Ursolic and Oleanolic Acid in ‘Aroma’ Apple Peel as Affected by Rootstock, Harvest Maturity, and Storage Method

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Abstract. Apples contain substances with health-promoting properties, among them, triterpenes, including oleanolic acid (OA) and ursolic acid (UA), which are thought to have anti-inflammatory activity as well as inhibiting initiation, promotion, and metastasis of cancer. This study evaluated effects of harvest time, rootstock, and storage method on two major triterpenes in apple peel, to enhance the understanding of the relative importance of cultivation and environmental factors influencing triterpene concentration. OA and UA concentrations in the semi-dwarfing ‘MM.106’ rootstock apples were significantly higher than in the dwarfing ‘M.9’ rootstock apples at the majority of harvest times over the 2 years of investigations. In 2012 at harvest time, highest concentrations of OA and UA in ‘MM.106’ rootstock apples were 63.4 and 416.3 $\mu\text{g}\cdot\text{cm}^{-2}$, respectively, while they were 59.7 and 380.9 $\mu\text{g}\cdot\text{cm}^{-2}$, respectively, in ‘M.9’ rootstock apples. UA remained fairly stable in most cases during storage, and no difference was found between regular atmosphere storage and controlled atmospheric (CA) storage. OA concentration decreased after harvest time. Furthermore, OA showed different responses to regular atmosphere storage and CA storage, and year-to-year difference was also observed. The two rootstocks showed only slight differences in the effect on total soluble solids (TSS) and fruit color, while the titratable acidity (TA) in apples from trees on ‘M.9’ rootstock had lower levels in most cases at the third harvest time as compared with apples from trees on ‘MM.106’ rootstock. OA concentration was positively correlated with UA concentration both directly at harvest and after storage. OA and UA concentrations were positively correlated with TA and TSS, while they were negatively correlated with red color.

Apples play a significant role in the diet in different countries around the world. Epidemiological studies have shown that consumption of apples is linked to reduced risk of cardiovascular disease, some cancers, asthma, and Alzheimer’s disease (Hyson, 2011). Health-promoting properties have been attributed to the presence of a range of secondary metabolites, including triterpenes. OA (3 β -hydroxy-olean-12-en-28-oic acid) and its isomer, UA (3 β -hydroxy-ursan-12-en-28-oic acid, UA), are the two major triterpenes in apple peel (Cefarelli et al., 2006). OA and UA are commonly found in the cuticular waxes of fruit and leaves and they are important components of the wax (Koch and Ensikat, 2008; Qi et al., 2006). Along with antioxidative activity, these compounds have also been reported to have anti-inflammatory activity, and inhibit initiation, promotion, and metastasis of cancer (Shanmugam et al., 2013). UA and its

derivatives have also raised much interest for the potential to act as chemopreventive and chemotherapeutic agents against different forms of cancer, and have entered Phase I clinical trials (Shanmugam et al., 2013).

Breeding for improved phytochemical and nutrient quality of horticultural products has become an interesting topic in recent years. Phytochemical concentration in melons, peppers, and tomatoes has been increased, including levels of vitamin C, β -carotene, zeaxanthin, and flavonoids (Crosby et al., 2007; Davuluri et al., 2005). Recent results from an investigation of 247 wild and domesticated apple accessions found that main bioactive compounds, including triterpenoids, had been determined, with the purpose of, e.g., assisting in innovative breeding strategies (Farneti et al., 2015). Due to lack of success of many governmental programs for increasing consumption of fruit and vegetables, it has been suggested that increased concentration of bioactive compounds and nutrients by breeding programs could lead to improved health (Patil et al., 2014). However, to elevate health substances within plants, a better understanding of the effects of cultivation and other environment factors is needed.

Rootstock is an important feature of apple trees in modern orchards. Due to genetic

factors, rootstock controls tree size (Webster, 2001), regulates mineral uptake and leaf mineral composition (Fallahi et al., 2001), and affects hydraulic resistance, water use of scion, and fruit quality (Cohen and Naor, 2002; Cohen et al., 2007; Fallahi et al., 2002). Furthermore, they may have resistance to soil-transmitted diseases (Modgil et al., 2012) and fire blight (Kaja et al., 2015). Many suggestions have been made to explain the separate features in the rootstocks that exert these differences such as the characteristics of the bark, production and movement of different plant hormones, and top-root relationships (Ferree and Warrington, 2003; Rom and Carlson, 1987). Dwarfing rootstock ‘M.9’ and semi-dwarfing rootstock ‘MM.106’ are commonly used in apple orchards in many countries. ‘M.9’ rootstock was introduced as one of the Malling apple rootstock series, a result of a successful trial in England, which evaluated rootstocks of different origin in the early 20th century. ‘M.9’ was a chance seeding found in France. The Malling series was later followed by the Malling–Merton rootstock series, from which ‘MM.106’ originate. This rootstock is a cross between ‘M.1’ (Malling series) and ‘Northern Spy’ (Ferree and Warrington, 2003; Rom and Carlson, 1987). In ‘M.9’, more limited supply of water and minerals was found as compared with ‘MM.106’. Plant hormones levels vary between these two rootstocks. Auxin and cytokinin were lower in ‘M.9’, whereas the level of abscisic acid was found to be higher than in ‘MM.106’ (Tromp et al., 2005). Although compounds with health benefits have been investigated more frequently during recent years, information about the effect of different cultivation factors, such as the rootstock, on triterpene concentration is still lacking.

