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FRUIT RIPENING / SCALD RELATIONSHIP IN APPLE

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Abstract

The ripening stage of apple fruits at harvest is the main factor influencing fruit quality during the cold storage period that lasts several months and give rise to physiological disorders in fruits of susceptible cultivars. In particular, superficial scald is connected to α-farnesene oxidation, leading to fruit browning. Therefore, the assessment of the optimal ripening stage at harvest is considered to be crucial to control the overall quality, the length of storage life and the scald incidence. However, the maturity indexes traditionally used in the horticultural practice do not strictly correlate with fruit maturity, and do not account for the variability occurring in the field. Hence, the present work focused on the determination of apple fruit ripening with the use of an innovative, non-destructive device, the DA-meter. The study was conducted on 'Granny Smith' and 'Pink Lady' cultivars, which differ in scald susceptibility. Pre- and post- harvest ripening behavior of the fruits was studied, and the influence of ripening stage and treatments with 1-MCP were evaluated in relation to scald development and related metabolites. I_{AD} was shown to be a reliable indicator of apple ripening, allowing cultivar-specific predictions of the optimal harvest time in different growing seasons. I_{AD} may also be employed to segregate apple fruits in maturity classes, requiring different storage conditions to control flesh firmness reduction and scald incidence. Moreover, 1-MCP application is extremely effective in reducing superficial scald, and its effect is influenced by fruit ripening stage reached at harvest. However, the relation between ethylene and α-farnesene was not entirely elucidated. Thus, ethylene can be involved in other oxidative processes associated with scald besides α-farnesene regulation.

Key words: Apple, Quality, Superficial scald, Maturity, I_{AD}, 1-MCP, Harvest date, Ethylene, α-farnesene

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1. Introduction

Apple fruit (*Malus domestica* Borkh.) is one of the main fresh consumed horticultural products, with a large marketing period from immediate on-farm sales to marketing after several month of storage. The ripening stage at harvest is the most important factor in maintaining superior fruit quality, especially since apples could be stored under cold temperatures for long periods (Shewfelt, 1999; Kader 1999). Apple fruits are commonly stored at low temperatures, between -1 and 4°C. However, under such storage conditions, sensory attributes are compromised, along with the possibility of physiological disorders related to chilling injury and oxidative stress. Among these disorders is the superficial scald, which emerges as a brown or black patch on the surface of the fruit skin. Several reports claimed that superficial scald in apples is considered as the most important cause of fruit loss during cold storage (Bain and Mercer, 1963; Ingle and D'Souza, 1989; Watkins *et al*., 1995; Eccher Zerbini *et al*., 1997; Kupferman, 2001; Kupferman 2002). Apple superficial scald is an expression of damage and death within the first hypodermal cell layers, affecting the external appearance of the fruit (Bain and Mercer, 1963; Ingle and D´ Souza, 1989; Watkins *et al*., 1995). Usually, scald symptoms develops after the removal of fruits from cold storage, though it may be visible prior to removal of fruits to warmer temperatures after extended storage and severe injury (Watkins *et al*., 1995; Bain and Mercer, 1963; Ingle and D'Souza, 1989; Kupferman, 2001). Thus, the development of strategies to improve scald control appears essential as this physiopathy is considered as a serious problem during storage and subsequent handling of apples.

1.1. Superficial scald etiology

Superficial scald is a type of chilling injury occurring in two stages: the damage inducing events are separated in time from symptom development, which involve cellular degradation (Watkins *et al*., 1995). Despite many years of investigation, the biochemical mechanisms underlying superficial scald is still under discussion. The prevailing hypothesis holds that oxidation products of the sesquiterpene α-farnesene, a naturally occurring volatile terpene in the apple fruit, are directly involved via generation of free radicals. These oxidation products, recognized as conjugated trienols (CTols), induce an

oxidative damage to the cell membranes, eventually leading to cell death in the first hypodermal cell layers (Bain and Mercer, 1963; Watkins *et al*., 1995; Mir and Beaudry, 1999; Whitaker and Saftner, 2000; Whitaker *et al*., 2000; Whitaker, 2004, 2008, 2013; Lurie and Watkins, 2012). A marked increase in α-farnesene synthesis occurs shortly after scald-susceptible fruits are placed in air storage, and oxidation of α-farnesene to conjugated trienols (CTols) takes place in the following six to eight weeks (Whitaker, 2004, 2008). Besides CTols, other oxidative products generated by α-farnesene oxidation, such as 6-methyl-5-hepten-2-one (MHO), can intensify scald symptoms (Watkins *et al*., 1995; Mir and Beaudry, 1999; Whitaker and Saftner, 2000; Whitaker *et al*., 2000; Whitaker, 2004, 2008, 2013; Lurie and Watkins, 2012).

Variation in the antioxidant defense mechanisms required to scavenge radicals and counteract oxidative stress is suggested to play a key role in susceptibility or resistance to scald (Whitaker, 2008; Whitaker, 2013). Some researchers have proposed that scald arises as a result of general oxidative stress, and that the autoxidation of α-farnesene is a secondary manifestation of unregulated free radical production (Rao *et al*.,1998; Rupasinghe *et al*., 2000). Since superficial scald is a symptom associated to a stress condition, the role of phenols was also considered, since their antioxidant activity may contribute against scald development. In addition, the oxidation of phenols by polyphenol oxidase may also contribute to the development of scald symptoms (Ju *et al*., 1996; Abdallah *et al*., 1997; Golding *et al*., 2001; Treutter, 2001; Lurie and Watkins, 2012, Whitaker, 2013).

The importance of ethylene in scald development has also been recognized, since it is a promoter of α-farnesene synthesis (Du and Bramlage, 1994; Fan and Mattheis, 1999; Ju and Curry, 2000; Watkins *et al*., 2000; Whitaker, 2008; Rupasinghe *et al*., 2000; Shekarchi *et al*., 2009; Lurie and Watkins, 2012; Whitaker 2013). Although the mode of action of ethylene has not been completely elucidated yet, the inhibition of ethylene synthesis is seen as a promising strategy for scald control.

1.1.1. Factors influencing apple scald incidence

Incidence and severity of superficial scald in apple fruits can be affected by several pre-harvest factors, ripening stage at harvest and storage conditions.

Scald susceptibility varies from year to year for a given cultivar as the consequence of the influence of weather and growth conditions. Fruits grown in warm and dry climates are more susceptible than those grown in cooler conditions. In addition, low night temperatures before harvest period reduce the occurrence of scald (Ferguson *et al*., 1999; Thomai *et al*., 1998; Kupferman, 2001; Diamantidis *et al*., 2002). Plant nutrition also influence the risk of scald incidence, that is higher in fruits with high nitrogen and low calcium, or high potassium content (Ingle and D´Souza, 1989; Ferguson *et al*., 1999; Kupferman, 2001). Moreover, other factors such as exposure to the sun, fruit position in the canopy, and fruit color development also influence fruit susceptibility to scald (Ingle and D´ Souza, 1989; Ferguson *et al*., 1998; Kupferman, 2001).

Aside from the pre-harvest conditions, other major factors that influence the occurrence of scald are the genetic background of each cultivar and the maturity stage of apples at harvest (Ingle and D'Souza, 1989; Watkins *et al*., 1995; Eccher Zerbini, 1997; Watkins *et al*., 2000; Erkan and Pekmezci, 2004; Whitaker, 2004; Lurie and Watkins, 2012). Different apple cultivars vary greatly in scald susceptibility, and are classified accordingly with their degree of susceptibility. 'Pink Lady', 'Gala', 'Empire', 'Golden Delicious', 'Fuji', 'Braeburn' were identified to be less susceptible to scald while 'Granny Smith', 'Delicious', 'Cortland', 'Low Rome' were classified to be more susceptible to scald (Ingle and D'Souza, 1989; Little and Holmes, 2000; Whitaker, 2004; Tsantili, *et al*., 2007; Trivedi, 2010, Lurie and Watkins, 2012). The underlying reasons in the variability in susceptibility to storage scald among apple cultivars are not yet elucidated. Furthermore, superficial scald susceptibility of apples is strongly associated to fruit maturity at harvest. Several works reported that delaying the harvest date reduces scald incidence, with more mature fruits exhibiting less symptoms compared to immature fruits (Ingle and D'Souza, 1989; Eccher Zerbini, 1997; Watkins *et al*., 2000; Erkan and Pekmezci, 2004; Calvo and Candan, 2010). However these reports are limited to maturity of fruits according to harvesting date, while the possibility of having heterogeneous fruits based on maturity stage in one harvest date was overlooked.

1.1.2. Control measures of superficial scald

The most effective postharvest scald control treatments are those related in preventing the oxidative process, and inhibiting ethylene synthesis. The most utilized agent in preventing

the oxidation process is the diphenylamine (DPA), which was reported to effectively control scald by means of postharvest dips, use of label, thermofogging or aerosol treatments (Du and Bramlage, 1994; Mir and Beaudry, 1999; Whitaker, 2004). However, the use of DPA was banned in some countries, following the increasing concern regarding DPA toxicity and leftover of residual (Lurie and Watkins, 2012).

Controlled atmosphere (CA) storage was also reported to greatly reduce the incidence of scald. It was demonstrated that both high carbon dioxide and low oxygen concentrations are effective in scald control (Kupferman, 2001; Zanella, 2003; Watkins and Nock, 2005; Lurie and Watkins, 2012). Low oxygen concentration could reduce or inhibit oxidation of α-farnesene to CTols, and 1 to 1.5% oxygen almost completely prevented scald incidence (Kupferman, 2001; Zanella, 2003; Watkins and Nock, 2005; Lurie and Watkins, 2012). Despite this, however, there is a limited range of oxygen concentration for apple storage, as the reported concentrations of oxygen, although reducing scald, were also found to cause fermentation on some apple cultivars (Watkins and Nock, 2005; Lurie and Watkins, 2012).

An alternative to the use of antioxidants or CA for scald control is the use of 1-methylcyclopropene (1-MCP) to inhibit ethylene responses. Since the importance of ethylene on scald development have been identified, several studies have been conducted to demonstrate the efficiency of 1-MCP in scald control and inhibition (Fun and Mettheis, 1999; Watkins *et al*., 2000; Watkins and Nock, 2005; Tsantili *et al*., 2007; Jung and Watkins, 2008; Shekarchi *et al.*, 2009; Trivedi *et al*., 2010). 1-MCP, by the inhibition of ethylene perception and autocatalytic production, is associated with lower α-farnesene and CTol accumulation, consequently limiting the availability of substrate for oxidation (Rupasinghe *et al*., 2000; Watkins *et al.*, 2000; Tsantili *et al*., 2007; Arquiza *et al*., 2005; Jung and Watkins, 2008, Shekarchi *et al*., 2009). The efficiency of 1-MCP as a scald control agent is also influenced by the fruit maturity stage at the time of application, storage conditions and the time-span between harvest and treatment (Watkins *et al*., 2000; Watkins and Nock 2005; Watkins, 2006; Jung and Watkins, 2008; Calvo and Candan, 2010).

1.2. Ripening stage at harvest and new approaches to define optimal harvest time

Fruit maturity stage at harvest is crucial as it determines both the final quality of the commodities, and their potential for storage. In apples, as in other fruits, the overall quality is defined by several attributes of the fruit, such as sugar content, aroma, skin color, according to its marketing destination. The challenge is to define a harvest period based on those attributes to ensure the best quality for the different markets. Thus, identifying the optimal ripening stage at harvest is recognized to be a main issue to define the storage life and quality of fruits after storage. Apples harvested too early on the season will not develop adequate sugars and flavor, and are more prone to shriveling and poor coloration. Likewise, less mature fruits are more susceptible to superficial scald and bitter pit (Watkins *et al*., 1995; Eccher Zerbini *et al*., 1997; Kader, 1999). On the other hand, fruits harvested late in the season could be overripe, with a higher tendency to become exceedingly soft and mealy. Such conditions decreases the potential of fruits for storability, while increasing the risk of other disorders such as senescent breakdown (Eccher Zerbini *et al*., 1997; Kader, 1999; Johnston *et al*., 2002). Hence, a reliable parameter that can indicate the proper harvest timing of apples is needed to prevent the consequences of anticipated or delayed harvesting.

Several indices have been used to identify the optimum physiological stage for harvest of fruit. Parameters commonly considered are related with changes in the skin color, texture, starch degradation, soluble sugars, acidity and aroma compounds, which in general defines the final quality of the fruit (Kader, 1999). Moreover, other attributes like respiration rate and ethylene production, related to fruit ripening stage, are used as physiological parameters to define the appropriate harvest period (Beaudry *et al*., 1993; Kader, 1999; De Castro *et al*., 2007).

In general, more than one parameter is needed to describe the optimal physiological stage for harvest. In some instances, parameters are combined and expressed as a single index, such as the Streif index, which expresses the relation between flesh firmness, soluble solids content and starch degradation. Streif index can be used as an indicator of physiological maturity during the last period of fruit growth, since it reduces seasonal variability (Streif, 1996; De Long *et al*.,1999, Zude-Sasse *et al*., 2002; Tijskens *et al*., 2008). However, the Streif index requires the destructive analysis of fruits. Destructive methods are commonly used because in general, they are reliable and descriptive. However, they do not allow the

possibility of repeated measurements on the same sample over time. In addition, sampling can only be performed in a limited quantity of fruits, hence may not represent the variability on tree or in the orchard.

In the last decades, research perspective has been focused in the use of non-destructive techniques to estimate fruit physiological stage. Such techniques make it possible to define maturity traits of large fruit samples, while, at the same time, enable to monitor physiological changes over time on the same sample. These methods comprise mechanical technologies, related to texture based on deformation; impact test or sonic and ultrasonic vibrations; electrochemical technologies based on gas detectors like electronic nose; and electromagnetic technologies based on optical properties (radiowave, microwave, ultraviolet, visible light, infrared, X-ray and gamma-ray radiation), fluorescence and magnetic resonance (Abbott, 1999). Among these techniques, Near Infrared Spectroscopy (NIRs) is largely diffused in the practice to estimate the physiological stage or quality parameters on fruits and vegetables (Liu and Ying, 2005; Nicolaï *et al*., 2007; Liu *et al*., 2008; Costa *et al*., 2009; Bertone *et al*., 2012).

