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The Effect of Ethylene Inhibition on "Ildrød Pigeon"

Apple Quality and Aroma Profile

PHD THESIS · 2012
Marta Jolanta Popielarz

The effect of ethylene inhibition on 'Ildrød Pigeon' apple quality and aroma profile

Ph.D. Thesis by
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Many of life's failures are men who did not realize how close they were to success when they gave up.

Thomas Edison

Preface

This Ph.D. project has been conducted in the Quality and Technology research group at the Department of Food Science, University of Copenhagen. Therefore grateful acknowledge goes to the University of Copenhagen for the funding sources that made my Ph.D. work possible.

Ph.D. project was carried under supervision of Mikael Agerlin Petersen and Torben Bo Toldam-Andersen. Mikael, thank you for sharing your 'aroma' experience with me. You were always organized and ready to help. The dissuasions with you were eyes opening, productive and inspiring. Thank you for your interest in my life and the help you showed during 'financial crises'. Torben, your advices and comments were always interesting and stimulating. Thank you for being direct and straight to the point at our meetings. Thank you for your time, which you spent on reading and 'tables/figures' check out. To both, Mikael and Torben, I send you my gratitude for the patience and the encouragement you have given me.

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I am grateful to Margaret for proof reading of the manuscript and also for being a good friend. Anders, thank you for a nice atmosphere in our office and for your 'jokes' - you remain 'the funniest guy in the world'. Frans, thank you for your magic powers - meaning statistic support and most of all for your patience. Jose, thank you for being a great colleague, who was always ready to listen and explain whatever needed. Abdel (my ethylene man) and Christina (my handyman lady), thank you for your great lab help – I promise to improve my baking skills.

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Thank you All

Marta Jolanta Popielarz
January 2012

SUMMARY

In recent decades, producers have observed an increasing trend of consumer demands for high quality stored apples. Apple quality involves many aspects including external appearance (color), as well as internal parameters namely firmness and flavour (taste and aroma). Development of suitable pre- and postharvest management that improves and maintains quality is a challenge. The objective of the presented thesis is to explore the practical approach to optimize horticultural management, and thus to ensure better overall apple quality after storage.

'Ildrød Pigeon' is a Danish apple cultivar, which market potential strongly depends on its distinct red color and characteristic flavour. There is however, no available literature about 'Ildrød Pigeon' aroma profile and only a little about changes in quality depending on horticultural practices. 'Ildrød Pigeon' apples are traditionally exposed to light for up to 2-3 weeks after harvest to ensure development of fruit red skin color. The length of light exposure is critical as apples can be sunburn, become overripe or even rotten, and firmness is strongly reduced. These exposed apples have impaired storability as they reach storage already very ripe.

The registration of the ethylene inhibitor SmartFresh® (1-MCP, 1-methylcyclopropene) in Denmark opened new alternatives to preserve apple quality and storability. 1-MCP binds to ethylene receptors and restrains ethylene action. This in turns affects ripening related processes like; color changes, softening, starch degeneration to sugar, acid degradation and aroma development. 1-MCP has been considered a "magic bullet" in postharvest technology as apple quality is maintained during storage, especially firmness. However, the suppression of aroma production, which contributes most to apple flavour, is one of 1-MCP drawbacks.

The experiments included in this PhD thesis were conducted to explore a range of the most important horticultural factors that affect final apple quality. Consequently, an optimized postharvest management is proposed with revised harvest time, new 1-MCP timing application and shorter light exposure period. As the outcome, apples with better quality and improved market potential were obtained. The color, ethylene production, aroma profile and firmness, which are 'Ildrød Pigeon' major quality contributors, were most important parameters in the experiments.

Outline of the thesis. The PhD thesis consists of 11 chapters;

Chapters 1-4 present an overview of already existing knowledge about apple physiological processes, especially during ripening. The role of ethylene in ripening, existing ethylene inhibitors and their properties are also described.

Chapter 5 refers to the popular ethylene inhibitor (1-MCP), describes its influence on apple quality parameters and includes a short explanation of the registration of the inhibitor in Denmark.

Chapter 6 introduces 'Ildrød Pigeon' as an apple variety with its traditional postharvest handling. GC-olfactometry technique and the detection frequency methods are described,

as they were used to identify 'Ildrød Pigeon' characteristic aroma compounds. Twelve aroma compounds (esters, aldehydes and alcohols) were identified as having the greatest impact on the overall flavour of the 'Ildrød Pigeon' apples.

Chapter 7 describes the effects of pre-harvest factors; mainly thinning practice (cropping level) and nitrogen fertilization, on 'Ildrød Pigeon' fruit growth and quality after storage. Maturity was delayed in the apples from non-fertilized trees as they had low 'fruit under' to 'fruit on the tree' ratio and low ethylene production after storage. Ethylene production decreased with higher crop load. Hand thinning practice led to an 8.4% increase in fruit size. There was also positive effect of nitrogen on apple size. Most of the principal aroma compounds were insensitive to nitrogen levels, however hexanal, 2-methyl-2-pentenal and isomer forms of 2,4-hexadienal had higher production in apples fertilized with moderate nitrogen level, while ethyl acetate and 1-heptanol were produced at the highest level in apples fertilized with high nitrogen.

In **chapter 8** the optimal length for ripening recovery after storage has been established between 5 to 8 days. The appropriate time at room temperature after cold storage is needed mostly to accelerate ripening and to enhance aroma development to meet consumer's quality demands.

The effects of harvest time, storage length in combination with 1-MCP treatment were described in **chapter 9**. The commercially harvested 'Ildrød Pigeon' apples seem to represent post-climacteric ripening stage; with decline ethylene concentration and lower firmness. 1-MCP application on those apples was not effective as its application after harvest was done when ethylene production already accelerated. Longer storage positively affected regeneration of volatile compounds. Harvest of 'Ildrød Pigeon' apples should be done earlier to delay the ethylene formation so that 1-MCP application can give anticipated responds.

Chapter 10 describes effects of postharvest light exposure combined with different timing of 1-MCP application on external and internal quality parameters. Due to 'Ildrød Pigeon' progressive softening during postharvest handling, it was desirable to optimize postharvest practice in a way, which assures adequate color development with a shorter postharvest sunlight exposure to achieve the better quality fruit. The main coloration occurred between day 2 and 4 of the light exposure. Color changes were inhibited by early 1-MCP application. Finally, sunlight exposure time was proposed to be shortened to an average of 8 days, after which 1-MCP should be applied. Apples exposed to a shorter light period and treated late with 1-MCP remained firmer, had lower weight loss and aroma composition similar to untreated apples. Early 1-MCP application strongly reduced many aroma compounds, including principal esters. The effect of 1-MCP on aroma is critical to the optimization of 1-MCP postharvest technology, as apple flavour will rely on the successful delay, but not complete inhibition, of the ripening processes.

Chapter 11 summarizes the results and gives general conclusions from performed experiments.

The results from several investigations carried out during the PhD study have been presented in the papers submitted to the international scientific journals (PAPER I-IV) or included in the conference proceedings (PAPERS V-VI).

List of publications

PAPER I *(submitted to HortScience)*
The pre-harvest management effects on fruit growth and aroma profile
By M. J. Popielarz, R. M. Callejón, T. B. Toldam-Andersen, M. A. Petersen

PAPER II (*submitted to The European Journal of Horticultural Science)*
Effect of light exposure and inhibition of ethylene action on the postharvest colour and quality of apples
By M. J. Popielarz, F. van den Berg, M. A. Petersen, T. B. Toldam-Andersen

PAPER III (*under preparation to submission to The American Journal of Horticultural Science)*
Timing of 1-MCP application and postharvest light exposure as tools to optimize aroma in apple
By M. J. Popielarz, R. M. Callejón, T. B. Toldam-Andersen, M. A. Petersen

PAPER IV
Comprehensive analysis of chromatographic data by using PARAFAC2 and PCA
By Amigo J.M., Popielarz M.J., Callejón R.M., Morales M.L., Troncoso A.M., Petersen M.A., Toldam-Andersen T.B. (2010)
In Journal of Chromatography A, 1217(26):4422-4429

PAPER V
Influence of light exposure after harvest on aroma profile of apples – 'Ildrød Pigeon'
By M. J. Popielarz, M. A. Petersen, T. B. Toldam-Andersen (2010)
In *Recent Advances in Food and Flavour Chemistry*. C.T. Ho, J.Mussinan, F. Shahidi and E. tratras Contis (Eds.). Proceedings of the 12th International Flavor Conference, Skiatos, Greece, pp.218-219.
http://pubs.rsc.org/en/content/chapter/bk9781847552013-00218/978-1-84755-201-3

PAPER VI
Influence of ethylene-blocking action, harvest maturity and storage duration on aroma profile of apples ('Ildrød Pigeon') after storage
By M. J. Popielarz, M. A. Petersen, T. B. Toldam-Andersen (2010)
In *Expression of multidisciplinary flavour science.* I.Blank, M.Wüst, Ch.Yeretzian (Eds.). Proceedings of the 12th Weurman Flavour Research Symposium. Interlaken, Switzerland, pp.211-214.
http://curis.ku.dk/ws/files/32643288/Weurman_2008_Popielarz_et_al.pdf

CONTENT

1. PHYSIOLOGY OF THE APPLE FRUIT

Apple fruits go through several growth stages until they become an edible product with acceptable quality. These physiological processes in fruit development include; cell division, seed development, cell enlargement, maturation and ripening.

1.1. Cell division and enlargement

Cell divisions and enlargement processes are crucial for the increasing weight and volume of fruits (Fleancu 2007). Cell division initiates fruit growth. The length of the cell division period varies depending on cultivar and growing conditions, but occurs within few weeks after blossoming. In 'Granny Smith' the cell division period was completed within 3 weeks of full bloom while for 'Cox' and unthinned 'Miller's Seedling' it lasted 6-7 weeks. Finally, cell division of thinned 'Miller's Seedling' was not completed before a period of 12 weeks (Denne 1960). It shows that an early pre-harvest thinning management, removing flowers/fruitlets from the tree to reduce internal competition for nutrients and assimilates, has an effect on apple physiology and growth. With increasing fruit/leaf-ratio both the fruit size and the concentration of dry matter and titratable acids in apple fruits decrease (Hansen 1977;Hansen 1993).Cell division is therefore the most critical phase for fruit number and size potential as fruits at that point are the most sensible to stresses and changes in growth conditions (Toldam-Andersen, oral communication).

Cell enlargement continues through and after the cell division until harvest. Nutrients, which support growing fruit come to a major extent from allocation of assimilates from the photosynthetic active leaves, particularly from leaves nearest the fruit (Hansen 1977). A limited photosynthesis takes place also in immature fruits which contain chloroplasts and are exposed to the light (Pavel & Dejong 1993). The products of photosynthesis are carbohydrates, which are used during cell growth and respiration. Fruit photosynthetic activity decreases to very low levels with the progressive fruit development and chlorophyll degradation (Fleancu 2007).

1.2. Maturation and ripening

Maturation and ripening are important stages of physiological growth for fruit chemical quality. Maturity is a stage of development, at which a plant or plant part possesses the prerequisites for use by consumers for a particular purpose and a certain level of maturity is needed to ensure proper completion of ripening (Kader 2002). The ripening is a physiological process, which refers to the stage of fruit development when it is ready for consumption. Ripening is the period from the final stage of maturation

through the early stage of its senescence, where fruit become over ripe and eventually decay (Watada et al. 1984).
The maturation starts with carpel changes and ends with full fruit expansion. Fruit size is genetically determined but is also affected by leaf area development, light intensity, photosynthesis products distribution and competition between shoots and fruits (Jackson 2003). Poor access to light on the tree results in lower photosynthetic potential of spurs, reduction in cell number and size of fruits. By thinning and canopy management procedures it is possible to reduce competition for assimilates, which allows remaining fruit to expand their cells, resulting in minor increasing fruit size and increased internal quality (Jackson 2003). By performing early thinning (at flowering stage) cell division and fruit size potential are affected, and by late thinning small effect on size is achieved but accumulation of assimilates (fx. sugar) is higher because of decreased fruit competition (Toldam-Andersen, oral communication).
The changes which occur during maturation can serve as indicators of maturity to evaluate potential harvest time (Fleancu 2007;Kader 2002). Fruits harvested late have lower storability and are more susceptible to physiological disorders (Fleancu 2007;Kader 2002). Immature fruits harvested too early are more resistant to mechanical damages but have lower edible quality, whereas fruits harvested overripe, have shorter shelf-life, become softer, maybe even mealy, and have declining aroma production. Fruit therefore has to be 'optimally' mature at harvest to have potential to develop the best edible quality (Preece & Read 2005). Optimal harvest time is then defined by maturity indices developed for each crop and cultivar.

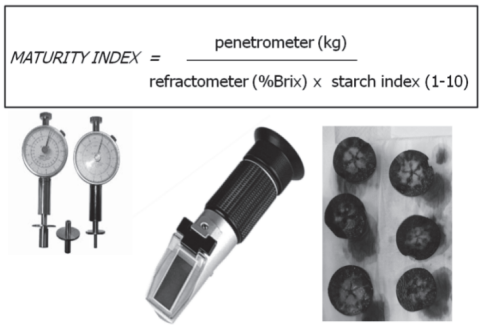


Figure 1 The equation for the 'Streif' maturity index based on firmness, soluble solid content and starch (Plotto et al. 1999)

Apple maturity is often evaluated based on firmness, soluble solid and starch content (Plotto et al. 1995;Preece & Read 2005). The evaluation of firmness and sugar can be easily done in orchard conditions using a simple penetrometer (kg) and a refractometer (% Brix, total soluble solids, TSS). Starch is commonly evaluated by dipping apple-halves in an iodine-iodine-potassium solution (Kader 2002). Starch in contact with iodine change color to black-blue and is evaluated with reference to a standard international scale from 1 to 10. Starch in apples is accumulated during the earlier phases of maturation and with the progressive maturation starch breaks down to fructose, glucose and sucrose (Kader 2002). When dipping centre cross-sectioned apple fruit halves in iodine-iodine-

potassium solution it can be easily seen how much starch remains in the apple. Each apple variety has individual levels of above-mentioned parameters when ready to harvest. Mature apples might be evaluated by individual parameters but an improved prediction is achieved, when several factors are combined into so called 'maturity index' (Preece & Read 2005), Figure 1). The maturity index for 'Ildrød Pigeon' is 0.17, while for 'Golden Delicious' and 'Elstar' is 0.10 and 0.30, respectively- Håndbog for frugt- og bæravlere (2006).

The ripening period is characterized by chemical and structural changes in the fruit leading to edible quality development. Fruits have different ripening mechanisms. For this reason, fruits can be divided into climacteric and non-climacteric. Climacteric fruits, like apples, pears, kiwi and peaches, are characterized by the increasing ethylene concentration and associated rising respiration rate in the ripening phase.

Non-climacteric fruits, like grapes, berries and citrus, show no changes in respiration rate during development and their ethylene production remains at a low level (Payasi & Sanwal 2010). Scientific evidence suggests however that low ethylene in non-climacteric, citrus fruits has some regulatory effect in the ripening (Barry & Giovannoni 2007).

In apples the ethylene burst comes just before the maximum respiration peak whereas in pears they occur simultaneously (Jackson 2003). Preclimacteric apples produce 0.1µl/kg/h ethylene and production increases markedly, up to 1000 fold, with ripening (Martinez-Romero et al. 2007). Depending on apple cultivar, concentration of endogenous ethylene varies from 25 to 2500µl/L in air (Burg & Burg 1962). Climacteric fruits are very sensitive to ethylene and when its synthesis in tissues exceeds 0.1µl/L, they immediately respond (Martinez-Romero et al. 2007). The processes related to ripening will be discussed in detail in chapter 3.

2. *ETHYLENE - A PLANT HORMONE*

Almost all kinds of plants produce ethylene in diverse concentrations and plant cells show different sensitiveness to it. Furthermore, it has been shown that ethylene takes part in a wide range of developmental responses (Khan & Singh 2007a;Khan & Singh 2007b;Lelievre et al. 1997;Sisler et al. 2006). Thus, ethylene plays an active role in seed germination, tissue differentiation, formation of roots and shoots, root elongation, lateral bud development, flowering initiation, anthocyanin synthesis, flower opening and senescence, pollination, fruit degreening and ripening, texture changes, volatile production (aroma formation in fruits), leaf and fruits abscission as well as reaction to biotic and abiotic stresses (Abeles et al. 1992;Sisler et al. 2006).

2.1. Ethylene – brief history

Probably the first who observed the effect of ethylene were Egyptians and Chinese. Egyptians used it to stimulate the ripening of figs (Sisler et al. 2006) and the Chinese noticed that pears ripened better in the near surrounding of burn incense. However, the earliest action of ethylene, which is known nowadays, was explained by Giarardin in 1864. He reported defoliation of trees near a leaking gas line and established that ethylene was one of the gas components. However, an effect of ethylene on plant development was reported, for the first time, by the Russian student Dimitry Neljubow in 1901. He was working with pea seedlings and observed that the epicotyl was short with a large diameter and horizontal germination when grown in lab conditions. Later he found out that coal gas was used for lamp light resulting in ethylene release as a side product, which caused this 'triple-response effect' in peas.

Further observation was described in 1910 when Cousin reported ethylene synthesis in oranges. He noticed that when oranges and bananas were transported together it did cause premature ripening of bananas. Additional, studies showed on the other hand that it was *Penicillium* mold on oranges, which produced ethylene (Abeles et al. 1992;Khan 2006). These observations started the discussion about the real effect and source of ethylene. Initially ethylene was recognized as an auxin by-product (Abeles et al. 1992;Khan 2006). Nevertheless, in 1934 ethylene started to be considered as a plant hormone. It was possible based on Gane's studies. He proved, by collecting gasses developed from 27kg of ripening apples for 4 weeks, that ethylene was a natural plant product (Khan 2006). That time there were still many skeptical opinions about ethylene as a plant regulator as there was no possible ways to detect ethylene production. More and more attention was paid to ethylene in the 1960's with the developing gas chromatography identification. It was shown then that ethylene production is associated with the respiratory climacteric peak and other ripening processes (Sisler et al. 2006).

2.2. Ethylene biosynthesis pathway

Ethylene (ethene) is a two-carbon compound with one double bond and a molar mass of 28.05. It is a flammable and colorless gas, which is soluble in lipids and water (Abeles et al. 1992). Ethylene in plants is synthesised in two systems described by McMurchie (1972). System I (auto-inhibitory system) functions during normal growth, and maturity development in pre-climacteric fruits, in the way that endogenous ethylene inhibits its synthesis. System II operates during fruit ripening and senescence. The main characteristics of this autocatalytic system is that ethylene production is ethylene induced (Oetiker & Yang 1995). The factor(s) affecting the transition from system I to II are yet not described. System I and II control ethylene production in climacteric fruits, whereas system I only in non-climacteric fruits (McMurchie et al. 1972). An ethylene burst in system II can be suppressed in fruits by storage under controlled atmosphere with low O_2 (inhibits ethylene synthesis and action) and high CO_2 (inhibits ethylene action), or by chemical ethylene inhibitors such as 1-MCP but it needs to be applied early when fruits are still in the pre-climacteric stage with low ethylene production (Oetiker & Yang 1995). Ethylene is stored in cells in the form of its precursor 1-amino-cyclopropane-1-carboxylic acid (ACC). Under stress or when triggered by a physiological signal ACC is converted to ethylene (Ferree & Warrington 2003). Shang Fa Yang established the synthesis pathway of ethylene, which includes transformation of methionine (MET) via S-adenosylmethionine (SAM or S-AdoMet) to ACC (Yang & Baur 1969). Methionine is additionally regenerated in the Yang cycle (Adam & Yang 1979).

Step 1 – Methionine (MET) conversion to S-adenosylmethionine (SAM)

Methionine (MET) has been established as the ethylene precursor in all higher plant tissues (Adams & Yang 1981). **SAM synthetase** catalyzes the transformation of MET to SAM. The conversion is energy-dependent and requires oxygen. With the rising respiration rate ATP is provided, which leads to higher ethylene production, even with a small pool of methionine (Barry & Giovannoni 2007;Martinez-Romero et al. 2007). In general MET is not occurring in plant cells in high amounts but it is recycled via 5'-methythioadenosine (MTA) from step 2.

Step 2 - S-Adenosylmethionine (SAM) conversion into 1-Aminocyclopropane-1-carboxylic acid (ACC)

ACC syntase (ACS) was suggested to be the rate-limiting enzyme in the ethylene synthesis. Its concentration rises when climacteric fruits are exposed to ethylene, wounding or water stresses (flood and drought). ACS was identified in both climacteric and non-climacteric fruits (Oetiker & Yang 1995). ACS is an unstable enzyme, encoded by a multi-gene family. In tomatoes 9 ACS genes were found (*LeACS1A, LeACS1B* and *LeACS2-8*). Among those, *LeACS1A* and *LeACS6* are expressed in tomato before ripening and *LeACS2* and *LeACS4* are highly correlated with the ripening processes (Oetiker & Yang 1995). However studies on tomato mutants (never ripe -Nr and ripening inhibitor -rin) have shown that only *LeACS2* is ethylene regulated while the others were unaffected (Oetiker & Yang 1995). One of the ACC derivatives in the ethylene biosynthesis pathway is malonyl-ACC (MACC). MACC synthesis plays a role in the ethylene autoinhibitory

system (Abeles et al. 1992). MACC might be responsible for low ethylene levels in pre-climacteric fruits (Bleecker et al. 1998;Lelievre et al. 1997).
In step 2 also 5'-methythioadenosine (MTA) is produced as a side product of the MET to SAM reaction. MTA is used to regenerate methionine (Yang & Baur 1969).

Step 3 – 1-Aminocyclopropane-1-caroxylic acid (ACC) conversion into ethylene
The enzyme ACC oxidase (ACO) takes part in the final step of the ethylene pathway, therefore it is also called "ethylene forming enzyme (EFE)" (Abeles et al. 1992). In apples and tomato fruits ACO is located in the external plasma of the cell wall of fruit tissues (Ramassamy et al. 1998). It is oxidizing ACC to ethylene in an oxygen dependent reaction. In anaerobic conditions ethylene formation is suppressed. ACO is encoded, like ACS, by a family of genes. In tomato five ACO genes were found; LeACO1-5 (Oetiker & Yang 1995). LeACO1 and LeACO4 increases in transcript abundance mostly during fruit ripening and can be blocked by 1-MCP, which indicates their regulation by ethylene. ACO production increases in the pre-climacteric stage, or by wounding and during senescence (Oetiker & Yang 1995).

2.3. Ethylene binding to the receptors

To understand the action of ethylene, which causes the response in plants, the concept of ethylene receptors was investigated. It was proposed that the receptor has an active centre containing a copper ion and amino-acid ligands in a hydrophobic packet (favourable condition for copper stabilization) (Bleecker et al. 1998;Dal Cin et al. 2006;Rodriguez et al. 1999). The Burgs (1962) were the first to propose that the ethylene receptor acts *via* a metal. They reported that compounds such as ethylene and propylene, butene (ethylene agonists) bind to silver ions in the same order as their ability to induce ethylene responses in plants (Burg & Burg 1962). Ethylene, by accepting electrons from the putative metal, changes the charge distribution and possibly starts the rearrangement of ligands associated with the metal (Bleecker 1999;Bleecker et al. 1998;Rodriguez et al. 1999). In other words, ethylene binding to a receptor may structurally rearrange it and serves as the initial signal to the transduction pathway (Figure 2).

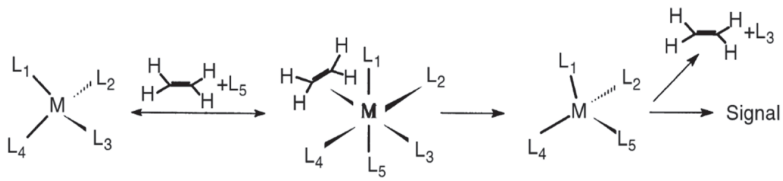


Figure 2 Possible mechanism of ethylene perception via the rearrangement of ligands in the active metal centre – the first step in the signal transduction pathway presented in Khan book (2006) proposed elsewhere (Clark et al. 1998;Rodriguez et al. 1999).

In apples, so far, two ethylene receptors have been isolated and their expression pattern recognized for early fruit development and abscission (Dal Cin et al. 2006). The theory about the ethylene regulatory system and receptor structure is primarily based on extensive research using Arabidopsis as model plant (Bleecker 1999;Bleecker et al. 1998). In Arabidopsis five receptors were identified and their action was explained by a **negative regulation model**. These functional receptors are divided into two sub-families based on sequence and structural similarities of the proteins; ETR1 and ETR2. Subfamily 1 contains ETR1 and ETS1 and subfamily 2 contains ETR2, EIN4 and ERS2. ETR1 and ERS1, which are placed in the endoplasmic reticulum, react directly on CTR1. CTR1 is the main negative ethylene regulator, which acts downstream from the receptors. The rest of the receptors (ETR2, EIN4 and ERS2) act more indirectly and less effective on CTR1.
Receptors are in the active (inhibitory) state in the absence of ethylene. In this mode, they allow active CTR1 protein to inactivate EIN2 (Figure.3a).
When ethylene is present - receptors are inactivated. They modulate CTR1 to 'off' mode and reverts EIN2 to its active form (Figure.3b). EIN2 initiates the activity of various responses in plants probably by a second messenger in the cell, which stimulates transcription of genes involved in ethylene responses (Bleecker 1999;Bleecker et al. 1998;Prange & DeLong 2003)
When dominant alleles (etr1-1, ein4-1) remain active, they are still keeping CTR1 in 'off' mode. Allele etr1-1 acts directly on CTR1 (Figure.3c) while ein4-1 is able to keep CTR1 activated indirectly via ETR1 and ERS1 (Figure.3d). ETR1 and ERS1 without supportive receptors (Figure.3e) or the supportive receptors without primary signalling receptors (Figure.3f) are incapable of maintaining sufficient activation of CTR1. EIN2 is then able to initiate ethylene response even if there is insufficient amount of receptors to keep active CTR1- but the response to ethylene might be weaker.

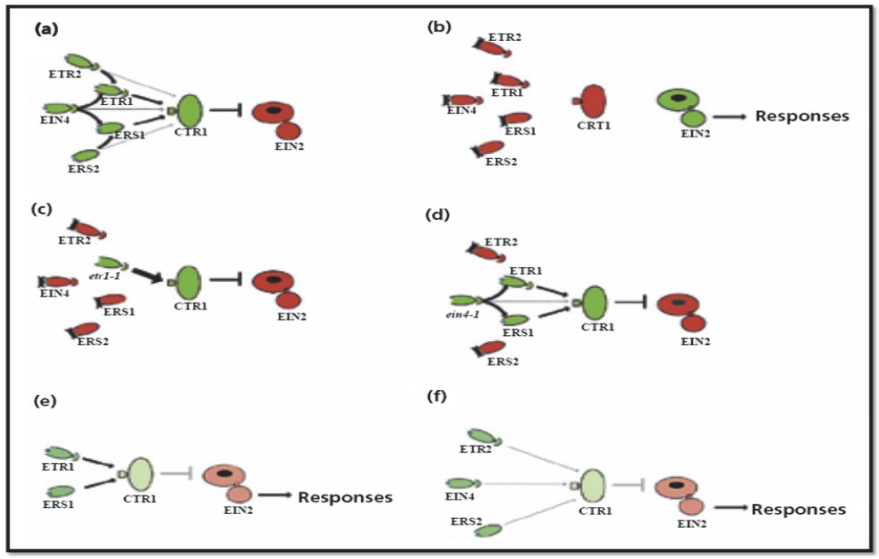


Figure 3 Model of ethylene action – explanation in the text. Red color presents inactivated forms and green color presents active receptors (Prange & DeLong 2003); CTR1 (constitutive triple response) is a negative regulator of the signal transduction pathway, EIN2 (ethylene insensitive) is a ethylene response initiator, ETR1 and ERS1 are primary signalling receptors, ETR2, EIN4 and ERS2 are subfamily 2 receptors.

3. *ETHYLENE INVOLVEMENT IN THE RIPENING PROCESSES*

The plant hormone ethylene takes part in many developmental processes in plants. However, its function in the activation of processes, which lead to the development of edible fruit quality during ripening, is the focus here. It can activate biochemical changes, which renders fruit attractive (Martinez-Romero et al. 2007). On the other hand, ethylene with progressive ripening also leads to loss in quality and to senescence (Figure 4). Therefore, it is also called a "plant aging agent". In this chapter, the focus is on the positive role of ethylene in the ripening processes.

The main changes associated with ripening in climacteric fruits include; softening, sugar accumulation and acid degradation, color changes (loss of green pigments and formation of carotenoids and anthocyanins) and aroma development (Martinez-Romero et al. 2007).

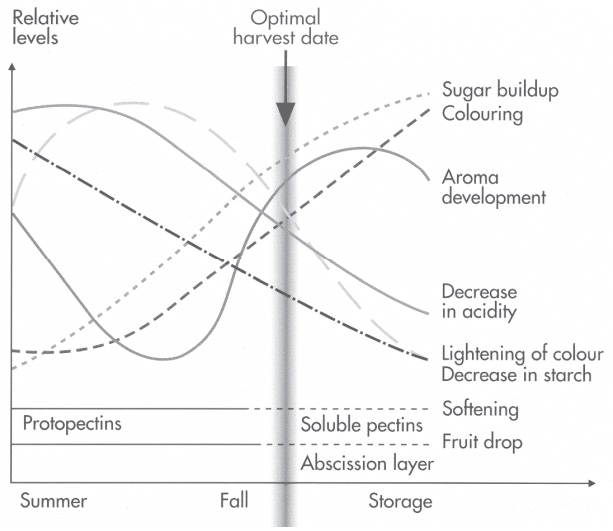


Figure 4 Physiological changes during climacteric fruits growth and storage (Werth 1997)

3.1. Texture

In fruit, climacteric and post-climacteric stages are accompanied with softening processes changing the texture (firmness) which is a very important determinant of quality and consumer acceptability (Payasi & Sanwal 2010). Texture is a creation of plant cell composition and their structure. Cell walls in fruits is a polysaccharide matrix; it consists of cellulose microfibrils embedded in a complex of hemicelluloses and pectin (Cosgrove 2001). Pectic polymers are constituents of the middle lamella, called 'the cell bonding agent', which is important for maintaining cell to cell adhesion (Cosgrove 2001;Harker et al. 1997). An increasing fraction of water soluble pectin is associated with softening in apple (Knee et al. 1975). There is however a number of wall modifying

enzymes taking part in this process; exo- and endo-polygalacturonase (PG), pectin methyl esterase (PME), glycosidases and rhamnogalacturonase (Johnston et al. 2002). PG enzyme causes pectin solubilisation and PG treatment of unripe apple discs caused disruption in the middle lamella similar to those observed in ripe apples (Ben-Arie et al. 1979). However experiments on transgenic tomatoes have given evidence that PG is not initiating softening (Giovannoni et al. 1989). The role of the PME enzyme, was considered to be modification of pectins in a way that they are easy to solubilise and depolymerise by other enzymes (Wakabayashi 2000). PME is therefore believed to have an indirect effect in softening. Glycosidases, such as β-galactosidase and α-L-arabinofuranosidase, might facilitate pectin solubilisation and removal of galactose and arabinose residues form pectin during softening. Both enzymes increase remarkably during fruit softening. However, softening rates were not explained by differences in β-galactosidase activity in experiments by Yoshioka et al. (1995). Not much is known about the role of rhamnogalacturonase except that it is active during apple ripening (Gross et al. 1995).

Extensive studies on the ethylene effect on ripening, lead to the conclusion that the hormone promotes apple softening. It was especially visible in experiments with ethylene inhibitors, where treated apples were firmer while ethylene production was reduced (Mir et al. 2001). The enzymes endo-PG, β-galactanase, α-arabinosidase and β-galactosidase were ethylene dependent in transgenic melons with suppressed ethylene synthesis, whereas exo-PG and PME were ethylene independent (Pech et al. 2008). Expression of some cell-wall modifying enzymes and proteins is therefore influenced by ethylene (Brummell 2006;Brummell & Harpster 2001;Lelievre et al. 1997;Tatsuki & Endo 2006) and so far, no single enzyme was identified as a major determinant in fruit softening.

Fruit texture might be affected by cell size. Positive correlation was found between tissue breakdown during storage and cell size (Letham 1961). Cell size might be influenced by water content - turgor. Transpiration is a physiological process in which water evaporates from the plant tissues so leaves and fruit temperature is regulated. However, in sever water stress fruit quality and appearance is deteriorated. Transpiration depends strongly on cuticle and wax structure as well as external factors (temperature, humidity, wind etc) (Preece & Read 2005). Positive correlation was found between cell turgor and firmness in apple cultivars stored at 0-2^{o}C for 6 months (Tong et al. 1999), however it was concluded that other cellular aspects influenced firmness in storage and it is unlikely that turgor may explain all variation in postharvest firmness (Johnston et al. 2002). Additional cellular factors might be cell shape and structure. Soft fruits have rounder cells, more cell separation and larger intercellular spaces than firm fruits. The same observation was noticed in early maturing apple varieties, which explains why these cultivars tend to soften rapidly in the storage (Kahn & Vincent 1990). It has also been indicated that fruits with bigger cells have more intercellular spaces and therefore have weaker tissues (Harker et al. 1997).

Apples can lose 25-50% of their harvest firmness, while melons, tomatoes and kiwifruits can soften 75% (Johnston et al. 2002;Johnston et al. 2001). This decline in firmness continues after harvest and is a main concern in storage handling.

3.2. Acidity and Sugar

Fructose, glucose and sucrose are main sugars in climacteric apples, - accumulated during maturation and during the post harvest climacteric ripening provided mainly from starch conversion. In apples, starch hydrolysis begins usually 2-3 weeks before the increase in ethylene production. Sugar content increases till harvest with fructose as the predominant in most cultivars fx. 'Golden Delicious' or glucose in 'Cox's Orange Pippin' (Corrigan et al. 1997;Jackson 2003). There is a rather indirect effect of ethylene on acidity and sugar content, as these components are used during the enhanced metabolic processes of the climacteric ripening.

Malic acid accounts for around 90% of the organic acids in apples (Ackermann et al. 1992). Some apple cultivars have small amounts of citric, quinic, galacturonic and chlorogenic acids (Jackson 2003). Organic acids are main substrates for the respiration. Citrate can be directly fed to Krebs cycle, while malate is catalyzed first by malic enzymes to pyruvate. Through the climacteric stage the metabolism of malate increases in the apple tissue and is related to the increasing respiration rate (Jackson 2003;Preece & Read 2005).

With decreasing level of organic acid the sweetness perception increases. The balance between sugar and acid content influences the taste perception. Corrigan (1997) presented that sugar to acid ratio varies from 12 to 36. In the same study sugar content differed from 11-15.7% and malic acid level between 0.35 to 0.95%. These parameters vary among cultivars (and climate zones) but so do taste expectations of consumers. 'Ildrød Pigeon' TA content varied in our experiments between 8.4-10.7g/L (0.84 and 1.07 %) while total soluble solids (TSS) was between 12.4-13.5%Brix dependent on measured influential factors (chapter 9).

3.3. Pigmentation

Color changes, in apples are due to chlorophyll breakdown, and carotenoid and phenolic pigment (anthocyanin, flavonols) synthesized in the cell cytoplasm and accumulated in the vacuole (Tanaka et al. 2008). For red cultivars the most important pigments are anthocyanins (Saure 1990). There are two stages during fruit development where anthocyanins are formed: 1) in small fruitlets during cell division, which does not result in persistent red color, and 2) during ripening (Jackson 2003;Saure 1990). When ripening progresses, chlorophyll degradation reveals existing pigments such as carotenoids and more anthocyanins are produced. Light is a crucial factor in anthocyanin synthesis as little or no pigment is produced without light exposure. UV light was found to induce anthocyanins production (Arakawa et al. 1986;Kondo et al. 2002).

