μl) were injected onto the RP-18 column (Hypersil, C18 (ODS), 5 μm, 250 mm x 4.6 mm) which was fitted with a direct connect prefillable guard column (Alltech) and was maintained at 30 °C using a Marathon column heater. The flow rate was maintained at 1.0 ml/min. In the 1998 season, the Shimadzu SPD-10AV VP programmable UV-VIS detector was used. The solvents and wavelength used for separation and monitoring the individual flavonoids and chlorogenic acid were as follows: (1) 10% acetic acid for 12 min followed by 10% acetic acid/acetonitrile (70/30) for 5 min monitored at 280 nm for catechin and epicatechin and (2) 10% acetic acid/acetonitrile (91.5/8.5) monitored at 366 nm for chlorogenic acid and the quercetin glycosides group, 530 nm for cyanidin 3-galactoside, and 280 nm for phloridzin. The chromatogram peaks of individual compounds were identified by comparing their retention times with the retention times of pure standards. Integrated peaks were calculated by comparison with standard solutions of known concentration. Standards used to quantify the HPLC data were cyanidin 3-galactoside, quercetin 3-galactoside, quercetin 3-rhamnoglucoside, quercetin 3-glucoside and quercetin 3-arabinoside obtained from Routh; quercetin 3-xyloside purchased from Plantech, Reading U.K.; quercetin 3-rhamnoside obtained from Sigma; (+)-catechin and (-)-epicatechin obtained from Aldrich and phloridzin

and chlorogenic acid obtained from Fluka. Analytic quality control was performed by including control samples with a known amount of flavonoids and chlorogenic acid in every series of analyses. All determinations were carried out in duplicate. When duplicates differed more than 10%, sample extraction and measurement were repeated. Flavonoids and chlorogenic acid data are expressed as both concentration (mg g dw) and amount (mg/apple) for both cultivars.

2.4. Statistical analysis

The data of 1998 were subjected to an analysis of variance (ANOVA) and the means were separated by the least significant difference (LSD) test at the 5% level using the statistical package Genstat 5, release 4.1 (Rothamstead, UK). Presented data points are the means of two replications in 1998 for 'Jonagold', while for 'Elstar' they were the means of two replications and for the outer fruit of two orchards.

3. Results

3.1. Fruit growth and fruit maturity characteristics

In order to relate the changes in flavonoids and chlorogenic acid content to fruit growth, the skin dry weight per apple for outer and inner fruit of both cultivars was determined during development. Since the results of both the 1997 and the 1998 seasons were similar only the results from 1998 are shown. The increase in skin dry weight, a function of the square of the radius, shows a clear sigmoidal pattern for 'Jonagold' and a slight sigmoidal pattern for 'Elstar' (Fig. 2a and b). For 'Jonagold' maximum skin dry weight was reached at the optimum harvest dates (OHD) but for 'Elstar' the OHD was about 20 days earlier (Fig. 3a and b). Whither there is a real difference in final skin weight between outer and inner fruits can not be decided from the data since differences were not statistically significant.

In 'Elstar', the outer fruit was significantly riper than the inner fruit as determined both by the streif and the starch index (Fig. 3a). In 'Jonagold', outer and inner fruit showed similar development both according to the streif index and according to the starch index (Fig. 3b). The OHD were 4 September and 2 October in 1997 and 8 and 29 September in 1998 for 'Elstar' and 'Jonagold' apples, respectively.

3.2. Changes in concentration and amount of total flavonoids

The total flavonoid concentration and that of the different classes of flavonoids and of chlorogenic acid are shown in Fig. 4a and b for 'Elstar' outer and inner fruit and in Fig. 4c and d for 'Jonagold' outer and inner fruit, respectively. The absolute amount per apple of the different flavonoids and chlorogenic acid are shown similarly in Fig. 5a and b for 'Elstar' outer and inner fruit and Fig. 5c and d for 'Jonagold' outer and inner fruit, respectively.

Although the concentration of total flavonoid decreased during development, the total amount per apple increased to 10 and 3 mg/apple, respectively, for outer and inner fruit of 'Elstar', and 20 and 8 mg/apple, respectively, for outer and inner fruit of 'Jonagold'.
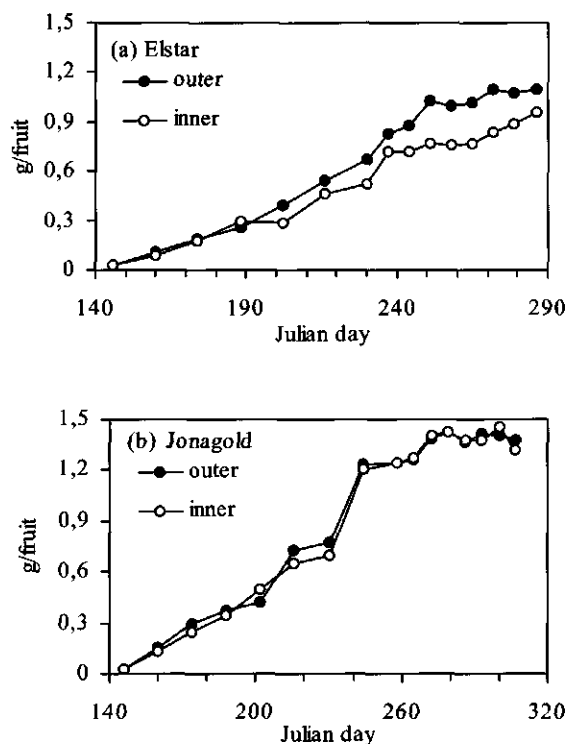

Fig. 2. The growth of 'Elstar' (a) and 'Jonagold' (b) apples from the outer and the inner positions of the tree canopy in 1998 expressed as skin dry weight. LSD (5%) for both time and position effects are 0.76 for 'Elstar'.

3.3. Quercetin glycosides

The determination of quercetin glycosides included quercetin 3-galactoside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside, quercetin 3-glucoside, quercetin 3-xyloside and quercetin 3-arabinoside. In both cultivars the highest concentration of quercetin glycosides was found at the early stage of development, both in outer and inner fruit, but thereafter generally decreased to a lower steady level during maturation and ripening (Fig. 4). Among the six types of measured quercetin glycosides, the quercetin 3-galactoside and quercetin 3-
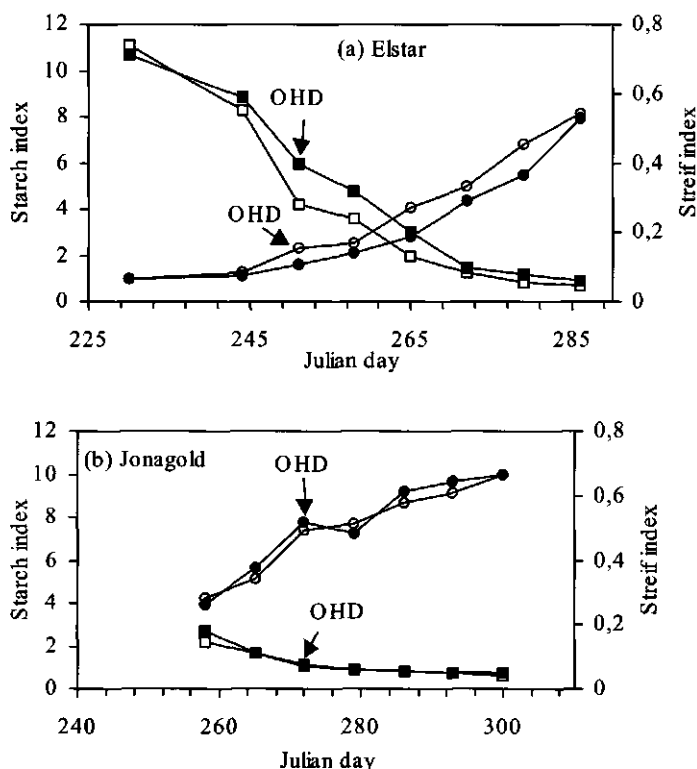
Fig. 3. Maturity development of 'Elstar' (a) and 'Jonagold' (b) apples from the outer and the inner positions of the tree canopy, according to the starch index (scale 1-10) and the streif index in 1998. LSD (5%) of both time and position effects are 0.067 and 1.0 for streif and starch index, respectively. OHD are at starch indexes 2 and 8 and streif indexes 0.30 and 0.08 for 'Elstar' and 'Jonagold', respectively. (O, ●), starch index for outer and inner fruit, respectively. (■, □), streif index for outer and inner fruit, respectively.

rhamnoside were the principal glycosides. There were some fluctuations in the ranking of the individual quercetin glycosides especially during ripening (data not shown). The total amount of quercetin glycosides (mg per fruit), in the outer fruit of both cultivars, generally increased during development with some small fluctuations during maturation and ripening (Fig. 5a and c). Such an increase was less clear in inner fruits. The absolute amount of quercetin glycosides in mature apples was higher in 'Jonagold' than in 'Elstar', and for both cultivars the absolute amount of quercetin glycosides was higher in the outer fruit than in the inner fruit (4 vs. 1 mg/apple for 'Elstar', 10 vs. 3 mg/apple for 'Jonagold' respectively; Fig. 5).

3.4. Catechins

The concentration of total catechins (catechin plus epicatechin) in both cultivars initially increased but then decreased to reach a steady level during maturation and ripening. At early

stages of development, the concentration of catechin rapidly decreased, while that of epicatechin increased, reaching a stable level at maturation and ripening (data for individual

Fig. 4. Changes in concentrations of flavonoids and chlorogenic acid in the skin of 'Elstar' outer (a) and inner (b) fruit and 'Jonagold' outer (c) and inner (d) fruit during development and ripening in 1998. Quercetin glycosides, the sum of quercetin 3-galactoside, quercetin 3-rhamnoglucoside, quercetin 3-rhamnoside, quercetin 3-glucoside, quercetin 3-xyloside and quercetin 3-arabinoside; catechins, the sum of catechin and epicatechin; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin glycosides. LSD (5%) of time effects are 2.47, 1.85, 0.61, 1.20, 0.61, 0.31 for total flavonoids, quercetin glycosides, catechins, phloridzin, cyanidin 3-galactoside and chlorogenic acid, respectively. ■, Total flavonoids; ◆, Quercetin glycosides; ▲, Catechins; ●, Phloridzin; O, Cyanidin 3-galactoside; ✱, Chlorogenic acid.
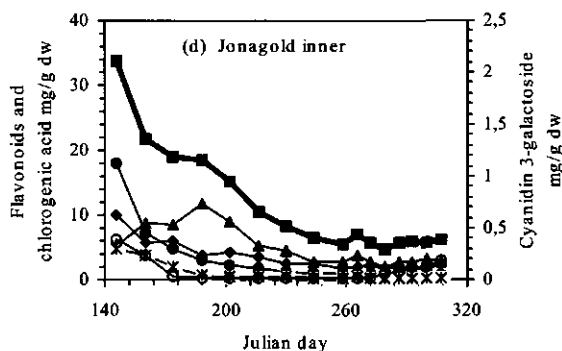
Fig. 4. *(Continued).*

catechins are not shown). The amount of total catechins in outer and inner fruits of both cultivars reached a maximum at early stage, but showed some decrease by mid season and remained relatively constant thereafter. There was no significant difference in the accumulation profile and contents of catechins between mature outer and inner fruit (2 and 4 mg/apple for 'Elstar' and 'Jonagold' respectively; Fig. 5).

3.5. Phloridzin

Early in the season, phloridzin was the main flavonoid in the skin at a concentration of 11.9 and 8.7 mg/g dw for 'Elstar' outer and inner fruit, respectively and 18.0 mg/g dw for 'Jonagold' both in outer and inner fruit (Fig. 4). This concentration, however, sharply decreased from the first sampling date, reaching a low stable level below 1.0 mg/g dw both in outer and inner fruit for both cultivars. The amount per fruit increased in early season and remained at a steady level thereafter (Fig. 5).

3.6. Anthocyanin

The concentration of anthocyanin (cyanidin 3-galactoside) in outer fruit of both cultivars was initially relatively high but gradually decreased to near zero around the middle of the season. Shortly before maturation, the concentration rose very rapidly (Fig. 4a and c). In the inner fruit of both cultivars the concentration at early stage of development was also relatively high, but the large increase during maturation did not occur (Fig. 4b and d). The amount per fruit of anthocyanin remained at a very low steady level until shortly before maturity. From there on, in the outer fruit the amount rapidly increased to reach a level during maturation and ripening of 1.8 and 2.4 mg/apple for 'Elstar' and 'Jonagold', respectively (Fig. 5a and c). In the inner fruit, the amount of anthocyanin remained at a very low steady level during development with only a small increase during maturation and ripening of up to 0.2 mg/apple for both cultivars (Fig. 5b and d).
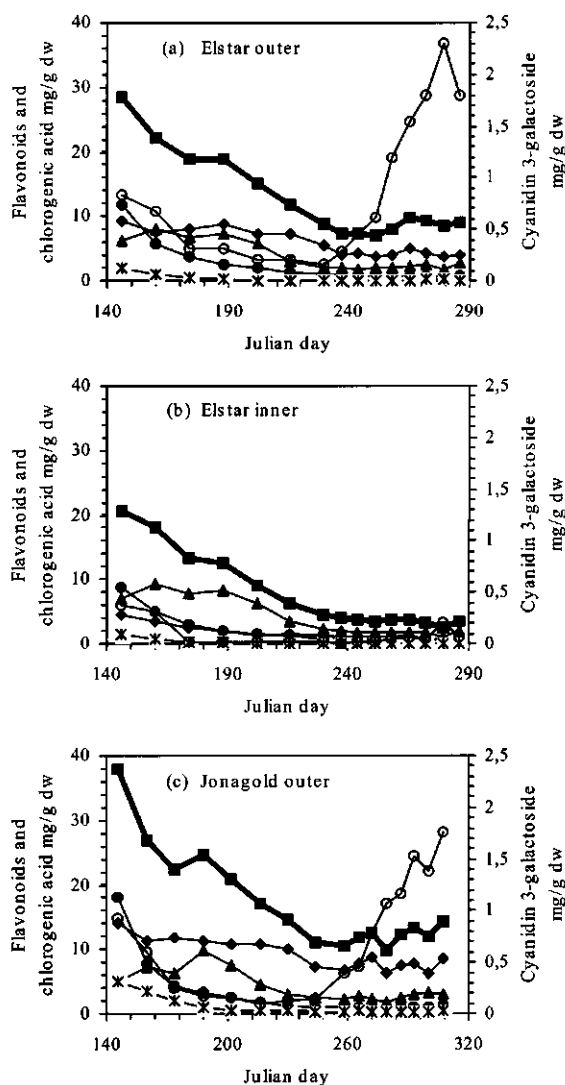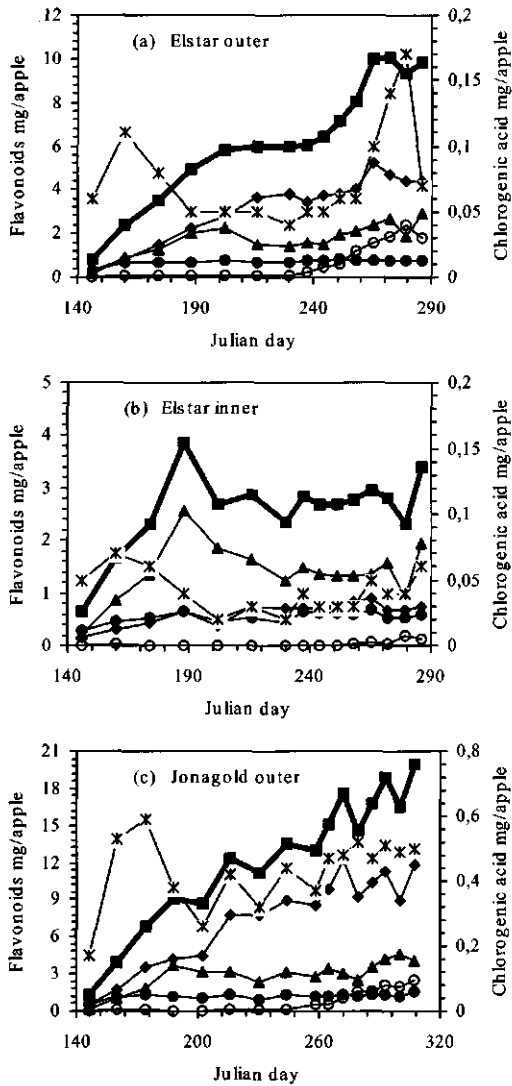
Fig. 5. Changes in amounts per apple of flavonoids and chlorogenic acid in the skin of 'Elstar' outer (a) and inner (b) fruit and 'Jonagold' outer (c) and inner (d) fruit during development and ripening in 1998. Quercetin glycosides, the sum of quercetin 3-galactoside, quercetin 3-rhamnoglucoside, quercetin 3-rhamnoside, quercetin 3-glucoside, quercetin 3-xyloside and quercetin 3-arabinoside; catechins, the sum of catechin and epicatechin; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin glycosides. LSD (5%) of time effects are 2.77, 0.95, 0.45, 0.13, 0.63, 0.06 for total flavonoids, quercetin glycosides, catechins, phloridzin, cyanidin 3-galactoside and chlorogenic acid, respectively. ■, Total flavonoids; ◆, Quercetin glycosides; ▲, Catechins; ●, Phloridzin; O, Cyanidin 3-galactoside; ✳, Chlorogenic acid.

Fig. 5. *(Continued)*.

3.7. Chlorogenic acid

In both cultivars the highest concentration of chlorogenic acid was at the early stage of development, but it gradually decreased thereafter to a very low steady level (Fig. 4). The amount per fruit was generally very l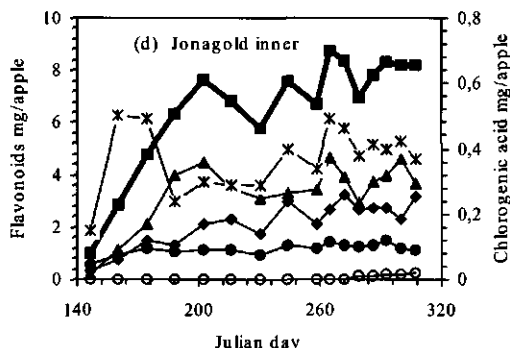ow, showing a peak early in the season and during late ripening especially in 'Elstar' fruit (Fig. 5). In both cultivars the concentration and amount of chlorogenic acid were slightly lower in inner fruit compared to that in outer fruit during development and ripening (Fig. 4 and 5). The final amount was much lower in 'Elstar' (0.07 and 0.06 mg/apple for outer and inner fruit, respectively; Fig. 5a and b) than in 'Jonagold' (0.50 and 0.37 mg/apple for outer and inner fruit, respectively; Fig. 5c and d).

4. Discussion

The accumulation profiles of flavonoids and cholorogenic acid in 'Elstar' and 'Jonagold' apples were qualitatively very similar during development and ripening. However, 'Jonagold' apples showed higher values of flavonoids and chlorogenic acid than 'Elstar' apples throughout fruit development as previously reported for mature fruit (Awad et al., 2000).

The dry weight of fruit skin continued to increase until September for both 'Elstar' and 'Jonagold' (Fig. 2a and b). A net increase in the amount of quercetin glycosides, catechins, phloridzin and chlorogenic acid with growth in combination with a decrease in concentration in the young fruit strongly suggest that the rate of accumulation of these compounds is highest in early season and tapers off (but does not cease) as the fruit grows (Fig. 4 and 5). Our results agree with those of studies on citrus fruit that suggest that the major flavonoids, naringin and hesperidin, are rapidly synthesised during the early phases of fruit development and that synthesis slows down in the later phases (Jourdan et al., 1985; Vandercook and Tisserat, 1989; Castillo et al., 1992). Hamauzu et al. (1999) found for 'Tsugaru' and 'Fuji' apples that the content per fruit of oligomeric procyanidins, catechin and epicatechin in the flesh increased by the middle stage of development and then leveled off or even decreased thereafter. They also found a stable amount of phloridzin in both cultivars during

development; however, the amount of chlorogenic acid increased in 'Fuji' apples. Our results deviate from the conclusion of Lister et al. (1994) in that the amount of quercetin glycosides and procyanidins, calculated on fresh weight basis, in skin of 'Granny Smith' and 'Splendour' apples increased steadily as the fruit surface area increased. However, it appears from their figures that the amount of quercetin glycosides leveled off at maturity for 'Granny Smith' and in one season for 'Splendour' apples. It can be deduced from our results that the decrease in the concentration of flavonoids and chlorogenic acid might be due to a dilution of these compounds caused by the progressive growth of the fruit, because there is increase in the total amount per fruit.

The higher concentration and amount of anthocyanin and quercetin glycosides in outer fruit than in inner fruit of both cultivars can be explained by differences in light conditions (quantity and quality) at the outer and inner positions of trees (Awad et al., 2001a). Light may control differences in enzyme levels channeling substrates into quercetin glycosides and anthocyanin and/ or determining substrate fluxes (Saure, 1990; Ju et al., 1997). The fruit maturity indices according to the starch index and the streif index show that fruit maturity and ripening clearly had advanced when fruits were harvested later both in outer and inner fruits. The differences in ripeness between outer and inner fruit were, however, slight (Fig. 3a and b). Thus the differences between outer and inner fruit in anthocyanin and quercetin glycosides contents are not merely explained by differences in fruit maturation (Awad et al., 2000; Awad et al. 2001a). Comparison of the amount of total flavonoids in outer (Fig. 5a and c) and inner (Fig. 5b and d) fruits of both cultivars shows similar accumulation in an early stage of development followed by a leveling off in the inner fruit. Since this is coinciding with a large increase in internal canopy shading this is consistent with our conclusion on the effect of light on flavonoids synthesis (Awad et al., 2001a).

Since accumulation of quercetin glycosides and cyanidin 3-galactoside do not influence accumulation of any of the other flavonoid classes, their biosynthesis seems to be regulated independently from the other classes, although they have the same biosynthetic pathway (Fig. 1). This might come about by physical separation on the cellular level, as suggested by the apparent on-off mechanism in anthocyanin formation (Awad et al., 2000) or be the consequence of channeling through different multi-enzyme complexes (Burbulis and Shirley, 1999).

4.1. Flavonoids content in relation to fruit healthiness

The quercetin glycosides were quantitatively predominant among the measured flavonoid classes in skin of mature apple especially of the outer fruit (averaged 4 and 10 mg/apple for 'Elstar' and 'Jonagold', respectively) followed by catechins (averaged 2 and 4 mg/apple for 'Elstar' and 'Jonagold', respectively) (Fig. 5 and c). Quercetin glycosides and catechins are considered most beneficial to human health and have, consequently, been used in most studies investigating physiological and biological effects of flavonoids (Hertog et al., 1993; Formica and Regelson 1995; Tijburg et al., 1997; Kohlmeier et al., 1997). Anthocyanin, phloridzin and chlorogenic acid are less important despite the fact that they show higher in

vitro antioxidant activities than vitamins C and E (Rice-Evans et al., 1997). We have shown here that quercetin glycosides, catechins, phloridzin and chlorogenic acid are mainly accumulated during development until the onset of maturation. On the other hand, the main accumulation of anthocyanin occurs during maturation. Furthermore, our results show that the accumulation of quercetin glycosides and anthocyanin are strongly light/tree position dependent while that of the other classes are independent of tree position. According to Lancaster et al. (2000), post-harvest UV-B irradiation at 20 °C for 72 h led only to a limited increase of quercetin glycosides, anthocyanin and chlorogenic acid in some apple cultivars. They found no effect of UV-B irradiation on the concentration of procyanidins (including catechin, epicatechin, procyanidin B2 and B5). Practically, pre-harvest improving of the concentration of anthocyanin and quercetin glycosides in fruit might be much easier and more economic compared to such post-harvest treatments. In order to improve both fruit healthiness and attractiveness before harvesting, manipulations of trees such as summer pruning, repositioning branches or covering orchard floor with reflecting films to improve light conditions within the tree, will increase quercetin glycosides and anthocyanin accumulation. According to our results these measures should be performed early during fruit development, prior to the maturation stage.

Acknowledgements

7

Formation of flavonoids, especially anthocyanin, and chlorogenic acid in 'Jonagold' apple skin: influences of growth regulators and fruit maturity

Mohamed A. Awad, Anton de Jager

Abstract

The influences of ethephon, (S)-trans-2-amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride (ABG-3168), gibberellins (GA_{4+7} and GA_3), alar, cycocel (CCC), prohexadione-Ca, seniphos, shikimic acid, plantacur-E and galactose on the accumulation of flavonoids and chlorogenic acid in 'Jonagold' apple skin were investigated, with emphasis on anthocyanin, in order to separate maturity-related from other development-related influences. Fruit maturation/ripening as determined by both streif index and starch index was not affected by these chemicals. Ethephon application greatly increased anthocyanin accumulation but not that of other flavonoid compounds and chlorogenic acid compared to the control. ABG and GA_3 applications significantly inhibited anthocyanin accumulation but not that of other flavonoid compounds and chlorogenic acid. ABG delayed the transition to fast anthocyanin accumulation by about two weeks compared to the control and to other treatments. The application of alar, CCC, prohexadione-Ca, GA_{4+7}, plantacur-E, shikimic acid, galactose and seniphos did not significantly influence the formation of anthocyanin, total flavonoids and chlorogenic acid in fruit skin. Percentage of blush increased during maturation and was higher in ethephon treated fruit and lower in ABG and GA_3 treated fruit compared with control. The results show that anthocyanin formation is dependent on developmental signals and independent of both fruit maturity/ripening and of the synthesis of other flavonoid classes and responds in a complicated way to ethylene.