Fruit ripening is a complex process, which includes a series of biochemical, physiological, and structural changes (Lelièvre et al., 1997). Apple fruit will experience various metabolic changes during maturation and storage, such as the change of texture (Johnston et al., 2001), anthocyanin and phenolic concentration (Burda et al., 1990; Ju and Bramlage, 1999), antioxidant activity (Ju and Bramlage, 1999), and wax composition (Veraverbeke et al., 2001). The total phenolics concentration in apple peel showed variable responses to storage, including an increase, decrease, or no change (Golding et al., 2001; Kevers et al., 2011; Rössle et al., 2010). Triterpenes are secondary metabolites and important components in the wax of the cuticle. A study on grapes showed that the young grapes had higher level of triterpenoids, which gradually decreased during ripening, but the neutral triterpenoids showed a slight increase (Pensec et al., 2014). However, changes in the triterpene concentration of apples during the ripening on the tree and after different storage methods have not yet been thoroughly investigated.

The aim of this study was to evaluate the effects of harvest time, rootstock, and storage method on two major triterpenes in apple

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peel, OA and UA, to enhance the understanding of the compositional evolution of triterpenes during apple fruit ripening and postharvest storage, which can provide valuable knowledge for prebreeding projects and thereby a sound basis for applied breeding programs. In addition, correlations between different quality parameters were determined.

Materials and Methods

Fruit material. Twenty-four 'Aroma' trees, on two different rootstocks (12 trees on a semi-dwarfing 'MM.106' and 12 trees on a dwarfing 'M.9'), were randomly chosen and divided into three replicate blocks by row, with each block containing four trees in an IP-orchard in Kivik, southeastern Sweden (N 56°6'23'', E 14°40'57'') during 2012 and 2013 seasons. Trees had been planted in 1998 and trained as slender spindle. A similar orchard management program, including fertilization, mechanical weed control, and integrated pest management was applied. Trees were not thinned because they showed an acceptable yield and fruit size as well as very slight differences in their crop load.

Fruit were harvested at three times as following: early harvest on 12 Sept. 2012 and 10 Sept. 2013, middle harvest on 17 Sept. 2012 and 2013, and late harvest on 24 Sept. 2012 and 2013 from interior and perimeter of the canopies to show an actual presentation for the total tree yield. The three harvest times were used to reflect different fruit maturity, where the second harvest time was the commercial harvest maturity stage. Fruit harvesting date was estimated according to our experience with this cultivar (Tahir, 2006) using Streif Index {firmness [$\text{kg}\cdot\text{cm}^{-2}$] $\times$ [total soluble solid (%) $\times$ starch hydrolysis index (degradation stage)]²} and previous results (Streif, 1996). Firmness was measured on opposite, peeled sides, at the equator of each fruit, by using a penetrometer (model FT 327; Effigi, Italy, plunger diameter of 11.1 mm and depth of 7.9 mm) and results expressed as $\text{kg}\cdot\text{cm}^{-2}$. Starch index was evaluated by dipping a slide of equator part of apple in an iodine solution [15 g potassium iodide (Grade 99.5%; MERCK, Darmstadt, Germany) and 6 g iodine (Grade: 99%; VWR International AB, Stockholm, Sweden) per liter] for 1 min, and using a grading scale where 1 = full staining and 9 = no stain (Ericsson, 1982). Fruit were harvested in the both seasons when their Streif Index was 0.22–0.24 at the first harvest, 0.16–0.18 at the second harvest, and 0.11–0.13 at the third harvest. Six medium-sized apples were harvested uniformly (totally 72 apples at each harvest time for three blocks: 6 apples/tree $\times$ 4 trees/block $\times$ 3 blocks = 72 apples). Each block and each replicate within each block were analyzed separately. Twenty-four apples of each block were separated. The 24 fruits of each block with three blocks (totally 72 apples) were then divided into three groups: first group of eight apples in each block (totally 24 apples in three

blocks) were analyzed immediately, the second group of eight apples in each block (totally 24 apples in three blocks) were stored in regular atmospheric (RA) storage; 2–3 °C and 85% to 90% RH for 4 months in 2012 and 3 months in 2013, and the third group of eight apples in each block (totally 24 apples in three blocks) were stored in CA storage; 2 °C with 2 kPa O₂ and 2 kPa CO₂ and 90% RH for 4 months in 2012 and 3 months in 2013. CA storage conditions with computer-controlled gas composition were established in 350-L chambers (Nino-laboratory, Onsala, Sweden) 3 d before placing the apples in the chambers. Fruits were stored in RA and CA for the same time to clarify the actual storage effect. Skin color was measured on eight fruit in each block; the peel of these fruit was used for triterpenes analysis, while their flesh was used to measure the TSS, pH, and TA.

Fruit color measurement. Color was measured at three locations on both the sunny (the red side of the fruit, facing away from the tree trunk) and shadow side (the green side of the fruit, facing the tree trunk) of fruit, using a Minolta Chroma meter CR-200 (Konica Minolta Inc., Osaka, Japan) with an 8-mm-diameter window. Color was expressed as hue angle (h°) (where 0° = red-purple, 90° = yellow, 180° = bluish-green, and 270° = blue) and L as lightness (0 = black and 100 = white) (McGuire, 1992).

