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Late Prohexadione-Calcium Application on Maturing Apple cv. 'Braeburn' Fruit Reduces Anthocyanins and Alters the Phenolic Content

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Summary

In this study the influence of prohexadione-Calcium (Pro-Ca) treatment on peel colour development and polyphenol metabolism of apples during advanced maturity was investigated. Apple trees were treated 3 weeks before technological maturity. Changes in the content of hydroxycinnamic acids, dihydrochalcones, flavonols, flavanols and anthocyanins were monitored six times during the advanced maturation until technological maturity of the fruits. To evaluate the effect of Pro-Ca on the coloration of apples, the changes in the chromaticy values a*, h° and the lightness coefficient L* were monitored. The parameters showed a significant difference in the intensity of red coloration between the treated and untreated apples. The application of Pro-Ca led to a decrease in the enzyme activities of the 2-oxoglutarate dependent dioxygenases flavanone-3-hydroxylase (FHT) and flavonol synthase (FLS), and the flavonoid pathway was negatively affected in general. Concomitantly, the concentrations of flavonols, anthocyanins and dihydrochalcones in the peel of treated apples decreased, whereas the contents of total phenolic compounds and hydroxycinnamic acids increased. Flavanol contents, however, remained unchanged. In apple pulp, slightly higher concentrations of all phenolic compounds were measured in the treated apples compared to the control on the majority of sampling dates. The results indicate that Pro-Ca modulates the polyphenol spectrum resulting in a pronounced decrease of red coloration during advanced maturation of apples. Pro-Ca therefore to some extent exhibits the potential to avoid undesired red coloration of apple fruit.

Key words. Anthocyanins – flavonoids – fruit colour – phenolic acids – prohexadione Calcium

Introduction

Prohexadione-Calcium (Pro-Ca) is a multi-functional plant bioregulator currently being used in a number of countries on apple and some other fruit trees (Rademacher and Kober 2003). Treating fruit trees with Pro-Ca resulted in the reduction of shoot growth (Medjoub et al. 2004), reduced incidence of insect pests (Krawczyk and Greene 2002), and reduced incidence of fire blight and other bacterial and fungal diseases (Rademacher and Kober 2003). The compound acts as a structural mimic of 2-oxoglutarate, thereby inhibiting 2-oxoglutarate-dependent dioxygenases. This leads to significant changes in the flavonoid spectrum of apple (Roemmelt et al. 2003a; Halbwirth et al. 2006). When applied in high dosage, anthocyanin formation and red coloration of fruits and flowers (Lancaster 1992; Lancaster et al. 1997) can also be inhibited (Rademacher et al. 1992). In apple fruits, Pro-Ca treatments caused a significant decrease in the formation of flavanols and flavonols, whilst hydroxycinnamic acid concentrations increased significantly (Mikulic-Petkovsek et al. 2009).

The accumulation of anthocyanins is influenced by many factors including light (Saure 1990), nitrogen fertilization (Treutter 2001), ethylene (Whale and Singh 2007) temperature and cultural practices (Lancaster 1992). Coloration of the apple peel is primarily based on the presence of anthocyanins, but other flavonoids, such as flavonols (quercetin 3-*O*-glycosides) and proanthocyanidins, also have some influence (Lister et al. 1994). Apple peel colour is of great commercial importance; hence different techniques have been used to improve coloration (Whale et al. 2008). However, in some green apple cultivars like 'Granny Smith', red coloration is not desirable and a deep green colour is usually preferred by the majority of its consumers.

Pro-Ca treatments can have divergent effects on peel colour development when applied once or twice in spring (Mata et al. 2006). An observed enhancement of red coloration in the case of cv. 'Fuji' might be the consequence of shoot growth inhibition or of the activation of the polyphenol metabolism by Pro-Ca as previously observed in apple leaves (Fischer et al. 2006).

In the present study, the red-coloured cultivar 'Braeburn' was selected as a model plant to investigate the potential of Pro-Ca treatment to alter the levels of fruit coloration, total and individual phenolic content and related enzyme activities in treated fruit compared to the control. The objective was to determine if the application of Pro-Ca during the advanced ripening in autumn can reduce the red coloration of apples and if it alters the phenolic metabolism in a similar way as has previously been reported for apple leaves. This is the first study on autumn application of Pro-Ca on apple fruit. The results will be interesting for a further detailed research on apple phenolic metabolism as well as for fruit producers, which strive to effectively reduce the undesired red colour formation in certain apple cultivars.

Materials and Methods

Chemicals

For the quantification of phenolic compounds the following standards were used: chlorogenic acid (5-caffeoylquinic acid), phloretin and rutin (quercetin-3-*O*-rutinoside) from Sigma Aldrich Chemie (Steinheim, Germany), cyanidin-3-*O*-galactoside chloride, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, (−)-epicatechin, *p*-coumaric acid, procyanidin B2 and phloridzin dihydrate from Fluka Chemie (Buchs, Switzerland), quercetin 3-*O*-arabinofuranoside and quercetin-3-*O*-xyloside from Apin Chemicals (Abingdon, UK) and (+)-catechin from Roth (Karlsruhe, Germany). Methanol for the extraction of phenolics was acquired from Sigma. Chemicals for the mobile phases were the high performance liquid chromatography–mass spectrometry (HPLC-MS) grade acetonitrile and formic acid from Fluka Chemie. Water for mobile phase was bidistilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA). For determination of the total phenolic content, Folin–Ciocalteu phenol reagent (Fluka Chemie GmBH), sodium carbonate (Merck, Darmstadt, Germany), gallic acid and ethanol (Sigma) were used. Substrates for enzyme assays were synthesised as described (Gosch et al. 2003).