1. Introduction

A protective role of fruit and vegetables against cancer and coronary heart diseases is partly attributed to constituents such as vitamins C and E, flavonoids, carotenoids, lycopene, selenium and dietary fibers. Flavonoids and phenolic acids are important classes of secondary plant metabolites showing antioxidant capacity in both in-vivo and in-vitro systems (Formica and Regelson, 1995; Steinmetz and Potter, 1996; Rice-Evans et al., 1997). Apple fruits, especially the skin, are rich in flavonoids and contain considerable amounts of hydroxycinnamic acid derivatives, mainly represented by chlorogenic acid (Nicolas et al., 1994). Their red colour is mainly due to the anthocyanin pigment cyanidin 3-galactoside that can also scavenge superoxide radicals in an in-vitro system (Yamasaki et al., 1996). Thus, flavonoids and phenolic acids contribute to both fruit colour and human health benefits.

In young fruit the concentrations of total flavonoids and chlorogenic acid is relatively high but gradually decrease during growth to a steady level during maturation and ripening (Lister et al., 1994; Awad et al., 2001b). Accumulation of anthocyanin, however, shows two peaks: the first in young fruitlets during cell division and the second in fully developed apples during maturation (Lister et al., 1994; Awad et al., 2001b).

It is an important question whether or not anthocyanin formation is maturity/ripening and therefore ethylene related. If it is not, then methods should be sought to increase red colour formation without accelerating maturity/ripening and thus decreasing storability of the fruit. Murphey and Dilley (1988) suggested that enhancement of anthocyanin biosynthesis

may require only a brief exposure to ethylene that may be insufficient to affect other fruit ripening characteristics. To test the relationship between maturity/ripening and anthocyanin formation, substances that known to accelerate maturity/ripening can be applied and anthocyanin formation measured, and substances known to stimulate anthocyanin formation can be applied and maturity/ripening processes measured. Alar might inhibit ethylene and delay maturity but enhance or retard red colour formation (Saure, 1990). Seniphos (a phosphorus-calcium mixture) reportedly decreases internal ethylene and improves colour without affecting maturation of 'Starking Delicious' apples (Larrigaudiere et al., 1996). Repeated applications of a vitamin E formulation (25% alpha-tocopherol) during growth reportedly increases red colour formation of 'Elstar' and 'Jonagold' apples without influencing maturity (Schmitz and Noga, 1998). Dipping 'McIntosh' apples in shikimic acid (a flavonoid precursors) considerably favoured anthocyanin formation in unripe but not in ripe fruit during irradiation (Faust, 1965b). Preharvest application of 0.25 M galactose or glucose enhanced anthocyanin and red colour formation, maintained firmness, increased pH and soluble sugar content in 'Fuji' apples (Bae and Lee, 1995). Little information is available, however, on the influence of such chemicals on flavonoids other than anthocyanin and chlorogenic acid in apples.

Ju et al. (1995) found that ethephon application (250 ppm) at 20 days before harvest increased anthocyanin accumulation and UDPGal:flavonoid-3-o-glycosyltransferase (UFGalT) activity during maturation, but had no effect on flavonoid concentrations and chalcone synthase (CHS) activity in both 'Delicious' and 'Ralls' apples. However, ethephon applied at 60 days before harvest induced high UFGalT activity and increased the accumulation of flavonoids but did not induce anthocyanin formation when the fruits were picked at 40 days before harvest time. Application of the ethylene inhibitor AVG (500 ppm) alone did not affect activity of CHS and UFGalT or the accumulation of anthocyanin and flavonoids, but when applied with ethephon, AVG partially counteracted the effect of ethephon. Prohexadione-Ca, an inhibitor of gibberellin metabolism, and possible growth retardant in apple, favoured the accumulation of eriodictyol and luteoliflavan, which do not normally occur in apple tissue (Roemmelt et al., 1999).

Thus, using these materials, the objectives of our study were (1) to examine the relation between maturity/ripening and anthocyanin and red colour formation in fruit skin and (2) to study the concomitant changes in flavonoid and chlorogenic acid concentrations.

2. Materials and methods

2.1. Experimental

Exp. 1.

In 1997, five-year-old 'Jonagold' apple trees trained as slender spindles on M.9 rootstock and planted in a single row system with spacing of 3.0 x 1.0 m at the experimental orchard in Wilhelminadorp were selected. The following chemical treatments were applied as

foliar spray: 480 ppm ethephon (2-chloroethyl phosphonic acid), 1000 ppm CCC (2-chloroethyltrimethyl ammonium chloride, cycocel), 1000 ppm GA_{4+7}, 0.2% plantacur-E (a vitamin E formulation containing 25% alpha tocopherol), 125 ppm prohexadione-Ca (3-oxido-4-propionyl-5-oxo-3-cyclohexane-carboxylate), 200 ppm shikimic acid and 0.5 M galactose. Ethephon, CCC and GA_{4+7} were applied twice, July 7 and August 13. Shikimic acid, prohexadione-Ca and plantacur-E were applied 3 times: July 7, August 18 and September 15. Galactose was applied on August 18 and September 15. All chemicals were combined with 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate) wetting agent using a manual sprayer until the entire tree was wet. The experimental design was a randomised complete block design with 3 replicates (3 trees for each). A control treatment sprayed only with water was included. At commercial harvest (7 October) samples of 15 fruits each, from the outer position of the tree canopy (5 fruits/tree), were collected for maturity determination, and for flavonoids and chlorogenic acid measurements.

Exp. 2.

In 1999, ten-year-old 'Jonagold' apple trees trained as slender spindles on M.9 rootstock and planted in a single row system with spacing of 3.0 x 1.25 m in a commercial orchard in the vicinity of Randwijk were used. The following chemical treatments were applied as foliar spray: 480 ppm ethephon, 2000 ppm alar (succinic acid-2,2-dimethylhydrazide), 500 ppm GA_3 (gibberellic acid), 500 ppm (S)-trans-2-amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride (ABG-3168, Abbott) and 1.0% seniphos (a phosphorus-calcium mixture). Alar, ABG and GA_3 were applied on 11, 24 and 31 August, respectively (week 15, 17 and 18 after full bloom, respectively). Ethephon and seniphos were applied on 9 September (week 19 after full bloom), about 4 weeks before the expected commercial harvest. GA_3 was used in this experiment instead of GA_{4+7} because the latter was not effective on flavonoid formation and fruit maturity in the previous experiment. All chemicals were combined with 0.1% Tween 20 wetting agent using a manual sprayer until the entire tree was wet. A control treatment sprayed only with water was included. The experimental design was a randomised complete block design with 3 replicates (4 trees for each). From about 4 weeks before expected commercial harvest and until 3 weeks thereafter, samples of 16 fruits each, from the outer position of the tree canopy (4 fruits/tree), were collected weekly for maturity determination and flavonoids and chlorogenic acid measurements.

2.2. Determination of fruit maturity

At each sampling date, the following fruit maturity characteristics were measured: flesh firmness at the non-blushed side (kg, Instron, 11 mm probe, 8 mm depth in 2 seconds), soluble solids (% brix) and titratable acidity (% malic acid in juice). Starch conversion stage was assessed after dipping a transverse fruit section into a solution of 1% (w/v) iodine and 4% (w/v) potassium iodide; the image was scored on a 1-10 scale (1 = completely black, 10 =

completely white). The streif maturity index (firmness/(starch x % brix)) was calculated (Streif, 1996). The percentage of blushed area of fruit skin was estimated by the colour measuring vision system 'Keurmeester' (AWETA Fruit Grading Company, Nootdorp, The Netherlands) adjusted to a specific setting for 'Jonagold' apples. The background colour of the skin (opposite the blush side) was measured spectrometrically (Minolta CR 200) using the 'a' value.

2.3. Determination of flavonoids and chlorogenic acid in fruit skin

After colour measurement, fruit were peeled and the combined peel of each sample was frozen immediately in liquid nitrogen and vacuum dried. The freeze-dried peel was ground and sieved to separate the skin from adhering fleshy parts. The dry skin samples were kept at -20 °C until extracted for flavonoids and chlorogenic acid. The extraction and the RP-HPLC quantification of these compounds were done as previously described by Awad et al. (2000) with some modifications (Awad et al., 2001b).

2.4. Statistical analysis of data

Data were subjected to analysis of variance (ANOVA) and the treatments means were separated by F-test and the least significant difference (LSD) test at the 5% level. Regression analyses were also done for anthocyanin concentration and blush percentage to determine significances over time and treatments using the statistical package Genstat 5, release 4.1 (Rothamstead, UK).

3. Results

3.1. Flavonoids and chlorogenic acid concentrations in fruit skin

In *Exp.* 1, ethephon application significantly increased the concentration of cyanidin 3-galactoside (anthocyanin) in the fruit skin at commercial harvest (Table 1a). The concentration of the other flavonoid classes as well as of total flavonoids was not significantly influenced by any of the applied chemicals. However, ethephon tended to enhance the concentration of both quercetin 3-glycosides, total flavonoids and chlorogenic acid ($P = 0.11$). GA_{4+7} tended to have a negative effect on chlorogenic acid concentration ($P = 0.11$).

In *Exp.* 2, at week 19 after full bloom the concentration of anthocyanin was very low (almost zero) but it gradually increased during fruit maturation in all treatments (Fig. 1a). Ethephon application accelerated the accumulation of anthocyanin and greatly increased the final concentration compared to the control and all other treatments. In contrast, ABG application delayed the onset of anthocyanin accumulation by about two weeks and greatly reduced the final concentration. GA_3 application also markedly reduced the accumulation of anthocyanin compared to the control whereas alar and seniphos applications had no influence. However, total flavonoids concentration showed no significant changes during maturation

(Fig. 1b). Chlorogenic acid concentration decreased during maturation and was slightly higher at harvest in the ethephon treated fruit compared to most other treatments (Fig. 1c).

Table 1a. Effects of the application of some growth regulators, plantacur E, shikimic acid and galactose during growing on the concentration of flavonoids and chlorogenic acid in 'Jonagold' apple skin at commercial harvest (exp.1, 1997)[a]

| Treatment | Flavonoids and chlorogenic acid (mg g dw) | | | | | |
	Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Control	0.83	0.84	3.02	4.6	9.2	0.22
Ethephon	2.03	0.82	3.32	6.1	12.3	0.33
CCC	0.90	0.96	2.91	4.2	9.0	0.20
Prohexadione-Ca	0.88	0.77	3.38	4.2	9.2	0.20
GA$_{4+7}$	0.54	0.97	2.61	4.5	8.6	0.16
Plantacur E	0.93	0.96	3.21	5.0	10.1	0.19
Shikimic acid	1.13	0.87	3.03	5.1	10.1	0.21
Galactose	1.10	0.79	3.10	4.5	9.4	0.21
F-test	**	NS	NS	NS	NS	NS
LSD $_{0.05}$	0.74	–	–	–	–	–

[a] Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3- glycosides. NS, not significant and (**) significant at $P = 0.01$; (–) not calculated. Presented data points are means of three replications.

3.2. Fruit maturity characteristics

In *Exp.* 1, at commercial harvest there were no significant differences in fruit maturity between the different treatments, as estimated by starch index and streif index (Table 1b). Soluble solids concentration was significantly lower in GA$_{4+7}$ treated fruits and the 'a' value was significantly higher (less green) in ethephon and shikimic acid treated fruits as compared to the control.

Table 1b. Effects of the application of some growth regulators, plantacur E, shikimic acid and galactose during growing on maturity characteristics of 'Jonagold' apples at commercial harvest (exp. 1, 1997)[a]

Treatment	% Brix	Acidity % malic acid	Firmness (Kg/cm^2)	a-value	Streif index	Starch index
Control	14.0	0.58	7.2	-11.4	0.060	8.5
Ethephon	14.4	0.52	7.0	1.80	0.057	8.9
CCC	13.9	0.56	7.1	-7.8	0.059	8.7
Prohexadione-Ca	13.5	0.57	7.2	-9.3	0.061	8.9
GA$_{4+7}$	13.0	0.52	7.4	-10.7	0.056	8.9
Plantacur E	13.4	0.56	7.0	-8.5	0.057	9.0
Shikimic acid	14.1	0.55	7.0	-4.8	0.056	8.9
Galactose	14.0	0.57	7.2	-9.6	0.063	8.3
F-test	*	NS	NS	**	NS	NS
LSD $_{0.05}$	0.94	–	–	6.2	–	–

[a] NS, not significant; *, * *, significant at $P = 0.05$ and 0.01, respectively; (–) not calculated. Presented data points are means of three replications.

In *Exp.* 2, fruit maturity as estimated by both streif and starch index clearly progressed during the period from 19 to 25 weeks after full bloom in all treatments. Malic acid content gradually decreased and soluble solids concentration slightly increased during maturation. However, there were little or no differences among treatments (Fig. 2). Fruit firmness gradually decreased throughout the sampling period and was not significantly affected by any of the treatments (Fig 3a). Percentage of blush clearly increased during maturation in all treatments (Fig. 3b). The effect of the different treatments on this characteristic was the same as for the anthocyanin concentration. The green colour of fruit decreased during maturation (higher 'a' values) and was slightly lower in ethephon treated fruit and higher in ABG treated fruit than in the control (Fig. 3c).

4. Discussion

Our results show that maturity/ripening related fruit characteristics were not significantly influenced by the different treatments while anthocyanin accumulation was clearly stimulated by ethephon and the onset of anthocyanin accumulation was clearly delayed about two weeks by ABG (Fig. 1a, 2 and 3; Table 1). This indicates that anthocyanin formation in apple skin is regulated independently from maturity/ripening associated changes. Anthocyanin formation is apparently regulated both by developmental signals (non-ethylene components) and by ethylene signalling. We observed that the changes in the potential of fruit to accumulate anthocyanin started at a certain stage of development, about 2-3 weeks before maturity in all treatments, except for the ABG treatment in which it started about two weeks later (Fig. 1a). This is an indication that under normal developmental conditions ethylene signalling in the apple skin is limiting for anthocyanin accumulation. We postulate that ABG may suppress the expression of the gene (s) related to anthocyanin biosynthesis by inhibiting the accumulation of the trigger, ethylene. Once the endogenous ethylene reaches a critical level it will trigger the gene (s) related to anthocyanin biosynthesis. In the ethephon treated fruit the transition to fast anthocyanin accumulation occurred at the same time as in untreated fruit (Fig. 1a). Also, ethephon application at mid season did not induce anthocyanin formation until shortly before maturation (Ju et al., 1995a). This suggests that the onset of fruit maturation results in a change in sensitivity and responsiveness of apples to ethylene (Firn, 1986). Indeed there is evidence that the number of ethylene binding sites/receptors increases during ripening of tomato fruit (Lelievre et al., 1997; Fu, 2000). Our data supports the suggestion of Murphey and Dilley (1988) that enhancement of anthocyanin biosynthesis may require only a brief exposure to ethylene that may be insufficient to affect other fruit ripening characteristics. However, in an other study a low concentration of ethephon (25 ppm) advanced ripening of 'McIntosh' apples by about one week (Greene et al., 1974). Therefore it is possible that the effect of ethephon on fruit maturation may depend upon cultivar and environmental conditions (Saure, 1990).
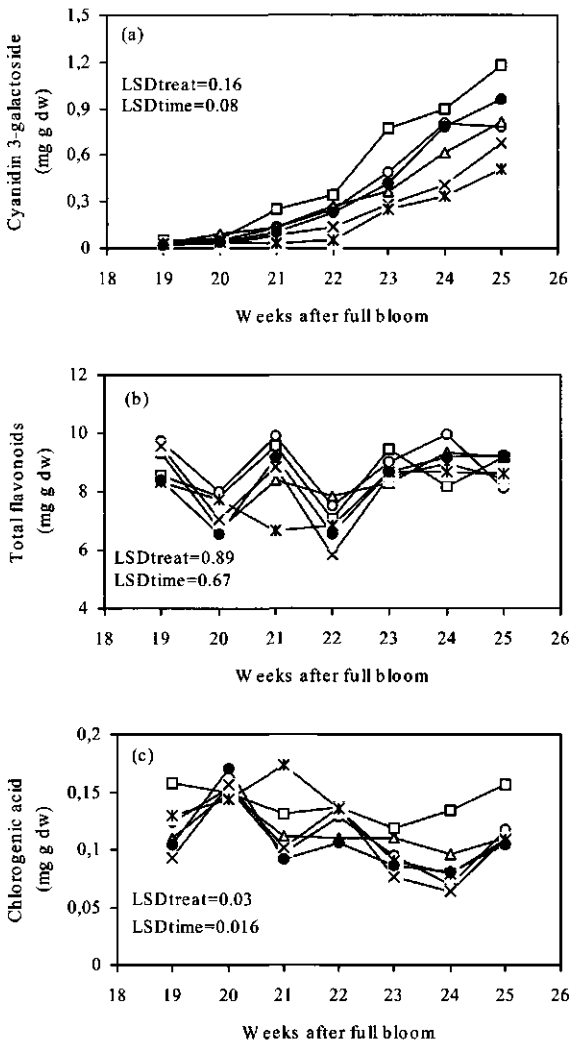
Fig. 1. Accumulation of (a) cyanidin 3-galactoside, (b) total flavonoids and (c) chlorogenic acid during maturation of 'Jonagold' apples as affected by ethephon, ABG, GA₃, alar, and seniphos application (exp. 2, 1999). Presented data points are the means of three replications. LSD (5%) of main effects are presented. □, Ethephon; ✳, ABG; ×, GA₃; Δ, Alar; O, Seniphos; ●, Control.

Fig. 2. Development of (a) starch index, (b) streif index, (c) titratable acidity and (d) % brix during maturation of Jonagold apples as affected by ethephon, ABG, GA₃, alar, and seniphos application (exp. 2, 1999). Presented data points are the means of three replications. LSD (5%) of main effects are presented. □, Ethephon; ✱, ABG; ✕, GA₃; Δ, Alar; O, Seniphos; ●, Control.

(a)

Firmness (Kg)

8
7
6
5
4

LSDtreat=0.38
LSDtime=0.40

18   19   20   21   22   23   24   25   26

Weeks after full bloom

(b)

% of blush

80
60
40
20
0

LSDtreat=8.7
LSDtime=3.2

18   19   20   21   22   23   24   25   26

Weeks after full bloom

(c)

a* value in green side

5
0
-5
-10
-15
-20

LSDtreat=2.3
LSDtime=2.1

18   19   20   21   22   23   24   25   26

Weeks after full bloom

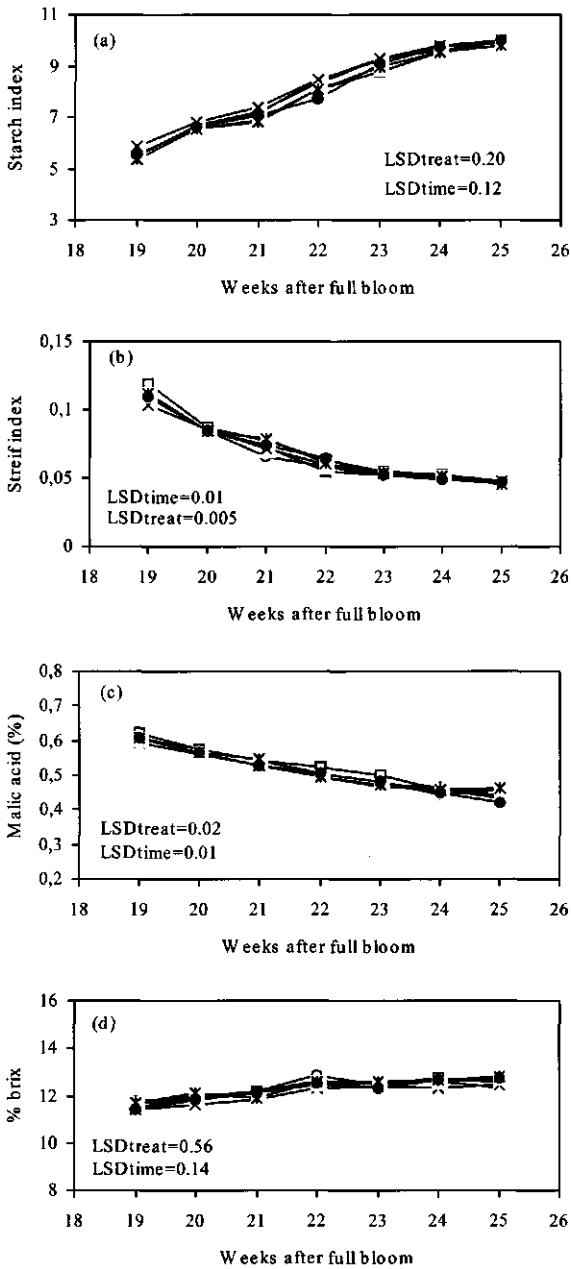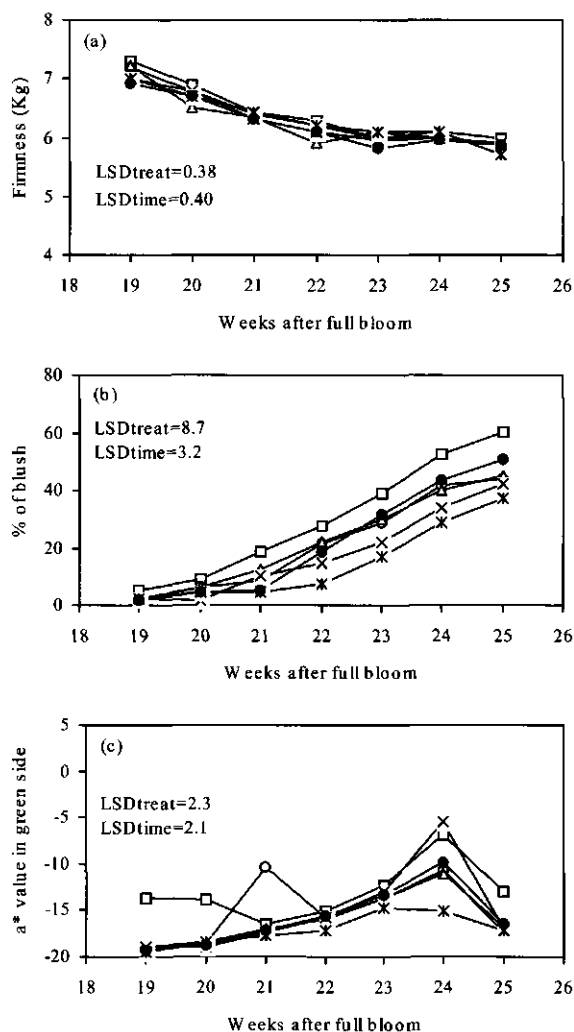Fig. 3. Development of (a) firmness, (b) % of blush and (c) background colour of the skin ('a-value') during maturation of Jonagold apples as affected by ethephon, ABG, GA₃, alar, and seniphos application (exp. 2, 1999). Presented data points are the means of three replications. LSD (5%) of main effects are presented. □, Ethephon; ∗, ABG; ×, GA₃; △, Alar; O, Seniphos; ●, Control.

Furthermore, our results show that none of the used chemicals influenced the formation of chlorogenic acid and other flavonoid classes except for anthocyanin (Fig. 1; Table 1a). This suggests independent regulation of different flavonoid classes and of chlorogenic acid, although they share the same biosynthetic pathway. This independent regulation has been observed before (Awad et al., 2001b) and might be caused by physical separation on the cellular level, as suggested by the apparent on-off mechanism in anthocyanin formation

(Awad et al., 2000) or be the consequence of channelling through different multi-enzyme complexes (Burbulis and Shirley, 1999).

Our results concerning ABG are conflicting with those of Ju et al. (1995, see Introduction). Although internal ethylene or ethylene production was not measured in our experiment, ABG is most likely acting through ethylene since structural analogs of rhizobitoxine (L-2-amino-4-(2-aminoethoxy)-trans-3-butenoic acid) are strong inhibitor of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) but did not affect the conversion of ACC to ethylene (Wang and Mellenthin, 1977; Yang, 1980). Regression analysis (exponential for anthocyanin and logistic for blush percentage) showed no significant differences in rates of accumulation of anthocyanin and blush percentage among treatments. Our results suggest that ethylene is normally present in excess of what is needed for achieving a maximal maturation rate, since maturation of untreated fruit processed at a similar rate as fruit treated with ABG or ethephon. We note that the increase in anthocyanin concentration in response to ethephon application is similar to the increase in blush percentage (Fig. 1a and 3b), suggesting that the increase is caused by an increasing in number of cells producing anthocyanin. In contrast, the decrease in anthocyanin concentration in response to ABG application that inhibits endogenous ethylene biosynthesis, is larger than the decrease in blush percentage (Fig. 1a and 3b). This suggests that not only fewer cells are involved in anthocyanin formation but also anthocyanin formation per cell is affected.

GA$_3$ reduced anthocyanin accumulation and blush percentage, to a lesser extent than ABG (Fig. 1a), without influencing fruit maturation (Fig. 1a, 2 and 3). GA$_3$ has been found to reduce anthocyanin formation in apples and in other fruits as well and its effects have been partially attributed to retarding ripening (see Saure, 1990). Application of the gibberellin inhibitors CCC and prohexadione-Ca did not significantly influence the formation of anthocyanin or fruit maturation (Table 1a, b). Alar seems to reduce anthocyanin formation without delaying fruit maturation (Fig. 1a, 2 and 3). This might be due to a decrease in ethylene production (see Saure, 1990). Seniphos neither influenced anthocyanin formation nor fruit maturation (Fig. 1a; 2 and 3). Literature information concerning both alar (see Saure, 1990) and seniphos (Larrigaudiere et al., 1996; Bertschinger et al., 1998) influences is rather scarce and conflicting. The lack of influence of plantacur-E on anthocyanin formation and blush percentage agrees with the results of Bertschinger et al. (1998) but disagrees with those of Schmitz and Noga (1998).

Shikimic acid and galactose applications had no clear effect on the formation of anthocyanin, other flavonoids and chlorogenic acid (Table 1a), suggesting that the endogenous level might already saturate the enzyme system. Alternatively, the penetration of externally applied shikimic acid and galactose under field conditions might be limited.

In conclusion our results show evidence that anthocyanin accumulation and red coloration of 'Jonagold' apples were enhanced by ethephon without substantially hastening maturation/ripening. The accumulation of other potential health flavonoids is apparently independent of ethylene and fruit maturation, stressing the importance of light conditions during growth for producing healthfulness fruits.