Preparation of samples for OA and UA analysis. From each group of 24 apples (first group at harvest, second group after RA storage, third group after CA storage), the apples were divided into four replicates per block, giving totally 12 replicates per sample (24 apples: 3 blocks $\times$ 4 replicates within each block = 12 replicates totally). Each apple was cut into 12 pieces and peeled carefully using a peeler. Each piece of peel was punched into 9-mm disks and 36 disks were collected for each replicate. Disks were stored in –20 °C until analysis. For OA and UA extraction, disks were dried in a freeze dryer for 24 h. Lyophilized disks were extracted with 8 mL ethanol for 1.5 h in an ultrasonic bath at room temperature. Thereafter, the extract solution was centrifuged at 18,000 g_n for 12 min. A 7000 HPLC system (Merck-Hitachi, Darmstadt, Germany) with an ultraviolet detector was used to separate and quantify OA and UA at 207 nm. A VYDAC column (Reverse Phase C18, CRT201 TP54; The Nest Group, Inc., Southborough, MA) was used for analysis with the mobile phase, methanol: acetonitrile: H₂O (40:40:20, v/v/v), with isocratic flow, at 1.5 mL·min^{–1} flow rate, and the injection volume was 10 µL. External standards OA and UA were purchased (Extrasynthese, Genay Cedex, France) and were used for the construction of a standard curve. OA and UA in the samples were identified by comparing retention times and spectra with the external OA and UA standards. The quantification of OA and UA concentrations in the samples was performed by calculation, comparing with the standard curve.

TSS, pH, and TA. From the apple flesh, 11 g juice was collected using a Waring blender

(Model 91-358, New Hartford, CT). Juice was centrifuged for 10 min at 5500 g_n and 2 mL supernatant was collected. The supernatant was mixed with 12 mL H₂O and titrated with 50 mM NaOH to endpoint pH 8.3 by using Titroline easy (SI Analytics GmbH, Mainz, Germany). TA values were presented as percentage malic acid (g/100 mL). TSS values were measured by thermostatic refractometer RFM 80 (Bellingham + Stanley Ltd, Tunbridge Wells, UK) and presented as percentage of sugar (g/100 mL).

Statistics. All statistics were performed by using Minitab 16.2.4.0 (Minitab Ltd., State College, PA). Data were presented as means $\pm$ SE of 12 independent replicates. Data were analyzed by general linear model to test the factor interaction, and statistical significance was considered at $P < 0.05$ by using Tukey's test. Evaluation of the year-to-year effect on fruit quality parameters for each rootstock was performed by analysis of variance.

Results

Rootstock effect on OA and UA concentrations at harvest time. Rootstocks affected OA and UA concentrations at different harvest times to different extent. At the first harvest in 2012, OA and UA in rootstock 'MM.106' were 61.9 and 416.3 µg·cm^{–2}, respectively, which were significantly higher than the concentrations in 'M.9' apples with 57.8 and 373.1 µg·cm^{–2}, respectively. At the second harvest in 2013, OA and UA levels in rootstock 'MM.106' apples were 53.3 and 327.4 µg·cm^{–2}, respectively, and they were significantly higher than the levels 48.8 and 296.5 µg·cm^{–2} found in 'M.9' apples (Table 1). The same trend was also found for OA and UA concentrations at the third harvest time in both 2012 and 2013. At the rest of the harvest times, no significant differences were found between the rootstocks in the OA and UA concentrations. At harvest time in 2012, the highest concentrations of OA and UA in 'MM.106' rootstock apples were 63.4 and 416.3 µg·cm^{–2}, respectively, while they were 59.7 and 380.9 µg·cm^{–2}, respectively, in 'M.9' rootstock apples. On average, both OA and UA concentrations were higher in 2012 than in 2013. The highest OA concentration in 2013 was 53.8 µg·cm^{–2}, which was 15.1% lower than the highest OA concentration in 2012. The same trend was found for the highest UA concentration in 2013, which was 21.4% lower than the highest UA concentration in 2012 (Table 1).

Harvest time and storage effects on OA and UA concentrations. The three harvest times reflect different maturity of the apples. In general, the harvest time showed inconsistent effect for OA and UA concentrations under different conditions.

At harvest time in 2012, different harvest times had no significant effect on the OA and UA concentrations. The OA concentration ranged from 59.8 to 60.2 µg·cm^{–2} and the UA concentration ranged from 385.9 to 394.7 µg·cm^{–2} (Table 2). After RA storage, fruit were harvested at the first and second harvest

Table 1. Oleanolic acid (OA) and ursolic acid (UA) ($\mu\text{g}\cdot\text{cm}^{-2}$) concentrations in peel of apple from rootstock 'MM.106' and 'M.9' at three harvest times in 2012 and 2013.