Anthocyanin synthesis in apples depends on sugar availability and involves two biosynthetic pathways (Golding et al. 2003;Wang et al. 2000). Anthocyanin pigments are assembled from two different metabolic streams in the cell, both starting from the C2 unit acetate or acetic acid derived from photosynthesis, one stream involves the shikimic acid

pathway to produce the amino acid phenylalanine (Figure 5). The other path (the acetic acid pathway) produces three molecules of malonyl-Coenzyme A (C3 unit). These two are coupled together by the enzyme chalcone synthase, which forms chalcone. The chalcone is subsequently isomerized to the prototype pigment naringenin, which is next oxidized by enzymes like flavonoid hydroxylase and bound to sugar molecules to form anthocyanins. The red-skinned apples contain mostly cyanidin-anthocyanins such as; cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, cyanidin-7-*O*-arabinoside, cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-xyloside (Mulabagal et al. 2007). Pigments are transported through the cytoplasm to the vacuole membrane via glutathione S-transferase (GST) (MacLean et al. 2007). Both phenylalanine ammonia-lyase (PAL) and CHS were considered key enzymes on flavonoid biosynthesis (MacLean et al. 2007). However, the activity of PAL is positively correlated with anthocyanin formation (Golding et al. 2003;Wang et al. 2000). It was found that ethylene initiates rapid anthocyanin accumulation during apple ripening by increasing the level of PAL enzyme in the apple skin (Faragher 1983). However, ethylene only stimulated anthocyanin production via PAL in unripe apples, but not when applied on already ripe fruits (Faragher 1983;Faragher & Brohier 1984;Wang et al. 2000). Some studies strongly point out an interaction between ethylene and anthocyanin synthesis (Blankenship & Unrath 1988;Faragher & Chalmers 1977;Larrigaudiere et al. 1996) but there is still some inconsistency (Mattheis et al. 2004). Anthocyanins, among other pigments, responded quickest both to ethylene promoting and suppressing. Additionally promotion of anthocyanin synthesis with the ethylene treatment requires light and ethylene treatment actually inhibits pigment formation in darkness (Craker & Wetherbee 1973).

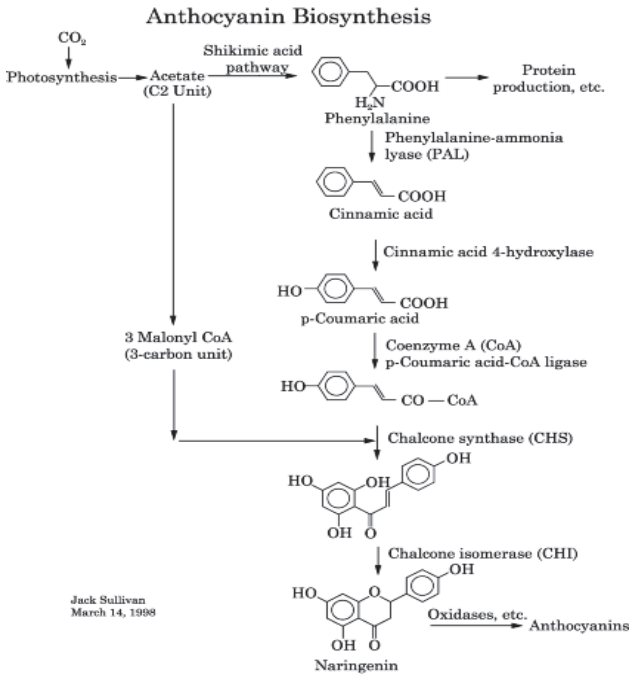


Figure 5 The anthocyanin biosynthesis pathways (http://www.carnivorousplants.org/cpn/samples/Science273anthocyanin.htm)

Still enhancement of anthocyanin synthesis could require only a very low ethylene level, which might be insufficient for initiating other ripening processes (Awad & de Jager 2002). On the other hand it was shown that ethylene played an enhancing role when used with methyl jasmonate (stimulator of anthocyanin accumulation) but there was no discernable effect of exogenous ethylene on anthocyanin production when applied alone (Mattheis et al. 2004). Therefore it was suggested that other factors than ethylene are involved in anthocyanin accumulation like temperature, fruit maturity and horticultural management (MacLean et al. 2006).

Optimal color development occurs at 20-25^{o}C, while temperature above 35^{o}C completely prevented anthocyanin accumulation because PAL enzyme is inactivated at high temperatures (MacLean et al. 2006). According to Uota (1952) a greater energy is required to synthesize pigment at higher temperatures. Cool temperature at nigh also stimulate red color development, while fruit at night temperature, above 15^{o}C, remain green (Toldam-Andersen, unpublished data). Carbohydrate supply and fruit to leaf ratio were found also influential on red color changes. Basically the more leaves per fruit the more sugar delivery and better anthocyanins synthesis (Jackson 2003).

3.4. Aroma compounds

300-350 different aroma compounds have been identified in various apple cultivars (Dixon & Hewett 2000). The specific cultivar aroma profile depends on a mixture of principal compounds, their thresholds and concentrations (Hansen et al. 1992;Song & Bangerth 1996;Song et al. 1997). Aroma compounds are synthesized largely in apple peel tissue (Pechous & Whitaker 2004) but also in apple flesh (Kondo et al. 2005). Esters, alcohols and aldehydes are quantitatively the major volatiles in apples (Argenta et al. 2006;Kondo et al. 2005), which arise from fatty and amino acids. Any changes in the ester substrate pool might depend on many factors like; the physiological state of the fruit, genetical potential, agronomic, environmental, post-harvest conditions and all other which influence the balance of the metabolic pathways

The concentration of aroma compounds increases during ripening and reaches the highest production during the climacteric peak (Dixon & Hewett 2000). Rising ethylene concentration and increased respiration might be needed to deliver the aroma compound precursors required. Production of esters in fruits has been shown by Schaffer et al. (2007) to be ethylene related. When the ethylene concentration in apples was reduced ester synthesis was inhibited but aldehydes were not affected (Schaffer et al. 2007).

The general volatile synthesis was observed to follow according to aroma compound groups; aldehydes being predominant in pre-climacteric stage, alcohols and then esters increasing with rising ethylene concentration (Mattheis et al. 1991). It is related to a major precursor of aroma volatiles; fatty acids, which further involvement in biosynthetic transformation lead to development of aldehydes, than alcohols and finally esters (Dixon & Hewett 2000). Almost no esters were present in 'Anna' apples at harvest but a great amount of the aldehydes, 2-hexenal, and the alcohols hexanol and 2-methylbutanol. These compounds decreased during ripening while esters accumulated (Lurie et al. 2002).

Aldehydes contribute to green, grass-like, herbaceous, leaf-like odour (Dixon & Hewett 2000). They are formed from fatty acid degradation by lipoxygenase (LOX) (Schaffer et al. 2007) and further hydroperoxide lyase (HPL) action (Riley et al. 1996) (Figure 6). The LOX enzyme is located in sub-cellular positions, which prevents aldehyde formation in intact apples (Sanz et al. 1997). During apple degreening, when chloroplasts decompose, the linoleic (18:2) and linolenic (18:3) acids are released from galactolipids (Paillard 1986). The concentration of these acids is higher in pre- rather than in post-climacteric apples (Dixon & Hewett 2000). During the tissue damage the LOX enzyme is released and C6-adehydes are significantly produced (Baldwin et al. 2000;Myung et al. 2006). Linoleic and linolenic acids are oxidized and formed fx. hexanal and cis-3-hexenal (Paillard 1986;Schaffer et al. 2007). These C6-aldehydes were found to protect injured cells from bacterial decomposition (Corbo et al. 2000).

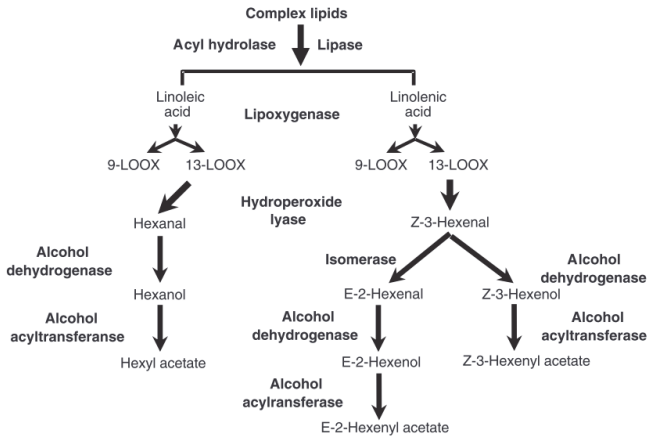


Figure 6 Lipoxygenase pathway; C6-aldehydes, alcohol and straight-chain ester production in vegetables and fruits (Baldwin et al. 2000;Hui 2010)

Alcohols might constitute 6-16% of the volatile profile (Paillard 1990).They arise from beta oxidation of fatty acids, together with acyl co-enzyme A (CoA) (Schaffer et al. 2007). In a further step, alcohol is catalysed to form esters by alcohol acyltransferase enzyme (AAT).Alcohols may also be produced from ester hydrolysis catalysed by the esterase. The activity of this enzyme increases during the climacteric phase (Goodenough 1983).The concentration of alcohols might limit ester formation or promote formation of some esters. When intact apples were exposed to hexanal vapour, immediately 1-hexanol formation was observed and after additional 5 hours hexyl acetate accumulation increased (Song et al. 1996). It confirms that hexanal needs to be converted, firstly to alcohol in order to transform into the later ester. Butyl acetates and butanoate ester concentrations increased when apples were exposed to 1-butanol. Ethanol and hexanol stimulated formation of ethyl and hexyl esters at the expense of butyl esters, indicating that ester synthesis is a selective response (Kollmannsberger & Berger 1992).

Esters are responsible for fruity, floral, fresh and apple-like aromas (Dixon & Hewett 2000). They account for 80% (Kakiuchi et al. 1986), 78-92% (Paillard 1990) of total content of volatile compounds. In fruit, straight chain esters are synthesised from fatty acids via the lipoxygenase pathway (Figure 6), whereas branched chain esters are produced from the metabolism of branched chained amino acids fx.leucine, isoleucine, valine (Rowan et al. 1996;Schaffer et al. 2007;Wyllie & Fellman 2000) (Figure 7).

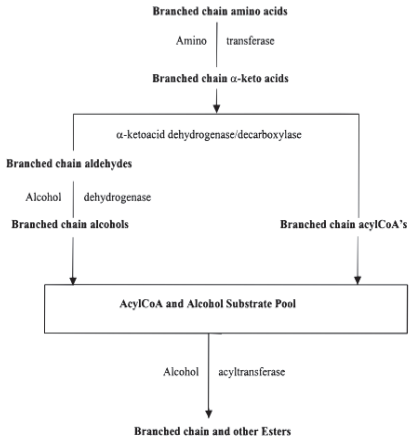


Figure 7 Scheme for the conversion of branched chain amino acid into branched-chain esters (Wyllie & Fellman 2000).

In general, esters are formed from alcohols and acyl co-enzyme A (CoA). Firstly, however, aldehydes are reduced by alcohol dehydrogenase (ADH) enzyme to the corresponding alcohols. Secondly, AAT catalyzes the connection of the acetyl moiety from acetyl CoA to these alcohols, forming esters. Ester biosynthesis can be controlled both by the availability of the necessary substrates and by the selectivity of the enzymes involved. Enzyme relations have been proven by accumulation of lipoxygenase-derived volatiles; C-6 aldehydes and reduction of alcohols, in an Arabidopsis mutant, which lacked ADH enzyme activity (Bate et al. 1998). 2-methylbutyl esters, which are normally not found in banana, were formed when fruit were postharvest exposed to 2-methylbutanol (Wyllie & Fellman 2000). This demonstrates that esters produced in the ripening banana are limited by the supply of alcohol precursor. In Wyllie & Fellman (2000) study, it was shown that different alcohol precursors were utilized to form esters but AAT exhibit preferences. Ethanol and propanol were clearly the least preferred substrates and the five and six carbon alcohols were the most reactive. This selectivity of the enzymes, involved in the transformation of fatty and amino acids, is not yet fully understood. It has been shown that there are significant differences in aroma profile between apple varieties; 'Red Delicious' and 'Granny Smith' depend on alcohol substrates exposure (Rowan et al. 1999;Rowan et al. 1996). 'Red Delicious' variety was unable to convert cis-3-hexanol to hexyl esters, while 'Granny Smith' could. Therefore, the fruit genetical potential is not out of importance. The AAT enzyme is established as a key enzyme in ester synthesis and is very important for the understanding of the action of ethylene in fruit ripening. AAT enzyme, but not yet ADH, activity and expression is proven to be regulated by ethylene (Defilippi et al. 2005;Schaffer et al. 2007). Schaffer et al. (2007) have created an apple fruit, which is an antisense mutant of ACO so does not produce internal ethylene. The expressions of the gene, which are potentially related to aroma compounds biosynthesis, were monitored in relation to exogenous ethylene exposure. The 17 out of 186 genes in the fruit skin were unregulated by ethylene treatment. As their project outcome, it was shown that ethylene selectively regulates the

expression of genes involved in aroma biosynthesis, mainly being involved in the final stage of aroma formation.

4. *ETHYLENE INHIBITORS*

Ethylene is physiologically active in plant cells in very small amounts (≤0.1ppm) and storage facilities have to be controlled to reduce ethylene production rates. It can be done by low temperature, reduced oxygen levels (controlled and modified atmosphere storage) or the use of ethylene scrubbers (Schaffer et al. 2007). For many years high levels of CO_2 was used in controlled atmosphere storage as a natural inhibitor of ethylene responses. It was suggested, that CO_2 compete with ethylene for the binding sites; today it is well known that higher CO_2 level is just suppressing ethylene synthesis (Dixon & Hewett 2000). It has become of a great importance to control ethylene responses to suppress some of the unwanted changes in fruits during storage (fx. softening). As a consequence of the work on different ethylene inhibitors, or agonists, researchers have made a major improvement in characterization of ethylene binding-sites and identification of ethylene signal transduction pathways.

The compounds, which interact with the ethylene receptors, might be divided into three classes. The first group is represented by 1-propylene and 1-butene. These are ethylene agonists, which means they turn on ethylene response signals by binding to the receptors (Sisler et al. 2006).

The second group is represented by diazocyclopentadiene (DACP) and 2,5-norbodiene (2,5, NBD). They compete with ethylene for the place in the receptors, when the hormone is present in relatively low levels. They bind impermanent to the binding-sites and therefore require continuous exposure. NBD is mainly used in scientific applications because of its unpleasant odour and the requirement for repeatable exposure limits its commercial implementation (Kepczynski 2006). DACP was used to extend postharvest life of ornamental plants. Interestingly it binds weakly to ethylene receptors, but when exposed to fluorescent light, its by-product becomes a very effective receptor blocker (Sisler & Serek 1997). Explosiveness and instability of DACP prevent the compound from use as a potential ethylene inhibitor in commercial applications.

Chemicals, like silver thiosulfate (STS) and cyclopropenes, which also bind to ethylene receptors and remain there for a long time preventing the activation of hormone action, represent a third group. The effect of these inhibitors diminishes, when the compounds diffuse or new receptors appear (Sisler & Blankenship 1990;Sisler & Serek 2003;Sisler et al. 1995). STS was commercially used to block ethylene action and protect ornamental plants against ethylene (Sisler et al. 1985). It was already reported in 1976 that silver ions are involved in ethylene responses in plants (Beyer 1976a;Beyer 1976b). Additionally, it was known that the ethylene receptor (ETR1) contains a copper ion that controls ethylene binding (Rodriguez et al. 1999). Therefore, the action of STS might include exchange of ions at the receptor sites. The binding between silver and the receptor is so strong that ethylene cannot bind or induce any processes at the receptor level (Rodriguez et al. 1999). Silver was found mobile in the phloem, when studied on carnations, and could accumulate in organs (Abeles et al. 1992;Sisler et al. 1985). The contamination risk of this heavy metal, eliminates STS from being used on vegetables and fruits.

4.1. Cyclopropenes

Cyclopropene (CP), 1-methylcyclopropene (1-MCP), 3-methylcyclopropene (3-MCP) and 3,3-dimethylcyclopropene (3,3-DMCP) are very effective in low concentrations and remain bonded to the receptors for many days (slow diffusion) (Sisler et al. 2006;Sisler & Serek 2003). Concentration-wise CP and 1-MCP are almost equally active; however, CP is unstable at room temperature. CP, 1-MCP and 3-MCP applied once protected bananas against ethylene at 23°C for 12 days, while 3,3-DMCP only worked for 7 days (Sisler et al. 2006;Sisler & Serek 2003). Still 1-MCP appeared to be the most useful among cyclopropenes, based on its stability at room temperature, concentration, efficiency, non-toxicity and odourless character. (Sisler et al. 2006;Sisler & Serek 2003).

The positions of substitutions and the double bond on the ring are important (Sisler et al. 2003). Methyl group adjacent to the double bond (1-MCP) was more effective at lower concentrations and allowed interact with receptor for longer time than methyl group substituted at 3-position (3-MCP) (Sisler et al. 2003). Therefore, a stabilizing effect of the ability of the compound to bind to the receptors is also due to the substitution in position 1. More cyclopropenes substituted in the position 1 with linear saturated side chains were tested on bananas (Sisler et al. 2003). The main observation was: the longer the side-chain the better activity. 1-DCP (1-decyclcyclopropene) showed the best performance when used in very low concentration (0.3nL/L) because banana fruits were protected for 36 days at 23°C in comparison to 12 days for 1-MCP (Sisler et al. 2003). This long lasting effect is suggested to be due to the side chain, which might anchor to the plant cell membrane and prevent the molecule from getting lost from the cell structure (Paliyath 2008).

5. *1-METHYLCYCLOPROPENE (1-MCP)*

Edward Sisler is considered to be the father of 1-MCP. Sisler found many ethylene antagonists, which began a new era in the control of ethylene responses in plants (Grichko et al. 2006). The discovery of 1-MCP came as a result of the cooperation of Edward Sisler and Sylvia Blankenship (Blankenship & Dole 2003). The finding of 1-MCP was a revolution in ethylene plant biology, which modernized the agricultural and horticultural world. 1-MCP reached the awarding patent in 1996 (Sisler, E.C., Blankenship,S.M. *Method of counteracting an ethylene response in plants*. US Patent 5,518,988). 1-MCP safety, toxicity and environmental profiles in regard to humans are very favorable (Blankenship & Dole 2003;Sisler & Serek 2003). It was accepted by USA Environmental Protection Agency (EPA) after series of tests on rats, where no signs of systematic toxicology were observed (U.S.Environmental Protection Agency 2008). Another positive attribute of 1-MCP is low rates of residues, which are considered below the detection limits (Blankenship & Dole 2003;Sisler & Serek 2003).
In 1999 1-MCP was released and distributed as EthylBloc® (Sisler & Serek 2003). At that time, EthylBlock® was allowed to be used only on ornamental plants. In the same year, AgroFresh Inc. launched the next product, SmartFresh™, on the market, with 1-MCP as active ingredient, which could be used on edible plants (Sisler & Serek 2003). The company created the *SmartFresh™ Quality System*, "where SmartFresh technology successfully controls fruit and vegetable ripening by controlling naturally occurring ethylene during storage and transport" (*www.smartfresh.com*). Basically, it is recommended to use the SmartFresh™ technology to maintain good quality during postharvest management.

5.1. 1-MCP registration in Denmark

SmartFresh™ was registered in Denmark in August 2008 by Danish Environmental Protection Agency (DEPA, Miljøstyrelsen). Denmark was importing apples, which were treated with 1-MCP, as it was registered for use in many countries much earlier than in Denmark. These fruit had become a treat to the Danish fruit market as they had better storability and maintained good quality for longer time compared to the local products. The 1-MCP registration raised consumer opposition voices, as Denmark is a country of a great organic awareness and use of chemical on fresh fruit became a concern. The Danish Veterinary and Food Administration (Fødevarestyrelsen) confirmed however that there are no safety concerns about eating apples treated with SmartFresh. The market associations needed, however, to stay behind the consumers' opinion. COOP, which is one of 'green brand chain-shops' in Denmark, decided to sell only untreated products. Shop associations ensure consumers that they supply free SmartFresh products as they have a trustful, yet only oral, agreement with producers, Danish as well as foreign, to deliver only untreated fruits and vegetables.

5.2. 1-MCP application

SmartFresh™ is produced in a form of powder with 0.014%, 0.14%, 0.63% or 3.3% of active compound (Jung & Lee 2009;Kondo et al. 2005). Under standard temperature, 1-MCP is a gas and it needs to be formulated with cyclodextrin into powder. It is released from the powder as a gas when in contact with water. Normally 1-MCP should be applied in a sealed environment at room temperature (20-25°C) for around 20 to 24 hours. It can also be used in lower temperatures but exposure time need to be longer, concentration higher and still 1-MCP effectiveness might be diminished (Acuna et al. 2011). The affinity of 1-MCP to the binding sites is 10-fold higher than ethylene (Blankenship & Dole 2003). It is proposed that 1-MCP binds to highly expressed receptors like ETR1 or to EIN4, and by that continuously activate CTR1 even when remaining receptors are inhibited by ethylene. 1-MCP must however, be bonded to the sites in a different way than ethylene, so it does not deactivate them and does not cause responds as ethylene does. It might be due to the time of compound-receptor binding. Ethylene dissolves from receptors in a couple of minutes while it takes hours, or even days in case of cyclopropenes (Sisler & Serek 1999). The full rearrangement of ligands in the receptor's metal active centre, which caused further ethylene responds in plants, is probably possible only when compound connects, causes the rearrangement and then dissolves from the receptor. Cyclopropenes bind to the metal site and remain there for long time so rearrangement cannot be completed and the receptor cannot transmit a signal (Figure 8).

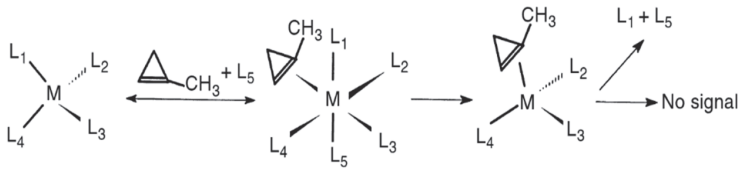


Figure 8 Ligand rearrangement cannot be completed as 1-MCP remain bound to the metal active site => no signal is transmitted along the transduction pathway (Sisler et al. 2006)

Many factors can influence apple response to 1-MCP. Each cultivar tends to behave differently due to different ripening development rate therefore different 1-MCP application conditions have been tested. Various 1-MCP concentrations have been used to delay ripening in apples (Blankenship & Dole 2003;Fan et al. 1999;Watkins 2008) - Table 1. Most experiments on apples have been performed in the time range of 20-24hours, but there are some studies performed with shorter exposure time (Table 1).

The time of 1-MCP application after harvest must be taken into account. 1-MCP, when used in appropriate time, binds to ethylene receptors so that the ethylene hormone cannot elicit its reaction to the already occupied sites. There is however, a possibility to delay the use of 1-MCP after harvest, but effectiveness might be aggravated. A delay of 1-MCP application up to 8 days after harvest strongly reduced treatment efficiency or did not affect it depending on cultivar, storage type and duration (Watkins & Nock 2005). Similarly, later harvested fruits are less responsive to 1-MCP than early harvested

(Watkins 2008;Watkins & Nock 2005). Still there is a great need to establish the most effective 1-MCP concentration and application conditions for each species and cultivar individually as they react differently. The understanding of these relationships is essential for a successful commercial utilization of 1-MCP (Watkins et al. 2000).

Table 1 Summary of some experiments carried out on apples where 1-MCP was applied – table concept based on Blankenship and Dole (2003)

Apple cultivars	1-MCP concentration	Temperature during treatment (°C)	Treatment length	Effects	References
'Anna'	0.1 and 1 µL/L	20	20h	Total volatiles were not reduced by treatment with 0.1 µL/L 1-MCP, but were 70% lower in fruits treated with 1 µL/L 1-MCP than in untreated fruits. Ethylene production was 50% by 0.1 µL/L 1-MCP and 95% by 1 µL/L 1-MCP. 1-MCP treated apples retained more alcohols, aldehydes, and β-damascenone volatiles than did untreated apples. Sensory panel preferred apples with less ripe aroma but firm (1µL/L 1-MCP).	(Lurie et al. 2002)
'Gala'	63 µmol/m^3	20	12h	Treatment with 1-MCP and then storage in air or CA or storage in CA without 1-MCP treatment reduced volatile production as compared to apples not treated with 1-MCP stored in air. The production of esters, alcohols, aldehydes, acetic acid, and 1-methoxy-4-(2-propenyl)benzene by 1-MCP-treated fruit stored in air plus 7 days at 20 °C increased after 20 or 28 weeks of storage.	(Mattheis et al. 2005)
'Gala', 'Delicious', 'Granny Smith', 'Fuji'	0.6 to 1 µL/L	20	18h	1-MCP+CA: delayed ripening, delayed softening and TA; SSC loss delayed only in 'Gala' but not in other cultivars.	(Bai et al. 2005)

'Gala'	0.625 µL/L	20	18h	1-MCP+CA caused volatile inhibition greater than 1-MCP alone. 1-MCP maintained firmness and acidity.	(Baldwin et al. 2003)
'Tsugaru', 'Hangro', 'Fuji' (early, mid- and late harvest)		20	8, 16 and 24h	1-MCP in unripe fruit inhibited ethylene, decreased respiration rates, maintained TA more effectively when more mature fruits were treated. 1-MCP effect on flesh firmness were similar for apples at mid- or late harvests. 1-MCP treatment of early-harvested fruit of the early-maturing 'Tsugaru' inhibited softening and loss of TA to a greater extent than for late harvested fruit. The same pattern of softening was found for 'Hongro' and 'Fuji'. 'Tsugaru' and 'Fuji' firmness was maintained after 8 h treatment with 1µL/L 1-MCP but 16h treatment was required for 'Hongro'. 2d 1-MCP treatment delay had no negative impact on fruit firmness	(Jung & Lee 2009)
'Fuji'	10 µL/L +0.2 mmol/L methyl jasmonate for 2min	-	12h	1-MCP treatment alone or with MJ inhibited ethylene production. MJ and MCP inhibited production and formation of many volatile alcohols and esters.	(Fan & Mattheis 1999)
'Redchief Delicious' (3 maturity stages)	0.7 µL/L +repeatable 1-MCP treatments (1/week, 1/2weeks, 1/month,1/year)	20 (4 storage temp. 0,5,10,15,or20 °C)	16h	1-MCP slowed softening at all temperatures relative to nontreated fruit, however with decreasing temp.1-MCP benefits became less pronounced. Effectiveness of 1-MCP declined slightly as harvest maturity increased. Efficacy of 1-MCP treatment	(Mir et al. 2001)

					increased with greater frequency of application at 5, 10, 15, and 20 °C, but not at 0 °C. 1-MCP application reduced, but did not prevent decay. Rate of decline in titratable acidity increased with storage temperature and 1-MCP had no significant effect on retarding on acid content. Rate of decline in titratable acidity increased with storage temperature and 1-MCP had no significant effect on retarding the decline in acid content. Application of 1-MCP resulted in greater retention of firmness than CA.	
40	'Cortland' and 'Empire'	0.6 µL/L	3, 13 and 23	0, 3, 6, 9, 12, 16, 24 and 48h	Cortland' apples treated with 1-MCP at 3 °C showed improved firmness retention (>63.0 N) with at least 9 h of treatment, whereas those treated at either 13 or 23 °C showed improved firmness retention with at least 6 h of treatment. Empire' apples treated with 1-MCP showed improved firmness retention (>67.5 N) with only 3 h of treatment regardless of temperature, but those treated at 3 °C for 3 h no longer had the full firmness advantage after an additional 7 days at 20 °C. No significant effect of 1-MCP on soluble solids concentration. Treatment with 1-MCP for 3 h at any of the temperatures significantly reduced	(DeEll et al. 2002)

				superficial scald in 'Cortland'.	
'McIntosh' (3 maturity stages)	1 and 0.625 µL/L (3, 7 and 10d after harvest)	0-1	24h	Apples treated with 1-MCP and held in air or CA storage were firmer than those not treated, but this difference in firmness was less with later harvests, more delay before 1-MCP treatment, and longer storage time. Apples treated with 1µL/L 1-MCP were firmer than those treated with 0.625 µL/L after 6 months of storage and/or 7 days at 22 °C. Ethylene and CO_2 production were reduced in apples treated with 1-MCP, especially in fruit from the first harvest and those treated 3 days after harvest. 1-MCP reduced the incidence of superficial scald, flesh browning, core browning and senescent breakdown.	(DeEll et al. 2008)
'Gala'	1 µL/L	0	24h	1-MCP reduced flavour volatiles. Consumers could distinguish a difference in treated and control apples, but no significant differences in overall liking scores.	(Marin et al. 2009)
'Law Rome', 'Golden Delicious'	160 mg/L sprayed on trees- 3,10,17 and 24d before harvest, 1 µL/L postharvest	0	24h	Pre-harvest treatment had minimal effect on maturity, IEC and firmness loss after storage were reduced by both treatments, positive effect of pre-harvest 1-MCP on postharvest quality decline in fruit harvested 3d or more after spraying while pre-harvest 1-MCP continued to have positive effect on quality of 'Golden	(McArtney et al. 2009)

Delicious' apples harvested up to 9 days after spraying – attached apples of some cultivars are capable of rapid generation of new ethylene receptors

6. *'ILDRØD PIGEON' – A DANISH APPLE CULTIVAR*[1]

'Ildrød Pigeon' is a unique, red Danish apple cultivar, which is cultivated almost exclusively in Denmark. It is also known under the synonyms 'Mørkerød Pigeon', 'Dueæble' or 'Juleæble'. These Danish names are based on the appearance of the apple or the usage purposes. The apples are grown for a niche market as they are used and sold mainly before and during Christmas (Figure 9). It is an old cultivar dating back to around 1840 from the island Funen and is most likely a seedling of the old French cultivar 'Pigeonnet Jèrusalem'. It was first distributed from a nursery orchard in Korsør, Sjælland in 1870. The cultivar has been widely grown all over the country since then and became one of the most popular cultivated cultivars in Denmark in the 1920 and -30'ies (Pedersen 1950).

Figure 9 'Ildrød Pigeon' apples are sold in shops during Christmas season

'Ildrød Pigeon' apples are egg or cone-shaped, usually quite small with length of 51-60mm and diameter of 52-56 mm. The skin color at harvest might be greenish only with a

[1] Internet Sources:
www.pometet.dk
http://www.sonneruplund.dk/0%20html/Pigeon%20Ildroed.html
http://www.havenyt.dk/spoergsmaal/frugt_og_baer/aebler/889.html
http://www.nordgen.org/nak/index.php?view=show&id=7459&PHPSESSID=g9e11gtt1b1eakmfgp18n12i75
http://www.meyersmadhus.dk/da/meyers_laekkerier/frugt_fra_lilleoe/pigeon.html

partial cover of red over color. These apples develop, when attached to the tree, a red cheek on the sun exposed side while the rest of the fruit skin remains green (Kühn et al. 2011). The apple flesh is white – sometimes with a rosy tint, especially if the fruit has been well exposed to light during development. 'Ildrød Pigeon' has a distinctive tart-sweet taste with a delicate bitterness and characteristic almond-like aroma. However, its aroma potential and profile have not been precisely described.

It is an excellent table cultivar but is also well used for desserts, salads, baking and mousse (Toldam-Andersen, unpublished data). Its shape, small size and intense red skin color makes it also great for decoration at Christmas.

'Ildrød Pigeon' cultivar is harvested in early to mid September and is kept in cold storage until December. Apples at harvest are in most cases not fully red. Therefore, after harvest the fruits are exposed to sun light to obtain bright red skin color, covering more than 50% of the fruit (Sakskøbing-storage facility; oral communication). Traditionally after harvest, the apples are placed between the tree rows in the orchard (Figure 10). They are rotated with a rubber broom at daily intervals to expose all sides to the sun. Light exposure might last up to 3 weeks (Kühn et al. 2011). During this time, the apples continue to ripen and thus lose storage potential. Often their quality is aggravated during storage, especially firmness (Kühn et al. 2011).

Figure 10 'Ildrød Pigeon' apples before and after light exposure (Frugt og Grønt, nr. 9/2008)

'Ildrød Pigeon' became an apple model cultivar for our experiments where optimization of this type of postharvest practice was studied in order to avoid storage loss. The softening of 'Ildrød Pigeon' is a major problem, which was observed in our experiments. Recently, it was published that 'Pigeon' apples are losing 45% of their firmness because of the sun exposure period and additional 20% during storage (Kühn et al. 2011). The use of the ethylene inhibitor (1-MCP), which would suppress acceleration of the ripening processes, might be a possible solution to maintain apple firmness. However, the timing of its application needs to be adjusted to allow fruits to develop red skin color.

6.1. 'Ildrød Pigeon' principal aroma compounds (PAPER III)

Apple odour formation is a critical factor for the fruit quality. The apple aroma profile is composed of a very complex mixture of volatiles. More than 300 aroma compounds have been detected in different apple varieties. Among these only about 20-40 are directly responsible for characteristic aroma perception (Vanoli et al. 1995). The aroma of apple varieties is suggested to be divided into groups depending on their important components (Dixon & Hewett 2000;Li et al. 2008). Ethyl butyrate, ethyl acetate and ethyl 2-methylbutyrate are characteristic for ester-like varieties fx. 'Delicious' and 'Golden Delicious'. Additionally, according to Petersen and Poll (1995) apple cultivars could be divided into three ester sub-groups, namely those dominated by butanoate esters ('Filippa' and 'McIntosh'), acetate esters ('Elstar', 'Cox Orange' and 'Golden Delicious') and those where both butanoate and acetate esters contribute to the total aroma profile ('Mutzu', 'Ingrid Marie' and 'Spartan'). The 'Jonathan' variety is representing an alcohol-like group with butanol, 3-methyl-1-butanol and hexanol as typical aroma components. Finally, a fifth group represented by aldehydes like 2-hexenal and low content of esters fx. the 'Granny Smith' cultivar (Li et al. 2008). Paillard (1979, 1990) categorized apple volatile pattern depend on fruit skin color and the relative presence of C-6 aldehydes. Paillard (1979) reported that yellow-skinned apple cultivars produce mainly acetic acid esters while red-skinned cultivars mostly produce butyric acid esters. Concentration of C-6 aldehydes in 'Cox's Orange Pippin' and 'Jonathan 'apples were 4 and 6 times greater than in 'Golden Delicious' for hexanal and 100 times greater for trans-2-hexenal (Paillard 1990).

The aroma profile of 'Ildrød Pigeon' needed to be evaluated. To determine relative odour potency of compounds and prioritize them, gas chromatography-olfactometry (GC-O) was used. Molecules from the sample are separated, depending on their chemical properties, when they travel through the capillary column of a GC. A GC-O is equipped with two detectors; human nose and a flame ionisation detector (FID). Basically, the effluent from the column is split into these two, and so allowing the instrument detector to register changes in aroma during the time of measure, while assessor perception is recorded simultaneously. Further steps require comparing and matching resolved compounds between the GC-MS chromatogram with the assessor's perceived ones. This can be problematic in case of a very complex sample. The ranking techniques are used to place perceived compounds according to their importance to overall aroma profile. There are several methods; detection frequency method (NIF, SNIF), dilution to threshold method (AEDA and CHARM) and intensity method (OSME) (Drake & Civille 2002;Plutowska & Wardecki 2008;Ruth 2001;Ruth 2004). Dilution techniques are based on a repeated number of sniffing to dilutions of the sample until odour is no longer detected. The need for sniffing replications makes these techniques time consuming (Plutowska & Wardecki 2008). Compound intensity methods involve indication of perceived compound descriptors and also compounds classification according to the previously introduced intensity scale. This required special panelists training and therefore direct intensity methods are difficult to perform properly.

In general, good correlation has been found between OSME, AEDA and SNIF, wherein detection frequency method was twice as fast as others (Le Guen et al. 2000). The detection frequency method is used because of its simplicity and reproducibility with a minimum number of 6-8 panelists (Pollien et al. 1997). Panelists sniff only one dilution. The method is repeatable and the results reflect the differences in sensitivity between the evaluators, which reflects the differences within given population (Plutowska & Wardecki 2008). The results are presented in an aromagram/olfactogram, where numbers of panellists perceiving the compound versus retention time is shown. The height of a peak represents the number of judges who indicated the presence of the compound – NIF (Nasal Impact Frequency). Based on NIF value the status of perceived compounds in order of their importance is made (Delahunty et al. 2006). In this method number of panelists perceiving a compound at the same time is used to describe the importance of the compound to overall aroma profile (Pollien et al. 1997). Pollien et al. (1997) showed that the detection frequency method does not require special panelist training and the results are repeatable with a minimum number of GC runs.

