Triterpenes and Phenolic Compounds in Apple Fruit (*Malus domestica* Borkh.)

Variation due to Cultivar, Sun Exposure, Rootstock, Harvest Maturity, Bruising, Fungi Inoculation, Ozone Treatment and Storage Conditions.

Yanrong Lv
Faculty of Landscape Architecture, Horticulture and Crop Production Science
Department of Plant Breeding
Alnarp

Doctoral Thesis
Swedish University of Agricultural Sciences
Alnarp 2016

Acta Universitatis agriculturae Sueciae
2016:5

Cover: Apple tree (photo: Marie E. Olsson), Molecular structure (made by Yanrong Lv)

ISSN 1652-6880
ISBN (print version) 978-91-576-8512-4
ISBN (electronic version) 978-91-576-8513-1

Print: SLU Service/Repro, Alnarp 2016

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Abstract

Apple (*Malus* × *domestica* Borkh.), a popular and widely cultivated fruit world-wide, contains bioactive triterpenes and phenolic compounds with potentially valuable pharmacological functions. This thesis investigated the effects of pre-harvest and postharvest factors on concentrations of these bioactive compounds in apples. It also studied the effect of ozone treatment, before or during storage, combined with cold storage on triterpene and phenolic compound concentrations in apples and the antifungal activity of triterpene-enriched crude extract.

Concentrations of two major triterpenes, oleanolic acid and ursolic acid, in apple peel varied significantly between cultivars, with the late-ripening cultivar 'Gloster' having higher concentrations than the earlier ripening 'Discovery' and 'Aroma'. The concentrations were higher in peel from the shaded side than from the sun-exposed side of both 'Discovery' and 'Gloster' apples. Harvest time and storage methods had minor effects on the concentrations, although some between-cultivar variation was observed. Inoculation with *Penicillium expansum* decreased oleanolic acid concentration in 'Discovery' and 'Gloster' at harvest in one study year and decreased ursolic acid concentration in 'Aroma' after cold storage. Oleanolic acid and ursolic acid concentrations in apple peel showed non-consistent changes in different cultivars after bruising. On four of six harvest occasions during a two-year study of 'Aroma', the concentrations were higher in peel of apples from rootstock 'MM106' than from 'M9'. Total polyphenolics concentration was higher in 'Amorosa' peel than in 'Santana' peel at harvest and after four months of storage.

Ursolic acid concentration in apple peel was almost unaffected by ozone treatment, but oleanolic acid concentration showed differing responses to varying ozone application. The concentrations of different bioactive compounds both increased and decreased after ozone treatment, but the changes were within the range of fluctuations observed in untreated apples during storage. Low-dose ozone treatment (0.5 ppm gaseous ozone one hour per day) during four months of cold storage did not affect total polyphenolics in 'Amorosa' and 'Santana' flesh, but increased total polyphenolics in 'Amorosa' peel and decreased them in 'Santana'. Short-term ozone treatment (2.5 ppm gaseous ozone and ozonated water, alone or combined) before one month of cold storage reduced total polyphenolics content in apple peel, while inconsistent responses were observed in apple flesh.

Triterpene-enriched water significantly inhibited mycelial growth of all pathogens studied, but inhibited conidia production differently in each pathogen.

Keywords: anthocyanin, antifungal activity, 'MM106', 'M9', flavonols, HPLC, inoculation, oleanolic acid, polyphenolics, ursolic acid

Author's address: Yanrong Lv, Department of Plant Breeding,
Swedish University of Agricultural Sciences, P.O. Box 101, SE 23053 Alnarp, Sweden
E-mail: Yanrong.Lv@ slu.se

Dedication

To my parents and sister for their unconditional love, support and understanding.

Wisdom equals knowledge plus courage. You have to not only know what to do and when to do it, but you have to also be brave enough to follow through.

Jarod Kintz

Contents

List of Publications

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

I Lv, Y., Tahir, I.I. and Olsson, M.E. (2015). Factors affecting the content of the ursolic and oleanolic acid in apple peel: influence of cultivars, sun exposure, storage conditions, bruising and *Penicillium expansum* infection. *Journal of the Science of Food and Agriculture*, DOI: 10.1002/jsfa.7332.

II Lv, Y., Tahir, I.I. and Olsson, M.E. Ursolic and oleanolic acid in 'Aroma' apple peel as affected by rootstock, harvest maturity and storage method. *HortScience*. (Submitted)

III Lv, Y., Tahir, I.I. and Olsson, M.E. Effect of ozone application during cold storage on bioactive compounds in apple fruit. (Manuscript)

IV Lv, Y., Tahir, I.I., Garkava-Gustavsson L. and Olsson, M.E. Triterpene-enriched extracts from apple peel inhibit the growth of post-harvest pathogens of fruit. (Manuscript).

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The contribution of Yanrong Lv to the papers included in this thesis was as follows:

I Planned the experiment together with the co-authors, performed all experimental work, and did the data analyses, was responsible for interpreting the results together with the co-authors and writing the the manuscript which was finalized with contributions from the co-authors.

II Participated in the planning of the experiment together with the co-authors, performed all experimental work, and did the data analyses, was responsible for interpreting and evaluating the results together with the co-authors and writing the manuscript, with contributions from the co-authors.

III Participated in the planning of the experiment together with the co-authors, performed all experimental work, and did the data analyses, was responsible for interpreting and evaluating the results together with the co-authors and writing the manuscript, with contributions from the co-authors.

IV Planed the experiment together with the co-authors, conducted the experimental work, evaluated and analysed the data together the co-authors, and wrote the manuscript with contributions from the co-authors.

Abbreviations

ANOVA	Analysis of variance
AOS	Active oxygen species
BA	Betulinic acid
CA	Controlled atmosphere
CVD	Cardiovascular disease
DMAPP	Dimethylallyl diphosphate
DVD	Diode array detector
eNOS	Endothelial nitric oxide synthase
FAO	Food and agriculture organization
GLM	General linear model
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HPLC-MS	High-performance liquid chromatography-mass spectroscopy
IPM	Integrated production management
IPP	Isopentenyl diphosphate
MA	Maslinic acid
MEA	Malt extract agar
NO	Nitric oxide
NRCS	Natural resources Conservation Service
OA	Oleanolic acid
RA	Regular atmosphere
RH	Relative humidity
TA	Titratable acidity
TESW	Triterpene-enriched suspended water
TSS	Total soluble solids
UA	Ursolic acid
USDA	United States Department of Agriculture
UV	Ultraviolet

1 Background

1.1 Apple

Apple (*Malus* × *domestica* Borkh.) is one of the most widely consumed fruits in the world. The suitable cultivation area for apple is relatively wide. Apples are produced in most countries of the temperate region and also in some tropical areas at high altitude (Ferree & Warrington, 2003). The majority of the apples produced globally are for domestic fresh use. In some countries, such as the United States of America, Germany and Australia, processed apple products also account for a large market (Ferree & Warrington, 2003). These processed products include slices, dried apple, apple sauce, juice and cider. Apples not only contain multiple nutrients, but also a variety of bioactive compounds (Eberhardt *et al*., 2000; Leontowicz *et al*., 2002). These bioactive compounds have become increasing attractive because of their pharmacological functions (Ikeda *et al*., 2008; Somova *et al*., 2003; Liu, 1995).

1.1.1 History and origin

The domesticated apple belongs to the Rosaceae family and it is the main fruit crop in temperate regions of the world (Velasco *et al*., 2010). The domesticated apple is considered to be an interspecific hybrid complex and is designated *Malus* × *domestica* Borkh. or *M*. *domestica* Borkh (Korban, 1984). It is suggested that Central Asia is the area of greatest diversity and the original source of the domesticated apple (Janick *et al*., 1996). The Tian Shan forest has been identified as the geographical area where apple was first domesticated, based on the considerable intraspecific morphological variability of wild apple populations in this region (Dzhangaliev, 2003; Vavilov, 1926). *Malus sieversii*, the wild apple of Turkestan (Kazakhstan, Kyrgystan, Uzbekistan, Turkmenistan and Tajikistan), and its close relatives are suggested to be the progenitors of *M*. *domestica*. In fact, wild apple domestication can be traced to Almaty (Kazakstan) (Vavilov, 1930). *Malus sieversii* is very diverse and has

all the qualities present in *M. domestica* Borkh (Forsline, 1995; Forsline *et al*., 1994). Recently, chloroplast diversity data suggested that *Malus sylvestris*, the wild apple native to Western Europe, may be the main maternal wild progenitor of domestic apples (Coart, 2006). The early evolution of the apple was complete about 5000-8000 years ago when humans began to occupy the area (Harris *et al*., 2002). In the late Neolithic or early Bronze Age, according to archaeological and molecular data, the seed of the central Asian wild apple was carried from central China to the West by travellers through the great trade route (Silk Road) which passed through India, Persia, Greek, Egypt and the Roman Empire (Ferree & Warrington, 2003; Harris *et al*., 2002). It has been suggested that the Romans spread the domestic apple to Europe and Britain. From the 13th century onwards, apples were grown increasingly widely in Europe (Morgan, 1993). During the 16th and 17th century, the French spread the apple to Canada and, in the same period, the Protestant settlers who left European countries planted apples along the eastern seaboard of America and these later spread to the Midwest and along the Pacific coast (Morgan, 1993).

The cultivated apple is a diploid (2n=2x=34), although some cultivars present as triploids (Pereira-Lorenzo *et al*., 2007) or have even higher somatic numbers of 68 or 85 (Chyi & Weeden, 1984). The delimitation of species within the Malus genus is still vague, because the great diversity, potential for hybridisation, polyploidy and presence of apomixes in the genus (Compbell *et al*., 1991). Janick *et al*. (1996) counted 37 species and Forsline *et al*. (2003) listed 27 primary species. According to the classification of Phipps *et al*. (1990), Harris *et al*. (2002) identified about 55 species. There are 36 species recorded in the USDA, NRCS (2012) Plants database (http://plants.usda.gov). Pereira-Lorenzo *et al*. (2009) indicated the origin and use of 27 primary species classified by Forsline *et al*. (2003), of which 21 species are from Asia (11 located mainly in China), 4 in North America and 2 in Europe. There are more than 10,000 documented cultivars of apple, but only a few major cultivars now dominate global fruit production (Janick, 1996). Great variation is present among cultivars for most traits, such as yield, fruit characteristics, plant size and disease resistance (Troggio *et al*., 2012).

1.1.2 Production

According to the FAO database (2013), global fruit production in 2013 was 610.15 million tons, of which apple production comprised 80.82 million tons, accounting for 13% of total fruit production. Asia had the highest apple production in that year, followed by Europe and then the Americas (Figure 1). As regards different countries, China produced about 39.68 million tons, which accounted for 49% of world production. The Americas, Turkey, Poland and

Italy are also top countries in apple production, with total output ranging from 2.22 to 4.08 million tons. However, Austria has the highest yield, 54 tons per hectare, which is 3.79-fold the yield in Asia.