Triterpenes	Rootstock	2012			2013		
		First harvest	Second harvest	Third harvest	First harvest	Second harvest	Third harvest
OA	'MM.106'	61.9 $\pm$ 0.9 a ² α ³	60.7 $\pm$ 1.1 a α	63.4 $\pm$ 0.5 a α	52.6 $\pm$ 0.7 a α	53.3 $\pm$ 0.8 a α	52.3 $\pm$ 0.8 a α
	'M.9'	57.8 $\pm$ 1.0 b α	59.7 $\pm$ 1.3 a α	56.9 $\pm$ 1.0 b α	53.8 $\pm$ 1.4 a α	48.8 $\pm$ 0.7 b β	47.0 $\pm$ 0.6 b β
UA	'MM.106'	416.3 $\pm$ 5.3 a α	390.9 $\pm$ 4.7 a α	415.0 $\pm$ 3.5 a α	325.4 $\pm$ 4.6 a α	327.4 $\pm$ 4.6 a α	326.5 $\pm$ 4.5 a α
	'M.9'	373.1 $\pm$ 6.2 b α	380.9 $\pm$ 6.6 a α	364.2 $\pm$ 4.9 b α	319.7 $\pm$ 5.0 a α	296.5 $\pm$ 3.5 b β	290.5 $\pm$ 3.5 b β

²Different letters indicate significant differences between the two rootstocks at the same harvest times by Tukey test ($P < 0.05$).

³Different letters indicate significant differences between harvest times with the same rootstock by Tukey test ($P < 0.05$).

Table 2. Oleanolic acid (OA) and ursolic acid (UA) ($\mu\text{g}\cdot\text{cm}^{-2}$) concentrations in apple peel at harvest time, after regular atmospheric (RA) storage, and controlled atmospheric (CA) storage in 2012 and 2013.

Harvest time	Harvest/storage	2012		2013	
		OA	UA	OA	UA
First harvest	At harvest	59.8 $\pm$ 0.8 a ² α ³	394.7 $\pm$ 6.0 a α	53.2 $\pm$ 0.8 a α	322.5 $\pm$ 3.4 a α
	RA storage	59.1 $\pm$ 0.7 a α	400.3 $\pm$ 5.0 a α	51.7 $\pm$ 0.5 a α	323.8 $\pm$ 2.7 a α
	CA storage	58.7 $\pm$ 0.6 a β	390.1 $\pm$ 3.8 a α	48.3 $\pm$ 0.8 b β	318.0 $\pm$ 4.0 a α
Second harvest	At harvest	60.2 $\pm$ 0.8 a α	385.9 $\pm$ 4.1 b α	51.1 $\pm$ 0.7 a $\alpha\beta$	311.9 $\pm$ 4.3 a α
	RA storage	60.9 $\pm$ 0.7 a α	402.0 $\pm$ 3.4 a α	48.4 $\pm$ 0.7 b β	309.0 $\pm$ 4.0 a β
	CA storage	61.7 $\pm$ 0.6 a α	393.2 $\pm$ 2.9 ab α	48.0 $\pm$ 0.5 b β	311.1 $\pm$ 3.4 a α
Third harvest	At harvest	60.2 $\pm$ 0.9 a α	389.6 $\pm$ 6.1 a α	49.7 $\pm$ 0.7 a β	308.5 $\pm$ 3.4 a α
	RA storage	56.3 $\pm$ 0.8 b β	380.6 $\pm$ 5.4 a β	52.0 $\pm$ 1.0 a α	314.0 $\pm$ 3.9 a $\alpha\beta$
	CA storage	60.4 $\pm$ 0.6 a $\alpha\beta$	390.8 $\pm$ 3.5 a α	51.2 $\pm$ 0.9 a α	313.0 $\pm$ 4.6 a α

²Different letters indicate significant differences between the three treatments at harvest, after RA and CA storage within the same harvest time and the same year at $P < 0.05$ by Tukey test.

³Different letters indicate significant differences between different harvest times within the same harvest or storage conditions and the same year at $P < 0.05$ by Tukey test.

times showed higher OA and UA concentrations than fruit harvested at the third harvest time, while highest OA and UA concentrations were 60.9 and 402.0 $\mu\text{g}\cdot\text{cm}^{-2}$, respectively, in fruit of the second harvest time. After CA storage, a similar trend was observed that the highest OA and UA concentrations were found in the fruit of second harvest time, but no significant difference was found for UA concentration in fruit at different harvest times (Table 2).

At harvest time in 2013, the highest OA concentration (53.2 $\mu\text{g}\cdot\text{cm}^{-2}$) was found in fruit at the first harvest time, but no differences were found on UA concentration in fruit at different harvest times, with concentrations ranging from 308.5 to 322.5 $\mu\text{g}\cdot\text{cm}^{-2}$ (Table 2). After RA storage, OA concentration in fruit of the first and third harvest time showed higher value, 51.7 and 52.0 $\mu\text{g}\cdot\text{cm}^{-2}$, respectively, than that of the second harvest time with 48.4 $\mu\text{g}\cdot\text{cm}^{-2}$; UA concentration in fruit of the first harvest time showed the highest value with 323.8 $\mu\text{g}\cdot\text{cm}^{-2}$. After CA storage, the highest OA concentration, 51.2 $\mu\text{g}\cdot\text{cm}^{-2}$, was found in fruit at the third harvest time, and it was significantly higher than the values at the first and second harvest time, while no differences were found for UA concentration in fruit at the different harvest times (Table 2).