On apples, NIRs technology applications focus on the determination or prediction of firmness, total soluble solids, starch degradation, background color, Streif index and sensory attributes (Peirs *et al*., 2000; McGlone *et al*., 2002; Mehinagic *et al*., 2004; Peirs *et al*., 2005; Liu and Ying, 2005; Zude *et al*., 2006). Furthermore, since chlorophyll degradation takes place during apple maturity, changes in chlorophyll content in apple skin measured by spectrophotometry, or as reflectance of the apple surface, is also a useful parameter to asses fruit physiological stage (Knee, 1972; Knee, 1980; Merzlyak *et al*., 2003; Zude-Sasse *et al*., 2002; Solovchenko *et al*., 2005). Likewise, through the analysis of chlorophyll content, qualitative parameters and ethylene production can be predicted or estimated (Nicolaï *et al*., 2007; Costa *et al*., 2009, Bertone *et al*., 2012, Betemps *et al*., 2012), showing the feasibility of use chlorophyll content as a ripening marker.

Most of the mentioned non-destructive methods are limited by the need of repeated calibrations and complex data processing (Nicolaï *et al*., 2007; Costa *et al*., 2009). To overcome these limitations, the DA-meter, a portable and non-destructive device to assess fruit ripening has been developed (Ziosi *et al*., 2008; Costa *et al*., 2009). This instrument is based on NIRs technology, which reads the absorbance difference between two wavelengths (A670 and A720) expressed as an Index of Absorbance Difference (I_{AD}),

correlated to the actual chlorophyll-a content in the flesh fruit (Ziosi *et al*., 2008). Since chlorophyll degradation is part of the natural maturity process, the I_{AD} provides an indication of the state of ripening using chlorophyll content as a physiological index. I_{AD} value usually ranges from 2.2 to 0.2, where a higher number indicates a less ripe fruit, and depends on the cultivar (Ziosi *et al*., 2008; Costa *et al*., 2009; Bonora *et al*., 2013a,b).

Up to now, the use of the DA-meter was mainly studied on stone fruits. Some studies on peaches, nectarines and apricots have demonstrated the correlation between the I_{AD} and several parameters of the maturation process, such as ethylene production, fruit quality traits, and transcription of ripening-related genes (Ziosi *et al*., 2008; Costa *et al*., 2009; Costa and Noferini, 2013; Bonora *et al*., 2013a,b; Shinya *et al*., 2013). It has also been demonstrated that fruits sorted at harvest according to the I_{AD} end up in homogenous ripening classes, with each class possessing a distinct quality behavior during shelf-life and consumption (Costa *et al*., 2009; Shinya *et al*., 2013). Up to present, few studies have been done regarding to the use of the I_{AD} as a ripening index on pome fruits. On apple fruits, the use of the I_{AD} has been recently studied for the determination of quality traits of 'Gala', 'Starking', 'Granny Smith' and 'Pink Lady' (Costamagna *et al*., 2013; Nyasordzi *et al*., 2013), demonstrating the relevance of this index for ripening and quality assessment.

The optimum harvest window for apple fruits has been long studied based on bloom date, degree-day, interaction of climatic variables, destructive techniques based on fruit quality parameters, even on molecular analysis (Beaudry *et al*., 1993; De Long *et al*.,1999; De Castro *et al*., 2007; Kaack and Lindhard Pedersen, 2010; Kaack and Pedersen, 2011; Hertog *et al*., 2011; Neuwald and Streif 2012). While such studies can provide the needed information on identifying the optimum harvest date, most of the information generated from the studies requires trained personnel and analytical procedures, which are also time consuming, and in some cases expensive to be employed in practical use.

Furthermore, some studies have shown the possible use of non-destructive methods based on VIS-NIRs technologies to estimate fruit maturity parameters and quality traits, and its potential use for optimal harvest prediction (Peirs *et al*., 2000; McGlone *et al*., 2002; Peirs *et al*., 2005; Zude *et al*., 2006; Bertone *et al*., 2012; Zude-Sasse *et al*., 2002). Therefore, the use of DA-meter could be a reliable, non-destructive, and easy-to-use alternative for optimal harvest timing prediction, with a special regard for the minimization of scald incidence.

2. Aims of the thesis

Superficial scald incidence is extremely variable, and among the several factors that influence it, the ripening stage at harvest is considered to be the most important one. Identifying the optimum ripening stage for harvest is considered to be crucial to the control of the overall quality of fruits at harvest and after storage, the length of storage life of the fruits, and the incidence of scald. Hence, the need of a simple, though efficient optimized method to evaluate the ripening stage and optimum harvest time of apple fruits are important in the industry. This will facilitate the management of apple fruits to attain improved quality through minimizing the incidence of fruit scald. Moreover, this can provide further understanding of the metabolism involved in scald development and the possible treatments that could be utilized to control scald incidence.

The present research work focused on apple fruit ripening determination with the use of an innovative, non-destructive device, the DA-meter, associated to a better understanding and control of superficial scald. To accomplish these purposes, studies were conducted 'Granny Smith' and 'Pink Lady'. The two cultivars differ in their degree of susceptibility to scald. Pre- and post- harvest ripening behavior of the fruits was studied, and the influence of ripening stage and treatments with 1-MCP were evaluated in relation to scald development and related metabolites.

This thesis includes six chapters (3 to 8) that were structured as a scientific papers, with a proper Title, Introduction, Materials and Methods, Results and Discussion, Conclusions, References, Figures and Tables. Chapters 3 and 4 focus on apple fruit ripening assessed with a non-destructive device (DA-meter) and optimal harvesting time prediction. Chapters 5 focus on post-harvest quality. Chapter 6 to 8 focus on apple fruit scald incidence and regulation, in the bases of the preliminary results obtained on the research presented on chapter 6, the latter research works were developed (chapters 7 and 8). Finally, General Conclusions are reported in Chapter 9.

Some of the results of the present research work were presented as a poster at the X Giornate Scientifiche SOI (2013), title: "Utilizzo del DA-meter per la gestione della conservazione dei frutti di melo e per il controllo del riscaldo". Furthermore, part of the

reported work have been done in collaboration with the Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige (Trento), Italy.

3. Definition of apple fruit ripening stage with the use of innovative (Index of Absorbance Difference -I_{AD}) and of traditional methods

3.1. Abstract

The degree of chlorophyll degradation in the fruit flesh can be exploited as an indicator of apple fruits maturity. New instruments, such as the DA-meter, allow to measure chlorophyll degradation non-destructively and, therefore, to monitor fruit maturity and quality attributes development. In the present work the DA-meter, which express chlorophyll content as an index of absorbance difference (I_{AD}), has been used, during pre-harvest and at harvest, to monitor fruit quality and maturity development in 'Granny Smith and 'Pink Lady' apples in two consecutive years. The I_{AD} trends were correlated with the traditional measurement of the major quality and maturity traits (flesh firmness, starch degradation, total soluble solids, Streif coefficient and ethylene production). The results show that I_{AD} is a reliable indicator of apple maturity in all the tested cultivars and it allows to group fruit in homogeneous maturity batches. Finally, I_{AD} results collected in two consecutive years are comparable, thus showing that I_{AD} can become a standard descriptor for maturity assessment.

3.2. Introduction

The achievement of physiological ripening at harvest is the crucial condition needed for fruits to fully express their quality during the storage, marketing and consumption. Fresh fruit quality is a combination of attributes related to fruit maturity and ripening. These two physiological stages are overlapping and they are influenced by several external and internal factors (Beaudry *et al*., 1993; Kader, 1999; Kays, 1999). Conventionally, apple fruits are harvested close to the onset of ripening, but they are not entirely ripe yet (Kader, 1999). Identify the proper harvest is fundamental to obtain the best fruit quality for consumption and it ensures the highest profits. Indeed, the harvesting time is also chosen

on the basis on final market (local or export) and the post-harvest strategies. However, fruits harvested too early do not reach an adequate organoleptic quality or skin colouration, and they are more prone to physiological disorders such as shrivelling, superficial scald and better pit (Watkins *et al*., 1995; Eccher Zerbini *et al*., 1997; Kader, 1999). On the other hand, fruits harvested too late are overripe and they tend to become softer and mealy during storage (Eccher Zerbini *et al*., 1997; Kader, 1999; Johnston *et al*., 2002). Based on these considerations, the precise determination of fruit maturity is essential to steer post-harvest and market strategies.

Several indices are used to establish the proper ripening stage such as changes in the skin colour, starch degradation, soluble sugars content and acidity. However, some of them, although very practical and user-friendly, can not be used to predict the final fruit quality (Kader, 1999). A more precise evaluation of fruit maturity and physiological stage can be obtained by measuring respiration rate and ethylene production by fruits (Beaudry *et al*., 1993; Kader, 1999; De Castro *et al*., 2007). However, these methods are time consuming and they require trained personnel and sophisticate instruments. To improve the assessment of the optimal harvesting time, the measurements of more biochemical and physiological parameters can be combined and expressed in a single descriptor, such as the Streif index, which express the relation between flesh firmness, soluble solids content and starch degradation. The Streif index provide more reliable information than the single parameters, reducing variability observed in different seasons or areas (Streif, 1996; DeLong *et al*.,1999, Zude-Sasse *et al*., 2002; Tijskens *et al*., 2008).

All the methods presented so far to asses fruit quality and maturity require destructive analysis. Therefore, they can not be repeated over time on the same samples to monitor the maturation dynamics and they can be performed only on a limited number of fruits which may not be fully representative of the whole batch.

To overcome these problems, in the last decades, research focused on the use of non destructive techniques to estimate fruit physiological stage and maturity. These methods include texture analysis by mechanical deformation, impact test or sonic and ultrasonic vibrations; gas analysis by electronic nose, and electromagnetic technologies based on optical properties (radiowave, microwave, ultraviolet, visible light, infrared, χ-ray and γ-ray radiation), fluorescence and magnetic resonance (Abbott, 1999). Among these techniques, near infrared spectroscopy (NIRs) is widely used to estimate physiological

stage or quality parameters on fruits and vegetables (Liu and Ying, 2005; Nicolaï *et al*., 2007; Liu *et al*., 2008; Costa *et al*., 2009; Bertone *et al*., 2012).

In apples, NIRs measurements showed a good correlations with the firmness, total soluble solids, starch degradation, background colour, Streif index and some sensory attributes (Peirs *et al*., 2000; McGlone *et al*., 2002; Mehinagic *et al*., 2003; Peirs *et al*., 2005; Liu and Ying, 2005; Zude *et al*., 2006). Furthermore, since chlorophyll degradation takes place in apple skin during maturity development, changes in its content, measured by non-destructive technologies, can be used to estimate fruit ripening stage (Merzlyak *et al*., 2003; Solovchenko *et al*., 2005; Nicolaï *et al*., 2007; Costa *et al*., 2009, Bertone *et al*., 2012, Betemps *et al*., 2012). The major drawback of all these non-destructive methods is the need of a continuous calibration and complex data processing (Nicolaï *et al*., 2007; Costa *et al*., 2009). To overcome these limitations, a newly portable device has been developed: the DA-meter (Ziosi *et al*., 2008; Costa *et al*., 2009). This instrument measures the changes in flesh chlorophyll content as a difference in absorbance at 720 and 670 nm (I_{AD}), which provides a reliable indication of the ripening state of fruits. The use of the DA-meter was mainly described for stone fruits, where the I_{AD} also correlated ethylene production and transcription of ripening-related genes (Ziosi *et al*., 2008; Costa *et al*., 2009; Bonora *et al*., 2013a,b; Costa and Noferini, 2013; Shinya *et al*., 2013). In peaches, I_{AD} allowed to cluster at harvest the fruits in homogenous ripening classes, which presented a differential development of fruit quality traits during shelf-life (Costa *et al*., 2009; Shinya *et al*., 2013). Up to now, few studies have been investigated the possible use of the I_{AD} as a ripening index for pomes fruits. Recently, I_{AD} was used to determine maturity and fruit quality in 'Gala', 'Starking', 'Granny Smith' and 'Pink Lady' apples (Costamagna *et al*., 2013; Nyasordzi *et al*., 2013).

The present study aims to investigate the use of the DA-meter for precisely monitoring the ripening stage of 'Granny Smith' and 'Pink Lady' apples during pre-harvest and at harvest. These two cultivars have been chosen as a model for apple fruits. In fact, they are among most produced and consumed apple varieties in Europe. In addition, they are characterized by very different organoleptic characteristics and ripening behaviour, which may represent the two extremes among apple cultivars.

The DA-meter measurements were correlated with the most widely used fruit quality and ripening parameters [flesh firmness (FF), total soluble solids (TSS), starch degradation, Streif index and ethylene production].

3.3. Materials and Methods

3.3.1. Plant material, cultivation conditions and harvesting time

Trials were conducted on 'Granny Smith' and 'Pink Lady' trees for two consecutive seasons (2011 and 2012). Trees were five years old, grafted on M9 rootstock, and trained as spindle system. Plantation density was 3788 trees ha^{-1} for 'Granny Smith' (0.8 x 3.3 meters) and 3030 trees ha^{-1} for 'Pink Lady' (1.0 x 3.3 meters). The orchards were located in Ravenna, Italy, at 44°26'38'-12°5'53''E for 'Granny Smith' and at 44°20'46'' N-12°2'26''E for 'Pink Lady', both orchards with an approximate North-South orientation. The orchards were conducted with standard cultural practices (i.e. fertirrigation, disease and pest control).

Fruits development was followed, *in planta*, with the DA-meter every week starting from approximately 159 and 169 after full bloom (DAFB) 'Granny Smith' and 'Pink Lady', respectively. Harvest was performed, in 'Granny Smith' at 187 and 190 DAFB for the season 2011 and 2012 respectively, and 'Pink Lady' at 194 and 201 DAFB for 2011 and 2012 respectively.

3.3.2. Non-destructive I_{AD} (Index of absorbance difference) measurement

The non destructive analyses were performed with a DA-meter (TR Turoni, Forli, Italy). This instrument is based on NIRs technology and it reads the absorbance differences between two wavelengths (λ=670 nm and 720 nm), which is expressed as I_{AD}. DA-meter measurements correlate to the actual chlorophyll-a content in the flesh fruit. In apple, I_{AD} values range from 0.2 to 2.2, being the higher number slinked with less ripe fruit. The I_{AD} was measured weekly on 500 fruits per cultivar, randomly selected. On each fruit, I_{AD} calculated as the average of two measures taken on the opposite equatorial side.

At 20, 10 and 5 days before harvest, 100 fruits, selected among the 500 chosen for I_{AD} measurements, were harvested to analyse their quality and maturation traits (flesh firmness, TSS, starch degradation, Streif index and ethylene production).

At harvest, 500 fruits were collected and analysed with the DA-meter. According to I_{AD} values, the fruits were grouped in 4 homogeneous ripening classes for 'Granny Smith' (>2.0, ≤2.0-1.8, ≤1.8-1.6, ≤1.6-1.4) and 5 classes for 'Pink Lady' (>1.2, ≤1.2-1.0, ≤1.0-0.8, ≤0.8-0.6, ≤0.6-0.4). For each ripening classes, 20 fruits were selected for the determinations of the standard quality traits and ethylene emission.