Acknowledgements

8

Flavonoid and chlorogenic acid concentrations in skin of 'Jonagold' and 'Elstar' apples during and after regular and ultra low oxygen storage

Mohamed A. Awad, Anton de Jager

Abstract

Apples are important dietary sources of potentially healthy phenolics. In three successive seasons, the changes in concentrations of flavonoids and chlorogenic acid in the skin of two apple cultivars Jonagold and Elstar during and after regular (RS) and ultra low oxygen storage (ULO) at 1°C, were quantified by reversed-phase high performance liquid chromatography (RP-HPLC) with UV-VIS detector. There were no significant differences in the concentrations of flavonoids and chlorogenic acid between fruits stored under ULO and RS conditions. During storage of both 'Jonagold' (3, 6 and 8 months) and of 'Elstar' (2, 4 and 6 months), and during 1 or 2 weeks shelf life, the concentrations of cyanidin 3-galactoside and quercetin glycosides were relatively constant, while the concentrations of catechins, phloridzin and chlorogenic acid showed only minor changes. Exposing 'Jonagold' and 'Elstar apples' to white light during shelf life following storage increased the concentration of cyanidin 3-galactoside but not any of the other flavonoid classes. An explanation for this might be that the synthesis of different flavonoid classes may have different spectral sensitivity characteristics. It is concluded that flavonoids present in apples are stable and possibly not subject to net metabolic turnover during storage and shelf life.

1. Introduction

Flavonoids are polyphenolic compounds derived from the shikimate pathway and phenylpropanoid metabolism and are widely distributed among plant kingdom. These non-nutrient compounds show anti-oxidative, antimutagenic and anticarcinogenic activities in different systems (Formica and Regelson, 1995; Rice-Evans et al., 1997). Many studies have shown that the occurrence of coronary heart disease and certain cancers are inversely associated with the intake of flavonoids (Formica and Regelson, 1995; Steinmetz and Potter, 1996; Hollman, 1997). The protective effect of fruits and vegetables consumption is partly attributed to their non-nutrient constituents such as vitamins C and E, carotenoids, lycopene, selenium, dietary fiber and more recently to flavonoids (Slater and Block, 1991; Steinmetz and Potter, 1996; Rice-Evans et al., 1997; Robards and Antolovich, 1997). In the Netherlands, Hertog et al. (1993) have estimated the average intake of potentially healthy flavonols and flavones as 23 mg/day (expressed as aglycones) and cited tea, onion and apples as main contributors for these compounds in the Dutch diet. However, reliable quantitative data on the intake of other flavonoids such as catechins and phenolic acids are not yet available. In apples, flavonoids are important constituents and located mainly in the skin (Lancaster, 1992; Awad et al., 2000). Additionally, phenolic substances play a crucial role in determining the quality characteristics of colour and taste such as bitterness and astringency of fresh apples and its processed products (Lea and Timberlake, 1974; Lancaster, 1992; Robards and Antolovich, 1997). There is even indication

that flavonoids and phenolic acids may influence fruit firmness (Lidster et al, 1986). The contents of flavonoid compounds in apples are shown to be specific for particular tissues and, genetically, developmentally, and environmentally determined in a very complex and yet unclear way (Lancaster, 1992; Awad et al., 2000). There is general agreement that the concentrations of phenolics decrease during fruit development to reach a low, more or less steady, level during maturation and ripening (Burda et al., 1990; Mayr et al., 1995).

However, the literature concerning changes in phenolics during storage is much more contradictory. Mosel and Herrmann, (1974) reported that in 'Boskoop' apples the concentrations of catechin, epicatechin and phenolic acids significantly decreased during cold storage. Piretti et al. (1994) found that the most important phenolics in 'Granny Smith' apple skin, epicatechin, quercetin glycosides, procyanidins and other, unknown phenolic compounds generally decreased from day 100 to the end of storage at day 205, both at regular and low oxygen storage (1.0% O_2 +2.0% CO_2). Further decreases were also found during one week of shelf life at 20 °C. Kolesnik et al. (1977) found that the concentration of anthocyanins and flavonols increased during storage while catechins and leucoanthocyanins decreased indicating different behaviour of individual phenolic compounds. More recently, Ju et al. (1996) reported in 'Delicious' and 'Ralls' apples stored for 4–5 months in cold storage that no changes occurred in the concentrations of simple phenols (mainly chlorogenic acid), flavonoid and anthocyanin. However, during 7 days at 20 °C following storage simple phenols and flavonoids rapidly decreased. They also found that anthocyanin decreased during shelf life but only in early picked fruit. Other reports have also shown the total phenol concentration in apples to be relatively stable during storage (Coseteng and Lee, 1987; Kang and Seung, 1987). In apple juice, Spanos et al. (1990) found that juice pressed from 'Granny Smith' apples stored for 9 months at 1 °C contained a much lower concentration of phenolic compounds, especially procyanidins and catechins, than juice pressed from fruit stored for 3 months. Storing the juice concentrates for 9 month at 25 °C caused loss of about 36% of cinnamic acids, loss of 50-60% of quercetin and phloretin derivatives, and a complete loss of procyanidin.

Though the concentration of individual phenolics in fruits is usually low (Robards and Antolovich, 1997), nowadays progress in chromatography analysis procedures allows separating and more accurate quantification of the individual fruit phenolics. Since flavonoids might possess health benefits, information on important dietary sources of these compounds would be useful. Therefore, the aim of the present study was to characterise quantitatively by RP-HPLC the changes in individual flavonoids and chlorogenic acid during and after regular (RS) and ultra low oxygen storage (ULO) of the two main apple cultivars grown in the Netherlands, Jonagold and Elstar.

2. Materials and Methods

2.1. Fruits and storage conditions

Mature apple fruits (*Malus domestica* Borkh L., cv. Jonagold and Elstar) were collected at commercial harvest from trees grafted on a M.9 rootstock grown in a commercial orchard in The Netherlands.

2.1.1. 'Jonagold'

In 1996, 4 boxes (about 12 kg of each) of fruits were picked from the periphery of a group of trees at two different dates classified as early (3 Oct.) and normal (10 Oct.) harvests. One box of each harvest was stored at 1 °C either in RS or in 1.2% O_2 + 5.0% CO_2 (ULO) for 8 months plus 2 weeks of shelf life. In 1997, at commercial maturity, six boxes of fruit were picked from the periphery of a group of trees, three boxes were stored (one box for each take-out) at 1 °C both at RS and ULO for 3, 6 and 8 months (referred to as short, moderate and long respectively) plus 1 or 2 weeks of shelf life. In 1998 the experimental set-up was the same as described for the previous season but with two replicates.

2.1.2. 'Elstar'

In 1997 and 1998, the experimental set-up was the same as previously described for 'Jonagold' but the fruits were stored at 1 °C both at RS and ULO (1.2% O_2 + 3.0% CO_2) for 2, 4, and 6 months (referred to as short, moderate and long respectively) plus 1 or 2 weeks of shelf life.

The fruits of both 'Jonagold' and 'Elstar' were stored in small containers each of 1.0 m^3 with static control of O_2 and CO_2. After each removal of fruit samples, the gas conditions were restored within a few hours. The shelf life room was kept dark, until otherwise stated, and was always at 10 °C during the first week and 20 °C during the second week for both cultivars and for every season.

2.1.3. Illumination treatment

To study the influence of light exposure during shelf life on flavonoid and chlorogenic acid concentrations, additional samples of fruit were prepared for each condition in 1997 and 1998 after 3 months of storage for 'Jonagold' and after 4 months for 'Elstar'. The fruit samples were held in a single layer (the calyx-end down) in a climate chamber at 10 °C during the first and 20 °C during the second week and continuously illuminated with white light from cool high-pressure sodium lamps (Philips SON-T, 400W) positioned about 1.5 m above the fruit. Total irradiance was about 90 to 95 W m^2 near the fruit surface. Then, fruits of each sample were peeled and the peel was prepared as described below for analysis.

2.1.4. Sample preparation

At harvest, after storage and after 1 or 2 weeks of shelf life, a sample of 15 fruits was

randomly taken from each box and completely peeled. The peel of each sample was immediately frozen in liquid nitrogen and freeze-dried. The freeze-dried peel was ground in an electric blender and sieved to separate the skin tissues from adhering fleshy parts. The dry skin samples were then kept at $-20\ ^{\circ}$C for later flavonoids and chlorogenic acid analysis.

2.2. Extraction and quantification of flavonoids and chlorogenic acid

The extraction and reversed-phase high performance liquid chromatography (RP-HPLC) quantification of flavonoids and chlorogenic acid were carried out as previously described by Awad et al. (2000) with some modifications. In the 1998 season, the Shimadzu SPD-10AV VP programmable UV-VIS detector was used. The solvents and wavelength used for separation and monitoring the individual flavonoids and chlorogenic acid were as follows: (1) 10% acetic acid for 12 min followed by 10% acetic acid/acetonitrile (70/30) for 5 min monitored at 280 nm for catechin and epicatechin and (2) 10% acetic acid/acetonitrile (91.5/8.5) monitored at 366 nm for chlorogenic acid and the quercetin glycosides group, 530 nm for cyanidin 3-galactoside, and 280 nm for phloridzin. The chromatogram peaks of individual compounds were identified by comparing their retention times with the retention times of pure standards. Integrated peaks were calculated by comparison with standard solutions of known concentration. Standards used to quantify the HPLC data were cyanidin 3-galactoside, quercetin 3-galactoside, quercetin 3-rhamnoglucoside, quercetin 3-glucoside and quercetin 3-arabinoside obtained from Routh; Quercetin 3-xyloside purchased from Plantech, Reading U.K.; Quercetin 3-rhamnoside obtained from Sigma; (+)-Catechin and (-)-epicatechin obtained from Aldrich and Phloridzin and chlorogenic acid obtained from Fluka. Analytic quality control was performed by including control samples with a known amount of flavonoids and chlorogenic acid in every series of analyses. All determinations were carried out in duplicate. When duplicates differed more than 10%, sample extraction and measurement were repeated.

2.3. Statistical analysis

All data were subjected to an analysis of variance (ANOVA) and the means were separated by an F-test or the least significant difference (LSD) test at the 5% level using the statistical package Genstat 5, release 4.1 (Rothamstead, UK).

3. Results

Table 1 summarises the concentrations of flavonoids and chlorogenic acid in the skin of 'Jonagold' and 'Elstar' apples at harvest. In 1996, early picked fruit of 'Jonagold' had a lower concentration of cyanidin 3-galactoside but rather similar concentrations of the other flavonoid

classes and chlorogenic acid compared with normal picked fruit. Generally, 'Jonagold' had higher values of flavonoids and chlorogenic acid than 'Elstar'.

Storage conditions appeared to have no significant effect on the concentrations of flavonoid and chlorogenic acid in both 'Jonagold' and 'Elstar' apples after storage or after 2 weeks of shelf life (Table 2). After storage and after shelf life, fruit stored for a long period had significantly lower concentrations of phloridzin and catechins than fruit stored for a short period. After shelf life, chlorogenic acid concentration was significantly lower in fruit stored for a long period than

Table 1. Initial concentrations of flavonoid and chlorogenic acid in the skin of 'Jonagold' and 'Elstar' apples[a]

		Flavonoids and chlorogenic acid (mg g dw)					
		Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Jonagold							
1996 season							
	Early	0.36	0.88	2.10	7.90	11.24	0.20
	Normal	0.81	0.78	2.20	6.84	10.63	0.16
1997 season		0.47	1.10	2.96	6.74	11.27	0.22
1998 season		0.79	0.91	2.20	8.71	12.61	0.33
Elstar							
1997 season		0.35	0.75	2.11	3.80	7.01	0.05
1998 season		0.37	0.81	1.60	4.00	6.78	0.06

[a] Data of 1996 for Jonagold were a single sample, for early and normal harvest date. In 1997 the data were a single sample but in 1998 were the means of two replicates for both Jonagold and Elstar of normal harvest date. Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3- glycosides.

in those stored for a short period. The interactions between storage conditions, storage duration and cultivars on the concentrations of flavonoid and chlorogenic acid were not significant.

Table 3 shows the changes in flavonoid and chlorogenic acid concentrations in 'Jonagold' and 'Elstar' apples during shelf life. Generally, in both 'Jonagold' and 'Elstar', no significant changes in flavonoid and chlorogenic acid concentrations occurred during the first week at 10 °C or during the second week at 20 °C. In 'Elstar', the concentration of catechins significantly decreased during the first week without a further decrease during the second week of shelf life and it was higher in fruit stored under ULO than in fruit stored under RS conditions. The concentrations of cyanidin 3-galactoside and phloridzin in 'Jonagold' were significantly lower in fruit stored for a long period than in those stored for a moderate period. Chlorogenic acid concentration was lower in 'Elstar' fruit stored under ULO than in those stored under RS condition. The interactions between storage conditions, storage duration and shelf life on the concentrations of flavonoid and chlorogenic acid were not significant.

Exposing 'Jonagold' and 'Elstar' apples to white light after storage and during shelf life significantly increased the concentration of cyanidin 3-galactoside but not of any of the other flavonoid classes. Chlorogenic acid concentration in both 'Jonagold' and 'Elstar' apples was not affected by the light exposure treatment (Table 4).

Table 2. Flavonoid and chlorogenic acid concentrations in the skin of 'Jonagold' and 'Elstar' apples as affected by storage condition and storage duration[a]

Variable		Flavonoids and chlorogenic acid (mg g dw)					
		Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
After storage							
Storage condition	RS	0.55	0.93	2.53	5.81	9.81	0.21
	ULO	0.54	0.95	2.52	6.20	10.21	0.18
Storage duration	Short	0.54	0.99a	2.76a	5.92	10.21	0.20
	Moderate	0.56	0.99a	2.35b	6.18	10.08	0.21
	Long	0.53	0.84b	2.46b	5.91	9.74	0.17
Cultivar	Jonagold	0.71	1.03	2.61	8.16	12.5	0.28
	Elstar	0.37	0.85	2.43	3.86	7.52	0.11
Significance							
Storage condition		NS	NS	NS	NS	NS	NS
Storage duration		NS	*	***	NS	NS	NS
Cultivar		***	**	*	***	***	***
Plus 2 weeks shelf life							
Storage condition	RS	0.49	0.93	2.43	5.84	9.69	0.23
	ULO	0.47	0.92	2.40	6.00	9.80	0.20
Storage duration	Short	0.45	0.97a	2.48	5.85	9.75	0.25a
	Moderate	0.56	0.97a	2.40	6.00	9.93	0.22ab
	Long	0.44	0.84b	2.37	5.91	9.55	0.18b
Cultivar	Jonagold	0.66	1.10	2.68	8.12	12.5	0.31
	Elstar	0.31	0.77	2.12	3.72	6.95	0.12
Significance							
Storage condition		NS	NS	NS	NS	NS	NS
Storage duration		NS	*	NS	NS	NS	*
Cultivar		***	***	***	***	***	***

[a] Data were the means of the 1997 and 1998 seasons. Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3- glycosides. NS, *, **, ***, not significant and significant at level $P = 0.05$, 0.01 and 0.001, respectively. Means followed by the same letter are not significantly different ($P = 0.05$).

Table 5 shows the effects of harvest date and storage conditions on the concentrations of flavonoid and chlorogenic acid in 'Jonagold' apples during the 1996 season. Fruit picked at a

normal date had higher concentrations of cyanidin 3-galactoside and chlorogenic acid than those picked early. There were no significant differences in the concentrations of flavonoid and chlorogenic acid between fruit stored under RS and ULO conditions for 8 months. During 2 weeks of shelf life the concentration of catechins decreased significantly.

Table 3. Changes in flavonoid and chlorogenic acid concentrations in the skin of 'Jonagold' and 'Elstar' apples during shelf life following storage[a]

Variable		Flavonoids and chlorogenic acid (mg g dw)					
		Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Jonagold							
Shelf life	0 week	0.74	0.98	2.49	8.35	12.55	0.27
	1 week	0.76	0.99	2.53	8.18	12.45	0.26
	2 weeks	0.69	1.03	2.66	8.14	12.52	0.27
Storage condition	RS	0.72	1.02	2.57	8.10	12.39	0.26
	ULO	0.74	0.98	2.55	8.36	12.63	0.27
Storage duration	Moderate	0.78	1.15	2.58	8.36	12.88	0.28
	Long	0.67	0.85	2.53	8.10	12.14	0.25
Significance							
Shelf life		NS	NS	NS	NS	NS	NS
Storage condition		NS	NS	NS	NS	NS	NS
Storage duration		*	***	NS	NS	NS	NS
Elstar							
Shelf life	0 week	0.36	0.84	2.38a	3.78	7.35	0.10
	1 week	0.37	0.86	2.10b	4.38	7.70	0.11
	2 weeks	0.31	0.83	2.12b	3.80	7.10	0.11
Storage condition	RS	0.35	0.84	2.09	4.10	7.37	0.13
	ULO	0.34	0.84	2.31	3.90	7.38	0.10
Significance							
Shelf life		NS	NS	**	NS	NS	NS
Storage condition		NS	NS	**	NS	NS	***

[a] Data were the means of the 1997 and 1998 seasons. Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3- glycosides. NS, *, **, ***, not significant and significant at level $P = 0.05$, 0.01 and 0.001, respectively. Means followed by the same letter are not significantly different ($P = 0.05$).

4. Discussion

At removal from storage, rather similar concentrations of flavonoid and chlorogenic acid were found in both 'Jonagold' and 'Elstar' apples stored under ULO and RS condition (Tables 2 and 5). These results are in agreement with those of Piretti et al. (1994) who found in 'Granny Smith' apples that the concentrations of catechin, epicatechin and quercetin glycosides decreased

during storage to a similar extent in fruit stored at 1.0% O_2 + 2.0% CO_2 as in those stored in air at 0 °C. In 'Conference' pears stored in CA with various concentrations of oxygen and carbon dioxide, the concentration of total phenolics was not affected by storage conditions (Veltman et al., 1999). The slight decrease in phloridzin and catechins concentrations and the relative constant concentrations of cyanidin 3-galactoside and quercetin glycosides during storage (Table 2) indicate a different behaviour of individual flavonoid compounds. Kolesnik et al. (1977) observed

Table 4. Flavonoid and chlorogenic acid concentrations in the skin of 'Jonagold' and 'Elstar' apples as affected by exposure to white light during shelf life following storage[a]

Variable		Flavonoids and chlorogenic acid (mg g dw)					
		Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Jonagold							
Light exposure	With	0.80	1.14	2.58	8.20	12.72	0.30
	Without	0.58	1.19	2.72	8.10	12.56	0.39
Significance		***	NS	NS	NS	NS	NS
Elstar							
Light exposure	With	0.40	0.74	2.17	3.67	6.97	0.14
	Without	0.31	0.72	2.10	3.73	6.86	0.14
Significance		*	NS	NS	NS	NS	NS

[a] Data were the means of the 1997 and 1998 seasons and of RS and ULO conditions. Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3- glycosides. NS, *, ***, not significant or significant at level P = 0.05, 0.01 and 0.001, respectively.

Table 5. Flavonoid and chlorogenic acid concentrations in the skin of 'Jonagold' apples as affected by harvest date and storage conditions (1996)[a]

Variable		Flavonoids and chlorogenic acid (mg g dw)					
		Cyanidin 3-galactoside	Phloridzin	Catechins	Quercetin 3-glycosides	Total flavonoids	Chlorogenic acid
Harvest	Early	0.25	0.71	1.58	5.38	7.91	0.20
	Normal	0.39	0.63	1.57	5.46	8.10	0.22
Storage condition	RS	0.32	0.68	1.55	5.71	8.27	0.24
	ULO	0.32	0.65	1.61	5.13	7.70	0.19
Shelf life	0 week	0.35	0.68	1.72	5.76	8.51	0.19
	2 weeks	0.30	0.65	1.44	5.08	7.46	0.23
Significance							
Harvest		*	NS	NS	NS	NS	*
Storage condition		NS	NS	NS	NS	NS	NS
Shelf life		NS	NS	*	NS	NS	NS

[a] The fruits were stored for 8 months followed by 2 weeks of shelf life. Catechins, the sum of catechin and epicatechin; quercetin 3-glycosides, the sum of quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-rhamnoglucoside and quercetin 3-arabinoside; total flavonoids, the sum of cyanidin 3-galactoside, phloridzin, catechins and quercetin 3- glycosides. NS, *, not significant and significant, respectively at level P = 0.05.

that the concentration of anthocyanin and flavonols increased during storage but catechins and leucoanthocyanins decreased. Albrigo and Childers (1970) and Duvenage and de Swardt (1973) suggested that during cold storage of 'Stayman' or 'Granny Smith' apples an enhanced polymerization process resulted in a higher level of high-molecular-weight flavanols. On the other hand, Burda et al. (1990) reported that the concentration of epicatechin, procyanindin B2 and phloridzin as major phenolics of three apple cultivars 'Golden Delicious', 'Empire' and 'RI Greening' in both the flesh and the skin remained at a relatively constant concentration during 6 months of cold storage. The rather stable concentration of cyanidin 3-galactoside during storage is in line with the observation of Lin et al. (1989) where cyanidin 3-galactoside was the most stable anthocyanin among the major anthocyanin types in 'Starkrimson' apples stored at 2 °C and 73 % relative humidity for about 7 months. Also Reay (1998) reported that both anthocyanin and quercetin glycosides concentrations did not change significantly in 'Gala' apples during storage at 1 °C for about 5 months on either the blush or the shaded side of immature or mature fruit. Chlorogenic acid did not change significantly during storage (Table 2) as also reported by Coseteng and Lee (1987). However, after 2 weeks of shelf life the long stored fruit had a lower concentration of chlorogenic acid than the short stored fruit.

Except for the concentration of catechins in 'Elstar' apples, the data show that both in 'Jonagold' and in 'Elstar', flavonoid and chlorogenic acid concentrations remained relatively constant during 2 weeks of shelf life (Table 3). These results conflict with those of Piretti et al. (1994) for 'Granny Smith' and of Ju et al. (1996) for 'Delicious' and 'Ralls' apples where a rapid decrease in flavonoid and phenolic acids concentrations during 7 days at 20 °C following cold storage was observed. This rapid decrease in flavonoids and in phenolic acids was associated in both cultivars with the development of superficial scald. Interestingly, Perez-Ilzarbe et al. (1997) found that in 'Granny Smith' apples stored at 4 °C for 10 days, during re-warming the fruit at 22 °C for 21 days the concentration of phenolic compounds significantly increased in the skin but not in the flesh. However, during cold storage their concentration remained stable in the skin while it decreased in the flesh.

White light exposure during 2 weeks of shelf life following storage increased anthocyanin concentration in both 'Jonagold' and 'Elstar' apples (Table 4). Arakwa (1988b) found in a post harvest experiment that anthocyanin synthesis was induced by white light in some apple cultivars including 'Jonagold' but not in 'Mutsu' and 'Golden Delicious'. Other flavonoid classes, however, did not increase in concentration with white light illumination (Table 4). Reay (1999) found that visible light alone without UV-B did not significantly increase the concentration of quercetin in 'Granny Smith' apples. This is an indication that the synthesis of different flavonoid classes may have different spectral sensitivity characteristics. The increase in anthocyanin concentration also indicates that both in 'Jonagold' and in 'Elstar' apples after 3 and 4 months of storage respectively, both under ULO or RS conditions, the system of anthocyanin synthesis

remains intact and active. Bishop and Klein (1975) showed that 'McIntosh' apples stored for about 8 months under controlled atmosphere condition showed no diminution in synthetic capacity over time, in contrast to fruit stored at regular cold storage.

Generally, the main conclusion that can be drawn from all these results is that there is only a small profit, if any, of ULO over RS conditions in preserving flavonoids concentration in the fruit and that the flavonoids and chlorogenic acid present in apples are rather stable during storage under ULO or RS conditions and even during shelf life, although relatively small decreases occurred in some components like catechins and phloridzin. These results are in line with the general conclusions of Dangelmayr et.al. (1983) and Zenner and Bopp (1987) who found flavonoid compounds in various plant tissues to be relatively stable and in most cases catabolism to be relatively low, if occurring at all. According to Baruah and Swain (1959) and Roberts (1960), flavonoid compounds are stable and not suitable substrates for polyphenol oxidases (PPO) enzymes. According to van Buren (1970) the major natural substrates found for the oxidative enzymes are hydroxycinnamic acid esters and monomeric and dimeric flavans and not the potentially most healthy compounds, quercetin glycosides. Also, in intact cells, phenolic compounds are located in the vacuoles and protected from the PPO or peroxidase enzymes in the chloroplasts and mitochondria by physical barriers (Mayer, 1987).

Acknowledgements

9

General discussion

1. Introduction

Flavonoids and hydroxycinnamic acids contribute largely to both fruit colour and, through fruit consumption, to human health. As was reviewed in the general introduction (Chapter 1), the role of pre and post harvest factors in the regulation of accumulation of flavonoids other than anthocyanin is poorly documented. Therefore, the research described in this thesis focused on the extent to which the contents of flavonoids and chlorogenic acid in the skin of apples varies, how they develop and how they can be manipulated. The first part of this thesis (Chapters 2-5) deals with variation in the concentration of flavonoids and chlorogenic acid on the level of fruits, trees, orchards, cultivars and mutants. The factor light (Chapter 3), plant nutrients (Chapter 4) and assimilate availability (Chapter 5) were studied for their possible contribution to part of these variations. The second part of the thesis (Chapters 6-8) deals with developmental aspects of flavonoids and chlorogenic acid during fruit growth phase, ripening phase and post harvest phase. In this general discussion an attempt is made to integrate the results.