Plant materials

The trial was conducted in 2010 on 10-year-old trees of striped 'Braeburn' cultivar clone Hillwell grafted on M9 rootstock, grown according to the system of integrated production at the fruit growing center Maribor Gacnik location (latitude 46° 61', longitude 15° 68'). Full bloom (> 80 % of the opened flower buds) occurred on 22 April 2010. On 16 September 2010 (147 DAFB), approximately three weeks before technological maturity (169 DAFB), five trees were sprayed with 1 g (2.5 kg ha^{-1}) Regalis (BASF 125 10W-10 % Pro-Ca, BASF, Germany) per tree, whereas the control trees were sprayed with water.

Colour measurements

At each sampling date colour measurements on 50 randomly chosen fruit from the outside of the tree canopy (10 fruit per tree) up to a height of 2 m from the ground were performed. The peel colour was measured using a portable colorimeter (CR-200 b Chroma; Minolta, Osaka; Japan) and L*, a*, and b* values were recorded. The colorimeter was calibrated with a white standard calibration plate before use. In the CIE L*a*b* system of colour representation, the L* value corresponds to a dark-bright scale and represents the relative lightness of colours with a range from 0 to 100 (0 = black, 100 = white). Hue angle (h°), the most appropriate way of representing changes in colour, was also calculated ($\tan^{-1}$ b*/a*) and expressed in degrees from 0 to 360, where 0° = red, 90° = yellow, 180° = green and 270° = blue (McGuire 1992).

Fruit sampling

Fruit sampling commenced five days after the treatment (152 DAFB) and was performed six times during the advanced fruit maturation until reaching technological maturity, which was evaluated using the starch iodine test. At the date of harvest the value of firmness was 60 N and the starch index was 2.8. The sampling dates were 5, 8, 12, 15, 19, and 22 d after the Pro-Ca treatment. At each sampling date 10 fruits were randomly harvested and combined in five samples (2 fruit per sample, n = 5). Immediately after harvest, apples have been transported to the laboratory, where peel was separated from pulp for the extraction of individual and total phenolic compounds. One part of the apple peel was kept frozen at −80 °C until enzymatic analysis.

Extraction and determination of phenolic compounds

The extraction of fruit samples (peel and pulp separately) was done as described by Mikulic-Petkovsek et al. (2010), with some modification. Apple samples were ground to a fine powder in a mortar chilled with liquid nitrogen. 5 g of pulp and 2 g of peel were extracted with 10 ml of methanol containing 3 % (v/v) formic acid and 1 % (w/v) 2.6-di-tert-butyl-4-methylphenol (BHT) in a cooled ultrasonic bath for 1 h. BHT was added to the samples in order to prevent oxidation. After extraction, the treated samples were centrifuged for 5 min at 12,000 g_n. The supernatant was filtered through a Chromafil AO-45/25 poly-

amide filter (Macherey-Nagel, Düren, Germany) and transferred to a vial before the injection into the HPLC system.

The individual phenolic compounds were analyzed using a Thermo Finnigan Surveyor HPLC system (Thermo Scientific, San Jose, CA) with a diode array detector at 280 nm, 350 nm and 530 nm. The hydroxycinnamic acids, dihydrochalcones and flavanols were detected at 280 nm, flavonols at 350 nm and anthocyanins at 530 nm. For the separation of phenolic compounds Phenomenex (Torrance, CA) HPLC column C18 (150 × 4.6 mm, Gemini 3 μ) protected with a Phenomenex security guard column operated at 25 °C was used. The injection volume for the fruit extract was 20 μl, and the flow rate maintained at 1 ml min^{-1}. The elution solvents were aqueous 1 % formic acid (A) and 100 % acetonitrile (B). Samples were eluted according to the linear gradient described by Marks et al. (2007): 0–5 min, 3 % to 9 % B; 5–15 min, 9 % to 16 % B; 15–45 min, 16 % to 50 % B; 45–50 min, 50 % isocratic; this step was followed by the washing and reconditioning of the column. The compound identification was achieved by comparing the retention times and their UV-VIS spectra from 200 to 600 nm, as well as by the addition of an external standard. The identity of the phenolic compounds was also confirmed and quantified using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with an electrospray ionization (ESI) operating in the negative and positive ion mode. For the analyses full-scan data dependent MS^n scanning from m/z 115 to 2000 was used. Quantification was achieved according to the concentrations of a corresponding external standard. For the compounds lacking standards, similar compounds were used as standards for the quantification. Therefore 4-*O*-*p*-coumaroylquinic acid was quantified in equivalents of *p*-coumaric acid, phloretin-2-*O*-xylosylglucoside in equivalents of phloridzin, quercetin-3-*O*-arabinopyranoside in equivalents of quercetin-3-*O*-arabinofuranoside and anthocyanins (cyanidin-3-arabinoside, cyanidin-3-glucoside, and cyanidin-3-xyloside) were quantified in equivalents of cyanidin-3-galactoside. Concentrations of the phenolic compounds were expressed in mg kg^{-1} of fresh weight (FW) for fruit peel and pulp separately.

Determination of the total phenolic content

Extracts of the samples were obtained in the same way as for the phenolic compounds, with the difference that no BHT was added. The total phenolic content of extracts was assessed using the Folin-Ciocalteu phenol reagent method, as described by Singleton and Rossi (1965). To 100 μl of sample extracts, 6 ml of bidistilled water and 500 μl of Folin-Ciocalteau reagent were added; after resting between 8 s and 8 min at room temperature, 1.5 ml of sodium carbonate (20 % w/v) and 1.9 ml bidistilled water was added. The extracts were mixed and allowed to stand for 30 min at 40 °C. After that the absorbance was measured in a spectrophotometer (Perkin Elmer, UV/VIS Lambda Bio 20) at 765 nm. A mixture of water and reagents was used as a blank. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg kg^{-1} fresh weight (FW) of fruit peel and pulp. Absorption was measured in three replications.