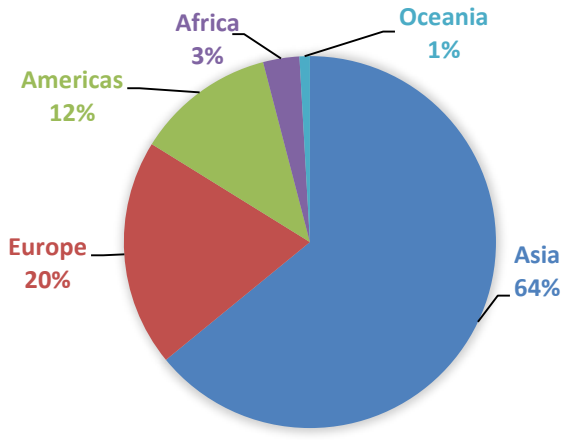


Figure 1. Global apple production distribution in 2013. Source: FAO (2013).

1.2 Health aspects of apple

Apples play a significant role in people's diet in many countries. In numerous epidemiological investigations, apples have been associated with a decreased risk of chronic diseases such as cancer, cardiovascular disease (CVD), asthma and Type II diabetes (Gossé *et al*., 2005; Boyer & Liu, 2004; Feskanich *et al*., 2000; Knekt *et al*., 2000; Knekt *et al*., 2002; Le Marchand *et al*., 2000). Other studies show that apples are a rich source of phytochemicals (Boyer & Liu, 2004; Lee, 2000). Due to their health benefits to humans, phytochemicals have gained much attention in the scientific field. One of the major roles of phytochemicals is antioxidant activity (Boyer & Liu, 2004; Lee, 2000). Almost all of the antioxidant activity in apples is due to phytochemicals, as the vitamin C in apples makes quite a low contribution to the antioxidant activity (Eberhardt *et al*., 2000). The high antioxidant activity of apples has effective functions in inhibiting cancer cell proliferation, decreasing lipid oxidation and lowering cholesterol, which has confirmed their role in potentially reducing the risk of chronic diseases (Boyer & Liu, 2004). In addition to their antioxidant effect, recent studies in humans and animals suggest that intake of apples can positively affect lipid metabolism (Ravn-Haren *et al*., 2013; Nagasako-Akazome *et al*., 2007;), weight management (De Oliveira *et al*., 2003) and

vascular function (Gasper *et al*., 2014). Furthermore, evidence of various mechanisms whereby triterpenoid compounds exert an effect on CVD has been found (Waldbauer *et al*., 2015; Han & Bakovic, 2015). Compared with apple flesh, apple peel shows more potent antioxidant activity and antiproliferative activity (Wolfe & Liu, 2003; Wolfe *et al*., 2003; Eberhardt *et al*., 2000).

1.2.1 Terpenoids and triterpenoids

Terpenoids

The terpenoids (also called isoprenoids or terpenes) are one of the largest families of phytochemicals, including more than 40,000 compounds (Goto *et al*., 2010). They are characterised by their biosynthetic origin from isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP) (Goto *et al*., 2010; Withers *et al*., 2007). According to the five-carbon unit present, terpenoids are classified into further groups: hemiterpenoid (C5), monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenoids (C25), triterpenoids (C30), tetraterpenoids (C40), and polyterpenoids (C>40) (González-Castejón & Rodriguez-Casado, 2011; Muffler *et al.*, 2011).

All organisms produce some terpenoids as part of primary or secondary metabolism (Goto *et al*., 2010; Withers *et al.*, 2007). Most of the bioactive terpenoids have been detected in higher plants and they are widely present in fruits and vegetables (González-Castejón & Rodriguez-Casado, 2011; Muffler *et al*., 2011). During the whole life cycle, the plant can synthesise many different terpenoid types under different environmental conditions (McGarvey & Croteau, 1995). Biologically, the terpenoids have unique functions in the interaction between plants and their environment (Rodríguez *et al*., 2011; Chappell, 1995). For example, cucurbitacin C, a bitter triterpenoid in the leaves of cucumber, is an important parameter in spider mite resistance (Chappell, 1995). Many terpenoids contribute to the quality of agricultural products and some have been used in food and cosmetics (Aharoni *et al*., 2005). In addition, many herbal plants used in folk medicine contain terpenoids (Goto *et al*., 2010; Qi *et al*., 2006; Liu, 1995).

Triterpenoids

Triterpenoids are a large group belonging to the terpenoid compounds. They are synthesised by the cyclisation of squalene, which is the precursor of all steroids and triterpenoid saponins (Phillips *et al*., 2006). The triterpenoids include the triterpenes, including naturally occurring terpenes, and natural degradation compounds such as terpene alcohol, acids, ketones, aldehydes,

esters, epoxides and hydrogenation products (Eggersdofer, 2005). Over 20,000 triterpenoids occur in nature and more than 100 distinct skeletons have been described (Liby *et al*., 2007; Xu *et al*., 2004). Triterpenoids have been found in different plant parts, such as sprouts, leaves, flowers, fruits, seeds, bark, cork and wood (Jäger *et al*., 2009; Muffler *et al*., 2011). These triterpenoids can be classified into different groups including cucurbitanes, cycloartanes, dammaranes, euphanes, friedelanes, holostanes, hopanes, isomalabaricanes, lanostanes, limonoids, lupanes, oleananes, protostanes, sqalenes, tirucallanes, ursanes and miscellaneous compounds (Bishayee, 2011; Setzer & Setzer, 2003). Many studies have identified the pharmacological effects of triterpenoids and their derivatives in preventing different cancers and immune diseases (Bishayee *et al*., 2011; Ríos, 2010; Liby *et al*., 2007).

Oleanolic acid (3β-hydroxy-olean-12-en-28-oic acid, OA) (Figure 2A.) and its isomer, ursolic acid (3β-hydroxy-ursan-12-en-28-oic acid, UA) (Figure 2B) are two triterpenic compounds widely distributed in the plant kingdom and in food products, such as olive oil (Guinda *et al*., 2010; Pérez-Camino & Cert, 1999). They usually exist in the form of free acid or aglycones of triterpenoid saponins (Mahato *et al*., 1988). As for their chemical structure, they only differ in the position of one methyl group. Together with betulinic acid (BA) and maslinic acid (MA), OA and UA are typical representatives of pentacyclic triterpenes and they are abundant in plants (Mazumder *et al*., 2011; Romero *et al*., 2010; Jäger *et al*., 2009).

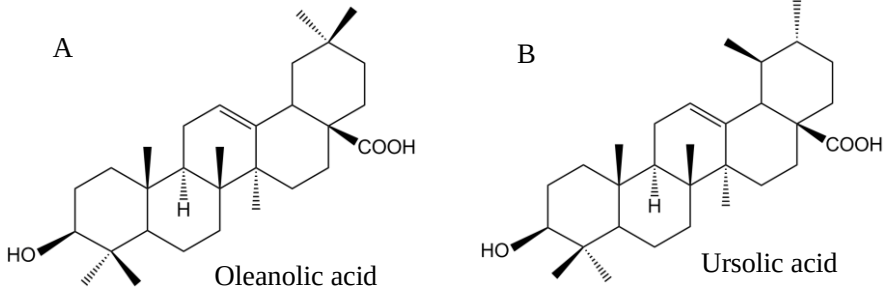


Figure 2. Structure of oleanolic acid (A) and ursolic acid (B).

Oleanolic acid and UA are important compounds of the wax and they are commonly found in the cuticular waxes of fruit and leaves (Koch & Ensikat, 2008; Qi *et al*., 2006; Liu, 1995). Ursolic acid is the constituent found in highest amounts in sweet cherry fruit wax (Peschel *et al*., 2007) and it is also found in cranberry and blueberry (Neto, 2011). Oleanolic acid, which is the second major triterpene in sweet cherry fruit wax (Peschel *et al*., 2007), is the most abundant constituent of grape wax (Radler & Horn, 1965). Both OA and

UA are also found in the fruit of apple (He & Liu, 2007), olive (Guinda *et al*., 2010; Romero *et al*., 2010) and persimmon (Zhou *et al*., 2010). Thirteen triterpenoids have been isolated and identified in the peel of 'Red Delicious' apple (He & Liu, 2007), and some studies report high concentrations of OA and UA in apple peel (Jäger *et al*., 2009; Cefarelli *et al*., 2006).

Health aspects of OA and UA

Oleanolic acid and UA have a wide spectrum of biological activity, and they are especially attractive because of their pharmacological functions including anti-inflammatory (Ikeda *et al*., 2008; Liu, 2005), anti-tumour (Liu, 1995; Young *et al*., 1995; Tokuda *et al*., 1986), hepatoprotective (Saravanan *et al*., 2006; Kim *et al*., 2004; Liu, 1995), anti-hyperlipidemic (Somova *et al*., 2003), antibacterial (Fontanay *et al*., 2008; Liu, 1995), anti-HIV (Kashiwada *et al*., 2000; Kashiwada *et al*., 1998; Xu *et al*., 1996) and anti-diabetic properties (Jang *et al*., 2009; Teodoro *et al*., 2008). Research also shows that UA can inhibit neointima formation in the rat carotid artery injury model, with daily doses of 6 mg/kg body weight for 10 days reducing both the ratio of intimal to medial areas and the degree of stenosis (Pozo *et al*., 2006). It has also been discovered that UA has analgesic effects in mice, an activity that may be related to the anti-inflammatory and antioxidant properties of this compound (Taviano *et al*., 2007). Recent intervention studies in human and animals also indicate that intake of apple can positively affect lipid metabolism (Ravn-Haren *et al*., 2013; Nagasako-Akazome *et al*., 2007), weight management (De Oliveira *et al*., 2003) and vascular function (Gasper *et al*., 2014). Furthermore, various mechanisms of the favourable effects of triterpenoid compounds on CVD have been studied (Koutsos *et al*., 2015). One mechanism in the prevention of CVD by fruit consumption is increased vascular nitric oxide (NO) bioavailability, which is due to enhanced endothelial nitric oxide synthase (eNOS) activity (Waldbauer *et al*., 2015).

1.2.2 Phenolic compounds

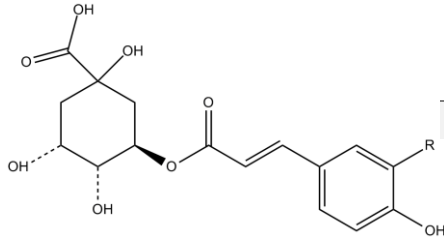
Plant phenolic compounds include a wide range of secondary metabolites that are synthesised from carbohydrates via the shikimate pathway and the acetate pathway (Bravo, 1998). Therefore the distribution of phenolic compounds is almost ubiquitous in the plant kingdom (Pereira *et al*., 2009). It has been estimated that among 100,000-200,000 existing second metabolites (Metcalf, 1987), 20% of the carbon fixed by photosynthesis is channelled into the phenylpropanoid pathway, generating the majority of natural phenolics (Weisshaar & Jenkins, 1998). The phenolic compounds include more than 8000 currently known phenolic structures (Harborne, 1989), and they have one

common structural feature, a phenol, which is an aromatic ring bearing at least one ‘acidic’ hydroxyl substituent (Harborne, 1989). Phenolic compounds are essential to the physiology of plants, mainly as regards: their contribution to plant morphology; providing plants with resistance to pathogens and predators; and protecting crops from pre-harvest seed germination (Bravo, 1998). Recent epidemiological studies have shown important antioxidant properties and other physiological effects of polyphenols and their probable role in the prevention of various diseases, such as cancer and CVD (Dai & Mumper, 2010; Cai *et al*., 2004; Morton *et al*., 2000). The antioxidant activities are related to the structure of phenolic compounds (Rice-Evans *et al*., 1996), in particular the reactivity of the phenol moiety, which depends on the number and position of hydroxyl groups and other substituents, and glycosylation of flavonoid molecules (Cai *et al*., 2004; Robbins, 2003).