2. Existing variation at harvest

2.1. Cultivars and mutants variation

Comparing two apple cultivars, significant genotypic variation was observed for the concentration of flavonoids and chlorogenic acid. 'Jonagold' apples contain significant higher concentrations (about 15-20%) (Chapters 2 and 6) and amounts (about 30-40%) (Chapter 6) of total flavonoids than 'Elstar' apples. The concentration of chlorogenic acid was about 3-fold higher in 'Jonagold' than in 'Elstar' apples. However 'Elstar' apples contained significant higher concentrations of some quercetin glycosides types as quercetin 3-rhamnoglucoside (about 40%) and quercetin 3-glucosides (about 60%) than 'Jonagold' apples. This might be relevant with respect to differences in bio-activity and antioxidant capacity of various flavonoid compounds. Cultivar variation in flavonoids and chlorogenic acid concentrations has also been reported by McRae et al. (1990) and Perez-Ilzarbe et al. (1991). If the potential maximum concentration of flavonoids in apple is mainly genetically determined breeding would be an important tool for increasing healthiness of apples. While the primary objective of plant breeding programs over the past 50 years has been to increase productivity by increasing yields, breeding or selecting for health promoting compounds such as nutrients, vitamins, carotenoids and phytochemicals have often been overlooked (Grusak and DellaPenna, 1999). At least the content of health promoting substances should be part of the quality evaluation of existing varieties.

In flowers, manipulation of flavonoid biosynthesis has become feasible after characterisation of several enzymes and cloning their corresponding genes. Recent advances in molecular biology as gene isolation, manipulation and transfer between species enable the alteration of plant properties with aesthetic and commercial value without changing the overall production characteristics (Mol et al., 1989; Bevan, 1993). Accordingly, De Vos et al.

(2000) up-regulated flavonoids biosynthesis in tomato fruits and potato tubers by means of genetic engineering. They introduced transgenic tomato lines that contain 70-fold higher levels of quercetin in their fruit peel compared to their parent plants. Likewise they produced transformed potato plants with up to 60-fold higher levels of kaempferol in the tuber, mainly in the flesh compared to their parent plants. It appears that genetic engineering provides opportunities for increasing flavonoids contents in fruit. However, the time frame of 10 to 15 years to deliver a commercial transgenic cultivar and public concern about genetically modified food must be considered.

The differences between mutants within a given cultivar show that the potential anthocyanin accumulation may increase several fold without influencing the concentrations of other flavonoid classes (Chapter 2). Since, however, the selection of these mutants is inherently based on the amount of red coloration, selection of mutants for higher levels of other potentially healthy flavonoid classes e.g. quercetin 3-glycosides might be considered, providing that such characteristics can be relatively easily determined.

2.2. Light as a key factor

The potentially maximal concentration of flavonoids in apples is apparently genetically determined but is largely influenced by developmental and environmental factors in particular the light conditions (Chapters 2 and 3). Chlorogenic acid concentration seems to be, however, only genetically and developmentally determined since its level was not significantly influenced by the studied environmental factors (Chapters 2-7). We found very low concentrations of anthocyanin, moderate concentration of quercetin 3-glycosides and relatively high concentrations of phloridzin, catechins and chlorogenic acid in the shaded, as compared to the illuminated side, skin of an individual fruit (Chapter 2). Fruit from the top of the canopy contained the highest percentage of blush and the highest concentrations of cyanidin 3-galactoside (anthocyanin) and quercetin 3-glycosides, followed by fruit from the outside of the canopy, and then those from the canopy interior. There were no significant differences in the concentrations of catechins, phloridzin and chlorogenic acid among fruit from the different canopy positions (Chapters 2, 3 and 6). Much of the within-tree variation in fruit quality can be attributed to two factors: position of the fruit in the canopy, which determines the light microclimate under which fruit develop, and bearing wood type (Volz et al., 1994). We showed that the intensity and the composition of the light are different in the exterior and interior regions of the tree canopy (Chapter 3) as previously reported (Looney, 1968; Proctor, 1975). Light in the interior of the canopy was poorer in UV-A, blue, green and red but relatively richer in far-red light than at all other positions. Consequently, the FR/R ratio was much larger at the interior of the canopy than at all other positions and there was a critical FR/R ratio of about 1 above which no anthocyanin and only minimal quercetin 3-glycosides were formed. There were large differences in flavonoid and chlorogenic acid concentrations in 'Elstar' fruit between two normally productive orchards differing mainly in growth vigour (Chapter 2). All these results show that light conditions are a main factor in the biosynthesis of flavonoids in apples. Over a wide range the relationship between light level

and quercetin glycosides concentration is linear. The same holds for anthocyanin though here a threshold appears below which no anthocyanin is formed (in the inside of the tree canopy) (Chapters 3 but also 2 and 6; Ju et al., 1995a and b; Ju et al., 1997). Catechins, phloridzin and chlorogenic acid are much less dependent on light conditions. This would suggest that genes controlling the biosynthesis of different phenolic classes might have a different sensitivity to light. Further, individual flavonoid and chlorogenic acid concentrations were not equally distributed within the apple fruit (Chapter 2). Precisely, the light dependent quercetin 3-glycosides and anthocyanin were almost exclusively found in the skin. This is in accordance with a protection role for these compounds against damaging effects of excessive light intensities (Merzlyak and Chivkunova, 2000). In order to improve both fruit healthiness and attractiveness before harvesting, manipulations of trees such as summer pruning, repositioning branches or covering orchard floor with reflecting films to improve light conditions within the tree, will increase quercetin 3-glycosides and anthocyanin accumulation. The maximum possible difference in flavonoid concentrations, based on difference between top fruit (optimal light conditions) and inner fruit (minimal light conditions) may be 3-fold for quercetin 3-glycosides and 2-fold for total flavonoids (Chapters 2 and 3).

2.3. The role of nutrients

Fruit nutrient composition is strongly associated with external and internal quality attributes of fruits such as colour, firmness, storability and disorder resistance (Bramlage, 1993; Johnson and Ridout, 1998). The current experiments with two types of 'Elstar' show frequent negative correlations between the concentration of N and Mg and the N/Ca ratio in fruit and the concentration of cyanidin 3-galactoside and total flavonoids in the skin (Chapter 4). In 'Elshof' mutant, only in one season out of three the concentrations of P and K in fruit were frequently negatively correlated with the concentration of anthocyanin and total flavonoids. Fruits from trees that received maximum N application contained about 9% less total flavonoids, 26% less anthocyanin and 9% less catechins than fruit from trees that received no N application. Similarly, ciders obtained from 'Dabinett' apple trees that received NPK fertilizer were less bitter and astringent than those from control trees, which was related to an overall decrease of 17% in fruit phenolic concentration (Lea and Beech, 1978). Only in standard 'Elstar' the concentration of Ca in fruit was positively correlated with the concentration of cyanidin 3-galactoside and total flavonoids in the skin (Chapter 4). The relationships between nutrition and chlorogenic acid concentration in apples are not consistent and further study is required. The consistent negative effect of N and the variable results with some other nutrients are in accordance with literature (see chapter 4).

It is concluded that, in addition to improving light conditions, the concentration of flavonoids in fruit skin could be further increased by optimising fertilization especially that of N, directed on preventing of excess N accumulation.

2.4. Assimilate supply is not a limiting factor

It is estimated that, under normal growth conditions, about 20% of all carbon photosynthesised by plants flows through the shikimate pathway and much of it might be used for the synthesis of the various secondary metabolites (Herrmann, 1995). According to Smith (1972), about 2% of the carbon fixed by plants is converted to flavonoids or closely related compounds. Flavonoid compounds are generally present in plants as glycosides, except flavan-3-ols (catechins) which are found in free rather than in glycosylated forms. If flavonoid synthesis is generally substrate limited one might expect that a treatment that causes a change in the availability of the flavonoid precursors, shikimic acid, acetate and sugar, might induce subsequent changes in the synthesis of flavonoids. In accordance with our results (Chapter 5), it is widely shown that differences in crop load can affect fruit development, the availability of assimilates, the content of sugars, acids and dry matter, and nutrients composition of apples (Poll et al., 1996; Volz and Ferguson, 1999; Wertheim, 2000).

However, in the present study, fruit thinning at different levels and times had no significant influence on accumulation of anthocyanin, other flavonoid classes and chlorogenic acid (Chapter 5). The lack of influence of crop load on flavonoids and chlorogenic acid formation could be explained by the fact that even at high crop load (low free assimilate level) carbon supply is not a limiting regulatory factor for secondary metabolite synthesis; a further increase in metabolite level does not take place. This does not correspond to results with pot-grown apples (Poll et al., 1996) or in-vitro grown shoots (Lux-Endrich et al., 2000). We have observed that exogenous application of two precursors, galactose and shikimic acid even during the last weeks before commercial maturity, where potentially carbon limitation for fruit growth occurs (Lakso et al., 1998), had no influence on the concentration of flavonoids and chlorogenic acid in 'Jonagold' apples at harvest (Chapter 7). Also removal of only the interior fruits (about one-third of total fruit) a few weeks before expected commercial maturity did not influence the concentration of flavonoids and chlorogenic acid of the remaining exterior fruits of either 'Jonagold' or 'Elstar' apples at harvest (Chapter 5). This treatment also did not influence other fruit characteristics. It is concluded that, under normal field conditions, assimilate availability is not a major regulatory factor for flavonoids and chlorogenic acid formation in apples. Sufficient number of well exposed leaves per fruit may promote primarily fruit characteristics such as weight, size, the content of sugar and acid, whereas anthocyanin and quercetin 3-glycosides formation may be significantly affected by light that actually strikes the fruit. Therefore it can be concluded that crop load variation does not contribute to the final level of flavonoids and chlorogenic acid in apples.

3. Pre and post harvest development

3.1. Fruit growth phase

Based on development of the amount ('net synthesis') of flavonoids and chlorogenic acid during fruit growth (Chapter 6), three different groups can be discerned. Quercetin 3-

glycosides (1) show a steady increase during the whole period but level off at the start of maturation (about 2 weeks before commercial harvest). Anthocyanin (2) shows a long period without any increase in amount followed by a peak resulting in the observed blush formation at harvest that is starting just when the increase in querectin 3-glycosides and fruit growth slows down. Other flavonoid compounds (3) show more or less an increase in amount in the first part of fruit growth and level off thereafter. Only chlorogenic acid shows a net decrease in amount that occurs rather early in season, followed by an increase (Chapter 6). The latter phenomenon is the only direct evidence for breakdown of any of the studied phenolics. The coincidence of slow down of net quercetin 3-glycosides synthesis and net increase in skin weight (or fruit growth) suggests a feed-back inhibition by end products but this is very speculative since none of the other classes shows such behaviour.

The accumulation of quercetin glycosides and anthocyanin are strongly tree position dependent while that of the other classes are independent of tree position (Chapters 2, 3 and 6). According to these results it is concluded that, any measure to improve light conditions within the tree canopy, for increasing especially anthocyanin and quercetin glycosides levels, should be performed during fruit development, prior to the maturation stage.

3.2. Ripening phase

Since the occurrence of the second peak in anthocyanin formation more or less parallels the maturation and ripening phase (like starch degradation and aroma production), anthocyanin formation itself is often considered as a ripening phenomenon, probably triggered by ethylene. Our results suggest, however, that there is no simple relation to ripening and consequently to ethylene (though we did not measure ethylene) (Chapter 7). This is concluded from the promotion of anthocyanin formation by ethephon (an ethylene releasing compound) application and the retardation of anthocyanin formation by ABG and GA_3 (known to lower or counteract endogenous ethylene), without significantly altering starch degradation and changes in streif index (combination of starch index, firmness and sugar concentration). These results indicate that anthocyanin formation is more sensitive to ethylene than ripening, as suggested by Murphy and Dilley (1988). Our results have also shown that the other flavonoid classes quercetin 3-glycosides, catechins and phloridzin and chlorogenic acid do not respond to any of the applied growth regulators (Chapter 7).

3.3. Post harvest phase

Concentrations of nutrients as vitamins in fruits and vegetables tend to decline during post harvest handling, storage processing and consumer preparation e.g. peeling. As much as 100% of a nutrient may be lost between the time of harvesting and consumption without detectable changes in other quality characteristics such as colour, flavour and texture (Buescher et al., 1999). Losses of vitamins in fruits and vegetables vary with the type of fruit and vegetable, physical damage, type and time of processing, temperature and storage environment (Shewfelt, 1990). The concentration of flavonoids and chlorogenic acid

generally decrease during fruit development to reach a low, more or less steady, level during maturation and ripening (Chapter 6; Burda et al., 1990; Mayr et al., 1995). However, the available literature concerning changes in phenolics during storage and shelf life is much more contradictory (Mosel and Herrmann, 1974; Burda et al., 1990; Piretti et al., 1994; Ju et al., 1996).

In the present study, the changes in individual flavonoids and chlorogenic acid during regular (RS) or ultra low oxygen (ULO) storage conditions at 1°C were determined in both 'Jonagold' and 'Elstar' apples (Chapter 8). It could convincingly be shown that during storage of both 'Jonagold' (3, 6 and 8 months) and of 'Elstar' (2, 4 and 6 months) and during 1 or 2 weeks shelf life, the concentrations of cyanidin 3-galactoside and quercetin glycosides were relatively constant, while the concentrations of catechins, phloridzin and chlorogenic acid showed only minor decreases. Moreover there were no significant differences in the concentration of flavonoids and chlorogenic acid between fruits stored under ULO compared to RS conditions. It is concluded that, following harvest, flavonoids present in apples are stable. There is no direct or indirect proof for breakdown (net metabolic turnover) during storage and shelf life.

4. Relational model of flavonoids accumulation

According to the foregoing results a conceptual model is developed (Fig. 1a) for the relationships between various factors and the accumulation of flavonoids in apples. The first factor determining the potential maximum level of flavonoids is the cultivar or the mutant cultivated. Light, interacting mainly with position in the tree, is the next dominating factor for flavonoids (especially quercetin 3-glycosides and anthocyanin) accumulation. Other factors as pruning, nutrition especially N, temperature, rain/irrigation, crop load, growth regulators and rootstock and interstock might influence flavonoids accumulation directly or indirectly mainly through modifying tree vigour and light conditions within the canopy and through altering fruit development and maturation.
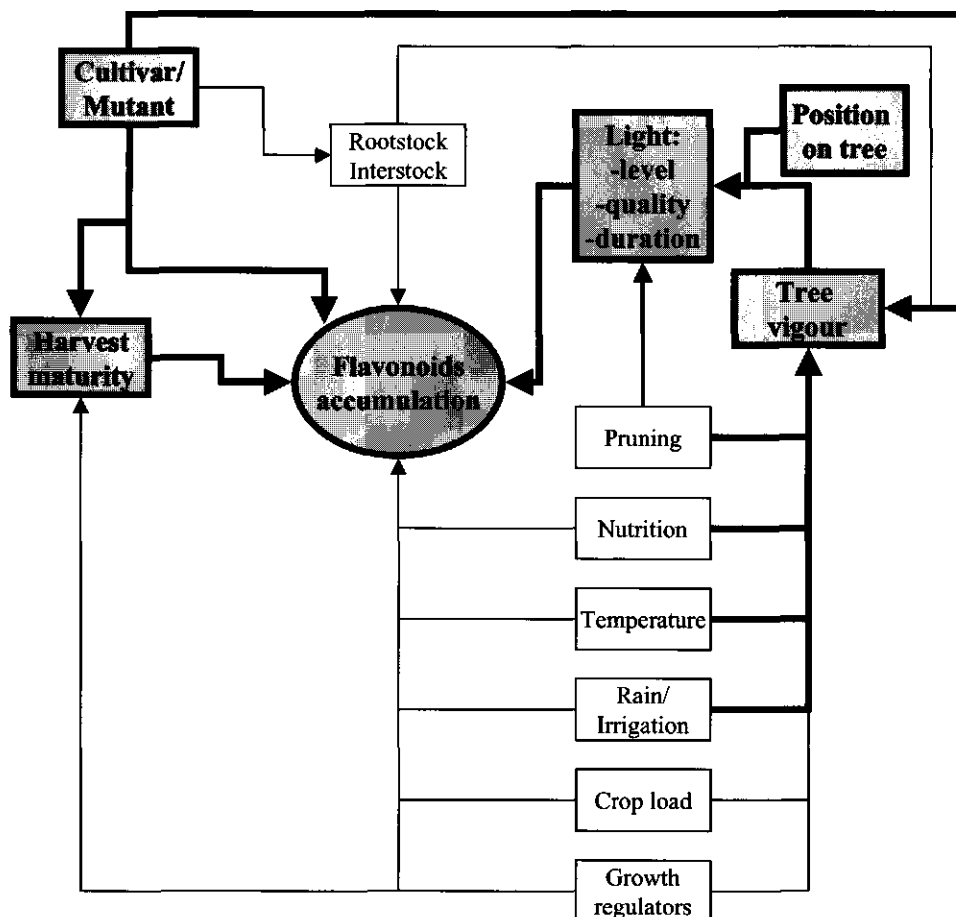
Fig. 1a. A conceptual model of the factors that influence flavonoids accumulation in apples.

5. Independent regulation of different phenolic classes

As presented in Figure 1 (Chapter 6), catechins, phloridzin and chlorogenic acid are part of the same biosynthesis pathway as quercetin 3-glycosides and cyanidin 3-galactoside (Lancaster, 1992) and one would expect competition for the same precursor molecules. However, accumulation of catechins, phloridzin and chlorogenic acid was apparently not affected by the synthesis of cyanidin 3-galactoside and quercetin 3-glycosides, even in fruit grown on the top and the outside of the tree canopy where there is a substantial accumulation of both of these two compounds (Chapters 2, 3 and 6). This rules out competition for precursors. Also the application of ethephon significantly increased and that of ABG significantly decreased the accumulation of anthocyanin without influencing the other flavonoid classes and chlorogenic acid (Chapter 7). These results strongly suggest

independent regulation of different flavonoid classes and of chlorogenic acid, although they share the same biosynthetic pathway. Perhaps synthesis of different flavonoid classes occurs in different cell types of the apple skin. The observation that even in the reddest area of apple skin anthocyanin only accumulates in some cells (Chapter 2) may support this suggestion. Alternatively, different independent multi-enzyme complexes, operating in the same cell, may channel substrate conversion towards either catechins, phloridzin and chlorogenic acid or quercetin 3-glycosides or cyanidin 3-galactoside (Burbulis and Shirley, 1999).

6. Practical aspects

As shown in previous chapters, the accumulation of flavonoids and chlorogenic acid in apples is clearly influenced by various internal and external factors. In Figure 1b, a schematic representation of the apple production chain is given. At each step a number of factors contribute to the final level of phytochemicals in fruit. In this paragraph the effects of these steps and the options for improving the final levels of flavonoids and chlorogenic acid for the consumer will be discussed.

Our results show that there is much room for optimising the level of potential health phytochemicals in apples. The first step would be cultivar selection either from already available genotypes or by developing new cultivars through classical breeding or molecular biology and gene technology. It appears that improvement of nutritional quality of horticultural products will be a rewarding activity for plant breeders as we enter the 21st century, especially in industrialised countries where sufficient food is available and the interest in healthy foods is increasing among the population (Bliss, 1999). However, before starting breeding for nutritional improvement careful assessment of existing cultivars and feasibility of breeding strategies must be considered. Selection of new mutants should be based on the level of potential health promoting flavonoid classes e.g. quercetin 3-glycosides and catechins.

Since environmental factors have a significant impact on determining the final level of flavonoids in fruit, the second step can be optimisation of climatic conditions and cultural practices especially those improving light conditions within tree canopy such as dwarfing root stocks, suitable planting system, optimum row orientation and proper training and pruning systems or covering orchard floor with reflecting films. A third step could be optimisation of the fertilization programme especially avoiding excess N and better timing of N-application.

Maturity at harvest is one of the most important factors determining the accumulation of anthocyanin in fruits. Thus during the maturation process fruit should be harvested at the time when enough anthocyanin and red colour are formed. This knowledge is, however, of very restricted use since waiting for colour means loss of fruit storability. Since the overall production of other flavonoid classes and chlorogenic acid is completed during fruit development before the onset of maturation (Chapters 6 and 7) this does not further increase total flavonoids content either. At harvest or later, sorting of fruit based on their blush might be a way to make healthiness classes, since blush is a good marker for exposure to light during growth and thus to some extent for the quercetin 3-glycosides level.

Additionally, also post harvest irradiation (Chapter 8; Dong et al., 1995; Lancaster et al., 2000) can be considered to improve flavonoids especially in green fruits from the canopy interior. This method will, however, not be very practical because of the space requirement of such treatment.

Light
Temperature
Humidity
Air pollutants

Nutrition
Soil moisture
Rootstock/Pruning
Crop load
Growth regulators

Maturity
Position on tree

Irradiation
Chemical treatments

Temperature
Atmosphere
Humidity
Duration
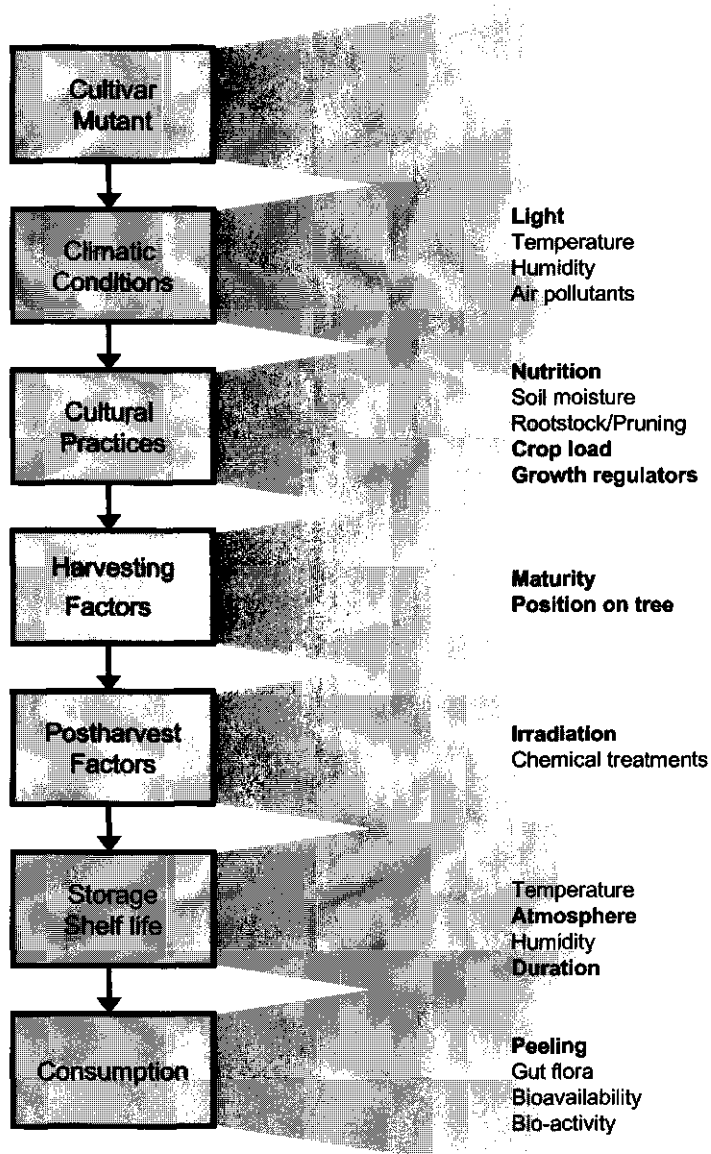
Peeling
Gut flora
Bioavailability
Bio-activity

Fig. 1b. Schematic representation of the apple production chain and possible causes for variation in levels of flavonoids and chlorogenic acid. The highlighted factors are those studied here.

Storage conditions and duration appear not to be an important consideration for flavonoids and chlorogenic acid retention (Chapter 8), thus applying the proper conditions for each cultivar to retain the most classical quality like firmness and acidity can be used.

Even when cultivar choice and cultivation methods succeed in getting high levels of flavonoids in fruit still the treatment by the consumer determines how much of these substances will be consumed. Many consumers still peel the fruit before consumption thereby removing almost all anthocyanin and quercetin 3-glycosides (Chapter 2). Also important vitamins like C and E and beta-carotene are mainly located in the peel (Buescher et al., 1999). On the other hand, pesticide and fungicide residues also are usually more concentrated in outer tissues, occasionally preventing their safe use as food. Thus, any promotion of apple consumption based on their healthy substances should imply or even promote consumption of the skin and this, in turn, should be facilitated by more safe, e.g. organic ways of farming. However, as amount per fruit, the flesh remain the main contributor for especially catechins and chlorogenic acid and about equal to the skin for phloridzin intake.

7. Future research

Because of the large sources of variation induced in the different steps of the production chain (Fig. 1b), a quantitative model would offer a practical and effective tool of partitioning variation and quantifying its components in a rational manner (Dekker et al., 2000). By developing such a model we would be able to predict and optimise the final level of healthy compounds and thus developing more accurate intake data and dietary recommendations. Such a model might also be integrated with light utilisation models in relation to qualitative and quantitative aspects of fruit production. A broad inventory of the levels of healthy compounds in existing cultivars is needed for better choice of cultivars and for breeding purposes. When a strategy is chosen to increase the levels of health protecting phytochemicals in apples, this has to be done in an integrated way also taking into account the other relevant quality attributes of apples. Especially sensory attributes like flavour and astringency may be influenced negatively by very high levels of flavonoids. Before plant improvement strategies should be pursued, more information on specific aspects of phytochemicals (e.g. the determination of their bio-availability and dose dependency and their mechanistic relation with human health) is desirable (Grusak and DellaPenna, 1999).

References

Albrigo, L.G. and Childers, N.F., 1970. Peel flavanols and their relation to superficial scald in Stayman apples. J. Amer. Soc. Hort. Sci. 95, 798-800.

Arakawa, O., 1991. Effect of temperature on anthocyanin accumulation in apple fruit as affected by cultivar, stage of fruit ripening and bagging. J. Horti. Sci. 66, 763-768.

Arakawa, O., 1988a. Photoregulation of anthocyanin synthesis in apple fruit under UV-B and red light. Plant Cell Physiol. 29, 1385-1389.