Neither RA storage nor CA storage did result in any difference in UA concentrations in any of the investigated years, but some differences were found for OA concentration (Table 2). In 2012, CA storage resulted in higher OA concentration (60.4 $\mu\text{g}\cdot\text{cm}^{-2}$) than RA storage (56.3 $\mu\text{g}\cdot\text{cm}^{-2}$) in fruit at the third harvest time; in 2013, RA storage resulted in higher OA concentration (51.7 $\mu\text{g}\cdot\text{cm}^{-2}$) than CA storage (48.3 $\mu\text{g}\cdot\text{cm}^{-2}$) in fruit at the first harvest time. For OA concentration at the

other cases in both years, no differences were found between RA storage and CA storage.

When comparing OA and UA concentrations in fruit at harvest time and after storage, a year-to-year difference was observed. For OA concentration in fruit in 2012, the value of the third harvest time after RA storage decreased by 6.5% as compared with the initial value of 60.2 $\mu\text{g}\cdot\text{cm}^{-2}$ at harvest time, while no differences were found between OA concentration at harvest time and after storage at the first and second harvest times (Table 2). For OA concentration in fruit in 2013, the value in fruit at the first harvest time after CA storage decreased by 9.2% as compared with that at harvest time with 53.2 $\mu\text{g}\cdot\text{cm}^{-2}$. A similar trend was found in fruit at the second harvest time, where both RA and CA storage apples showed reduced OA concentration by 5.3% and 6.1% as compared with 51.1 $\mu\text{g}\cdot\text{cm}^{-2}$ at harvest time. For UA concentration, the only difference between the value at harvest time and after storage was found in fruit at the second harvest time in 2012, where the UA concentration after RA storage increased by 4.3% as compared with that 385.9 $\mu\text{g}\cdot\text{cm}^{-2}$ at harvest time (Table 2).

TSS and TA. Different harvest times showed different effects on TSS of 'M.9' and 'MM.106' apples. At harvest time in 'M.9' apples, the highest TSS was 12.36%, which was found at the second harvest time in 2012, and 11.86% at the third harvest time in 2013, and the same trend was found after RA and CA storage (Table 3). Both at harvest time and after RA storage in 'MM.106', the third harvest time in 2012 and the second harvest time in 2013 showed highest TSS concentration (Table 3). The rootstock did not result in any clear tendency for TSS concentration (Table 3).

In general, the TA showed a decreasing trend from the first harvest to the third harvest time at harvest or after storage, although the differences were not always significant (Table 3). When the different rootstocks were compared, at harvest time, 'MM.106' apples showed higher TA concentration than 'M.9' apples in 2013, but the same trend was only found at the second harvest time in 2012. After RA and CA storage in 2012, 'MM.106' apples had higher TA concentration (0.69% and 0.83%) than 'M.9' apples (0.60% and 0.71%) at the third harvest time, and the same trend was found in 2013 (Table 3).

Fruit color. The hue angle of the sunny side was significantly smaller than that of the shaded side, which indicated that the sunny side was significantly redder than the shaded side (Table 4). At harvest time of 2012, the hue angle of the sunny side showed no difference between the three harvest times in 'M.9' apples, but on the shaded side, the third harvest time apples showed the smallest hue angle. For 'MM.106' apples at harvest time, lower values for hue angle were found for both the sunny and shaded side for the third harvest time, as compared with the first two harvest times. After RA storage and CA storage of 2012, the third harvest time of 'M.9' and 'MM.106' apples showed the lowest hue angle for both the sunny and shaded side, and similar tendency was found in 2013, though the third harvest values were not always significantly different from the second harvest (Table 4).

The two different rootstocks resulted only in slight differences concerning fruit color (Table 4). In 2012, the hue angle of 'M.9' apples' shaded side was larger than 'MM.106' apples at the third harvest time, both at harvest time and after RA storage, and the same tendency was found on the sunny

side of the third harvest time apples after CA storage. In 2013, the hue angle of 'MM.106' apples' sunny side at harvest time was larger than 'M.9' apples at the first and second harvest occasions, while no difference was found between the rootstocks after storage (Table 4).

Comparing the RA storage and CA storage, there was no difference in the hue angle, except for the third harvest time apples in 2012, where the RA stored 'M.9' apples had a significantly larger hue angle than the CA stored 'M.9' apples (Table 4, significances are not shown).

Correlation. Pearson correlation was used to express the relation of pairs of variables among triterpene concentration and fruit quality indexes. OA concentration was positively correlated with UA concentration both

at harvest time ($r = 0.917$; $P = 0.000$), after RA storage ($r = 0.908$; $P = 0.000$), and after CA storage ($r = 0.950$; $P = 0.000$) (Table 4). OA and UA concentrations were positively correlated with TA and TSS, and also with the color hue angle. OA and UA concentrations did not show any significant correlation with pH at harvest time, but after RA and CA storage, both OA and UA were positively correlated with pH (Table 5).

The year-to-year influence. The year-to-year influence was found to be significant for the OA and UA concentrations, fruit color, TA, and pH, with some exceptions. Year did not show significant effect on OA concentration at the third harvest time in 'MM.106' apples after cold storage (Table 6). It also did not show any significant effect on pH at harvest for the third harvest time in

'MM.106' apples and for the second harvest time in 'M.9' apples (Table 6). The year-to-year influence on hue angle of the 'M.9' apples was stronger than the influence on 'MM.106' apples. For the TSS, the year difference was significantly different in four cases of nine in 'MM.106' apples and seven cases of nine in 'M.9' apples (Table 6).