Phenolic compounds can be divided into 16 different classes depending on their basic chemical structure, which ranges from simple phenols (C6) to highly polymerised compounds such as lignins ((C6-C3)n) (Harborne, 1989). The two major groups of phenolic compounds present in apple fruit are: (1) phenolic acids and related compounds and (2) flavonoids (Spanos & Wrolstad, 1992).

Phenolic acids and related compounds

Hydroxycinnamic acid esters constitute one of the major subgroups of phenolic acids in various apple varieties (Tsao *et al*., 2003). Chlorogenic acid, the ester of caffeic with quinic acid, and p-coumaroylquinic acid are two classic compounds in the hydroxycinnamic acid ester group (Figure 3) (Tsao *et al*., 2003). The browning occurring in apple juice and cider is mainly due to oxidation of chlorogenic acid by oxidative enzymes (Nicolas *et al*., 1994). Chlorogenic acid is present in relatively high concentrations in both the peel and flesh of most apple cultivars (Marks *et al*., 2007; Pérez-Ilzarbe *et al*., 1991).



Hydroxycinnamic acids	R
Chlorogenic acid	OH
p-coumaroylquinic acid	H

Figure 3. Structure of two major hydroxycinnamic acids in apple fruit.

Flavonoids

Flavonoids are the most common and widely distributed group of plant phenolic compounds (Bravo, 1998). Over 4,000 flavonoids have been identified and they are widely distributed in different tissues of plants, such as leaves, seeds, bark and flowers (Heim *et al*., 2002). Flavonoids have a diphenylpropane skeleton (C6-C3-C6) and the three-carbon bridge between the phenyl groups is usually cyclised with oxygen (Figure 4) (Spanos & Wrolstad, 1992). The flavonoids can be divided into 13 sub-groups, according to differences in the number of substituent hydroxyl groups, degree of unsaturation and degree of oxidation of the three-carbon bridge (Bravo, 1998; Spanos & Wrolstad, 1992). Flavonoids are referred to as glycosides when they contain one or more sugar groups (or glucosides in the case of a glucose moiety), and as aglycones when no sugar group is present (de Rijke *et al*., 2006). The 3 position on flavonoids is the preferred glycosylation site and the 7 position is less frequent (Rice-Evans *et al*., 1996). In apple, anthocyanidin, flavanol (also named flavan-3-ols) flavonols (mainly quercetin glycosides), and dihydrochalcones are the major sub-groups of flavonoids (Chinnici *et al*., 2004; Guyot *et al*., 1998; Spanos & Wrolstad, 1992).

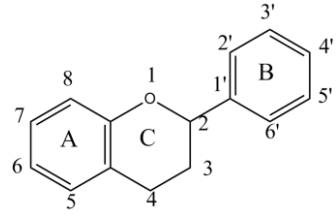


Figure 4. Flavonoid skeleton.

Anthocyanins are generally accepted as the most important group of water-soluble pigments and they usually occur in the vacuole of plants (Clifford, 2000). It is estimated that there are more than 500 diverse structures of anthocyanins in nature (McGhie *et al*., 2007). They are responsible for the blue, purple, red and intermediate hues in many plant tissues (Wu *et al*., 2006; Clifford, 2000). Anthocyanins have a C6-C3-C6 skeleton and they are glycosylated (Figure 5), polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium (McGhie & Walton, 2007; Stintzing & Carle, 2004). The most widespread anthocyanin is cyanidin, especially cyanidin 3-glucoside (Mazza, 2007; Kong *et al*., 2003), but cyanidin 3-galactoside is the dominant cyanidin in apple peel (Tsao *et al*., 2003; Mazza & Velioglu, 1992). The

structure of anthocyanins is highly influenced by the pH, temperature, co-pigmentation and other factors, which makes some corresponding changes to the colour or colour intensity of the tissues (Castañeda-Ovando *et al*., 2009; Cooke *et al*., 2005; Clifford, 2000). Furthermore, structural variations, such as different hydroxylations and glycosylation, may modulate their antioxidant activities (Stintzing *et al*., 2002; Wang *et al*., 1997).

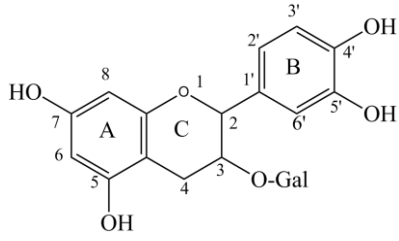


Figure 5. Structure of anthocyanin.

Catechin, epicatechin, gallocatechin, procyanidins and their polymers are major flavanols (also named flavan-3-ols) in apple (Awad, 2001). (+)-Catechin and (−)-epicatechin are the two isomers which are often found in food plants (Figure 6) (Tsao, 2010). They are abundant monomeric flavanols in apple peel and flesh, where epicatechin is present in much higher concentrations than catechin (Marks *et al*., 2007). Catechin and epicatechin can form polymers, and they are often referred to as proanthocyanidins, because acid-catalysed cleavage of the polymeric chains can produce anthocyanidins (Tsao, 2010). Procyanidin B1 and B2 are trimers which are commonly found in apples (Tsao *et al*., 2003; Schieber *et al*., 2001).

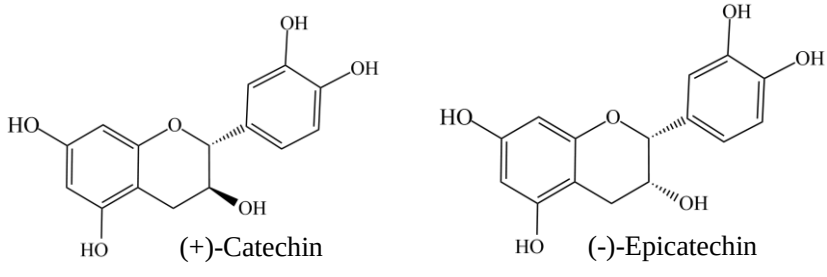


Figure 6. Structure of catechin and epicatechin.

Quercetin is the most common aglycon of the flavonols group and the glycosides rutinoside, glucoside, galactoside, arabinoside, xyloside and

rhamnoside are quite common in apple (Marks *et al*., 2007; Awad *et al*., 2000; Spanos & Wrolstad, 1992). Quercetin 3-glycosides are mainly found in the apple skin (Awad *et al*., 2000), but have also been detected in apple pulp in low concentrations (13.7-16.2 mg/kg fresh weight) (Chinnici *et al*., 2004). Some quercetin 3-glycosides, such as quercetin-3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside and quercetin 3-rhamnoside, have also been found in apple pomace and juice (Schieber *et al*., 2001).

Phloridzin (phloretin 2´-glucoside) and phloretin 2´-xyloglucoside are two dominant dihydrochalcones reported in apple peel and flesh, and are present in particularly high concentrations in the peel (Marks *et al*., 2007; Vrhovsek *et al*., 2004; Tsao *et al*., 2003; Sanoner *et al*., 1999). The concentration of dihydrochalcones in apple is relatively lower than that of other phenolic compounds, but they can be used as characteristic markers of apples and derived products (such as apple juice, cider, and pomace) due to their uniqueness to apple and can also be used to identify apple cultivars (Tomas-Barberan & Clifford, 2000; Versari *et al*., 1997; McRae *et al*., 1990).

Health aspects of phenolic compounds

Phenolic compounds are ubiquitous in the plant kingdom and they can be found in different parts of fruits and vegetables, although with concentration differences among different species and different organs within the same species (Morton *et al*., 2000; Robards *et al*., 1999). Numerous epidemiological surveys have shown that intake of fruit and vegetables is associated with a reduced risk of CVD, certain cancers, diabetes and age-related functional decline (Liu, 2003; Rice-Evans *et al*., 1997). It has been suggested that these health functions are due at least to some extent to their antioxidant content, and phenolic compounds often account for a large part of the antioxidant content (Morton *et al*., 2000). A direct linear relationship between the phenolic content and total antioxidant activity was observed in 11 common fruits tested by Sun *et al*. (2002), indicating that phenolics may be the major contributor to the total antioxidant activity of fruit. The major phenolic compounds isolated from olive oil have been confirmed to have a dual action, donation of a proton to HO● and inhibition of xanthine oxidase. Therefore, the phenolics can increase defence in scavenging reactive oxygen species and can also suppress xanthine oxidase activity, which is a factor known to influence carcinogenesis (Owen *et al*., 2000). Previous studies also suggest that consumers should get antioxidants from a diverse diet containing a balanced combination of phytochemicals to improve their nutrition and health (Liu, 2003).

1.3 Variation in concentrations of bioactive compounds

1.3.1 Genetic variation

There is a very diverse range of apple cultivars available for consumption. For the apple fruit, apart from differences in appearance, texture and flavour, there is significant variation among different cultivars in the concentration and composition of bioactive compounds such as triterpenes and polyphenols (McGhie *et al*., 2011; Volz & McGhie, 2011). On analysing the triterpenes in seven apple cultivars, McGhie *et al*. (2011) identified 43 compounds; 'Fuji' had the highest total amount and 'Granny Smith' had the lowest. Furthermore, the apple peel contained different chemical structures of ursenoic acid and the proportion of these compounds varied between cultivars (McGhie *et al*., 2011). Among apple genotypes in New Zealand, 46-97% of the variation in the concentration of total polyphenols is due to genotype differences (Volz & McGhie, 2011). A study of different apple cultivars, representing eight of the most widely cultivated varieties in Western Europe, showed that the mean concentration of total polyphenols was between 66.2 and 211.9 mg/100 g of fresh weight depending on the variety (Vrhovsek *et al*., 2004). The concentration of anthocyanins also shows cultivar differences in apple (Mulabagal *et al*., 2007).

1.3.2 Pre-harvest factors (cultivation methods, rootstock)

The appearance of fruit is not only a critical criterion in consumer decision making, but also a primary index for grading and pricing the products. These appearance indices, such as size, shape and colour, can be modulated by the pre-harvest factors (Kays, 1999). In addition, the texture of the fruits is influenced by pre-harvest factors (Sams, 1999). Many of the disorders occurring during ripening and storage of fruits are also related to pre-harvest factors (Ferguson *et al*., 1999).