To identify the most important aroma compounds for 'Ildrød Pigeon' it was decided to use dynamic headspace sampling and a trap containing 200mg Tenax TA. The thermal desorption of the aroma compounds was done on a Short Path Thermal Desorption unit (model TD-4, Scientific Instrument Services Inc. NJ). The detection frequency method (Pollien et al. 1997) was used for recording detected odors over a group of assessors. The number of assessors detecting an odor simultaneously (NIF) is used as an estimate of the odor's intensity.

There were 7 trained judges, who were recorded for 40 min during sniffing. The assessors were asked to indicate onset and end of each perceived odor and give a description of the odor quality. Compounds perceived by at least 3 panelists at the same time were assumed to be characteristic for 'Ildrød Pigeon'. Twelve characteristic 'Ildrød Pigeon' aroma compounds were defined as they were indicated by at least 43% of population perceiving a compound **(**NIF values equal to or above 3): **butanal, ethyl acetate, methyl 2-methyl butyrate, hexanal, 2-methyl-2-pentenal, cis-3-hexenal, 2-hexenal, butyl butyrate, 2,4-hexadienal 1, 2,4-hexadienal 2, 1-heptanol and 6-methyl-5-hepten-2-ol** (Figure 11).

In several cases panelists indicated perception of two compounds fx t-2-hexenal and butyl butyrate and two 2,4-hexadienal isomer forms. Where else two compounds were included as they co-eluted at time interval pointed by panelists fx. butanal and ethyl acetate, 2-methyl 2-pentenal and cis-3-hexenal, 1-heptanol and 6-methyl 5-hepten 2-ol. The co-elution of these peaks or compounds aroma blending occurring closely might raise evaluation of odorants in overall aroma profile. However, these issues are unavoidable no matter of technique used.

The composition of various aroma compounds influences the flavour perception and in 'Ildrød Pigeon' aldehydes, straight and branched chain butyrates and alcohols are considered to make a major contribution to the overall aroma quality (Figure 11).

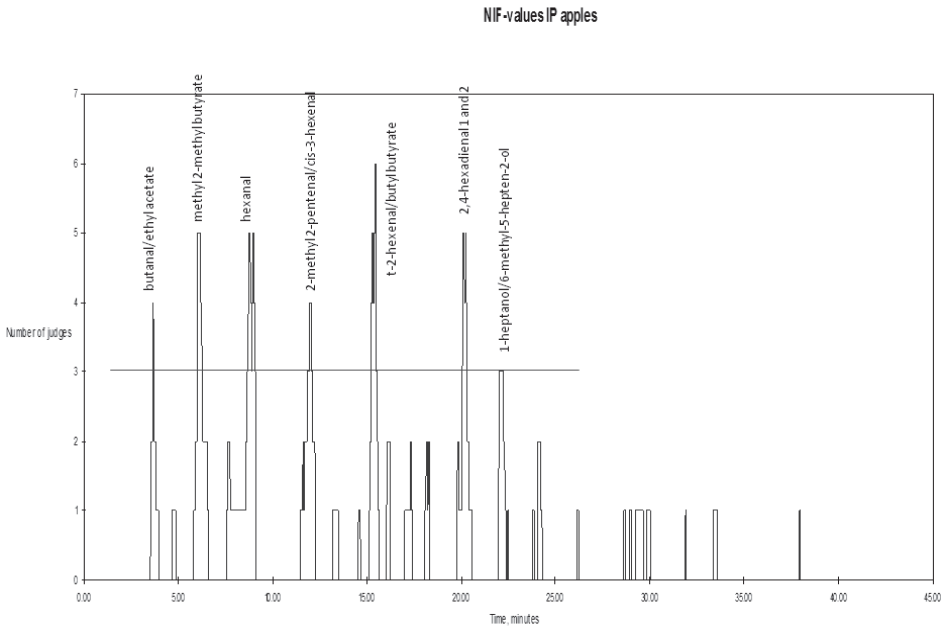


Figure 11 Aromagram, where principal 'Ildrød Pigeon' compounds are presented indicated by more than 3 panelists.

7. *PRE- HARVEST FACTORS AFFECTING APPLE QUALITY*

The quality changes during maturity and ripening might be influenced by many pre- and postharvest factors; tree age (Tahir et al. 2007), rootstock vigour (Lo Bianco et al. 2008), thinning and pruning practice (Link 2000), irrigation (Kafkas et al. 2009;Lo Bianco et al. 2008;Mpelasoka & Behboudian 2002), fertilization (Kafkas et al. 2009;Nava et al. 2008;Raese 1977), crop load, light access, harvest time (Echeverria et al. 2004a;Echeverria et al. 2004c;Vanoli et al. 1995) and storage conditions.

There is limited information available on the effects of pre-harvest practices on apple post-storage quality, especially aroma development. The combined influence of some of the mentioned postharvest factors will be discussed further in this thesis. However, this chapter is dedicated to pre-harvest thinning and nitrogen fertilization effects on color, firmness, total soluble solids, malic acid content, ethylene and aroma development.

In this chapter two experiments are presented; experiment I (PAPER I) and experiment II. The set up of the experiment I was described in PAPER I. Basically, half of the apple trees were thinned during fruit growth (the early July). At harvest apples were picked; from thinned (50% fruit load reduction) and unthinned trees; from three nitrogen levels; 0, 50 and 100 kgN/ha. Apples were kept in storage for 60 days after harvest. The quality parameters (firmness, ethylene production and aroma profile) were measured 5, 8 and 15 days after removing from cold storage to room temperature.

Apples for experiment II were harvested only from thinned trees. Bags were put on apples while on the trees in mid-summer, to prevent anthocyanin production and to create apples with pale skin on which the effect on red over color of a postharvest light exposure would be easily detected. Bags were removed directly after harvest. Apples, bagged and nonbagged, were picked from three nitrogen levels; 0, 50 and 100kgN/ha (thinned trees). After harvest, one set of samples were treated with 1-MCP immediately, another set was treated with 1-MCP after light exposure and a third set remained as control apples (untreated). The sunlight exposure lasted for 6 days. After sunlight exposure, apples were transferred to cold storage for 5 weeks (short storage for 'Ildrød Pigeon'). Maturity index was determined for bagged and non-bagged apples. The color development during sunlight exposure depend on nitrogen level was measured only on bagged apples and expressed as a/b ratio. It was hypothesized that the nitrogen fertilizer effect on anthocyanin formation would be more pronounced during light exposure if pigment development had been inhibited by bagging apples while on the trees. The evaluation of nitrogen effect on other quality parameters is presented based on measurements on nonbagged apples – these under natural development.

'Ildrød Pigeon' apples should be harvested when the harvest index parameters reach the following levels; starch 3-4, firmness 7-8 kg, TSS 11-13 % Brix, corresponding to a harvest index of 0.17 - Håndbog for frugt- og bæravlere (2006). Apples harvested on 2.09.08 were used in experiment II to evaluate nitrogen fertilization effect on quality parameters. The harvest indexes can be seen on Figure 12.

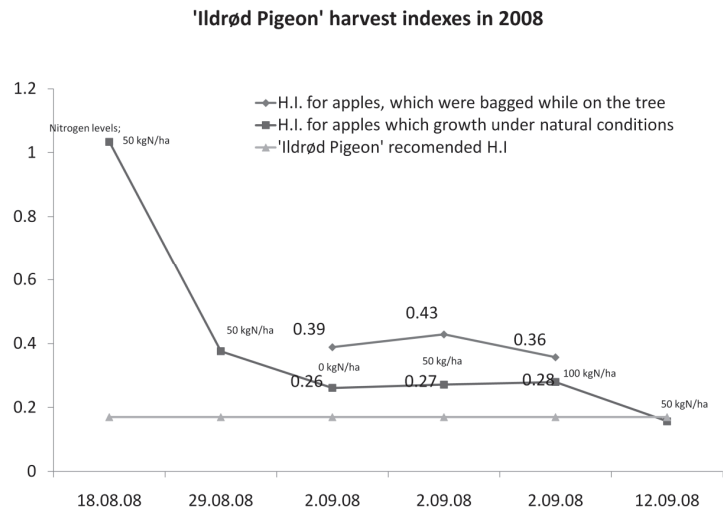


Figure 12 'Ildrød Pigeon' harvest indexes measured in 2008. Harvest index of bagged apples done only at date 2.09.08 but at three nitrogen levels. Harvest index of nonbagged apples at the middle nitrogen level (50 kgN/ha), done also at 0 and 100kgN/ha on 2.09.08.

Harvest index of bagged apples was higher than non-bagged apples on 2.09.08 (Figure 12). Bagging therefore, seems to delay maturity of apples while on the trees. According to Fan & Mattheis (1998) bagging practice delays the onset of fruit ripening by delaying ethylene concentration rise. Nitrogen fertilizer had no effect on anthocyanin production (Figure 13). After six days of light exposure there was a non-significant tendency of poorer color development in previously bagged apples, which were fertilized with 100kgN/ha (Figure 13). However, in the study of Kühn et al. (2011) 'Pigeon' apples from trees receiving high N level were significantly less red than apples from trees receiving lower N level. According to Strissel et al. (2005) anthocyanin synthesis and PAL activity seems to be suppressed by high nitrogen levels. In 'Elstar' apples negative correlation between nitrogen level in the fruits and total flavonoid and anthocyanin content was also observed (Awad & De Jager 2002).

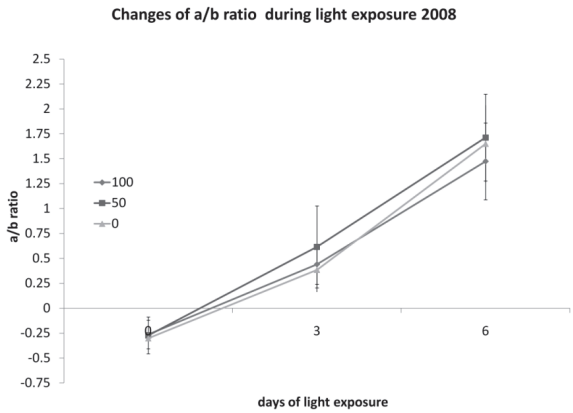


Figure 13 Changes in a/b ratio (which present direct respond to the changing anthocyanin content) depending on nitrogen fertilization levels of bagged apples. Vertical bars represent standard deviations.

In experiment II, with increasing nitrogen level the fruit size increased; 79, 81 and 85g, respectively for non fertilized, middle and high nitrogen level. Weight losses measured after storage showed that apples fertilized with 50 kgN/ha lost around 6.5% of their weight, while the loss caused by other nitrogen levels were 5.5-5.6%. Despite these losses, there was no effect of nitrogen on apples firmness after storage in experiment II. In experiment I (PAPER I) the effect of nitrogen fertilization on texture was minimal; apples from the moderate nitrogen level were slightly firmer (49N/cm^2) than other levels (46N/cm^2). There was also no relationship between the decrease of firmness of 'Pigeon' apples and the N supply in the study by Kühn et al. (2011). Several attempts have been made to correlate apple firmness after storage to pre-storage factors. Mineral content of fruit is not a good predictor of firmness (Johnson 2000). In general, softer apples at harvest are expected to be softer after storage. When leaf boron content, skin greenness and fruit nitrogen variables were included as variables into a firmness prediction model, 76% of the variation was explained in comparison to 55% variation explained by firmness at harvest alone (Johnson & Ridout 1998).

Nitrogen fertilization effects on sugar and acidity content was insignificant in experiment II. Apples from trees treated with 0 and 100kgN/ha had average sugar contents of 13.5 and 13.0% Brix, while apples from the moderate nitrogen level had 12.9% Brix. TA content was 8.16 g/L in apples from unfertilized trees while apples from high and moderate N level had 8.0 g/L. In a study of Kamamura et al. (2000) apple fruits from high N fertilized trees had lower soluble solid content, were softer and had poorer color. Similar results were obtained by Raese et al. (2007) in 'Golden Delicious' apples, where additionally titratable acidity was lower in apples from high nitrogen level trees. In a study of Kühn et al. (2011) the correlation between sugar content of 'Pigeon' and N fertigation (application of fertilizer through an irrigation system) was not found. The influence of some pre-harvest factors on quality might be more pronounced in big-fruited apple rather than fruits with small size genetic potential. In experiment I (PAPER I), despite the strong reduction of fruit load (50%), thinning resulted in 8.4% average

increase in fruit size (from 77g to 84g). The size compensation after thinning can in optimal cases result in almost similar yield levels (Hansen 1982;Knight 1980), which combined with a more favorable price on larger fruits results in an economic surplus. For 'Ildrød Pigeon' apples under 45mm diameter producers get 5.11kr/kg. However, increase in size, to 45-50 and 50-60mm, lead to higher prices, 8.23 and 7.40kr/kg respectively (Sakskøbing-storage facility; oral communication). A 3g increase in fruit weight of an 'Ildrød Pigeon' apple leads in average to approximately 1mm increase in fruit diameter (Toldam-Andersen, unpublished data 2011). Furthermore, based on a normal relationship between diameter and fruit weight for 'Ildrød Pigeon', the fruits from experiment I from thinned and unthinned trees can be estimated as apples with 50-60mm diameter (Toldam-Andersen, unpublished data 2011), so the effect of thinning was rather small. However, according to Toldam-Andersen (unpublished data 2011) the effect of late thinning on sugar content surplus is more beneficial.

'Ildrød Pigeon', in general, is a small fruited apple variety with an average fruit diameter of 50-60mm (Pedersen 1950) and therefore apples have only a small genetic potential to produce larger apples. The importance of the genetic potential for fruit size in relation to the effect of thinning was demonstrated by Hansen (1989). Large size fruit crops showed strong ability to react on crop load changes by increased size, dry matter and acid content.

In experiment II, the internal ethylene production after storage was lowest (669µL/L) in non-fertilized apples. Apples from medium and high fertilized trees produced moderate to high levels of ethylene with 1186 and 1410µL/L, respectively. The same ethylene trend was observed in experiment I described in PAPER I. It is known that extensive nitrogen levels retard fruit maturation by decreasing starch degradation and skin pigmentation (Goode & Higgs 1977;Kühn et al. 2011) but in our study retard maturation was observed as lower ethylene concentration in non-fertilized apples. Insufficient assimilate delivery, especially during the later part of fruit growth, may due to a reduced leaf area development at low N level, cause slower ripening. In contrast at high N levels increased competition for assimilates may periodically occur, when shoot growth increases due to augmented levels of fertilizers in the early season, resulting in high levels of early fruit drop and thus a more favorable assimilate availability during the later ripening (Toldam-Andersen & Hansen 1995). In PAPER I delay of maturity was confirmed in apples from non-fertilized trees as lower ethylene level and low fruit under to fruit on the tree ratio (fruit drop).

It needs to be mentioned that fruit variability, even among apples from the same growing and storage conditions, is a challenge in case of results understanding. All analyses presented in experiments were carried out on individual apples. Even though we controlled some horticultural pre-harvest aspects, as well as conditions during postharvest treatments, there is still additional variation in fruits from the same tree. These fruits can vary visually as well as internally. Differences in sweetness, crispness and flavour perception exists within fruits from top and bottom and side to side (Dever et al. 1995). Differences in aroma pattern were also observed on individual apples of three cultivars dependent on position on the tree by Petersen et al. (2007). This additional variability might have increased the standard deviation in our experiments and in some cases diminished the influence of investigated factor.

Fatty acids and amino acids are substrates for the synthesis of straight and branched chain ester in fruits (Rowan et al. 1996;Wyllie & Fellman 2000). It was shown that nitrogen fertilization affected amino acid levels in grapes, which lead to changes in wine quality (Ough et al. 1968;Spayd et al. 1994). Apple aroma composition in relation to nitrogen fertilization was evaluated. In experiment II, many compounds were insensitive to elevated nitrogen levels. However, volatiles listed in Table 2 reacted significantly on the nitrogen levels (19 out of 36 compounds).

Table 2 Aroma compounds which significantly reacted on different nitrogen levels ($p \leq 0.05$). Values in a row marked with the same letter are not significantly different
*: 'Ildrød Pigeon' principal aroma compounds (chapter 6.1)

Nitrogen Level (kgN/ha)	**0**	**50**	**100**
Compounds	**Relative Area x 10^3**		
propanal	4.6±1.9 b	6.2±2 a	5.9±2 a
2-methylbutanal	1.3±0.7 b	1.6±1.2 ab	2.2±1.6 a
hexanal*	156±70 b	395±152 a	191±138 b
2-methyl-2-pentenal*	15±7 b	30±16 a	15±9 b
2-hexenal*	488±155 b	603±137 a	596±233 a
octanal	0.6±0.1 b	0.8±0.3 a	0.6±0.1 b
2,4-hexadienal 1*	2.6±0.9 b	3.4±1.7 a	2.4±1 b
2,4-hexadienal 2*	6.5±2.3 b	8.5±4.2 a	5.9±2.3 b
decanal	0.9±0.4 b	1.3±0.6 a	0.7±0.2 b
methyl acetate	3.4±1.9 a	1.8±0.8 b	4.1±2.3 a
ethyl acetate*	90±46 a	21±12 b	74±59 a
propyl acetate	7.3±4.5 ab	4.7±3 b	7.7±5.2 a
butyl acetate	7.8±5.1 a	4.2±2.6 b	7.2±4.7 a
1-hexanol	719±397 a	512±288 b	680±411 a
2-hexen-1-ol	2.3±1 b	3.8±1 a	2.9±1.6 b
1-heptanol*	2.5±1 a	1.8±0.8 b	2.4±1.2 a
2-propanone	22.2±7 a	16.2±4.1 b	17.6±2.8 b
2-ethyl-furan	2±0.6 b	2.7±1.6 a	1.7±0.6 b
farnasene	4.9±5.5 b	9.5±7.9 ab	11.4±10.8 a

The low N and high N levels caused high production of acetate esters, 1-hexanol and 1-heptanol in comparison to medium nitrogen level. In contrast 2-hexen-1-ol, hexanal, 2-methyl-2-pentenal, 2,4-hexadienal, 1 and 2- decanal, octanal and 2-ethylfuran had higher production in apples treated with 50kgN/ha. In non-fertilized apples, production of propanone was higher but propanal, 2-hexenal, 2-methyl butanal and farnesene was the lowest. There is limited information about nitrogen fertilization effects on aroma profile in apple; however in strawberries esters and hexanal production increased with elevated nitrogen doses (Ojeda-Real et al. 2009). In peaches, concentration of α-decalactone which is a major constituent of peach aroma, was lowest in high nitrogen while cis-3-hexenol increased with fertilizer levels. These studies suggest also that with extensive nitrogen fertilization, peach flavour, mainly sweetness and aroma, is aggravated (Huijuan et al. 1999). The same observation was obtained in case of 'd'Anjou' pears, where flavour ratings decreased with increasing fertilization (Raese 1977). Production of 'Ildrød

Pigeon' principal compounds presented in Table 2, except 2-hexenal, 1-heptanol and ethyl acetate, was higher in apples harvested from trees fertilized with moderate nitrogen level. The production of aroma compounds was also investigated in PAPER I (experiment I), where cropping level additionally to nitrogen doses influenced aroma composition. Ethylene production was low in apples from unfertilized trees. Additionally, there was a trend of decreased production of ethylene, hexanal, butanal, farnesene and 3-octanol with higher crop load. 'Ildrød Pigeon' is a small fruited cultivar and as it was described in details in PAPER I the effect of crop load on 'Ildrød Pigeon' apple fruit growth and aroma compounds was rather weak. Nevertheless, experiment II results were similar in the case of most compounds, despite diverse experimental setups. In both experiments I and II ethyl, propyl, butyl acetate and 1-hexanol, followed the same trend being most abundant 5 days after storage in apples fertilized with 0 and 100 kgN/ha. Hexanal and 2,4-hexadienal, 2-hexen-1-ol had highest production in apples from 50kgN/ha. Hexyl acetate, methyl 2-methylbutyrate, 1-pentanol, 3-octanol and butanal were insensitive to nitrogen levels. Formation of volatile aroma compounds is a complex process, which as it was presented, might be affected to a different degree by variety of factors.

8. *APPLE AROMA RECOVERY AFTER STORAGE*

The main reason for cold storage after harvest is to maintain quality by slowing down all metabolic processes in apple fruits, for example by controlling the temperature. After storage appropriate ripening recovery time is needed, especially to provoke aroma development before putting apples on the market. Different lengths of ripening period have been reported necessary to obtain marketing quality in different cultivars; 7 days for 'Gala', 'Delicious', 'Granny Smith', 'Fuji' (Bai et al. 2005;Baldwin et al. 2003;Marin et al. 2009), 8 days for 'Jonagold' (Mir et al. 1999) while 14 days for 'McIntosh' (Deell et al. 2008). Volatile production during ripening after storage might, apart from cultivar, depend on harvest time. 'Starkspur Golden' apples had highest total volatile production after a long period of 33 days at room temperature after storage when early picked (158 and 173 DAFB), while apples harvested later (181 DAFB) achieved maximum aroma production after 10 days (Vanoli et al. 1995).

Therefore, suitable length of this period needs to be investigated for 'Ildrød Pigeon' apples. Based on aroma pattern and ethylene production after cold storage it was possible to evaluate the period in which these give optimal level. Aroma analyses were carried out 5, 8 and 15 days after removal from 8 weeks of cold storage to room temperature. The highest ethylene and aroma production were observed at day 5 and/or 8 days at room temperature. Most of the volatiles then showed a decreasing trend until day 15. Ethylene trend was parallel with production of the acetates in agreement with observations for other apple varieties (Defilippi et al. 2005;Lopez et al. 2007). Only methyl 2-methylbutanoate differed with a significant increase during ripening. These results agree with Defilippi et al. (2005) who observed that ripening had a negative effect on the synthesis of the acetates while the rest of the esters increased. The decrease in hexyl acetate also confirms the findings of previous reports for 'Fuji' (Altisent et al. 2008;Echeverria et al. 2004b;Echeverria et al. 2004c) and Delicious apples (Fellman et al. 2003). E.g. in our case the substrate for hexyl acetate; 1-hexanol minimally decreased as it was observed in 'Fuji' apples (Echeverria et al. 2004b). Furthermore, butyl acetate decreased significantly with progressive ripening (15 days), which is in agreement with observations of others (Tough and Hewett, 2001). This result may be explained by insufficient availability of its precursor, 1-butanol, which also decreased significantly after 8 days. Propyl acetate showed another tendency, increasing during the first 8 days and then decreasing until day 15. Lopez et al. (2007) also found significant increases of propyl acetates in Pink Lady® apples after 7 days at 20ºC along with increases of 1-propanol. In our study, 1-propanol and 1-pentanol increased significantly during the whole period after storage, reaching the highest amounts at 15 days (Table 3). On the contrary, the rest of the alcohols determined in our samples reached the lowest amounts at 15 days (Table 3).

The ripening period after storage had also a mixed effect on aldehyde production. Propanal and 2-methyl butanal increased within the ripening period, while hexanal remained constant. The rest of the aldehydes had decreasing tendencies between day 8

and 15. According to several authors (Defilippi et al. 2005) the volatile profile of apples is dominated by aldehydes at harvest and early phases of ripening, representing even up to 90%. Therefore, decreasing production of aldehydes might indicate advances in stage of ripening.

Table 3 Aroma compounds production at 5, 8 and 15 days at room temperature after storage. ↑↓ - Arrows point out rising or decreasing compounds tendencies between days.

	DAYS AFTER STORAGE				
	5		**8**		**15**
Ethylene (µL/L)	1335a		1260a		992b
	MEAN RELATIVE AREA X 10^3				
Esters					
ethyl acetate	93±63a		102±54a	↓	62±56b
propyl acetate	13±4.2b	↑	17±5a	↓	14±5.2b
butyl acetate	13±6.2a	↓	11±4b	↓	5.4±3.2c
hexyl acetate	6±2a	↓	5±2b	↓	3.5±2c
methyl 2-methyl butyrate	13±11c	↑	35±22b	↑	57±35a
Alcohols					
1-propanol	152±67c	↑	258±98b	↑	361±157a
1-butanol	1276±695a		1367±610a	↓	1059±420b
1-pentanol	30±10c	↑	44±14b	↑	75±27a
1-hexanol	741±243ab		769±190a	↓	692±197b
3-octanol	3.7±1.9a		3.3±1.8a	↓	1.4±0.9b
2-hexen-1-ol	3.4±1.4a		3.4±1.4a		3.1±1.4a
1-heptanol	3.4±0.9a		3.7±0.9a	↓	2.7±1.2b
Aldehydes					
propanal	6±2.5b	↑	9±4.1a		9.5±6.5a
butanal	83±51a		80±39a	↓	35±22b
2-methyl butanal	2.7±1.7b	↑	3.7±1.7a		3.7±2.1a
hexanal	337±184a		363±183a		351±176a
2,4 hexadienal	2.1±0.7a		2±0.7a	↓	1.6±0.8b
benzaldehyde	2.5±0.8a		2.4±0.9a	↓	2.0±1.1b
Others					
farnesene	25±18ab		28±14a	↓	20±14b
hexanoic acid	9.7±8.6b		11±10b	↑	15±11a

Finally, in our experiment hexanoic acid increased during ripening while farnesene decreased between day 8 and 15. The development of the physiological disorder 'superficial scald' (browning) was found to be related to the presence of oxidised forms of farnesene (Rupasinghe et al. 1998). This sesquiterpene production in apples was found to be stimulated by higher ethylene during ripening (Rupasinghe et al. 1998;Rupasinghe et al. 2003). In our experiment with decreased ethylene production farnesene also decreased.

9. *APPLE QUALITY DEPENDS ON HARVEST, 1-MCP AND STORAGE*

Determination of the appropriate harvest time is crucial for fruit storability (Echeverria et al. 2004c). It is especially important for climacteric fruits, as their development at harvest time must allow the fruit to develop postharvest to a product of edible quality. Harvest cannot be done too early, as fruits will be immature and unable to form good quality after they are detached from the parental plants. On the other hand, harvest cannot be performed too late as apples might be overripe and lose their storage potential as fruit in the climacteric phase undergo rapid loss of firmness and eating quality (Mattheis et al. 1995).

The effect of harvest time, storage length and 1-MCP application is presented in the following chapter. Apples were harvested at two developmental stages: early harvest (harvest index=0.38) on 29.08.08 and at the commercial harvest time (harvest index=0.16) on 12.09.08 (Figure 12). No postharvest light exposure was performed here as focus was mainly on the effects of maturity stage at harvest and storage length combined with the ethylene action inhibitor. Immediately after each harvest, apples were treated with 1μL/L1-MCP in air for 20 hours and then transferred to cold storage (1.5^{o}C). Control/untreated samples were kept in cold storage directly after each harvest. Samples were kept in storage both for 4 weeks (short storage) and for 10 weeks (long storage) after each harvest. Before analysis, apples were finally kept for 5 days at room temperature to obtain the optimal aroma production.

9.1 Quality parameters

Ethylene concentration, in control fruit from first harvest, was higher than their corresponding fruits from second harvest. Storage length was positively correlated to ethylene concentration (Figure 14). 1-MCP suppressed ethylene to practically zero in apples from the first harvest. When 1-MCP was applied to apples after second harvest, ethylene synthesis was also lower but not to such an extent as in apples from the first harvest (Figure 14). Ethylene production of apples from the second harvest was only 1.4-1.6 fold higher in the control than in treated apples. It might be that ethylene production was accelerating when apples harvested in the second harvest. In that case, ethylene molecules already had occupied most of its receptors. Thus, 1-MCP could not bind to them and therefore inhibition was not as strong as at the first harvest. Longer storage prompted ethylene synthesis in all corresponding samples.

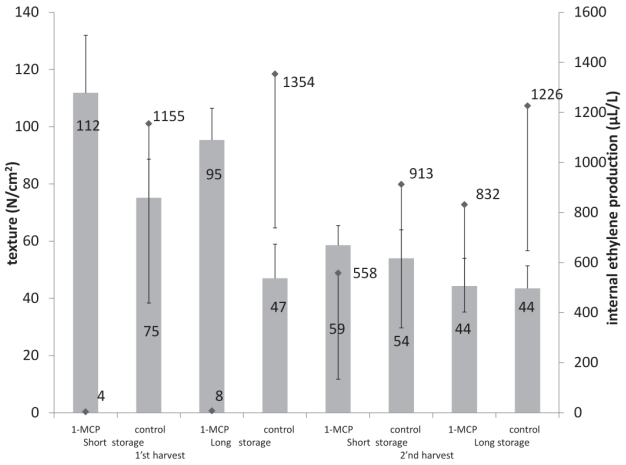


Figure 14 Texture (N/cm^2, columns) and internal ethylene concentration (µL/L, ♦) depend on harvest time, 1-MCP treatment and storage length. Upper and down bars represent standard deviation for texture and ethylene respectively. Abbreviations; 1'st harvest = early harvest, 2nd harvest= commercial harvest time.

Weight loss was significantly affected by harvest time as fruits from second harvest lost 7.13% of the initial weight in comparison to apples from the first harvest (4.95%). However, it might be due to a pronounced effect of storage time. Apples from commercial harvest (2nd harvest), including 1-MCP treated samples, when kept in storage for 10 weeks lost significantly more than other treatments (Figure 15). These losses were doubled from short to long storage (Figure 15). However, in all samples 1-MCP prevented weight losses in comparison to the controls. 1-MCP also decreased loss of firmness, except fruit from second harvest kept in long storage. These apples were softest (44N/cm^2) as well as control apples from the first harvest kept in long storage (47N/cm^2). Our results suggest the storage period is a crucial factor in progressive transpiration and softening processes but still harvest time is an important factor. This is in agreement with the study of Mir et al. (2001), where 'Redchief Delicious' apples independent of 1-MCP application tended to soften more rapidly with delayed harvest.

'Ildrød Pigeon' apples generally soften rapidly during storage. The average firmness at second harvest was 107N/cm^2 and dropped to 44N/cm^2 after 10-week of storage. It counts for around 41% of texture loss. There is therefore a good reason for testing the application of 1-MCP. A treatment with the ethylene inhibitor, at first harvest, greatly improved apple firmness (Figure 15). However, from a marketing point of view consumers might not accept such a hard 'Ildrød Pigeon' apple at Christmas. On the other hand, 1-MCP application on apples from second harvest only slightly improved their firmness. It might be that the commercially recommended harvest time of 'Ildrød Pigeon' (harvest index = 0.17) is too late if 1-MCP is to be applied. The maintenance of fruit firmness is the most appreciated apple's respond to 1-MCP application. Most studies report that softening was prevented or delayed by 1-MCP application (Deell et al. 2005;Defilippi et al. 2004;Fan et al. 1999;Saftner et al. 2003;Watkins 2006;Watkins 2008). Firmness of 'Redchief Delicious' was kept even at room temperature (20^{o}C) for more than 100 days but with

multiple 1-MCP applications (Mir et al. 2001). The difference in firmness of 'Gala', 'Ginger Gold', 'Jonagold' and 'Delicious' treated and untreated apples exceeded 10N/cm^2 after 6 month of storage (Fan et al. 1999). This favourable feature of 1-MCP is especially appealing in case of varieties like 'McIntosh', which has very low storability because of its extensive softening (Watkins 2006;Watkins 2008). The same applied for 'Ildrød Pigeon' cultivar as it lost firmness rapidly during post-harvest stage.

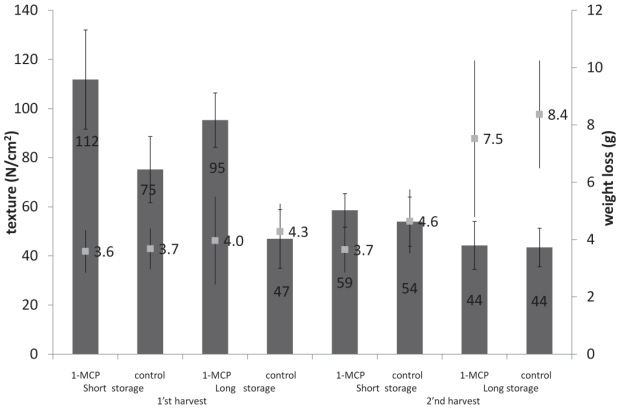


Figure 15 'Ildrød Pigeon' texture (N/cm^2, columns) and weight loss (g, ■) depend on harvest time, storage period and 1-MCP application. Vertical bars represent standard deviation

The titratable acidity was significantly higher in apples from the first than second harvest, (10.50 and 9.34g/L respectively, (Figure 16). Acidity decreased during storage, except in control samples from second harvest. This corresponds with other findings where prolonged cold storage (from 3 to 7 months) of 'Fuji' apples resulted in the loss of half of their initial malic acid content independent of harvest time (Echeverria et al. 2004c). Titratable acidity of 'Redchief Delicious' apples also decreased during storage independent of harvest time and there was no effect of 1-MCP treatment (Mir et al. 2001). In contrast, in our study 1-MCP inhibited usage of acid, as a source for metabolic processes in all samples (Figure 16). Overall total soluble solids content was higher in apples from second harvest and neither storage nor 1-MCP treatment significantly affected it. This higher content of sugar in apples from second harvest is probably due to the extended period of attachment to the tree, in which accumulation of translocated sugars into the fruit were continued. Higher soluble solids content at harvest was observed in apples picked 195 DAFB (days after full blooming) in comparison to harvest at 185 DAFB (Echeverria et al. 2004c). In the same experiment, there was no effect of cold storage period (3, 5, 7 months) on sugar content (Echeverria et al. 2004c).

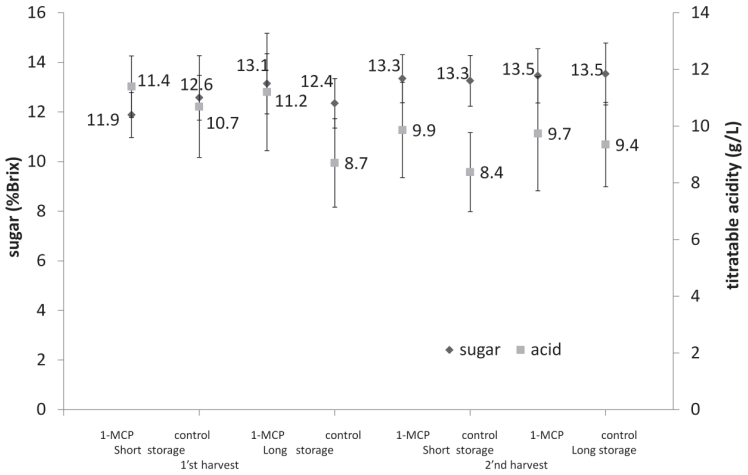


Figure 16 Sugar (%Brix) and titratable acidity (mL/L) depend on harvest time, storage length and 1-MCP treatment. Vertical bars represent standard deviation

The relationship between organic acid, soluble solids content (SSC) and ethylene is not yet obvious (Watkins et al. 2000). Generally, less ripe fruits (lower ethylene production) contain higher acid level and lower level of soluble solids than riper apples. Malic acid, as the principal organic acid in apple, decreases during storage as it is used for respiration (Ackermann et al. 1992;Defilippi et al. 2004). 1-MCP-treated apples and transgenic fruits, in which ACC synthase or ACC oxidase activity was suppressed, also showed also reduced loss of acids (Defilippi et al. 2004). These results might indicate that ethylene is involved somehow in organic acid metabolism. 1-MCP retards the loss of titratable acidity (TA) in apples but the effect depends also on storage conditions. Treated apples of four different cultivars had higher TA when stored in air, but the results from CA stored apples were inconsistent (Defilippi et al. 2004;Watkins et al. 2000). 1-MCP treated apples of 'Fuji', 'Gala', 'Ginger Gold', 'Jonagold', and 'Delicious' varieties, stored in air, had higher TA and greater or equal SSC in comparison to control (Fan et al. 1999). A higher content of acid might be due to low respiration rate during storage. SSC is usually related to starch breakdown to sugars, which normally starts before the climacteric peak and continues in storage (Brookfield et al. 1997;Watkins et al. 2000). Soluble solids in 1-MCP treated apples is reported as higher, lower or at the same level as in untreated fruits (Watkins 2006). Total soluble solids of 'McIntosh' and 'Delicious' remained unaffected after 60 and 120 days in air or controlled atmosphere storage (Rupasinghe et al. 2000). In another study, SSC was the lowest in 'McIntosh' and 'Law Roma' but the highest in 'Delicious' and 'Empire', all 1-MCP treated and stored in air (Watkins et al. 2000). Total soluble solids content is taking into consideration as ethylene-independent; however, the behavior of each individual sugar to ethylene action remains unclear (Defilippi et al. 2004). As it was observed in our experiment, the maturity stage, meaning the progress of starch hydrolysis at harvest, might play a role in the sugar changes during storage.