Arakawa, O., 1988b. Characteristics of color development in some apple cultivars: changes in anthocyanin synthesis during maturation as affected by bagging and light quality. J. Jpn. Soc. Hort. Sci. 57, 373-380.

Arakawa, O., Hori, Y., Ogata, R., 1985. Relative effectiveness and interaction of ultraviolet-B, red and blue light in anthocyanin synthesis of apple fruit. Physiol. Plant. 64, 323-327.

Autio, W.R., 1991. Rootstocks affect ripening and other qualities of Delicious apples. J. Amer. Soc. Hort. Sci. 116, 378-382.

Awad, M.A., Wagenmakers, P.S., de Jager, A., 2001a. Effects of light on flavonoid and chlorogenic acid levels in the skin of Jonagold apples. Sci. Hortic. 88, 289-298.

Awad, M.A., de Jager, A., van der Plas, L.H.W., van der Krol, A.R., 2001b. Flavonoid and chlorogenic acid changes in skin of Elstar and Jonagold apples during development and ripening. Sci.Hortic. *In Press.*

Awad, M.A., de Jager, A. and van Westing, L.M., 2000. Flavonoid and chlorogenic acid levels in apple fruit: Characterisation of variation. Sci. Hortic. 83, 249-263.

Awasthi, R.P., Kaith, N.S., Dev, G., 1995. Influence of rate and method of potassium application on growth, yield, fruit quality and leaf nutrient status of apple. J. Potassium Res. 11, 356-364.

Bae, R.N., Lee, S.K., 1995. Effects of some treatments on the anthocyanin synthesis and quality during maturation of Fuji apple. J. Korean Soc. Hort. Sci. 36, 655-661.

Barritt, B.H., Drake, S.R., Konishi, B.S., Rom, C.R., 1997. Influence of sunlight level and rootstock on apple fruit quality. Acta Hort. 451, 569-577.

Baruah, P. and Swain, T., 1959. Action of potato phenolase on flavonoid compounds. J. Sci. Food Agric. 10, 125-130.

Berhow, M.A., Vandercook, C.E., 1992. The reduction of naringin content of grapefruit by applications of gibberellic acid. Plant Growth Regulation, 11, 75-80.

Bertschinger, L., Stadler, W., Lemmeneier, L., 1998. First results from testing new vitamin E formulations for improving red colour formation and scab regulation in Swiss apple orchards. pp 220-231. In G. Noga and M. Schmitz (Editors), Antioxidants in higher plants: biosynthesis, characteristics, actions and specific functions in stress defence. Shaker Verlag, Aachen, 1998, Germany.

Bevan, M., 1993. Gene regulation in plants- present knowledge and future prospects. Agro-food-Industry Hi-Tech. March/April, 7-10.

Bishop, R.C., Klein, R.M., 1975. Photo-promotion of anthocyanin synthesis in harvested apples. HortScience. 10, 126-127.

Blankenship, S.M, 1987. Night temperature effects on rate of apple fruit maturation and fruit quality. Sci. Hortic. 33, 205-212.

Blanpied, G.D., Forshey, C.G., Styles, W.C., Green, D.W., Lord, W.J., Bramlage, W.J., 1975. Use of ethephon to stimulate red color without hastening ripening of McIntosh apples. J. Amer. Soc. Hort. Sci. 100, 379-381.

Bliss, F.A., 1999. Nutritional improvement of horticultural crops through plant breeding. HortScience 34, 1163-1167.

Block, G., Patterson, B., Subar, A., 1992. Fruit, vegetables and cancer prevention: a review of the epidemiological evidence. Nutr. Cancer 18, 1-29.

Bongue-Bartelsman, M., Phillips, D. A., 1995. Nitrogen stress regulates gene expression of enzymes in the flavonoid biosynthetic pathway of tomato. Plant Physiol. Biochem. 33, 539-546.

Bors, W., Heller, W., Michel, C., Saran, M., 1990. Flavonoids as antioxidants: determination of radical-scavenging efficiencies. Methods Enzymol. 186, 343-355.

Boynton, D., Cain, J.C., 1943. A survey of the relationships between leaf nitrogen, fruit color, and per cent of full crop in some New York McIntosh apple orchards. Proc. Amer. Soc. Horti. Sci. 40, 19-22.

Bramlage, W.J., 1993. Interactions of orchard factors and mineral nutrition on quality of pome fruit. Acta Hortic. 326, 15-28.

Buescher, R., Howard, L., Dexter, P., 1999. Postharvest enhancement of fruits and vegetables for improved human health. HortScience 34, 1167-1170.

Burbulis, I.E., Shirley, B.W., 1999. Interactions among enzymes of the arabidopsis flavonoid biosynthetic pathway. Plant Biol. 96, 12929-12934.

Burda, S., Oleszek, W. and Lee, C.Y., 1990. Phenolic compounds and their changes in apples during maturation

and cold storage. J. Agric. Food Chem. 38, 945-948.

Camm, E.L., Towers G.H., 1973. Phenylalanine ammonia layse. Phytochemistry 12, 961-973.

Castillo, J., Benavente, O., Del Rio, J.A., 1992. Naringin and Neohesperidin levels during development of leaves, flower buds, and fruits of Citrus aurantium. Plant Physiol. 99, 67-73.

Chalmers, D.J., Faragher, J.D., Raff, J.W., 1973. Changes in anthocyanin synthesis as an index of maturity in red apple varieties. J. Hortic. Sci. 48, 387-392.

Chalmers, D.J., Faragher, J.D., 1977. Regulation of anthocyanin synthesis in apple skin. I. Comparison of the effects of cyclohexamide, ultraviolet light, wounding and maturity. Aust. J. Plant Physiol. 4, 111-121.

Cheng, F.S., Weeden, N.F., Brown, S.K., 1996. Identification of co-dominant RAPD markers tightly linked to fruit skin color in apple. Theor. Appl. Genet. 93, 222-227.

Cook, N.C., Samman, S., 1996. Flavonoids: chemistry, metabolism, cardioprotective effects, and dietary sources. J. Nutr. Biochem. 7, 66-76.

Coseteng, M.Y. and Lee, C.Y., 1987. Changes in apple polyphenoloxidase and polyphenol concentrations in relation to degree of browning. J. Food Sci. 52, 985-989.

Creasy, L.L., 1968. The role of low temperature in anthocyanin synthesis in McIntosh apples. Proc. Amer. Soc. Hortic. Sci. 93, 716-724.

Croft, K.D., 1998. The chemistry and biological effects of flavonoids and phenolic acids. Ann. N.Y. Acad Sci. 854, 435-442.

Curry, E.A., 1997. Temperatures for optimum anthocyanin accumulation in apple tissue. J. Hortic. Sci. 72, 723-729.

Dangelmayr, B., Stotz, G., Spribille, R. and Forkmann, G., 1983. Relationship between flower development, anthocyanin accumulation and activity of enzymes involved in flavonoid biosynthesis in *Matthiola incana*. R. Br., Z. Naturforsch. 38c, 551-555.

Dayton, D.F., 1959. Red color distribution in apple skin. Proc. Amer. Soc. Hort. Sci. 74, 72-81.

De Jager, A., Roelofs, F.P.M.M., 1996. Prediction of optimum harvest date of Jonagold apple. pp 21-30. In A. de Jager, D. Johnson and E. Hohn (Editors), Determination and prediction of optimum harvest date of apples and pears. Brussels, Luxembourg, 1996, (EC. COST 94).

Dekker, M., Verkerk, R., Jongen, W.M.F., 2000. Predictive modelling of health aspects in the food production chain: a case study on glucosinolates in cabbage. Tren. Food Sci.Technol. 11, 174-181.

De Vos, R., Bovy, A., Busink, H., Muir, S., Collins, G., Verhoeyen, M., 2000. Improving health potential of crop plants by means of flavonoid pathway engineering. pp 25-26. In Martens, S., Treutter, D., and Forkmann, G. (Editors), Polyphenols communications 2000, Freising-Weihenstephan (Germany), September 11-15, 2000.

Diener, H.A., Naumann, W.D., 1981. Influence of day and night temperatures on anthocyanin synthesis in the apple skin. Gartenbauwissenschaft, 46, 125-132.

Dixon, R.A., Paiva, N. L., 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7, 1085-1097.

Dong, Y.H., Mitra, D., Kootstra, A., Lister, C., Lancaster, J., 1995. Post harvest stimulation of skin color of Royal Gala apple. J. Amer. Soc. Hort. Sci.120, 95-100.

Downs, R.J., Siegelman, H.W., Butler, W.L., Hendricks, S.B., 1965. Photoreceptive pigments for anthocyanin synthesis in apple skin. Nature 205, 909-910.

Duvenage, A.J. and de Swardt, G.H., 1973. Superficial scald in apples: the effect of maturity and diphenylamine on the flavonoid content in the skin of two cultivars. Z. Pflanzenphysiol. 70, 222-234.

Estiarte, M., Filella, I., Serra, J., Penuelas, J., 1994. Effects of nutrient and water stress on leaf phenolic content of peppers and susceptibility to generalist herbivore Helicoverpa armigera (Hubner). Oecologia, 99, 387-391.

Faragher, J.D. and Chalmers, D.J., 1977. Regulation of anthocyanin synthesis in apple skin. III. Involvement of phenylalanine ammonia-lyase. Austral. J. Plant Physiol. 4, 133-141.

Faragher, J.D., 1983. Temperature regulation of anthocyanin accumulation in apple skin. J. Exp. Bot. 34, 1291-1298.

Faragher, J.D., Brohier, R.L., 1984. Anthocyanin accumulation in apple skin during ripening: regulation by ethylene and phenylalanine ammonia-lyase. Sci. Hortic. 22, 89-96.

Faust, M., 1965a. Physiology of anthocyanin development in McIntosh apple. II. Relationship between protein synthesis and anthocyanin development. J. Amer. Soc. Hort. Sci. 87,10-20.

Faust, M., 1965b. Physiology of anthocyanin development in McIntosh apple. I. Participation of pentose phosphate pathway in anthocyanin development. J. Amer. Soc. Hort. Sci. 87,1-9.

Faust, M., 1973. Effect of growth regulators on firmness and red color of fruit. Acta Horti. 34, 407-420.

Firn, R.D., 1986. Growth substance sensitivity: The need for clearer ideas, precise terms and purposeful experiments. Physiol. Plant. 67, 267-272.

Fletcher, L.A., 1932. Effect of thinning on size and color of apples. Proc. Amer. Soc. Hort. Sci. 29, 51-55.

Formica, J.V. and Regelson, W., 1995. Review of the biology of quercetin and related bioflavonoids. Food

Chem. Toxic. 33, 1061-1080.

Gao, Y., Cahoon, G.A., 1993. Cluster shading effects on fruit quality, fruit skin color, and anthocyanin content and composition in Reliance (*Vitis* hybrid). Vitis 33, 205-209.

Gao, G., Sofic, E., Prior, R.L., 1996. Antioxidant capacity of tea and common vegetables. J. Agric. Food Chem. 44, 3426-3431.

Greene, D.W., Lord, W.J., Bramlage, W.J., Southwick, F.W., 1974. Effects of low ethephon concentrations on quality of McIntosh apples. J. Amer. Soc. Hort. Sci. 99, 239-242.

Grusak, M.A., DellaPenna, D., 1999. Improving the nutrient composition of plants to enhance human nutrition and health. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 133-161.

Guyot, S., Marnet, N., Laraba, D., Sanoner, P., Drilleau, J.F., 1998. Reversed-phase HPLC following thiolysis for quantitative estimation and characterization of the four main classes of phenolic compounds in different tissue zones of a French cider apple variety (*Malus domestica* Var. Kermerrien). J. Agric. Food Chem. 46, 1698-1705.

Hahlbrock, K., Scheel, D., 1989. Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 347-369.

Hamauzu, Y., Iijima, E., Banno, K., 1999. Changes in catechin and procyanidin contents during fruit development of two apple cultivars. J. Jpn. Soc. Hort. Sci., 68, 1184-1193.

Heinicke, D.R., 1966. Characteristics of McIntosh and Red Delicious apples as influenced by exposure to sunlight during the growing season. Proc. Amer. Soc. Hort. Sci. 89, 10-13.

Herrmann, K.M., 1995. The shikimate pathway: early steps in the biosyntheis of aromatic compounds. Plant Cell 7, 907-919.

Hertog, M.G.L., Holman, P.C.H., Katan, M.B., 1992. Content of potentially anticarcenogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. J. Agric. Food Chem. 40, 2379-2383.

Hertog, M.G.L., Holman, P.C.H., Katan, M.B. and Kromhout, D., 1993. Intake of potentially anticarcenogenic flavonoids and their deteminants in adults in The Netherlands. Nutrition and Cancer 20, 21-29.

Holcroft, D.M., Gil, M.I., Kader, A.A., 1998. Effect of carbon dioxide on anthocyanins, phenylalanine ammonia lyase and glucosyltransferase in the arils of stored pomegranates. J. Amer. Soc. Hort. Sci. 123, 136-140.

Holcroft, D.M., Kader, A.A., 1999. Controlled atmosphere-induced changes in PH and organic acid metabolism may affect color of stored strawberry fruit. Postharvest biol. Techol. 17, 19-32.

Hollman, P.C.H., 1997. Determinations of the absorption of the dietary flavonoids quercetin in man. PhD thesis. Wageningen Agriculture University, Wageningen,The Netherlands.

Holton, T.A., Cornish, E.C., 1995. Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7, 1071-1083.

Hung, C.Y., Murray, J.R., Ohmann, S.M., Tong, C.B.S., 1997. Anthocyanin accumulation during potato tuber development. J. Amer. Soc. Horti. Sci. 122, 20-23.

Jackson, J.E., 1980. Light interception and utilization by orchard systems. Hort. Rev. 2, 208-267.

Jaetak, Y., Minsoon, H., Jinsu, K., Seongyong, C., Kyungwhan, K., 1998. Effect of low temperature on the fruitletcoloring in Fuji apple during early growing season. J. Agro-envir. Sci. 40, 80-85.

Jiang, Y., Fu, J., 2000. Ethylene regulation of fruit ripening: molecular aspects. Plant Growth Regulation 30: 193-200.

Johnson, D.S., Samuelson, T.J., 1990. Short-term effects of changes in soil management and nitrogen fertilizer application on Brmley's seedling apple trees. II. Effects on mineral composition and storage quality of fruit. J. Horti. Sci. 65, 495-502.

Johnson, D.S., Ridout, M.S., 1998. Prediction of storage quality of cox's Orange Pippin apples from nutritional and meteorological data using multiple models selected by cross validation. J. Horti. Sci. Biotech. 73, 622-630.

Jones, D.H., 1984. Phenylalanine ammonia-layse: regulation of its induction, and its role in plant development. Phytochemistry 23, 1349-1359.

Jourdan, P.S., McIntosh, C.A., Mansell, R.L., 1985. Naringin levels in citrus tissues. Plant Physiol. 77, 903-908.

Ju, Z., 1998. Fruit bagging, a useful method for studying anthocyanin synthesis and gene expression in apples. Sci. Hortic. 77, 155-164.

Ju, Z., Liu, C., Yuan, Y., Wang, Y., Liu, G., 1999a. Coloration potential, anthocyanin accumulation, and enzyme activity in fruit of commercial apple cultivars and their F1 progeny. Sci. Hortic. 79, 39-50.

Ju, Z., Duan, Y., Ju, Z., 1999b. Effects of covering the orchard floor with reflecting films on pigment accumulation and fruit coloration in Fuji apples. Sci. Hortic. 82, 47-56.

Ju, Z., Yuan, Y., Liu, C., Wang, Y., Tian, X., 1997. Dihydroflavonol reductase activity and anthocyanin accumulation in Delicious, Golden Delicious and Indo apples. Sci. Hortic. 70, 31-43.

Ju, Z, Yuan, Y., Liu, C, Zhan, S. and Wang, M., 1996. Relationships among simple phenol, flavonoid and anthocyanin in apple fruit peel at harvest and scald susceptibility. Postharvest Biol. Technol. 8, 83-93.

Ju, Z., Liu, C., Yuan, Y., 1995a. Activities of chalcone synthase and UDPGal:flavonoids-3-o-glycosyltransferase

in relation to anthocyanin synthesis in apple. Sci. Hortic. 63, 175-185.

Ju, Z., Yuan, Y., Liou, C., Xin, S., 1995b. Relationships among phenylalanine ammonia-lyase activity, simple phenol concentrations and anthocyanin accumulation in apple. Sci. Hortic. 61, 215-226.

Kalt, W., Prange, R.K., Lidster, P.D., 1993. Post harvest colour development of strawberries: influence of maturity, temperature and light. Can. J. Plant Sci. 73, 541-548.

Kalt, W., McDonald, J.E., 1996. Chemical composition of lowbush blueberry cultivars. J. Amer. Soc. Hort. Sci. 121, 142-146.

Kang, S.D. and Seung, L.K., 1987. Nature and control of apple scald during cold storage. J. Kor. Soc. Hort. Sci. 28, 343-351.

Kangasjarvi, J., Talvinen, J., Utriainen, M., Karjalainen, R., 1994. Plant defence systems induced by ozone. Plant, Cell and Envir. 17, 783-794.

Kasperbauer, M.J., 1987. Far-Red light reflection from green leaves and effects on phytochrome-mediated assimilate partitioning under field condition. Plant Physiol. 85, 350-354.

Khan, M.I., 1980. Gibberellic acid bioassay based on the inhibition of anthocyanin production in tomato seedlings. Biol. Plant. 22, 401-403.

Knee, M., 1972. Anthocyanin, carotenoid, and chlorophyll changes in the peel of Cox's Orange Pippin apples during ripening on and off the tree. J. Exp. Bot. 23, 184-196.

Krause, J., Reznik, H., 1976. Investigation of flavonol accumulation in *Fagopyrum esculentum* Moench as influenced by P-and N-deficiency. Z. Pflanzenphysiol. 70, 392-400.

Koes, R.E., van Blokland, R., Quattrocchio, F., van Tunen, A.J., Mol, J.N.M., 1990. Chalcone synthase promoters in petunia are active in pigmented and unpigmented cell types. Plant Cell 2, 379-392.

Koes, R.E., Quattrocchio, F., Mol, J.N.M., 1994. The flavonoids biosynthetic pathway in plants: function and evolution. BioEssays 16, 123-132.

Kohlmeier, L., Weterings, K.G.C., Steck, S., Kok, F.J., 1997. Tea and cancer prevention: an evaluation of the epidemiologic literature. Nutrition and Cancer 27, 1-13.

Kolesnik, A., Elizarova, L.G., Starodubsteva, T.V., Afanasyeva, V.S. and Erokhina, T.S., 1977. Changes in polyphenols during storage of fruits and vegetables. Prikl. Biokhim.Mikrbiol. 13, 333-339.

Kubasek, W.L., Shirly, B.W., Mckillop, A., Goodman, H.M., Briggs, W., Ausubel, F.M., 1992. Regulation of flavonoid biosynthetic genes in germinating arabidopsis seedlings. The Plant cell 4, 1229-1236.

Kubo, Y. Taira, S., Zshio, S., Sugiura, A., Tamana , T., 1988. Color development of four apple cultivars grown in the southwest of Japan, with special reference to fruit bagging. J. Jpn. Soc. Horti. Sci. 57, 191-197.

Kuhnau, J., 1976. The flavonoids: a class of semi-essential food components: their role in human nutrition. World Rev. Nutr. Diet. 24, 117-120.

Lakso, A.N., Bepete, M., Goffinet, M.C., Grappadelli, L.C., 1998. Aspects of carbon supply and demand in apple fruits. Acta Hort. 466, 13-18.

Lancaster, J.E., 1992. Regulation of skin color in apples. Crti. Rev. Plant Sci. 10, 487-502.

Lancaster, J.E., Reay, P., Norris, J., Butler, R., 2000. Induction of flavonoids and phenolic acids in apple by UV-B and temperature. J. Hort. Sci. Biotech. 75, 142-148.

Lancaster, J.E., Grant, J.E., Lister, C.E., Taylor, M., 1994. Skin color in apples-influence of copigmentation and plastid pigments on shade and darkness of red color in five genotypes. J. Amer. Soc. Hort. Sci. 119, 63-69.

Larrigaudiere, C., Pinto, E., Vendrell, M., 1996. Differential effects of ethephon and seniphos on color development of Starking Delicious apple. J. Amer. Soc. Hort. Sci. 121, 746-750.

Lawanson A. O., Akindole, B.B., Fasalojo, P.B., Akpe, B.L., 1972. Time course of anthocyanin formation during deficiencies of nitrogen, phosphorus and potassium in seedling of *Zea mays* Linn. var.E.S. 1. Z. Pflanzenphysiol. 66, 251-253.

Lea, A.G.H., Beech, F.W., 1978. The phenolic of ciders: effect of cultured conditions. J. Sci. Food Agric. 29, 493-496.

Lea, A.G.H. and Timberlake C.F., 1974. The phenolics of ciders 1. Procyanidins. J. Sci. Food Agric. 25, 1537-1545.

Lelievre, J.M., Latche, A., Jones, B., Bouzayen, M., Pech, J.C., 1997. Ethylene and fruit ripening. Physiol. Plant. 101, 727-739.

Lidster, P.D., Dick, A.J., DeMarco, A., McRae, K.B., 1986. Application of flavonoid glycosides and phenolic acids to suppress firmness loss in apples. J. Amer. Soc. Hort. Sci. 111, 892-896.

Lin, T.Y., Koehler, P.E. and Shewfelt, R.L., 1989. Stability of anthocyanins in the skin of Starkrimson apples stored unpackaged, under heat shrinkable wrap and in-package modified atmosphere. J. Food Sci. 54, 405-407.

Lister, C.E., Lancaster, J.E., Walker, J.R.L., 1996. Developmental changes in Enzymes of flavonoid biosynthesis in the skins of red and green apple. J. Sci. Food Agric. 71, 313-320.

Lister, C.E., Lancaster, J.E., Sutton, K.H., 1994. Developmental changes in the concentration and composition of

flavonoids in skin of a red and a green apple cultivar. J. Sci. Food Agric. 64, 155-161.

Looney, N.E., 1968. Light regimes within standard size apple trees as determined spectrophotometrically. Proc. Amer. Soc. Hort. Sci. 93, 1-6.

Lux-Endrich, A., Treutter, D., Feucht, W., 2000. Influence of nutrients and carbohydrate supply on the phenol composition of apple shoot cultures. Plant cell, Tissue and Organ Culture 60, 15-21.

Magness, J.R., 1928a. Relation of leaf area to size and quality in apples. Proc. Amer. Soc. Hort. Sci. 25, 285-288.

Magness, J.R., 1928b. Observations on color development in apples. Proc. Amer. Soc. Hort. Sci. 25, 289-292.

Magness, J.R., Batjer, L.P., Regeimbal, L.O., 1940. Correlation of fruit color in apples to nitrogen content of leaves. Proc. Amer. Soc. Hortic. Sci. 37, 39-42.

Margna, U., 1977. Control at the level of substrate supply- an alternative in the regulation of phenylpropanoid accumulation in plant cells. Phytochemistry 16, 419-426.

Margna, U., Margna, E., Vainjarv, T., 1989. Influence of nitrogen on the utilization of L-phenylalanine for building flavonoids in buckwheat seedling tissues. J. Plant Physiol. 134, 697-702.

Marini, R.P., Barden, J.A., 1987. Summer pruning of apple and peach trees. Hortic. Rev. 9, 351-375.

Matern, U., Grimmig, B., 1994. Natural phenols as stress metabolites. Acta Hortic. 381, 448-462.

Mayer, A.M., 1987. Polyphenol oxidases in plants. Recent progress. Phytochemistry 26, 11-20.

Mayr, U., Treutter, D., Buelga, S., Bauer, H. and Feucht, W., 1995. Developmental changes in the phenol concentrations of Golden Delicious apple fruits and leaves. Phytochemistry 38, 1151-1155.

McRae, K.B., Lidster, P.D., De Marco A.C., Dick, A.J., 1990. Comparison of the polyphenol profiles of the apple fruit cultivars by correspondence analysis. J. Sci. Food Agric. 50, 329-342.

Meheriuk, M., 1990. Skin color of Newtown apples treated with calcium nitrate, urea, diphenylamine, and a film coating. HortScience 25, 775-776.

Merzlyak, M.N, Chivkunova, O.B., 2000. Light-stress-induced pigment changes and evidence for anthocyanin photoprotection in apples. J. Photochem. Photobiol. 55, 155-163.

Miller, T.W., Fellman, J.K., Mattheis, J.P., Fallahi, E., Mattinson, D.C., 1996. The Delicious dilemma: is redder better? Good Fruit Grower. 47, 85-87.

Mink, G.I., 1973. The apple industry in Japan. Hortsci.8, 81-86.

Mohr, H., Herrel, H.D., 1983. Coaction between phytochrome and blue/UV light in anthocyanin synthesis in seedlings. Physiol. Plant. 58, 408-414.

Mol, J., Jenkins, G., Schafer, E., Weiss, D., 1996. Signal perception, transduction, and gene expression involved in anthocyanin biosynthesis. Crit. Rev. Plant Sci. 15, 525-557.

Mol, J.N.M., Stuitje, A.R., van der Krol, A., 1989. Genetic manipulation of floral pigmentation genes. Plant Molecular. Biol.13, 287-294.

Morgan, D.C., Stanley, C.J., Volz, R., Warrington, I.J., 1984. Summer pruning of Gala apple: the relationships between pruning time, radiation penetration, and fruit quality. J. Amer. Soc. Hort. Sci. 109, 637-642.

Mosel, H.D. and Herrmann, K., 1974. Changes in catechins and hydroxycinnamic acid derivatives during development of apples and pears. J. Sci. Food Agric. 25, 251-256.

Murphey A.S., Dilley, D.R., 1988. Anthocyanin biosynthesis and maturity of McIntosh apples as influenced by ethylene-releasing compounds. J. Amer. Soc. Hort. Sci. 113, 718-723.

Nagy, S., 1980. Vitamin C contents of citrus fruit and their products: a review. J. Agric. Food Chem. 28, 8-18.