Discussion

Rootstock has been used for modifying the growth of deciduous fruit trees for a long time. Rootstocks can influence scion vigor, cropping, fruit quality, climatic adaptability, and susceptibility to pests and diseases (Ferree and Warrington, 2003; Webster, 1995). The results of this investigation showed that rootstock was the factor which gave the most consistent changes in the concentration of UA and OA of the investigated factors. In four of six harvest times (three per year), 'MM.106' gave higher UA and OA concentration than 'M.9'. A previous investigation of 'Pink Lady' apples on 'MM.106' and 'M.9' rootstocks showed differences in g_s , number of fruit, fresh weight of fruit, flesh firmness, and acidity between the two rootstocks (Talluto et al., 2008). Since the rootstock 'MM.106' has been described as semi-dwarfing while 'M.9' is described as dwarfing, different growth patterns impact hormones metabolism and translocation, supply of minerals and water, as well as light interception and photosynthesis production and thereby the size, quality, and anatomy of fruit and leaves (Gheyas et al., 1996; Tromp et al., 2005). However, the dwarfing growth of the rootstock is probably the effect of a range of complex interactions between different features and resulting in differences in multiple quality traits. Two rootstocks used in this investigation have been described as having different sensitivities to plant pathogens. 'M.9' has been described as susceptible to fire blight and woolly aphid, and 'MM.106' has been described as intermediate resistant to fire blight and resistant to woolly aphid, respectively (Rom and Carlson, 1987). Triterpenoids, and in some cases specifically OA or UA, have been linked to insecticidal and antifungal properties. Neem (*Azadirachta*

Table 3. Total soluble solids (TSS) and titratable acidity (TA) of apple fruit of three harvest times directly at harvest, after regular atmospheric (RA) storage, and after controlled atmospheric (CA) storage in 2012 and 2013.

Harvest/storage	Harvest times	TSS (%)			
		2012		2013	
		'MM.106'	'M.9'	'MM.106'	'M.9'
At harvest time	First	11.68 b ² α ²	11.48 b α	11.78 ab α	10.91 b β
	Second	11.73 b β	12.36 a α	12.03 a α	11.16 b β
	Third	12.64 a α	11.46 b β	11.24 b β	11.86 a α
After RA storage	First	12.83 ab α	12.53 a α	12.55 a α	11.97 ab β
	Second	12.48 b α	12.54 a α	12.61 a α	11.63 b β
	Third	13.60 a α	11.73 b β	11.20 b β	12.39 a α
After CA storage	First	12.63 b α	12.83 b α	13.23 a α	11.90 ab β
	Second	12.41 b β	13.69 a α	12.83 a α	11.59 b β
	Third	14.62 a α	12.10 b β	11.82 b β	12.24 a α

Harvest/storage	Harvest times	TA (%)			
		2012		2013	
		'MM.106'	'M.9'	'MM.106'	'M.9'
At harvest time	First	1.06 a ² α ²	1.02 a α	0.97 a α	0.88 a β
	Second	1.07 a α	0.98 a β	0.86 b α	0.79 b β
	Third	1.02 a α	0.97 a α	0.89 b α	0.79 b β
After RA storage	First	0.76 a β	0.82 a α	0.64 a α	0.64 a α
	Second	0.67 b α	0.62 b α	0.63 a α	0.62 a α
	Third	0.69 b α	0.60 b β	0.63 a α	0.56 b β
After CA storage	First	0.86 a β	0.99 a α	0.76 a α	0.68 a β
	Second	0.89 ab α	0.86 b α	0.74 ab α	0.72 a α
	Third	0.83 b α	0.71 c β	0.69 b α	0.61 b β

²Different letters indicate significant differences between three harvest times within the same treatment (harvest/storage type) and the same year at $P < 0.05$ by Turkey test.

³Different letters indicate significant differences between two rootstocks within the same treatment (harvest/storage type) and the same year at $P < 0.05$ by Tukey test.

Table 4. Fruit color on the sunny and shadow side of apple peel expressed as hue angle (h°) at harvest time, after regular atmospheric (RA) storage, and after controlled atmospheric (CA) storage in 2012 and 2013.

Harvest/storage	Harvest times	Hue angle (°)							
		2012				2013			
		Sunny		Shadow		Sunny		Shadow	
At harvest time	First	42.1a ² α ²	47.6 a α	101.3 a α	105.7 a α	52.9 a α	40.0 a β	87.7 a α	83.9 a α
	Second	47.4 a α	44.8 a α	106.4 a α	100.8 ab α	30.3 b α	24.9 b β	66.1 b α	68.5 b α
	Third	30.5 b α	41.1 a α	67.2 b β	93.7 b α	26.5 b α	23.2 b α	55.4 b α	58.1 c α
After RA storage	First	49.9 a α	55.8 a α	91.2 a α	98.4 a α	27.5 a α	35.9 a α	78.3 a α	74.5 a α
	Second	50.2 a α	54.1 a α	93.8 a α	90.6 b α	30.5 b α	25.4 b α	69.9 ab α	74.0 a α
	Third	31.4 b α	37.7 b α	64.0 b β	79.8 c α	23.5 b α	22.5 b α	58.8 b α	50.8 b α
After CA storage	First	52.1 a α	56.6 a α	92.2 a α	95.1 a α	40.9 a α	34.6 a α	83.5 a α	81.3 a α
	Second	52.6 a α	56.6 a α	94.0 a α	98.1 a α	31.1 b α	27.7 b α	71.0 b α	71.5 a α
	Third	24.4 b β	29.2 b α	63.2 b α	68.8 b α	22.9 c α	24.3 b α	63.8 b α	59.9 b α

²Different letters of a/b indicate significant differences between three harvest times within the same fruit side and the same year at $P < 0.05$ by Turkey test.