9.2 Aroma compounds

There is a general trend in volatile production in the experimental samples. The concentration of most aroma compounds was highest in untreated apples from commercial harvest while the lowest concentration was observed in 1-MCP treated apples from the early harvest. Both untreated apples from early harvest and 1-MCP treated apples from commercial harvest showed similarities in aroma pattern. Production of aroma components was positively stimulated by prolonged storage period. If to take into consideration ethylene and aroma profile during ripening, it looked like experimental apples were analyzed at the three developmental stages after storage; early (pre-climacteric), moderate (climacteric) and advanced ripening (post-climacteric). In two last stages, the storage length plays a very important role in enhancing overall apple aroma.

Ester production was greatest in untreated fruits from commercial harvest stored for 10 weeks (Table 5), except hexyl esters. Hexyl esters were produced in similar high levels in apples from the early harvest (control) and the commercial harvest (control and 1-MCP treated) after cold storage for 10 weeks. Generally, longer storage accelerated ester synthesis. What is more, it might look like longer storage overcame the action of 1-MCP in apples from the second harvest. Ester production is stimulated by ethylene (Mattheis et al. 2005;Song & Bangerth 1996) and as ethylene production was higher, the ester production raised from short to long storage. Such an effect of longer storage is positive in case of production of esters, which are responsible for fruity aroma odour and whose occurrence in apples is appreciated. Despite the storage length, the inhibitory effect of 1-MCP on early harvested apples was very strong. Most of the acetate esters were produced at minimal levels in these apples, while butyl acetate, butyl butyrate and methyl 2-methylbutyrate esters were totally inhibited at this developmental stage. The untreated apples from early harvest stored for 4 weeks, despite high internal ethylene concentration, were not able to accelerate ester synthesis. It is suspected that appropriate time in the storage is necessary to trigger ester formation. Production of **alcohols** followed a trend similar to esters, having the lowest concentration in early harvested fruits except controls kept for 10 weeks. The comparable production pattern of alcohols and esters was also observed in 'Gala' apples (Mattheis et al. 2005). However, in the current study the alcohol synthesis in apples from early harvest treated with 1- MCP was not so drastically inhibited as the ester synthesis. It suggests that ethylene affected alcohol production but not as direct as it affected esters. The increased alcohol synthesis in corresponding samples from the early and the commercial harvest was remarkable (Table 5). Commercially harvested, 1-MCP treated apples kept for 4 and 10 weeks had 244 and 132 fold higher 1-butanol production in comparison to the apples from early harvest. Production of 1-hexanol in commercially harvested, 1-MCP treated apples kept for 4 and 10 weeks was 48 and 26 fold higher in comparison to the apples from early harvest. Opposite results were obtained by Lurie et al. (2002) as 1-hexanol decreased with the ripening progression. 1-butanol however in intact 'Gala' apples showed an increasing trend within prolonged storage (Mattheis et al. 2005), as it did in our study. The long storage had a positive effect on all alcohol compounds. The concentration of 2-ethyl-1-

hexanol and octanol in early harvested apples was positively affected by longer storage, but the effect was reversed in apples from the commercial harvest.

Aldehydes showed the most varying respond to applied conditions. Propanal, butanal and 2-methylbutanal had low concentrations in 1-MCP treated apples from the early harvest and their production was greater in other samples especially in these from longer storage. Decanal production was enhanced by longer storage in apples from the early harvest but this tendency was reversed in the apples from the commercial harvest. Octanal production was enhanced by longer storage except in 1-MCP treated apples from commercial harvest. Aldehydes; 2-methyl-2-pentenal, two isomers of 2,4-hexadienal cis-3-hexenal and trans-2-hexenal represent characteristic aroma constituents of 'Ildrød Pigeon'. These aldehydes had higher production in short rather than in long stored apples, exceptions were cis-3-hexenal and trans-2-hexenal in 1-MCP treated fruit from early harvest. In the short stored apples the internal ethylene concentration was always lower in comparison to the concentration of the long stored apples and various aldehydes responded with higher or lower biosynthesis suggesting various aldehyde sensitivity to this plant hormone. 2-methyl-2-pentenal and isomer forms of 2,4-hexadienal had greater concentrations in apples from short storage/lower ethylene concentration. They are amino acid and fatty acid lipoxygenase degradation products, respectively. It might be that rising ethylene concentration more distinctly affects the aldehydes, which contain fewer than six carbons. In opposite, lower ethylene concentration promotes some C-6 aliphatic aldehydes; cis-3-hexenal and trans-2-hexenal. In 1-MCP treated apples from the first harvest cis-3-hexenal production was 4 and 10 fold higher than in other corresponding samples. Despite high production of cis-3-hexenal, trans 2-hexenal concentration in 1-MCP treated apples from the first harvest was at levels similar to other apples from the long storage. These two compounds are LOX-derived compounds produced in response to the wounding. trans-2-Hexanal is produced form linolenic acid (18:3). Firstly, 18:3 is oxidised by LOX to 13-HPOT, which is secondly cleaved by HPL resulting in synthesis of cis-3-hexenal and trans-10-ODA. Trans-10-ODA is autooxidised to traumatic acid, known as wound signal (Zimmerman & Coudron 1979). Cis-3-hexenal is isomerized, probably enzymatically, to more stable form of trans-2-hexenal (Baldwin et al. 2000). Myung et al. (2006) suggested that factor which regulates the isomerisation of cis-3-hexenal to trans-2-hexenal is activated upon wounding and therefore cis-3-hexenal is rapidly converted to trans-2-hexenal. However, the factor at some time after wounding returns to deactivated form allowing cis-3-hexenal to accumulate. It is still unclear which factors regulate cis-3-hexenal transformation, if this conversion is induced by the enzyme or is it a spontaneous isomerization or if the isomerisation factor expression depends on the fruit developmental stage. In our study, apples from the short storage periods had higher cis-3-hexenal accumulation followed by higher trans-2-hexenal production (except 1-MCP treated apples from early harvest). In these samples, short storage accelerated and intensified the lipoxygenase pathway but the conversion factor was not blocked. Moreover, cis-3-hexenal accumulation was even more enhanced in 1-MCP treated sample from the short storage. In 1-MCP treated fruit from early harvest the strong action of the ethylene inhibitor led to lack of sufficient ethylene, triggered cis-3-hexenal synthesis, and additionally inactivated the isomerisation factor so trans-2-hexenal production was lower

despite substrate availability. It is hypothesized that the isomerisation factor action is blocked when internal ethylene concentration is not sufficient, so it might be also related to early, pre-climacteric stage of ripening. Such an effect of the low ethylene concentration on these aldehydes is, to our best knowledge, observed for the first time.

Volatile compounds correlations The substrate availability, the competition for the substrates, the enzyme activity and specificity are factors, which have a great importance on the formation of volatile compounds. In our study some butyrate and acetate esters were produced simultaneously and were not in competition for acid substrate fx. greater emission of butyl acetate and butyl butyrate was correlated with higher production of propyl acetate and propyl butyrate (r=0.88/0.93) (Table 4). There was a strong dependence observed between the corresponding aldehydes and alcohols as well as between the relevant acetate and butyrate esters (Table 4). Acid content was very important but more pronounced in the formation of butyrates than acetates. In the study of Kondo et al. (2005) the high correlation of acetate esters was found to be related to the corresponding alcohol rather than to acetic acid. Results suggest that butyrate ester production is dependent on the butyric acid accessibility while acetates biosynthesis might require more involvement from the essential enzymes. The C6-aldehyde straight chain ester synthesis might be more related to enzyme activity in apples rather than to substrate availability (Table 4). The correlation between hexanal and hexanol was low and the synthesis of hexyl acetate from acetic acid was less pronounced than hexyl butyrate from butyric acid (Table 4). Despite the importance of potential substrates for hexyl ester production their levels cannot always be related to the formation of a specific aroma compound, which indicates the important role of AAT enzyme in the hexyl ester production (Figure 17). In the study of Lara et al. (2006) stronger correlation was found for butyl acetate rather than hexyl acetate with the alcohol precursor. The enzyme involvement depends also on the developmental stage of the fruit. Echeverria et al. (2004d) claimed that enzyme activity is less significant than substrate availability during on-tree maturation while Li et al. (2006) suggested LOX and ADH enzymes as most important in the upstream pathway of straight-chain ester biosynthesis during post-storage ripening.

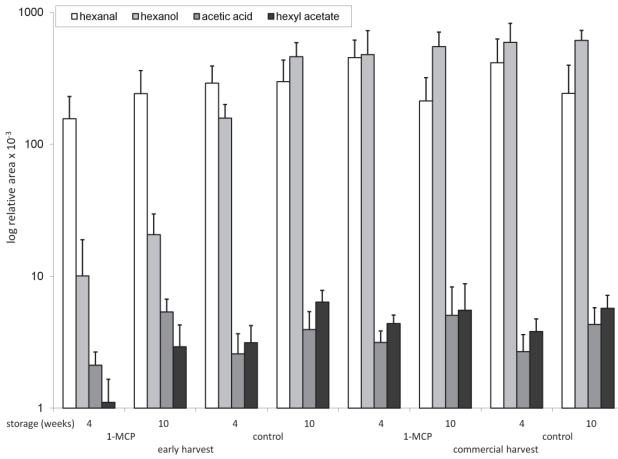


Figure 17 The production of hexyl acetate and its potential substrates. Upper bars show standard deviation (n=12)

Table 4 The Pearson correlation coefficients between pair of aroma compounds (n=96).
*, **, *** and # - donates significant at 0.0001, 0.001, 0.005 and 0.05
ns – not significant

	propanol												
propanal	.89*	propanal											
propyl acetate	.50**	.58*	propyl acetate										
propyl butyrate	.90*	.87*		propyl butyrate									
butanol					butanol								
butanal					.84*	butanal							
butyl acetate			.88*		.62*	.86*	butyl acetate						
butyl butyrate				.93*	.94*	.93*	.86*	butyl butyrate					
hexanol									hexanol				
hexanal									.35**	hexanal			
hexyl acetate									.55*	.21ns	hexyl acetate		
hexyl butyrate									.59*	.21ns		hexyl butyrate	
acetic acid			.33#				.45***				.32**		acetic acid
butanoic acid				.88*				.77*				.47***	

The time of harvest influences the volatile formation during postharvest ripening and determines the regeneration of aroma volatile after storage. With advancing maturity, the time required to regenerate aroma volatile to the optimal level after storage decreases (Fellman et al. 2003). Immature fruit produce less volatile compounds at harvest and lose the capacity for volatile production during storage (Fellman et al. 2003). In our study, apples harvested early and 1-MCP treated had very poor aroma and ethylene formation after storage. It indicates their early maturity stage. Additionally, the production of 2-ethylfuran, 5-ethyl-2-furanone and 3-pentanone was higher in fruit 1-MCP treated early harvest, and in untreated fruit from early harvest kept short in cold storage. These might be indicators of pre-climacteric stage as fx. 2-ethylfuran concentration was highest in freshly harvested apples and decreased or disappeared with progressive ripening (Lurie et al. 2002). In contrast, 10-weeks of storage of early harvested, control fruits improved aroma production to a level similar to apples 1-MCP treated apples from second harvest. It indicates that, despite picking fruits 2 weeks earlier than recommended time, volatiles could obtain the concentration similar to fruit picked at optimal time, which were 1-MCP treated. In the study of Hansen et al. (1992) with the cultivar 'Jonagold' similar results were obtained with low and delayed volatile production in early picked apples compared to later picked apples. Song & Bangerth (1996) showed that 'Golden Delicious' apples picked 4 weeks too early had poor aroma production and strongly inhibited respiratory peak while fruits harvested 2 weeks before optimal time obtained normal aroma production pattern after few days at 20^{o}C.

In the current study based on internal ethylene concentration as well as firmness and aroma profile it can be concluded that 1-MCP treated fruit harvested early from both storage regimes represent pre-climacteric fruit stage, with very low ethylene concentration, very firm apples and low aroma production. The internal ethylene concentration of control apples from early harvest is higher in comparison to the control apples from the commercial harvest, which suggests climacteric (the early harvest) and post-climacteric (the commercial harvest) stages of these fruit, respectively. Therefore, the aroma composition of 'Ildrød Pigeon' fruit picked at commercial harvest seemed to be influenced rather by storage than by 1-MCP. For apples, which were harvested at commercial time (harvest index=0.16), it was too late to apply 1-MCP as ethylene production accelerated and ethylene bound to the receptors preventing 1-MCP action. For that reason, 1-MCP application was less effective and became a needless cost. Early harvested apples had higher harvest index, equal 0.38 and 1-MCP treatment performed on these fruit kept them very firm through storage, suppressed strongly ethylene production and prevented aroma compound formation. 1-MCP untreated early harvested apples stored for 4 weeks had, despite higher ethylene level, low capacity to regenerate volatile compounds to an optimal level, probably because of too short postharvest storage period. However, 1-MCP untreated early harvested apples stored for 10 weeks developed quality parameters generally lower but still comparable to 1-MCP treated apples from the second harvest. Harvest index of 'Ildrød Pigeon' needs to be revised if fruit is to be treated with 1-MCP to obtain better overall quality. The 'Ildrød Pigeon' variety shows extensive softening during storage, and could thus benefit from 1-MCP application if applied at the appropriate moment. Typical 'Ildrød Pigeon' storage last for 9-11 weeks and as it was

observed, such a storage period enhanced production of most measured aroma compounds in comparison to short storage period, which limits the post-storage aroma recovery. 'Ildrød Pigeon' harvest index should be lower than 0.38 but not reaching 0.16. Apples for the experiment II presented in the chapter 7 were harvested at harvest index 0.27, treated with 1-MCP, exposed to sunlight for 6 days and stored for 5 weeks. Apples 1-MCP treated before light exposure and untreated had ethylene concentration of 618 and 1463μL/L and firmness at levels 80 and 69N/cm^2, respectively. Those results might be a starting point in the evaluation of appropriate harvest time in the new postharvest management, in which 1-MCP is to be applied.

Table 5 Aroma compounds production dependent on harvest time, 1-MCP application and storage length. Letters follow means represent significant difference between treatments ($p \leq 0.05$). The concentrations of aroma compounds in the samples on the right from the red line were less affected by 1-MCP application than the samples on the left.

	early harvest				commercial harvest			
	1-MCP		Control		1-MCP		Control	
COMPOUNDS	4 weeks	10 weeks	4 weeks	10 weeks	4 weeks	10 weeks	4 weeks	10 weeks
ethylene (µL/L)	4d	8d	1155bc	1354ab	558c	832bc	913ab	1126a
Aldehydes	RELATIVE AREA X 10^3							
propanal	1c	2c	1c	4b	3b	4b	4b	6a
butanal	1e	1de	2de	31b	11dc	37b	19c	82a
2-methylbutanal	0.9d	1.5cd	1.8bcd	2.8a	1.7cd	2.6abc	1.9abcd	2.7ab
hexanal	156d	242cd	291c	298bc	454a	213cd	415ab	243cd
2-methyl-2-pentenal	33a	29ab	35a	21bc	35a	12c	31a	14c
cis 3-hexenal	103a	108a	27b	12b	28b	6b	18b	10b
2-hexenal	532c	665bc	1154a	775b	1349a	633bc	1263a	631bc
octanal	0.57cd	0.85ab	0.48d	0.84ab	0.95a	0.79abc	0.75abc	0.65bcd
2,4-hexadienal 1	5a	4b	5ab	2c	5a	2c	4b	2c
2,4-hexadienal 2	11a	8bc	12a	6cd	12a	4d	10ab	5d
decanal	0.77de	1.07abc	0.84cde	1.1ab	1.3a	0.63e	1bcd	0.8de
Esters								
methyl acetate	nd	1.3d	0.1d	12c	0.9d	27a	0.5d	19b
ethyl acetate	3d	7cd	9cd	29c	13cd	65b	17cd	204a
propyl acetate	nd	0.1e	nd	8c	3d	12b	5d	17a
methyl 2-methylbutyrate	nd	nd	nd	7bc	1d	13b	3cd	25a
butyl acetate	nd	nd	nd	7b	2cd	10b	4c	19a
butyl butyrate	nd	nd	nd	2b	nd	3b	nd	7a
hexyl acetate	1.1e	2.9d	3.1d	6.4a	4.9bc	5.5ab	3.8cd	5.7a
hexyl butyrate	nd	nd	0.1d	2.4b	1.4c	2.1bc	2.1bc	4.1a

hexyl 2-methylbutyrate	nd	nd	nd	2.7a	1.2c	2.8a	1.8bc	2.0ab
Alcohols								
2-butanol	1.2e	2.3cd	1.4de	5.0a	2.3cd	3.2bc	2.9c	4.3ab
propanol	0.1d	0.8d	7.3d	78ab	61c	71bc	111ab	150a
2-methylpropanol	5e	21d	39c	94a	50bc	86a	60b	93a
1-butanol	2d	6d	91d	529bc	488c	790ab	776b	1078a
1-pentanol	0.6e	1.3de	6d	23bc	20c	29ab	27ab	32a
1-hexanol	10e	21e	158d	462c	479bc	549abc	592ab	613a
2-hexen-1-ol	3.4cd	3.3cd	4.1bc	5.0ab	5.2ab	5.9b	4.7bcd	2.6e
1-heptanol	0.11e	0.26e	1.43d	2.23c	2.71ab	2.46bc	2.96ab	3.12a
6-methyl-5-hepten-2-ol	nd	nd	nd	1.4b	0.47d	1.77ab	0.84c	1.94a
2-ethyl-1-hexanol	0.8c	1.8a	0.2d	1.1bc	1.7a	1.0bc	1.4ab	1.2b
octanol	0.3d	0.8c	0.9c	1.4ab	1.6ab	1.2bc	1.7a	1.5ab
Ketones								
2-propanone	11.6c	11.5c	10.9c	11.3c	16.4b	13.0c	20.6a	12.2c
2-butanone	1.6bc	1.5bc	0.6d	3.4a	1.9b	2.1b	1.6bc	0.9cd
6-methyl-5-hepten-2-one	nd	1.3b	nd	3.7a	1.3b	nd	nd	2.8a
3-pentanone	3.4a	2.2b	0.5c	0.3c	nd	nd	nd	0.4c
5-ethylfuranone	25a	16a	7b	3b	6b	1b	4b	3b
Acids								
acetic acid	2.1d	5.4a	2.6d	3.9bc	3.1cd	5.1ab	2.7d	4.3abc
butyric acid	nd	0.8c	nd	2.2b	0.5d	2.0b	nd	5.6a
hexanoic acid	1.5c	4.1bc	4.4b	6.2ab	5.6ab	3.6bc	6.2ab	7.4a
Others								
2-ethylfuran	4.3a	3.1b	3.2b	1.5cd	3.0b	0.9d	2.3bc	1.2d
farnesene	nd	nd	3d	18ab	9cd	13bc	18ab	22a

10. *1-MCP APPLICATION TIME AND APPLE QUALITY*

'Ildrød Pigeon' apples are traditionally postharvest exposed to sunlight for up to 2-3 weeks to develop intense red color. However, such a long sun exposure causes additional losses; rotten apples, sunburn apple surface, advanced ripening with low firmness and low storability. In the study of Kühn et al. (2011) 'Pigeon' apples lost 45% of firmness from harvest until the end of the sun exposure period with further 20% reduction during storage until marketing. Shorter light exposure needs to be evaluated to prevent such a high firmness loss but to allow good fruit colorization. Additionally, a new 1-MCP timing application could be implemented into the postharvest treatment to maintain better apple quality during storage.

Apples to this experiment were harvested at harvest index 0.29 despite recommended index level 0.17. The details of the experimental set up were described in PAPER II and PAPER III. To control pigmentation changes 'Ildrød Pigeon' fruit were monitored systematically from harvest through 8 days sunlight exposure time using a colorimeter. Many different color indexes are used to describe pigmentation in apples. Both a/b ratio and hue (^{o}) have a very good correlation with anthocyanin content. Iglesias et al. (2008) claim that in 'Gala' apples this correlation could be a good predictor of maturity (maturity index), that could easily be used as rapid, cheap and non-destructive estimation of anthocyanin content in situ. The a/b ratio represents direct rising anthocyanin production (Iglesias et al. 2008).

Additionally in the experiment bags were put on apples at the end of July. Both practices, bagging and postharvest light exposure, are used commercially to improve visual fruit quality. Bagging of 'Ildrød Pigeon' apples prevented anthocyanin production and resulted in fruit with paler, greener skin. 'Ildrød Pigeon' apples when attached to the tree develop red cheek while the rest of the apples skin remains green (Kühn et al. 2011). This red cheek is also noticeable on non-bagged apples in Figure 18. The statistical discrimination between bagged and non-bagged apples at harvest is not strong as the spots on non-bagged apples, where color was monitored, were chosen excluding the area where red coloration already existed (Figure 19, day 0). That is a reason for only small difference in a/b ratio between bagged and non-bagged apples at harvest day. Bags were put on fruit to create experimental apples with paler skin on which anthocyanin development would be more pronounce during sunlight exposure. The color measurements at the first day (harvest) were used to monitor color changes over time of sunlight exposure dependent on 1-MCP treatment rather than to point out the effect of bagging at harvest time.

Bagging have been extensively used on several fruit crops to improve skin color (Arakawa 1991), reduced mechanical damage (Amarante et al., 2002b) and reduced sunburn of the skin (Bentley and Viveros 1992). The apple bagging of apples has been a conventional practice to improve visual quality in China and Japan (Huang et al., 2009; Whale and Singh 2007; Amarante et al., 2002a; Amarante et al., 2002b; Wang et al.,2000; Fan and Mattheis 1998) and has been used extensively in the Pacific Northwest of North America, mostly for 'Fuji' apples (Fan & Mattheis 1998). Despite the fact that both

practices; bagging and postharvest sunlight exposure, are used in horticultural management, their effects on aroma quality parameters has not been extensively studied.

Bagged apples (B) after bags removal at harvest

Nonbagged apples (NB) at harvest

Figure 18 Apples, bagged and nonbagged, at harvest time – 2.09.2009

10.1 Quality parameters (PAPER II)

10.1.1 External quality parameter - Color development

Bags are put on apples during fruit development and removed 2-3 weeks before harvest to enhance the red over color. Bagging influences apple firmness, disorders and reduce soluble solids, acids, starch and calcium content (Amarante et al. 2002;Fan & Mattheis 1998;Wang et al. 2000;Whale & Singh 2007). However there are conflicting results using different apple cultivars and time of bags removal (Fan & Mattheis 1998).
Apples, exposed to sun light postharvest developed significantly more color than the apples from cold storage/non-light treatment, independent of 1-MCP treatment. The main changes in anthocyanin production during sun light exposure started to occur after day 2 in all samples as a rapid decrease in L value and increased a/b ratio. The pre-harvest bagging effect on L value diminished from harvest to the end of light exposure. Red color changes (a/b ratio) increased and changes were stronger for bagged apples (Figure 19). It is in agreement with the study of Kühn et al. (2011), where it was shown that a green apple/shaded apple sides, which in our case might respond to bagged apples, rapidly changed color between day 1and 3 of postharvest sun exposure.
1-MCP application inhibited postharvest red color development. Apples with early 1-MCP treatment and control initially had similar a/b ratio, but at the end of the sun light treatment the red color (a/b ratio) increased less compared to the control (Figure 19). The 1-MCP treated apples showed the same trend of rapid color change at day 2-4 as control,

however, the ethylene inhibitor suppressed color development between day 6 and 8 resulting in a lower final color value (Figure 19). Similar results were obtained by Golding et al. (2003), where 1-MCP treated apples exposed to light produced color, but not at the same extend as untreated apples. Any inhibition of anthocyanin development is considered as negative, especially for red apple varieties, where the red color is an essential quality parameter. 1-MCP didn't affect color of 'Queen Cox' and 'Bramley' apple varieties (Dauny & Joyce 2002) and didn't influence anthocyanins content in peel of 'Empire' apples stored at CA (Fawbush et al. 2009), but these cultivars were not exposed postharvest to light and color changes during storage were rather unexpected.
In the experiment, maximum a/b ratio was observed at 8 days of exposure. However, because measurements were not carried after that day, it is not certain if anthocyanins would stabilize after that time. In case of the apple cultivar 'Delicious' anthocyanin accumulation reached maximum already after 3 days from bag removal (Ju 1998). Therefore, we could expect that more than 8 days of sun exposure will bring only minor further changes in anthocyanin production.

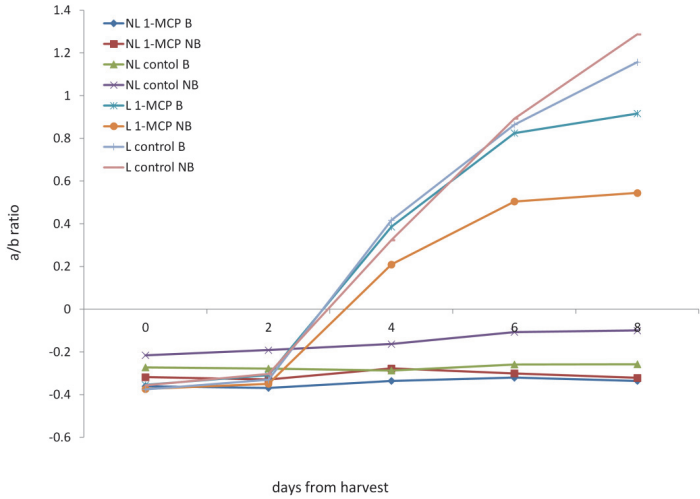


Figure 19 Changes in a/b ratio of apple samples (n=10) exposed to light for 8 days or kept in cold storage after harvest. Abbreviations; L: samples exposed to light after harvest, NL: no light = apples kept in cold storage after harvest, B: bagged apples, NB: nonbagged apples. There are 8 samples presented, as late 1-MCP sample is not included. *See experimental design explained in PAPER II.*

The main color change of 'Ildrød Pigeon' apples occurred between day 2 and 4 and a/b ratio increased slowly during the next days (Figure 19). The same pattern was observed at day 4 after bag removal and UV-B exposure on 'Fuji' apples (Fan & Mattheis 1998). Also at the study of Kühn et al. (2011) the main color changes occurred between day 1 and 3 for shaded apple sides and between day 6 and 9 for blush side of the fruits. These results suggest that 3 weeks of 'Ildrød Pigeon' light exposure can be definitely shortened.

10.1.2 Internal quality parameters

Early ethylene inhibitor application limited post-storage weight loss of light exposed apples to 6.7g. Late 1-MCP application and controls had higher losses (7.0g and 7.8g). Apples, which were treated with 1-MCP and kept in cold storage, lost significantly less (4.3g) in comparison to control fruit, which have lost 6.1g. Ethylene production in 1-MCP treated samples kept in cold storage was very low (2µl/L), while untreated fruit had production on the level of 1079 µl/L. Early 1-MCP application and light exposition led to an ethylene production of 171µl/L. Late 1-MCP application caused higher ethylene synthesis but not significantly different from the controls 1055 and 1363 µl/L. The sunlight exposure associated with higher temperature seems to enhance ethylene synthesis, strongly in early 1-MCP treated apples and slightly in control apples, exposed to light and kept in cold storage. The time of 1-MCP application had a strong influence on apple firmness. Early 1-MCP treated apples exposed to sun light had an average firmness of 80N/cm^2, while 1-MCP late application led to a firmness of 47N/cm^2. The average firmness of control apples exposed to light was the lowest – 37N/cm^2. Fruit, which were treated with 1-MCP and kept directly in cold storage, were 67N/cm^2 firm, while control apples kept directly in cold storage after harvest were 38N/cm^2 firm.
Bagged apples exposed to light lost significantly more weight after storage (7.8g) than non-bagged fruits (6.5g). Bagged fruit kept in cold storage lost insignificant more weight than non-bagged (5.4g and 5.0g respectively). Bagged apples were slightly firmer than non-bagged fruits exposed to light (58 and 51N/cm^2) and those kept in cold storage (54 and 52N/cm^2). There was no statistically significant bagging effect on ethylene synthesis of 'Ildrød Pigeon' apples exposed to the light but there was a trend of higher ethylene production in bagged apples.

Kühn et al. (2011) documented strong firmness reduction due to the long sunlight exposure of 'Pigeon' apples. This strong softening was however not evident in our study if to compare control samples exposed to light and from cold storage. It is therefore an indication that shorter sunlight exposure (8days) does not cause such a progressive softening as 2-3 weeks of sunlight exposure. However, with no doubts 'Ildrød Pigeon' belongs to apple cultivars characterized by significant softening during storage. It was beneficial to use 1-MCP even after sun exposure, to reduce further softening during storage. Delayed 1-MCP application improved apple firmness by 27%. Apples, which traditionally reach consumers at Christmas, are probably even softer than our control apples as they are exposed to sun for 2-3 weeks not 8 days. In the study of Parker et al. (2010) 8 days delayed 1-MCP application from harvest of 'Cortland' and 'Empire' mature fruit resulted in increasing softening after cold storage compared to earlier treatment. Yet, it seems likely the effect of delayed 1-MCP treatment might have various responses on different apple cultivars. For example, 5 days postponement of the inhibitor treatment increased internal ethylene production and reduced firmness of 'Cox' and 'Bramley' but had no effect on 'Gala' apples (Johnson 2008). Probably the effectiveness of 1-MCP depends on the cultivar as well as its individual ripening rate and developmental stage at harvest. It is hypothesized, that for some apple varieties, 1-MCP

treatment from harvest should occur in narrow harvest to storage period, while other varieties will respond well on 1-MCP despite late application.
The conditions in the bags and bag types might influence structure of the apple cuticle affecting water losses. An intense light exposure increases fruit temperature (can cause sun burn) and thus also results in higher water loss. Bagging causes changes in the cuticle structure and enhances permeability of gasses (Nilsson & Gustavsson 2007; Amarante et al. 2002;Fan & Mattheis 1998). According to Farhoomand et al. (1977) 'Delicious' shaded apples on the tree had higher ethylene production compared to the outside fruit with red blush. Bagging practice delayed the increase in the internal ethylene concentration at the onset of fruit ripening (Fan & Mattheis 1998b). Bagging practice resulted alike to shading. It might suggest that shading and bagging delay the climacteric peak which explains the delayed internal ethylene concentration peak in our study. It is additionally confirmed by the findings described in chapter 7, where noticed, based on harvest indexes, that bagging delayed maturity. Nilsson and Gustavsson (2007) noticed higher emitted ethylene production of shaded 'Aroma' apples but it was not associated with the enhanced internal ethylene concentration. It points out that shaded apples have higher ethylene production as a result of lower resistance to ethylene diffusion due to differences in intercellular space, volume and cell size between exposed to light and shaded fruit. The conditions in the bags might influence structure of the apple skin as well as a wax coating composition (Li et al. 2006; Amarante et al. 2002a; Fan and Mattheis 1998b;). The resistance to gas diffusion changed during maturation and ripening of 'McIntosh' apple and was associated with the deposition of culticular wax and differentiation of lenticels structure in apple (Park et al. 1993). Therefore, bagging might influence apple cuticle structure, which consists of 1) cutin and 2) wax. Waxes are embedded in the cutin and form a continuous layer. The cuticle thickness is not uniform on apples (Homutova and Blazek 2006). It is thicker on the shaded than on the sun-exposed side of apple peel (Babos et al. 1984).

Firmness vs. volatile correlation. In chapter 3.1, which is related to apple firmness and softening during ripening, the role of PME enzyme was shortly presented. Softening is due to activity of some enzymes, including the PME enzyme. PME is taking part in the softening process by removing methyl groups from pectin and cleave ester linkages between polysaccharide chains in the cell wall (Cosgrove 2001). In a study of Supriyadi et al. (2003), PME treatment resulted in synthesis of methyl esters in the presence of acetyl-CoA and methanol. When they tried to find the origin of methanol it was found that it was due to methyl groups cleaved off from pectin. PME enzyme catalyzed also the formation of methyl hexanoate in the presence of methyl pectin and hexanoyl-CoA in a model system (Supriyadi et al. 2003).We have observed the correlation between apple firmness, methyl acetate and butyrate. The correlation performed on all collected data from our experiment, resulted in negative correlations of -0.71 and -0.59 ($p<0.0001$) between apple firmness vs. methyl actetate and methyl butyrate, respectively. The methyl groups cleaved from proteins might be selective to straight chain esters as the correlation between firmness and methyl 2-methylbutyrate was only -0.23 ($p<0.07$). The correlation coefficient between firmness and methyl acetate from the study described in subchapter

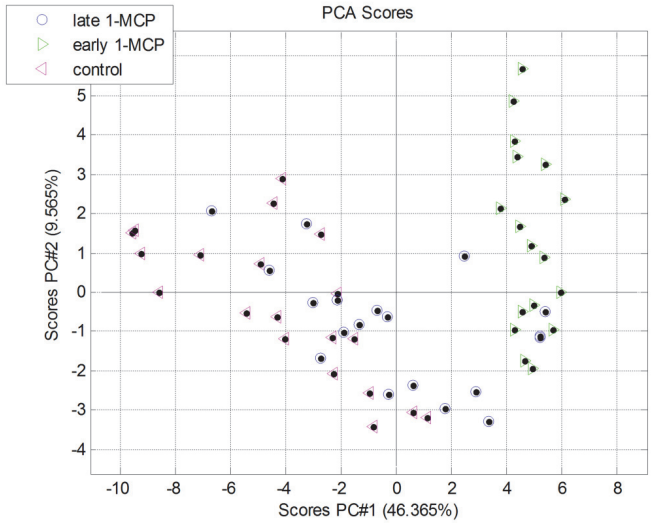
9.2 was also found -0.58 ($p<0.0001$). It seems like an interesting observation but there is a need for model improvement. Samples treated early with 1-MCP and two other treatments (late 1-MCP and controls) formed separate groups depend on firmness level. Early 1-MCP apples are represented by high firmness and low aroma production. Ideally, the firmness measurement on naturally developing apples should be included throughout the maturity and ripening stages in order to obtain representative variability in firmness combined with detection of methyl chain esters. These might assure the reliability of the proposed correlation. Additionally, the activity of PME enzyme should be monitored.

Summarizing, early 1-MCP treatment suppressed color development preventing the red skin color improvement. Apples treated early with 1-MCP had very low ethylene production after storage and were very firm. When 1-MCP was performed after 8 days of light exposure apple skin color development occurred without any disturbances. Late 1-MCP treated fruit had higher internal ethylene production, softer apples, and greater weight losses but these parameters were still better in comparison to the control apples. By performing late 1-MCP application firmness was improved by 27% and weight loss reduced by 10%.

10.2. Aroma compounds (PAPER III)

In the experiment, 46 aroma compounds were analyzed after 11 weeks of cold storage followed by 5 days at room temperature. To compare the aroma profile of untreated apples exposed to light with the samples treated with 1-MCP a Principal Component Analysis (PCA) was used. PCA is a multivariate statistical technique to explore and simplify visualization of complex data matrix. Nowadays, when more and more responds are included in experimental design, PCA is a powerful tool to analyze and understand data. PCA focuses on the covariance/pattern of variables to describe samples (Munck 2005). PCA expresses data in a graphical way by highlighting similarities and differences between samples based on measured variables.
In the PCA plots presented in Figure 20, PC1 and PC2 together explain more than 55% of the variance in the model. The PCA scores plot shows a difference between samples aroma profile dependent on 1-MCP treatment; PC1separates samples from the early 1-MCP application from these from late 1-MCP treatment and controls. Samples with the delayed 1-MCP treatment are shifted towards controls, which suggest similarities in their aroma profiles. The PCA loadings plot shows the distribution of aroma compounds depending on 1-MCP treatment (Figure 20). The concentration of compounds located to the right in the plot PC1 is higher in samples treated early with 1-MCP. This means that 2-propanone**,** cis-3-hexenal, 2-methyl-2-pentenal, 2-ethylfuran, and isomers of 2,4-hexadienal had higher relative areas in these samples. In our previous experiments (chapter 9.2) these compounds had also higher concentrations in 1-MCP treated apples and possible explanation was given. On the left side in the PC1 loadings plot all alcohols, esters, acids and ketones, except 2-propanone, are located. These compounds are high in concentration in corresponding samples in the PCA score plot; e.g. late 1-MCP treated

and controls. The control apples exposed to light had the highest concentration of 10 monitored esters and other esters; methyl acetate, methyl butyrate and hexyl acetate remained on the same level in late 1-MCP treated as in the control apples. Ethyl propionate and methyl butyrate were not detected in early 1-MCP treated apples (Table 6). The production of most alcohols, except 2-butanol, was highest in control apples and lower in late treated fruit but remained by far lowest in the early treated apples. Aldehydes; hexanal, t-2-hexenal, octanal, decanal concentrations were high in both late 1-MCP treated and control apples or in case of propanal and butanal were higher in control. Some aroma components like; 2-hexen-1-ol and acetic acid were insensitive to 1-MCP application. In general, the control apples exposed to light had greater concentration of volatile compounds and late 1-MCP treated apples had lower yet similar aroma production to controls. Early 1-MCP treatment apples significantly inhibited aroma compounds, except 'Ildrød Pigeon' principal aldehydes.