Enzyme preparations

For determination of chalcone synthase and dihydroflavonol synthase activities, enzyme preparations were obtained according to Claudot and Drouet (1992). For FHT and FLS the protocol of Thill (2010) was used: 0.2 g plant material was ground with 0.2 g Polyclar AT, 0.2 g silica sand and 3 ml of extraction buffer and then centrifuged for 10 min at 4 °C and 10,000 g. The extraction buffer was a 0.1 M HEPES buffer pH 7.3 containing 1.5 g sucrose 1.5 g polyethylene glycol 20000, 2 g Na-ascorbate, 0.015 g dithioerythritol and 0.36 g $CaCl_2$ per 100 ml. Enzyme preparations were generally subjected to gel chromatography to remove low-molecular compounds with the exception of those used for the demonstration of the competitive FHT inhibition of Pro-Ca.

Analyses of enzyme activity

Enzyme assays were performed as described previously (Slatnar et al. 2010). FHT assays showing the competitive FHT inhibition of Pro-Ca were performed according to Fischer et al. (2006). All values represent an average of three independent biological repetitions.

Statistics

The data was analyzed using the Statgraphics Plus 4.0 program (Manugistics. Inc.; Rockville, Maryland, USA). Data from all the analyses was tested for any differences among treatments using one-way analysis of variance (ANOVA). Significant differences among treatments were determined independently for each sampling date using the least significant difference test (LSD), whereas the differences between sampling dates for each treatment were tested using the Duncan test. In both cases, the significance level was 0.05. In diagrams, the means as well as their standard errors are presented (mean ± SE). For the determination of the association between the hue angle and total anthocyanins and a* and total anthocyanins Spearman's rank correlation was calculated.

Results and Discussion

To evaluate the potential of Pro-Ca applications of preventing undesired red fruit coloration during advanced fruit maturity, the effect of Pro-Ca was determined as the altered levels of fruit coloration, polyphenol contents and related enzyme activities in treated fruit compared to the control.

The hue angle, which represents the most appropriate way of evaluating red colour changes (McGuire 1992), decreased during advanced fruit maturation (Fig. 1A), indicating an increasing colour intensity. This is in agreement with the results of Whale and Singh (2007), who monitored colour development of 'Pink Lady' apple. In our experiment, the measured control values were over 31 % lower than on the first sampling date. However, in the Pro-Ca treated apples the decline in hue angle was lower than 25 %. Pro-Ca significantly influenced the hue angle, indicated by significantly higher values of treated apples compared to the control 8 d after the treatment until technological maturity at all sampling dates. Similarly, an influence of Pro-Ca treatment on the a* parameter was also determined at the same sampling dates (Fig. 1B). The a* parameter increased from 15.97 to 25.90 in the Pro-Ca treated apples and from 17.46 to 28.78 in the controls. A higher a* value is in connection with the increasing red coloration of apples (McGuire 1992). The reduction of the hue angle and the increase of a* were significantly ($P \leq 0.001$) correlated with the increase in concentrations of total anthocyanins ($r_s = -0.72$, and 0.74 respectively) (Fig. 4), therefore the development of red colour during advanced maturity can be ascribed to the increased anthocyanin concentration.

The lightness coefficient L* decreased during advanced maturity. This indicates darker hues of the fruit peel. Unlike the parameters a* and h°, significant differences in L* value between the treatments were measured only three days before and at technological maturity (Fig. 1C). At that time the apples of the control were significantly darker than the apples treated with Pro-Ca. An increase in the peel darkness was probably the consequence of the increased anthocyanin concentration, due to a greater proportion of darker red vacuoles and several layers of red cells (Lancaster et al. 1997).

Anthocyanins were only present in the apple peel and the main anthocyanin was cyanidin-3-*O*-galactoside. The total anthocyanin content increased during advanced ripening (Fig. 1D) and varied between 93.74 and 193.84 mg kg^{-1} FW. The average value measured was comparable to earlier reports (Jakopic et al. 2007). Pro-Ca significantly reduced the content of anthocyanins 5, 12 and 15 d after treatment (Fig. 1D). Treated apple peels contained from 59.41 to 130.25 mg anthocyanins per kg FW. The biggest difference between means was