Kays (1999) divided pre-harvest factors into six groups: (1) biological factors; (2) physiological factors; (3) environmental/cultural factors; (4) mechanical damage; (5) extraneous matter; (6) genetic variation and aberrations. The range and abundance of phytochemicals in the fruits can vary under different pre-harvest conditions. Studies on the concentration of ascorbic acid for three sour berry cultivars ('Kelleris 16', 'Újfehértói Fürtös' and 'English Morello') revealed significant differences between various rootstocks (Kopytowski & Markuszewski, 2010). For some apple cultivars in New Zealand, such as 'Pacific Queen', 'Pacific Rose' and 'Granny Smith', the polyphenolic content showed significant variation between regions due to

environmental effects (McGhie *et al*., 2005). The red colour of apple, which is mainly due to the presence of anthocyanins, is directly regulated by light, temperature and cultivar (Iglesias *et al*., 2008), and it is also related to soil fertility. The concentration of flavonoids in apple skin can be increased by optimising fertilisation, especially nitrogen (Awad & de Jager, 2002). For the apple cultivar 'Cox's Holsteiner', integrated production (IP) results in apples which contain more triterpenes than organic apples (Ellgardt, 2006). Large differences in flavonoid and chlorogenic acid concentrations may arise depending on apple position on the tree and the orientation of the orchard (Awad *et al*., 2000).

1.3.3 Harvest factors (harvest time)

There is significant variation in the concentration of phytochemicals during the growth and maturation of fruit. Ripening is the result of a complex of biochemical, physiological and structural changes in fruit (Lelièvre *et al*., 1997). Dilution of phenolic compounds has been found to occur during apple fruit enlargement from 30 to 120 days after full bloom, while for catechin, epicatechin, procyanidin B2 and chlorogenic acid, the concentration per unit weight remained almost stable from 120 to 150 (ripening) days after full bloom (Zhao *et al*., 2015). Another study showed that apples or pears harvested at the same degree of maturity, but at different harvest dates, showed no significant differences in antioxidant capacity, total phenolics or ascorbic acid content due to the different harvest date (Kevers *et al*., 2011). That means the ripening stage may be more important than the specific harvest date for fruits which have reached the same degree of ripeness (Kevers *et al*., 2011). The wax composition of apple peel did not change much with picking date for the cultivars 'Elstar', 'Jonagold' and 'Jonagored' in a study by Veraverbeke *et al.* (2001). In contrast, a study on 'Delicious' apple fruit showed that during ripening, all four components of wax (hydrocarbons and wax esters, free alcohols, free fatty acids and diols) increased, coinciding with the climacteric rise in ethylene (Ju & Bramlage, 2001). Therefore, cultivar may also influence wax composition changes during fruit ripening.

1.3.4 Postharvest factors (storage methods)

Storage is an essential part of orderly fruit marketing (Thompson, 2003). Like other aerial parts of terrestrial plants, the apple is covered by cuticle (Müller & Riederer, 2005) and it is the first protective barrier against abiotic and biotic environmental stress (Buschhaus & Jetter, 2011). The major components of cuticle are cutin and wax (Müller & Riederer, 2005; Pollard *et al*., 2008).

Cuticular wax is predominantly comprised of very long chain aliphatic lipids, with a hydrocarbon backbone of 21 to >40 carbon atoms, and also cyclic compounds, such as pentacyclic triterpene acids, as well as other secondary metabolites, including hydroxycinnamic acid derivatives and flavonoids (Müller & Riederer, 2005; Kunst & Samuels, 2003;). During storage, the wax layer as a physical barrier is especially important in limiting water and weight loss in apples (Veraverbeke *et al*., 2001). Previous studies have found changes in the composition of wax during apple storage (Morice & Shorland, 1973; Veraverbeke *et al*., 2001), and decreases in the concentrations of some ester fractions during long-term controlled atmosphere (CA) cold storage (Veraverbeke *et al*., 2001). One study on apples showed that long-term storage, both regular atmosphere (RA) cold storage and CA cold storage, did not influence flavonoid concentrations (van der Sluis *et al*., 2001). A study on 'King Jonagold' apple showed that total phenolics increased in the first three months of storage, followed by a decrease in the next six months of storage under all storage conditions tested (RA, CA and pre-treatment with 1-MCP plus CA storage) (Kevers *et al*., 2011). Therefore, storage conditions may play an important role for the content of bioactive compounds in apples. It has been suggested that fruits should be consumed soon after purchase and with their peel intact, as domestic storage and peeling can decrease the bioactive compound content (Kevers *et al*., 2011).

1.4 Postharvest disease of apple

Losses of plant products from field to consumer are estimated to amount to up to 25% of total production in industrialised countries and more than 50% in developing countries (Kader, 2005; Spadaro & Gullino, 2004). During storage, the losses of apples are about 5-25%. Generally, all postharvest diseases of fruit and vegetables are caused by fungi and bacteria, while viruses are not an important cause (Coates, 1997). For apples, *Botrytis cinerea*, *Penicillium expansum* and the *Gloeosporium* group are considered to be the major pathogens responsible for most economic losses, although physiological disorders, such as bitter pit, water core and scald, are other important causes of loss (Jijakli & Lepoivre, 2004). In addition, *Monilinia fructigena*, *Neofabraea* spp. and *Colletotrichum* spp. cause serious losses in stored apples (Neri *et al*., 2009; Xu & Robinson, 2000).

According to how infection is initiated, post-harvest diseases can be classified into two main groups: latent (or quiescent) infection) disease and wound pathogen disease. With latent infection, mycelial development is arrested after infection and resumes only as the host plant reaches maturity

and/or senescence, or the environmental or nutritional conditions become suitable for the pathogen's growth. The latent infection diseases in apples include bitter rot, caused by *Colletotrichum gloeosporium* or *C. acutatum*, and bull's-eye rot, caused by *Neofabraea* spp. (Verhoeff, 1974). Wound pathogen disease infections are initiated during and after harvest and occur through surface wounds created by mechanical or insect injury. Common post-harvest diseases resulting from wound infections include blue and green mould caused by *Penicillium expansum* (Coates, 1997).

1.5 Ozone treatment of postharvest fruit and vegetables

The use of ozone to prevent decay in fresh fruit and vegetables during handling and storage has been widely studied. Ozone, triatomic oxygen, is a highly reactive form of oxygen (Forney, 2003). It has been approved as a food additive by the US Food and Drug Administration (2001). The commercial use of ozone involves many fruits, including apple, kiwi, peach, table grapes and others (Suslow, 2004). Two major modes of application of ozone are treating water with ozone and adding ozone to the storage atmosphere (Forney, 2003). Ozone treatment may affect quality parameters of the treated fruits and vegetables. Inactivation of *Escherichia coli* OI57:H7 by ozone has been confirmed to be sufficiently fast for practical application in cider production (Steenstrup & Floros, 2004). Apples inoculated with *B. cinerea* and treated with ozone (450 ppb, 48 h) developed lesions later and the lesions were considerably smaller than in the controls (Sharpe *et al*., 2009). A study of kiwi fruit showed that reducing sugar was lower in cold storage with ozone treatment than in RA cold storage between 15 and 29 weeks of storage, and that RA cold storage better conserved organic acids (citric, quinic and malic acids) than in ozone-treated fruits at 29 weeks (Barboni *et al*., 2010). A study on tomato showed that short-term gaseous ozone treatment (10 μL/L; 10 mins) increased the total phenolics content immediately after treatment and after 6 days of storage at 20 °C (Rodoni *et al*., 2010). Short-time (0-30 mins) ozone treatment (8±0.2 mL/s flow rate) of fresh-cut pineapple, banana and guava was also investigated, and the results showed that total phenolics and flavonoid concentrations in pineapple and banana increased significantly when ozone treatment was applied up to 20 mins, but decreased in guava (Alothman *et al*., 2010). On the whole, the effect of ozone on fruit quality is inconsistent in different fruit species, and the ozone concentration, application method and period seem to be very important in optimising the treatment effect.

2 Aim and objectives

The main aim of this thesis was to determine the effects of different pre-harvest and postharvest factors on triterpene concentrations in apple peel. Specific objectives were to:

- Investigate the effects of cultivar, sun exposure, storage conditions, bruising and *Penicillium expansum* infection on the concentrations of oleanolic acid and ursolic acid in apple peel (Paper I)

- Investigate the effects of rootstock, harvest maturity and storage method on the concentrations of oleanolic acid and ursolic acid in 'Aroma' apple peel (Paper II).

A second aim was to investigate the effect of post-harvest ozone treatment combined with cold storage on triterpene and phenolic compounds concentrations in apple fruit. Specific objectives were to:

- Investigate the effect of ozone application during cold storage on the concentrations of triterpenes and phenolic compounds in apple fruit (Paper III).

The antifungal activity of triterpene-enriched crude extract was also tested, in order to:

- Investigate the inhibiting effect of triterpene-enriched extracts from apple peel on the growth of post-harvest pathogens of fruit (Paper IV).

3 Materials and methods

3.1 Plant and fungal materials

3.1.1 Plant materials

Five apple cultivars commonly grown in Swedish commercial orchards, ‘Amorosa’, ‘Aroma’, ‘Discovery’, ‘Gloster’ and ‘Santana’, were used in the studies. These cultivars were obtained from a commercial Integrated Pest Management (IPM) orchard in Kivik, south-east Sweden (56°6’23’’N, 14°40’57’’E). Fruit harvesting date was estimated according to the Streif Index [firmness/(total soluble solid × starch hydrolysis)] (Streif, 1996).

In Paper I, three apple cultivars, an early-ripening cultivar ‘Discovery’, a mid-ripening cultivar ‘Aroma’ and a late-ripening cultivar ‘Gloster’, were harvested during 2012 and 2013 seasons at full maturity.

In Paper II, apples of ‘Aroma’ from trees grafted onto two rootstocks (‘M9’ and ‘MM106’) were harvested three times as follows: early harvest on 12 September 2012 and 10 September 2013; medium harvest on 17 September 2012 and 2013; and late harvest on 24 September 2012 and 2013.

In Paper III, two apple cultivars, ‘Amorosa’ and ‘Santana’, were harvested in September 2014.

In Paper IV, ‘Gloster’ apples, which were used for triterpene-enriched extract preparation, were harvested in October 2014. ‘Aroma’ apples harvested from a commercial orchard in September 2015 were used for *in vivo* inoculation tests.

3.1.2 Fungal materials

In Paper I, in order to prepare the spore suspension for inoculation, *Penicillium expansum* was collected from naturally infected apples that were showing typical symptoms of blue mould and the pathogen was cultivated on Petri

dishes with potato dextrose agar (PDA), and then identified under microscope according to spore morphology. The *P. expansum* spores of suspension conidia were collected from 10-day-old cultures and suspended in 5 mL sterile distilled water containing 0.05% (v/v) Tween 80. The suspension was filtered and spore concentrations were adjusted to 1×10^5 conidia/mL.