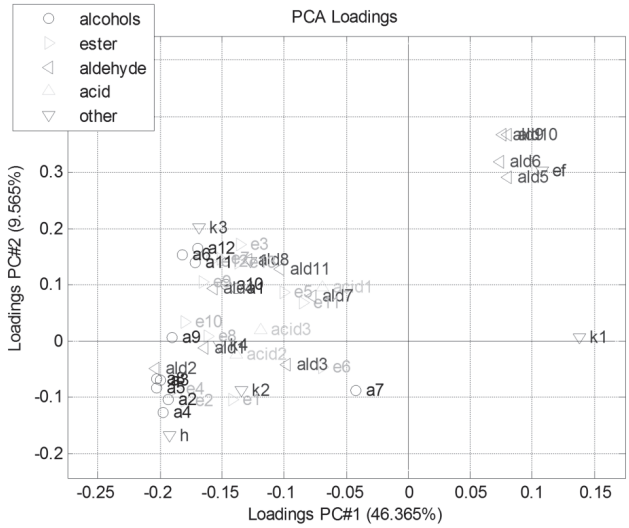


Figure 20 PCA scores and loadings plots of samples exposed to light (early, late 1-MCP and controls) according to their aroma profile (46 aroma compounds) after storage. Abbreviations used here are explained in Table 6

Bagging practice reduced production of some aroma compounds. Compounds; 2-butanol, propanol, 2-methylpropanol, 3-octanol, 6-methyl-5-hepten-2-ol, 2-ethyl 1-hexanol, 1-octanol, 5-octen-1-ol, ethyl propionate, ethyl butyrate, butyl 2-methylbutyrate, hexyl acetate, butyl butyrate, hexyl 2-methylbutyrate, propanal, butanal, hexanal, octanal, decanal, acetic acid, farnesene and 3-octanone, had higher concentration in non-bagged apples exposed to light. The rest of the compounds were insensitive to the bagging treatment when exposed to light. Aroma compounds are synthesized in the apple flesh and peel (Defilippi et al. 2005b; Pechous et al. 2005). The levels of volatiles, their precursors and aroma-related enzymes vary between peel and flesh, which indicates that the mechanism of regulation may also differ among tissues. Volatile production enzyme activity levels and precursors availability are higher in the peel than in the flesh (Defilippi et al. 2005b; Echeverria et al. 2004). Additionally, Defillipi et al. (2005b) reported that in flesh tissue, the (AAT) enzyme is a more important biochemical step than in the peel, in which the supply and metabolism of substrates, amino acids and fatty acids, seems to be more critical. It is suggested that bagging, as it affects apple cuticle structure, it might lead to poorer accumulation of fatty acid and amino acids accumulation, which constitutes as volatile substrates for straight and branched chain esters respectively. In pears, the concentration of two characteristic aroma compounds, γ- and δ- decalactone, were lowest in non-bagged fruit (Jia et al. 2005), while in grapes cv. 'Perla' ethyl acetate was predominant in non-bagged fruit while limonene, *trans*-2-hexenal, 3-hexanol and 2-hexen-1-ol were predominant in bagged fruit (Signes et al. 2007). Mattheis et al. (1996) reported that emission of ester and alcohol volatiles was lower in bagged 'Fuji' fruit and that postharvest volatile emission was negatively correlated with bagging duration during

development. It is worth to notice that bagging in our study reduced production of farnesene, which oxidation products are associated with superficial scald occurrence in apples (Shoji et al. 1998).

Light, as a crucial factor for apple pigmentation, might also have an effect on aroma pattern in apples. To obtain a full factorial experimental design, in order to evaluate the light effect on aroma compounds, late 1-MCP treated samples were removed from the statistical data set, as they had no representation in cold storage. Statistical data included, therefore, samples from early 1-MCP treatment and untreated/control samples from both light exposure and cold storage. Light and/or the temperature during light exposure showed positive effect on most aroma compounds. All measured alcohols had higher aroma production when exposed to light. The majority of aldehydes, acids, ketones and other compounds showed increasing trend if exposed to light in both 1-MCP treated and control samples. Most of esters also reacted with higher or sustainable synthesis when exposed to light in both control and treated samples. Higher temperature caused by sun exposure might provoke ripening processes and therefore production of these aroma compounds was enhanced. In 'Kent' strawberries methyl and ethyl butyrate production was stimulated after storage when exposed for 3 days at 20^{o}C in light, but in dark or at 10^{o}C none of these aroma compounds were detectable (Artur et al. 1995). Ethyl acetate and ethyl propionate had higher production in treated apples kept in cold storage, while methyl 2-methylbutyrate and butyl acetate synthesis was greater in control apples also kept in cold storage (data not shown). In case of butyl acetate it might be an effect of bagging combined with lack of light. These were all fruits, where anthocyanin development was low (bagged/control/cold storage apples). It was shown in the study of Miller et al. (1998) that with high sun exposure anthocyanin production increases but at the expense of acetate esters (hexyl, butyl and 2-methylbutyl acetates) in apples. In their experiment, 2-methyl butylacetate was clearly the major acetate ester. Its concentration was highest when exposed to reduced percentage of full sunlight, but at full sun exposure it decreased significantly, while anthocyanin content increased. It suggests that some ester precursors or esters might be utilized as anthocyanin substrates. It is very likely as acetates and/or acetic acid are necessary substrates for the further anthocyanin biosynthesis. Moreover, during esterification free coenzyme A (CoA) is formed (Shalit et al. 2001) and it is a necessary substrate for red pigment production. Flavonoid and aroma compounds might therefore depend on the same substrate, which means that they could influence one another (Siegelman & Hendricks 1958). The apple samples with the highest production of butyl acetate was those, which were bagged while on the trees to restrain anthocyanin production and additionally, a/b ratio after storage was similar to the one at harvest (Figure 19) as apples were kept in cold storage and pigment did not increase because of lack of light access. Therefore, production of butyl acetate, which in the biosynthesis might cross with the anthocyanin pathway, was significantly enhanced here. This tendency was not observed for any other acatetes in our study. It is not clear, which acetates react with a poorer production when apples are exposed to extended sunlight.

Volatile compounds correlations. The actual apple aroma composition could be controlled by both the selectivity and activity of enzymes involved or by the substrate

pool availability (Echeverria et al. 2004). In our study high correlation was found between ethylene and alcohols rather than aldehydes, which suggest ethylene supportive role in alcohol synthesis. The good correlations were found between aldehydes and the corresponding alcohols, which conversion constitutes to the ester formation. Hexyl and propyl acetates showed however stronger dependence to the alcohol and aldehyde pool than butyl acetate. In case of the formation of butyrate esters alcohols, aldehydes and butanoic acid availability were important. Butyrate esters were by far most dependent to the acid pool availability than acetates. Acetate esters showed weak or no-significant correlations to acetic acid. There was also high correlation found between butyl butyrate and hexyl butyrate, which reflects lower competition for their acid substrate and shows cooperative pathway activity. Ethylene regulates ester production via alcohol acyltransferase enzyme (AAT) (Schaffer et al., 2007; Defilippi et al., 2005a; Defilippi et al., 2005b; Dandekar et al. 2004; Defilippi et al. 2004). Ester synthesis is additionally limited by the availability of alcohol as ester substrates (Schaffer et al., 2007; Defilippi et al., 2005a; Defilippi et al., 2005b). Alcohol production decreased after ethylene inhibition in 'Delicious' and 'Golden Delicious' apples (Kondo et al., 2005). Furthermore, followed by alcohols the ester production declined in 'Gala' apples (Bai et al., 2004; Marin et al., 2009). The final apple aroma composition is probably the effect of the balance between the enzyme activity and selectivity properties combined with the availability of the substrates, although little is known about the substrate specificity of the enzymes taking part in volatile formation in apples. AAT in strawberries showed strong activity towards heptanol, octanol, nonanol and geraniol in combination with acetyl-CoA, butyryl-CoA and hexanoyl-CoA (Aharoni et al. 2000). In melon 4 AAT genes were isolates and showed different substrate preferences fx. CmAAT-1 showed high preference towards 2-hexanol with acetyl-CoA and hexanol with hexanoyl-CoA (El-Sharkawy et al. 2005). If the enzyme selectivity is specific for each fruit species or is even cultivar dependent than the flavor production partly depends on the genetic potential.

In conclusion, the results indicate that early 1-MCP treatment inhibited many aroma compounds. Most of the aroma compounds in late treated apples remained in moderate concentration or were on the similar level of the control apples. There were some exceptions fx. aldehydes, which are characteristic 'Ildrød Pigeon' constituents, whose concentration was actually enhanced by the early 1-MCP application. Taking into consideration external and internal quality changes, the application of 1-MCP after shorter period of light exposure is a good solution to maintain 'Ildrød Pigeon' quality during storage. These apples were firmer, had lower weight loss and moderate internal ethylene concentration, which provoked aroma synthesis to the similar levels of control apples. In general, late 1-MCP treated fruit had better quality parameters then control apples. It is, however, still of a great importance to conduct further sensory analysis to investigate consumer responds to the quality changes of 'Ildrød Pigeon' depend on 1-MCP treatment. The sensory characteristics of fruit can be grouped into categories: flavour (texture, smell and taste) and appearance. The appearance attracts consumer and determines the fruit purchase. Flavour on the other hand is the most important for the

eating enjoyment and consumer satisfaction by affecting taste and smell. Flavour, therefore often decides whether the consumer will re-purchase fruit. In case of 'Ildrød Pigeon' intense red skin color will visually attracts the consumers. It is however important to establish if and to which extend consumers perceive the differences in the apple red coloration depend on the various length of the postharvest sunlight exposure period. Secondly, the consumer firmness acceptability and preferences should be investigated. The consumer might be interested in very firm and crispy apples with the poorer aroma or in opposite the 'Ildrød Pigeon' consumers might be used very soft, overripe fruit with intense aroma. Alternatively, consumers should be introduced to firmer fruit with still intense aroma obtained by the postharvest management proposed in this thesis.

Table 6. Aroma compounds production dependent on 1-MCP treatment divided into apples exposed to light and kept in cold storage directly after harvest. Compounds mark bold are characteristic 'Ildrød Pigeon' compounds.
a-c letters following means represent significant difference only between 1-MCP application of samples exposed to light, while A-D letters indicate significant difference between samples kept directly in storage or exposed to light after harvest (1-MCP treated and control) ($p \leq 0.05$). Values are averages of bagged and non-bagged apples.

		Apples exposed to light after harvest			Apples kept in cold storage after harvest	
		late 1-MCP	early 1-MCP	control	early 1-MCP	control
Aroma compounds	*Abbrev.*	RELATIVE AREA x 10^3				
Alcohols						
2-butanol	a1	16b	15bB	22aA	8.8C	16B
propanol	a2	194b	22cC	409aA	8.6C	219B
2-methyl-1-propanol	a3	233b	65cB	312aA	43C	204B
butanol	a4	1586b	119cB	2474aA	53C	1807B
1-pentanol	a5	88b	10cC	156aA	5.3C	99B
3-octanol	a6	34b	3.3cB	54aA	2.2B	25B
2-hexen-1-ol	a7	41a	38aAB	45aA	33B	43AB
1-heptanol	a8	7.7b	1.9cB	12aA	0.9B	11A
6-methyl-5-hepten-2-ol	a9	11b	1.9cC	19aA	0.1C	12B
2-ethyl-1-hexanol	a10	9.2a	4bB	9aA	3.2B	4.9B
1-octanol	a11	6.7b	1.9cC	10aA	1.5C	7.4B
5-octen-1-ol	a12	6b	0.7cC	10aA	0.4C	7.6B
Esters						
methyl acetate	e1	52a	9.2bB	55aA	9.5B	47A
ethyl acetate	e2	294b	27cC	440aA	71C	313B
ethyl propionate	e3	2.6b	nd	4.4aA	1.9B	4.6A
propyl acetate	e4	39b	9.4cC	69aA	5.7C	40B
methyl butyrate	e5	1.8a	nd	2aA	nd	1.4A
methyl 2-methylbutyrate	e6	35.6b	5.3cC	66aB	0.9C	103A
ethyl butyrate	e7	5.6b	0.7cC	10aA	nd	5.6B
butyl acetate	e8	21b	1.6cB	35aB	0.7B	239A
butyl butyrate	e9	23b	4.8cB	47aA	2B	21A
butyl 2-methylbutyrate	e10	9.1b	4.8cC	14aA	3.3C	7.8B

hexyl acetate	e11	21ab	17bB	22aA	9.8C	21AB
hexyl butyrate	e12	12b	4.0cC	17aA	4.0BC	13AB
hexyl 2-methylbutyrate	e13	7.2a	3.3bB	8.5aA	3.7B	6.8A
Aldehydes						
propanal	adh1	6.9b	4.3cC	9.8aA	3.1C	8B
butanal	adh2	176b	10cC	338aA	4.8C	224B
2-methylbutanal	adh3	10b	9.7bB	13aA	7.5B	14A
hexanal	adh4	925a	574bC	972aA	344D	806B
2-methyl-2-pentenal	adh5	67b	96aA	65bB	79AB	65B
cis-3-hexenal	adh6	69b	209aA	61bB	212A	38B
trans-2-hexenal	adh7	1497a	1188bC	1357aB	1102C	1558A
octanal	adh8	2.7a	2bB	2.7aA	1.7B	2B
2,4 hexadienal 1	adh9	9.8b	17aA	11bBC	13B	8.8C
2,4 hexadienal 2	adh10	19b	34aA	20bBC	25B	17C
decanal	adh11	3.4a	1.7bB	3aA	2AB	2.1AB
Acids						
acetic acid	acid1	62a	34aA	49aA	28A	31A
butyric acid	acid2	21b	3.1cB	44aA	1.2B	35A
hexanoic acid	acid3	23ab	13bB	26aA	7.2B	30A
Others						
2-ethyl-furan	ef	7.7b	23aA	8bB	17A	6.6B
1-hexene	h	751b	177cC	938aA	97D	844B
farnesene	f	33b	nd	48aA	nd	44A
2-propanone	k1	37b	57aA	38bB	43B	37B
2-butanone	k2	11a	9.2bC	15aA	5.8D	11B
3-octanone	k3	3.2b	2.2bB	4.7aA	4.2A	2.5B
6-methyl-5-hepten 2-one	k4	8.2b	6.9bC	11aA	4.7D	8.2B

11. *CONCLUSIONS*

The apple variety 'Ildrød Pigeon' was used as a model cultivar in the experiments to investigate effects of 1-MCP treatment. 'Ildrød Pigeon' is a cultivar with progressive softening during postharvest period especially because of its unique postharvest light treatment, which ensures red color development, but causes quality aggravation by the ripening acceleration. As there is limited information about this cultivar, it was decided to explore the effect of some pre- and postharvest factors on various quality parameters. The main parameters observed were; internal ethylene internal concentration, color development, firmness and aroma profile. The development of these is particularly critical for 'Ildrød Pigeon'.

Pre-harvest management; thinning (cropping level adjustment) and nitrogen fertilization levels (0, 50 and 100kgN/ha) seem to have only small influence on aroma profile, weight loss, firmness, sugar content and titratable acidity after storage. High nitrogen fertilization showed only a slight tendency to suppress color development. Increased nitrogen doses, however, positively affected fruit size. There was also 8.4% increase in apple size when trees were thinned. This is, however, not an increase, which could cause economical benefits for a producer. Apples from non-fertilized trees had lower (delayed) fruit drop and lower ethylene production after storage. It might be because of an insufficient assimilates delivery during growth, which cause low competition and slow down developmental processes. There was very weak observed effect of nitrogen on apple firmness, sugar and acidity. It is hypothesized that 'Ildrød Pigeon', as a small-fruited cultivar, has limited genetic potential to increase fruit size in response to an augmented assimilate availability provided by pre-harvest practices such as late hand thinning. Further studies need to be done to evaluate timing of thinning effect on quality of 'Ildrød Pigeon' apples. The nitrogen fertilizer influencd fruit flavour. The production of some 'Ildrød Pigeon' principal aroma compounds, except 1-heptanol and ethyl acetate, was highest at the moderate nitrogen level. 1-Heptanol, 1-hexanol and all acetate esters had higher levels in apples from non-fertilized and fertilized with high nitrogen dose trees. In apples from non-fertilized trees, the level of propanone was higher but propanal, 2-hexenal, 2-methyl butanal and farnesene was the lowest. The concentration of ethylene, hexanal, butanal, farnesene and 3-octanol was found to decrease with the rising crop load.

The evaluation of characteristic aroma compounds showed that 'Ildrød Pigeon' belongs to apple varieties in which predominant compounds are aldehydes, esters and alcohols. The principal compounds found in 'Ildrød Pigeon' are; butanal, ethyl acetate, methyl 2-methylbutyrate, hexanal, 2-methyl-2-pentenal, cis-3-hexenal, 2-hexenal, butyl butyrate, isomers of 2,4-hexadienal, 1-heptanol and 6-methyl-5-hepten-2-ol. In various experiments carried out for the project, it was found that the aldehydes 3-hexenal, 2-methyl-2-pentenal and isomers of 2,4-hexadienal continuously had higher production in apples, in which ethylene internal production was strongly reduced by 1-MCP. Opposite, the production of principal esters was suppressed to

different degree dependent on 1-MCP timing treatment. The aroma synthesis is an important parameter for the apple quality. The fruit should be kept at a proper time at room temperature after storage to accelerate ripening and to provoke aroma development before reaching the consumers. The time at room temperature after cold storage, which is needed to obtain optimal 'Ildrød Pigeon' aroma development was established to be between 5 and 8 days. At this point, the concentrations of the majority of the measured aroma compounds were highest. Therefore, in all experiments presented in the thesis, apples were taken out from storage and left for 5 days at room temperature before analysis.

'Ildrød Pigeon' apples are traditionally picked in early, mid-September at harvest index around 0.17. There was only very small effect of 1-MCP on apple quality when harvest at that time. It seems like early stages of ripening had already occurred at traditional harvest time. In this case, some of the ethylene receptors were probably already occupied by ethylene and therefore 1-MCP treatment was not as effective as anticipated. This low 1-MCP effectiveness reflected also less softening reduction in apples as all fruits harvested at harvest index 0.38 had much higher firmness. The appropriate harvest time is important as with longer storage additional softening, weight loss and decrease in titratable acid content was observed. Most aroma compounds were produced in lowest concentration when harvested early and were additional suppressed by 1-MCP treatment. In general, commercial harvest of 'Ildrød Pigeon' was done too late to observe a suitable 1-MCP effect. However, harvest at index 0.38 combined with 1-MCP treatment seems to be too early as there was a very strong inhibition of ethylene concentration and aroma compounds. Additionally, apples were probably too firm to be accepted by consumers. Thus, 'Ildrød Pigeon' apples should be picked at a harvest index below 0.38, but not as low as 0.16.

An improved quality, in terms of firmness and aroma composition, was observed when 'Ildrød Pigeon' apples were picked at index 0.29 and exposed to sun light for 8 days. The main 'Ildrød Pigeon' color change occurred between day 2 and 4 of light exposure and increased slowly until day 8. Therefore, 2-3 weeks, which are traditionally used for pigment promotion, could be shortened to about 8 days (the actual climate has to be considered), after which 1-MCP application should be performed. The early 1-MCP application prior to light exposure suppressed color development. These early 1-MCP treated fruit were also very firm and would probably not be accepted by consumers. The quality of apples treated later with 1-MCP was better compared to untreated apples. The fruits remained 27% firmer and had lower weight loss after storage than control samples. The aroma profile of late 1-MCP treated fruits was closer to untreated fruits, which were rich in fruity esters, rather than to early 1-MCP, which were rich in aldehydes. Additionally, the early 1-MCP application suppressed the principal aroma compounds of 'Ildrød Pigeon'. There were however compounds; 2-methyl-2-pentenal, 3-hexenal, 2,4-hexadienal isomers and 2-ethyl furan, which reacted with higher concentration when 1-MCP was used directly after harvest i.e. when ethylene production was strongly suppressed. It suggests that their production is enhanced when ethylene synthesis is limited or inhibited.

Summarizing, ‘Ildrød Pigeon’ postharvest handling production was optimized to obtain fruit with better quality after storage, mainly in terms of firmness, color and aroma composition. As the outcome of the project the ‘Ildrød Pigeon’ modified postharvest management was proposed to improve external and internal quality of the cultivar by adjustment of harvest time, shortened sunlight exposure combined with a delay1-MCP treatment. 1-MCP application after a shorter period of sun exposure seems to be new a “magic bullet” to sustain ‘Ildrød Pigeon’ quality during storage. The evaluation of the consumer satisfaction, in terms of fruit flavour, must be an indication of the delivery of the highest fruit quality on the market. Therefore, additional consumer sensory tests are suggested to establish consumer acceptance.

Despite the fact that ‘Ildrød Pigeon’ might be a niche apple cultivar the delay of 1-MCP application must be taken into consideration in the apple commercial postharvest practice as fruits are typically accumulated for several days to fill a room before applying CA storage. 1-MCP should be applied in the narrow window between harvest and storage, and this should be established for each cultivar individually as each has a unique ripening rate. The optimization of 1-MCP postharvest technology in terms of fruit flavour quality must rely on the successful delay of the ripening processes, but not their complete inhibition.

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PAPERS

PAPER I

The influence of pre-harvest management on apple fruit growth and aroma profile

Submitted to HortScience

The Influence of Pre-harvest Management on Apple Fruit Growth and Aroma Profile

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The Influence of Pre-harvest Management on Apple Fruit Growth and Aroma Profile

Additional index words. fruit growth, hand thinning, cropping load, aroma compounds, ethylene

Abstract. Apple fruit growth parameters and aroma development are affected by many pre-harvest parameters like; hand thinning and nitrogen fertilization. The crop load can affect size and assimilate concentration but especially in varieties with large size fruit. In small fruited apple varieties like 'Ildrød Pigeon' effects was less pronounced. Decreasing the cropping level by thinning in our experiment increased fruit size by 8.4% and increased firmness to a small degree. Increasing nitrogen level in unthinned trees affected fruit number by decreasing fruit number per cm^2 trunk cross section area (TCSA). Nitrogen fertilized apple trees also had enhanced maturity development observed as increased fruit drop just before harvest. This was in agreement with ethylene production after storage, which was lowest in non-fertilized compared to the elevated nitrogen levels. Furthermore, ethylene concentration was related to the levels of aroma compounds; especially acetates. Aroma compounds like; E,E-farnesene, hexanal and 3-octanol obtained lowest production at highest cropping level. The effect of cropping level and nitrogen fertilizer, as cultivation techniques, on 'Ildrød Pigeon' growth and aroma profile was observed. Additionally, the internal ethylene concentration and aroma compounds production were monitored during the final ripening after storage being the greatest at 5 and 8 days at room temperature.

The fruit cultivation and the choice of appropriate growing techniques are intended to maximize fruit yield and fruit quality. Fruit size is in particular interest of growers as it determines the fruit price while fruit quality components attached to the taste and the enjoyment values; firmness and aromatic content become important as ones, which influence consumer fruit re-purchase choice. Development of apple aroma compounds is one of the processes closely associated to ripening, and thus to ethylene production (Martinez-Romero, 2007). Many studies are related to appropriate harvest time and storage conditions and their influence on aroma development. Much less is known about possible effects on aroma related to the pre-harvest physiology of the fruit growth and development (Poll, 1996). In large fruited species, such as apples and pears, the cropping level affects fruit development significantly, while in small fruited species effects may be small or insignificant (Hansen, 1982, 1993; Toldam-Andersen and Hansen, 1993, 1995). Among apple cultivars focus is normally on those with largest fruits, however, in a few cases rather small fruited cultivars, like variety 'Ildrød Pigeon', are grown for niche market use. 'Ildrød Pigeon' market potential is based on its characteristic aroma profile and distinct red color. Both these fruit quality contributors change strongly during the final fruit development and ripening. It is therefore very important to understand how pre-harvest factors and ripening conditions affect these. Based on the limited genetic potential for fruit size (fruit sink) in this cultivar, we hypothesize that the effects of the fruit load might resemble the weak relationships that are typical for other small fruited species. Nitrogen fertilizer level affects both vegetative and generative components in fruit crops in dynamic and complicated ways, which makes it one of the most important pre-harvest factors to study in relation to effects on fruit sink, fruit development and fruit quality.

The main study objective was to investigate the effects of the nitrogen fertilization in combination with the crop load on the fruit growth and quality, mainly firmness and volatile formation during ripening.

Materials and Methods

Apples of the variety 'Ildrød Pigeon' were picked in mid-September 2008 from 5-years old trees (M9 rootstock), grown on sandy loam at the experimental station Pometet, Copenhagen University. The 7.5 gN per tree of calciumnitrate $Ca(NH_3)_2$ was distributed by hand in April shortly before budbreak for middle and high N levels and the procedure was repeated in the last week of June for high nitrogen level trees resulting in 15 $gN \cdot tree^{-1}$. These amounts equal to 50 and 100$kgN \cdot ha^{-1}$ distributed in the tree row. All trees (including the 0 $kgN \cdot ha^{-1}$ treatment) were additionally fertilized in March with P and K with a total amount of 12 $kgP \cdot ha^{-1}$ and 63 $kgK \cdot ha^{-1}$. Before 2007 the apples trees were fertilized with NPK at commercial levels. In 2007 the three N fertilizer levels (0, 50 and 100 $kgN \cdot ha^{-1}$) was established with P and K as additional nutrition's continued at the standard levels mentioned above. Leaf samples collected in the early September 2007 showed no deficiencies in makro-elements and N level varied among the treatments from 2 to 2.15% of dry matter which is within recommended ranges. Each nitrogen level was repeated in the orchard in two blocks of 15 apple trees. Each block and N level was separated by a border tree. Hand thinning was done in first week of July (after natural fruit drop) on every second tree (45 trees) while the rest remained un-thinned (45 trees). Hand thinning was aiming at 50% reduction in fruit load. At harvest time the fruit loadings for thinned and unthinned trees were 5.9 and 11.9 $fruits \cdot cm^{-2}$ expressed as fruits per trunk cross section area (TCSA). 5 trees of each treatment and block were selected. From each tree 6 apples were picked from the top (light exposed) and 6 from the bottom (shadow) creating 2 samples per tree. These were taken to the

laboratory to make representative samples by taking 2 randomly chosen apples from each tree sample. In total 24 representative samples were prepared so each contained 10 apples (9 fruits needed for analyses and 1 extra). After, samples were transferred to cold storage (1.5 ^{o}C) for 60 days. After the storage period apples from each treatment were exposed to room temperature (24 ^{o}C) for 5, 8 and 15 days and then analyzed. Each day of analysis 3 apples were taken out randomly from each bag and analyzed. All the measurements were done on individual apples.

Fruit quality parameters. To measure internal ethylene concentration (IEC) a 1 mL gas sample was taken from the core cavity of the apple using a gas tight syringe with a 0.4 x 40 mm needle. The gas sample was injected into a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector and packed column Porapak Q; 80/100Mesh (length 1.82 m, 15.2 cm coil of 3 mm OD stainless steel) using helium as a carrier gas (30 ml·min^{-1}); the injection port and the own temperature set to 100 ^{o}C; the detector temperature set to 140 ^{o}C. Ethylene concentration was calculated from standard curve obtained from analyses of known ethylene/air mixtures and presented in μL·L^{-1}.

The flesh firmness was measured on a Texture Analyser TA.XT.plus (Exponent, Stable MicroSystems). Puncture measurements were done on opposite sides of each apple after skin removal. The instrument was set up to penetrate the apple flesh at 1 mm·s^{-1} with the contact force of 5 g, for a distance of 7 mm, using a 10 mm diameter probe. The maximum force used (N·cm^{-2}) was recorded. Since there was no significant difference in firmness between 5, 8 and 15 days after storage the firmness averages were made from all post-storage periods to evaluate effect of nitrogen levels and cropping load.

After these measurements, juice was pressed from each individual apple using a juice extractor Philips HR1861. The aroma compounds were measured using dynamic headspace sampling from

10 ml of juice (4-methyl-1-pentanol added as internal standard) with nitrogen as purge gas (70mL·min^{-1} for 20min) according to Petersen et al. (2007). The traps were thermally desorbed using a Perkin Elmer ATD 400 and analyzed on a Hewlett Packard G1800A GC-MS system including a DBWax column (30 m x 0.25 mm x 0.25 µm). The column flow rate was 1.0 mL min^{-1} using helium as a carrier gas. The column temperature programme was: 10 min at 45 °C, from 45 °C to 240 °C at 6°C min^{-1}, and finally 10 min at 240 °C. The MS was operating in the electron ionisation mode at 70 eV and mass-to-charge ratios between 15 and 300 were scanned. Volatile compounds were tentatively identified by matching their mass spectra with those of a commercial database (Wiley275.L, HP product no. G1035A). Amounts of aroma compounds were expressed as peak area divided by peak area of internal standard (relative areas x 10^3).

Statistical analysis. Significance testing was done by two-way ANOVA with cropping levels and nitrogen levels as factors (InfoStat versión 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). To determine treatment effect and mean separation Fisher's Least Significant Difference at $p \leq 0.05$ was calculated. Neither fruit position on the canopy (top and bottom) nor nitrogen blocks showed significant difference on any of observed variables. Outliers were removed from the aroma dataset if problems occurred (fx. water vapor in aroma trap which enable chromatogram integration).

Results and Discussion

The aimed level of thinning with a 50% reduction in fruit load per tree was achieved very closely with an average reduction in fruit number per tree of 43, 52 and 53% when expressed relative to the tree size as fruits per TCSA for each nitrogen level (Table 1). This strong reduction only resulted in 8.4% average increase in fruit size, from 77g to 84g. The size compensation after thinning can in optimal cases result in almost similar yield levels (Hansen, 1982; Knight, 1980),

which combined with a more favorable price on larger fruits results in an economic surplus. However, Ildrød Pigeon is a small apple variety with a fruit diameter of only 50-60mm (Petersen, 1950), and therefore 'Ildrød Pigeon' apples may only have a small genetic potential to produce larger apples. The importance of the genetic potential for fruit size in relation to the effect of thinning was demonstrated by Hansen (1989). Large size fruit crops showed strong ability to react on changes in fruit to leaf ratio both by increased size, dry matter and acid content.

Table 1. Physiological changes of 'Ildrød Pigeon' apples at harvest time depending on fertilization and cropping level practice. Means in the same column followed by different letters show significant difference at $p \leq 0.05$ (Fisher's test).

cropping level	nitrogen fertilizer ($kgN \cdot ha^{-1}$)	total fruits	fruit size (g)	total fruits weight ($kg \cdot tree^{-1}$)	fruits under the tree at harvest	fruits on the tree at harvest	fruits under/on ratio	fruits cm^{-2} (TCSA)
thinned trees	0	102de	81ab	8.4d	12b	90d	0.13b	6.7c
	50	72e	85a	6.2e	13b	59e	0.22a	5.2c
	100	98d	85a	8.3d	13b	85d	0.15ab	5.8c
unthinned trees	0	240a	76bc	18.0a	28a	212a	0.14b	15.0a
	50	138c	76c	10.4c	21a	117c	0.18ab	10.0b
	100	186b	79bc	14.6b	25a	161b	0.16ab	10.8b

In our study apples from thinned trees were significantly firmer after storage (45 and 49 $N \cdot cm^{-2}$ unthinned and thinned respectively). This effect might be related to a larger assimilates availability, which results in formation of thicker cell walls (Johnson, 1994).

Only weak negative correlation between the fruit size and amount of fruits per TCSA was observed on unthinned trees in the present study. Nitrogen fertilization affected number of apples per tree negatively when evaluated on the unthinned trees. This was also the case when expressed as $fruits \cdot cm^{-2}$ TCSA (Table 1). The unthinned non-N fertilized trees kept higher level

of fruits most likely because of low competition for assimilates at the early stage of fruit development due to low levels of shoot growth. The middle and high N levels had got the same level of N at the beginning of April resulting in a comparable competition for assimilates in these two treatments reflected in the final crop load. There was no effect of nitrogen level on fruit size (Table 1). Effects of nitrogen on apple development might be complex as it is influencing both shoot and leaf growth as well as flower development and fruit set (Poll, 1996). Low nitrogen levels may results in high fruit numbers in unthinned trees due to lower, early season fruit drop while high nitrogen levels may cause an increase in fruit drop because of competition from shoot growth (Toldam-Andersen and Hansen, 1995). The nitrogen level may also affect fruit quality and maturation. Fruits from trees fertilized with a moderate nitrogen level (50 $kg \cdot ha^{-1}$) were insignificantly firmer when measured after storage with 49 $N \cdot cm^{-2}$, while low and high (0 and 100 $kg \cdot ha^{-1}$) were both at firmness of 46 $N \cdot cm^{-2}$.

'Ildrød Pigeon' apples create an early abscission layer causing pre-harvest fruit drop. Thus, the relation of fruits number under and on the tree might indicate differences in maturity (Table 1). The apples from 50 $kgN \cdot ha^{-1}$, thinned trees had the highest under/on tree ratio while non-fertilized had the lowest. This suggests differential maturity stages with a delayed maturity at the low nitrogen level. Also the not fertilized trees, especially the unthinned, were the most loaded ones so fruits had to compete for assimilate sources (Table 1). This is a situation, which slows down fruit development and may delay maturity (Denne, 1960). This delay in non-fertilized, high cropping trees was also confirmed by the lowest ethylene concentration after storage (Fig. 1A). It is worth to notice that average ethylene production was higher at elevated nitrogen level at both cropping levels. In the study of Fallahi et al. (1987) no effect of N on internal ethylene was observed in attached apple fruit, however high N applications increased ethylene production

after storage. Ethylene as a plant hormone influences aroma compound development during ripening. Aroma analyses were done 5, 8 and 15 days after removal from cold storage to room temperature. The period between 5 and 8 days at room temperature appeared to be the climacteric peak period for ethylene and most of the monitored aroma compounds. The average ethylene level at day 5 was 1335 $\mu L \cdot L^{-1}$ and at day 8 was 1260 $\mu L \cdot L^{-1}$ regardless of pre-harvest factors. At day 15 ethylene synthesis was the lowest (992 $\mu L \cdot L^{-1}$). A decreasing trend of aroma production, similar to that of ethylene, was observed throughout the room temperature period for the compounds; ethyl acetate, butyl acetate, hexyl acetate, 1-butanol, 1-hexanol, 3-octanol, 1-heptanol, butanal, 2,4-hexadienal, benzaldehyde and E,E-farnesene. In contrast, the branched chain ester methyl 2-methylbutyrate and the compounds 1-propanol, 1-pentanol, propanal and hexanoic acid were continuously increasing during the room temperature period reaching the highest level at day 15. Aroma compounds relative areas presented in Table 2 are averages from the analysis at day 5 and 8 after storage. For the majority of the measured compounds no significant differences were found between these days; only propyl acetate, 1-propanol, 1-pentanol and propanal showed average 30, 69, 49 and 50% higher values at day 8 compared to day 5.

The aroma of 'Ildrød Pigeon' is very characteristic and thus a central quality parameter. In this study aroma production was not affected by apple position on the tree and the effects of nitrogen fertilization and cropping level were small (Table 2).