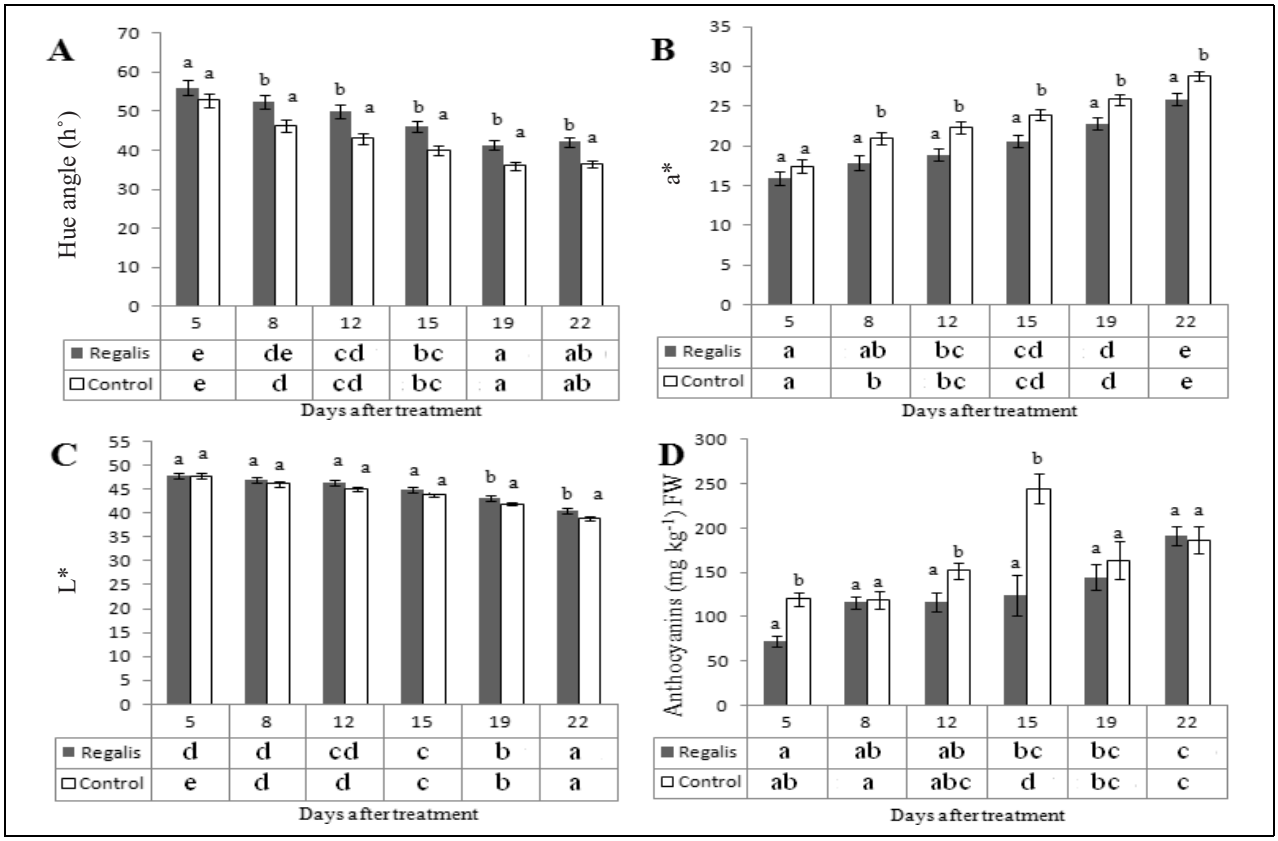


Fig. 1. Colorimetric parameters hue angle (h°) (A), a* (B), L* (C) and the content of anthocyanins of the 'Braeburn' apples (D) treated with prohexadione-Calcium ■ and control □ at different sampling dates.
Different letters above each column denote significant differences between treated and untreated apples on each sampling date (LSD test, $\alpha \leq 0.05$). Different letters in the table denote significant differences among sampling dates for each treatment (Duncan test, $\alpha \leq 0.05$). Vertical bars represent SE.

detected 15 d after treatment, when an almost 50 % lower content of anthocyanins was measured in the treated apples compared to the control. At technological maturity, however, there was no significant difference between the treated and untreated apples.

In our study 19 polyphenols were identified in apple peel belonging to five groups: hydroxycinnamic acids, flavonols, dihydrochalcones, flavanols and anthocyanins. In apple pulp, 13 polyphenols from the groups of hydroxycinnamic acids, dihydrochalcones, flavanols and flavonols were determined. Rutin (quercetin-3-*O*-rutinoside), quercetin-3-*O*-arabinofuranoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-xyloside were detected and quantified only in the apple peel. In general, apple peel contained significantly higher amounts of phenolic compounds than apple pulp, which agrees with the results reported by Lata et al. (2009) and Veberic et al. (2010). The content of flavonols in the peel was as much as 1000 fold higher compared to pulp.

Total phenolic content in apple peel was quite constant during advanced fruit maturation, whereas a significant decrease was observed in the pulp regardless of the treatment (Fig. 2A, 2B). Also, in the study of Zheng et al. (2012) a decrease of total phenolics was noted during the final ripening stages. However, in their study whole apples where analyzed and apple pulp represents the major part of the fruit. Simultaneously, an increase in fruit weight and diameter has been reported, probably due to increased cell size. Therefore, the reduction of total phenolics could be due to the dilution effect which is probably much more pronounced in the apple pulp. Pro-Ca treated apples contained statistically higher amounts of total phenolic compounds in the pulp 8, 12, 15 and 19 d after the treatment and 19 and 22 d after the treatment in the peel. Our results are different from those reported by Mikulic-Petkovsek et al. (2009) who found lower total polyphenol contents in Pro-Ca treated apples compared to the control during the major part of the growing period and also at technological maturity. The reason for different results obtained in our study could be ascribed to a different influence of the Pro-Ca treatment on the total phenolics applied during the advanced ripening or early in the spring.

Among hydroxycinnamic acids, chlorogenic acid, caffeic acid and 4-coumaroylquinic acids were determined. A significantly higher content of total hydroxycinnamic acids in the peel of treated apples compared to the control was found 8 and 19 d after the treatment (Fig. 3A). In apple pulp, higher contents of hydroxycinnamic acids in the Pro-Ca treated apples were measured at the majority of sampling dates, however, the differences were significant only 8 d after treatment (Fig. 3B).