Nicolas, J.J., Richard-Forget, F.C., Goupy, P.M., Amiot, M.J., Aubert, S.Y., 1994. Enzymatic browning reaction in apple and apple products. Crit. Rev. Food Sci. Nutr. 34, 109-157.

Nilson, E.T., Orcutt, D.M., 1996. The physiology of plants under stress. Abiotic factors. John Wiley & Sons, INC., pp 515-544.

Noro, S., 2000. Studies on coloring and skin disorders on apples. Bull. Aomori. Apple Exp. Stn. Kuroishi, Aomori 036-0332, Japan. 31, 80-84.

Noro, S., Ichinohe, H., Obara, N., 1991. Effects of controlled air temperature on development of anthocyanin, sugars and citramalic acid during maturation of the apple cultivar Starking Delicious. Bull. Aomori. Apple Exp. Stn. Kuroishi, Aomori 036-0332, Japan. 27, 122-124.

Noro, S., Kudo, N., Kitsuwa, T., 1988. Differences in sugar and organic acids between red and yellow apple cultivars at time of coloring, and effect of citramalic acid on development of anthocyanin. J. Jpn. Soc. Hort. Sci. 57, 381-389.

Palmer, J.W., Avery, D.J., Wertheim, S.J., 1992. Effect of apple tree spacing and summer pruning on leaf area distribution and light interceptio. Sci. Hortic. 52, 303-312.

Parchomchuk, P., Meheriuk, M., 1996. Orchard cooling with pulsed overtree irrigation to prevent solar injury and improve fruit quality of Jonagold apples. HortScience 31, 802-804.

Patil, B.S; Alva, A.K. 1999. Enhancing citrus nutraceuticals through variable nutrient rates. HortScience 34, 520.

Phillips, R., Henshaw, G.G., 1977. The regulation of synthesis of phenolics in stationary phase cell cultures of *Acer pseudoplatanus*. L. J. Exp. Bot. 28, 785-794.

Perez-Ilzarbe, J., Hernandez, T., Estrella, I. and Vendrell, M., 1997. Cold storage of apples (cv. Granny Smith) and changes in phenolic compounds. Z. Lebensm Unters Forsch 204, 52-55.

Perez-Ilzarbe, J., Hernandez, T., Estrella, I., 1991. Phenolic compounds in apples: varietal differences. Z. Lebensm. Unters. Forsch. 192, 551-554.

Piretti, M.V., Gallerani, G. and Pratella, G.C., 1994. Polyphenol fate and superficial scald in apple. Postharvest Biol. Technol. 4, 213-224.

Poll, L., Rindom. A., Toldam-Andersen, T.B., Hansen, P., 1996. Availability of assimilates and formation of aroma compounds in apples as affected by the fruit/leaf ratio. Physiol. Plant. 97, 223-227.

Pratt, C., 1988. Apple flower and fruit: Morphology and anatomy. Hort. Rev. 10, 273-307.

Proctor, J.T.A., Lougheed, E.C., 1976. The effect of covering apples during development. HortScience 11, 108-109.

Proctor, J.T.A., Kyle, W.J., Davies, J.A., 1975. The pentration of global solar radiation into apple trees. J. Amer. Soc. Hort. Sci. 100, 40-44.

Raese, J.T., Drake, S.R., 2000. Effect of calcium spray materials, rate, time of spray application, and rootstocks on fruit quality of Red and Golden delicious apples. J. Plant Nut. 23, 1435-1447.

Raese, J.T., Drake, S.R., 1997. Nitrogen fertilization and elemental composition affects fruit quality of Fuji apples. J. Plant Nut. 20, 1797-1809.

Raese, J.T., Staiff, D.C., 1990. Fruit calcium, quality and disorders of apples (*Malus demostica*) and pears (Pyrus communis) influenced by fertilizers, pp. 619-623. In: M.L. van Beusichem (ed.), Plant nutrition-tree physiology and applications. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Reay, P.F., 1999. The role of low temperatures in the development of the red blush on apple fruit (Granny Smith). Sci. Hortic. 79, 113-119.

Reay, P.F., Fletcher, R.H., Thomas, V. G., 1998. Chlorophylls, carotenoids and anthocyanin concentrations in the skin of Gala apples during maturation and the influence of foliar applications of nitrogen and magnesium. J. Sci. Food Agric. 76, 63-71.

Rengel, Z., Kordan, H.A., 1987. Effects of growth regulators on light-dependent anthocyanin production in *Zea mays* seedlings. Physiol. Plant. 69, 511-516.

Rice-Evans, A.C., Miller, N.J. and Paganga,G. 1997. Antioxidant properties of phenolic compounds. New Trends Plant Sci. Rev. 2, 152-159.

Robards, K. and Antolovich, M., 1997. Analytical chemistry of fruit bioflavonoids. Analyst 122, 11R-34R.

Roemmelt, S., Treutter, D., Speakman, J.B., Rademacher, W., 1999. Effects of prohexadione-Ca on the flavonoids metabolism of apple with respect to plant resistance against fire blight. Acta Hort. 498, 359-363.

Sannomaru, Y., Katayama, O., Kashimura, Y., Kaneko, K., 1998. Changes in polyphenol content and polyphenoloxidase activity of apple fruits during ripening process. J. Jpn. Soc. Food Sci. Techn. 45, 37-43.

Saure, M.C., 1990. External control of anthocyanin formation in apple. Sci. Hortic. 42, 181-218.

Saure, M.C., 1987. Summer pruning effects in apple- a review. Sci. Hortic. 30, 253-282.

Schmitz, M., Noga, G., 1998. Alpha-tocopherol reduced environmental stress and improved fruit quality. Acta Hort. 466, 89-94.

Schupp, J.R., Ferree, D.C., 1989. Root pruning for growth control in apple trees. Acta Hortic. 243, 103-109.

Sharples, R.O., 1973. Orchard and climatic factors. In: Fidler, J.C., Wilkinson, B.G., Edney, K.L., Sharples, R.O. (Editors), The biology of apple and pear storage. CAB, Farnham Royal, UK, pp. 173-225.

Shewfelt, R.L., 1999. What is quality? Postharvest Biol. Tech. 15, 197-200.

Shewfelt, R.L., 1990. Sources of variation in the nutrient content of agricultural commodities from the farm to consumer. J. Food Qual. 13, 37-54.

Shirley, B.W., 1996. Flavonoids biosynthesis: new functions for an old pathway. New Trends Plant Sci. Rev. 1, 377-382.

Siegelman, H.W., Hendricks, S.B., 1958. Photocontrol of anthocyanin synthesis in apple skin. Plant Physiol. 33, 185-190.

Slater, T.F. and Block, G., 1991. Antioxidant vitamins and B-carotene in disease prevention. Amer. J. Clin. Nutr. 53, 189-196.

Smith, H., 1972. The photocontrol of flavonoid biosynthesis. In Mitrakos, K., and Shropshire, W. (Ed.), Phytochrome. Academic Press, London, pp. 433-481.

Smock, R.M., 1969. Laboratory studies on the effects of chemicals on the coloration of apples. Proc. Amer. Soc. Hort. Sci. 94, 49-51.

Smock, R.M., 1966. Laboratory studies of anthocyanin development in McIntosh apples. Proc. Amer. Soc. Hort. Sci. 88, 80-88.

Spanos, G.A., Wrolstad, R.E. and Heatherbell, D.A., 1990. Influence of processing and storage on the phenolic composition of apple juice. J. Agric. Food chem. 38, 1572-1579.

Stafford, H.A. (Ed.), 1990. Flavonoids metabolism. CRC Press, Boca Raton, FL, pp. 101-132.

Steinmetz, K.A. and Potter, J.D., 1996. Vegetables, fruit, and cancer prevention: a review. J. Amer. Diet. Assoc. 96, 1027-1039.

Streif, J., 1996. Optimum harvest date for different apple cultivars in the Bodensee area. pp 15-20. In A. de Jager, D. Johnson and E. Hohn (Editors), Determination and prediction of optimum harvest date of apples and pears. ECSC-EC-EAEC, Brussels; Luxembourg, 1996, (EC. Cost 94).

Sun, B.H., Francis, F.J., 1967. Apple anthocyanins: Identification of cyanidin 7-arabinoside. J. Food. Sci. 32, 647-648.

Takahama, U., Oniki, T., 1997. A peroxidase/phenolics/ascorbate system can scavenge hydrogen peroxide in plant cells. Physiol. Plant. 101, 845-852.

Tan, S.C., 1980. Phenylalanine ammonia-lyase and the Phenylalanine ammonia-lyase inactivating system: effects of light, temperature and mineral deficiencies. Aust. J. Plant Physiol. 7, 159-167.

Tijburg, L.B.M., Mattern, T., Folts, J.D., Weisgerber, U.M., Katan, M.B., 1997. Tea flavonoids and cardiovascular diseases: A review. Crit. Rev. Food Sci. Nutr. 37, 771-785.

Tobin, E.M., Silverthorne, J., 1985. Light regulation of gene expression in higher plants. Ann. Rev. Plant Physiol. 36, 569-593.

Tomas-Barberan, F.A., Cantos, E., Ferreres, F., 2000. UV-irradiation as a method to increase phenolics content and improve quality and health-promoting properties of harvested fruits. pp 487-488. In Martens, S., Treutter, D., and Forkmann, G. (Editors), Polyphenols communications 2000, Freising-Weihenstephan (Germany), September 11-15, 2000.

Tromp, J., 1999. Maturity of apple cvs Cox's Orange Pippin and Elstar as affected by mid-season and late-season temperature. Gartenbauwissenschaft 64, 145-151.

Unrath, C.R., 1972. The quality of Red Delicious apples as affected by overtree sprinkler irrigation. J. Amer. Soc. Hort. Sci. 97, 58-61.

Unrath, C.R., 1973. Interaction of irrigation, evaporative cooling, and ethephon on fruit quality of Delicious apple. HortiScience 8, 318-319.

Uota, M., 1952. Temperature studies on the development of anthocyanin in McIntosh apples. Proc. Amer. Soc. Hortic. Sci. 59, 231-237.

van Buren, J., 1970. Fruit phenolics. In: A.C. Hulme (Editor), The biochemistry of fruits and their products. Academic Press, pp. 295-296.

van dercook, C.E., Tisserat, B., 1989. Flavonoid changes in developing lemons grown in vivo and in vitro. Phytochemistry, 28, 799-803.

van der Meer, I.M., Stuitje, A.R., Mol, J.N.M., 1993. Regulation of general phenylpropanoid and flavonoids gene expression. In: Verma, D.P.S. (Ed.), Control of gene expression. CRC Press, Boca Raton, FL, pp. 125-155.

Veltman, R.H., Larrigaudiere, C., Wichers, H. J., van Schaik, A.C.R., van Der Plas, L.H.W. and Oosterhaven, J., 1999. PPO activity and polyphenol content are not limiting factors during brown core development in pears (Pyrus communis L. cv. Conference). J. Plant Physiol. 154, 697-702.

Vestrheim, S., 1970. Effects of chemical compounds on anthocyanin formation in McIntosh apple skin.J. Amer. Soc. Hort. Sci. 95, 712-715.

Volz, R.K., Ferguson, I.B., 1999. Flower thinning method affects mineral composition of Braeburn and Fiesta apple fruit. J. Hort. Sci. Biotech. 74, 452-457.

Volz, R.K., Ferguson, I.B., Hewett, E.W., Woolley, D.J., 1994. Wood age and leaf area influence fruit size and mineral composition of apple fruit. J. Hort. Sci. 69, 385-395.

Wagenmakers, P.S., Callesen, O., 1995. Light distribution in apple orchard systems in relation to production and fruit quality. J. Hort. Sci. 70, 935-948.

Wagenmakers, P .S., 1991. Planting systems for fruit trees in temperate climates. Crit. Rev. Plant Sciences 10, 369-385.

Walter, T.E., 1967. Factors affecting fruit colour in apples: A review of world literature. Rep. East. Malling Res. Stn. 1966, 70-82.

Wang, H., Gao, G., Prior, R.L., 1996. Total antioxidant capacity of fruits. J. Agric. Food Chem. 44, 701-705.

Wang, C.Y., Mellenthin, W.M., 1977. Effect of aminoethoxy analog of rhizobitoxine on ripening of pears. Plant Physiol. 59, 546-549.

Weeks, W.D., Southwick, F.W., Drake, M., Steckel, J.E., 1958. The effect of varying rates of nitrogen and potassium on the mineral composition of McIntosh foliage and fruit color. Proc. Amer. Soc. Horti. Sci. 71, 11-19.

Wertheim, S.J., 2000. Developments in the chemical thinning of apple and pear. Plant Growth Regulation. 31, 85-100.

Wertheim, S.J., 1987. Jonagold kleurt pas bij veel blad per vrucht. Fruitteelt 77, 15-16 (in Dutch).

Wertheim, S.J., De Jager, A., Duyzens, M.J.J.P., 1986. Comparison of single-row and multiple-row planting

system with apple, with regard to productivity, fruit size and colour, and light conditions. Acta Hort. 160, 243-258.

Williams, M.W., Billingsley, H.D., 1974. Effect of nitrogen fertilizer on yield, size and colour of golden Delicious apple. J. Amer. Soc. Hort. Sci. 99, 144-145.

Workman, M., 1963. Color and pigment changes in Golden Delicious and Grimes Golden apples. J. Amer. Soc. Hort. Sci. 83: 149-161.

Yamasaki, H., Sakihama, Y., Ikehara, N., 1997. Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H_2O_2. Plant Physiol. 115, 1405-1412.

Yamasaki, H., Uefuji, H., Sakihama, Y., 1996. Bleaching of the red anthocyanin induced by superoxide radical. Arch. Bioch. and Biophy. 332, 183-186.

Yang, S.F., 1980. Regulation of ethylene biosynthesis. HortScience 15, 238-243.

Yao, J.L., Dong, Y.H., Kvarnheden, A., Morris, B., 1999. Seven MADS-box genes in apple are expressed in different parts of the fruit. J. Amer. Soc. Hort. Sci. 124, 8-13.

Youljae, Y., Hanchan, L., 1999. Effect of pre-harvest defoliation on fruit color and tree physiology in apples. J. Korean Soc. Hort. Sci. 40, 209-212.

Zenner, K. and Bopp, M., 1987. Anthocyanin turnover in *Sinapis alba* L. J. Plant Physiol. 126, 475-482.

Zhang, D., Quantick, P.C., Grigor, J.M., 2000. Changes in phenolic compounds in Litchi (*Litchi chinensis* Sonn.) fruit during postharvest storage. Postharvest Biol. Technol. 19, 165-172.

Zornoza, P., Esteban, R.M., 1984. Flavonoids content of tomato plants for the study of the nutritional status. Plant and Soil 82, 269-271.

Summary

The ultimate objective of the production, handling and distribution of fresh fruits and vegetables is to satisfy consumers requirements. In general the attractiveness of fruits and vegetables to consumers is determined both by visible (e.g. colour) and invisible (e.g. healthiness) quality attributes. Flavonoids and hydroxycinnamic acid derivatives, secondary metabolites, contribute largely to both fruit colour and, through fruit consumption, to human health and it is, therefore, very useful to study factors that affect these substances with the aim of further improving the relevant fruit attributes. Flavonoids and hydroxycinnamic acid derivatives are widespread in the plant kingdom, comprise a large group of naturally occurring antioxidants that form part of the human diet. There is considerable evidence for the role of antioxidant constituents of fruits and vegetables in the maintenance of health and disease prevention. Recent studies have shown that the majority of the antioxidant activity of a fruit or vegetable may originate from the flavonoids and other phenolic compounds. Apple fruit are rich in flavonoids such as flavonols (quercetin 3-glycosides), flavanols (catechin, epicatechin, gallocatechin, procyanidins and its polymers), dihydrochalcone glycosides (phloritin glucoside (phloridzin) and phloritin xyloglucoside), and cyanidin 3-glycoside (anthocyanins). Apple fruits also contain considerable amounts of hydroxycinnamic acid derivatives mainly represented by chlorogenic acid. The red colour of apples is primarily a consequence of the flavonoid pigments anthocyanins which are located in the vacuole. Despite the importance of flavonoids for the intrinsic quality of apples very little is known of their regulation in fruit. The aim of the work described in this thesis was therefore, to obtain knowledge on the extent to which the contents of flavonoids and chlorogenic acid in the skin of apples varies, how they develop during fruit growth phase, ripening phase and post harvest phase and how they can be manipulated.

Chapter 1 contains a review of the literature. It appears that the accumulation of flavonoids and phenolic acids in plants is under control of many internal and external factors.

In Chapter 2 the extent of natural variation in flavonoids and chlorogenic acid concentration due to within fruit, within tree, between orchards, between cultivars and among mutants was determined. Considerable variation was observed among these variables. Individual flavonoids and chlorogenic acid concentrations were not equally distributed within the fruit. Quercetin 3-glycosides and anthocyanin were almost exclusively found in the skin. The sun-exposed skin of individual fruit had much higher cyanidin 3-galactoside (anthocyanin) and quercetin 3-glycoside concentrations than the shaded skin, while phloridzin, catechins and chlorogenic acid were similar in the skin of both sides (Chapter 2). Significant genotypic variation was observed for the concentration of flavonoids and chlorogenic acid. 'Jonagold' apples contain significant higher concentrations (about 15-20% higher) (Chapters 2 and 6) and amounts (about 30-40% higher) (Chapter 6) of the total flavonoids than 'Elstar' apples. Chlorogenic acid concentration was about 3-fold higher in 'Jonagold' than in 'Elstar' apples. However 'Elstar' apples contained significant higher concentrations of some quercetin glycosides types as quercetin 3-rhamnoglucoside (about

40% higher) and quercetin 3-glucosides (about 60% higher) than 'Jonagold' apples. This might be relevant with respect to differences in bio-activity and antioxidant capacity of various flavonoid compounds. As far as the potential maximum concentration of flavonoids in apple is genetically determined breeding would be an important tool for increasing healthiness of apples. The differences between basic forms and coloured mutants within a given cultivar (for example Jonagold-Jonaprince; Elstar-Elshof) show that the potential anthocyanin accumulation (but only that) may increase several fold without influencing the concentrations of other flavonoid classes (Chapter 2). Microscopic study showed that the most blushed mutants had a higher number of red cells per cell layer and more cell layers containing red cells than the standard cultivar and the less blushed mutants. It is striking to observe coloured and completely uncoloured cells as neighbours. Since the selection of coloured mutants is inherently based on the amount of red coloration, selection of mutants for higher levels of other potential healthy flavonoid classes e.g. quercetin 3-glycosides could be considered, providing that such characteristics can be relatively easily determined. The concentrations of anthocyanin, quercetin 3-glycosides and total flavonoids were highest in fruit borne in the top of the tree followed by fruit from the outer tree parts, whereas the lowest concentrations were found in fruit from the inner tree. Terminal fruit contained the highest concentrations of these compounds, including catechins, compared to lateral and spur fruit. Phloridzin and chlorogenic acid were not affected by the position of the fruit in the tree nor by the bearing wood type. The maximum possible difference in flavonoid concentrations, based on difference between top fruit (optimal light conditions) and inner fruit (minimal light conditions) may be 3-fold for quercetin 3-glycosides and 2-fold for total flavonoids (Chapters 2 and 3). There were significant differences in flavonoid and chlorogenic acid concentrations in 'Elstar' fruit between two normally productive orchards differing mainly in growth vigour and internal shading. All these results show that light conditions are a main regulatory factor in the biosynthesis of flavonoids in apples.

In Chapter 3 the natural distribution of light within the tree canopy in relation to the concentration of flavonoids and chlorogenic acid in fruit skin was analysed. The concentrations of cyanidin 3-galactoside and quercetin 3-glycosides and the percentage of blush in the fruit skin were directly related to light level in the direct vicinity of the fruit. Light in the interior of the canopy was poorer in UV-A, blue, green and red (R) but richer in far-red (FR) light than at all other positions. Consequently, the FR/R ratio (with large influence on formative processes) was much larger at the interior of the canopy than at all other positions. There was a critical FR/R ratio of about 1 above which no anthocyanin and only low amounts of quercetin 3-glycosides were formed.

In Chapter 4 the relationships between the fruit nutrients N, P, K, Ca and Mg and concentrations of flavonoids and chlorogenic acid in fruit skin were studied with two types of 'Elstar'. In an experiment with the mutant 'Elshof' with the 5 nutrients applied at 5 rates in 4 replications, only N and Ca applications resulted in higher concentration of these nutrients in the fruit, but sufficient variation was present among treatments to correlate the concentration of the other nutrients with those of flavonoids and chlorogenic acid. Negative correlations were frequently found between the concentration of N and Mg and the N/Ca ratio in fruit

during growth, and anthocyanin and total flavonoids concentration at maturity in 1996, 1997 and 1998. In 1997, these correlations were weakest but still significant. In that season, P and K concentration were frequently negatively correlated with the concentration of anthocyanin and total flavonoids. The concentration of Ca was not related to the concentration of anthocyanin and total flavonoids. In a study in 1996 with standard 'Elstar', we used the variation in nutrient concentration due to differences in fruit position on tree. The concentrations of N and K and the N/Ca ratio in fruit at maturity were negatively and that of Ca was positively correlated with the concentration of anthocyanin and total flavonoids. Magnesium concentration was negatively correlated with anthocyanin concentration but not with total flavonoids. As a consequence of the relation with position of the fruit in the tree an interaction with the influence of light may, however, be expected. Multiple regression models mainly containing N as factor accounted for up to 40% and 30% of the variance in anthocyanin and total flavonoids concentration of 'Elshof' mutant apples, and for up to 70% and 65% of the variance in anthocyanin and total flavonoids concentration of standard 'Elstar' apples. The relationships between plant nutrients and chlorogenic acid concentration in apples were not consistent and further study is required. It is concluded that, in addition to improving light conditions, the concentration of flavonoids in fruit skin could be further increased by optimising fertilization especially that of N, directed at preventing excess N accumulation.

In Chapter 5 we tested the concept that under condition of high carbon supply, plants may increase the formation of their secondary metabolites, like phenolic compounds. In field experiments crop load was manipulated by applying flower or fruit thinning at different stages of development and at different severity. At a low crop load, fruit weight, soluble solids, acidity and firmness were significantly higher than at high and moderate loads. However, the concentrations of flavonoid and chlorogenic acid were similar at the different levels of crop load. Time of thinning had no significant influence on the concentration of flavonoids and chlorogenic acid in fruit skin and had no further effect on fruit quality characteristics such as weight, soluble solids, acidity and firmness. Removal of only the interior fruits (about one-third of total fruit) at about 4 weeks before expected commercial harvest had no influence on the concentration of flavonoids and chlorogenic acid or on the quality characteristics of the remaining exterior fruits of either 'Elstar' or 'Jonagold'. The results indicate that, within the 'normal' range of conditions, assimilate availability is not a major regulatory factor in flavonoids and chlorogenic acid formation in apples. These results are in agreement with the lack of any influence of the supply of precursors in the orchard (Chapter 7).

In Chapter 6 the changes that take place in the concentration and the amount of individual flavonoids and chlorogenic acid in the skin of 'Elstar' and 'Jonagold' apples during development and ripening were investigated. In both cultivars, the concentration on a dry weight basis of quercetin glycosides, phloridzin and chlorogenic acid was highest early in the season but decreased at different rates during fruit development to reach a steady level during maturation and ripening. Catechins (catechin plus epicatechin) concentration showed a similar pattern, but a temporary increase was observed in an early stage of development. The concentration of cyanidin 3-galactoside (anthocyanin) was relatively high early in the season, gradually decreased to a very low steady level during growth, but started to increase near

maturation, especially in the outer fruit. On a fruit basis the amount of quercetin glycosides increased during development and was about two times higher in 'Jonagold' compared to 'Elstar', both in outer and inner fruit. These compounds were the most abundant flavonoids in the skin of both cultivars and their accumulation showed a strong dependency on fruit position on tree. The amount of the second most abundant flavonoid type, catechins, increased during development to a maximum and then showed some decrease by mid season which was independent of fruit position on tree. The amount of phloridzin increased only early in the season reaching a steady level during development and ripening, and was independent of fruit position on tree. The amount of chlorogenic acid in both cultivars initially increased, but subsequently decreased to reach a low steady level and was slightly higher in outer than in inner fruit. The latter phenomenon is the only direct evidence for (net) breakdown of any of the studied phenolics. Although anthocyanin concentration was relatively high at early stages of development, significant accumulation on a fruit basis only occurred during maturation and ripening. The accumulation of anthocyanin, similar to that of quercetin glycosides, showed a strong dependency on fruit position on tree. The results indicate that, in general, the overall production of total flavonoids, with the exception of anthocyanin, and chlorogenic acid in apple skin is completed during fruit development before the onset of maturation.

Chapter 7 reports the influence of exogenous application of a number of chemicals that are precursors of flavonoids or are known to affect ripening on the accumulation of flavonoids and chlorogenic acid in 'Jonagold' apple skin with emphasis on anthocyanin. One aim was to identify a possible substrate limitation and another to separate the formation of anthocyanin from other related maturity/ripening events. Since the occurrence of the second peak in anthocyanin formation more or less parallels the maturation and ripening phase (like starch degradation and aroma production), anthocyanin formation itself is often considered as a ripening phenomenon triggered by ethylene. Our results suggest, however, that there is no simple relation to ripening and consequently to ethylene production (though we did not measure ethylene). This is concluded from the promotion of anthocyanin formation by ethephon (an ethylene releasing compound) and the retardation of anthocyanin formation by ABG and GA_3 (known to lower or counteract endogenous ethylene), without significantly altering starch degradation and changes in streif index (combination of starch index, firmness and sugar concentration). Our results have also shown that the other flavonoid classes quercetin 3-glycosides, catechins and phloridzin and chlorogenic acid do not respond to any of the applied chemicals. It is concluded that anthocyanin formation is dependent on developmental signals and independent of both fruit maturity/ripening and of the synthesis of other flavonoid classes and responds in a complicated way to ethylene.