3.3.3. Destructive analysis for fruit quality traits determination

Flesh firmness (FF) was measured using a Fruit Texture Analyzer penetrometer (FTA Güss, South Africa). After eliminating a skin thin layer, each fruit was compressed at the two equatorial sides using a pressure tester fitted with a 11 mm plunger tip, at 2 mm at a rate of 0.1 mm/s.

Starch content was assessed with the Lugol test. The measurements were visually evaluated using the Ctilf starch conversion chart for apples, expressed in a scale from one to ten.

Total soluble solids (TSS) were measured with a digital refractometer (DBR-95, Italy), part of the mesocarp was squeezed and the juice drops were used to measure the percentage of soluble solids at the two equatorial side.

Streif Coefficient was calculated by the equation described by Streif (1996), were the index is defined as [FF/(TSS x Starch degradation)].

3.3.4. Ethylene assessment

Ethylene production was measured by placing the whole fruit in a 0.8 L jar tightly sealed with a lid equipped with a rubber stopper, and left at room temperature for 1 h. The measures were performed on a 10 ml gas aliquot sampled from the fruit headspace . The analysis were carried out using a Dani HT 86.01 (Dani, Milan, Italy) packed-gas chromatograph fitted with FID and a Porapak Q column (Supelco, Bellefonte, PA, USA). Oven temperature was set at 80°C, and for the injector and detector at 180°C. N_2 was used

as the carrier gas, 16 mL min-1 flow rate. Ethylene concentration was expressed as nanoliter/ gram of fresh weigh /1h.

3.3.5. Statistical analysis

Data were processed by ANOVA and the means were compared by the Tukey's HSD test at significant level of 0.05 by the software STATISTICA 7 (StatSoft Inc., Tulsa, USA). Differences between means were analyzing by T-test for independent samples by variables (p value < 0.05). Relation between I_{AD} and fruit attributes and ethylene content was evaluated using the Pearson Product-Moment Correlation (95% confidence).

3.4. Results and Discussion

3.4.1. In planta pre-harvest monitoring of maturity evolution measured by I_{AD}

The I_{AD} evolution during the last month before harvest showed a decrease from 1.96 to 1.80 for 'Granny Smith' and from 1.60 to 1.10 for 'Pink Lady' (Table 1). This reduction corresponds with an increase of the fruit maturity stage as indicated by the results of destructive analysis (Table 2). The I_{AD} evolution showed a significant correlation with FF and starch degradation in 'Granny Smith' in the 2 years of analysis (Figure 1 A, C, respectively). In 'Pink Lady', the correlation between I_{AD} and FF or starch content is not as clear as in 'Granny Smith' (Figure 1 B, D , respectively). However, it should be noted that, in our experiments, FF and starch degradations did not vary significantly in relation to 'Pink Lady' maturity evolution. The I_{AD} values did not significantly correlate with TSS in none of the two cultivars (Figure 1 I, F). In fact, in both cultivar, TSS showed only little differences which indicated, that, in the last month before commercial harvest, the total sugar content was already fully established and only little increases occurred.

The I_{AD} showe3d a robust correlation with the Streif coefficient in both cultivars (Figure 1, G, H). Interestingly, this parameter was the one that better describe the maturity development in apple thus strengthening the evidence that I_{AD} can be an easy to use indicator of maturity stage to predict the harvesting time. Finally, the ethylene analysis

showed that in ‘Granny Smith’ this parameter does not correlate neither with maturation or I_{AD} due to the very low emission (Figure 1, I). In ‘Pink Lady’, ethylene emission absent at the initial maturity stages, while thereafter, an emission burst was observed (Figure 1, L). Therefore, the ethylene evolution during maturity development did not show a linear correlation with I_{AD}. The Pearson’s correlation coefficient showed a good linkage between I_{AD} and ethylene.

Our results confirmed the studies previously conducted on ‘Starking’ apples (Nyasordzi *et al*., 2013). I_{AD} correlated also in ‘Granny Smith’ and ‘Pink Lady’ with the internal quality changes occurring immediately before harvest. Based on these evidences, we propose the use of I_{AD} as a fast, non-destructive, easy to use and robust indicator of internal quality changes in order to establish the most appropriate harvesting time to fully express apple fruit quality after storage. The efficacy of I_{AD} can be explained with its strong correlation with the content of chlorophyll-a, which decrease during the increase of maturation (Merzlyak *et al*., 2003; McGlone *et al*., 2002; Zude *et al*., 2006; Bertone *et al*., 2012). Differently from previous works where the non-destructive instrumentations to detect the reduction of chlorophyll contents needed a continues calibration and complex statistical analysis (Zude-Sasse *et al*., 2002; Zude *et al*., 2006; Bertone *et al*., 2012), the DA-meter provides a ready to use data that can be easily interpreted also by non-trained personnel.

3.4.2. Fruit maturity distribution at harvest measured by I_{AD}

The heterogeneity of apple fruit maturation at harvest was estimated by I_{AD} (Figure 2 and 3). In ‘Granny Smith’ the I_{AD} values ranged between 1.4 and 2.1 in both years (Figure 2). In this cultivar nearly 90 % of fruits presents an I_{AD} between 2.0 and 1.6., and the majority of fruits clustered in 4 different I_{AD} classes (higher than 2.0; 2.0-1.8; 1.8-1.6 and 1.6-1.4). In ‘Pink Lady’, I_{AD} varied between 0.4 to 1.6 showing a more heterogeneous maturity distribution at harvest (Figure 3). In this cultivar, the main I_{AD} classes chosen for further analysis were 5 (higher than 1.2; 1.2-1.0; 1.0-0.8; 0.8-0.6 and 0.6-0.4). In both cultivars, the distribution of maturity showed the same trend in the two consecutive years, although in 2012, the harvest was delayed of 4 and 7 DAFB in ‘Granny Smith’ and ‘Pink Lady’, respectively. This delay resulted in a higher percentage of fruits clustering in classes of lower I_{AD} (more mature). For each mentioned I_{AD} classes standard quality parameters (FF, TSS, starch index degradation and Streif coefficient) and ethylene emission were assessed.

In ‘Granny Smith’, the fruits with different I_{AD} values, also showed significantly different FF values (Figure 4, A). In ‘Pink Lady’, this correlation was weaker due to the inner characteristic of this cultivar that have been selected for the ability of the fruits to preserve an almost constant FF during maturation (Figure 4, B). Indeed, the fruits in lower I_{AD} classes, starting from 1.0-0.8, presented significantly different FF values from the ones in the higher I_{AD} classes.

Concerning starch degradation, in ‘Granny Smith’ the different I_{AD} classes showed also significantly different starch content for the 2011 harvest. In 2012, a similar trend was observed, but the differences were not always statistically significant (Figure 4, C). In ‘Pink Lady’, in 2011, the I_{AD} classed generally did not statistically differ for the starch content, only the classes with lower I_{AD} (from 0.8-0.6) differed from the least mature fruits (I_{AD}>0.8) (Figure 4, D). In 2012, significant differences in starch content were observed among all I_{AD} classes (Figure 4, D).

A general, increase in TSS was observed in relation to I_{AD} decrease in both cultivars (Figure 4 E, F). In ‘Granny Smith’, the differences in TSS were significant only between the two boundary classes (I_{AD}>2.0 and 1.6<I_{AD}<1.4) (Figure 4, E). In ‘Pink Lady’, in 2012, the comparison of TSS among the different I_{AD} classes showed the same trend as in ‘Granny Smith’ (Figure 4, F). In 2011, significant differences were observed between I_{AD} >1.2 and 1.2<I_{AD}<1.0 and between 1.0<I_{AD}<0.8 and 0.6< I_{AD}<0.4.

In ‘Granny Smith’ in 2011, different I_{AD} classes showed significantly different Streif coefficient among the three least mature I_{AD} classes. In 2012, the differences in Streif coefficient were statistically significant only among the I_{AD} classes higher or lower than 1.8 (Figure 4, G). In ‘Pink Lady’ differences between I_{AD} classes in relation to Streif coefficient were significant in the most mature classes (Figure 4, H). In both cultivar, the differences observed in the Streif coefficient among 2011 and 2012 were mainly due to the differences observed in TSS and starch, whereas, FF did not differ among the two years.

Concerning ethylene emission, ‘Granny Smith’ presented an almost absent ethylene emission and therefore this parameter showed statistical difference only among the boundary I_{AD} classes (Figure 4, I). In ‘Pink Lady’, the fruits clustering in all I_{AD} classes higher than 0.8 showed an extremely low emission of ethylene (Figure 4, J). Whereas, the fruits in the classes with an I_{AD} lower than 0.8 produced significant amount of ethylene and a sharp increase when I_{AD} was lower than 0.6 (Figure 4, J).

In this study, ‘Granny Smith’ fruit quality parameters were more constant on the two seasons than on ‘Pink Lady’ where some differences were detected especially on starch degradation. Starch degradation and TSS were the parameters with the higher variability between the two years. These differences on starch degradation were also reported in previous studies and they are mainly related with the different dates of the beginning of start degradation, between the seasons (Brookfield *et al*., 1997; De Castro *el al*., 2007).

In our experiments, in both season, the clustering of fruits according to I_{AD} confirmed the subdivision in maturity groups categorized on the bases of standard quality traits, such as FF, starch degradation and Streif coefficient, which are fair indicators of ripening evolution. Whereas, other parameters, such as TSS, did not show significant differences in relation to the maturity classes categorized on the bases of other quality traits.

3.5. Conclusions

Our data confirm that the chlorophyll degradation can be taken as an index to define harvest and asses apple fruit quality, although it is rarely used to examine the maturity distribution at harvest (McGlone *et al*., 2002; Zude *et al*., 2006; Bertone *et al*., 2012 Nyasordzi *et al*., 2013).

The results obtained demonstrated that the I_{AD} could represent a reliable indicator for monitoring fruit ripening evolution both *in planta* during the growing season and at harvest. Therefore, I_{AD} can be used, in field, for a precise prediction of the most appropriate harvesting time in order to fully express the fruit quality potentialities. Whereas, at harvest, I_{AD} can be used to cluster the fruit in uniform maturity classes in order to decide the most suitable post-harvest strategy for each of them. The major advantages of I_{AD} is that this parameter can be obtained with a portable instrument directly in the orchard and, since, the instrument is non-destructive, easy to use and fast, a high number of fruits can be measured thus providing a more representative evaluation of the maturity stage in field. The I_{AD} also allows to describe the fruit heterogeneity in a single tree or in a given fruit batch.

In comparison with the standard parameters, which have limitations related to their climate and environment-dependency, which make the different years not comparable, I_{AD} showed, in our study, a good stability among the different seasons.

Establish the proper harvest by commercial criteria, such as starch degradation, TSS or colouration of the skin, varies from region to region. Each of these parameters, when used alone, are not enough reliable for defining the best harvesting time. In fact, it has been observed that delaying the harvest for achieving a high percentage of red coloured surface fruit, without considering, for example, the starch degradation, in some areas could compromised the quality and increase losses during storage (De Castro *el al*., 2007).

Based on these considerations, future researches should evaluate the use of I_{AD} in different regions and climates to verify if this parameter is less dependent that the standard index from environmental and cultivation conditions.

Further studies are needed to optimize this instrument for the prediction of the best harvesting time. Studies should be focus on direct monitoring fruit maturity in the field, and the possibility to get on real time a vision of the actual state of the fruit maturity for each specific orchard, these could allow to implement management decisions at the single orchard level before fruit harvest.

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3.7. Figures and Tables

Table 1. I_{AD} mean values in relation to DAFB, for the season 2011 and 2012 on 'Granny smith' and 'Pink Lady' apples. Different letters, in the same column, indicates significant differences at $P \leq 0.05$.

	2011		2012	
	DAFB	Fruit Maturity (I_{AD})	DAFB	Fruit Maturity (I_{AD})
'Granny Smith'	159	1.96 a	162	1.91 a
	169	1.92 b	169	1.88 b
	175	1.89 c	176	1.86 c
	179	1.87 d	183	1.83 d
	187	1.82 e	190	1.80 e
'Pink Lady'	169	1.60 a	173	1.57 a
	175	1.55 b	180	1.50 b
	182	1.44 c	187	1.33 c
	187	1.33 d	191	1.21 d
	194	1.12 e	201	1.10 e

Table 2. Changes in maturity parameters in ‘Granny Smith’ and ‘Pink Lady’ apples during the last month before harvest. Flesh firmness (FF), total soluble solids (TSS), starch degradation, Streif coefficient and ethylene emission are reported for season 2011 and 2012. Different letters, in the same columns, indicate significant differences at $P \leq 0.05$.