³Different letters of a/b indicate significant differences between two rootstocks within the same fruit side and the same year at $P < 0.05$ by Tukey test.

Table 5. Pearson correlation between oleanolic acid (OA) and ursolic acid (UA) and fruit quality at harvest time, after regular atmospheric (RA) storage, and after controlled atmospheric (CA) storage.

	Harvest/storage	UA	TA	TSS	pH	Hue-S	Hue-Sh
OA	At harvest time	0.917***	0.638***	0.374***	-0.111	0.344***	0.454***
UA			0.711***	0.343***	-0.135	0.339***	0.489***
OA	After RA storage	0.908***	0.401***	0.388***	0.606***	0.502***	0.396***
UA			0.401***	0.441***	0.756***	0.612***	0.503***
OA	After CA storage	0.950***	0.539***	0.361***	0.633***	0.432***	0.283***
UA			0.615***	0.366***	0.744***	0.508***	0.389***

TA = titratable acidity; TSS = total soluble solids; Hue-S = Hue angle on sunny side; Hue-Sh = Hue angle on shadow side.

Table 6. Differences between years shown by *P* value of one-way analysis of variance for each parameter of two rootstocks.

Parameters	'MM.106'								
	At harvest time			After cold storage			After ULO storage		
	First	Second	Third	First	Second	Third	First	Second	Third
OA	0.000	0.000	0.000	0.000	0.000	0.072	0.000	0.000	0.000
UA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Hue-S	0.039	0.000	0.340	0.019	0.000	0.004	0.023	0.000	0.348
Hue-Sh	0.017	0.000	0.058	0.030	0.000	0.269	0.068	0.000	0.906
TSS	0.703	0.212	0.000	0.076	0.684	0.000	0.020	0.145	0.000
TA	0.001	0.000	0.000	0.000	0.050	0.010	0.000	0.000	0.000
pH	0.000	0.036	0.782	0.000	0.000	0.000	0.000	0.019	0.000

	'M.9'								
	At harvest time			After cold storage			After ULO storage		
	First	Second	Third	First	Second	Third	First	Second	Third
OA	0.027	0.000	0.000	0.001	0.000	0.003	0.000	0.000	0.000
UA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Hue-S	0.039	0.105	0.000	0.000	0.000	0.000	0.000	0.000	0.011
Hue-Sh	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.000	0.022
TSS	0.017	0.000	0.065	0.042	0.002	0.012	0.005	0.000	0.554
TA	0.000	0.000	0.000	0.000	0.956	0.036	0.000	0.000	0.000
pH	0.013	0.947	0.000	0.000	0.000	0.000	0.000	0.000	0.000

OA = oleanolic acid; UA = ursolic acid; Hue-S = hue angle on sunny side; Hue-Sh = hue angle on shadow side; TSS = total soluble solids; TA = titratable acidity; ULO = ultra-low oxygen.

indica) as an insecticide contains a range of different triterpenoids. In commercial products, the active ingredient azadirachtin is used (Nisbet, 2000; Salehzadeh et al., 2002). UA isolated from *Bretonia salicina* was found to have antifungal activity against six investigated plant pathogenic fungi, though the minimum inhibitory concentration values varied (Mahlo et al., 2013). OA was found to have the highest antifungal activity against *Aspergillus*, *Candida*, and *Penicillium* sp., when several different compounds were isolated from *Ficus drupacea* (Yessoufou et al., 2015). Therefore, the higher concentration of OA and UA found in 'MM.106' in this investigation could possibly be linked to the previously described higher resistance to plant pathogens in the 'MM.106' rootstock than in 'M.9'.

Previous investigations have shown that rootstock can also influence the acidity of fruit. The fruit from trees with the rootstock of sour orange in 'Valencia' orange had higher TSS and TA (Economides, 1977), and the same result was found on 'Lapithkiotiki' lemon (Georgiou, 2009). A study on 'Delicious' apple showed that fruit from trees on 'M.27 EMLA' or 'M.9' had significantly higher TSS than fruit from trees on 'M.7 EMLA', 'MAC 9', or 'OAR 1', but this result was only shown in one of the investigated years (Autio, 1991). In the present study, the

fruit from rootstock 'MM.106' had higher TA concentration at harvest in 2013, and the same tendency was found in late-harvested fruit after storage in both investigated years, but no clear tendency was found on TSS concentration. Fruit maturity rate has previously been shown to be affected by rootstock (Castle, 1995; Rutkowski et al., 2005), and the difference in TA could be linked to differences in maturity processes between the scions on different rootstocks, since the acidity of the fruit is known to change during maturity. It has been reported that rootstock can change nutrient uptake and thereby control the tree size. Dwarfing rootstock 'M.9' has been found to have a higher proportion of nonfunctioning phloem as compared with 'MM.106' (Rom and Carlson, 1987). As translocation of some minerals, such as phosphorus, occurs in the phloem (Rom and Carlson, 1987), the nonfunctioning phloem in 'M.9' might reduce phosphorus transport. It has been reported that declined leaf phosphorus concentration was correlated with decreased juice TA (Dris et al., 1999). Therefore, fruit quality traits, such as TA concentration, might be also related to differences in tree mineral nutrition levels induced by the rootstock.