Table 2. Aroma compounds production depends on cropping level (fruits·cm^{-2}) and nitrogen dozes (kgN·ha^{-1}). Means of compound production at day 5 and 8 after storage. Letters in the row indicate significant differences between treatments ($p \leq 0.05$)

	THINNED TREES			UNTHINNED TREES		
Nitrogen fertilization (kgN·ha^{-1})	0	50	100	0	50	100
Cropping level (fruits·cm^{-2})	6.7	5.2	5.8	15.0	10.0	10.8
Aldehydes	RELATIVE AREA x 10^3					
propanal	7.9a	6.9a	8.7a	6.3a	7.5a	7.6a
butanal	86.2ab	61.1b	100.1a	57.7b	91.0a	85.4ab
2-methyl butanal	2.7ab	3.8a	3.6a	2.2b	3.4ab	3.5a
hexanal	349.9a	349.0a	429.2a	205.9b	352.3a	414.6a
2,4-hexadienal	1.9c	2.5a	2.0c	1.9c	1.8c	2.3ab
benzaldehyde	2.7a	2.2a	2.7a	2.3a	2.5a	2.3a
Esters						
ethyl acetate	102.7ab	97.1ab	117.7a	80.6b	99.5ab	81.7b
propyl acetate	17.6a	14.4ab	14.8ab	16.2ab	14.1ab	13.5b
methyl 2-methyl butyrate	27.1a	25.1a	19.6a	22.1a	29.2a	16.8a
butyl acetate	13.7a	9.5b	12.1ab	12.7ab	12.7ab	11.9ab
hexyl acetate	5.6a	5.0a	5.3a	6.3a	5.6a	6.2a
Alcohols						
1-propanol	229.1a	175.7a	218.8a	173.4a	212.5a	210.2a
1-butanol	1430.6a	985.2b	1459.5a	1089.8ab	1460.8a	1446.8a
1-pentanol	36.7a	33.4a	37.9a	32.1a	40.2a	38.2a
1-hexanol	739.9ab	639.1b	782.4ab	686.1ab	817.1a	829.8a
3-octanol	3.3ab	3.8a	4.1a	2.2b	3.5a	4.2a
2-hexen-1-ol	3.1b	2.9b	3.1b	3.3b	3.7ab	4.4a
1-heptanol	3.4ab	3.1b	3.6ab	3.5ab	3.8a	3.8a
Others						
E,E-farnesene	39.6a	31.8a	28.9a	15.1b	23.4ab	31.1a
hexanoic acid	9.4ab	6.8b	14.7a	5.8b	11.2ab	11.6ab

Production of the compounds; methyl 2-methylbutyrate, hexyl acetate, propanal, benzaldehyde, propanol, pentanol and hexanol were not significantly different. Hexanal in apples from not fertilized, unthinned trees (cropping level 15.03 fruits·cm^{-2}) was produced in significantly lower levels than from other treatments (Table 2, Fig. 1B). However, hexanal and hexanol

concentration increased with elevated nitrogen. Ethyl acetate production was the highest in thinned, high fertilized apples. In contrast butyl acetate and propyl acetate reached the highest production in apples from non fertilized, thinned trees (cropping level 6.67 fruits·cm^{-2}). Interestingly, both butanol (Fig. 1C) and butanal, which are butyl acetate possible precursors, behaved the same way reaching the lowest production at the treatments 50kgN·ha^{-1}/thinned and 0kgN·ha^{-1}/unthinned trees. Butanol and butanal correlation was high (r^2=0.80) (Fig. 2A), while the correlation between butyl acetate and butanol or butanal was weaker; r^2=0.31 and 0.37 respectively (data not shown). A similar observation was obtained in the case of the relationships between propanal, propanol (Fig. 2B) and propyl acetate (data not shown). Aldehydes, as propanal and butanal, are directly converted by alcohol dehydrogenase action to their respective alcohols propanol and butanol, which might explain the high correlation. Next step involves formation of propyl and butyl acetate via alcohol acetyltransferase (AAT). Propyl and butyl acetate correlations with precursors are weaker as secondary substrate (acetic acid) and/or enzyme availability might be limited. The rest of the monitored aldehyde and alcohol compounds reacted variously on cropping level and nitrogen level and their concentrations are presented in Table 2. As the effect of the crop load on size was very weak in the small fruited 'Ildrød Pigeon' a weak or no effect may also be expected on aroma. In the study by Poll et al. (1996) the large fruited cultivar 'Jonagold' was used and the effect of fruit to leaf ratio on aroma compounds was found in parallel to the effect on fruit size and contents of soluble solids and acids. In general fruit species and cultivars that are genetically coded for small fruit size, express no or only weak effect of fruit to leaf ratio on fruit development (Hansen, 1993; Toldam-Andersen and Hansen, 1993).

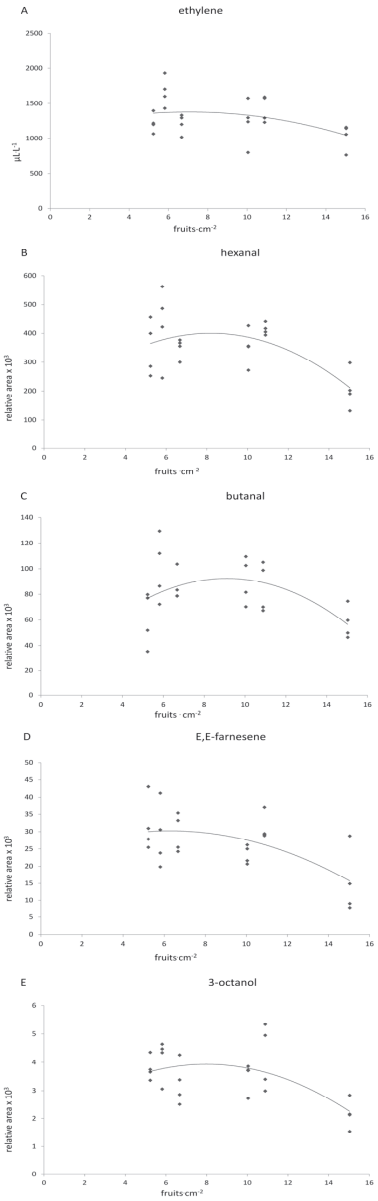
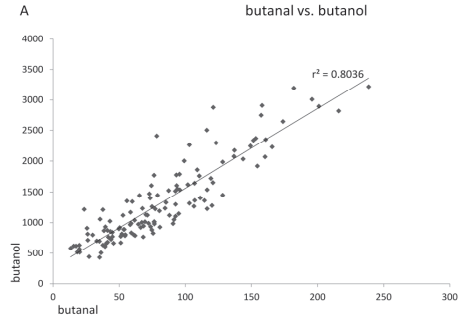


Fig.1. Production of (**A**) ethylene (in µL L^{-1}) and (**B**) hexanal, (**C**) butanal, (**D**) E,E-farnesene and (**E**) 3-octanol (in relative area x 10^3) depend on cropping level (fruits·cm^{-2}). Each crop load is represented by four data points; two from sampling day 5 and two from day 8 at room temperature after removal from cold storage. At each day the two data points represents the average aroma production of apples from the bottom (two nitrogen blocks, n=6) and the average aroma production of apples from the top of the canopy (two nitrogen blocks, n=6).



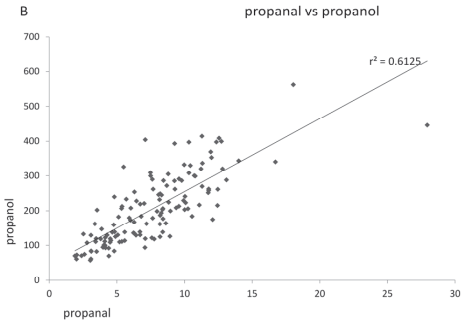


Fig. 2. Relationships between (**A**) butanal and butanol, (**B**) propanal and propanol. Volatile compound production (expressed as relative area $x10^3$) measured on single fruits from the N and thinning treatments at 5 and 8 days after storage.

As it was mentioned above, apples from not fertilized trees had delayed maturity development with ethylene production at the lowest level. Hexanal, E,E-farnesene and 3-octanol followed the same pattern as ethylene development in relation to cropping level (Fig. 1B, 1D and 1E). E,E-Farnesene is an aroma compound, which production in the apple skin is strongly related to ethylene synthesis (Dauny and Joyce, 2002). Low concentration of E,E-farnesene at high crop load might be a valuable observation here as it was noticed in other study that superficial scald occurrence during storage was associated with the higher amounts of E,E-farnesene (Dauny and Joyce, 2002). With higher cropping level the competition for assimilates might increase and production of these compounds decreased. In the study of Poll et al. (1996) was shown that with low fruit to leaf ratio, the sugar and acid pools are larger. These are main substrates in aroma compound synthesis and favour their accumulation. Additionally intensity of aroma synthesis is related to ripening stage. A higher aroma formation at a low cropping level may then also result from fruits being in an advance ripening stage and vice versa.

Conclusions

The choice of different cultivation techniques fx.; thinning and nitrogen fertilization, is intended to maximize fruit quality to handle the rising consumer demands. Lately, more attention is paid to the fruit aromatic content as a very important attribute of the fruit taste. The relationship between pre-harvest factors like; nitrogen fertilization, cropping level on fruit growth and aroma compound production in small fruited varieties like 'Ildrød Pigeon' is still very often overlooked. In our study hand thinning improved fruit size by 8.4% and increased fruit firmness to a small degree. Increasing nitrogen levels in unthinned trees affected fruit number by decreasing fruit amount per TCSA. Nitrogen fertilized apple trees had enhanced maturity development observed as increased fruit drop just before harvest. Additionally, the non-fertilized apples compared to these from elevated nitrogen levels had lowest ethylene production after storage, which indicated developmental delay. Ethylene and aroma compounds; E,E-farnesene, hexanal and 3-octanol production diminished with the increasing cropping level. Therefore, the relevant compromise of growing techniques becomes the growers' task to seek in order to maximize yield and to obtain the best overall quality fruit.

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PAPER II

Effect of light exposure and inhibition of ethylene action on the postharvest colour and quality of apples

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Effect of light exposure and inhibition of ethylene action on the postharvest colour and quality of apples

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Summary

The influence of the timing of 1-MCP application on apple fruit colour development and quality after storage were investigated. 'Ildrød Pigeon' represents apple cultivars which are exposed to sunlight after harvest for up to two or three weeks to develop an intense red colour on all sides of the fruit. Because of such a long exposure after harvest losses are unavoidable as apples become overripe and quality is diminished. To create experimental conditions in which postharvest red colour development would be clear to study, bags were put on the apples while on the trees to inhibit pre-harvest anthocyanin production. At harvest the bags were taken off and apples were exposed to light for 8 days. 1-MCP treatment was performed before or after the light exposure. A similar set of samples was prepared and stored directly without any light exposure. Main colour changes occurred between days two and four during the light exposure. 1–MCP negatively affected red colour change but only during the last days of sun exposure. This suggests other regulatory factors are involved in anthocyanin production besides ethylene and light. Bagged apples were firmer and developed a more intense red colour during postharvest sunlight exposure

than nonbagged, had higher internal ethylene concentration and greater weight loss after storage. Early 1-MCP strongly inhibited ethylene production and slowed down firmness loss. However, the delayed 1-MCP application (after light exposure) affected these parameters more moderately and the overall quality was better than in control (no 1-MCP treatment) apples. Therefore, taking into consideration sufficient pigment synthesis and quality maintenance during storage, the delayed 1-MCP application could be the optimal postharvest practice.

Keywords. bagging - colour development - light exposure - postharvest quality - 1-MCP

Introduction

For apple growers it is extremely important to achieve the best fruit quality. In terms of consumer perception; colour, size, firmness and taste are the most dominant quality parameters (DOBRZAŃSKI and RYBCZYŃSKI 2002; NILSSON and GUSTAVSSON 2007). All these, except size, are to a high degree determined during ripening. Ethylene is a plant hormone strongly involved in the regulation of ripening. When ethylene is bound to receptors located in apple cell membranes, it promotes first a wide range of ripening related processes and later senescence (LELIEVRE et al. 1997). The action of ethylene can be blocked by certain compounds like 1-MCP which, by linking to receptors, inhibit ethylene action (BLANKENSHIP and DOLE 2003; WATKINS 2006). Additionally, by blocking ethylene perception the production is suppressed, as ethylene is synthesized via an autocatalytic pathway (LELIEVRE et al. 1997). By 1-MCP application ripening processes are thus retarded and fruit quality is maintained during and after storage. An inhibitory effect is normally obtained by a 1-MCP treatment shortly after harvest either prior to or at the beginning of storage (MIR et al. 2001). Better understanding is required of changes in endogenous ethylene production and its effects on

colour development and other quality parameters. This could help growers in postharvest management to improve colour as well as quality after storage. Putting bags on apples during fruit development and removing them two to three weeks before harvest is one of possible method to enhance the red colour. It has been a practice for many years in some countries (e.g. Japan) (AMARANTE et al. 2002; FAN and MATTHEIS 1998; WANG et al. 2000; WHALE and SINGH 2007). These prior studies indicated that the fruit bags influence apple firmness and disorders plus reduce soluble solids, acids, starch and calcium content. However, there were conflicting results using different apple cultivars and times for bag removal (FAN and MATTHEIS 1998).

In the current study the variety 'Ildrød Pigeon' was used. It is a small-fruited (diameter of 50-60mm), red coloured apple with a characteristic aroma. It is harvested in mid-September and in order to obtain a full cover of red colour the apple fruit are traditionally exposed to sunlight for up to three weeks after harvest (KÜHN ET AL. 2011). The sunlight exposure practice is necessary as light is crucial for anthocyanin production. Apples are then stored until November-December to be sold as a traditional specialty at Christmas time. It can be difficult to maintain good quality of apple cultivars similar to 'Ildrød Pigeon' since the apples after the postharvest sunlight exposure are already very ripe. Additionally, 'Ildrød Pigeon' is a variety with progressive softening, which loses 45% of firmness during postharvest light exposure, followed by an additional 20% reduction during storage (KÜHN ET AL. 2011). 'Ildrød Pigeon' was therefore used as a model apple cultivar, which might benefits from 1-MCP application, due to its postharvest handling, which causes strong firmness reduction. The objectives for current study were: 1) to evaluate colour development during light exposure and determine the influence of 1-MCP on the rate of colour change with the aim of shortening the duration of postharvest

sunlight exposure; 2) to determine how the timing of 1-MCP affects changes in fruit quality, mainly loss of firmness, during storage. Based on the current study we present an optimized postharvest practice with a new and delayed timing of 1-MCP application, which lead to a better quality product after storage.

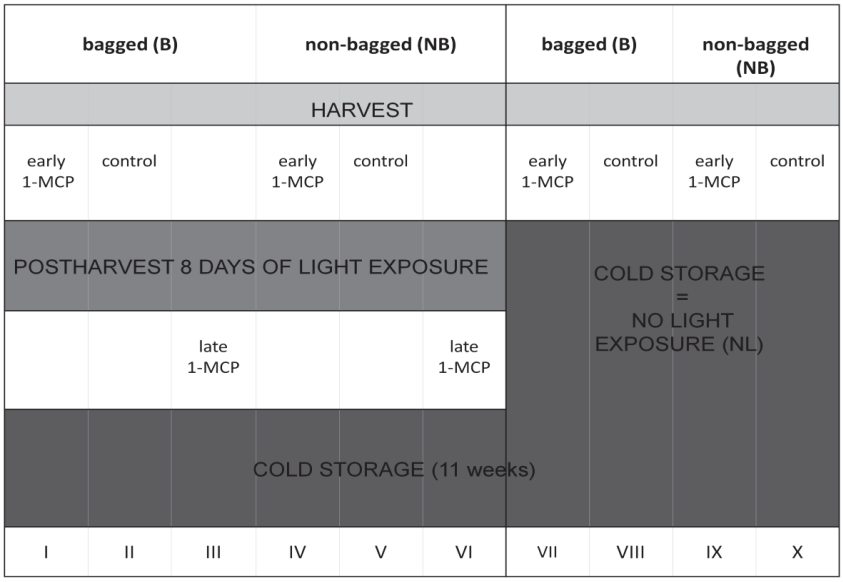
Materials and Methods

Plant material

Apples of the 'Ildrød Pigeon' variety were obtained from the 'Pometet' experimental orchard of the University of Copenhagen. At the end of July bags were put on seven apples from each tree to prevent anthocyanin development. The bags were made of two-layer paper: an outer-grey layer to eliminate light and inside a blue cover with wax (Kabayashi, 'Fuji apple bag', Japan). The fruit were picked from fourteen apple trees on 2nd of September 2009, when non-bagged apples reached a firmness of 10 N cm^{-2}, a starch index of 3 (scale 1-10) and total soluble solids of 11.3%Brix. In total ten sample-sets were prepared during the experiment: five samples with apples, which had been bagged, and five samples, which developed normally on the trees without bags (Table 1); each sample consisted of ten apples. Among these, six sets were exposed postharvest to sunlight for eight days and four samples were not exposed to sunlight but directly moved to cold storage (Table 1). During sun exposure the apples were placed in trays protected from birds with thin netting. Additionally, a 1-MCP treatment was carried out at one of two stages; directly after harvest ('early 1-MCP', Table 1 – four samples; I, II, VII and IX) and after light exposure ('late 1-MCP'; Table 1 – two samples; III, VI). Late 1-MCP application was performed after light exposure to ensure natural colour development, and to observe if the ethylene action inhibitor, applied at this late stage, could have an effect on fruit storability and

quality parameters (texture, ethylene production, and weight loss). The remaining four samples were control fruit not treated with 1-MCP (Table 1- four samples; II, V, VIII, X).

Table 1. Treatments (N=10; I-X) applied in the experiment (with abbreviations); early 1-MCP (treatment after harvest/before light exposure) and late 1-MCP (treatment after light exposure). Sample size was ten apples (n=10) for all treatments.



1-MCP treatment

Treatment with 1-MCP lasted for 20 hours at room temperature. The 1-MCP application was carried out by releasing 1-MCP gas to the samples placed into a box covered tightly by a plastic bag. A vial with 0.075 g 1-MCP (0.14% active component) was prepared, which achieves a concentration of approximately 1000 nL L^{-1} (BLANKENSHIP and DOLE, 2003). A small bottle with 1-MCP was placed in the box with samples next to a small ventilator to ensure adequate distribution of 1-MCP. Untreated samples were placed in the same temperature conditions in the lab next door. Finally, after all the treatments, samples were transferred to small ventilated cold

storage chambers (1.5^{o}C) for 11 weeks. After storage all samples were placed at room temperature for 5 days of shelf life to allow ripening. Analyses were carried out on each individual apple.

Colour measurement

The colour analysis was carried out on the side of the apple exposed to sunlight. Colour measurements were done at harvest and every second day of exposure (total five times) - also on apples from samples not exposed to sunlight (samples VII-X, Table 1). Colour measurements were done on three spots on the apple: from the stem end (pedicel), middle and blossom end (sepal/stigma). The changes in colour were monitored using a colourimeter (Minolta, Japan) equipped with a CR-300 measuring head. The instrument was standardized against a white tile before each determination series. CIELab is the most commonly used colour space and represents colour as perceived by humans. In this system L* is a lightness factor while a* and b* are chromaticity co-ordinates, which when positive indicate intensity of red and yellow colouration, respectively.

Ethylene measurement

For the ethylene evaluation a 1 ml gas sample was taken from the core cavity of the apple using a gas tight syringe with a 0.4 mm diameter x 40 mm length needle. The gas sample was injected into a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector and packed column Porapak Q; 80/100Mesh (length 1.82 m, 15.2 cm coil of 3 mm OD stainless steel) using helium as carrier gas (30 ml min^{-1}), the injection port and own temperature were set to 100^{o}C, detector temperature set to 140^{o}C. Ethylene concentration was calculated from standard curves obtained from analyses of ethylene/air standard mixtures.

Texture and weight

The flesh firmness was measured on a Texture Analyser TA.XT.plus (Exponent, Stable MicroSystems). Puncture measurements were done on opposite sides of each apple. The instrument was set up to penetrate the apple at a controlled speed of 1 mm s^{-1} with a touch force of 5 g (0.049 N), for a penetration distance of 7 mm, using a 10 mm diameter probe. The maximum force (N cm^{-2}) was recorded. Apples were weighed at harvest and after storage plus 5 days of shelf-life to evaluate weight loss (grams).

Statistical calculations

The statistical analyses were carried out by using JMP® 7.0 (2007, SAS Institute Inc.) and InfoStat (InfoStat versión 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). Data were subjected to analysis of variance. The treatment's means were separated by *F*-tests and the least significant difference (LSD) tests at the level 0.05.

To create the full factorial experimental design, which evaluated the colour changes during 8 days of sun exposure, samples treated late with 1-MCP were not included into the model (samples III and IV, Table 1). The complete combination of treatments: bagging (bagged or non-bagged apples), light conditions (exposed to sunlight or kept directly after harvest in cold storage) and 1-MCP application (early or control), were evaluated. Additionally, day of colour measurement was used as a factor to determine colour changes over time.

To evaluate influence of factors on quality parameters, the experiment was divided into two full factorial parts: samples exposed to light (samples I-VI, Table 1) and samples kept in storage (samples VII-X, Table 1). The main purpose of apples treated with 1-MCP after sun exposure ('late 1-MCP') in this experiment was to evaluate potential profits coming from late 1-MCP application on storability and quality changes, while at the same time allowing colour

development without any interference of the ethylene action inhibitor. Therefore, these samples were included into a complete factorial experiment, which consists only of samples exposed to light after harvest. Treatments: bagging (bagged or non-bagged) and 1-MCP application (early, late or control) were tested. A second full factorial tested bagging and 1-MCP application effects on quality parameters of fruit placed in cold storage directly after harvest.

Results

Colour evaluation

Apples exposed to sunlight for eight days after harvest developed significantly more colour than the apples placed directly in cold storage/non-light conditions, independent of 1-MCP treatment. Almost no changes occurred in a/b ratio during the initial eight days in cold storage (Table 2). Postharvest exposure of apples to sunlight enhanced their red colour significantly. The length of sun exposure was an important factor in colour development (Table 2). The main changes in anthocyanin production started to occur after day 2 in all samples (Table 2) manifested as a rapid increase in the a/b ratio (Table 2) and decrease in L value. The L value remained higher and the red colour (a/b ratio) increased less in early 1-MCP treated compared with control apples. The early 1-MCP treated apples showed the same trend of rapid colour change at days 2-4 as the control fruit, however, the ethylene action inhibitor suppressed colour development between day 6-8, resulting in a significantly lower final a/b value. Bagging management improved red colour development in early 1-MCP treated apples in comparison to nonbagged, however not to the a/b level of controls, for which bagging did not enhance red colour development.

Table 2. Development of color during eight days of light exposure or in cold storage depend on the bagging practice (B for bagged or NB for non-bagged) and early 1-MCP treatment.

Light conditions	1-MCP treatment	Bagging	Average a/b ratio during light exposure and storage days after harvest				
			0 ^{de}	2 ^{de}	4 ^{de}	6 ^{d}	8 ^{d}
Cold storage (no light)	1-MCP	B	-0.36	-0.37	-0.34	-0.32	-0.34
		NB	-0.32	-0.33	-0.30	-0.31	-0.30
	control	B	-0.27	-0.28	-0.29	-0.26	-0.26
		NB	-0.22	-0.19	-0.16	-0.11	-0.10
			0 ^{f}	2 ^{ef}	4 ^{c}	6 ^{b}	8 ^{a}
Light	1-MCP	B	-0.35	-0.31	0.39	0.82	0.92
		NB	-0.37	-0.35	0.21	0.50	0.54
	control	B	-0.38	-0.33	0.42	0.86	1.16
		NB	-0.36	-0.30	0.32	0.89	1.29

Letters a-f for days present least significant differences groups over exposure time depend on storage and light treatment (LSD ≤ 0.05, n=10).

Internal quality parameters - Apples exposed to light

Apples, which had been bagged pre-harvest lost more weight after storage (7.8 g) than non-bagged fruit (6.5 g). Early ethylene inhibitor application limited post storage weight loss in comparison to controls, 6.8 g and 7.8 g respectively. Late 1-MCP application had comparable loss as early treated apples, 7.0 g (Fig. 1). Bagged apples were firmer than non-bagged fruits in case of late 1-MCP treatment and control samples (Fig. 2). Apples treated early with 1-MCP had an average firmness of 80 N cm^{-2}, while late application fruit had an average firmness of 47 N cm^{-2}. The control apples average firmness was 37 N·cm^{-2} (Fig. 2). Bagging had no significant effect on ethylene synthesis but there was a trend of lower ethylene concentration in the non-bagged apples. Early 1-MCP application resulted in marked suppression of ethylene production (171 µL L^{-1}). Internal ethylene concentration in late 1-MCP treated apples was insignificantly lower than in controls, 1055 and 1363 µL L^{-1}, respectively (Fig. 2).

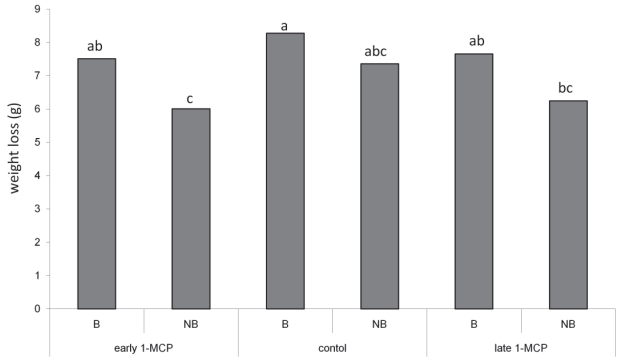


Fig. 1. Weight loss of apple fruit exposed to sunlight determined after storage (treatment groups I-VI, Table 1). Letters a-c indicates least significant difference groupings between treatments (LSD≤0.05, n=10).

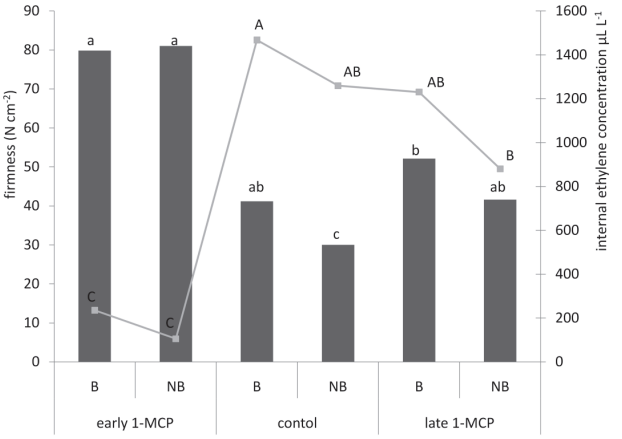


Fig. 2. Firmness (N cm^{-2}, columns) and internal ethylene concentration (µL L^{-1}, ■) of apple fruit postharvest exposed to light measured after storage (treatment groups I-VI, Table 1). Letters indicate least significant difference groups between treatments (LSD ≤0.05, n=10).

Internal quality parameters - Apples kept in cold storage

1-MCP application reduced weight loss during cold storage in comparison to the controls; 4.3 and 6.1 g respectively. No effect of bagging on apple weight loss and firmness was observed. 1-MCP treated apples were firmer than controls: 67 and 38 N cm^{-2} respectively. Internal ethylene concentration was very low in 1-MCP treated apples, 2 µL L^{-1}, while untreated apples had a significantly higher production of 1079 µL L^{-1}. Bagging influenced ethylene production in untreated apples in a way that non-bagged apples had a significantly greater concentration than bagged, 1315 and 843 µL L^{-1} respectively.

Discussion

In apples the breakdown of chlorophyll and anthocyanin production are important aspects of ripening (WATKINS 2006). The anthocyanin accumulation can be influenced by many factors (temperature, position on the tree, horticultural practices etc.) but light is essential (DOBRZAŃSKI and RYBCZYŃSKI 2002; WHALE and SINGH 2007). 'Ildrød Pigeon' fruit when attached to the tree develop red cheek while the rest of apple remains green (KÜHN et al. 2011). In our experiment the spot on the apples to monitor colour changes over time was chosen avoiding these red cheeks. There was no colour improvement on apples from cold storage, which is explained by a critical lack of light for anthocyanin formation. The main colour change of 'Ildrød Pigeon' fruit exposed to light occurred between days two and four and improved slowly until day eight (Table 2). The same pattern was observed at day four after bag removal and UV-B exposure on 'Fuji' apples (FAN and MATTHEIS 1998). Our results suggest that the traditional three weeks of 'Ildrød Pigeon' light exposure can be shortened. During such a long sun exposure apples are rotated to develop uniform red colour on all sides but sun burn and over ripening may occur, reducing storability by increasing the risk of postharvest loss and

physiological disorders. In the study by Kühn et al (2011) it was observed that most of post-harvest colour development occurred within first 6-9 days and was enhanced on the shaded side of fruit. Bagging practice may reduce starch and sugar while the effect on firmness is inconsistent (AMARANTE et al. 2002). The conditions in the bags, as well as their type, might influence structure of the apple cuticle affecting water loss. It was noticed that bagged apples exposed to light had higher mass loss, which could be influenced by cuticle deposition and lower resistance to gas diffusion (AMARANTE et al. 2002; FAN AND MATTHEIS 1998). Additionally, intense light exposure increases fruit temperature (possibly causing sun burn) and thus results in higher water and firmness loss. Here, bagged apples were slightly firmer but with greater weight loss after storage. Bagging reduced internal ethylene production (FAN and MATTHEIS 1998), but this was significant reduction only in apples from cold storage but not when exposing apples postharvest to sunlight. Bagging is performed to improve colour development of apples by increased anthocyanin synthesis after bag removal (FAN and MATTHEIS 1998). Also in the current study bagged apples developed a slightly more intense red colour except control fruit exposed to sunlight. The explanation might be given by the study of Wang et al. (2000) who found that anthocyanin accumulation and phenylalanine ammonia lyase (PAL) activity were higher in mature bagged apples than non-bagged fruit. PAL is a key enzyme which, together with sugar availability, controls the complex biochemical pathway of anthocyanin production. Ethylene initiates rapid anthocyanin accumulation during apple growth by increasing the level of the PAL enzyme in the apple skin (FARAGHER 1983). Ethylene however stimulated anthocyanin production via PAL on unripe apples, but not when applied on already ripe fruits (FARAGHER 1983; FARAGHER and BROHIER 1984; WANG et al. 2000). With increased ripening anthocyanins production decreases, even with relatively high PAL

activity. It suggests that there is an additional factor regulating anthocyanin production in ripe apples. Even though some studies point out an interaction between ethylene and anthocyanins synthesis, there is still some inconsistency in findings (MATTHEIS et al. 2004). Mattheis et al (2004) showed ethylene played an enhancing role when used with methyl jasmonate (a stimulator of anthocyanins accumulation) but there was no discernable effect of exogenous ethylene on anthocyanins production when applied separately. In the current experiment early application of the ethylene action inhibitor did not affect the main colour changes at 2 to 4 days, but anthocyanins development was inhibited somewhere around 6 to 8 day of light exposure and did not reach the colour of control apples. These results are in agreement with a study where another undefined regulatory factor, in addition to ethylene, was suggested to be involved in pigments synthesis (MACLEAN et al. 2006). Successful use of 1-MCP should be delayed in case of processes involved in pigment metabolism but applied in the appropriate time to maintain a good quality during storage, especially in case of cultivars which exhibit strong firmness reduction. Apples treated with 1-MCP maintain firmness longer during storage (PARKER et al. 2010; WATKINS 2006). In our study, 1-MCP application was very effective in apples treated directly after harvest and placed in cold storage. These apples were very firm, had strongly inhibited ethylene concentration and less weight loss in comparison to controls under the same conditions. However, in the case of the 'Ildrød Pigeon' apple cultivar light exposure after harvest is required and therefore the timing of 1-MCP application between harvest and storage is important. Early picked fruit might respond effectively to the treatment perhaps due to a larger number of free ethylene receptors. Conversely, the increasing level of ethylene found during ripening in some varieties may be sufficient to reduce the effectiveness of 1-MCP. 'McIntosh' apples had a greater loss in flesh firmness after storage when 1-MCP usage was delayed three

days compared to fruit treated immediately after harvest (DEELL et al. 2008). Delaying 1-MCP for eight days from harvest of ‘Cortland’ and ‘Empire’ mature fruit resulted in increased softening after cold storage compared to earlier treatment (PARKER et al. 2010). It seems like the effect of delayed 1-MCP treatment might have various responses in different apple cultivars. For example five days postponed inhibitor treatment increased internal ethylene concentration and reduced firmness of ‘Cox’ and ‘Bramley’ but had no effect on ‘Gala’ apples (JOHNSON 2008). When 1-MCP application was performed eight days after harvest on ‘Ildrød Pigeon’ it resulted in higher internal ethylene production, softer apples and higher weight loss than in fruit from early 1-MCP treatment, but still not to the level of controls.

‘Ildrød Pigeon’ apples reaching consumers at Christmas time are represented in this study by non-bagged, 1-MCP untreated and exposed to sunlight apples. These fruit are our references in an attempt to optimize the postharvest light and 1-MCP treatment practice. The effect of early application was very strong - meaning poorer colour development, an inhibited ethylene production and very firm apples. The optimization of 1-MCP technology must rely on the successful delay of the ripening processes but not their complete inhibition. For that reason 1-MCP application after light exposure might be a good alternative. However, the sun exposure time should be shorter than 3 weeks as main changes occurred at day 2-4 and were increasing slowly till day 8. The delayed 1-MCP application is also important from commercial point of view as apples are normally collected in the room for couple of days to fill the storage before applying CA condition. Apples treated with 1-MCP after sunlight exposure could synthesize pigments without any limitations. Furthermore, during the later storage period the late 1-MCP treatment was able to maintain apples 27% firmer than controls with a lower weight loss and the ability to develop an ethylene production to a level which allows a continued ripening, essential

for aroma production. Further sensory studies are needed to establish consumer preferences towards 1-MCP treated fruit as well as to evaluate if consumer can distinguish the red colour intensity differences, which were statistically significant in the current study, between the apples from different timing of 1-MCP treatments.

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PAPER III

Timing of 1-MCP Application and Postharvest Light Exposure as Tools to Optimize Aroma in Apple

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Timing of 1-MCP Application and Postharvest Light Exposure as Tools to Optimize Aroma in Apple

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Subject Category: Postharvest Biology

Timing of 1-MCP Application and Postharvest Light Exposure as Tools to Optimize Aroma in Apple

Additional index words. apple aroma, volatile compounds, principal flavour compounds, bagging, postharvest light exposure, 1-MCP, ethylene inhibitor

Abstract. 1-MCP (1-methylcyclopropene) application has lately been incorporated into horticultural practice to maintain fruit quality through storage and marketing. The effect of 1-MCP treatment timing on the final flavour quality was investigated. Also the influence of pre-harvest bagging and postharvest sunlight exposure, which are commercially used to improve visual fruit quality, was studied to evaluate their effect on aroma composition. Twelve aroma compounds were identified by GC-olfactometry as having the highest impact on the overall flavour of the 'Ildrød Pigeon' apples. Application of 1-MCP immediately after harvest had the strongest reductive effect on the production of especially esters and alcohols. Among these were important principal 'Pigeon' aromas; ethyl acetate, methyl 2-methylbutyrate, butyl butyrate, butanal, 1-heptanol and 6-methyl-5-hepten-2-ol. Also aldehydes were identified as principal pigeon aromas and the most important ones butanol, hexanal and trans-2-hexanal were also significantly reduced. On the other hand, production of 2-methyl-2-pentenal, cis-3-hexenal and isomers of 2,4-hexadienal was enhanced in apples treated early with 1-MCP. These are also identified as principal pigeon aromas but generally found in low amounts. Delay of the 1-MCP application until after a postharvest light exposure period of eight days affected aroma production only to a moderate degree. In bagged apples there was a tendency of higher ethylene concentration; however production of most of aroma

compounds were higher in non-bagged fruits. Bagging therefore delayed the increase in the internal ethylene concentration and consequently delayed upcoming ripening processes like aroma development. Postharvest sunlight exposure, associated with higher temperature, in comparison to direct postharvest cold stored conditions, provoked higher internal ethylene concentration. Thus, ripening processes accelerated and production of most aroma compounds increased, when exposed to sunlight.

In general, delay of 1-MCP application must be taken into consideration in the commercial postharvest practice of apples as fruits are typically accumulated for several days to fill a storage room before applying controlled atmosphere or, as in case of 'Ildrød Pigeon', are postharvest exposed to sunlight. The effect of 1-MCP on aroma is critical to the optimization of 1-MCP postharvest technology, as apple flavour will rely on the successful delay, but not complete inhibition, of the ripening processes.