	2011						2012					
	DAFB	FF (N)	TSS (%)	Starch (1-10)	Streif Index	Ethylene (nL/gFWh)	DAFB	FF (N)	TSS (%)	Starch (1-10)	Streif Index	Ethylene (nL/gFWh)
‘Granny Smith'	169	81.9 a	10.4 b	4.8 c	1.7 a	0.02 a	169	81.5 a	10.8 b	5.3 c	1.5 a	0.02 b
	179	74.0 b	11.2 a	6.1 b	1.1 b	0.03 a	176	79.1 ab	11.2 b	6.5 b	1.1 b	0.03 ab
	182	70.5 c	11.2 a	7.4 a	0.9 c	0.03 a	183	70.3 b	12.3 a	7.7 a	0.8 c	0.05 a
‘Pink Lady'	175	85.9 a	13.0 b	6.7 b	1.0 a	0.11 b	180	87.2 a	12.1 b	7.0 c	1.1 a	0.06 b
	182	85.5 a	13.7 a	7.2 a	0.9 b	0.17 b	187	85.7 a	12.8 a	7.8 b	0.9 b	0.10 b
	187	81.7 b	13.7 a	7.5 a	0.8 c	0.58 a	191	83.4 b	12.7 a	8.2 a	0.9 b	0.32 a

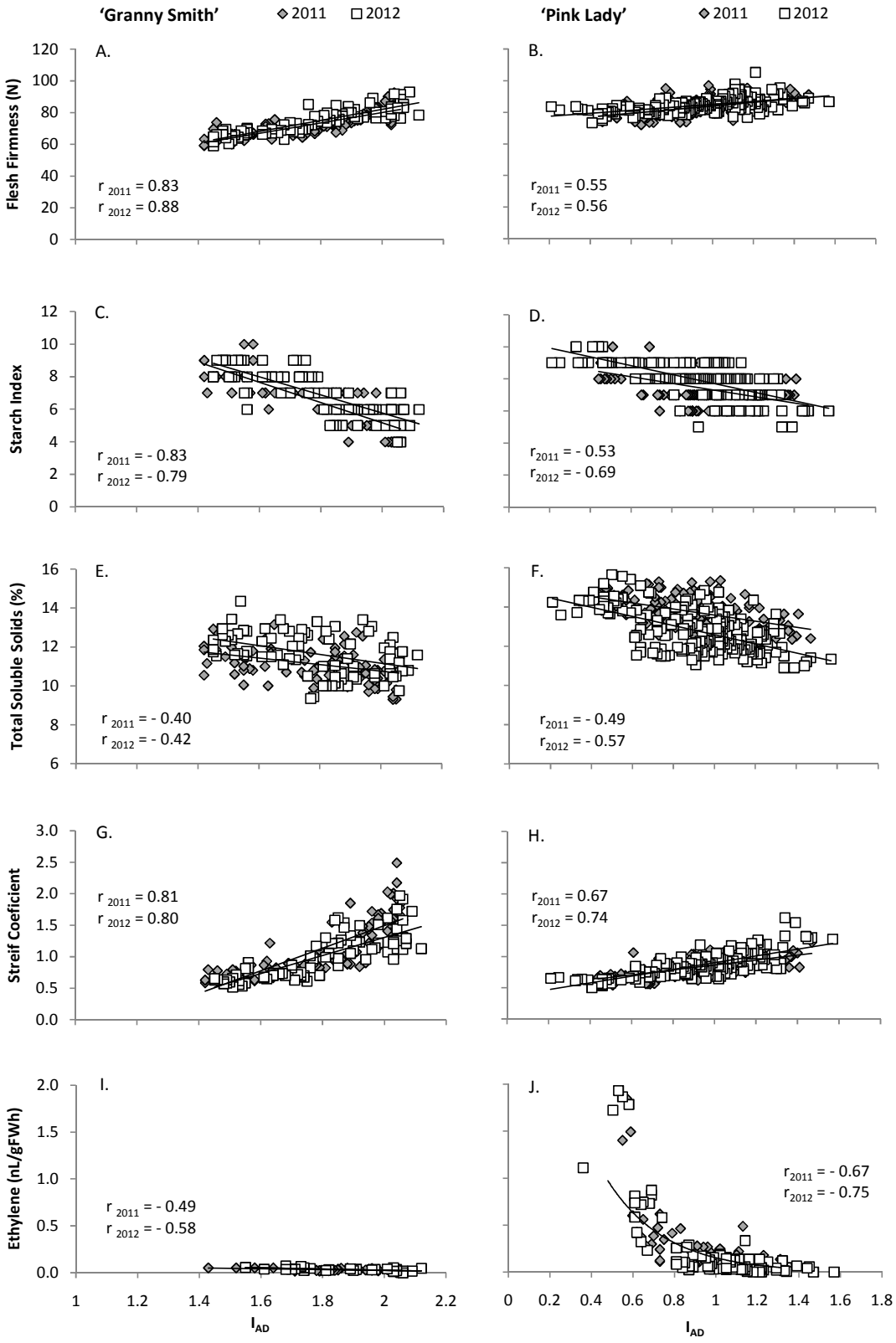

Figure 1. Relation between I_{AD} and the main maturity parameters during pre-harvest in 'Granny Smith' and 'Pink Lady'. Panels A, B: Flesh Firmness, C, D: Starch Index, E, F: total soluble solids (TSS), G, H: Streif coefficient and I, J: ethylene emission. Solid lines

indicate the correlation between parameters, and *r* = correlation coefficient for season 2011 and 2012.

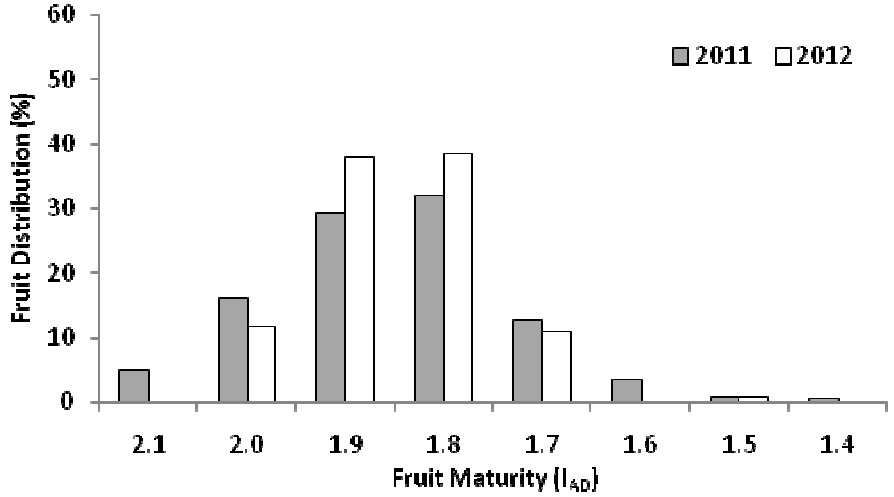

Figure 2. Distribution of I_{AD} classes at harvest in 'Granny Smith' apples. Fruits were harvested at 187 and 190 DAFB for the season 2011 and 2012, respectively.

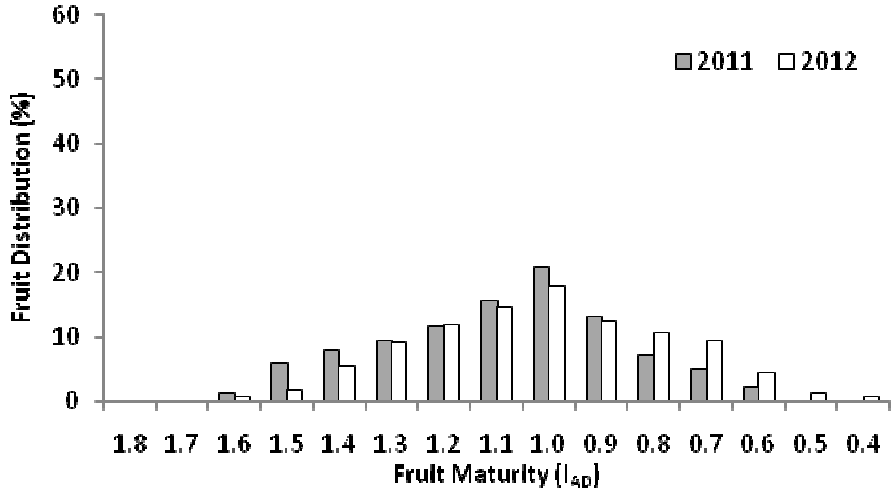

Figure 3. Distribution of I_{AD} classes at harvest in 'Pink Lady' apples. Fruits were harvested at 187 and 190 DAFB for the season 2011 and 2012, respectively.

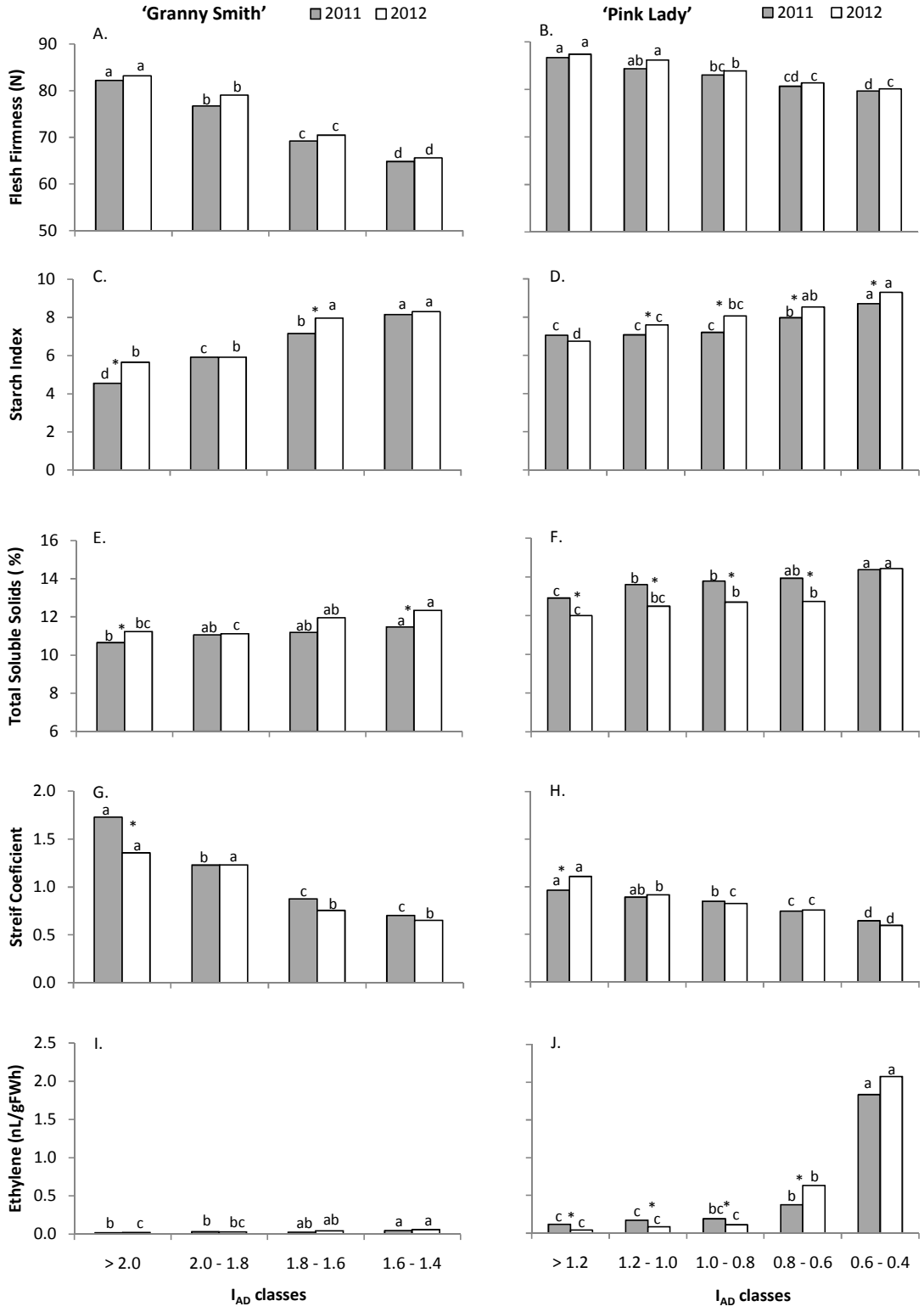

Figure 4. Fruit maturity parameters in different I_{AD} classes at harvest on 'Granny Smith' and 'Pink Lady' apples. Panels A, B: Flesh Firmness, C, D: Starch Index, E, F: total

soluble solids (TSS), G, H: Streif coefficient and I, J: ethylene emission. Different letters indicate significant differences between I_{AD} classes at $P \leq 0.05$. The asterisk indicates differences between 2011 and 2012, among the same parameter.

4. Optimization of the DA-meter, a non destructive tool to monitor apple fruit maturity, to predict the best harvesting time

4.1. Abstract

A precise definition of the ripening stage directly in the orchard is essential to predict the best harvesting time to allow fruits to fully express their organoleptic potential. The DA-meter allows to measure chlorophyll-a degradation which is a reliable indicator of apple maturity development. In the present work the DA-meter, which express chlorophyll content as an index of absorbance difference (I_{AD}), has been used, during pre-harvest to monitor, directly *in planta*, fruit maturity development in 'Granny Smith and 'Pink Lady' during two consecutive years. The aim of the research was to develop a simple model, based on the I_{AD} monitoring, to predict the optimal harvesting time and fruit maturity heterogeneity at harvest. The data collected confirm the cultivar-specific behaviour of the I_{AD} before harvest. Moreover, I_{AD} was found to be consistent, in the same cultivar, year after years. The variation of I_{AD} during pre-harvest time showed a constant decrease that can be modelled by a linear regression ($R^2 \geq 0.9$) which can be used to predict, with up two weeks of advance, the apple quality at harvest.

4.2. Introduction

The quality of the fruits as defined in terms of visual appearance (shape, skin colour, size), texture, flavour and aroma is the most appreciated by consumers. However, nowadays, the fruit nutritional value and safety are also considered a priority. Therefore, providing the market with high quality fruits should be one of the major drivers to increase fruit consumption and expand profit for the producers and distributor. The cultivation methods, the harvesting time and the post-harvest management are the three major factors influencing the development of fruit maturation and final quality. The definition of most

appropriate harvesting time is particularly important since in apple fruits, the maturity stage at harvest affects the quality at consumption, the storage strategy and shelf-life duration. It has been demonstrated that fruit harvested too early on the season will not develop adequate sugars and flavour, and are more prone to shrivelling and to develop insufficient skin coloration, (Watkins *et al*., 1995; Eccher Zerbini *et al*., 1997; Kader, 1999). In addition, fruit harvested with a suboptimal maturity are more prone to storage disorders as superficial scald and bitter pit. On the other hand, fruits harvested late in the season could be overripe, with a higher tendency to become exceedingly soft and mealy. Such conditions decreases the potential of fruits for storability, while increasing the risk of other disorders such as senescent breakdown (Eccher Zerbini *et al*., 1997; Kader, 1999; Johnston *et al*., 2002).

Nowadays, two major approaches exist to predict harvesting time: i) the monitoring quality traits, such as flesh softening, starch degradation, accumulation of soluble solids, fruit skin colouring, etc. or ii) the use of prediction models. The monitoring of quality traits needs the destruction of the fruits during the analysis and some of these parameters are influenced by the climate conditions and location and, therefore, they cannot be used as an universal scale (Beaudry *et al*., 1993; Kader, 1999; Kays, 1999, De Castro *et al*., 2007). Concerning the models to define the optimal harvest window, they are based on detailed description of the environmental and physiological process, blooming date, degree-days, climatic variables interactions, fruit quality parameters measured by destructive techniques and molecular analysis (Beaudry *et al*., 1993; De Long *et al*.,1999; De Castro *et al*., 2007; Kaack and Lindhard Pedersen, 2010; Kaack and Pedersen, 2011; Hertog *et al*., 2011; Neuwald and Streif, 2012). Although these models are able to precisely define the ripening stage and the optimal harvesting time, they are time consuming and they require sophisticate analysis, equipped laboratories and trained personnel. Moreover, they do not allow the real-time monitoring of the maturation process. Therefore, most of these models are restricted to research studies and their practical application is still difficult.

Based on these considerations, new reliable and easy to use approaches to predict the most appropriate harvesting time are sought. The use of non-destructive devices, such as the DA-meter (TR Turoni, Forli, Italy), has shown the potentiality to be a useful tool to monitor fruit quality and maturation development in order to predict harvesting time (Costa *et al*., 2009; Costa and Noferini, 2013; Costamagna *et al*., 2013, Nyasordzi *et al*., 2013).