In Chapter 8 the changes in individual flavonoids and chlorogenic acid during regular (RS) or ultra low oxygen (ULO) storage conditions at 1°C are reported in both 'Jonagold' and 'Elstar' apples. It could convincingly be shown that during storage of both 'Jonagold' (3, 6 and 8 months) and of 'Elstar' (2, 4 and 6 months) and during 1 or 2 weeks shelf life, the concentrations of cyanidin 3-galactoside and quercetin glycosides were relatively constant, while the concentrations of catechins, phloridzin and chlorogenic acid showed only minor decreases. Moreover there were no significant differences in the concentration of flavonoids

and chlorogenic acid between fruits stored under ULO compared to RS conditions. It is concluded that, following harvest, flavonoids present in apples are stable. There is no direct or indirect proof for breakdown (net metabolic turnover) during storage and shelf life.

In Chapter 9 the practical applications of the findings made in this study were discussed. Our results show that there is much room for increasing the level of potential health phytochemicals in apples. The first step would be cultivar selection either from already available genotypes or by developing new cultivars through classical breeding or molecular biology and gene technology. We showed that light has a significant impact on the final level of flavonoids in fruit. Therefore, the second and more proximate option would be the optimisation of light conditions within tree canopy by measures such as choice of root stocks, planting system, row orientation and training and pruning systems or covering the orchard floor with reflecting films (though the latter is not promoting the visual aspect of the orchard). A third step could be optimisation of the fertilization programme especially avoiding excess N and better timing of N-application. A further possibility is to sort fruit in healthiness classes. As long as a simple method to detect non-destructively quercetin 3-glycosides is lacking, sorting of fruit based on their blush might be a way to make healthiness classes, since blush is a good marker for exposure to light during growth and thus to some extent for the quercetin 3-glycosides level. Even when cultivar choice and cultivation methods succeed in getting high levels of flavonoids in fruit still the treatment by the consumer determines how much of these substances will be consumed. Many consumers still peel the fruit before consumption thereby removing almost all anthocyanin and quercetin 3-glycosides (Chapter 2). Promotion of fruit on the basis of healthiness is, in our opinion, however, only useful if it is accompanied with a guarantee of absence of pesticides, as is most credible, at least to the public, in organic farming.

Because of the large influence of a number of factors at several steps of the production chain, a quantitative model e.g. integrated with light distribution models, would offer a practical and effective tool for estimating the effect of certain measures and to predict and maximise the final level of healthy compounds in apples enabling the development of more accurate intake data and dietary recommendations.

Samenvatting

Bij de productie, bewaring en afzet van vers fruit komt het er uiteindelijk op aan om aan de wensen van de consument te voldoen. De aantrekkingskracht van fruit voor potentiële kopers wordt 1 bepaald door uiterlijke eigenschappen, zoals kleur, en door innerlijke eigenschappen, zoals stoffen die de gezondheid bevorderen. Flavonoiden en derivaten van hydroxycinnamonzuur, plantenstoffen uit de zogenaamde secundaire stofwisseling, leveren een belangrijke bijdrage aan beide genoemde eigenschappen en het is daarom de moeite waard om factoren te bestuderen die deze stoffen beïnvloeden met het doel om kleur- en gezondheidseigenschappen van appelen verder te verbeteren. Secundaire plantenstoffen zijn wijd verbreid in het plantenrijk en omvatten een grote groep van natuurlijke antioxydanten die via groente en fruit in het menselijk dieet terecht komen. Het is zeer waarschijnlijk dat deze antioxydanten een belangrijke rol spelen in het bevorderen van de gezondheid en het voorkomen van ziekten. Recent onderzoek heeft aangetoond dat het grootste deel van de antioxydantwerking van fruit en groente waarschijnlijk afkomstig is van flavonoiden en andere fenolen. Appelen zijn rijk aan flavonoiden zoals flavonolen (quercetin-3-glycosydes), flavanolen (catechine, epicatechine, gallocatechine en procyanidine en diens polymeren), dihydrochalcone-(floritine-glucoside [floridzine] en floritine-xyloglucoside) en cyanidine-3-glycoside (anthocyaninen). Appelen bevatten ook aanzienlijke hoeveelheden derivaten van hydrocinnamonzuur, hoofdzakelijk chlorogeenzuur. De rode kleur van appelen wordt vooral veroorzaakt door anthocyanen, flavonoid pigmenten die zich in de vacuole bevinden. Ondanks het kennelijke belang van flavonoiden voor de kwaliteit van fruit is er weinig bekend van de regulatie van deze groep stoffen in fruit. Het doel van het werk dat in dit proefschrift is beschreven, is daarom het verwerven van meer inzicht in de natuurlijke variatie in gehalten van deze stoffen in appelen, met name in de schil, de vorming van deze stoffen tijdens de ontwikkeling van de vrucht, en de mogelijkheden om het niveau van deze stoffen vooral tijdens de teelt te beïnvloeden.

In hoofdstuk 1 wordt een overzicht gegeven van de literatuur. Daaruit komt naar voren dat de vorming van flavonoiden en fenolzuren in planten wordt beïnvloed door een groot aantal interne en externe factoren.

In hoofdstuk 2 wordt de natuurlijke variatie in gehalten aan flavonoiden en chlorogeenzuur beschreven voor zover die te maken heeft met plaats in de vrucht (schil, of vruchtvlees), verschillen tussen standaardrassen en hun mutanten, de plaats van de vrucht in de boom en de verschillen tussen boomgaarden. Er werd een aanzienlijke variatie aangetroffen. Het gehalte aan specifieke flavonoiden en aan chlorogeenzuur bleek af te hangen van het type weefsel. Quercitine-3-glycosiden en anthocyaan werden vrijwel uitsluitend in de schil aangetroffen en in veel hogere concentraties aan de 'zonkant' dan aan de 'schaduwkant' van de vrucht. floridzine, catechinen en chlorogeenzuur, daarentegen, werden zowel in schil als vruchtvlees aangetroffen en in de schil evenveel in de zon- als de schaduwkant van de vrucht. Jonagold-appels bevatten een 15-20% hogere concentratie en een 30-40% grotere hoeveelheid aan flavonoiden en chlorogeenzuur dan die van Elstar-appels (zie ook H 6). Daarentegen bevatten Elstar-appels een 40% hogere concentratie van quercetin-3-

rhamnoglucoside en een 60% hogere concentratie van quercetin-3-glucosides dan Jonagold-appels. Dit is relevant omdat verschillende flavonoidverbindingen verschillen in bioactiviteit en antioxydantcapaciteit. Deze resultaten vormen een aanwijzing voor genetische verschillen tussen rassen en bieden perspectieven voor veredeling gericht op hogere antioxydantactiviteit van appelrassen. Verschillen tussen standaardrassen en kleurmutanten binnen één ras (Jonagold-Jonaprince; Elstar-Elshof) berusten waarschijnlijk uitsluitend op een (meestal) sterke verhoging van het anthocyaangehalte zonder enige invloed op de andere flavonoidklassen. Uit anatomisch onderzoek bleek dat de kleurmutant Red Jonaprince zowel meer roodgekleurde cellen per cellaag als meer cellagen met roodgekleurde cellen bezit. Frappant is de scherpe scheiding tussen onmiddellijk aangrenzende cellen met en zonder roodgekleurde vacuoles. De gedachte dringt zich op dat er ook mutanten kunnen bestaan met en hoger gehalte aan b.v. quercetin-3-glycosiden. Het is echter evident dat de herkenning van zulke mutanten een veel groter probleem is dan bij gebloste mutanten. De gehalten aan anthocyaan, quercetin-3-glycosiden en totaal-flavonoiden waren het hoogst in vruchten afkomstig uit de top van de boom en vervolgens in vruchten uit de buitenzijde van de boom. De laagste gehalten werden gevonden in vruchten afkomstig uit het midden van de boomkroon. Uit onderzoek naar de invloed van het type vruchthout bleek dat terminale vruchten van het éénjarige hout hogere gehalten aan flavonoiden en van catechinen hadden dan laterale vruchten van het éénjarige hout en dan vruchten van de kortloten. Gehalten aan floridzine en chlorogeenzuur bleken niet af te hangen van type vruchthout of van de positie in de boom. Gehalten in appelen uit de top van de boom waren voor quercitine-3-glycosiden maximaal het drievoudige en voor totaal flavonoiden maximaal het tweevoudige van die in appelen uit de binnenzijde van de boomkroon. Tussen twee normaal producerende boomgaarden, die alleen duidelijk verschilden in groeikracht en inwendige beschaduwing, werden duidelijke verschillen gevonden in flavonoid- en chlorogeenzuurgehalte van Elstar-appels. Al deze resultaten wijzen in de richting van licht als de belangrijkste externe factor voor de biosynthese van flavonoiden in appelen.

Hoofdstuk 3 is gewijd aan een analyse van de lichtverdeling in de kroon van appelbomen in relatie tot het gehalte aan flavonoiden en chlorogeenzuur in appelen. Er bleek een directe relatie te zijn tussen de concentratie van cyanidine-3-galactoside (anthocyaan) en quercitine-3-glycosiden en het percentage roodgeblost oppervlak van de vrucht enerzijds en het lichtniveau in de directe nabijheid van de vrucht anderzijds. Vergeleken met andere posities bezat licht in de binnenzijde van de boomkroon minder van de componenten ultraviolet (UV-A), blauw, groen en rood (R) maar meer verrood (VR), zodat de VR/R verhouding (van grote invloed op formatieve processen) hier veel hoger was. Boven een kritische drempelwaarde van de VR/R verhouding van 1 werden geen anthocyaan en weinig quercitine-3-glycosiden aangetroffen.

Hoofdstuk 4 is gewijd aan de relatie van de plantenvoedingsstoffen N, P, K, Ca en Mg in de vrucht met gehalten van flavonoiden en chlorogeenzuur in de vruchtschil. In een experiment met de Elstar-mutant 'Elshof' waarbij de vijf voedingsstoffen op vijf niveaus in vier herhalingen werden toegediend, werden alleen het N- en het Ca-gehalte duidelijk beïnvloed. Er was niettemin tussen alle objecten voldoende variatie in de gehalten in de

vrucht van de andere nutriënten om de correlatie tussen de concentratie van elk van de genoemde nutriënten met die van flavonoiden en chlorogeenzuur te kunnen bestuderen. In de meeste gevallen werd een negatieve correlatie gevonden tussen de gehalten aan N, Mg en de N/Ca-verhouding gedurende de ontwikkeling in de vrucht en de gehalten aan anthocyaan en flavonoiden bij de pluk in 1996, 1997 en 1998. In 1997 waren deze correlaties relatief zwak maar wel significant. In dat jaar vertoonden ook de P- en de K-concentratie, maar niet de Ca-concentratie, een negatieve correlatie met het gehalte aan anthocyaan en totaal flavonoiden. In een andere proef in 1996 met standaard Elstar werd gebruik gemaakt van de variatie in nutriëntgehalten in de vrucht tengevolge van verschillen in positie in de boom. De gehalten aan N, K en de N/Ca verhouding bij pluk waren negatief gecorreleerd, en die van Ca positief, met de anthocyaan- en totaal flavonoidgehalten op dat tijdstip. Mg-gehalte vertoonde alleen een (negatieve) correlatie met het anthocyaangehalte. Door de verschillen in positie van de vruchten in de boom is er wel interactie met de invloed van licht te verwachten. Multipele regressiemodellen, met hoofdzakelijk N als factor, verklaren in de proeven met de mutant 'Elshof' tot 40% van de variatie in anthocyaan- en tot 30% van de variatie in totaal flavonoidgehalte en in het experiment met standaard Elstar tot 70% van de variatie in anthocyaan en tot 65% van de variatie in totaal flavonoidgehalten. De relatie tussen nutriënten en chlorogeenzuur in appelen was niet consistent en verder onderzoek is op dit punt aan te bevelen. De conclusie is gerechtvaardigd dat de concentratie aan flavonoiden in de schil van appelen behalve door licht verder kan worden verhoogd door optimalisering van de voeding, met name door maatregelen die (te) hoge N-gehalten voorkomen.

In hoofdstuk 5 is de veronderstelling getoetst dat de synthese van fenolen, met name flavonoiden, beperkt wordt door de beschikbaarheid van assimilaten. In een experiment met Elstar werd de vruchtdracht gemanipuleerd door gedurende de eerste negen weken na bloei op verschillende tijdstippen in verschillende mate bloem- of vruchtdunning toe te passen. In de objecten met lage vruchtdracht waren gehalten aan suikers en zuur en de hardheid duidelijk hoger dan in de objecten met normale of hoge vruchtdracht. De concentraties aan flavonoiden en chlorogeenzuur in de schil waren in al deze objecten niet verschillend. Ook het tijdstip van dunnen leidde niet tot verschillen noch in de concentraties van flavonoiden en chlorogeenzuur, noch in vruchtkenmerken van het fruit als gewicht, suikergehalte, zuurgehalte en hardheid. In een aparte proef met Elstar en Jonagold bleek verwijderen van alle vruchten uit de binnenzijde van de boom, ongeveer 30% van het totale aantal, één maand vóór de pluk, evenmin invloed te hebben op de concentraties van flavonoiden en chlorogeenzuur of op de vruchtkenmerken van de resterende vruchten. Deze resultaten tonen aan dat beschikbaarheid van assimilaten geen grote rol speelt in de vorming van flavonoiden en chlorogeenzuur in de schil van appelen, althans binnen de hier gestelde grenzen. Deze resultaten worden ondersteund door het feit dat toediening in de boomgaard van voorlopers van flavonoiden geen enkel invloed had (zie H 7).

In hoofdstuk 6 staan de veranderingen centraal die plaats vinden in concentratie en hoeveelheid flavonoiden en chlorogeenzuur in de schil van Elstar- en Jonagold-appels tijdens ontwikkeling en de rijping van de vrucht. In beide cultivars waren de concentraties van quercetin-3-glycosiden, floridzine en chlorogeenzuur, op basis van drooggewicht, het hoogst

in de beginfase van de ontwikkeling, en daalden deze – zij het met verschillende snelheid – gedurende de ontwikkeling van de vrucht om een stabiel niveau te bereiken gedurende de laatste weken vóór de pluk inclusief de rijping. De concentraties van catechinen (catechine en epicatechine) lieten ook een dergelijk patroon zien maar hier trad in een vroeg stadium een tijdelijke piek in de concentraties op. De cyanidine-3-galactosideconcentratie (anthocyaan) was relatief hoog aan het begin van de ontwikkeling, daalde vervolgens tot een zeer laag niveau maar vertoonde weer een duidelijke stijging aan het einde van de vruchtontwikkeling. De hoeveelheid quercetine per vrucht nam gedurende de gehele ontwikkeling van de vrucht toe en was ongeveer tweemaal zo hoog in Jonagold als in Elstar, zowel in vruchten afkomstig van de buitenzijde als van binnenzijde van de boomkroon. Quercetinen maakten bij beide cultivars het grootste aandeel uit van flavonoiden in de schil en waren sterk afhankelijk van de positie in de boom. Catechinen, kwantitatief op de tweede plaats, vertoonden aanvankelijk een toename in totale hoeveelheid, maar namen na het midden van het seizoen weer wat in hoeveelheid af. Catechinen werden niet beïnvloed door de positie aan de boom. De hoeveelheid floridzine per vrucht nam in beide cultivars alleen vroeg in het seizoen toe, bleef verder constant en was onafhankelijk van de positie in de boom. De hoeveelheid chlorogeenzuur per vrucht nam in beide cultivars aanvankelijk toe maar daalde vervolgens tot een stabiel lager niveau dat enigszins hoger was in vruchten aan de buitenkant van de boom dan uit de binnenzijde. De afname in hoeveelheid tijdens de ontwikkeling is alleen voor chlorogeenzuur gevonden en is de enige aanwijzing voor (netto) afbraak van fenolen. Hoewel de anthocyaanconcentratie in een vroeg stadium relatief hoog was, trad pas aan het eind van de ontwikkeling, tijdens de rijping, een duidelijke accumulatie op. Deze ophoping vertoonde, net als die van quercetine glycosiden, een sterke afhankelijkheid van positie aan de boom. Deze resultaten tonen aan dat, in het algemeen gesproken, met uitzondering van anthocyaan, de productie van flavonoiden en chlorogeenzuur in de schil tot stilstand komt vóór het begin van de rijping.

In hoofdstuk 7 worden de resultaten behandeld van een studie naar de invloed van toediening van chemicaliën die ofwel kunnen dienen als voorloper van flavonoiden, of die de rijping van de vrucht beïnvloeden, op de ophoping van flavonoiden en chlorogeenzuur in de schil van Jonagold-appels. Doel daarvan was om een mogelijke substraatbeperking te identificeren en om anthocyaanvorming te onderscheiden van rijpingsverschijnselen. Omdat de tweede piek in anthocyaanproductie samenvalt met volgroeien en rijpen van de vrucht zoals gekarakteriseerd door zetmeelafbraak en aromaproductie, wordt blosvorming zelf ook vaak als een rijpingsproces opgevat dat wordt aangezet door ethyleen. Onze resultaten laten echter zien dat er geen eenvoudige relatie is tussen rijping, dus waarschijnlijk ook niet met de ethyleenproductie (hoewel niet door ons gemeten), en anthocyaanvorming. Deze conclusie volgt uit de waarneming dat anthocyaanvorming wordt gestimuleerd door ethephon (een bron van ethyleen) en wordt geremd door gibberellinezuur (GA_3) en ABG (een analoog van AVG, bekend als ethyleenremmer) terwijl een aantal rijpingsindicatoren in de vrucht, zoals zetmeelafbraak en Streifindex (een combinatie van zetmeelafbraak, suikergehalte en hardheid) niet werden beïnvloed. De andere flavonoidgroepen, quercetin-3-glycosiden, catechinen, floridzine en chlorogeenzuur, werden niet beïnvloed door de toegediende

chemicaliën. Geconcludeerd wordt dat anthocyaanvorming enerzijds bepaald wordt door het ontwikkelingsstadium maar anderzijds enigszins los kan staan van rijping en onafhankelijk geschied van de vorming van andere flavonoiden. Er is een ingewikkelde relatie met ethyleen.

Hoofdstuk 8 is gewijd aan de veranderingen in gehalten aan flavonoiden en chlorogeenzuur in vruchten van Elstar en Jonagold tengevolge van bewaring gedurende een aantal maanden in gewone koeling of in een zogenaamde ULO-regiem (Ultra Low Oxygen). Duidelijk bleek dat gedurende bewaring van Jonagold (3, 6 en 8 maanden) en Elstar (2, 4 en 6 maanden) en gedurende daaropvolgende 'uitstalling' van 1 respectievelijk 2 weken de concentraties van cyanidine-3-galcatoside en quercetine glycosiden min of meer constant bleven terwijl de concentraties van catechinen, floridzine en chlorogeenzuur slechts een geringe daling vertoonden. Bovendien waren deze resultaten gelijk voor beide typen bewaring. Hieruit mag worden geconcludeerd dat na de oogst flavonoiden in de vrucht stabiel blijven. Er zijn dus geen directe of indirecte aanwijzingen voor het optreden van afbraak van flavonoiden in deze fase.

Hoofdstuk 9 bevat een bespreking van de praktische toepassingen van de resultaten van het gepresenteerde onderzoek. Het blijkt dat er veel mogelijkheden zijn om het gehalte aan potentiële gezondheidsstoffen in appels te verhogen. De verschillen tussen rassen tonen aan dat veredeling en selectie goede perspectieven bieden, mogelijk via een herevaluatie van bestaande rassen, maar meer door het ontwikkelen van nieuwe rassen via klassieke veredeling of met toepassing van gentechnologie. De omgevingsfactor licht blijkt een duidelijke invloed te hebben op het gehalte aan flavonoiden in de vrucht. Een tweede mogelijkheid, die dichter bij de directe praktijk ligt, is daarom het optimaliseren van de lichtcondities in de boom door maatregelen zoals de keuze van de onderstam, het plantsysteem, de rij-oriëntatie, de boomvorm, de snoeiwijze en mogelijk ook de toepassing van reflecterende folie op de grond (hoewel de laatste maatregel niet direct omgevingsvriendelijk is). Een derde maatregel, die ook binnen het bereik van de teler ligt, is het optimaliseren van de voedingstoestand van de boom. Met name het vermijden van hogere N-giften en een mogelijk een betere 'timing' van de N-bemesting kunnen het gehalte aan flavonoiden verder bevorderen ten opzichte van de huidige situatie. Als vierde mogelijkheid kan worden gedacht aan het sorteren van fruit in gezondheidsklassen. Zolang geen eenvoudige methode beschikbaar is om bijvoorbeeld niet-destructief op quercetinen te sorteren, is sorteren op percentage geblost oppervlak een redelijke vervanger omdat de rode blos een maat is voor de blootstelling aan licht en zo ook tot op zekere hoogte voor het gehalte aan het quercetine-3-glycoside gehalte.

Echter, wanneer door al deze maatregelen een aanzienlijke verhoging van de gehalte aan gezondheidsbevorderende stoffen zou worden bereikt, hangt het nog van de consument af of deze stoffen ook daadwerkelijk worden geconsumeerd. Veel consumenten hebben nog steeds de gewoonte om het fruit te schillen vóór consumptie waardoor alle anthocyaan (een krachtige antioxydant) en vrijwel alle quercetine-3-glycosiden worden verwijderd. Een publiciteitscampagne gericht op de gezondheid van fruit, op basis van het gehalte aan gezondheidsstoffen, lijkt zinvol, maar ons inziens alleen als deze samengaat met een garantie van afwezigheid van residuen van gewasbeschermingsmiddelen. Vanwege de grote invloed van verschillende factoren op het productieproces van flavonoiden, met name de quercetine-

3-glycosiden, zou een kwantitatief model, bijvoorbeeld geïntegreerd met een lichtverdelingsmodel, van praktische waarde zijn om het effect van bepaalde maatregelen in te schatten en het uiteindelijke gehalte aan gezondheidsbevorderende stoffen te voorspellen en te maximaliseren.

Curriculum Vitae

The author of this dissertation, Mohamed Abdel-Ghani Awad, was born in Bani-ebiad, Dekernis, Egypt on 24 January 1967. After finishing high school examination in 1984, he joined the Faculty of Agriculture, Mansoura, University. He obtained his B.Sc. degree in Agriculture Sciences (Plant Production Section) in June 1988 with general grade "Excellent with Honour Degree". A few months later, he was asked by the Horticulture Department of that faculty to work as a teaching assistant. After joining the army for a period of 14 months, starting in October 1988, he took the responsibility of doing research and teaching within this department. In 1991, he started his M.Sc. study in Pomology within the Horticulture Department.

In September 1991, he got a scholarship from NUFFIC (Netherlands Organisation for International Co-operation in Higher Education) to study in the field of post-harvest fruit quality at the Fruit Research Station in Wilhelminadorp, The Netherlands. During the period from September 1991 till October 1993, he did the experimental research work required for his M.Sc. degree in Wilhelminadorp under the supervision of Dr. A. de Jager. In October 1994, he obtained his M.Sc. degree in Pomology from Mansoura University. His M.Sc. titled: Physiological Studies on Superficial Scald in Jonagold apple. In October 1995, he got a scholarship from the Egyptian Ministry of Higher Education to study in The Netherlands for his Ph.D. In September 1996, he arrived for a second time to The Netherlands and started his Ph.D. work at the Fruit Research Station in Wilhelminadorp/Randwijk and Wageningen University. During his M.Sc. and Ph.D. work he published a list of 8 full papers. He was also participated in several international symposia and submitted a number of abstracts. Currently he is one of the staff members of the Department of Pomology, Faculty of Agriculture, Mansoura University.

His Address in Egypt is:
M. A. Awad
Department of Pomology
Faculty of Agriculture
Mansoura University
El-Mansoura/Egypt
Fax 20-50347900

Publications

FULL PAPERS

Awad, M.A., de Jager, A., Van Westing, L.M., 2000. Flavonoid and chlorogenic acid levels in apple fruit: characterisation of variation. *Sci. Hortic.* 83, 249-263.

Awad, M.A., de Jager, A., 2000. Flavonoid and chlorogenic acid levels in skin of Jonagold and Elstar apples during and after regular and ultra low oxygen storage. *Postharvest Biol. Technol.* 20, 15-24.

Awad, M.A., Wagenmakers, P.S., de Jager, A., 2001. Effects of light on flavonoid and chlorogenic acid levels in the skin of Jonagold apples. *Sci. Hortic.* 88, 289-298.

Awad, M.A., de Jager, A., van der Plas, L.H.W., van der Krol, A.R., 2001. Flavonoid and chlorogenic acid changes in skin of Elstar and Jonagold apples during development and ripening. *Sci. Hortic. In Press.*

Awad, M.A., de Jager, A., Dekker, M., Jongen, W.M.F., 2001. Formation of flavonoids and chlorogenic acid in apples as affected by crop load. *Sci. Hortic. In Press.*

Awad, M.A., de Jager, A., 2001. Relations between fruit nutrients and the concentration of flavonoids and chlorogenic acid in Elstar apple skin. *Sci. Hortic. In Press.*

Awad, M.A., de Jager, A., 2001. Formation of flavonoids, especially anthocyanin, and chlorogenic acid in Jonagold apple skin: influences of growth regulators and fruit maturity. *Sci. Hortic. In Press.*

Awad, M.A., de Jager, A., Roelofs, F.P., Scholtens, A., 1993. Superficial scald in Jonagold apples as affected by harvest date and storage conditions. *Acta Hortic.* 326. 245-249.

Awad, M.A., 1994. Physiological studies on superficial scald in Jonagold apple. *M.Sc. thesis, 138 pp.* Faculty of Agriculture, Mansoura University, El-Mansoura, Egypt.

Jager, A.de., Awad, M., Westing, L. van., 1997. New FPO-research on colour and health-substances in apples: the best of the apple is often thrown away. *Fruitteelt (Den Haag),* 87 (27) 14-15. (In Dutch).