The final fruit quality depends strongly on the production of aroma compounds. More than 300 compounds have been identified in different apple varieties. Esters, alcohols and aldehydes are quantitatively the major volatiles in apples (Kondo et al., 2005). In this very complex mixture of volatiles about 20-40 compounds are directly responsible for characteristic aroma perception (Vanoli et al., 1995). There are general trends of volatile synthesis through the physiological stages of fruit development under natural conditions: Aldehydes are quantitatively the largest aroma group in mature apples, then aldehyde concentration declines and alcohol biosynthesis ensues upon the climacteric peak. Finally, ester synthesis is closely related to the advance in climacteric and post-climacteric stages of ripening (Lanciotti et al., 1999). The production of aroma compounds is therefore strongly related to ripening, which accelerates with rising internal ethylene concentration. In a study by Danekar et al. (2004) on transgenic ethylene-suppressed apples a reduction

of esters was found, while aldehyde and alcohol production was only slightly affected. The influence of ethylene on especially ester production in apples has been confirmed by Defilippi et al. (2005a) and Schaffer et al. (2007).

The apple varieties are suggested to be divided into groups dependent on aroma components, which contribute most to the flavour quality (Li et al., 2008; Dixon and Hewett 2000). Ethyl butyrate, ethyl acetate and ethyl 2-methylbutyrate are characteristic for ester-like varieties as 'Delicious' and 'Golden Delicious'. Additionally, according to Petersen and Poll (1995) apple cultivars could be divided into three ester sub-groups, namely those dominated by butyrate esters ('Filippa' and 'McIntosh'), acetate esters ('Elstar', 'Cox Orange' and 'Golden Delicious') and those, where both butyrate and acetate esters contribute to the total aroma profile ('Mutzu', 'Ingrid Marie' and 'Spartan'). The 'Jonathan' variety is representing an alcohol-rich group with butanol, 3-methyl-1-butanol and hexanol as typical aroma components. Finally, there is a fifth group represented by 'Granny Smith' characterized with aldehydes like 2-hexenal, and low content of esters (Li et al., 2008).

In the current study the 'Ildrød Pigeon' variety was used. It is a small-fruited, red colored apple with a characteristic aroma. 'Ildrød Pigeon' aroma is described by consumers as characteristically intense and special but there is no available information about the contributing principal compounds. Apples are harvested in mid-September and in order to obtain a full cover of red color, fruits are traditionally exposed to sun light for up to three weeks after harvest (Kühn et al., 2011). Apples are then cold stored until November-December to be sold as a traditional specialty at Christmas time. Good quality maintenance of apples, which are postharvest exposed to sunlight is an issue since the apples after the exposure are already very ripe. Lately, 1-MCP (1-methylcyclopropene) treatment has been incorporated into horticultural practice to maintain fruit quality

through storage and marketing. The postharvest gaseous application of 1-MCP has been shown to affect many physiological characteristics of apples, such as reducing ethylene production and respiration, enhancing fruit firmness and acidity retention, and reducing peel greasiness (wax layer) and reducing various physiological disorders (DeEll et al., 2007; Watkins 2006; Blankenship and Dole 2003; Fan et al., 1999a; Fan et al., 1999b). One of the drawbacks of using 1-MCP is aroma reduction. Concentration of aroma compounds is, however, also strongly dependent on the genetic potential of the cultivar, harvest time, storage and post-storage recovery period (Popielarz et al., 2010; Schaffer et al., 2007; Kondo et al., 2005). The apple is a fruit with many cultivars, each with unique ripening rates and storage potential and therefore 1-MCP application conditions must be established for each cultivar individually to assure a successful commercial utilization of 1-MCP (Watkins et al., 2007;Watkins et al., 2000). One very important condition is the time of 1-MCP application. The current guidelines for 1-MCP treatment require most apple cultivars to be treated within three to ten days after harvest (Parker et al., 2010). 1-MCP binds to the ethylene binding sites and should therefore be used while the receptors are free, i.e. before the endogenous ethylene concentration starts to rise. In return, it will prevent the hormone to elicit its reaction to the already occupied sites, when its production increases. 1-MCP should be used in a narrow time window between harvest and storage, but it is still cultivar dependent. For example, delaying postharvest 1-MCP treatment from one to seven days after harvest reduced its efficacy in terms of keeping firmness in 'Golden Delicious' and 'Law Rome' but not in 'Gala' apples (Parker et al., 2010), while 1-MCP delay was optimal with 3 days for the 'McIntosh' cultivar (DeEll et al., 2008). According to Watkins (2008) such a variation in the optimal delay from harvest for different cultivars must be taken into account in the commercial practice as apple fruits are typically accumulated for several days to fill a room before applying CA

storage. Watkins (2008) indicated that despite the extensive literature about 1-MCP technology, relatively little information concerning commercial aspects of its use is available. The primary objective in the current study was to evaluate the effect of the timing of 1-MCP application on 'Ildrød Pigeon' volatiles to assess any limitation in the ability to sustain aroma production when ethylene action is being suppressed. It is a complex issue as the reaction of different volatiles, even from the same aroma group, variously depends on ethylene action. Nevertheless, this information may help to optimize the commercial postharvest management for the delivery of the highest quality of fruit, in terms of aroma, to the consumer.

The second part of the study relates to the effects of pre-harvest bagging and postharvest sunlight exposure on post-storage aroma composition. Light is well established as a crucial factor for anthocyanin development (Whale and Singh, 2007), however it has also been reported that the postharvest volatile production may change depending on the manipulation of sunlight availibilty during fruit development (shading) (Miller et al., 1998). Acetate ester production increased with reduced sunlight intensity and decreased with intensified sunlight exposure (Miller et al., 1998). The pre-harvest bagging has been extensively used on several fruit crops to improve skin color (Arakawa 1991), reduce mechanical damage (Amarante et al., 2002b) and reduce sunburn of the skin (Bentley and Viveros 1992). The bagging of apples has been a conventional practice to improve visual quality in China and Japan (Huang et al., 2009; Whale and Singh 2007; Amarante et al., 2002a; Amarante et al., 2002b; Wang et al., 2000; Fan and Mattheis 1998) and has been used extensively in the Pacific Northwest of North America, mostly for 'Fuji' apples (Fan & Mattheis 1998). These studies indicated that the fruit bags reduce soluble solids, acids, starch, and increase mass loss while the effect on firmness is inconsistent (Amarante et al., 2002a; Amarante et al., 2002b; Fan & Mattheis 1998).

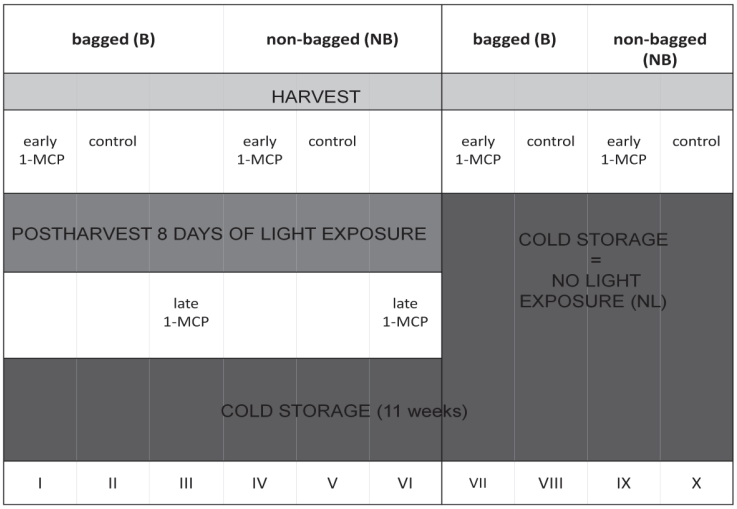
Despite the fact that both bagging and postharvest sunlight exposure are used in horticultural management, very few studies have described the effects on aroma quality parameters. Mink (1973) suggested that bagging might lead to lack of flavour due to the changes in the apple aroma composition. Bagging, in fact, reduced the volatile content of 'Fuji' apples (Mattheis et al, 1996) but was shown to improve the characteristic peach aroma compounds γ- and δ- decalactone (Jia et al., 2005). In grapes cv. 'Perla', acetate esters were predominant in non-bagged fruits while limonene, *trans*-2-hexenal, 3-hexanol and 2-hexen-1-ol were predominant in bagged fruits (Signes et al., 2007). Therefore, our secondary goal was to investigate the pre-harvest bagging and the postharvest sunlight exposure effects on post-storage apple aroma composition.

Material and Methods

Plant material. Apples of the 'Ildrød Pigeon' variety were obtained from 'Pometet', the experimental orchard of University of Copenhagen. At the end of July (after fruit drop) bags were put on seven apples/tree distributed in various positions of the tree canopy to prevent anthocyanin development. The bags were made of two-layer paper: an outer-grey layer to eliminate light and inside a blue cover with wax (Kabayashi, 'Fuji apple bag', Japan). Fruits were picked from fourteen apple trees when non-bagged apples reached a firmness of 10 $N \cdot cm^{-2}$, a starch index of 3 (scale 1-10) and a total soluble solids of 11.3 % brix. In total, ten sample-sets were prepared for the postharvest experiment: five samples with apples, which had been bagged, and five samples, which developed normally on the trees without bags; each sample consisted of ten apples. Among these, six samples were exposed postharvest to sunlight for eight days and four samples were not exposed to sunlight but directly moved to cold storage (Table 1). During sun exposure the apples were placed in trays protected from birds with thin netting. Additionally, a 1-MCP

treatment was carried out at one of two stages; directly after harvest - before light exposure ('early 1-MCP', Table 1 – four treatments; I, IV, VII and IX) and after light exposure ('late 1-MCP'; Table 1 – two treatments; III, VI). Late 1-MCP application was performed after light exposure to ensure natural color development, and to observe if the ethylene action inhibitor, applied at this late stage, could have an effect on fruit aroma quality parameters. The remaining four samples were controls not treated with 1-MCP (Table 1- four treatments; II, V, VIII, X).

Table 1. Treatments (N=10; I-X) applied in the experiment (with abbreviations); early 1-MCP (treatment after harvest/before light exposure) and late 1-MCP (treatment after light exposure). Sample size was ten apples (n=10) for all treatments.



Treatment with 1-MCP was carried out by releasing 1-MCP gas into a box covered tightly by a plastic bag. A vial with 0.075 g 1-MCP (0.14%) was prepared, which results in a concentration of approximately 1000 $nL \cdot L^{-1}$ when water is added (Blankenship and Dole, 2003). The vial with 1-MCP powder was put in a small flask that was placed in the box together with a small ventilator to ensure adequate distribution of 1-MCP. Finally water was added to the vial with 1-MCP, shacked and the plastic bag was closed immediately after. 1-MCP treatment lasted for 20hours at room temperature ($20^{o}C$). During the early

1-MCP application all the remaining sample sets intended to be light exposed were kept in the same temperature conditions in the lab next door. Sample sets I – VI were exposed for eight days to sunlight. The average max and min temperatures for the period were: 19.4 and 12.5 °C. Day length/total sun radiation lasted 13 hours per day with an average precipitation of 2.3 mm/day (metrological data for the region obtained from The Danish Meteorological Institute). Finally, after light exposure the 'late 1-MCP' treatment was performed as described for the 'early 1-MCP' with the remaining sets (I, II, IV and V) kept next door at similar temperature conditions. After the light exposure samples were transferred to the cold storage (1.5 °C) for 11 weeks. After storage all samples were placed at room temperature for five days for the final ripening to occur. Analyses were carried on each individual apple.

GC-MS analysis. Analyses of volatiles were carried out on juice pressed from each individual apple. Aroma was trapped using dynamic headspace sampling from 20 ml of juice (added 4-methyl-1-pentanol as internal standard) with nitrogen as purge gas (70 $ml \cdot min^{-1}$ for 20 minutes) into a trap containing 200 mg Tenax TA. The volatiles collected in the traps were liberated using an automatic thermal desorption device (ATD 400, Perkin Elmer, Norwalk, USA). Traps were desorbed by heating to 250 °C with a hydrogen flow of 60 $mL \cdot min^{-1}$ for 15 min and volatiles were focused in a cold trap which subsequently was flash-heated to 300°C and held for 4 min. A split ratio of 1:50 was applied to transfer the volatiles to a GC–MS for separation and identification. The GC–MS used was a 7890A GC-system interfaced with a 5975C VL MSD with Triple-Axis detector (Agilent Technologies, Palo Alto, California) equipped with a DB-Wax capillary column (30 m × 0.25 mm × 0.25μm) (J&W Scientific). The column flow rate was 1.0 $mL \cdot min^{-1}$ using hydrogen as a carrier gas. The temperature of the oven was held at 40 °C for 10 min and then increased by 8 °C $\cdot min^{-1}$ up to 240°C, which was kept

constant for 5 min. The mass spectrometric detector operated in the electron ionization mode at 70 eV and scanned mass/charge ratios (m/z) between 15 and 300. Aroma compounds were tentatively identified by matching mass spectra with these from a commercial database (Wiley275.L, HP product no. G1035A). Peak areas divided by internal standard area were used as relative measures of concentration of compounds. GC-MS chromatograms obtained from control apples were used to identify compounds detected by the panelists in the GC-O part. The identities of the characteristic compounds were confirmed by comparing the retention times and mass spectra of the chromatographic peaks with authentic standards purchased from Aldrich (Sigma-Aldrich).

GC-O analysis. An additional set of 15 apples was harvested, at the same time as apples for the 1-MCP timing experiment, to be used for GC-O analysis (nonbagged, no 1-MCP treatment). They were cold stored, as previously described, and removed for 5 days to room temperature prior to GC-O sampling. Juice was pressed from all these apples combined in one portion using a juice processor. The aroma compounds were measured by GC-MS according to the earlier described method. For GC-O, thermal desorption of the aroma compounds from the traps was done on a Short Path Thermal Desorption unit (model TD-4, Scientific Instrument Services Inc. NJ). Desorption temperature was 250 °C which held for 4 minutes with a helium flow of 10 ml/min. Separation was performed with a Hewlett-Packard 5890 GC with the following conditions: capillary column, DB Wax; 30 m x 0.25 mm i.d. x 0.25 μm film thickness; carrier gas, helium; start flow, 1 ml·min^{-1}; column pressure, 88kPa (constant); oven program, 45 °C for 10 min, 6 °C ·min^{-1} to 240 °C, constant at 240 °C for 10 min; detector temperature 250 °C; air flow 345 ml·min^{-1}; hydrogen flow 35 ml·min^{-1}. For the GC-O analysis, the effluent from the column was split approximately 1:4 with the minor part going to the FID and the major part going to

an olfactory detector outlet ODO-1 from SGE, Ringwood, Victoria, Australia. The detection frequency method (Pollien et al., 1997) was used for recording detected odors over a group of seven assessors. The number of assessors detecting an odour (NIF-Nasal Impact Frequency) was used as an odor's intensity estimator. Compounds perceived by at least 3 panelists at the same time, were considered important.

Internal ethylene concentration. For the ethylene evaluation a 1 ml gas sample was taken from the core cavity of the apple using a gas tight syringe with a 0.4 mm diameter x 40 mm length needle. The gas sample was injected into a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector and packed column Porapak Q; 80/100Mesh (length 1.82 m, 15.2 cm coil of 3 mm OD stainless steel) using helium as carrier gas (30 $ml \cdot min^{-1}$), the injection port and oven temperature were set to 100 ^{o}C, detector temperature was set to 140 ^{o}C. Ethylene concentration was calculated from standard curves obtained from analyses of commercial ethylene/air standard mixtures.

Statistical analysis. The statistical analyses were carried out using JMP® 7.0 (2007, SAS Institute Inc.) and InfoStat (InfoStat versión 2008, Grupo InfoStat, FCA, Universidad National de Córdoba, Argentina). Data were subjected to analysis of variance. The treatment's means were separated by *F*-tests and the least significant difference (LSD) tests at the level 0.05.

The experiment was divided into two full factorial parts in order to evaluate the influence of main factors and their interactions on volatiles composition.

The first complete factorial experiment included only samples, which were exposed to sunlight after harvest (treatments I-VI, Table 1) in order to evaluate the effects of 1-MCP time application (early, late or control) and bagging (bagged and non-bagged) on aroma compounds production.

In the second factorial experiment, which evaluates the effect of sunlight exposure on aroma changes, late 1-MCP treated samples (treatments III and VI, Table 1) were excluded from the model as they have no representation in cold storage conditions. These late 1-MCP treated samples were prepared to evaluate potential benefits from delayed 1-MCP application rather than monitoring the light effect on aroma composition.

Results and Discussion

Principal aroma compounds. Twelve principal aroma compounds were identified in 'Ildrød Pigeon' apples by at least three panelists during GC-O (Table 2). The aroma compounds belong to 3 groups; esters, aldehydes and alcohols. The esters were represented by ethyl acetate, methyl 2-methylbutyrate and butyl butyrate. Hexanal, *trans*-2-hexenal, butanal, 2-methyl-2-pentenal, cis-3-hexenal and two isomer forms of 2,4-hexadienal were representing aldehydes and were detected by four or more judges. Finally, the alcohols; 1-heptanol and 6-methyl-5-hepten-2-ol were identified by three judges. The panelists' odour descriptions, retention times and NIF values are given in Table 2. In three cases, the principal aroma compounds were well separated and the descriptors used by the judges were in good agreement with literature indications (Table 2). However, for butanal and ethyl acetate the judges were not able to separate two compounds eluted close in retention time. For other compounds the literature description of the two suggested compounds are alike, thus separation is clearly a difficult task. In case of *trans*-2-hexenal and butyl butyrate the panel description looks like a combination of literature descriptions of the two odors. With these identification limitations of the principal flavors, 'Ildrød Pigeon' appears to have both esters and aldehydes as being important for the special 'Pigeon flavour'. Thus its complex aroma profile combines all the aroma groups listed by Petersen and Poll (1995) and Li et al. (2008).

Effect of time of postharvest 1-MCP application on principal aroma compounds in apples exposed to sunlight after harvest. In the present study the average concentration of ethylene in the early 1-MCP treated apples was significantly lower than ethylene concentration in the control and the late 1-MCP treated apples (Table 3). The early 1-MCP treatment also significantly affected the levels of principal aroma compounds since production of 6 out of the 12 components decreased drastically. The compounds reduced to a minimum in early 1-MCP treated apples were: ethyl acetate, methyl 2-methylbutyrate, butyl butyrate, butanal, 1-heptanol and 6-methyl-5-hepten-2-ol (Table 3). In addition *trans*-2-hexenal remained at quite high concentration in all treatments being, however significantly lower in early 1-MCP treated apples (Table 3). Relative concentration of hexanal decreased almost by half in early 1-MCP treated samples in comparison to the control and the late 1-MCP treated apples. The rest of the principal aldehydes; 2-methyl-2-penenal, *cis*-3-hexenal and the two isomers of 2,4-hexadienal, reacted with significantly higher relative concentrations when apples were treated early with 1-MCP. The most attention in previous reports, related to delayed 1-MCP application, was paid to the textural quality of apples (Parker et al., 2010; DeEll et al., 2008; Watkins and Nock 2005). No attention was given to the influence on aroma. After 1-MCP treatments 'McIntosh' apples from early harvest treated 3 days after harvest had the lowest internal ethylene concentration and were firmer. These parameters were gradually reduced with later harvests and delay of 1-MCP treatment (until 10 days). A delay of 1-MCP application up to 8 days after harvest strongly reduced treatment efficiency (internal ethylene concentration, firmness and soluble solid concentration) or did not affect it depending on cultivar, storage type and duration (Watkins & Nock 2005). 1-MCP treatment directly after harvest has become a common practice described in scientific publications related to the fruit postharvest management. However, the early 1-

MCP application strongly reduced the concentration of most of 'Ildrød Pigeon' principal esters. In agreement with this the production of esters, contributing to the characteristic aroma of 'Gala', was also reduced by 1-MCP treatment in a study by Mattheis et al. (2005). However, in contrast Lurie et al. (2002) found that hexanal and *trans*-2-hexenal concentrations remained at levels similar to these found at harvest time after storage in 'Anna' apples despite of 1-MCP treatment. Biosynthesis of C_6 aldehydes is regulated by lipoxygenase (LOX) activities, and this enzyme increases rapidly during tissue wounding (Myung et al., 2006; Lanciotti et al., 1999). The LOX pathway may also become active during ripening when cell membranes become more permeable (Echeverria et al., 2004; Sanz et al., 1997). Defilippi et al. (2005b) reported that *trans*-2-hexenal is accumulated via an ethylene-independent pattern. In our study, different aldehydes responded with higher or lower biosynthesis depends on different 1-MCP time application. These results confirm that sensitivity to ethylene action varies among the pathways of volatile production in apple fruit. Interestingly, production of 2-methyl-2-pentenal, *cis*-3-hexenal and isomers of 2,4-hexadienal was positively correlated to ethylene inhibition. It might indicate that expression of regulators of the synthesis of these aldehydes is enhanced by ethylene suppression. 2-Methyl-2-pentenal and isomer forms of 2,4-hexadienal are amino acid and fatty acid degradation products, respectively. C-6 aliphatic aldehydes; *cis*-3-hexenal and *trans*-2-hexenal arise from linolenic acid oxidation. In early 1-MCP treated apples *cis*-3-hexenal concentration was around 3 fold higher than in other samples. Despite high *cis*-3-hexenal concentration, *trans*-2-hexenal concentration was at a lower level than in other samples. These two compounds are LOX-derived compounds produced in response to wounding. Firstly, linolenic acid is oxidised by LOX to 13-hydroperoxyoctadecatrienoic acid (13-HPOT), which is secondly cleaved by hydroperoxide lyase (HPL) resulting in synthesis of *cis*-3-hexenal and 12-oxo-*trans*-10-

dodecenoic acid (*trans*-10-ODA), which is autooxidised to traumatic acid, known as wound signal compound (Zimmerman & Coudron 1979). *cis*-3-Hexenal is isomerized, probably enzymatically, to the more stable *trans*-2-hexenal (Baldwin et al., 2000). Myung et al. (2006) suggested that a factor, which regulates the isomerisation of *cis*-3-hexenal to *trans*-2-hexenal, is activated upon wounding and therefore *cis*-3-hexenal is rapidly converted to *trans*-2-hexanal. However, some time after wounding the isomerisation factor returns to its deactivated form allowing *cis*-3-hexenal to accumulate. It is still unclear which factors regulate *cis*-3-hexenal transformation; if this conversion is induced by an enzyme, if it is a spontaneous isomerization or if the isomerisation factor expression depends additionally on the fruit developmental stage. In our study, early 1-MCP treatment suppressed the internal ethylene concentration, triggered *cis*-3-hexenal synthesis but most likely inactivated the isomerisation factor so *trans*-2-hexenal production was lower despite substrate availability. The isomerisation factor's action might be blocked when internal ethylene concentration is at insufficient levels.

Postharvest sunlight exposure and pre-harvest bagging effects on apple overall volatile composition. The internal ethylene concentration after storage was unaffected by light, air temperature or both during the postharvest sunlight exposure, but tended in both 1-MCP treated and control apples to be a slightly higher in the light exposed (Table 2). This positive trend in the effect of the postharvest light exposure was reflected in the production of most volatile compounds. All measured alcohols had significantly higher aroma production when exposed to light. Most of the esters reacted with higher or sustained synthesis when exposed to light and only methyl 2-methylbutyrate and butyl acetate decreased (Table 2). The majority of aldehydes, acids, ketones and other compounds also showed an increasing trend or remained at similar synthesis levels when

exposed to light (Table 2). In comparison to direct postharvest cold storage, postharvest light exposure associated with the higher temperature, might provoke ripening processes to be accelerated. Consequently, the production of aroma compounds will be enhanced. In 'Kent' strawberries methyl and ethyl butyrate production was stimulated after storage when exposed for 3 days at 20 °C in light, but in dark at 10 °C none of these aroma compounds were detectable (Artur et al., 1995). It is suggested that aroma compound production is triggered by light. However, Miller et al. (1998) showed that with high sun exposure anthocyanin production in apples increased, but it was at the expense of acetate esters (hexyl-, butyl-, 2-methylbutyl acetates). In their experiment, 2-methylbutyl acetate was clearly the major acetate ester and its concentration was highest when sunlight intensity reaching fruit surface was reduced by half. At full sun exposure 2-methylbutyl acetate decreased significantly, while anthocyanin content increased. Some ester precursors or esters might be utilized as anthocyanin substrates. Moreover, during esterification free coenzyme A (CoA) is formed (Shalit et al., 2001) and it is a necessary substrate for both red pigment production and aroma production. Flavonoid and aroma compounds might therefore depend on the same substrate and in this way influence one another (Siegelman and Hendricks 1958). In our experiment the bagged apples kept in cold storage had the highest production of butyl acetate (data not shown). Bags inhibited light access when fruit were on the tree and there was additional lack of light during cold storage. This tendency, however, was not observed for any other acetate esters in our study, in contrary light increased and bagging reduced aroma production. The pre-harvest bagging showed a trend of higher ethylene production in bagged apples (Table 4). There were 22 aroma compounds, which had significantly greater production in nonbagged apples after storage (all presented in Table 4), along with farnesene, of which oxidation products are associated with superficial scald occurrence in apples (Shoji et al. 1998). In

contrast, bagged apples exposed to postharvest sunlight had only a higher concentration of 2-propanone after storage in late 1-MCP treatment and controls (Table 4). Bagging effects in general were most pronounced in late 1-MCP treated and control samples, while in early 1-MCP samples bagging effects were diminished because of the strong 1-MCP suppression effect on aroma compounds (Table 4).

The rise in internal ethylene concentration at the onset of the climacteric phase stimulates the ripening processes. According to Farhoomand et al., (1977) 'Delicious' shaded apples had higher ethylene production compared to non-shaded fruits with red blush, as shading delayed the increase in the internal ethylene concentration at the onset of fruit ripening. The bagging practice delayed the climacteric peak which explains higher internal ethylene concentration in our bagged apples (Table 4) while ethylene concentration already had started to decline in non-bagged apples regardless of 1-MCP treatment. The conditions in the bags, as well as their type, might influence structure of the apple skin as well as reduce cuticle deposition (Amarante et al., 2002a; Fan and Mattheis 1998). Aroma compounds are synthesized in the apple flesh and peel (Defilippi et al., 2005b; Pechous et al., 2005) but activity of responsible enzymes and precursor availability are higher in the peel than in the flesh (Defilippi et al., 2005b; Echeverria et al., 2004). Since peel structure of the apple is affected by bagging, while on the tree, then fatty acid and amino acids accumulation may be lower in the peel and this in consequence results in lower straight chain and branched chain esters synthesis. Shoji et al. (1998) reported that content of *trans*-2-hexenal, 2-methylbutan-1-ol, hexanal, propanol, *trans*-3-hexenol, hexyl propionate and amyl acetate was significantly lower in peel of bagged than of nonbagged 'Hokuto' apples. Mattheis et al. (1996) reported that emission of ester and alcohol volatiles was lower in bagged 'Fuji' fruit and that postharvest volatile emission was negatively correlated with bagging duration during development.

Conclusion

In ‘Ildrød Pigeon’ twelve aroma compounds (aldehydes, esters and alcohols) have been identified as these contributing the most to overall sensory quality. Overall pre-harvest bagging diminished and postharvest sunlight exposure enhanced volatile formation in ‘Ildrød Pigeon’. Results suggested that bagging practice delays internal ethylene concentration and as a consequence delays ripening processes related to ethylene. The effects of 1-MCP treatment and the application timing on the aroma properties of ‘Ildrød Pigeon’ apples have been established in the current experiment. The next step should involve the sensory characteristics of 1-MCP-treated apples. The flavour perception is complex as more sensory parameters are involved fx. firmness. A key to increased fruit consumption is to provide apples with high quality including better flavour. Treatment with the ethylene inhibitor 1-MCP leads to substantial suppression of a major part of the cultivar characteristic volatiles, which is a factor that must be taken into consideration when 1-MCP is used in commercial apple production. The use of 1-MCP relies on the successful delay of ripening processes, but not their complete inhibition like it was shown in case of the early 1-MCP treatment in our study. The delayed 1-MCP application in ‘Ildrød Pigeon’ did not reduce aroma production as strong as when 1-MCP treatment was done directly after harvest. In case of apple cultivars, like ‘Ildrød Pigeon’, which are postharvest exposed to sunlight, delayed 1-MCP application might also be appropriate for logistic and practical reasons. The realistic postharvest management approach allows for harvesting apples, placing them for several days in the orchard tree rows to be exposed to sunlight, then transport to storage facilities, treat with 1-MCP and store.

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Table 3. Aroma compound production dependent on 1-MCP treatment divided into apples exposed to light and kept in cold storage directly after harvest. Compounds marked in bold are the 'Ildrød Pigeon' principal aroma compounds. Means (n=20) are calculated of both bagged and non-bagged apples.

	Apples exposed to the sunlight after harvest			Apples kept in the cold storage after harvest	
	late 1-MCP	early 1-MCP	Control	early 1-MCP	control
Ethylene ($\mu L \cdot L^{-1}$)	1060a	170bB	1360aA	2B	1080A
Aroma compounds	RELATIVE AREA x 10^3				
Alcohols					
2-butanol	16b	15bB	22aA	8.8C	16B
propanol	194b	22cC	409aA	8.6C	219B
2-methyl-1-propanol	233b	65cB	312aA	43C	204B
butanol	1586b	119cB	2474aA	53C	1807B
1-pentanol	88b	10cC	156aA	5.3C	99B
3-octanol	34b	3.3cB	54aA	2.2B	25B
2-hexen-1-ol	41a	38aAB	45aA	33B	43AB
1-heptanol	**7.7b**	**1.9cB**	**12aA**	**0.9B**	**11A**
6-methyl-5-hepten-2-ol	**11b**	**1.9cC**	**19aA**	**0.1C**	**12B**
2-ethyl-1-hexanol	9.2a	4bB	9aA	3.2B	4.9B
1-octanol	6.7b	1.9cC	10aA	1.5C	7.4B
5-octen-1-ol	6b	0.7cC	10aA	0.4C	7.6B
Esters					
methyl acetate	52a	9.2bB	55aA	9.5B	47A
ethyl acetate	**294b**	**27cC**	**440aA**	**71C**	**313B**
ethyl propionate	2.6b	nd	4.4aA	1.9B	4.6A
propyl acetate	39b	9.4cC	69aA	5.7C	40B
methyl butyrate	1.8a	nd	2aA	nd	1.4A
methyl 2-methylbutyrate	**35.6b**	**5.3cC**	**66aB**	**0.9C**	**103A**
ethyl butyrate	5.6b	0.7cC	10aA	nd	5.6B
butyl acetate	21b	1.6cB	35aB	0.7B	239A

butyl butyrate	**23b**	**4.8cB**	**47aA**	**2B**	**21A**
butyl 2-methylbutyrate	9.1b	4.8cC	14aA	3.3C	7.8B
hexyl acetate	21ab	17bB	22aA	9.8C	21AB
hexyl butyrate	12b	4.0cC	17aA	4.0C	13B
hexyl 2-methylbutyrate	7.2a	3.3bB	8.5aA	3.7B	6.8A
Aldehydes					
propanal	6.9b	4.3cC	9.8aA	3.1C	8B
butanal	**176b**	**10cC**	**338aA**	**4.8C**	**224B**
2-methylbutanal	10b	9.7bB	13aA	7.5B	14A
hexanal	**925a**	**574bC**	**972aA**	**344D**	**806B**
2-methyl-2-pentenal	**67b**	**96aA**	**65bB**	**79AB**	**65B**
cis-3-hexenal	**69b**	**209aA**	**61bB**	**212A**	**38B**
trans-2-hexenal	**1497a**	**1188bC**	**1357aB**	**1102C**	**1558A**
octanal	2.7a	2bB	2.7aA	1.7B	2B
2,4 hexadienal 1	**9.8b**	**17aA**	**11bBC**	**13B**	**8.8C**
2,4 hexadienal 2	**19b**	**34aA**	**20bBC**	**25B**	**17C**
decanal	3.4a	1.7bB	3aA	2AB	2.1AB
Acids					
acetic acid	62a	34aA	49aA	28A	31A
butyric acid	21b	3.1cB	44aA	1.2B	35A
hexanoic acid	23ab	13bB	26aA	7.2B	30A
Others					
2-ethylfuran	7.7b	23aA	8bB	17A	6.6B
1-hexene	751b	177cC	938aA	97D	844B
farnesene	33b	nd	48aA	nd	44A
2-propanone	37b	57aA	38bB	43B	37B
2-butanone	11a	9.2bC	15aA	5.8D	11B
3-octanone	3.2b	2.2bB	4.7aA	4.2A	2.5B
6-methyl-5-hepten-2-one	8.2b	6.9bC	11aA	4.7D	8.2B

a-d letters following means represent significant difference only between 1-MCP application of samples exposed to light, while A-D letters indicate significant difference between samples kept directly in storage or exposed to light after harvest (1-MCP treated and control) ($p \leq 0.05$).

Table 2. Panelist and reference odour descriptors of the principal aroma compounds identified in 'Ildrød Pigeon' based on NIF (Nasal Impact Frequency) value.

PRINCIPAL COMPOUNDS	R.T.	NIF	PANELISTS DESCRIPTION	References [a]
BUTANAL	3.04	4	green, solvent like, apple like and fresh	pungent odour
ETHYL ACETATE	3.15	4		pleasant, ethereal-fruity, brandy-like odour, pineapple
METHYL 2-METHYL BUTYRATE	5.28	5	green, apple like, sweet, chewing gum, candies like and pleasant	sweet, fruity, apple-like
HEXANAL	7.84	5	green, leafy, green apple, apple and tree like	fatty, green, grassy, powerful, penetrating characteristic fruity
2-METHYL 2-PENTENAL	10.79	4	fresh, green like, leaves like, wet soil like, unpleasant	powerful, grassy-green, slightly fruity odour
***cis*-3-HEXENAL**	11.04	4		grassy, green, herbaceous, leaf, sweet
***trans*-2-HEXENAL**	14.13	6	sweet, cinnamon, almond, marcepan, nail polish, chemical like and fresh apples	sweet, fragrant, almond, fruity green, leafy, apple, plum, vegetable
BUTYL BUTYRATE	14.33	6		fresh, apples
2,4-HEXADIENAL 1/2	18.79/ 18.88	5/5	mushrooms, wood like, orange like and strong chemical.	fresh, green, floral, citrus odour.
1-HEPTANOL	20.15	3	fried potatoes, fermented and green.	unripe, green, earthy, oily
6-METHYL-5-HEPTEN-2-OL	20.29	3		flowery, green, mushroom-like

[a] Plotto et al. (2000), Burdock (2005), Komthong et al.(2007)

Table 4. Means (n=10) of aroma compounds production in the apples exposed postharvest to the sunlight dependent on 1-MCP and bagging treatments (only volatile compounds presented, which reacted significantly on bagging practice as the main factor).

	late 1-MCP		early 1-MCP		control	
Bagging	B	NB	B	NB	B	NB
Treatments	III	VI	I	IV	II	V
	RELATIVE AREA x 10^3					
Ethylene ($\mu L \cdot L^{-1}$)	1230ab	880b	236c	106c	1468a	1259ab
Alcohols						
2-butanol	13.0d	17.9c	11.4d	18.2cb	20.5b	23.4a
propanol	167.1c	217.4c	26.4d	18.5d	349.5b	468.6a
2-methyl -1-propanol	184.6c	275.7b	58.7d	70.6d	237.6b	385.9a
3-octanol	24.0c	43.7b	3.28d	nd	39.6b	68.86a
6-methyl-5-hepten-2-ol	7.5c	14.5b	2.1c	1.9c	16.0a	22.6a
2-ethyl- 1-hexanol	3.7b	14.2a	4.2b	3.8b	4.0b	14.7a
1-octanol	4.5cd	8.7b	1.8d	2.0cd	6.2bc	13.8a
Esters						
ethyl propionate	2.2b	3.0b	nd	nd	3.4b	5.4a
ethyl butyrate	4.6bc	6.4b	0.7c	nd	7.1b	13.2a
butyl butyrate	23.0bc	22.9bc	4.8c	nd	35.6b	57.9a
butyl 2-methyl butyrate	7.6cd	10.2bc	5.6de	4.2e	11.8b	16.4a
hexyl acetate	16.6bc	24.2a	13.04c	19.9ab	20.8ab	23.5a
hexyl 2-methyl butyrate	3.8b	9.6a	3.5b	1.9b	7.3ab	9.8a
Aldehydes						
propanal	5.8cd	8.0bc	3.8d	4.8d	8.2b	11.3a
butanal	160.3c	189.2bc	15.4d	5.3d	258.2b	417.2a
hexanal	716.4bc	1112.4a	559.1c	587.2c	920.7ab	1024.1a
octanal	2.1b	3.3a	2.3b	1.8b	2.2b	3.2a
decanal	1.9b	4.5a	2.0b	1.3b	2.1b	3.8a
Others						
acetic acid	30.9c	92.7a	22.7c	44.8bc	27.2c	85.5ab
farnesene	18.4c	44.8ab	nd	nd	28.7c	69.1a
2-propanone	42.5c	32.5d	60.0a	55.2ab	48.6bc	28.2d
3-octanone	2.7b	3.9b	1.8c	4.0ab	3.6b	5.9a

a-e letters following means represent significant difference as an effect of interaction between 1-MCP application and bagging practice of ($p \leq 0.05$).
nd - not detected

ADDITIONAL PAPER BY AUTHOR

PAPER IV

Comprehensive analysis of chromatographic data by using PARAFAC2 and PCA

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Comprehensive analysis of chromatographic data by using PARAFAC2 and principal components analysis

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ABSTRACT

The most straightforward method to analyze an obtained GC–MS dataset is to integrate those peaks that can be identified by their MS profile and to perform a Principal Component Analysis (PCA). This procedure has some important drawbacks, like baseline drifts being scarcely considered or the fact that integration boundaries are not always well defined (long tails, co-eluted peaks, etc.). To improve the methodology, and therefore, the chromatographic data analysis, this work proposes the modeling of the raw dataset by using PARAFAC2 algorithm in selected areas of the GC profile and using the obtained well-resolved chromatographic profiles to develop a further PCA model. With this working method, not only the problems arising from instrumental artifacts are overcome, but also the detection of new analytes is achieved as well as better understanding of the studied dataset is obtained. As a positive consequence of using the proposed working method human time and work are saved. To exemplify this methodology the aroma profile of 36 apples being ripened were studied. The benefits of the proposed methodology (PARAFAC2 + PCA) are shown in a practitioner perspective, being able to extrapolate the conclusions obtained here to other hyphenated chromatographic datasets.