The DA-meter is a portable device based on Visible/Near Infrared (Vis/NIR) spectroscopy. The DA-meter has been used to assess fruit maturity, which do not required calibration and offers a direct estimation of the actual chlorophyll-a content expressed as the index of absorbance difference (I_{AD}) between two wavelengths (λ=670nm and λ=720nm) (Ziosi *et al*., 2008; Costa *et al*., 2009). Previous studies demonstrated that monitoring the changes on chlorophyll content in apple skin by Vis/NIR spectroscopy allowed the estimation of fruit physiological stage during apple maturity (Knee, 1972; Knee, 1980; Merzlyak *et al*., 2003; Zude-Sasse *et al*., 2002; Zude, 2003; Solovchenko *et al*., 2005). The DA-meter was mainly used on peaches, nectarines and apricots where the I_{AD} correlated with ethylene production, fruit quality traits, and transcription of ripening-related genes (Ziosi *et al*., 2008; Costa *et al*., 2009; Bonora *et al*., 2013a,b; Shinya *et al*., 2013). On stone fruits, the use of the I_{AD} allowed to divide at harvest the fruits in homogenous ripening classes, each class present a diverse quality behavior during shelf-life and consumption preferences (Costa *et al*., 2009; Shinya *et al*., 2013). Up to now, few studies have been performed to test the use of the I_{AD} as a ripening index on pomes fruits (Costamagna, *et al*., 2013; Nyasordzi *et al*., 2013). On apple fruits the use of the I_{AD} have been recently study on 'Gala', 'Starking', 'Granny Smith' and 'Pink Lady' to determine quality traits and its shown the potential of the use of this index for ripening and quality assessment (Costamagna, *et al*., 2013; Nyasordzi *et al*., 2013).

The use of I_{AD} was successfully used to assess apple fruits quality at harvest. The I_{AD} was cultivar dependent, but independent from the growing seasons, thus suggesting that this index can be used, in different years, and probably in different cultural or geographic conditions, as an universal parameter to describe fruit quality and maturation (according to the data reported on chapter 3). Preliminary results also showed that I_{AD} can be used, directly in field conditions, to follow the development of maturation and quality. Recently, a model to predict the appropriated harvest period of apple fruits was developed by measuring chlorophyll degradation with NIR and Vis/NIR technologies (Bertone *et al*., 2012). However, the model robustness and reliability still need to be validated in relation to the seasonal variability and geographical location (Bertone *et al*., 2012). Hence, the aim of this work was to optimize the use of I_{AD} during pre-harvest to predict the i) optimal harvesting time, and ii) fruit maturity heterogeneity at harvest.

4.3. Materials and Methods

4.3.1. Plant material, cultivation conditions and harvesting time

Trials were conducted on ‘Granny Smith’ and ‘Pink Lady’ trees for two consecutive seasons (2011 and 2012). Trees were five years old, grafted on M9 rootstock and trained as spindle system. Plantation density was 3788 trees ha^{-1} for ‘Granny Smith’ (0.8 x 3.3 meters) and 3030 trees ha^{-1} for ‘Pink Lady’ (1.0 x 3.3 meters).The orchards were located in Ravenna, Italy, at 44°26’38’-12°5’53’’E for ‘Granny Smith’ and at 44°20’46’’ N-12°2’26’’E for ‘Pink Lady’, both orchards with an approximate North-South orientation. The orchard surface for ‘Granny Smith’ and ‘Pink Lady' was 4 and 3 hectares, respectively. Each orchard was delimited and geo-referenced by the use of Global Position System (GPS, Garmin® eTrex® Vista HCx). The orchards were conducted with standard cultural practices (i.e. fertirrigation, disease and pest control).

Fruits development was followed, *in planta*, with the DA-meter every week starting from approximately 157 and 164 after full bloom (DAFB) for‘Granny Smith’ and ‘Pink Lady’, respectively. Harvest was performed, in ‘Granny Smith’ at 187 and 190 DAFB for the season 2011 and 2012 respectively, and ‘Pink Lady’ at 194 and 201 DAFB for 2011 and 2012 respectively. Commercial harvesting time was decided on the based on flesh firmness (FF) and starch degradation in ‘Granny Smith’, and red blush development and total soluble solid contents (TSS) in ‘Pink Lady’ in accordance with the optimal range for each parameter and cultivar (Fadanelli, 2008), which correspond to a mean I_{AD} value 1.8 and 1.1 for ‘Granny Smith’ and ‘Pink Lady’ respectively (according to the data reported on chapter 3).

4.3.2. Monitoring of apple fruit maturity during pre-harvest

Fruit ripening progression was assessed in relation to the full bloom date from approximately one month before the expected harvest. Along this period, fruit maturity behaviour was assessed, directly *in planta*, by the use of the DA-Meter. The I_{AD} was detected every four days starting from approximatively150 DAFB. I_{AD} measurements were postponed, up to seven days, in case of adverse climatic conditions. In each time point, the

I_{AD} was measured on 300 apple fruits randomly chosen inside the orchard. Fruit were randomly selected in the middle canopy position, boundary rows were discriminate, and a minimum of six rows were sampled. To determine the minimal number of fruits needed to record a reliable measure of I_{AD} random subsamples of 100 and50 fruits were processed independently.

4.3.3. Development and validation of a prediction model for optimal harvesting time

In both years, the I_{AD} data collected during the different sampling times (DAFB) were processed to obtain a regression equation to model I_{AD} development in relation to time. The regression equation was used to predict the fruit maturity at harvest. The model was validated, by plotting the measured I_{AD} values in relation to the regression line per each studied year, furthermore to validate the model independently of the season, the model obtained in 2011 was validated with the real measures in 2012. The prediction of the harvest date was forecast for a I_{AD} value 1.8 and 1.1 for 'Granny Smith' and 'Pink Lady' respectively.

The prediction of the heterogeneity of fruit maturity distribution at harvest was analysed by plotting the percentage of each forecasted I_{AD} maturity class against the results obtained with fruits sampled at commercial harvest.

4.3.4. Statistical analysis

Collected data were processed by Tukey's HSD test at a level of significance of 0.05. The interactions between factors were assessed with a multiple factor ANOVA test. Comparison between means on time was performed by t-test for independent samples by variables (p value < 0.05). Linear regression to model the development of I_{AD} in relation to time (expressed as DAFB) was evaluated by the coefficient of determination (R^2), root mean square error (RMSE) and p value were. Statistical analysis were performed by STATISTICA 7 software (StatSoft. Inc., Tulsa, OK, USA).

4.4. Results and Discussion

4.4.1. Assessment of fruit maturity on tree before harvest

The I_{AD} was measured on the fruits of the two cultivars, 'Granny Smith' and 'Pink Lady' during different time points before harvest, and the minimal number of fruits needed to obtain a reliable evaluation of the mean I_{AD} value was evaluated (Table 1). In 'Granny Smith', a sample size of 50 fruits generally resulted representative of the mean I_{AD} values at the different time points before harvest, even though a sample of 100 fruits gave a more robust estimation of the I_{AD} value (Table 1). In 'Pink Lady', 100 was the minimal number of fruits be measured in order to obtain a reliable estimation of the I_{AD} value (Table 1). In the time span considered, the I_{AD} values in 'Granny Smith' presented lower variability in comparison with 'Pink Lady', decreasing from above 1.97 to 1.80 and from 1.60 to 1.10, respectively (Table 1). The evolution of the I_{AD} values during the last period before harvest can be explained by its correlation with the chlorophyll degradation during fruit ripening fruit (Knee, 1972; Knee, 1980; Merzlyak *et al*., 2003; Zude-Sasse *et al*., 2002; Solovchenko *et al*., 2005; Costa *et al*., 2009; Bertone *et al*., 2012; Nyasordzy *et al*., 2013). Our results corroborate the previous findings obtained on 'Starking' apples (Nyasordzy *et al*., 2013).

I_{AD} changes *in planta* during the last month before commercial harvest are characterized by an initial trend where little variations occur over time. Successively, once a certain I_{AD} value, which is specific for each cultivar, is reached, the reduction of I_{AD} becomes linear. This break point was nearly 1.9 for 'Granny Smith' and 1.6 for 'Pink Lady'. The trend in I_{AD} decrease in 'Granny Smith' presents a smaller slope in comparison with 'Pink Lady' (Figures 1A and 2A).

4.4.2. Prediction of harvesting time

The measurement of I_{AD} values performed during 2011 and 2012 showed that the variation of I_{AD} during time (DAFB) follows a linear regression, which varies according to the cultivar (Figures 1, B-C and 2, B-C). The data allowed to calculate the linear regression describing the observed decrease of I_{AD}. This regression can be calculated, for both cultivars, by taking in consideration on the first three I_{AD} values recorded after the break

point (I_{AD}=1.9 for 'Granny Smith' and I_{AD}=1.6 for 'Pink Lady') (Figures 1, B-C and 2, B-C). On the present study, the slope of the linear regression was similar or equal between years.

The linear regression calculated on these three points allowed to predict the harvest day with a coefficient of determination of R^2 0.99 and 0.98 for 'Granny Smith' for the season 2011 and 2012 respectively (Figure 1, B-C). Also in 'Pink Lady', the coefficient of determination of R^2=0.95 and 0.99 for 2011 and 2012, respectively(Figure 2, B-C). The model was validated by plotting the measured I_{AD} values in relation to the regression line calculated, each years, by using the first three I_{AD} after the break point (Figure 1, B-C and 2 B-C). In addition, to verify if the I_{AD} values, in a specific orchard, follow a linear reduction independently form the season, the regression line calculated 2011 was validated with the real measures obtained in 2012 (Figures3 and 4).

The model calculated with the data collected in 2011 perfectly predicted, in both cultivars, the I_{AD} trend in 2012 (Figure 3 and 4). Therefore, our results show that, in a specific orchard, the harvesting time can be predicted by simply determining when the I_{AD} reaches the break point. This break point occurred, independently form the year, at 165 and 175 DAFB 'Granny Smith' and 'Pink Lady', respectively.

The heterogeneity of fruit ripening detected before the harvest provided other useful information. The maturity distribution, expressed as I_{AD} classes, is reported for the two studied cultivars on three representative days before harvest, which were the ones used to estimate the maturity trend (Figure 5). Fruit maturity distribution can be described by a Gaussian curve with the amplitude dependent by the cultivar. In 'Granny Smith', the curve presents a lower fruit ripening heterogeneity and this trend did not change overtime (Figure 5A). In 'Pink Lady', fruits showed a higher ripening homogeneity (Figure 5B).These information allowed also to estimate the fruit maturity distribution at harvest expressed as I_{AD} classes (Figure 6) by the maturity rate. For 'Granny Smith' from the fruit maturity distribution measured at 169 DAFB, the predicted fruit maturity at harvest was estimated with a R^2 above 0.98, RMSE 0.1 and low *p*-value. In 'Pink Lady', the measured fruit maturity distribution assessed at 182 DAFB predicted the fruit maturity distribution at harvest with a R^2 above 0.97, RMSE around 0.3-0.4 and low *p*-value. The coefficient of determination, RMSE and *p*-value are reported in Table 2 for season 2011 and 2012.

4.5. Conclusions

I_{AD} index could represent a decisional support tool for pre-harvest and post-harvest management. Indeed, I_{AD} allows to monitor the progression of apple fruit maturity directly in the orchard and it can be used to predict the best harvesting time and the heterogeneity of fruit maturity at harvest. The results presented in this work confirm the cultivar-specific behaviour of the I_{AD} evolution before harvest and its consistency over different growing seasons. Similar maturity trends were observed on for the two studied apple cultivars. The maturity evolution during time can be described by a linear regression. This information is specific for each cultivar and do not appear related to the growing season. The results reported on this study also highlight the variability of the maturity stage infield conditions and at harvest.

4.6. References

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4.7. Figures and Tables

Table 1. Fruit maturity expressed as I_{AD} at different DAFB, for the season 2011 on 'Granny Smith' and 'Pink Lady' apples. Different letters indicates significant differences at P=0.05 along the season, * indicates significant differences at P=0.05 between different samples size 300, 100 and 50.

	2011	**FruitMaturity (I_{AD})**		
Cultivar	**DAFB**	**300**	**100**	**50**
'Granny Smith'	**152**	1.97 ± 0.11a	1.97 ± 0.09a	1.93 ± 0.11a*
	159	1.96 ± 0.10a	1.96 ± 0.07a	1.96 ± 0.09a
	166	1.94 ± 0.08b	1.93 ± 0.08b	1.95 ± 0.08a
	169	1.92 ± 0.09c	1.91 ± 0.10c	1.87 ± 0.09b*
	175	1.90 ± 0.09d	1.88 ± 0.08d	1.89 ± 0.08b
	179	1.88 ± 0.08e	1.86 ± 0.08e	1.88 ± 0.09b
	187	1.83 ± 0.18f	1.81 ± 0.08f	1.87 ± 0.29b
'Pink Lady'	**159**	1.68 ± 0.16a	1.72 ± 0.16a	1.59 ± 0.18a*
	169	1.61 ± 0.17b	1.57 ± 0.19b	1.55 ± 0.17b
	175	1.55 ± 0.16c	1.50 ± 0.18c*	1.46 ± 0.17c*
	179	1.51 ± 0.20d	1.46 ± 0.22d	1.45 ± 0.22c
	183	1.41 ± 0.23e	1.37 ± 0.24e	1.19 ± 0.20d*
	187	1.31 ± 0.23f	1.29 ± 0.20f	1.29 ± 0.24e
	190	1.23 ± 0.22g	1.21 ± 0.24g	1.10 ± 0.23f*
	194	1.12 ± 0.17h	1.09 ± 0.15h	1.11 ± 0.18f

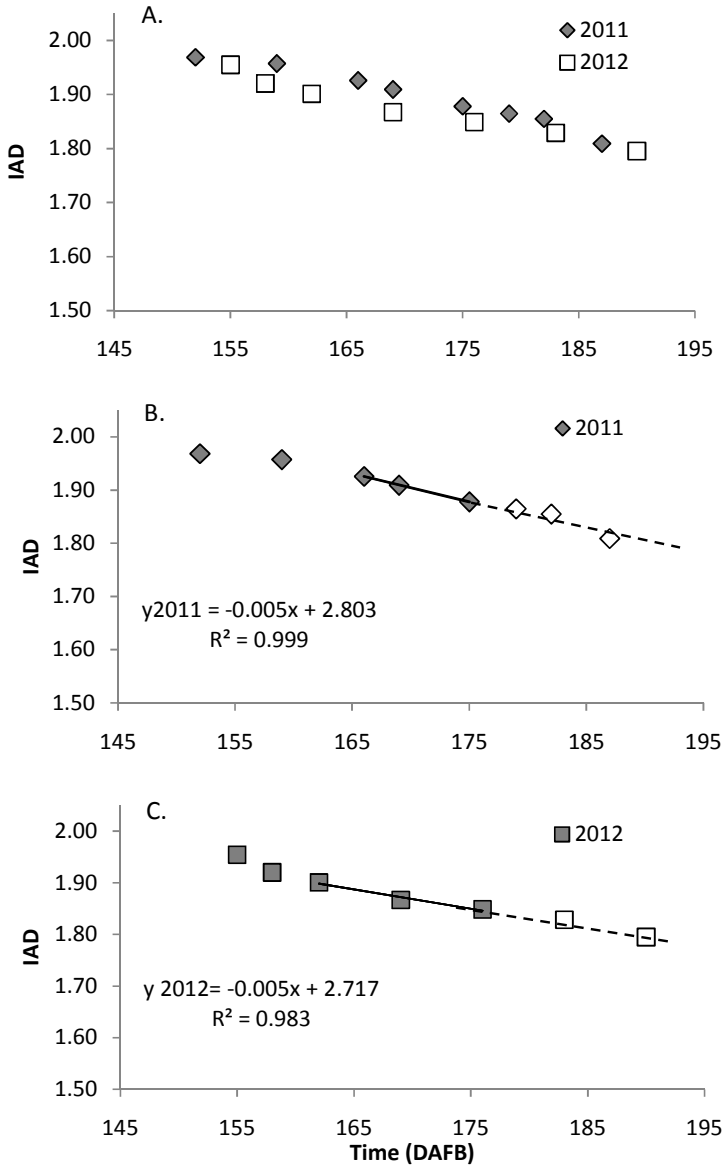

Figure 1.Evolution of I_{AD} values during time for the 'Granny Smith' apple fruit during the year 2011 and 2012 (A). The regression lines for the year 2011 (B) and 2012 (C) are reported. The regression lines were calculated by considering only 3 DAFB (solid lines). The predicted trend is expressed by the dotted line. White symbols are the measured I_{AD} mean values. Linear equations of the regression lines, and coefficients of determination (R^2) are reported.