Harvest maturity has been considered a critical factor for storage of different fruits. Previous studies have confirmed that the

harvest maturity can influence fruit quality and phytochemical concentration. Studies on apples have found that early harvested fruit had higher firmness (Bulens et al., 2012; Kweon et al., 2013), and also higher TA, but lower TSS (Kweon et al., 2013). In accordance with this, in the present study at harvest in year 2012, and most often after RA or CA storage in both years, the fruit of the first or second harvest time had higher TA concentration, whereas the fruit of the second or third harvest time had higher TSS as compared with the first harvest time fruit at harvest. A previous study on the cuticular wax of 'Delicious' apple showed that the hydrocarbons and diols (more than half was UA) increased significantly during fruit development (Ju and Bramlage, 2001), but the wax compounds were detected just once per month from June to September, and the large increase of UA was found between August and September. Another study on apple showed that wax composition did not change much with different picking date (Veraverbeke et al., 2001). In our study, 'Aroma' apples, which were collected every week during the fruit ripening in September, showed inconsistent UA concentration of fruit of both rootstocks 'M.9' and 'MM.106' with different harvest times. At harvest, the first harvest time apples showed higher UA concentration than that at the second harvest time, but the third harvest time 'MM.106' apples also showed higher UA concentration. This result indicates that UA concentration could remain stable to some extent during the fruit maturation.

Effects of the two main storage methods, RA storage and CA storage, on fruit quality, including antioxidant concentration, have been widely studied. Some previous studies have confirmed that the triterpene concentration in apple fruit can show variability for different cultivars and also for different investigated seasons (Lv et al., 2015a; Morice et al., 1973). For example, a study on the surface wax composition of apple fruits showed that the petroleum ether-insoluble acids (PEIA; which were mainly UA according to the author) in three cultivars (Dougherty, Granny Smith, and Sturmer) reacted differently in RA storage. The PEIA in 'Dougherty' and 'Granny Smith' showed stable concentration or increased after 70-d RA storage; while for 'Sturmer', the PEIA decreased in the first investigated year and maintained relative stable in the second investigated year (Morice et al., 1973). Our previous study also showed that UA concentration in 'Gloster' remained stable after cold storage, while OA and UA concentrations in 'Aroma' were unchanged in one investigated year, but changed in the other investigated year (Lv et al., 2015a). In our present study, no difference was found between RA and CA storage in the UA concentration in any of the investigated years; while the OA concentration showed different response to RA and CA storage in the two investigated years. After RA and CA storage, some decreased values were found for OA concentration as

compared with the value at harvest time. The postharvest changes of most wax components have been found to be associated with ethylene production and ripening (Dong et al., 2012), though in this investigation the storage methods seemed to have quite small influence on maturation processes that affected the UA concentration, but some influence was observed for OA concentration.

Year-to-year variations in the climate are known to influence different quality traits. For example, in apple, TSS, TA, flesh firmness, and color have been found to vary between years (Robinson et al., 1991; Rutkowski et al., 2005). In this investigation, higher average concentrations of OA and UA as well as higher levels of TA were found in 2012 but not in 2013, while the hue angle showed that the apples were redder (lower values of hue angle) in 2013 than in 2012. These recorded differences between years might indicate different climate conditions and cultivation effects between the 2 years and could explain the different pattern in OA and UA levels during the ripening period, as registered at the three harvest times. Strong and complex interaction between rootstock and climatic conditions and also the year-to-year variations have been reported for Japanese plum quality (Daza et al., 2008). The present result also confirmed the effect of complex interaction for rootstock/storage method and harvest time/storage method on triterpenes concentration in apple peel.

Conclusions

The results showed that in the investigated cultivar, the effects of the rootstock gave the largest and most consistent differences on the concentrations of the OA and UA, while storage and harvest time (reflecting ripening stage) gave minor and inconsistent changes. The rootstock 'MM.106' should be considered as a good resource if higher concentrations of triterpenes should be promoted as compared with rootstock 'M.9'. Different harvest time showed minor effect on OA and UA concentrations in 'MM.106' apples, while if 'M.9' apples are consumed directly after harvest, the first harvest time gave higher OA and UA concentrations. Apples harvested at the second harvest time (commercial harvest) can keep stable or increased OA and UA concentrations after RA and CA storage as compared with the levels at harvest time. For the early- or late-harvested apples, OA and UA concentrations in different rootstocks might show inconsistent changes after storage. In conclusion, previous results demonstrating major significant differences between three investigated apple cultivars in the concentration of UA and OA (Lv et al., 2015b), together with present results indicate that there is a potential to use the variability of scion/rootstock genotype combination. The relative stability in the concentration of UA and OA while subjected to changes in harvest time and environmental factors, during the two investigated years and different storage

methods are also good prerequisites for successful breeding for increased levels of these desirable quality traits.

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