In Paper IV, four pathogens (*P. expansum*, *Monilinia fructigena*, *Colletotrichum acutatum* and *Neofabraea perennans*) were used. The pathogens were isolated from stored apple fruits showing typical symptoms of diseases caused by these four pathogens. Infected fruit were surface-sterilised for 30 s with 70% ethanol and rinsed with sterile distilled water. Small parts from the growing margin of the fruit rot lesion were transferred to PDA for *P. expansum* and *M. frutigena* and to 1% (m/v) malt extract agar (MEA) for *C. acutatum* and *N. perennans*. The plates were then held in an incubator at 24 °C for two weeks. Pure cultures were obtained by transferring the hyphal tips to new PDA/MEA plates and incubating at 20 °C for 3 weeks. Thereafter, the pathogens were maintained at 4 °C separately as pure cultures.

3.2 Experiment conditions and treatments

3.2.1 Storage conditions (Papers I & II)

The three apple cultivars used in Paper I ('Discovery', 'Aroma' and 'Gloster') were stored in RA cold storage (2-3°C, 85-90% relative humidity (RH)). The early-ripening cultivar 'Discovery' was stored for two months, while 'Aroma' and 'Gloster' were stored for three months.

In Paper II, 'Aroma' apples from two rootstocks ('M9' and 'MM106') were stored in RA cold storage (2-3°C, 85-90% RH) or CA cold storage (2 kPa O_2 and 2 kPa CO_2 90% RH) for four months in 2012 and three months in 2013.

3.2.2 *In vivo* inoculation test (Papers I & IV)

In Paper I, apple fruits were washed with distilled water to remove naturally occurring fungi and wounded with a 1 mL pipette tip (4 mm diameter, 4 mm depth) on the sun-exposed side and the shaded side. The fruits were then inoculated by injecting 20 μL of freshly prepared conidial pathogen suspension (1×10^5 conidia/mL) into the wounded points. Inoculated fruits were left at room temperature (20 °C) for 96 h before the lesion area diameter was examined.

In Paper IV, apples were wounded at two opposite sides and then 20 µL of spore suspension collected from treated and control plates was applied to the wound points. Within 30 minutes of inoculation, inoculated apples were

transferred to a coldroom and stored at 3-4 °C for 10 days. Thereafter, the inoculated apples were transferred to room temperature, mimicking shelf life, and decay lesion diameter was measured on day 2 and day 8 at room temperature for *P. expansum* and on day 5 and day 16 for the other three pathogens.

3.2.3 Ozone treatment before or during cold storage (Paper III)

In Paper III, 'Amorosa' and 'Santana' fruits were stored (i) after three different ozone treatments and then used after 36 h, 4 days, 8 days and one month of storage for a detailed time study, and (ii) 0.5 ppm ozone gas was pumped into the storage room for one hour per day during four months of cold storage (2°C and 85% RH). In the ozone treatment experiment (i), fruits were placed in sealed plastic jars (volume 40 L) and then subjected to one of three different ozone treatments: (a) 2.5 ppm ozone was pumped into 10 L water in the sealed plastic jar for 2 hours; (b) 2.5 ppm ozone as gas was pumped into the sealed plastic jar for 2 hours; and (c) 2.5 ppm was pumped into 10 L water for 2 hours, then the apples were removed from the water treatment and 2.5 ppm ozone as gas was pumped into the sealed plastic jar for 2 hours. Ozone gas was generated by an ozone generator using oxygen.

Figure 7. The sealed plastic jar connected with ozone generator.

3.2.4 Triterpene-enriched crude extract preparation and treatments on culture plates (Paper IV)

In Paper IV, fresh 'Gloster' apples were cleaned and peeled carefully. Fresh peels were stored at -20 °C until extraction. The frozen peels were lyophilised

in a freezer dryer and then milled to powder. A 1 g portion of peel powder was extracted with 8 mL 99.5% ethanol for 1.5 h in an ultra-sonic bath at room temperature. After centrifuging the ethanol extract, an aliquot of 6 mL supernatant was transferred to a 10 mL glass tube for evaporation until all ethanol evaporated. Triterpene-enriched suspended water (TESW) was obtained by adding 1 mL sterilised Millipore water to the concentrated supernatant. A volume of 0.5 mL TESW (TESW-1) or 1 mL TESW (TESW-2) was applied to pathogen culture plates. Plates receiving 1.0 mL sterilised Millipore water were used as positive controls and plates without any treatment were used as blank controls. The plates were placed in an incubator at 23°C. The diameter of the mycelial radial growth of the four pathogens was measured after 3 and 7 days of incubation. The experiment was repeated three times (giving in total 12 replicate plates for each pathogen). After mycelium measurement, all dishes were placed under ultraviolet (UV) light for 2 h and then moved back to room temperature for 5 days. Spores were collected from UV light-treated plates and suspended with 5 mL 0.05% Tween 80. The spore concentration was determined with a haemacytometer under light microscopy.

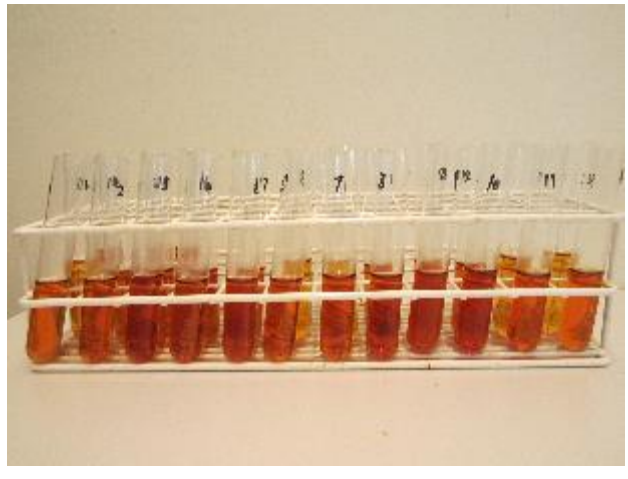


Figure 8. The 99.5% ethanol supernatant from apple peel.

3.3 Chemical analyses and fruit quality index determination

For all studies (Papers I-IV), OA and UA concentrations in apple peel were quantified by high-performance liquid chromatography (HPLC). For the OA and UA extraction, lyophilised peel discs were extracted in 99.5% ethanol for 1.5 h in an ultrasonic bath at room temperature. The extracted samples were

then centrifuged and aliquots of 1 mL of the supernatant were used for the HPLC analysis. A 7000 HPLC system equipped with a diode array detector (DAD) and a reverse phase C18 VYDAC column was used. Detection was carried out at 207 nm.

In Papers III and IV, major phenolic compounds were quantified by high-performance liquid chromatography-mass spectroscopy (HPLC-MS). In Paper III, major phenolics in apple peel and flesh were extracted with 80% methanol with 3% formic acid, and then identified by an Agilent Technologies 1260 Infinity HPLC-MS system equipped with a DAD (G4212B). Detection was carried out at 280 nm (catechin, procyanidin B2, phloretin 2´-xylglucoside and phloridzin), 320 nm (chlorogenic acid and p-coumaroylquinic acid), 360 nm (flavonols) and 530 nm (cyanidin 3-galactoside). All single phenolic compounds were quantified using external standards. In Paper IV, only the major phenolic in apple peel were analysed, in the same way as described above.

In Papers I and II, fruit colour was determined using a Minolta Chromameter CR-200 with an 8 mm diameter window and results were expressed as hue angle (h°).

In Papers I, II and III), total soluble solids (TSS) were measured by thermostatic refractometer and presented as percentage by weight (g)/volume (100 mL); titratable acidity (TA) and pH were measured using a titration unit and TA was presented as percentage malic acid by weight (g)/volume (100 mL).

3.4 Statistics

Differences in triterpene and phenolic compounds were analysed with general linear model (GLM) and analysis of variance (ANOVA) using Minitab. Mean comparisons were performed by Tukey's test, with significance level set at $P<0.05$. Pearson correlation was used to analyse the correlation between triterpene content and fruit quality index.

4 Results and discussion

4.1 Variation in triterpenes and phenolic compounds between apple cultivars

The major triterpenes in apple peel varied significantly between the five apple cultivars investigated in Papers I and III. The highest OA and UA concentrations at harvest time were found in ‘Gloster’ (Figure 9), with 84.7 and 482.0 µg/cm^2, respectively. The OA concentration in ‘Aroma’ was 27.5% lower than that in ‘Gloster’, followed by ‘Amorosa’ >‘Discovery ’>‘Santana’. The order of magnitude of the UA concentration in peel of the other cultivars was ‘Amorosa’ >‘Santana’ >‘Aroma’ >‘Discovery’, but no significant difference was found between ‘Amorosa’, ‘Santana’ and ‘Aroma’. The UA concentration in ‘Discovery’ was 42.2% lower than in ‘Gloster’.

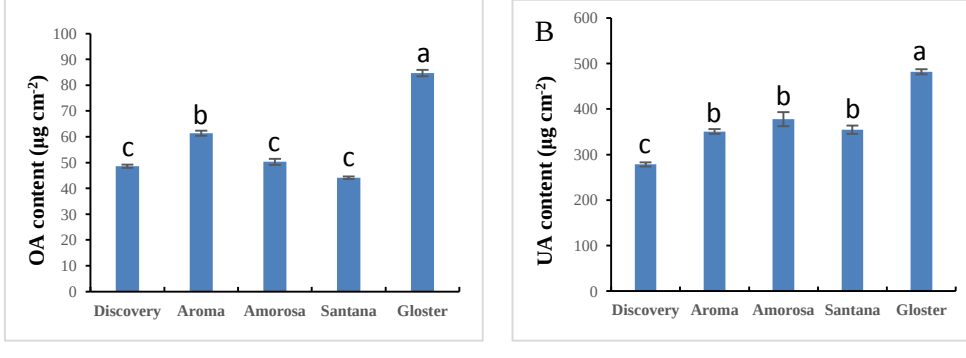


Figure 9. Oleanolic acid (OA) and ursolic acid (UA) (µg cm^{-2}) in the peel of different apple cultivars. Different letters indicate significant differences between cultivars according to Tukey’s test (p<0.05). (A) OA content; (B) UA content).

Previous studies have also reported cultivar differences in triterpene concentration in apple peel (McGhie, 2011; Frighetto, 2008). The triterpenes are one of the dominant groups of compounds in cuticular wax (Buschhaus & Jetter, 2011). Therefore, the composition and structure of cuticular wax might influence the amount of triterpenes. The abundant amount of triterpenes in 'Gloster' might be due to the fact that it had thicker peel than the other cultivars investigated based on the dry weight of peel per unit area (data not shown). On the other hand, 'Discovery' is an early-ripening cultivar and 'Gloster' is a late-ripening cultivar. It has been shown previously that these two cultivars have differing resistance to fungal disease, with 'Gloster' developing smaller lesions than 'Discovery' after fungal inoculation. Since the triterpenes have some anti-fungal activity (Yessoufou, 2015; Mahlo, 2013), the difference in concentration between cultivars is most likely related to fruit resistance to disease.