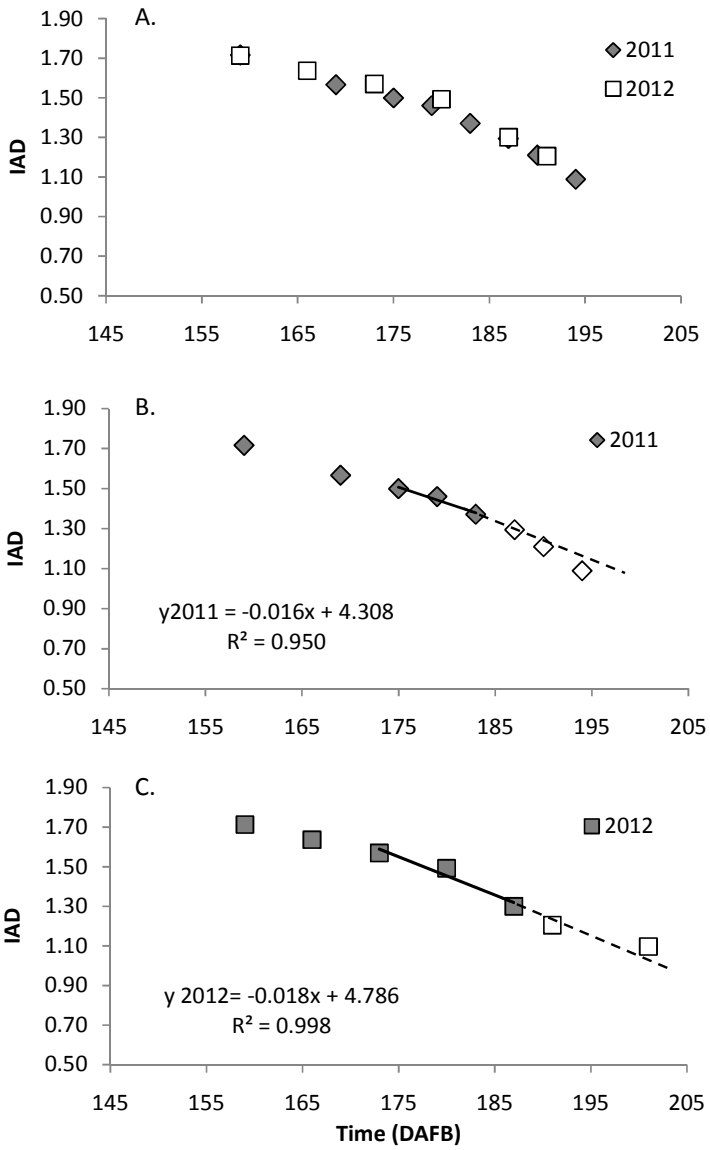

Figure 2. Evolution of I_{AD} values during time for the 'Pink Lady' apple fruit during the year 2011 and 2012 (A). The regression lines for the year 2011 (B) and 2012 (C) are reported. The regression lines were calculated by considering only 3 DAFB (solid lines). The predicted trend is expressed by the dotted line. White symbols are the measured I_{AD} mean values. Linear equations of the regression lines, and coefficients of determination (R^2) are reported.

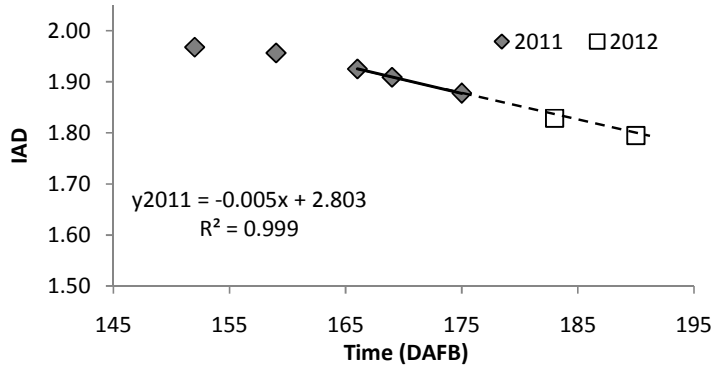

Figure 3. Validation of the 2011 maturity regression model for 'Granny Smith' with the data measured in 2012. The regression lines were calculated by considering only 3 DAFB (solid lines). The predicted trend is expressed by the dotted line. White squares (□) are the I_{AD} values measured in 2012. Linear equation of the regression line, and coefficient of determination (R^2) are reported.

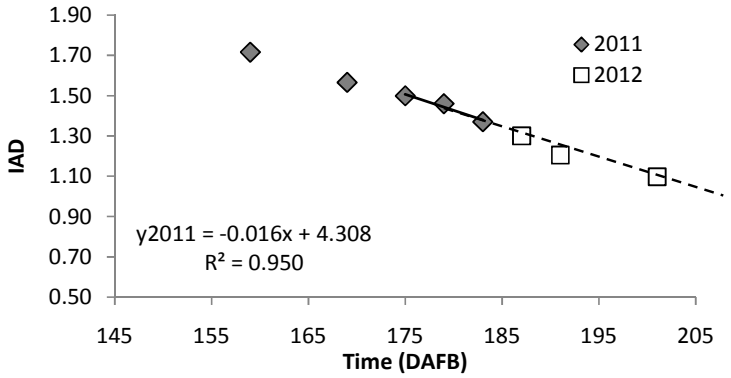

Figure 4. Validation of the 2011 maturity regression model for 'Pink Lady' with the data measured in 2012. The regression lines were calculated by considering only 3 DAFB (solid lines). The predicted trend is expressed by the dotted line. White squares (□) are the I_{AD} values measured in 2012. Linear equation of the regression line, and coefficient of determination (R^2) are reported.

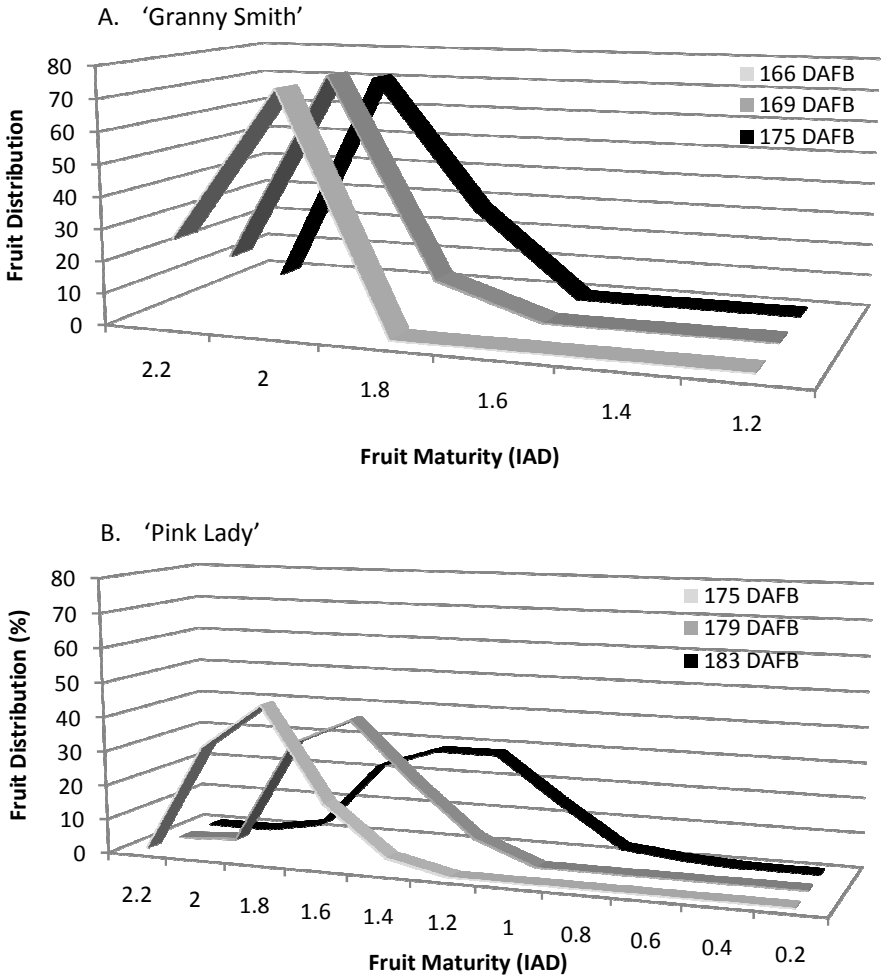

Figure 5. Apple fruit maturity distribution, expressed as percentage of fruits in the different I_{AD} classes, evaluated at three time points before harvest in 'Granny Smith' (A) and 'Pink Lady' (B). Data refers to the 2011 season.

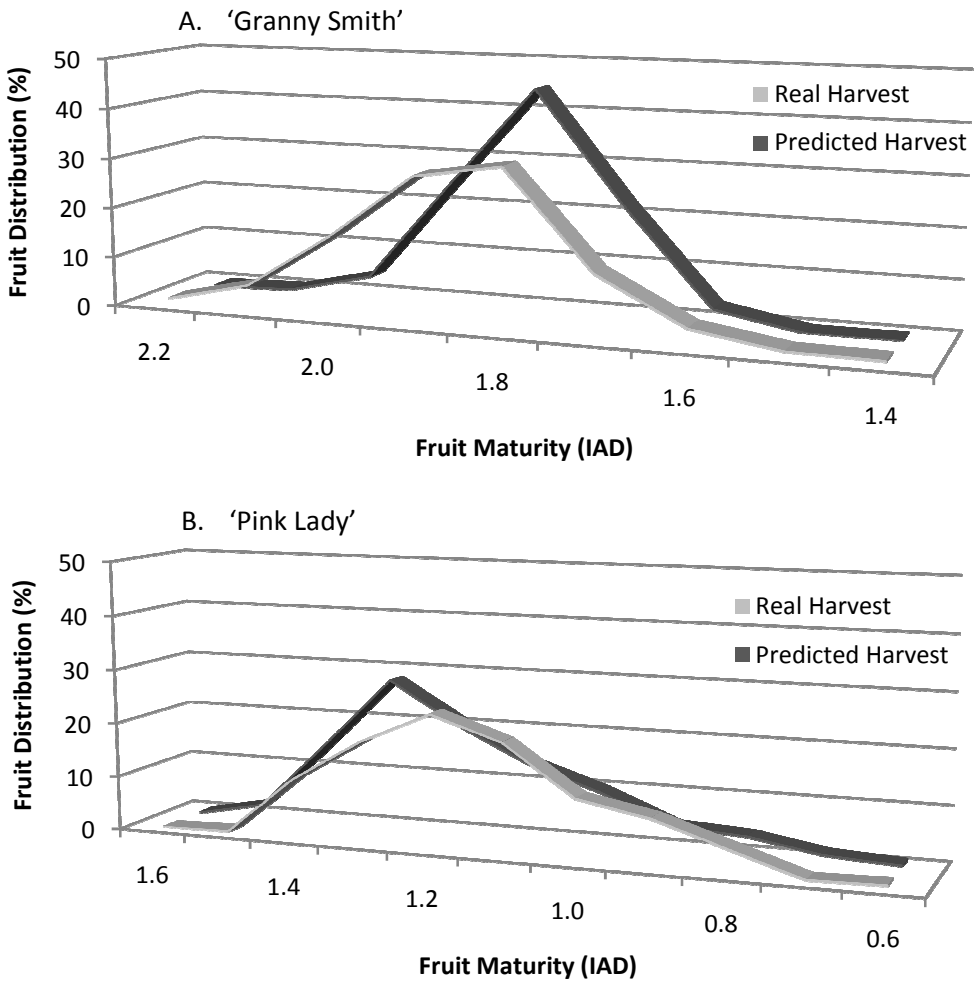

Figure 6. Comparison between the predicted and measure apple fruit maturity distribution at harvest. The maturity distribution is expressed as percentage of fruits in the different I_{AD} classes, (A): 'Granny Smith' and (B): 'Pink Lady'. Data refers to the 2011 season. The prediction of the heterogeneity of fruit maturity distribution at harvest was analysed by plotting the percentage of each forecasted I_{AD} maturity class against the results obtained with fruits sampled at commercial harvest.

Table 2. Regression coefficients, RMES and *p*-value for the linear regression model described by the I_{AD} for the growing season 2011 and 2012 on 'Granny Smith' and 'Pink Lady' apple fruits.