Awad, M., de Jager, A., 1998. Make the most of fruit quality. *Fruitteelt (Den Haag),* 88 (7) 14-15.(In Dutch).

De Jager, A; Awad M. A; Roelofs, F.P.M., 1992. Voorspelling van het risico op scald in daarvoor gevoelige. *Annual Report of PFW.* 116-119 (In Dutch).

De Jager, A; M.A. Awad, 1992. Scald gronding aangepakt. *Groenten + Fruitteelt,* 29, 6-8 (In Dutch).

Roelofs, F.P.M., de Jager, A., Awad, M.A., Scholtens, A., 1993. Scald is vrijwel helmaal te voorkomen. *Fruitteelt (Den Haag),* 83 (3) 14-15 (In Dutch).

ABSTRACTS

Awad, M.A., de Jager, A., Dekker, M., Jongen, W., van der Krol, A., van der Plas, L., 2000. Flavonoids and chlorogenic acid in apples as affected by mineral nutrition and crop

load. In: Martens, S; Treutter, D; Forkmann, G., eds. Polyphenols communications 2000. XXth International conference on polyphenols. Freising-Weihenstephan (Germany), September 11-15, 2000, pp 657-658.

Awad, M.A., Jongen, W., van der Plas, L., de Jager, A., 2000. Control of the content of flavonoids in apples as quality factors for colour and healthiness. Abstract book, Food science study tour 2000, Denmark-Sweden, pp 12.

Roelofs, F.P.M., de Jager, A., Awad, M.A., Scholtens, A., 1993. The effect of oxygen and carbon dioxide concentration on the incidence of scald in Jonagold apple. PostHarvest 93, International symposium. Kecskemet, Hungary, 30th August – 3rd September.

Awad, M.A., de Jager, A., Roelofs, F.P.M., 1993. Prediction and control of scald in Jonagold apple. International symposium on the quality of fruit and vegetables: the influence of pre-and post-harvest factors and technology. Kreata, Greece, 20–24 September.

الثمار مثل مؤشر النشا " Starch index " أو التغيرات في مؤشر Streif index (معادلة تشمل كمية النشا وتركيز السكريات ودرجة الصلابة). وهذه النتائج تدل على أن عملية تخليق الأنثوسيانين أكثر حساسية للايثيلين مقارنة بعمليات النضج الأخرى. وعلى الجانب الآخر لم تؤثر الكيماويات المستخدمة على تركيز باقي أنواع الفلافونات الأخرى أو حمض الكلوروجينيك. والخلاصة أن عملية تخليق الأنثوسيانين تعتمد على إشارة معينة مرتبطة بالنمو ولكنها لا تعتمد على اكتمال النمو والنضج أو على تخليق باقي مجموعات الفلافونات الأخرى ولكنها تستجيب للايثيلين بطريقة معقدة.

الفصل الثامن:

وفيه تم دراسة التغيرات التي تحدث في تركيز الفلافونات وحمض الكلوروجينيك في قشرة ثمار التفاح "الالستار" و"الجوناجولد" أثناء التخزين البارد العادي أو تحت ظروف منخفضة جدا من الأكسيجين على درجة حرارة 1 درجة مؤوية ولمدد مختلفة. وكذلك تم دراسة التغيرات التي تحدث في تركيز هذه المركبات على درجة حرارة الغرفة خلال فترة أسبوعين بعد التخزين كمدة محاكية لعملية التسويق. ولقد أظهرت النتائج أن مركبات الكويرزاتين جلايكوزيد 3 والأنثوسيانين كانت ثابتة خلال فترات التخزين المختلفة بينما أظهر تركيز الكاتيكينز والفلوريديزين وحمض الكلوروجينيك انخفاضا قليلا فقط. وعلاوة على ذلك لم يلاحظ أي فرق معنوي في مستوى هذه المركبات بين ظروف التخزين المختلفة. والخلاصة هي أن الفلافونات وحمض الكلوروجينيك كانت ثابتة أثناء التخزين ولم توجد أي مؤشرات مباشرة أو غير مباشرة تدل على حدوث هدم لهذه المركبات أثناء أو بعد التخزين.

الفصل التاسع:

في هذا الفصل تم مناقشة النتائج المعروضة في الفصول السابقة في إطار عام مع إيضاح ومناقشة الجوانب التطبيقية للنتائج المتحصل عليها. فقد ظهر أن هناك مساحة كبيرة لزيادة وتحسين مستوى المركبات الصحية في ثمار التفاح وذلك من خلال تطبيق عددا من الخطوات.

وأول هذه الخطوات هي اختيار الصنف المناسب سواء من بين الأصناف أو السلالات المتوفرة حاليا أو من خلال تطوير أصناف جديدة عن طريق اتباع برامج التربية التقليدية أو من خلال استخدام البيولوجيا الجزئية وتكنولوجيا الجينات. ونظرا لأن العوامل البيئية لها تأثير قوى على تحديد مستوى الفلافونات في ثمار التفاح، فإن الخطوة التالية تكون من خلال توفير ظروف النمو وتطبيق المعاملات الزراعية المناسبة وخاصة تلك التي تعمل على تحسين الظروف الضوئية داخل هيكل الشجرة مثل استخدام الأصول المقصرة للتطعيم، استخدام نظام الزراعة الأمثل، و تطبيق نظام التربية والتقليم الأمثل، أو قد نلجأ إلى تغطية أرض البستان تحت الأشجار بأغطية بلاستيكية عاكسة للضوء. ويعتبر تطوير برامج التسميد وبصفة خاصة تجنب الإضافة الزائدة للنيتروجين مع اختيار الوقت المناسب للإضافة من الخطوات المهمة. هذا ويمكن فرز الثمار عند الحصاد أو فيما بعد اعتمادا على نسبة اللون الأحمر بها وبالتالي يمكن تحديد درجات صحية مختلفة للثمار وذلك لأن نسبة اللون الأحمر تدل على درجة تعرض الثمار للضوء خلال موسم النمو وبالتالي إلى حد ما تعكس محتوى الثمار من مركبات الكويرزاتين جلايكوزيد 3.

وفى النهاية فإن طريقة الاستهلاك لثمار التفاح تبقى ذات أهمية قصوى في تحديد الكمية النهائية التي يمكن أن يحصل عليها الفرد من هذه المركبات الصحية. ويلاحظ أن هناك نسبة كبيرة من المستهلكين تقوم بتقشير الثمار قبل تناولها مما يعنى فقدانهم إلى كل الأنثوسيانين ومركبات الكويرزاتين جلايكوزيد 3 ونسبة كبيرة من مركبات الكاتيكينز والفلوريديزين وحمض الكلوروجينيك. ولكن على الجانب الآخر يجب تأمين خلو الثمار عموما من المبيدات الضارة وذلك عن طريق تشجيع طرق الإنتاج الأكثر أمنا مثل الزراعة البيولوجية لزيادة وتشجيع استهلاك ثمار الفاكهة.

وبصفة عامة فنظرا لوجود مصادر عديدة للتباين في مستوى الفلافونات وحمض الكلوروجينيك في قشرة ثمار التفاح خلال مراحل الإنتاج المختلفة فإن العمل على تطوير نموذج رياضي من الممكن أن يستخدم كأداة للتقسيم الكمي للتباين إلى مصادره المختلفة وتحديد العلاقة بين هذه المصادر. وبتطوير هذا النموذج الرياضي يمكننا أن نتنبأ أو أن نحسن من مستوى الفلافونات والمركبات الصحية الأخرى في الثمار وبالتالي يمكننا كذلك وضع توصيات غذائية وصحية أكثر دقة للمستهلكين. ويمكن كذلك ربط هذا النموذج الرياضي مع النماذج التي تصف الاستغلال الأمثل للضوء وعلاقة ذلك بنواحي الإنتاج الكمي والكيفي لثمار الفاكهة عموما.

تم بحمد الله عز وجل

الفصل الخامس:

في هذا الفصل تم اختبار النظرية التالية: انه تحت ظروف توفر الكربوهيدرات ونواتج التمثيل الضوئي فان الأشجار ربما تزيد من تكوين الفلافونات كطريقة مثلى لتخزين الكربون الزائد. في عددا من التجارب الحقلية تم تعديل معدل حمل الثمار على الأشجار عن طريق خف الأزهار أو الثمار في أوقات ودرجات مختلفة. ولقد ظهر أنة عند مستوى الحمل المنخفض فان وزن الثمار ونسبة المواد الصلبة الذائبة والحموضة وصلابة الثمار كانت أعلى معنويا مقارنة بالحمل العادي أو المتوسط. ولكن على الرغم من ذلك لم يتأثر تركيز الفلافونات وحمض الكلوروجينيك في قشرة الثمار بمستوى الحمل. لم يكن هناك أي تأثير معنوي لوقت إجراء عملية الخف سواء على خواص جودة الثمار أو على تركيز الفلافونات وحمض الكلوروجينيك في قشرة الثمار. إزالة كل الثمار الداخلية (تشكل حوالي ثلث الثمار الكلية على الشجرة) عند حوالي 4 أسابيع من الموعد المتوقع للحصاد التجاري لم يؤثر على تركيز الفلافونات وحمض الكلوروجينيك أو خواص الجودة للثمار الخارجية المتبقية على الأشجار لكلا الصنفين "الاستار" و"الجوناجولد". والخلاصة هي أن مدى توفر الكربوهيدرات أو نواتج التمثيل الضوئي(assimilates) ليس عاملا رئيسيا في تكوين الفلافونات وحمض الكلوروجينيك في قشرة ثمار التفاح. وهذه النتائج تتفق مع عدم وجود أى تأثير لرش الثمار أثناء النمو بحمض "الشيكيميك" أو سكر الجلاكتوز كمصادر للتخليق الحيوي للفلافونات (انظر الفصل السابع).

الفصل السادس:

وفيه تم دراسة التغيرات التي تحدث في تركيز وكمية الفلافونات وحمض الكلوروجينيك في قشرة ثمار التفاح "الاستار" و"الجوناجولد" أثناء مراحل النمو والنضج. في كلا الصنفين خلال مراحل النمو المبكرة كان تركيز مركبات الكويرزاتين جلايكوزيد 3 والفلوريديزين وحمض الكلوروجينيك أعلى ما يمكن ثم انخفض بمعدلات مختلفة خلال مراحل النمو التالية ليصل إلى مستوى ثابت خلال مرحلة اكتمال النمو والنضج. أظهر تركيز الكاتيكينز سلوكا مشابها ولكن ارتفع بدرجة ملحوظة ومؤقتة خلال مراحل النمو المبكرة. أبدى تركيز الأنثوسيانين ارتفاعا نسبيا في بداية موسم النمو ثم انخفض تدريجيا الي مستوى قريب من الصفر ليرتفع مرة أخرى وبشكل سريع بالقرب من مرحلة اكتمال النمو خاصة في الثمار الخارجية والمعرضة للضوء. وبحساب الكمية لكل ثمرة فان مركبات الكويرزاتين جلايكوزيد 3 أظهرت زيادة تدريجية خلال مراحل النمو واحتوت ثمار الصنف "الجوناجولد" على ضعف الكمية الموجودة في ثمار الصنف "الاستار". ومثلت هذه المركبات اكثر الفلافونات وجودا في قشرة ثمار كلا الصنفين وأظهرت اعتمادا كبيرا على موقع الثمار على الأشجار. وعلى العكس من ذلك فان كمية مركبات الكاتيكينز (تمثل ثاني أكثر الفلافونات) أظهرت ارتفاعا ملحوظا عند منتصف موسم النمو ولم تتأثر بموقع الثمار على الأشجار. أظهرت الفلوريديزين زيادة في الكمية فقط خلال مراحل النمو المبكرة ثم انخفض إلى مستوى ثابت خلال مراحل النمو التالية والنضج ولم يتأثر بموقع الثمار على الأشجار. أبدى حمض الكلوروجينيك ارتفاعا نسبيا في الكمية في بداية موسم النمو ثم انخفض تدريجيا إلى مستوى قليل وثابت بعد ذلك وكانت الكمية أكثر ارتفاعا في الثمار الخارجية مقارنة بالثمار الداخلية. وهذه الظاهرة تمثل دليلا على حدوث هدم لأحد المركبات الفينولية التي تم دراستها. على الرغم من أن تركيز الأنثوسيانين كان مرتفعا نسبيا خلال مراحل النمو المبكرة إلا أن التراكم في الكمية لكل ثمرة لم يحدث إلا خلال مرحلة اكتمال النمو والنضج حيث أظهرت اعتمادا كليا على موقع الثمار على الأشجار. والجدير بالذكر أن الزيادة في كمية الكويرزاتين جلايكوزيد 3 والأنثوسيانين خاصة في الثمار الخارجية لم تؤثر على مستوى مركبات الكاتيكينز والفلوريديزين وحمض الكلوروجينك في هذه الثمار مما يدل على عدم وجود تنافس بين هذه المركبات على مصادر التخليق الحيوي. وبصفة عامة أظهرت الدراسة أن الإنتاج العام لمجموع المركبات الفلافونية وحمض الكلوروجينيك في قشرة ثمار التفاح يكتمل خلال مراحل النمو وقبل النضج.

الفصل السابع:

في هذا الفصل تم دراسة تأثير بعض منظمات النمو (والتي تؤثر على عمليات اكتمال النمو النضج) وكذلك دراسة تأثير بعض الكيماويات الأخرى (والتي تمثل مصدرا للتخليق الحيوي للفلافونات) على تراكم الفلافونات و حمض الكلوروجينيك في قشرة ثمار الصنف "الجوناجولد". وكان الهدف الرئيسي لهذه الدراسة هو فصل عملية تخليق الأنثوسيانين عن باقي العمليات المرتبطة باكتمال النمو والنضج وكذلك معرفة أي إمكانية لوجود نقص في مصادر التخليق الحيوي للفلافونات وحمض الكلوروجينك. هذا ولقد أظهرت النتائج أن العلاقة بين عملية تخليق الأنثوسيانين والنضج والإيثيلين ليست علاقة بسيطة. حيث وجد أن " الإيثيفون" شجع تخليق الأنثوسيانين بدرجة كبيرة ولكن الـ "GA$_{4+7}$, GA$_3$, ABG" قللت تخليق الأنثوسيانين دون التأثير على عمليات نضج

ولقد ظهر أن الطفرات تختلف فقط في تركيز الأنثوسيانين دون المركبات ت الفلافونية الأخرى وحمض الكلوروجينيك مقارنة بالصنف الرئيسي. وأظهرت الدراسة الميكروسكوبية أن الطفرة الأكثر احمرارا من الناحية الظاهرية تحتوى كذلك على عدد أكبر من الخلايا المنتجة لصبغة الأنثوسيانين في طبقة القشرة وكذلك على عدد أكبر من الطبقات المحتوية على هذه الخلايا المنتجة لصبغة الأنثوسيانين مقارنة بالصنف الرئيسي. ومن الجدير بالذكر وجود الخلايا الملونة مجاورة للخلايا الغير ملونة ومعرضة لنفس ظروف الإضاءة. ونظرا لأن اختيار الطفرات يتم عموما اعتمادا على درجة اللون الأحمر فإنه يمكن الوضع في الاعتبار مستقبلا الاختيار على أساس محتوى الفلافونات خاصة وأن قياسها سهل نسبيا.

وجد أن تركيز مركبات الكويرزاتين جلايكوزيد 3 والأنثوسيانين وكذلك تركيز مجموع الفلافونات الكلية أعلى ما يمكن في قشرة الثمار الموجودة في قمة الشجرة ثم الثمار الموجودة على الأجزاء الخارجية للشجرة بينما كان التركيز أقل ما يمكن في قشرة الثمار الموجودة في الأجزاء الداخلية للشجرة . كذلك كان تركيز هذه المركبات بالإضافة إلى الكاتكينز أعلى في قشرة الثمار المحمولة قميا مقارنة بالثمار المحمولة جانبيا على خشب الحمل الحديث (أفرع حديثة عمرها عام) أو الثمار المحمولة على دوابر معمرة. لم يتأثر تركيز الفلوريزين وحمض الكلوروجينيك بموقع الثمار على الأشجار أو بنوع خشب الحمل. وبصفة عامة فإن أعلى فرق محتمل بناء على مقارنة الثمار الموجودة في قمة الشجرة (المعرضة لأفضل ظروف إضاءة) والثمار الموجودة في الأجزاء الداخلية للشجرة (المعرضة لأقل ظروف إضاءة) يصل إلى ثلاثة أضعاف مركبات الكويرزاتين جلايكوزيد 3 وضعفين لمجموع الفلافونات الكلية (الفصل الثانى والثالث). وجد اختلاف كبير في تركيز هذه المركبات بين بستانين متمرين بشكل طبيعي من الصنف "الاستار" ومختلفين بصورة رئيسية في درجة النمو الخضري للأشجار ودرجة التظليل. كل هذه النتائج السابقة توضح مدى أهمية الضوء كعامل رئيسي في التخليق الحيوي للفلافونات.

الفصل الثالث:

وفيه تم تحليل العلاقة بين التوزيع الطبيعي للضوء بداخل أجزاء الشجرة المختلفة وتركيز الفلافونات وحمض الكلوروجينيك في قشرة الثمار. أظهرت النتائج أن هناك ارتباط موجب بين مستوى الضوء وتركيز مركبات الكويرزاتين جلايكوزيد 3 والأنثوسيانين وكذلك النسبة المؤوية للون الأحمر في قشرة الثمار. في الأجزاء الداخلية للشجرة انخفضت جودة الضوء بدرجة كبيرة وخاصة في الأشعة الفوق بنفسجية والطيف الأزرق والأخضر والأحمر ولكنه كان غنيا نسبيا في الطيف الأحمر البعيد وذلك مقارنة بجودة الضوء الموجود في أجزاء الشجرة الأخرى. ونتيجة لذلك كانت النسبة بين الطيف الأحمر البعيد (FR) إلى الطيف الأحمر (R) أكبر في الأجزاء الداخلية مقارنة بباقي أجزاء الشجرة. ولقد وجد أنه عندما تكون نسبة (FR/R) حوالي 1 أو أعلى فإنه لا يتكون أي أنثوسيانين في قشرة ا لثما ر بينما يتكون فقط مستوى قليل من الكويرزاتين جلايكوزيد 3.

الفصل الرابع:

وفيه تم دراسة العلاقة بين المحتوى المعدني في أنسجة الثمار (N, P, K, Mg and Ca) وتركيز الفلافونات وحمض الكلوروجينيك في قشرة الثمار للصنف "الا ستار " والطفرة التابعة له "الاسهوف". أظهرت النتائج وجود ارتباط سالب بين تركيز الـ "N/Ca, N, Mg" في أنسجة الثمار أثناء النمو وبين تركيز مجموع الفلافونات الكلية والأنثوسيانين في قشرة ثمار الطفرة "الاسهوف". عند اكتمال النموذلك خلال ثلاث مواسم دراسية (1996، 1997، 1998) . وفي دراسة مع الصنف" الاستار" خلال موسم 1996 تم استخدام التباين في تركيز المحتوى المعدني الراجع إلى اختلاف موقع الثمار على الشجرة . وأظهرت النتائج وجود ارتباط سالب بين تركيز الـ "N/Ca, N, K" في أنسجة الثمار وبين تركيز مجموع الفلافونات الكلية والأنثوسيانين في قشرة الثمار عند اكتمال النمو . بينما أظهر تركيز الـ " Ca" ارتباطا موجبا مع تركيز الفلافونات والأنثوسيانين . أبدى تركيز الـ "Mg" ارتباطا سالبا مع تركيز الأنثوسيانين فقط ولكن دون تركيز مجموع الفلافونات الكلية . شرحت معادلات تحليل الانحدار المتعدد حوالي 4.3-.% من التباين في تركيز الأنثوسيانين ومجموع الفلافونات الكلية على التوالي في قشرة ثمار الطفرة "الاسهوف" وبين 7-65 .% من التباين فى تركيز الأنثوسيانين ومجموع الفلافونات الكلية على التوالي في قشرة ثمار الصنف "الاستار" .اتضح أن تركيز الـ"N" أهم عامل في هذه المعادلات . العلاقة بين حمض الكلوروجينيك والمحتوى المعدني في أنسجة الثمار لم تكن واضحة وتحتاج إلى المزيد من البحث. والخلاصة هي أنه بالإضافة إلى تحسين الظروف الضوئية بداخل الأشجار فإنه يمكن زيادة تركيز الفلافونات في الثمار عن طريق ضبط برنامج التسميد وخاصة تجنب الإضافة الزائدة للنيتروجين .

بسم الله الرحمن الرحيم

الملخص العربي

إن الهدف النهائي من عمليات الإنتاج والتداول والتوزيع لثمار الفاكهة الطازجة والخضراوات هو تحقيق متطلبات واحتياجات المستهلكين. وبصفة عامة فان جاذبية الثمار والخضراوات للمستهلكين تحدد بواسطة المواصفات الظاهرية مثل اللون والموصفات الغير ظاهرية مثل القيمة الصحية. وتلعب المركبات الفلافونية دورا كبيرا في تحديد لون الثمار وكذلك قيمتها الصحية.

فاللون الأحمر لثمار التفاح يرجع إلى وجود صبغة الأنثوسيانين وهى أحد أهم الصبغات الفلافونية ومن خلال الاستهلاك فإنها تفيد صحة الإنسان. وتنتشر هذه المركبات في المملكة النباتية حيث تضم مجموعة كبيرة من مضادات الأكسدة الطبيعية والتي تُكون جزءً من غذاء الإنسان. ويوجد العديد من الإيضاحات حول دور مضادات الأكسدة الموجودة في الثمار والخضراوات في حفظ صحة الإنسان والحماية من الإصابة بالعديد من الأمراض مثل أمراض السرطان وأمراض القلب. ولقد أوضحت الأبحاث الحديثة أن الفلافونات والمركبات الفينولية عموما تشكل أهم مضادات الأكسدة الموجودة في الثمار والخضراوات. وتعتبر ثمار التفاح مصدرا غنيا لهذه المركبات مثل: الكويرزاتين جلايكوزيد 3 ، والسيانيدين جلايكوزايد 3 ، والكاتيكينز ، والفلوريديزين (Quercetin 3-glycosides, Cyanindin 3-glycosides, Catechins and Phloridzin). و تحتوى كذلك ثمار التفاح على كمية كبيرة من الأحماض الفينولية مثل حمض الكلوروجينيك (Chlorogenic acid) . هذا ويرجع اللون الأحمر لثمار التفاح إلى وجود الصبغات الفلافونية وهى صبغات الأنثوسيانينات والتي توجد في الفجوات العصارية لبعض خلايا القشرة.

وعلى الرغم من أهمية المركبات الفلافونية والأحماض الفينولية لخواص جودة ثمار التفاح إلا أنه لا توجد معلومات كافية عن وسائل التحكم في تكوينها في الثمار. ولذلك فان الهدف من هذه الدراسة هو الحصول على معلومات حول مدى التباين الطبيعي الموجود في تركيز هذه المركبات وكيفية تطورها أثناء النمو والنضج والتخزين والتسويق وكذلك دراسة تأثير عدد من المعاملات الزراعية على تركيز هذه المركبات في ثمار التفاح. وفيما يلي عرض ملخص لمحتويات فصول الرسالة:

الفصل الأول:

في هذا الفصل تم دراسة المعلومات المتوفرة في المراجع حيث ظهر أن تكوين المركبات الفلافونية والأحماض الفينولية في النبات بصفة عامة يخضع لعوامل خارجية وداخلية عديدة. وينتهي هذا الفصل بعرض تفصيلي للمشروع البحثي وأهدافه والفصول المختلفة لهذه الرسالة.

الفصل الثاني:

وفيه تم تقدير مدى التباين الطبيعي في تركيز المركبات الفلافونية وحمض الكلوروجينيك الراجع إلى التباين داخل الثمرة وداخل الشجرة و بين البساتين وبين الأصناف وبين الطفرات المشتقة منها. حيث أظهرت النتائج وجود تباين معنوي جدا بين هذه العوامل المختلفة. وجد أن تركيز المركبات الفلافونية وحمض الكلوروجينيك تتوزع بدرجة غير متساوية بداخل أجزاء الثمرة الواحد. حيث وجد أن مركبات الكويرزاتين جلايكوزيد 3 والأنثوسيانين لا توجد إلا في القشرة . كذلك الجانب المعرض للشمس احتوى على تركيز مرتفع جدا من مركبات الكويرزاتين جلايكوزيد والأنثوسيانين مقارنة بالجانب الغير مواجه للشمس لنفس الثمرة. بينما وجد أن مركبات الكاتيكينز والفلوريديزين وكذلك حمض الكلوروجينيك لا تختلف في جانبي الثمرة.

احتوت قشرة ثمار الصنف "الجوناجولد" على حوالي 2-15.% من تركيز الفلاقونات الكلية (mg/g dw) وحوالي 4.3-.% من كمية الفلافونات الكلية (mg/fruit) أعلى من ثمار الصنف "الاستار" (الفصل الثاني والسادس). وكذلك كان مستوى حمض الكلوروجينيك حوالي ثلاثة أضعاف في قشرة ثمار "الجوناجولد" مقارنة بثمار الصنف "الاستار". ولكن قشرة ثمار الصنف "الاستار" أكثر احتواء على بعض أنواع مركبات الكويرزاتين جلايكوزيد 3 بدرجة أعلى من قشرة ثمار الصنف "الجوناجولد". وهذه الاختلافات في تركيز الفلافونات ربما تكون ذات أهمية كبيرة إذا أخذ في الاعتبار الاختلافات في كفاءة الفلافونات المختلفة كمضادات أكسدة. وعموما إذا كان المحتوى النهائي للمركبات الفلافونية في ثمار التفاح يحدد بواسطة العوامل الوراثية بصفة رئيسية فإنه يمكن استخدام وسائل التربية والتهجين كأداة لإنتاج أصناف أغنى في محتواها من المركبات الفلافونية والأحماض الفينولية عن الأصناف المتوفرة حاليا.