Phenolic compounds were investigated in 'Amorosa' and 'Santana' (Paper III). At harvest time, 'Amorosa' peel had a higher total polyphenolics concentration than 'Santana', and the same trend was found after four months of cold storage (Table 1). Total flavonols were the largest group within total polyphenolics in 'Santana' peel, accounting for 40.8% after four months of storage. Total flavonols were the second largest group in 'Amorosa' peel, accounting for 34.1% after four months of storage. As regards total flavonols concentration in apple peel, no significant differences were found between 'Amorosa' and 'Santana' at harvest time or after four months of storage, even though a higher total flavonols concentration was found in 'Santana' than in 'Amorosa' after one month of storage. Seven quercetin glycosides were found in the peel of both apple cultivars. Quercetin 3-galactoside and quercetin 3-arabinopyranoside constituted 70% of the flavonols in 'Amorosa' and 48.8% in 'Santana' after four months of storage. 'Amorosa' had a significantly higher quercetin 3-galactoside concentration than 'Santana' during the whole study period, while 'Santana' had higher concentrations of quercetin 3-rutinoside, quercetin 3-glucoside, quercetin 3-xyloside and quercetin 3-rhamnoside than 'Amorosa'. At harvest time, quercetin 3-arabinopyranoside and quercetin 3-arabinofuranoside showed no difference between 'Amorosa' and 'Santana', but higher concentrations of both flavonol compounds were found in 'Amorosa' after four months of storage.

Total procyanidins were the largest group of total polyphenolics in 'Amorosa' peel and constituted 36.9% of total polyphenolics content after four months of storage (Table 1). Total procyanidins represented the second largest group in 'Santana' peel, comprising 27.0% of total polyphenolics content after four months of storage. 'Amorosa' peel showed significantly higher total

procyanidins concentration than ‘Santana’, both at harvest time and after one and four months of storage. Similarly, epicatechin and procyanidin B2, two procyanidins, were found in significantly higher amounts in the peel of ‘Amorosa’ than ‘Santana’ during the whole study period. Cyanidin 3-galactoside was the only anthocyanin detected in the peel of both apple cultivars. It constituted 12.5% and 9.4% of total polyphenolics content in ‘Amorosa’ and ‘Santana’, respectively, after four months of storage. A higher cyanidin 3-galactoside concentration was found in ‘Amorosa’ than ‘Santana’ during the whole study period.

Two hydroxycinnamic acid esters with quinic acid, chlorogenic acid and p-coumaroylquinic acid, were found in the peel of both ‘Amorosa’ and ‘Santana’ (Table 1). Total hydroxycinnamic acids accounted for 9.5% and 10.8% of total polyphenolics content in ‘Amorosa’ and ‘Santana’ respectively, after four months of storage. Chlorogenic acid constituted the major component of total hydroxycinnamic acids and did not show any significant difference between ‘Amorosa’ and ‘Santana’ at harvest time and after four months of storage. On the other hand, total hydroxycinnamic acids concentration was higher in the peel of ‘Amorosa’ than ‘Santana’ at harvest time and after four months of storage.

Phloretin 2´-xylglucoside was the dominant dihydrochalcone, together with phloridzin, identified in apple peel of both cultivars (Table 1). Similar results have been reported for some other apple cultivars (Lister, 1994; McRae, 1990; Pérez-Ilzarbe, 1991). Phloretin 2´-xylglucoside was present in higher concentrations in the peel of ‘Amorosa’ than ‘Santana’ at harvest time and after one month of storage, but no differences were found between the two cultivars after four months of storage. The total dihydrochalcone concentration was higher in the peel of ‘Santana’ than ‘Amorosa’ during the whole study period.

Apple fruits, especially the peel, are rich in flavonoids and hydroxycinnamic acid derivatives (Awad, 2001; Lancaster, 1992). These polyphenolic compounds also contribute to the quality aspects of apples, such as fruit colour and taste (Awad, 2000). The chemical composition and concentrations of polyphenolic compounds have been found to be affected mainly by genetic variation, although they can also be influenced to some degree by culture and growing conditions (Tsao, 2003; Awad, 2000; McRae, 1990;). A previous study has demonstrated that the concentration of polyphenolic compounds in apple fruit can vary considerably between different apple cultivars in Sweden (Ahmadi-Afzadi, 2015). In this thesis, ‘Amorosa’ had a higher total polyphenolics content in apple peel than ‘Santana’, both at harvest time and after four months of cold storage. The same trend was found

Table 1. The content (mg kg^{-1} fresh weight) of phenolic compounds in the peel of 'Amorosa' and 'Santana' at harvest time, after 1 month storage and after 4 months storage.

	At harvest		After 1 month storage		After 4 months storage		% (After 4 months of storage)	
	Amorosa	Santana	Amorosa	Santana	Amorosa	Santana	Amorosa	Santana
Chlorogenic acid	100.4±1.0 A a	95.2±5.4 A b	95.7±2.2 B a	113.4±0.4 A a	102.5±10.3 A a	98.1±9.1 A b	8.1	9.9
p-coumaroylquinic acid	18.0±0.6 A a	10.4±0.1 B a	13.8±0.1 A b	11.2±0.2 B a	17.2±0.5 A a	8.4±0.5 B b	1.4	0.9
Total hydroxycinnamics	118.4±1.2 A a	105.6±5.4 B b	109.5±2.3 B a	124.6±0.6 A a	119.7±10.0 A a	106.5±9.0 B b	9.5	10.8
Epicatechin	213.0±7.5 A ab	66.2±3.3 B a	226.2±17.6 A a	71.1±4.8 B a	188.0±16.1 A b	62.7±9.8 B a	14.9	6.3
Procyanidin B2	264.2±4.3 A a	166.3±3.3 B c	273.8±14.9 A a	181.4±0.5 B b	277.8±21.8 A a	203.7±6.6 B a	22.0	20.6
Total procyanidins	477.2±6.7 A a	232.5±2.3 B b	500.1±30.6 A a	252.5±4.2 B ab	465.8±33.5 A a	266.4±15.2 B a	36.9	27.0
Cyanidin 3-galactoside	125.3±12.3 A b	83.5±8.0 B a	145.1±2.9 A a	85.2±2.9 B a	158.0±8.3 A a	92.7±9.2 B a	12.5	9.4
Total anthocyanin	125.3±12.3 A b	83.5±8.0 B a	145.1±2.9 A a	85.2±2.9 B a	158.0±8.3 A a	92.7±9.2 B a	12.5	9.4
Quercetin 3-rutinoside	4.7±1.0 B a	7.6±2.6 A b	3.3±0.1 B b	13.5±1.0 A a	2.8±0.6 B b	6.0±0.9 A b	0.2	0.6
Quercetin 3-galactoside	167.0±2.9 A ab	91.3±11.3 B b	144.8±12.7 A b	124.3±23.4 B a	174.2±13.3 A a	92.3±10.0 B b	13.8	9.3
Quercetin 3-glucoside	26.3±1.3 B b	55.6±4.0 A b	23.5±1.1 B b	74.2±9.2 A a	30.6±1.5 B a	53,6±7.2 A b	2.4	5.4
Quercetin 3-xyloside	28.8±2.7 B b	38.4±3.2 A c	28.1±0.8 B b	51.7±0.6 A a	37.2±1.9 B a	43.5±1.6 A b	2.9	4.4
Quercetin 3-arabinopyranoside	108.2±10.2 A b	96.2±5.1 A b	105.7±6.7 B b	123.2±4.6 A a	127.5±8.8 A a	104.3±11.4 B b	10.1	10.6
Quercetin 3-arabinofuranoside	17.9±1.7 A b	18.3±1.8 A b	18.2±0.7 B b	23.5±0.9 A a	22.5±1.4 A a	19.8±0.9 B b	1.8	2.0
Quercetin 3-rhamnoside	21.7±0.6 B b	77.7±5.6 A b	21.6±1.6 B b	107.4±6.0 A a	35.7±1.3 B a	83.3±10.0 A b	2.8	8.4
Total flavonols	374.5±18.9 A b	385.0±16.9 A b	345.0±23.2 B b	517.8±26.2 A a	430.4±26.7 A a	402.6±38.1 A b	34.1	40.8
Phloretin 2´-xylglucoside	54.9±4.2 B b	75.0±3.9 A ab	53.8±0.8 B b	85.3±1.4 A a	70.1±4.3 A a	64.4±7.4 A b	5.6	6.5
Phloridzin	4.9±0.5 B b	60.3±4.3 A a	3.3±0.1 B b	60.5±3.2 A a	18.0±3.6 B a	55.2±4.5 A a	1.4	5.6
Total dihydrochalcones	59.9±3.8 B b	135.3±6.1 A a	57.1±1.0 B b	145.8±3.7 A a	88.1±4.9 B a	119.6±7.8 A b	7.0	12.1
Total polyphenolics	1155.2±22.0 A b	941.9±23.5 B b	1156.8±46.5 A b	1125.9±35.5 A a	1262.1±57.5 A a	987.8±57.9 B b	100	100

Different letters (a, b) indicate significant differences between different stages (at harvest, after 1 month of storage, and after 4 months of storage) for each compound by Tukey test ($p<0.05$).
Different letters (A, B) indicate significant differences between cultivars within the same stage for each compound by Tukey test ($p<0.05$).

for epicatechin, procyanidin B2 and cyanidin 3-galactoside during the whole study period. These differences between cultivars are most likely connected with their genetic background.

4.2 Effect of sun exposure, bruising and *Penicillium expansum* inoculation on the triterpene concentration in apple peel

The red skin colour of apple fruit is mainly due to the presence of anthocyanin (Saure, 1990). Paper III confirmed that cyanidin 3-galactoside is the major anthocyanin in apple peel. The biosynthesis of anthocyanins in plant tissues is light-dependent (Mancinelli, 1985). However, the effect of light on triterpene synthesis in apple peel is still unclear.

In Paper I, three apple cultivars with obvious sun-exposed and shaded sides were investigated for triterpene concentration in both sides of the fruit. The results showed that OA and UA concentrations were higher in the peel of the shaded side than of the sun-exposed side for both 'Discovery' and 'Gloster' (Figure 2 in Paper I). One study of grapefruit showed that fruit wax composition varied with canopy position and orientation (McDonald *et al*., 1993). It also showed that terpenoid concentration, as weight percentage of total surface wax, was higher in grapefruit harvested from the interior canopy and in the shaded side of exterior fruit, although the differences were not statistically significant. Furthermore, the wax platelet size on fruit peel from the shaded side was smaller than that on peel from the sun-exposed side. In 'Aroma' apple, the wax structure of the sun-exposed side usually has clumps, while the shaded side often has nail-like crystals (Tahir *et al*., 2009). Curry (2008) suggested that epicuticular wax from the sun-exposed and shaded sides of apple fruit differs both qualitatively and quantitatively. The triterpene concentration difference observed in Paper I between the sun-exposed and shaded side of apple fruit might due to the wax structure and compositional differences between sides. On the other hand, since the light can influence the skin colour, it might modify triterpene synthesis to some extent in wax formation in apple peel.

Terpenoids, including triterpenes, constitute one of the largest families of natural products in both primary and secondary metabolism. Apart from their physiological and metabolic functions, some specific terpenoids can also play a role in communication and defence activities in planta (Goto *et al*., 2000). Previous studies have verified the antifungal activity of triterpene compounds in *in vitro* tests on different fungi species (Yuan *et al*., 2009; Kumar *et al*., 2007; Smania *et al*., 2003). In Paper I of this thesis, *P. expansum* inoculation and bruising effects on the triterpene concentration in apple peel were studied.