Our results are in agreement with Roemmelt et al. (2003a, b) who found higher content of hydroxycinnamic acids in Pro-Ca treated leaves. Since the effect of Pro-Ca on apple fruit in our study was visible for a longer period in comparison with those reported for the leaves, this could be the reason for the higher content of total hydroxycinnamic acids in the peel meassured 19 d after the treatment. Our results are similar to those of Mikulic-Petkovsek et al. (2009), who also found higher content of hydroxycinnamic acids in Pro-Ca treated fruits. However, at technological maturity they also did not find significant differences between the treated and untreated fruits. Hydroxycinnamic acid accumulation may be explained as a consequence of the FHT inhibition which on the one hand provokes an upstream accumulation of flavonoids and related precursors, while, on the other hand, the production of cinnamic acid precurors is accelerated to make up for the depletion of downstream flavonoids (Roemmelt et al. 2003a; Fischer et al. 2006). An increase of hydroxycin-

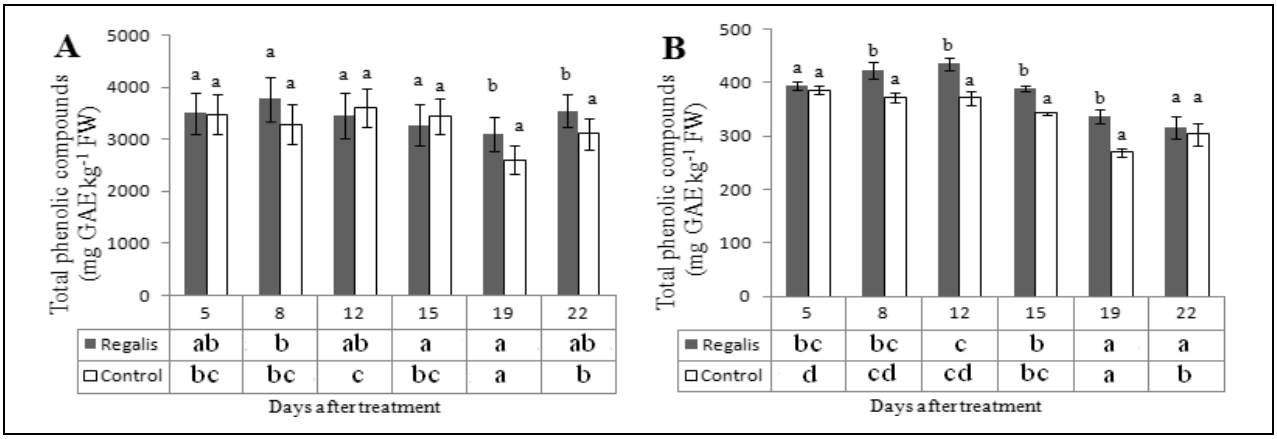


Fig. 2. Content of total phenolic compounds (mg GAE kg^{-1} FW) in the peel (A) and in the pulp of 'Braeburn' apples (B) treated with prohexadione-Calcium ■ and control □ at different sampling dates. Different letters above each column denote significant differences between treated and untreated apples on each sampling date (LSD test, $\alpha \leq 0.05$). Different letters in the table denote significant differences among sampling dates for each treatment (Duncan test, $\alpha \leq 0.05$). Vertical bars represent SE.