Cultivar	Year	R^2	RMES	*p* value
'Granny Smith'	2011	0.999	0.113	< 0.00001
	2012	0.983	0.106	< 0.00001
'Pink Lady'	2011	0.950	0.404	< 0.00001
	2012	0.998	0.325	< 0.00001

5. Relationship between fruit ripening stage at harvest, fruit quality and storage disorders

5.1. Abstract

Apple fruit physiological stage at harvest affects the final quality after storage and it also influence the susceptibility to cold storage disorders, as superficial scald. At harvest, fruits usually present a high heterogeneity of maturity stage, thus making the post-harvest handling complex. The present study aimed in testing a non-invasive method to group the fruits in homogenous maturity classes to be differentially processed during post-harvest. Trials were carried out for two seasons on 'Granny Smith' and 'Pink Lady' cultivars. At commercial harvest, the fruits were grouped in maturity classes according to their index of absorbance difference (I_{AD}). This parameter was used as a maturity marker since it evaluates chlorophyll-a degradation in fruit skin and flesh and correlates with the major quality and maturity traits. The evolution of I_{AD} was monitored in the different maturity classes during six months of post-harvest storage and it was correlated with the flesh firmness, starch degradation, sugar content, ethylene production and scald incidence. During storage, I_{AD} reduction and flesh softening followed a linear regression model with high determination coefficient for both cultivars (R^2 values over 0.85). I_{AD} evolution did not correlate with the changes of any of the other maturity traits during post-harvest. The different I_{AD} classes were also characterized by a different scald incidence, being the most mature fruits less susceptible in both cultivars. Our results suggest that I_{AD} can be a reliable marker to follow maturation during post-harvest and to steer the storage strategy to reduce the incidence of scald or excessive softening.

5.2. Introduction

Fruit ripening stage at harvest affects fruit quality and susceptibility to cold storage disorders, such as superficial scald or excessive softening. Scald is one of the most important problem occurring in apples during the cold storage and it heavily reduces the marketability of the fruits (Watkins *et al*., 1995; EccherZerbini *et al*., 1997; Kupferman, 2001; 2002). Superficial scald susceptibility is cultivar dependent, being 'Granny Smith', 'Cortland', 'Rome Beauty', 'Delicious' the most susceptible cultivars, whereas 'Pink Lady', 'Idared', 'Braeburn' the most resistant ones (Ingle and D'Souza, 1989; Tsantili, *et al*., 2007; Trivedi *et al*., 2010, Lurie and Watkins, 2012). Softening is another undesirable natural process, influenced by several pre-harvest and post-harvest factors. Therefore, a precise monitoring and/or prediction of this process during storage is very important in the whole commercial chain (Johnston *et al*., 2002). Unfortunately, the conditions favourable to the reduction of softening are also the ones increasing the susceptibility to scald. Indeed, to reduce the risk of excessive softening, fruits are harvested unripe which is the one of the main conditions increasing scald incidence (Watkins *et al.*, 1995*;* Eccher Zerbini, 1997*;* Lurie and Watkins, 2012). Therefore, the precise evaluation of fruit maturity at harvest is essential to steer the post-harvest strategies to minimize the occurrence of storage disorders.

Flesh firmness, total soluble solid, starch content, skin colour, ethylene emission are the major parameters currently used to define the fruit maturity and quality. However, all these analysis are destructive, time consuming and they need specific different instruments. In addition, these parameters differ from cultivar to cultivar and they are influenced by the climatic conditions during pre-harvest. Finally, any of these parameters alone is a reliable descriptor of fruit maturity.

In the last decades, not destructive methods based on Vis-NIRs technologies have been developed to estimate fruit maturity parameters and quality traits (McGlone *et al*., 2002; Peirs *et al*., 2000 and 2005; Zude-Sasse *et al*., 2002; Zude *et al*., 2006; Bertone *et al*., 2012). The most reliable equipments are able to evaluate the reduction in chlorophyll content as a result of the natural maturity process (Zude, 2003; Zude *et al*., 2006; Nicolaï *et al*., 2007; Costa *et al*., 2009, Bertone *et al*., 2012, Betemps *et al*., 2012). The DA-meter (TR Turoni, Forli, Italy) is an instrument based on this principle and allows to follow fruit

chlorophyll degradation through a new index called Index of Absorbance Difference (I_{AD}) (Ziosi *et al*., 2008). The DA-meter measure the absorbance in the wavelength window between 670 and 720 nm, which overlaps with the absorbance peak of the chlorophyll-a (Ziosi *et al*., 2008; Costa *et al*., 2009). In this way, the DA-meter provides an indication of the maturity stage related to the chlorophyll degradation involved in this process. So far, the use of the DA-meter was mainly studied on stone fruits to confirm the use of I_{AD} as a maturity index. In stone fruits, I_{AD} correlates with ethylene production, fruit quality traits, and transcription of ripening-related genes which attend the progression of the ripening process (Ziosi *et al*., 2008; Costa *et al*., 2009; Bonora *et al*., 2013a,b; Costa and Noferini, 2013; Shinya *et al*., 2013). Moreover, the DA-meter consents to segregate stone fruits at harvest by in homogenous ripening classes with diverse quality attributes and different behaviour during shelf-life (Costa *et al*., 2009; Infante *et al*., 2011; Costa and Noferini, 2013; Shinya *et al*., 2013). On apple fruits, the use of the I_{AD} have been recently studied on 'Gala', 'Starking', 'Granny Smith' and 'Pink Lady' to determine quality traits and its potential of the use of this index for ripening and quality assessment (Costamagna *et al*., 2013; Nyasordzi*et al*., 2013).

Hence, the general aim of this research was to investigate the I_{AD} as a maturity marker to improve the post-harvest management in apple fruits. I_{AD} was tested as a possible predictor for the length of storage in order to preserve quality attributes and minimize scald incidence and softening. The experiments were performed on two apple cultivars with diverse storage behaviour 'Granny Smith' and 'Pink Lady'.

5.3. Materials and Methods

5.3.1. Maturity assessment at harvest by DA-meter and fruit sorting

The experiments were carried out, for two consecutive years, on 'Granny Smith' and 'Pink Lady', which are known to have a different susceptibility to superficial scald. At harvest, about 600 fruits of each variety were grouped in different uniform ripening classes according to the I_{AD} distribution. Two classes were obtained in 'Granny Smith' (I_{AD} = 2.0-1.8 and I_{AD}< 1.8-1.6) and three in 'Pink Lady' (I_{AD} = 1.2-1.0, I_{AD}< 1.0-0.8, I_{AD}< 0.8-0.6).

The fruit belonging to different I_{AD} classes (20 fruit each) were also characterized for ethylene emission and quality parameters as described in the next sessions [i.e. flesh firmness (FF), total soluble solids (TSS), starch degradation].

5.3.2. Fruit quality traits and superficial scald incidence

Fruits belonging to different ripening classes (expressed as I_{AD}) were stored at 0.5 °C, 95% RH for six month. Every month, 20 fruits each 'Granny Smith' and 'Pink Lady' were sampled in all the different maturity classes and I_{AD}, FF, TSS and starch content were determined.

Flesh firmness (FF) was determined with a Fruit Texture Analyzer penetrometer (FTA Güss, South Africa); on the two equatorial sides using a pressure tester fitted with a 11 mm plunger tip, at 2 mm at a rate of 0.1 mm/s.

Total soluble solids (TSS) were assessed with a digital refractometer (DBR-95, Italy) on juice drops.

Starch content was determined with the Lugol test visually evaluating the starch conversion by the Ctilf (scale from 1 to 10).

Superficial scald incidence was detected on 50 fruits per class that were removed from storage after 2, 4 and 6 months. The symptoms were visually evaluated after 7 days of shelf life at 20°C. Superficial scald incidence was expressed as the percentage of injured fruits.

5.3.3. Ethylene emission

At harvest, ethylene production was determined by placing the whole fruit in a 0.8 L jar tightly sealed with a lid equipped with a rubber stopper, and left at room temperature for 1 h. A 10 ml gas sample of the head-space was taken and was injected in a Dani HT 86.01 (Dani, Milan, Italy) packed-gas chromatograph fitted with FID and a Porapak Q column (Supelco, Bellefonte, PA, USA). Oven temperature was set at 80°C, and for the injector and detector at 180°C. N_2 was used as the carrier gas at16 mL min^{-1} flow rate. Ethylene concentration was calculated and expressed as nanoliter per gram of fresh weigh per 1 h.

5.3.4. Statistical Analysis

Statistical analysis was performed using STATISTICA software Version 5.0 (Statsoft Inc., Tulsa, OK, USA). Analysis of variance and comparisons between means were performed using the ANOVA procedure by the Tukey's HSD test at *P* value = 0.05. Coefficient of determination for parameters at harvest and along storage were graphically represented by Excel software by linear regression model. Relation between I_{AD} and FF was described by linear correlation and Pearson Product-Moment Correlation was defined (95% confidence).

5.4. Results and Discussion

5.4.1. Maturity stage at harvest, I_{AD} and quality parameters

At harvest, 'Granny Smith' fruits were divided in two I_{AD} classes, while 'Pink Lady' fruits were grouped in three classes. For each class, FF, TSS, starch index and ethylene emission were determined (Table 1 and 2 for 'Granny Smith' and 'Pink Lady', respectively). In both cultivars as the I_{AD} decreased FF decreased, and TSS, starch index and ethylene content increased, but not in the same strength. 'Granny Smith' I_{AD} classes at harvest were well identified by differences on FF, TSS and starch content between classes, but no differences were observed regarding ethylene content since very low emissions were measured in these fruits (Table 1).

In 'Pink Lady', I_{AD} classes showed significant differences on FF and TSS, but only slight differences were observed on starch content. Regarding ethylene emission, the differences among classes started to be significant only for I_{AD} value lower than 0.8 (Table 2). Similar mean values at harvest were observed by Nyasordzi *et al*. (2013), with I_{AD} among 2.0 and 1.8 for 'Granny Smith', and I_{AD} about 0.8 and 0.5 for 'Pink Lady'. FF and ethylene emission, in each maturity class, did not differ from year to year. On the other hand, TSS and starch content were different in the different years (Table 1 and 2). The differences in starch index value from one year to other were linked with the date the starch conversion started in the different years, rather than to the rate of conversion (De Castro *et al*., 2007).

5.4.2. Relation between I_{AD} at harvest and quality parameters during storage

The effect of maturity stage at harvest, expressed as I_{AD} classes, was evaluated during cold storage. No difference on fruit behaviour along storage was observed between years for any of the study parameters. Along the storage, a decrease in I_{AD} values was observed, describing a linear regression model with high determination coefficient for both cultivars (R^2 values over 0.85) (Figure 1A and 2A). The differences between I_{AD} classes at harvest were mostly maintained throughout the storage (Figure 1A and 2A). Changes on I_{AD} along cold storage were specific for each cultivar and I_{AD} maturity class. In 'Granny Smith', after six month of storage I_{AD} of fruit belonging to the class 2.0-1.8 decrease about 10% (mean 2011-2012), while the class 1.8-1.6 decrease by 20% (Figure 1A). Meanwhile, for the same period of storage, the I_{AD} of 'Pink Lady' fruits showed a decrease of about 44% (mean 2011-2012) for the class 1.2-1.0, 41% for the class 1.0-0.8, and 53% for the class < 0.8-0.6 (Figure 2A). These results conformed those obtained in similar trials by Nyazordzi *et al*. (2013).

Changes in TSS during storage were only moderate, and the differences of TSS between I_{AD} classes along cold storage did not show any trend in both cultivars (data not shown). Starch content expressed by starch conversion Ctifel index, reached the maximum value after three and two months in cold storage for the less and more mature class respectively on 'Granny Smith' apples. In 'Pink Lady', the maximum value was recorded after two months in all the I_{AD} classes (data not shown).

In both cultivars, FF evolution during the six months of cold storage is described by a linear regression, with a high determination coefficients (R^2 values above 0.9) (Figure 1B and 2B). In 'Granny Smith', differences in FF between I_{AD} classes were maintained along storage showing a decreased of about 30% (mean 2011-2012) for both maturity classes (Figure 1B). In 'Pink Lady', at harvest, the differences in FF among the I_{AD} classes were limited, but become more pronounced during the storage. The highest decrease of FF was recorded in the most mature fruits (lowest I_{AD} value at harvest) (Figure 2B). After six months of storage FF decreased of about 25% for the I_{AD} class 1.2-1.0 (mean value of two years), 28% for the class 1.0-0.8, and 35% for the class 0.8-0.6 (Figure 2B). In a previous studies, FF at harvest was found to determine the final FF after storage, were the rate of softening depends on the harvest date and was not influenced by seasonal conditions, since the rate of softening was the same in the studied years (Kvikliene *et al*., 2005).

Fruit softening is an undesirable natural process considered a major quality problem along storage, since texture is the primary limiting factor for acceptability (Shewfelt, 1999, Johnston *et al*., 2002). Since both I_{AD} and FF follow a linear decrease during the cold storage, the correlation between these two parameters was evaluated by Pearson's product-moment correlation coefficient (Figure 3 and 4 for 'Granny Smith' and 'Pink Lady', respectively). The correlation coefficients (*r*) was above 0.7 and it was cultivar dependent showing similar or equal trend along the seasons. Correlation between chlorophyll degradation (measured by the I_{AD}) and the reduction of FF could be explained by the parallelism of two metabolic process: chloroplast degradation and pectin conversion on the maturity process (Harker *et al*., 1997; Johnston *et al*., 2002). Also other studies have been reported the use of Vis-NIR for apple fruit flesh firmness evaluation, and its estimation was improved when spectral measurements were combined with other non destructive measurements as acoustic measurements (Peirs *et al*., 2000; Moshou *et al*., 2005; Peng and Lu, 2006; Zude *et al*., 2006; Nicolaï *et al*., 2007). Linear relationship was reported between firmness and chlorophyll fluorescence which was also cultivar depended (Song *et al*., 1997; Moshou *et al*., 2005). Nyasordzi *et al*. (2013) reported that the I_{AD} value at harvest was useful to predict the softening during shelf life, but in their work, different cultivars were evaluated together in a mixed bunch of fruits.

5.4.3. Superficial scald incidence in different I_{AD} classes

In both cultivars, the incidence of scald was negatively correlated with maturity and fruits with lower I_{AD} value (more mature) were less prone to this disorder (Figure 5 and 6). Our data confirm the finding reported in previous studies (Watkins *et al*., 1995; Eccher Zerbini, 1997; Erkan and Pekmezci, 2004; Lurie and Watkins, 2012). An other factor positively influencing scald incidence was the duration of the storage and, in both cultivar, after 6 months of storage, in the least mature fruits the incidence was approximately 100% (Figure 5 and 6). In 'Granny Smith', the most mature fruits developed scald only after 4 months, whereas the less mature one presented a very high incidence also after 2 months. In 'Pink Lady', scald incidence was observed only after 4 months of storage and also in this case, it was highly related with fruit maturity at harvest.