Inoculation with *P. expansum* decreased OA concentration in 'Discovery' and 'Gloster' at harvest time in 2013, and decreased UA concentration in 'Aroma' after cold storage in 2012. Therefore *P. expansum* might have caused changes in both the synthesis and degradation of triterpenes, but further studies are required to confirm the specific metabolic reactions. Bruising increased the OA concentrations in 'Discovery' to some extent, but no effect was found on 'Aroma'. Furthermore, OA and UA concentrations showed inconsistent changes in 'Gloster' after bruising. Therefore, it can be concluded that the change in triterpene concentration induced by bruising showed cultivar variation. Increased OA concentration in 'Discovery' after bruising was most likely due to the fruit defence reaction induced by the mechanical injury.

4.3 Effect of rootstock, harvest time and storage method on the triterpene concentration in apple peel

Various studies have demonstrated the effect of rootstock on different features such as vegetative growth and yield of apple trees (Amiri *et al*., 2014), leaf mineral uptake (Fallahi *et al*., 2001), and yield efficiency and fruit quality (Fallahi *et al*., 2002; Kviklys *et al*., 2013; Kviklys *et al*., 2014). The rootstock effect on the triterpene concentration in apple peel was investigated in Paper II. On four out of six harvest occasions (three harvests per year) during the two-year study, OA and UA concentrations in the peel of apples from rootstock 'MM106' were higher than in peel from 'M9'. In another study, the rootstock 'MM106' and 'M9' showed differences in the fresh weight of fruit, fruit firmness and flesh acidity in 'Pink Lady' apples (Talluto *et al*., 2008). Different kinds of rootstocks also influence the composition of phenolic compounds in 'Ligol' apple fruit (Kviklys *et al*., 2014). For all phenolic compounds tested in that study, super-dwarf rootstocks ('P 61' and 'P 22') gave the highest concentrations, while dwarf rootstocks ('M.9', 'P 62') and semi-dwarf ('M.26') gave the lowest. It has been suggested that the accumulation of certain phenolic compounds is mainly due to rootstock genotype, although the environmental conditions, crop load and fruit weight also contribute to the rootstock-dependent variation in phenolic compounds (Kviklys *et al*., 2014). In accordance with this suggestion, it may be possible that the triterpene concentration differences between 'MM106' and 'M9' observed in Paper II might be related to the rootstock genotype. On the other hand, the dwarfing mechanisms in apples include the top-root relationship and plant hormone production and distribution (Simons, 1987). This might also contribute to the triterpene concentration difference by regulating their biosynthesis.

Harvest time can determine fruit maturity, and therefore fruit quality is likely to be affected by different harvest times. For the UA concentration in the peel of apples at harvest, no significant difference was found between different harvest times in any of the study years, and the same trend was observed for UA concentration after CA cold storage (see Table 2 in Paper II). After RA storage in 2012, the UA concentration in apples on the third harvest time was significantly lower than that on the first and second harvest time After RA storage in 2013, the UA concentration in apples on the second harvest time was significantly lower than on the first time (Table 2 in Paper II). For the OA concentration in the peel of apples at harvest time, no significant difference was found between the different harvest times in 2012, but in 2013 the OA concentration in the peel on the first harvest time was significantly higher than that on the third harvest time (Table 2 in Paper II). After RA and CA storage, no consistent trend was found for OA concentration in the peel of apples picked at different harvest times in the two study years (Table 2 in Paper II). These results indicate that for apples consumed directly at harvest or after CA storage, different harvest times during the fruit ripening period do not influence the UA concentration in fruit peel. For the apples kept at RA storage, those from the first harvest time maintained a higher UA concentration after storage than apples harvested on the second or third times. For the OA concentration, some differences were found between different harvest times, with apples from the first harvest time showing consistently high values directly after harvest or after storage.

A previous study on 14 varieties of apples showed that fruit harvested at the same degree of maturity showed no significant differences in total phenolics and ascorbic acid content (Kevers *et al*., 2011). The wax composition in the apple peel of three cultivars (‘Elstar’, ‘Jonagold’ and ‘Jonagored’) also did not change much with picking date (Veraverbeke *et al*., 2001). In contrast, one study on ‘Delicious’ apple found that all four components of wax increased during fruit ripening and that this coincided with the climacteric rise in ethylene (Ju & Bramlage, 2001). These results indicate that ripening stage may be more important than specific harvest date for some apple cultivars when they have reached a particular degree of ripeness (Kevers *et al*., 2011). Furthermore, the specific wax compositional changes, including OA and UA, might be influenced by cultivars.

The effects of RA and CA storage methods on OA and UA concentrations in apple peel were studied in Paper II (see Table 2 in Paper II). No difference was found for UA concentration in the peel of apples after RA and CA storage. RA and CA storage showed inconsistent effect on OA concentration in the peel of apples from different harvest times and study years. When the UA

concentration in peel of apples at harvest time and after storage were compared, it was found that it remained stable after storage in most cases, and only the UA concentration in the peel of apples on the second harvest time increased, by 4.3%, after RA storage in 2012. The OA concentration in apple peel decreased in some cases after RA and CA storage compared with at harvest. Some wax component changes during storage have been confirmed to be associated with ethylene production and fruit ripening (Dong *et al*., 2012). In this thesis, cultivar variation in OA and UA concentrations in apple peel were also observed during RA storage (Table 4 in Paper I).

4.4 Effect of ozone treatment on the concentration of triterpenes and phenolic compounds

The use of ozone on fresh fruits and vegetables for post-harvest sanitisation and decay control has been investigated widely (Boonkorn *et al*., 2012; Ozkan *et al*., 2011; Gabler *et al*., 2010; Palou *et al*., 2002). However, most studies have focused on the antimicrobial effect of ozone treatment on post-harvest fruits and vegetables, and research on the changes in bioactive compounds in the fruit due to ozone treatment is quite limited. Therefore, the effect of ozone treatment before and during apple storage on triterpenes and phenolic compounds was studied.

Treatment of 'Santana' apples with ozone before and during storage did not have any influence on the OA and UA concentrations in the peel after one month or four months of cold storage (Figure 1 and Tables 1 & 2 in Paper III). After one month of cold storage, the OA concentration in the peel of 'Amorosa' increased by 16.8% to 23.6% due to different ozone treatments before storage (Table 1 in Paper III). In contrast, low doses of ozone treatment during four months of storage decreased the OA concentration in the peel slightly (Figure 1 in Paper III). The UA concentration in the peel of 'Amorosa' was generally not influenced by either of the ozone treatments before storage, or by low doses of ozone treatment during storage (Figure 1B and Table 2 in Paper III). A previous study of carrot found that immediately after different periods of treatment (1000 nL/L ozone treatment for 1, 2, or 4 days), most headspace terpene volatiles concentrations were elevated compared with the control (Forney *et al*., 2007). Paper III showed that the UA concentration in apple peel was almost unaffected by ozone treatment, but the OA concentration showed different responses to different ozone applications. The results indicated that UA was not sensitive to ozone treatment and that OA could be influenced by ozone treatment with cultivar-specific differences, although the changes were within the range of natural variation occurring during storage.

The effects of three kinds of short-term ozone treatments (gaseous ozone, ozonated water and a combination of ozonated water and gaseous ozone treatments) before storage on phenolic compounds in apple peel were studied. After one month of cold storage, total polyphenolic concentration in the peel of 'Amorosa' and 'Santana' remained stable after ozonated water and gaseous ozone combination treatment, compared with untreated control, but the individual gaseous ozone or ozonated water treatments slightly decreased the total polyphenolics content in the peel of both cultivars (Table 3C in Paper III). After one month of cold storage, total flavonols concentration in the peel of both 'Amorosa' and 'Santana' was decreased significantly by all three kinds of ozone treatments. However, the concentration of total hydroxycinnamics and total procyanidins in the peel of 'Amorosa' and the total anthocyanin concentration in the peel of 'Amorosa' and 'Santana' did not change after ozone treatments (Table 3C in Paper III). In contrast, in a previous study, short-term gaseous ozone treatment (10 μL/L, 10 mins) of tomato increased the total phenolics content immediately after ozone treatment and after six days of storage at 20 °C (Rodoni *et al*., 2009). Another study showed that when short-term gaseous ozone treatment (8±0.2 mL/s, 0-30 mins) was applied to fresh-cut pineapple, banana and guava, the total phenolics and flavonoids concentrations in pineapple and banana increased when ozone treatment was applied up to 20 mins, but the concentrations in guava decreased (Alothman *et al*., 2010). Therefore, short-term ozone treatment before storage can increase the concentration of phenolic compounds in some fruit, but no effect and decreased concentrations of phenolic compounds have also been observed.

Low doses of ozone treatment during four months of storage affected the content of phenolic compounds in the peel of 'Amorosa' and 'Santana' differently (Table 4 in Paper III). In general, the total polyphenolics content in the peel of 'Amorosa' increased by 6.7% after ozone treatment during storage, but the opposite result was observed for 'Santana', with 7.1% decreased total polyphenolics content after ozone treatment. Ozone treatment increased the concentrations of total procyanidins, total flavonols and total dihydrochalcones in the peel of 'Amorosa' by 6.9%, 10.8% and 11.9%, respectively. For the individual compounds, the increased total polyphenolics in 'Amorosa' could be attributed to the increase in the concentration of epicatechin (10.9%), quercetin 3-galactoside (15.0%), quercetin 3-rhamnoside (39.2%) and phloretin 2´-xylglucoside (14.0%). In the peel of 'Santana', the concentration of total anthocyanin, total flavonols and total dihydrochalcones was decreased by 22%, 9.8% and 8.9%, respectively, by ozone treatment during storage, but total hydroxycinnamic acids concentration was increased by 9.5%. The individual compounds which decreased in concentration included procyanidin B2,

quercetin 3-xyloside, quercetin 3-arabinopyranoside, quercetin 3-arabinofuranoside and phloridzin. These results indicate that the effect of ozone treatment on phenolic compounds is cultivar-dependent. In previous investigations, similar cultivar-related sensitivity to ozone treatment has been found for strawberry growth (Drogoudi & Ashmore, 2002) and on quality parameters of strawberries grown in an ozone-enriched atmosphere (Keutgen & Pawelzik, 2008). One study on 'Autumn Seedless' table grapes showed that flavan-3-ols (also referred to as flavanols) and total polyphenolics content increased after cold storage and during the retail period when continuous gaseous ozone was applied during storage (Artés-Hernández *et al*., 2007).