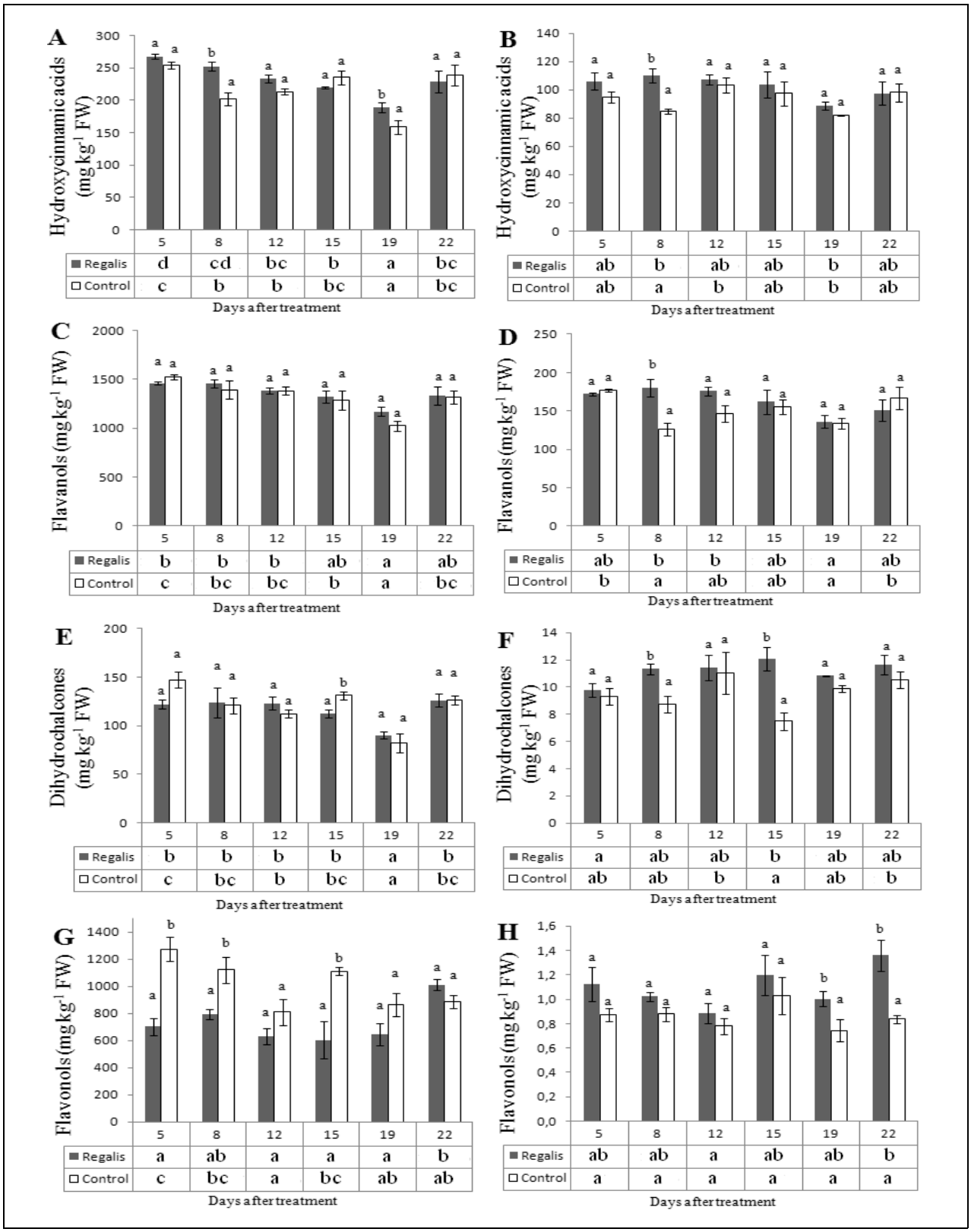


Fig. 3. Content of total hydroxycinnamic acids, flavanols, dihydrochalcones and flavonols (mg kg^{-1} FW) in the peel (A, C, E, G) and in the pulp (B, D, F, H) of 'Braeburn' apples treated with prohexadione-Calcium ■ and control □ at different sampling dates.
Different letters above each column denote significant differences between treated and untreated apples on each sampling date (LSD test, $\alpha \leq 0.05$). Different letters in the table denote significant differences among sampling dates for each treatment (Duncan test, $\alpha \leq 0.05$). Vertical bars represent SE.

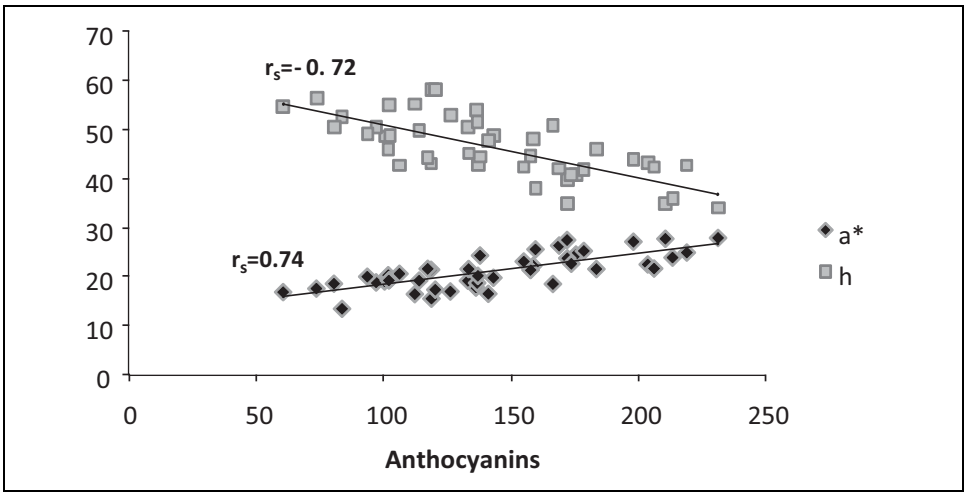


Fig. 4. Relationship between the colorimetric parameters hue angle (h°) and the content of anthocyanins and a* and the content of anthocyanins of 'Braeburn' apples during advanced ripening (r_s: Spearman's rank correlation coefficient ($P \leq 0.001$)).

namic acids caused by the Pro-Ca treatment was also found by Gosch et al. (2003) in *Actinidia arguta* leaves.

The following flavanols were determined in apple peel and pulp: catechin, epicatechin, procyanidin B1 and procyanidin B2. The content of total flavanols in the peel of the treated apples and controls were similar on the majority of sampling dates and no significant influence of Pro-Ca treatment was noted (Fig. 3C). In the pulp, significantly higher content of flavanols in the treated apples 8 d after the treatment was measured, whereas on the other sampling dates no significant differences were found (Fig. 3D). Our results differ from those reported by Mikulic-Petkovsek et al. (2009), who found a decrease in the flavanols content in apples treated with the Pro-Ca on the majority of sampling dates and also at technological maturity. It must be considered, however, that in their study the Pro-Ca application was done in the spring. Therefore, this might be the reason for different results of the influence of Pro-Ca on the content of flavonols compared to our study. Similarly Roemmelt et al. (2003a) also reported a significant decrease in oligomeric flavanols of apple leaves.

Among dihydrochalcones, phloretin and phloridzin (phloretin 2'-*O*-β-D-glucoside) were analyzed in apple peel and pulp. 15 d after the treatment, a statistically lower amount of total dihydrochalcones in the peel of the treated apples compared to the control was measured (Fig. 3E). At that sampling date, the treated apples contained statistically less phloridzin, whereas for phloretin no significant differences between means were noted (data not shown). However, at other sampling dates no significant differences in total dihydrocalchones content between the treated apples and the control were detected. In the pulp, on the contrary, a higher content of total dihydrocalchones in treated apples was detected. The difference between means was significant 8 and 15 d after the treatment with Pro-Ca (Fig. 3F). A decrease in phloridzin content was also observed in Pro-Ca treated fruits (Mikulic-Petkovsek et al. 2009); however, at technological maturity, no differences could be determined between treated and untreated fruits. Ruehmann and Treutter (2003) reported a significant reduction of phloridzin in treated apple leaves.

Among flavonols, hyperin (quercetin-3-*O*-galactoside), quercitrin (quercetin-3-*O*-rhamnoside), isoquercitrin (quercetin-3-*O*-glucoside) and quercetin-3-*O*-xyloside were identified in apple peel and pulp. In the peel, rutin (quercetin-3-*O*-rutinoside) and quercetin-3-*O*-arabinofuranoside were present as well. Quercetin-3-*O*-galactoside was the major flavonol in apple peel accounting for approx. 50 % of the total flavonols analyzed, whereas the content of rutin was lowest. Similar findings were also reported by Jakopic et al. (2010) in 'Fuji' apples. Pro-Ca caused a significant decrease of total flavonols in apple peel as early as five days after the treatment (Fig. 3G). The most pronounced differences in the contents of total flavonols were detected in the peel of the treated and untreated apples fifteen days after treatment. However, at technological maturity, no differences in the content of flavonols between the treatments were detected. In the pulp, on the other hand, 19 d after the treatment and at technological maturity, total flavonols in treated apples was higher than controls (Fig. 3H). Similary, Roemmelt et al. (2003a) also reported a significant decrease of the flavonols hyperin, isoquercetin, rutin and quercetin in Pro-Ca treated apple leaves. Similar effects were reported in other fruit species (Gosch et al. 2003) and rose leaves (Schlangen et al. 2003).