1. Introduction

1.1. Classical chromatographic data analysis

When one wants to face the analysis of a chromatographic dataset, the first attempt is to integrate the peaks to obtain the area (or sometimes height) for each individual peak. Despite the fact that nowadays chromatographic systems have a high reproducibility and they are often coupled to very powerful detectors (e.g. Time of Flight mass spectrometry detector; TOF-MS), there are still problems that arise from the common sources of variability of such systems. This variability is mainly promoted in the different parts of the global chromatographic experiment (chromatographic device, detector and/or experimental conditions) [1], which are reflected in the signal and, therefore, causing problems directly linked to the final purposes of chromatography (to achieve a perfect separation and individual detection of the different analytes). The problems can be listed according to the effect they cause in the signal, being the methods to avoid/solve/model them as milestones of chromatography:

(a) Baseline drifts: Considering classification purposes by using the so-called fingerprint of each sample (the chromatogram itself), non-reproducible baseline drifts may be a big issue, generating loss of robustness and misinformation in the classification. Some methods to correct these drifts have been reported in the literature [2,3]. However, in complex systems they present several drawbacks that make their use tedious.
(b) Peak shift between samples: In the routine analysis, when already programmed routines are used with fixed windows, the fact that the peaks may change their elution time may generate an important source of error. This promotes that the researcher has to check for the correct definition of the window in the software.
(c) Low signal-to-noise ratios: This issue plays a special role when new compounds are to be found, or when the analyte in consideration is in low concentration. In this aspect, the quality of the detector and its limit of detection is usually improved by increasing its capability (additional columns or better detection devices, like TOF in mass detectors [4–6]). Dealing with GC–MS

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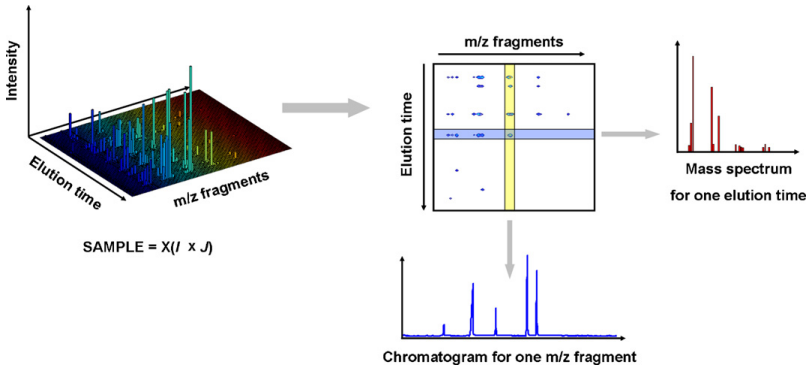

Fig. 1. Visualization of a chromatographic landscape for one sample.

devices, one of the most common alternatives is to seek for specific ions. But this choice can be tedious and sometimes not so evident.

(d) Overlapping/co-elution: It can be stated that this is one of the main worries to tackle in chromatographic datasets. To solve this problem, two alternatives can be selected [7]: try to improve the classical chromatographic parameters (mobile phase composition and steepness of gradients, temperature programs, etc.) to achieve a perfect separation of the co-eluted peaks, or resolve each chromatographic run into the contribution of the different components, by decomposing the original signal in a sum of different profiles by means of modelling [8]. Obviously, these two alternatives are not problem-free. The first one is time consuming and it is very common to find that other co-eluted episodes still remain after re-programming the methods. Meanwhile, the success of the second choice is directly linked to the selective nature of the elution profiles. If one peak is totally embedded (i.e. located just below another peak so that one profile is simply a different scale of the other), peak fitting and modelling becomes problematic [8]. There is a third alternative, which is to seek for specific spectral channels. This is very useful when the detector is a mass spectrometer. Sometimes, however, two co-eluted components may have very similar mass spectra and, therefore, finding specific ions will not be an easy task.

1.2. Analysis by using hyphenated chromatographic devices and multi-way techniques

All these problems make the chromatographic analysis tedious and sometimes there are not simple solutions for any specific peak/co-elution problem. Nevertheless, hyphenated chromatography has widely demonstrated its usefulness for the detection, quantification and/or identification of compounds, becoming one of the most important developments in different fields of chemistry in the last 40 years [9]. The reliability of the coupled detectors (that offer the possibility of obtaining rich spectral information in each elution time) joined with the separation power of the different high resolution chromatographic methods has changed many routines in analytical chemistry, in such a way that nowadays almost all reference methods for the determination and quantification of analytes in complex matrices, refer to a chromatographic routine coupled with, for example, a mass detector [10].

By using hyphenated techniques, one sample can be visualized as a chromatographic landscape (matrix **X** in Fig. 1) in which there is one spectrum for each elution time and vice-versa (Fig. 1).

With this perspective several curve resolution techniques have been proposed for peak purity and resolution of co-elution problems [11]. The final purpose of all of these is to achieve the pure chromatographic and spectral profiles when problems like co-elution appear and considering that resolution techniques do not assume any empirical function (e.g. Gaussian or Lorentzian functions) to model the chromatographic peaks [11–13]. Nevertheless, as Manne pointed out in his theorems [14], re-written by de Juan and Tauler [11] this resolution would not be possible if the chromatographic profile of a minor component is totally embedded in the peak of a major component and if not being totally embedded their spectral pattern is highly similar (mass spectrum of isomeric compounds) [15].

One way of solving these drawbacks is to study several chromatographic runs (i.e. similar type of samples involved in an experiment) together [16], since each new sample may offer selective information for the resolution. With this scenario, two main sample arrangements are usually defined in the literature. The first one is to create augmented data matrices (i.e. multi-set structures) for further application of an extended version of multivariate curve resolution (MCR) [11,13,17–19]. Another possibility is the arrangement of the different samples as a three-dimensional array, $\underline{\mathbf{X}}$ ($I \times J \times K$) were I and J refers to the elution profile and the spectral channel, respectively and K represents the samples (Fig. 2). This new structure of the dataset can be studied by using a family of resolution models called three-way methods [10,20]. The main purpose of the three-way methods can be extrapolated from the bi-dimensional curve resolution methods. That is, to obtain the pure elution as well as the pure spectral profiles for each component. But now, there is "additional" information. That is, the relative influence of each component in each sample; i.e. the concentrations.

Among three-way methods, Parallel Factor Analysis 2 (PARAFAC2) [21,22] has specially demonstrated its usefulness in chromatographic datasets [23]. Several important features of PARAFAC2 deserve to be recaptured. Firstly, PARAFAC2 is able to quantify and identify the chromatograms and the spectra of pure analytes in non-specific signals [1], by profiting the fact that each analyte has a unique spectral signature. The different analytes can be resolved, then, because of their differences in both elution and spectral direction. Secondly, an important feature is that the mathematical decomposition is not depending on the initial estimates and always reaches a unique solution. However, unique solutions may not correspond to 'real chemical solutions' if the rank is wrongly estimated (i.e. if the right number of components to perform the PARAFAC2 model has not been determined). These two features are also common to other three-way methods, like

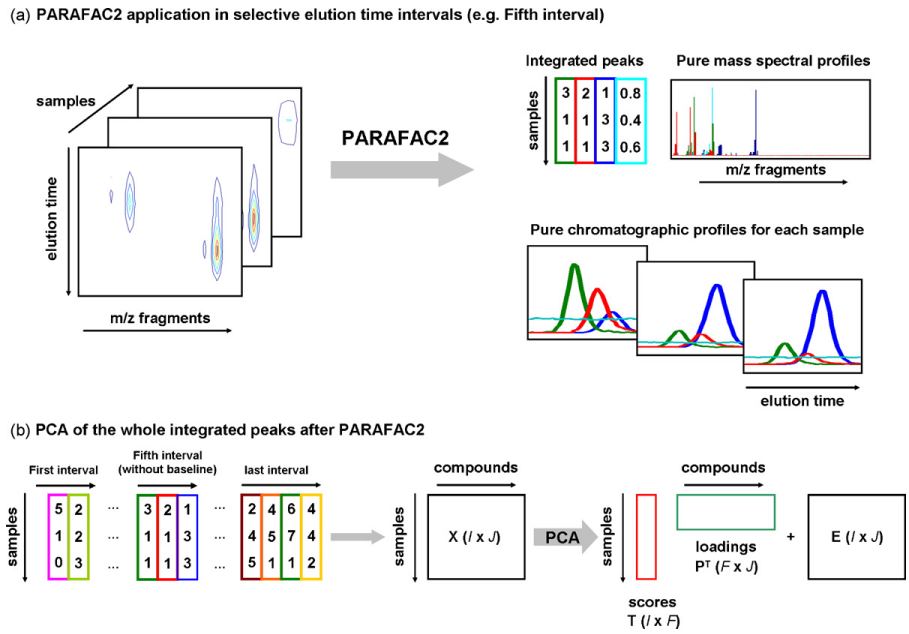

Fig. 2. Working methodology proposed. (a) PARAFAC2 model in selective areas of the chromatographic profile and (b) application of PCA to the solved chromatographic profiles.

PARAFAC. Nevertheless, the most important feature of PARAFAC2 compared to PARAFAC, is that PARAFAC2 does not assume that the shape or length of the elution profile of an analyte is the same in each sample [1,23]. PARAFAC model assumes that the elution time for each analyte does not change sample to sample. Nevertheless, changes in elution time from run to run are commonly found in chromatography [10]. PARAFAC2, on the contrary, assumes that the spectral and sample profiles, together with the cross-product of the elution profile are invariant in every experiment [10]. Therefore, PARAFAC2 calculates an individual elution profile for each sample.

One of the main drawbacks of all factor analysis methods and, specially, three-way methods is that they can only be applied in local areas of the chromatographic profiles where the sources of variability (i.e. analytes and also physical variations like baseline drifts) do not exceed a determined number. This number depends on the quality of the dataset in the selected interval as well as on the levels of co-elution of the different analytes. Nevertheless, nowadays the capability of Personal Computers in computing time makes this problem ineligible, and, therefore, a complete dataset can be studied by dividing it in different blocks and applying individual PARAFAC2 models [23–25].

With this background, the objective of this work is two-fold: firstly, to demonstrate that the combination of dividing a chromatographic dataset into different parts and applying individual analysis of PARAFAC2 is a powerful working methodology, not only to overcome the problems usually encountered in a chromatographic dataset, but also to check to what extent PARAFAC2 can be useful modeling of artifacts (like co-elution and baseline drifts) and detection of new and/or unexpected analytes in the dataset. The second one is also to demonstrate that the combination of the final results obtained with PARAFAC2 and PCA analysis helps to extract relevant information and features in hyphenated chromatographic dataset in an efficient manner.

1.3. Effects of different length of ripening time on aroma profile of Malus domestica apples

To illustrate the benefits of the proposed working methodology, this work deals with problems encountered in a GC–MS aroma profile dataset of 36 apples being ripened in different intervals of time. The combination of PARAFAC2 and PCA demonstrates the capability to solve the main issues concerning common chromatographic artifacts (co-elution of peaks, elution time shifts, baseline drifts, etc.) in the dataset as well as classification of apples according to their aroma profile. In that sense, the problems arising from the instrumental artifacts are overcome. Moreover, the classification of the different ripening times and the detection of new analytes are achieved.

To establish the appropriate length of room temperature exposure (ripening time) after cold storage to achieve full aroma profile of *M. domestica* apples is an important issue to assure that the product is sold in the markets in the proper time with the proper quality. One way to study aroma profile changes is to store a representative amount of apples at room temperature and analyze samples after different storage times by gas chromatography–mass spectrometry (GC–MS) [26,27].

The most straightforward method to analyze the obtained GC–MS dataset is to integrate the major peaks (the ones that can be identified by their MS profile) and to perform a Principal Component Analysis (PCA). This alternative presents some of the drawbacks discussed above, the others being time consuming.

2. Data description

2.1. Material

36 apples of Ildrød Pigeon variety (*M. domestica*) were collected from Pometet, Copenhagen University's experimental orchard.

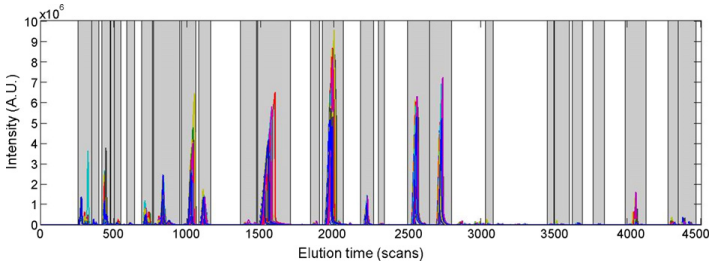

Fig. 3. Chromatographic profile obtained for the 36 samples and its division into 25 parts for further analysis with PARAFAC2. The plot depicts the sum of all the mass fragments for each elution time. The elution time for each interval is defined in Table 1.

After harvesting they were submitted to cold storage for 60 days. After the storage period, they were exposed to room temperature (ripening period) for 5, 8 and 15 days and analyzed (12 apples for each ripening period).

2.2. GC–MS aroma profile

Apple juice was pressed from each individual apple using a commercial juice extractor. The aroma compounds were determined using dynamic headspace sampling from 10 ml of juice (50 ppm of 4-methyl-1-pentanol added as internal standard) with nitrogen as purge gas (70 ml/min for 20 min). Aroma compounds were trapped on traps containing 200 mg Tenax TA. The traps were thermally desorbed using a PerkinElmer ATD 400 and analyzed on a Hewlett Packard G1800A GC/MS system including a DBWax column (30 m × 0.25 mm × 0.25 μm). The column flow rate was 1.0 ml/min using helium as carrier gas. The column temperature program was: 10 min at 45 °C, from 45 to 240 °C at 6 °C/min and finally 10 min at 240 °C. The MS was operating in the electron ionization mode at 70 eV and mass-to-charge ratios between 15 and 300 were scanned (in full scan mode).

2.3. Software

PARAFAC2 and PCA analyses were performed using PLS-Toolbox (PLS-Toolbox v 5.3, *Eigenvector Research Inc. USA*) working under MATLAB® software (*The Mathworks, Inc. USA*).

3. Working methodology

3.1. PARAFAC2 in local intervals of the chromatographic profile

Once the GC–MS aroma profile for the 36 apples was measured, the final structure of the hyphenated chromatographic data was interpreted as a three-way array, $\underline{\mathbf{X}}$ ($I \times J \times K$), in which the three modes accounted for elution time (I scans), spectral domain (J m/z fragments) and samples (K), respectively [23] (Fig. 2).

PARAFAC2 decomposes the three-way array into a set of matrices containing different information. An example is shown in Fig. 2a considering three samples of the interval no. 5. The set of three samples is modeled by PARAFAC2 assuming that four analytes are present in the interval (three chemical compounds plus the baseline influence). The final results are a matrix containing the relative concentration for each one of the analytes modeled in each sample (integrated peaks matrix), one matrix containing the pure mass spectral profiles for each analyte and a set of matrices containing the pure chromatographic profiles for each sample, respectively [28,29]. In the example shown in the Fig. 2a it can be seen how the analyte colored in red is perfectly modeled despite the fact that is totally co-eluted with blue and green analytes, being able to obtain specific information for this analyte.

As it has been pointed out in the previous section, the efficiency of PARAFAC2 (in terms of interpretability and computation time) is increased by working in local intervals of the chromatographic profile wherein the sources of variability (number of chemical components and artifacts like baseline drifts) remain under certain levels [1,10,23,25,30,31]. Therefore, the chromatographic dataset was divided into 25 different parts and each part was analyzed by independent PARAFAC2 models as indicated in Fig. 3. The intervals were selected accordingly to previous knowledge about the analysis of aroma compounds in some varieties of apples [32–34]. The number of peaks in each interval depended on the resolution (co-elution) of the compounds in the chromatogram.

The correct number of factors (chemical and physical components) for each PARAFAC2 model was determined by calculating the explained variance of the model (Eq. (1)) and by visual appearance of the chromatographic and spectral profiles as well as the residuals. The explained variance was calculated as follows:

$$\%\mathrm{VAR} = 100 \times \left(1 - \frac{\mathrm{SSE}}{\mathrm{SSX}}\right) \quad (1)$$

where SSE and SSX account for the sum of the squares of the residuals and the elements in the three-way array, respectively.

To identify the components giving rise to the mass spectral profiles obtained for each PARAFAC2 model, the 'NIST MS Search 2.0' software (*NIST/EPA/NIH Mass Spectral Library, NIST Scientific and Technical Databases, Gaithersburg, MD 20899-8380*) that contains a library containing 190,825 spectra of 163,198 compounds was used. The identification for each mass spectrum obtained by PARAFAC2 was performed by checking the similarity with the spectrum in the database.

3.2. PCA analysis

After obtaining the best PARAFAC2 model for each interval, a general Principal Component Analysis (PCA) was performed by using the final relative areas of the well resolved peaks (and not considering the baseline influence, as indicated in Fig. 2b) to seek for effects of different length of ripening time on aroma profile of *M. domestica* apples.

PCA [35,36] is a variable reduction technique that condenses all the information of the chemical variables, i.e. compound relative areas (usually highly correlated) into a few latent variables or Principal Components (PCs) that contain the uncorrelated information. This is done by decomposing the data matrix $\mathbf{X}$ (I,J) into two sub-matrices as follow (Fig. 2b):

$$\mathbf{X} = \mathbf{T}\mathbf{P}^{\mathrm{T}} + \mathbf{E} \quad (2)$$

where $\mathbf{T}$ (I,F) and $\mathbf{P}^{\mathrm{T}}$ (F,J) are the so called scores and loadings matrices, depending of the number of PCs selected (F). The scores matrix compiles all the useful information about the samples; whereas

Table 1
Classification of the obtained chromatographic profiles by PARAFAC2 in all the intervals selected accordingly with their mass spectrum.

	Interval 1	Interval 2	Interval 3	Interval 4	Interval 5
Elution time range (scans)	'255–351'	'350–400'	'420–480'	'475–505'	'505–550'
No. components	5	3	3	4	4
Explained variance (%)	99.86	98.80	99.98	99.46	99.83
Component 1	2-Propanamine (1)[a]	Propanal (6)	Butanal (8)	Baseline	Benzene (12)
Component 2	Heptane (2)	2-Propanone (7)	Ethyl acetate (9)	2-Methyl-butanal (10)	2-Propanol (13)
Component 3	Ethylether (3)	Baseline	Baseline	Pentanal (11)	Ethanol (14)
Component 4	Dimethylpropanol (4)	–	–	Interferent (Fig. 5a)	Baseline
Component 5	Pentane (5)	–	–	–	–
	Interval 6	**Interval 7**	**Interval 8**	**Interval 9**	**Interval 10**
Elution time range (scans)	'590–640'	'690–770'	'764–950'	'960–1060'	'1080–1160'
No. components	2	4	3	1	1
Explained variance (%)	97.82	99.89	99.72	99.87	99.52
Component 1	Propyl acetate (15)	Methyl 2-methylbutanoate (16)	Toluene (NI)	Hexanal (20)	2-Butanol (21)
Component 2	Baseline	Baseline	Unclassified	–	–
Component 3	–	Propanenitrile (17)	1-Propanol (19)	–	–
Component 4	–	Trichloromethane (18)	–	–	–
Component 5	–	–	–	–	–
	Interval 11	**Interval 12**	**Interval 13**	**Interval 14**	**Interval 15**
Elution time range (scans)	'1360–1480'	'1470–1700'	'1840–1900'	'1920–2060'	'2180–2270'
No. components	2	1	3	1	2
Explained variance (%)	95.33	99.96	99.34	93.53	99.92
Component 1	2-Methyl-2-pentenal (NI)	1-Butanol (22)	Baseline	Heptene (25)	1-Pentanol (26)
Component 2	Interferent		(E)-2-Hexenal (23)	–	Baseline
Component 3	–		methanesulfonic acid (24)	–	–
Component 4	–		–	–	–
Component 5	–		–	–	–
	Interval 16	**Interval 17**	**Interval 18**	**Interval 19**	**Interval 20**
Elution time range (scans)	'2300–2340'	'2500–2650'	'2650–2800'	'3980–4120'	'4270–4340'
No. components	2	1	1	3	3
Explained variance (%)	99.43	99.96	99.95	99.76	99.64
Component 1	Hexyl acetate (27)	4-Methyl-1-pentanol (IS)	1-Hexanol (29)	Farnesene (30)	Baseline
Component 2	Baseline	–	–	Baseline	Hexanoic acid (31)
Component 3	–	–	–	2,3,3-Trimethyl-1,4-pentadiene (NI)	2-(2-Butoxyethoxy)-ethyl acetate (32)
Component 4	–	–	–	–	2-Hexenyl acetate (33)
Component 5	–	–	–	–	–
	Interval 21	**Interval 22**	**Interval 23**	**Interval 24**	**Interval 25**
Elution time range (scans)	'3760–3840'	'3620–3690'	'3450–3495'	'3500–3600'	'3030–3080'
No. components	4	2	2	3	2
Explained variance (%)	98.59	92.30	99.03	99.42	99.62
Component 1	Baseline	Butanoic acid (37)	1-Octanol (38)	Baseline	Acetic acid (41)
Component 2	Cyclohexyl isothiocyanate (34)	Baseline	Baseline	1,3,5-Trimethylcyclohexane (39)	Baseline
Component 3	3-Methylbutanoic acid (35)	–	–	Ethylcyclohexane (40)	–
Component 4	2-Methylbutanoic acid (36)	–	–	–	–

[a] The number between brackets is the numeric index label for PCA analysis and Fig. 6.

the loadings matrix contains the corresponding connexion with the variables (chemical compounds). The super-index in the loadings matrix denotes the transpose of the matrix. The information not explained in the scores and loadings matrices is collected in the residuals matrix **E** (I,J), that contains the information not relevant for explaining the variance of the samples and variables (i.e. the noise).

4. Results

4.1. Performance of the local PARAFAC2 models and identification of the components

Once the whole dataset was divided according to Fig. 3, consecutive PARAFAC2 models were run. The final results obtained

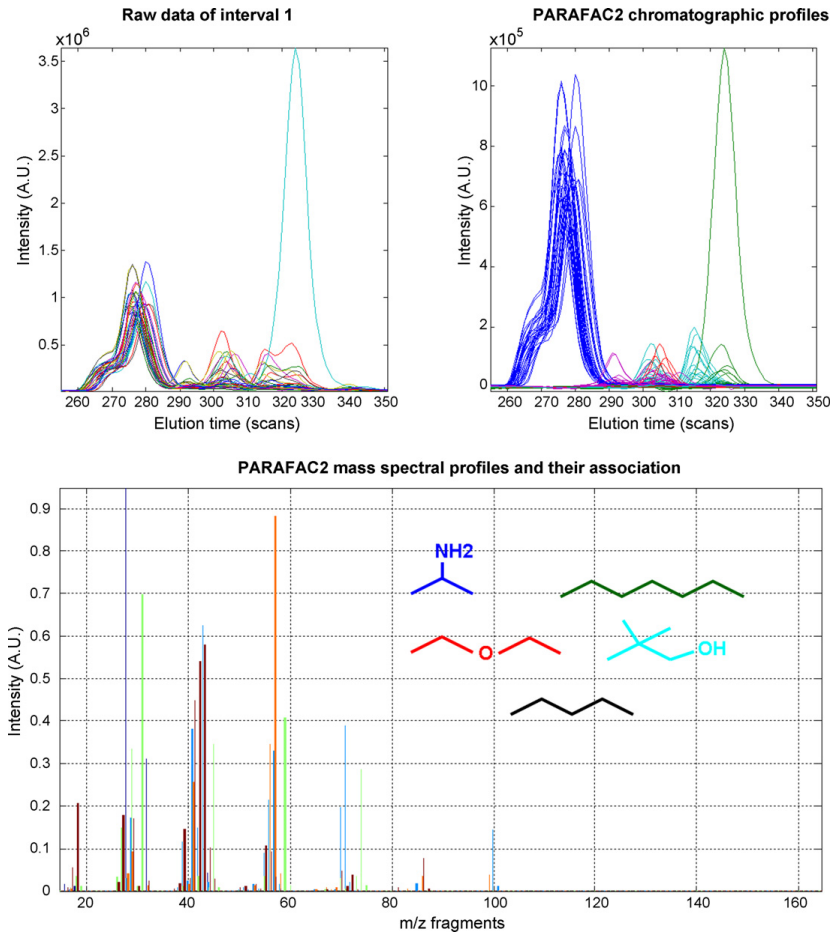

Fig. 4. Example of the results obtained by using PARAFAC2 in the first interval selected. Upper part: left plot is the raw data; whereas right plot is the chromatographic profiles obtained. Bottom part: the mass spectral profiles obtained and their corresponding component (blue, 2-propanamine; green, heptane; red, ethyleter; cyan, dymethylpropanol; black, pentane). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

with PARAFAC2 are shown in Table 1. The explained variance in the entire valid final models (a total of 25, one model for each interval) was higher that 95%, indicating a correct fitting between the raw data and the PARAFAC2 results (see Table 1). A total of 41 analytes were correctly modeled, assessing their belonging to the mass spectral profiles by using the database mentioned in Section 3.1 (Fig. 4). It is also noteworthy that the baseline effects, as well as some interfering effects, were also modeled in 16 of the 25 areas studied.

One of the arguable points in this methodology is the difficulty in dividing the chromatogram in different individual intervals. Sometimes it is not easy to select intervals in where there is noise or the different elution time shifts between samples are large enough to misclassify different peaks. Nevertheless, since no *a priori* knowledge is needed, PARAFAC2 is also able to model interferences that come from closely eluting peaks (Fig. 5a), being able to eliminate this interference once the model has been obtained, making the task of defining the correct window of analysis much more flexible.

PARAFAC2 was also able to model almost embedded peaks (Fig. 5b and c). This result is important in order to assure further qualitative and quantitative analysis of the obtained peaks, making the task of finding selective *m*/*z* ions unnecessary. Also its capability of modeling low signal-to-noise peaks is remarkable (Fig. 5d), being able to differentiate between isomeric compounds in low concentrations (Table 1, interval 21).

4.2. PCA with integrated peaks

As indicated in Fig. 2, PARAFAC2 also calculated the area for each analyte in each sample. This area has to be understood as the sum of the intensities of the resolved chromatographic profile obtained with PARAFAC2 for each analyte in each sample. The PCA analysis was performed with the relative after sample to sample correction with the internal standard area (without the interferences and baseline effects) and autoscaling the data. As it can be observed in the scores plot of Fig. 6, there was separation between different ripening times. This separation was basically explained by the

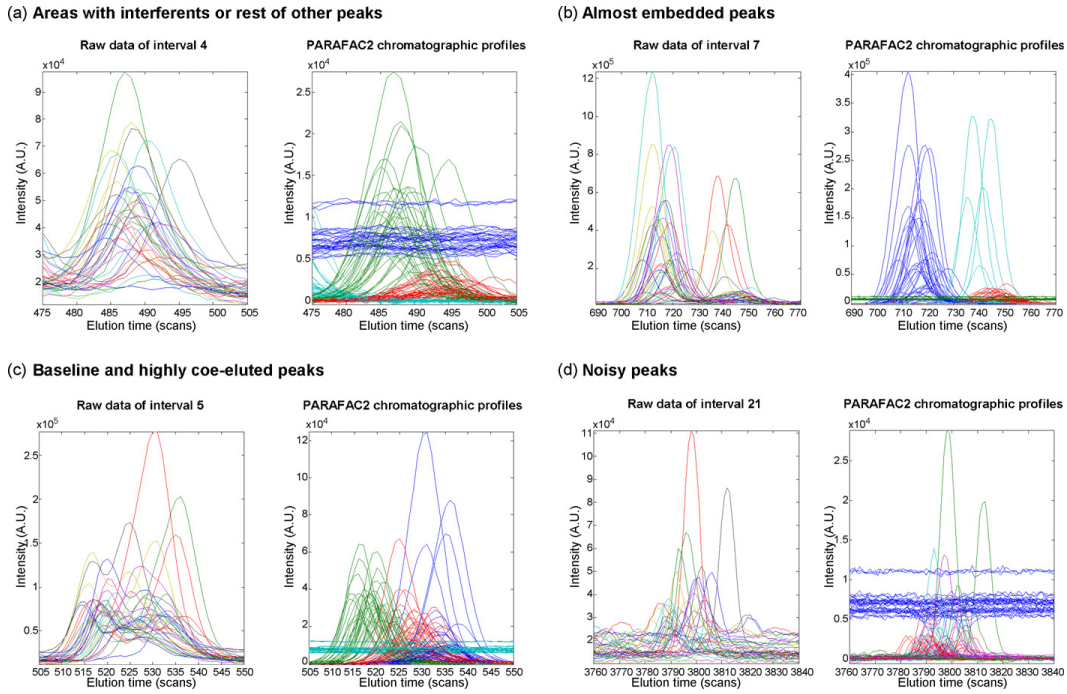

Fig. 5. PARAFAC2 results obtained in several intervals denoting different chromatographic problems. In all the cases, the left plot corresponds to the raw data; whereas the right plot corresponds to the resolved chromatographic profiles obtained by PARAFAC2.

first and the second principal components (the model with two PCs explained more than 60% of the variance). In that sense, it can be said that the first PC basically explained the different concentration of analytes in apples being ripened 15 days (all the red triangles of the scores plot are located in the positive part of the first PC in Fig. 6; whereas the distribution of the other two classes are in between negative and positive part of PC1, giving a slight indication of evolution in the concentration between the three ripening time. On the other hand, PC2 clearly showed intrinsic differences between ripening times. In the same way as in PC1, all the apples ripened during 15 days (red triangle in the scores plot of Fig. 6) were located in the positive part of the PC2 scores. These observations (and more precisely, the diagonal direction of the plane formed by PC1 and PC2) indicated small changes in aroma profile from day three to eight and bigger change to 15 days, indicating that apples have post storage self life.

PCA model also demonstrated that the period at room temperature after cold storage has an important effect on aroma profile of Ildrød Pigeon apples. According to the loadings plot, almost all variables were located in the positive part of the first PC. This reaffirmed the observation in the scores plot that first PC basically explained the different concentration between ripening times.

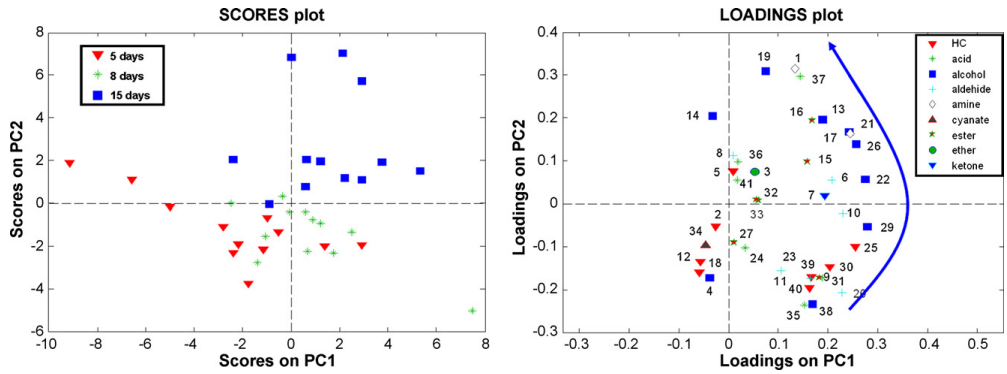

Fig. 6. PCA analysis of the peaks obtained with the PARAFAC2 analysis. Left: Scores plot denoting the ripening time. Right: loadings plot.

Alcohol concentration and distribution plays an important role in the development of aroma profile, being considered as precursors of esters [37]. There is a remarkable trend in the alcoholic profile (blue arrow in the loadings plot of Fig. 6) obtained in this work, since the heaviest alcohols (octanol; pentanol) were located in the negative part of PC2 and the lighter (ethanol; butanol; propanol) were located in the positive part of PC2.

It has to be enhanced that most of the aldehydes and hydrocarbons were located in the negative part of PC2. They remained constant during the first 8 days at room temperature, and then showed a significant decrease in apples ripened for 15 days. Among them, farnesene is responsible for "green odour" of some apple varieties [38]. It was clearly associated to the apples ripened for 5 and 8 days, decreasing in importance in the apples ripened for 15 days. These trends of loosing hydrocarbons and aldehydes with ripening time could be related to intensity of respiration associated with the metabolic processes taking place in the apple after harvesting and during room temperature storage.

Esters which are associated with the fruity perceptions [38–41], were represented in the current study by 4 compounds (see Table 1 and Fig. 6 legend). Hexylacetate (number 27 in Table 1 and Fig. 6) is usually considered to be one of the most important esters giving fruity and green odour in some varieties of apples at harvest [42]. The results found in the analysis denoted a decreasing effect on the synthesis of hexylacetate during ripening. Highest amounts of this component were found in samples analyzed after 5 and 8 days. On the contrary, higher concentrations after 15 days of ripening were found of methyl-2-methylbutanoate and propylacetate. For comparison, Echeverria et al. [42,43] also found higher levels of propyl acetate in the latest maturity stages in 'Fuji' aples, while methyl 2-methylbutyrate and 2-hexenyl acetate were not found. Also in accordance with the present study, levels of ethyl acetate were found to be highest in the more unripe apples by Echeverria et al. [42,43]. The same tendency was found for ethyl 2-methylbutanoate which was not found in the present study.

5. Conclusions

The combination of individual PARAFAC2 models in selected areas of the chromatographic profile and PCA has clearly demonstrated to be a very useful strategy in chromatography to obtain rich information to define the addressed problem. In that sense, problems like co-elution, as well as retention time shifts and baseline drifts have been overcome by PARAFAC2. Moreover, more than 41 compounds have been considered for further analysis with PCA. After the appropriate preprocessing of the data, data treatment was performed by Principal Component Analysis (PCA). This denoted that, in fact, important changes in aroma compounds were observed during exposure to room temperature after storage.

But, maybe, the most important conclusion that has to be extrapolated from the presented results is that with this working methodology a step forward in the comprehensive analysis of chromatographic data has been taken. Apart from the abovementioned benefits (detection of minor components, baseline modeling, resolution of co-eluting peaks, further classification), it has to be highlighted that human time has been saved (e.g. no need for manual integration or curve deconvolution of co-eluted peaks) transforming this time into computing time.

In that sense, we encourage the readers to use multi-way (e.g. PARAFAC or PARAFAC2) or multi-set (MCR) methodologies when they are needed. And also it has to be remarked these methods are not exclusive from GC–MS datasets, but they can be applied in any hyphenated separation techniques (e.g. HPLC-UV-DAD and LC–MS) where peak shift and peak-shape changes and baseline contributions are often even bigger issues [23].

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