The ozone tolerance of plant tissue has been suggested to be related to the presence of phenolic compounds, and ozone can induce or enhance the production of the phenolics in plants (Forney, 2003). The auto-decomposition of ozone can produce different free radicals, such as hydroperoxyl (H2O●), hydroxyl (●OH), and superoxide (●O2-) (Hoigné & Bader, 1983). When plants and fruits are exposed to ozone, the phenolic compounds together with other antioxidant compounds can react with the free oxygen radicals and thereby prevent oxidative injury to other molecules such as lipids and proteins (Larson, 1995; Moldau, 1998). Therefore, the phenolic compounds might show temporarily reduced concentrations after ozone treatment. This was demonstrated in the present thesis, where the total polyphenolics concentration was decreased by ozone treatment after one month of storage. On the other hand, ozone treatment might cause oxidative stress by the formation of the active oxygen species (AOS), which can activate the plant defence mechanism, including the defence genes (Koch *et al*., 1998). In summary, the final polyphenolics concentration depends on the balance of synthesis and decomposition of these compounds and is related to the plant defence mechanism. However, the cultivar-dependent sensitivity to ozone and the concentration and treatment time of ozone should be considered in practical application.

4.5 Antifungal effect of triterpene-enriched crude extract from apple peel

Triterpene-enriched suspended water was applied to culture plates of four post-harvest pathogens to investigate the antifungal activity. The two treatments tested were 0.5 mL TESW (TESW-1) and l mL TESW (TESW-2).

In *in vitro* investigations, TESW-1 showed significant inhibition of mycelial growth compared with the water control for all four pathogens (Table 2 in Paper IV). TESW-2 showed significant inhibition of mycelial growth of all

four pathogens during incubation for three and seven days. Furthermore, TESW-2 showed a significantly higher inhibition effect than the water control on all four pathogens after three days of incubation, and the same trend was observed for three pathogens after seven days of incubation, but not for *N. perennans*. TESW-1 showed significant inhibition of mycelial growth of *P. expansum*, *M. fructigena* and *C. acutatum* after three days of incubation, but significant inhibition was only observed for *M. fructigena* after seven days of incubation. For TESW-1 treated plates, a significantly higher inhibition effect than with the water control was found for *M. fructigena* and *C. acutatum* after three days of incubation and for *P. expansum*, *M. fructigena* and *C. acutatum* after seven days of incubation.

Treatment with TESW inhibited conidial production to different extents for the four pathogens (Table 3 in Paper IV). The production rate of conidia of *P. expansum*, N. perennans and *C. acutatum* was significantly lower when they were collected from plates treated with TESW-1 and TESW-2 compared with plates given the water treatment, but no significant differences were observed between TESW-1 and TESW-2 treatments.

Previous studies have shown that triterpene-enriched plant extracts from some leaves have antifungal activity on various plant and zoonotic pathogens. For example, UA isolated from *Breonadia salicina* leaves demonstrated antifungal activity on seven plant pathogens, including *P. expansum* and *C. gloeosporioides* (Mahlo *et al*., 2013). The crude stem bark extract of *Ficus drupacea* L. also showed antifungal activity on seven fungi, including two *Penicillium* species, and this crude extract contained oleanolic acid (Yessoufou *et al*., 2015). In Paper IV, the triterpene-enriched crude extracts from apple peel also showed similar antifungal activity on post-harvest pathogens. Pharmacological properties and cytotoxic activities of triterpenes on different cancer cell lines have been widely studied. For UA, it has been verified that two hydrogen-bond forming groups, a hydroxyl group at position 3 and a carboxylic acid group at position 28, exhibit cytotoxic activity (Ma *et al*., 2005). Ursolic acid treatment also resulted in the induction of apoptosis and blockage of the cell cycle progression in the G1 phase in a study by Hsu *et al*. (2004). These observations may be of relevance in the context of this thesis. Until now, no clear conclusion has been drawn on the antifungal mechanism of triterpenes or their derivatives. Based on the results in Paper IV, it can be hypothesised that the antifungal mechanism of triterpene might have some similarity or relationship with apoptosis induction mechanisms, but further studies are required.

5 Conclusions and future perspectives

This thesis investigated pre-harvest and post-harvest effects on the concentrations of triterpenes and phenolic compounds in apples, in order to understand how different factors affect these bioactive compounds after harvest. In addition, the antifungal activity of apple peel crude extract was investigated. The key findings and main conclusions are as follows:

- Cultivar is the dominant factor in terms of levels of triterpene and phenolic compounds in apple fruit. Therefore, breeding programmes, including selection of existing varieties and development of new varieties, can be an effective way to increase the concentrations of these bioactive compounds in apple fruit.

- Sun exposure can have an impact on the concentration of triterpenes in apple peel. Since light is the major factor for sun and shade effects, further research is required on the mechanism of light effects on triterpene synthesis.

- Bruising increased the oleanolic acid concentration in 'Discovery', and the change in triterpene concentration induced by bruising showed cultivar variation. The increased oleanolic acid concentration after bruising was most likely a fruit defence reaction induced by the mechanical injury.

- The triterpene concentration was higher in fruit from rootstock 'MM106' than 'M9'. This difference might be related to the rootstock genotype.

- Ursolic acid concentration in apple peel was not affected by different harvest times in apples consumed directly after harvest. After cold storage, a high concentration of triterpene could be maintained when the fruit was harvested about one week earlier than the commercial harvest time, but the triterpene concentration differences between harvest times were relatively small.

- Ursolic acid concentration in apple peel can remain stable after cold storage in most cases, while oleanolic acid concentration in apple peel decreases to a limited extent in some cases after cold storage. For ursolic acid concentration in apple peel, no difference was found between regular atmospheric (RA) and controlled atmospheric (CA) cold storage, but oleanolic acid concentration showed differing responses to RA and CA cold storage. These results indicate that storage methods have only a small influence on ursolic acid concentration and some influence on oleanolic acid concentration.

- In the peel of apple fruit, ursolic acid concentration was not sensitive to ozone treatment, but oleanolic acid concentration could be affected by ozone treatment and the response showed cultivar-specific differences. Thus changes in triterpenes might be due to induction of the defence system by ozone treatment.

- Ozone treatment did not result in any major changes in the concentrations of triterpenes or phenolic compounds, and the changes found were within the range of natural variation occurring during storage.

- Low-dose ozone treatment during storage did not affect total polyphenolics content in the flesh of ‘Amorosa’ and ‘Santana’, but it increased it in ‘Amorosa’ peel, and decreased it in ‘Santana’. Short-term ozone treatment before storage reduced total polyphenolics content in apple peel in some cases in both cultivars after one month of storage, while an inconsistent response was observed for total polyphenolics in apple flesh. These results indicate that total polyphenolics have tissue-specific and cultivar-dependent sensitivity to ozone treatment. The concentration and

treatment time of ozone should also be considered in practical application.

- Triterpene-enriched suspended water (TESW) showed significant inhibition of mycelial growth and also a higher inhibition effect than the water control for all pathogens studied. TESW inhibited conidia production to differing extents in different pathogens. The antifungal mechanism of triterpenes might have some similarity or relationship to the apoptosis induction mechanism, but further studies are required.

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Acknowledgements

It is not an easy journey to arrive the end of doctoral studies. It is long because of the frustration and failure in the experiments; it is short because of the company, support and encouragement from all lovely people around. Now I would like to express my acknowledgements to all those people who accompanied me on my PhD journey.

To my supervisor Marie Olsson, who provided me with the opportunity for doctoral studies and also guided me to the destination. I am very grateful to you for accepting me as a PhD student. Your encouragement and trust always gave me confidence and strength. Thanks for all meetings, discussions and patient manuscript modification to improve my scientific and writing abilities. Thanks also for your professional expertise and rigorous attitude to improving my academic knowledge and research motivation.

To my co-supervisor, Dr. Ibrahim Tahir, thank you for all kind support and expert advice on experiment analysis and manuscript writing. I am grateful for all you have taught me during the fungal experiment. Sincere thanks to you for helping me with the apple harvest and transportation.

To my co-supervisor, Dr. Larisa Gustavsson, thank you for many helpful suggestions for the experiment. I am also thankful to you for correcting manuscripts in a timely way and for your willingness to help.

I am grateful to Karl-Erik Gustavsson. Many thanks for your conscientious and responsible assistance in the laboratory. Thank you also for bringing humour and relaxed working environment. Thanks to you, I now have a deeper understanding of ‘never give up’. I have learned so much from you, both in analytical skills and positive attitude towards life.

I am thankful to Jan-Eric Englund for his professional and patient help with statistics.

Many thanks to Eva Johansson and Rumune Kuktaite for organising some activities in the group so that I could experience Swedish culture.

I am thankful to Maria Luisa Prieto-Linde for helping with laboratory materials. Thank you also for the warm invite with great food at your home.

I am thankful to Staffan Andersson and Lars Mogren, for your helpful advice with the lab work and your knowledge of Swedish culture. Also thanks Lotta for sharing the happy experience of glögg cooking.

Many thanks to Helen Lindgren and Annelie Ahlman all management of laboratory supplies and equipment so that I could focus on the experiment.

I'm thankful to Salla Marttila for the helping of microscope adjustment.

I am thankful to Camilla Stjärnäng for the helping of financial reports, and to Helena Persson Hovmalm, Erland Liljeroth and Cecilia Alsved for all administrative procedures about PhD education. Thanks to Christina Johansson for assistance on the accommodation.

I am thankful to Anna and Kaul Wisen in Kivik orchard for the management and transportation of my apple fruit.

Many thanks to Li-Hua Zhu and Xue-Yuan Li for inviting me to your home many times to spend the traditional Chinese festivals together with rich food. Your sweet dessert made me feel home warmth.

I'm thankful to Ann-Sofie Fält for inviting me for home party, discussion and hospitality.

Special thanks to Lijie Zhong for your friendship and constant support. Thanks for your company and help, not only in the laboratory but also in daily life and many travels. You can always understand and enlighten me more than a sister. I am not lonely because you bring the sunshine to my life. Thanks for everything and I wish you all the best every day.

I am thankful to Dr. Liaqat Ali, for your support and help with laboratory work. Your encouragement made me brave. Your friendship and hospitality made my campus life interesting.

Many thanks to Dr. Marjan Ghasemkhani and Dr. Masoud Ahmadi-Afzadi for your friendship and always enthusiastic help.

I would like to thank all the faculty and staff members of Plant Breeding Department and the previous colleagues in Biosystems and Technology Department in Alnarp for the friendly working environment.

Many thanks to the previous and present PhD friends, Abel, Alphonsine, Bahromiddin, Bill, Cheng, Dharani, Elaine, Emelie, Evelyn, Esayas, Faiza, Faraz, Firuz, Kibrom, Helle, Joel, Linda-Marie, Li-Ying, Mahbubjon, Maja, Marufkul, Mbaki, Mehboob, Michael, Mira, Samareh, Santosh, Sonja, Rui, Tiny, Tora, Xiao-Ming, Xiu-Juan, Zakir, Zeratsion,for sharing events and experiences with me.

I would like to thank Gösta och Anna-Birgit Henrikssons fund for providing me the travel grant for international conferences. I am grateful to Chinese Scholarship Council for providing PhD scholarship for my PhD study in Sweden.

I am grateful to my supervisor, Xiao-Lin, Ren, during my Master study in China. You have supported and encouraged me for the abroad study. Thanks to you and Hui-Ling Zhou together to be my guarantors for the abroad study.

Finally, I would like to thank my parents and sister for the unconditional love, support, encouragement and understanding.