A decrease in the anthocyanin formation in treated apples is the consequence of the structural similarity between Pro-Ca and 2-oxoglutaric acids. This leads to a temporary blockage of the 2-oxoglutarate-dependent dioxygenases flavanone 3-hydroxylase (FHT), flavonol synthase (FLS) and anthocyanidin synthase (ANS) which play a key role in the biosynthesis of anthocyanins and other flavonoids (Halbwirth et al. 2006). The competitive inhibition by Pro-Ca was demonstrated in our study by measuring FHT activity in the absence of surplus 2-oxoglutarate and using enzyme preparations which were not

Table 1. Flavanone 3-hydroxylase, flavonol synthase, chalcone synthase/chalcone isomerase and dihydroflavonol 4-reductase activity of apples treated with prohexadione-Calcium (Pro-Ca) (as % of the respective control value) at different sampling dates.

		Days after treatment		
		5	15	22
FHT activity (% rel.)	Pro-Ca	59 %	53 %	98 %
FLS activity (% rel.)	Pro-Ca	87 %	68 %	97 %
CHS/CHI activity (% rel.)	Pro-Ca	100 %	84 %	84 %
DFR activity (% rel.)	Pro-Ca	98 %	60 %	75 %

subjected to gel chromatography as described previously (Fischer et al. 2006). The FHT inhibition in Pro-Ca treated apples could be observed until 15 d after the treatment. At technological maturity, however, there were no differences in the levels of anthocyanins between the treated and untreated apples (Table 1). This can be explained by the 10 to 15 d half-life of Pro-Ca and its rapid metabolisation in plant tissues (Halbwirth et al. 2003). However, the effect of Pro-Ca on apple fruit was visible for a longer period than on the leaves, where the inhibition was completely suspended 10 d after treatment (Fischer et al. 2006). Pro-Ca did not result in a complete blockage of FHT in the fruits, but in an approx. 50 % inhibition. This was also observed for leaves and creates a bottleneck leading to the observed changes in the polyphenol spectrum. In contrast to the leaves (Fischer et al. 2006), Pro-Ca treated fruits generally showed a universal reduction of all enzymes of the flavonoid pathway tested (Table 1). This is in agreement with the changes observed in the polyophenol spectrum, which was characterized by a strong decrease of anthocyanin and flavonol content and an increase of early intermediates such as hydroxycinnamic acids.

Conclusions

In the present study we were able to demonstrate, that prohexadione-Calcium significantly reduced the content of flavonols, anthocyanins and the red coloration of apple peel, when applied during advanced maturation of the apples. Contrary to that, total phenolic content in apple peel and pulp increased. The impact on the content of other polyphenol groups was only slightly noticeable and differed in apple peel and pulp. Moreover, it has been determined that the physiological response of the treated apples in our experiment was quite different from the response which was noticed in young developing leaves and fruits by the researchers, who applicated Pro-Ca in two replications in spring time. Thus it was demonstrated, that Pro-Ca to some extent has the potential to avoid undesired red coloration of apple fruit.

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References

Claudot, A.C. and A. Drouet 1992: Preparation and assay of chalcone synthase from walnut tree tissue. Phytochem. **31**, 3377–3380.

Gosch, C., I. Pnuhl, H. Halbwirth, K. Schlangen, S. Roemmelt, C. Andreotti, G. Costa, T.C. Fischer, D. Treutter, K. Stich and G. Forkmann 2003: Effect of prohexadione-Ca on various fruit crops: flavonoid composition and substrate specificy of their dihydroflavonol 4-reductases. Europ. J. Hort. Sci. **68**, 144–151.

Fischer, T.C., H. Halbwirth, S. Römmelt, E. Sabatini, K. Schlangen, C. Andreotti, F. Spinelli, G. Costa, G. Forkmann, D. Treutter and K. Stich 2006: Induction of flavonoid gene expression in apple (*Malus × domestica*) after the application of a dioxygenase enzyme inhibitor. Physiol. Plant. **128**, 604–617.

Halbwirth, H., T. C. Fischer, S. Roemmelt, F. Spinelli, K. Schlangen, S. Peterek, E. Sabatini, C. Messina, J.-B. Speakman, C. Andreotti, W. Rademacher, C. Bazzi, G. Costa, D. Treutter, G. Forkmann and K. Stich 2003: Induction of antimicrobial 3-deoxyflavonoids in pome fruit trees controls fire blight. Zeitschr. Naturf. **58c**, 765–770.

Halbwirth, H., T.C. Fischer, K. Schlangen, W. Rademacher, K.-J Schleifer, G. Forkmann and K. Stich 2006: Screening for inhibitors of 2-oxoglutarate-dependent dioxygenases: Flavanone 3β-hydroxylase and flavonol synthase. Plant Sci. **171**, 194–205.

Jakopic, J., R. Veberic and F. Stampar 2007: The effect of reflective foil and hail nets on the lighting, colour and anthocyanins of 'Fuji' apple. Sci. Hortic. **115**, 40–46.

Jakopic, J., F. Stampar and R. Veberic 2010: Influence of hail net and reflective foil on cyanidin glycosides and quercetin glycosides in 'Fuji' apple skin. HortScience **45**, 1447–1452.

Krawczyk, G. and G.M.Greene 2002: The impact of plant growth regulator Apogee on insect pest populations and fruit quality. PA Fruit News **82**, 18–24.

Lancaster, J.E. 1992: Regulation of skin Colour in Apples. Crit. Rev. Plant. Sci. **10**, 487–502.

Lancaster, J.E., C.E. Lister, P.F. Reay and C.M.Triggs 1997: Influence of pigment composition on skin colour in a wide range of fruit and vegetables. J. Am. Soc. Hort. Sci. **122**, 594–598.

Lata, B., A. Trampczynska and J. Paczesna 2009: Cultivar variation in apple peel and whole fruit phenolic composition. Sci. Hort. **121**, 176–181.

Lister, C.E., J.E. Lancaster, K.H. Sutton and J.R.L. Walker 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.

Marks, S.C., W. Mullen and A. Crozier 2007: Flavonoid and chlorogenic acid profiles of English cider apples. J. Sci. Food Agric. **87**, 719–728.

Mata, P.A., J. Val and A. Blanco 2006: Differential effects of prohexadione-Calcium on red colour development in 'Royal Gala' and 'Fuji' apples. J. Hortic. Sci. Biotech. **81**, 84–88

McGuire, R.G. 1992: Reporting objective colour measurements. HortScience. **27**, 1254–1255.

Medjoub, R., J. Val and A. Blanco 2004: Prohexadione-Ca inhibits vegetative growth of 'Smoothee Golden Delicious' apple trees. Sci. Hort. **101**, 243–253.

Mikulic-Petkovsek, M., F. Štampar and R. Veberic 2009: The effect of prohexadione-Calcium content in developing fruits and leaves of apple trees. J. Food Agric. Environ. **7**, 369–375.

Mikulic-Petkovsek, M., A. Slatnar, F. Stampar and R. Veberic 2010: The influence of organic/integrated production on the content of phenolic compounds in apple leaves and fruits in four different varieties over a 2-year period. J. Sci. Food Agric. **90**, 2366–2378.

Rademacher, W., K.E. Smith-Temple, D.L. Griggs and P. Hedden 1992: The mode of action of acylcyclohexanediones-a new type of growth retardant. In: Karssen, C.M., L.C. Van Loon and D. Vreugdenhil (eds.): Current Plant Science and Biotechnology in Agriculture. Dordrecht: Kluwer Academic, 571–575.

Rademacher, W. and R. Kober 2003: Efficient Use of Prohexadione-Ca in Pome Fruits. Europ. J. Hort. Sci. **68**, 101–107.

Roemmelt, S., N. Zimmermann, W. Rademacher and D. Treutter 2003a: Formation of novel flavonoids in apple (*Malus* × *domestica)* treated with the 2-oxoglutarate-dependent dioxygenase inhibitor prohexadione-Ca. Phytochem. **64**, 709–716.

Roemmelt, S., T.C. Fischer, H. Halbwirth, S. Peterek, K. Schlangen, J.-B. Speakman, D. Treuter, G. Forkmann and K. Stich 2003b: Effect of dioxygenase inhibitors on the resistance-related flavonoid metabolism of apple and pears: Chemical, biochemical and molecular biological aspects. Europ. J. Hort. Sci. **68**, 129–136.

Ruehmann, S. and D. Treutter 2003: Effect of N-nutrition in apple on the response of its secondary metabolism to prohexadione-Ca treatment. Europ. J. Hort. Sci. **68,** 152–159.

Saure, M.C. 1990: External control of anthocyanins formation in apple. Sci. Hort. **42**, 181–218.

Schlangen, K., C. Gosch, S. Roemmelt, J. Knott, T.C. Fischer, G. Forkmann, D. Treuter, K. Stich and H. Halbwirth 2003: Can prohexadione-Ca induce antimicrobial flavonoids in Rose? Europ. J. Hort. Sci. **68**, 137–143.

Singleton, V.L. and J.A. Rossi 1965: Colorimetry of total phenolics with phosphomolybdic-phospholungistic acid reagents. Am. J. Enol. Vitic. **16**, 144–158.

Slatnar, A., M. Mikulic-Petkovsek, H. Halbwirth, F. Stampar, K. Stich and R. Veberic 2010: Response of the phenylpropanoid pathway to *Venturia inaequalis* infection in maturing fruit of 'Braeburn' apple. J. Hortic. Sci. Biotech. **85**, 465–472.

Thill, J. 2010: Biochemische und molekularbiologische Untersuchungen zur Flavonoidbiosynthese in *Onobrychis viciifolia*. Ph.D thesis, Technical University of Vienna.

Treutter, D. 2001: Biosynthesis of phenolic compounds and its regulation in apple. Plant Growth Regulation **34**, 71–89.

Veberic, R., V. Schmitzer, M. Mikulic-Petkovsek and F. Stampar 2010: Impact of shelf life on content of primary and secondary metabolites in apple (*Malus domestica* Borkh.). J. Food Sci. **75**, 461–468.

Zheng, Hu-Zhe, Kim, Young-ll, Chung and Shin-Kyo 2012: A profile of physicochemical and antioxidant changes during fruit growth for the utilisation of unripe apples. Food Chem. **131**, 106–110.

Whale, S.K. and Z. Singh 2007: Endogenous ethylene and color development in the skin of 'Pink Lady' apple. J. Amer. Soc. Hort. Sci. **132**, 20–28.

Whale, S.K., Z. Singh, M.H. Behboudian, J. Janes and S.S. Dhaliwal 2008: Fruit quality in 'Cripp's Pink' apple, especially colour, as affected by preharvest sprays of aminoethoxyvinylglycine and etephon. Sci. Hort. **115**, 342–351.

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