Finally, our study confirmed that the development of scald is cultivar dependent being 'Granny Smith' more susceptible than 'Pink Lady' (Figure 5 and 6). The scald

symptomatology is shown in Figures 5B and 6B for ‘Granny Smith’ and ‘Pink Lady’ respectively. ‘Pink Lady’ fruits were less affected than ‘Granny Smith’, did not present any scald symptom until four month under cold storage. In both cultivar, the symptoms showed also a clear relation with the maturity classes.

In our study, scald incidence was more influenced by the $I_{AD,}$ the cultivar or the length of storage than by the different years. This result shows the potentiality of using the I_{AD} to segregate apple fruits, in different years, in maturity classes with differential behaviour along storage.

5.5. Conclusions

The use of the DA-Meter presents a high potentiality as a tool to divide apple fruits on different maturity classes, which behave differently during storage. This allow to tailor the post-harvest management according the fruit maturation expressed as I_{AD}. Indeed, I_{AD} correlated with FF reduction during cold storage and with scald incidence, being the less mature fruits more susceptible. In addition, I_{AD} trend remained similar or equal in the different seasons. Concerning with the relation between the I_{AD} and losses on flesh firmness, the use of the DA-meter technology by real time monitoring could be a useful device since the I_{AD} could be use as a predictor index for long term storage.

Based on these considerations, the development of an in line DA-meter device to be placed in the sorting machine is a high research priority to improve and optimise the post-storage procedure and management.

5.6. References

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5.7. Figures and Tables

Table 1. Characterization of 'Granny Smith' I_{AD} classes assessed at harvest. Values indicates means ± standard error (n=20). Different letters indicates significant differences between classes, and *differences between years, at P=0.05.

	I_{AD} class at Harvest	I_{AD}	FF (N)	TSS (%)	Starch (1-10)	Ethylene (nl/gFWh)
2011	2.0 - 1.8	1.92 ± 0.01a	80.4 ± 0.76a	10.6 ± 0.14b*	4.3 ± 0.20b*	0.05 ± 0.01a
	1.8 - 1.6	1.64 ± 0.01b	72.4 ± 0.73b	11.1 ± 0.18a*	7.2 ± 0.19a*	0.05 ± 0.01a
2012	2.0 - 1.8	1.89 ± 0.02a	81.7 ± 0.81a	11.6 ± 0.19b	5.8 ± 0.17b	0.03 ± 0.01a
	1.8 - 1.6	1.68 ± 0.01b	71.4 ± 0.63b	12.2 ± 0.12a	8.5 ± 0.14a	0.05 ± 0.01a

Table 2. Characterization of 'Pink Lady' I_{AD} classes assessed at harvest. Values indicate means ± standard error (n=20). Different letters indicates significant differences between classes, and *differences between years, at *P*=0.05.

	I_{AD} class at Harvest	I_{AD}	FF (N)	TSS (%)	Starch (1-10)	Ethylene (nl/gFWh)
2011	1.2 - 1.0	1.07 ± 0.02a	84.4 ± 1.10a	13.7 ± 0.12b	7.5 ± 0.14b*	0.1 ± 0.01b
	1.0 - 0.8	0.91 ± 0.02b	83.5 ± 0.49ab	13.9 ± 0.09ab	8.3 ± 0.15a	0.5 ± 0.08b
	0.8 - 0.6	0.64 ± 0.03c	80.8 ± 0.73b	14.2 ± 0.09a*	8.56 ± 0.20a	4.6 ± 0.58a
2012	1.2 - 1.0	1.15 ± 0.02a	85.9 ± 0.83a	13.1 ± 0.06b	8.7 ± 0.10a	0.1 ± 0.02b
	1.0 - 0.8	0.89 ± 0.01b	85.0 ± 0.66ab	13.6 ± 0.08a	8.8 ± 0.09a	0.1 ± 0.02b
	0.8 - 0.6	0.66 ± 0.01c	82.6 ± 0.57b	13.6 ± 0.10a	8.8 ± 0.09a	3.3 ± 0.56a

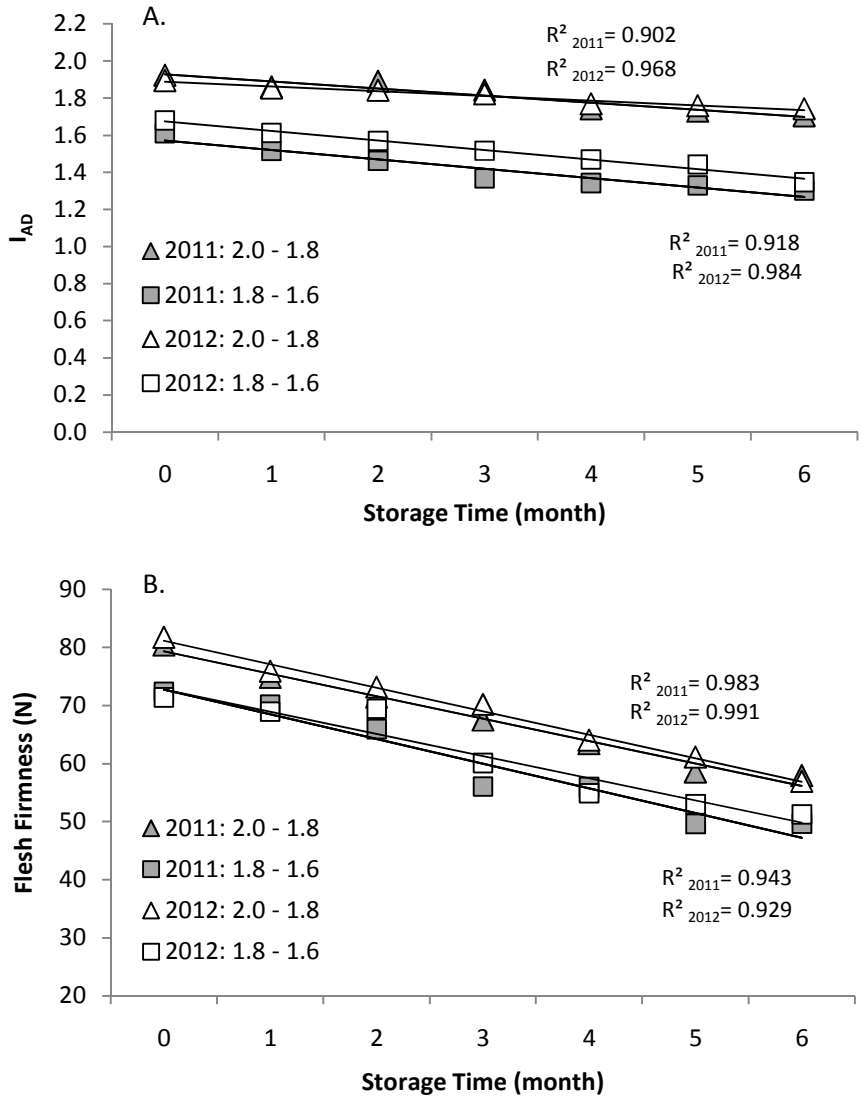

Figure 1. Changes in I_{AD} (A) and Flesh Firmness (B) of 'Granny Smith' apples during storage in relation to different maturity classes defined at harvest (I_{AD} 2.0-1.8 and I_{AD} 1.8-1.6). The data refer to the seasons 2011 and 2012. Each point is the mean of 20 fruits. Determination coefficients (R^2) are reported for each I_{AD} class for 2011 and 2012.

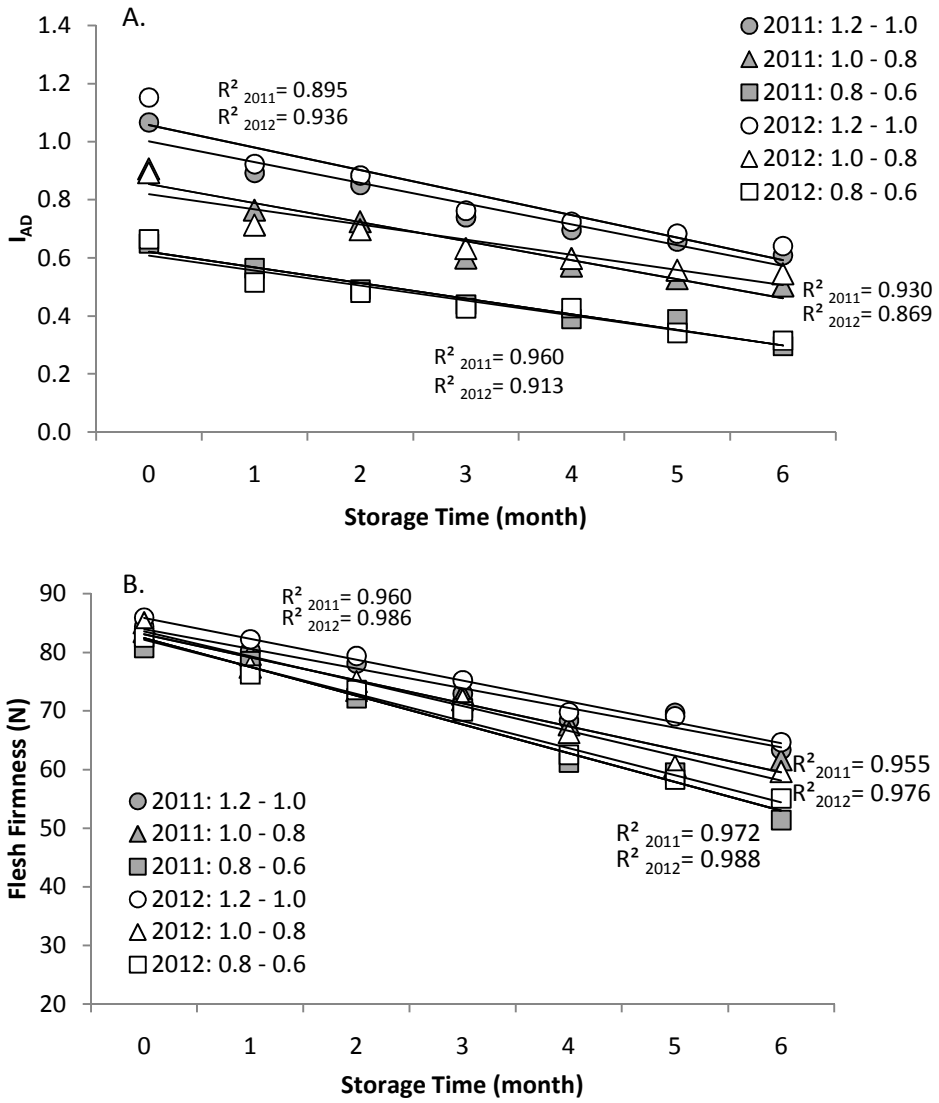

Figure 2. Changes in I_{AD} (A) and Flesh Firmness (B) of 'Pink Lady' apples during storage in relation to different maturity classes defined at harvest (I_{AD} 1.2.-1.0, I_{AD} 1.0-0.8 and I_{AD} 0.8-0.6). The data refer to the seasons 2011 and 2012. Each point is the mean of 20 fruits. Determination coefficients (R^2) are reported for each I_{AD} class for 2011 and 2012.

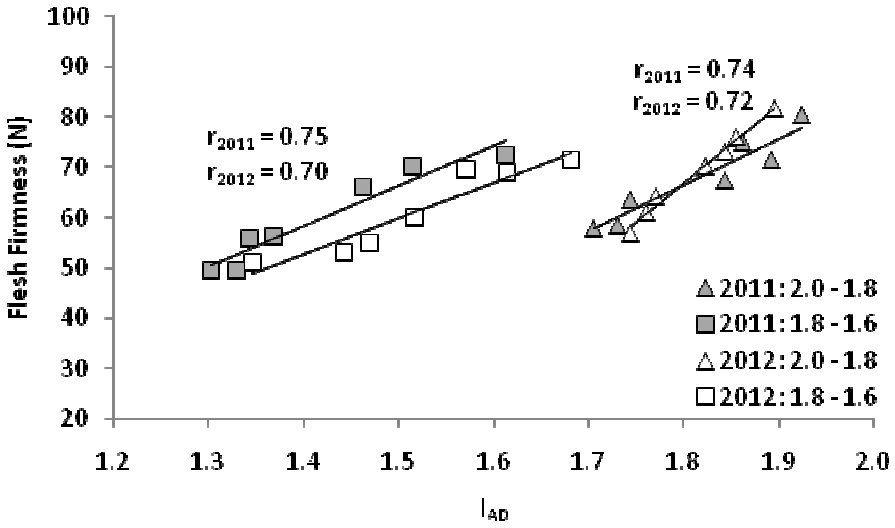

Figure 3. Linear correlation between Flesh Firmness and I_{AD} during storage for the two maturity classes assessed at harvest for 'Granny smith' apples (I_{AD} 2.0-1.8 and I_{AD} 1.8-1.6). The data refer to the seasons 2011 and 2012. Each point is the mean of 20 fruits, and r indicates the correlation coefficient for n=140.

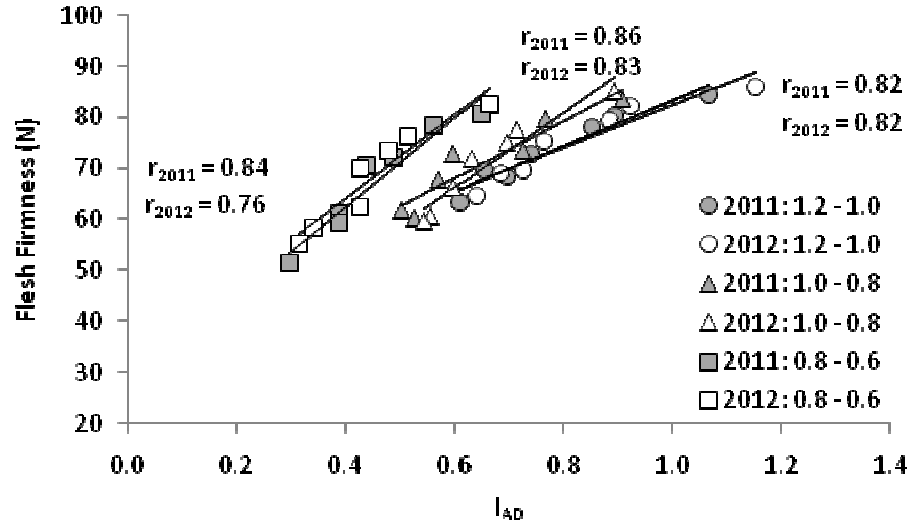

Figure 4. Linear Correlation between Flesh Firmness and I_{AD} during storage for two maturity classes assessed at harvest for 'Pink Lady' apples (I_{AD} 1.2-1.0, I_{AD} 1.0-0.8 and I_{AD} 0.8-0.6).The data refer to the seasons 2011 and 2012. Each point is the mean of 20 fruits, and r indicates the correlation coefficient for n=140.

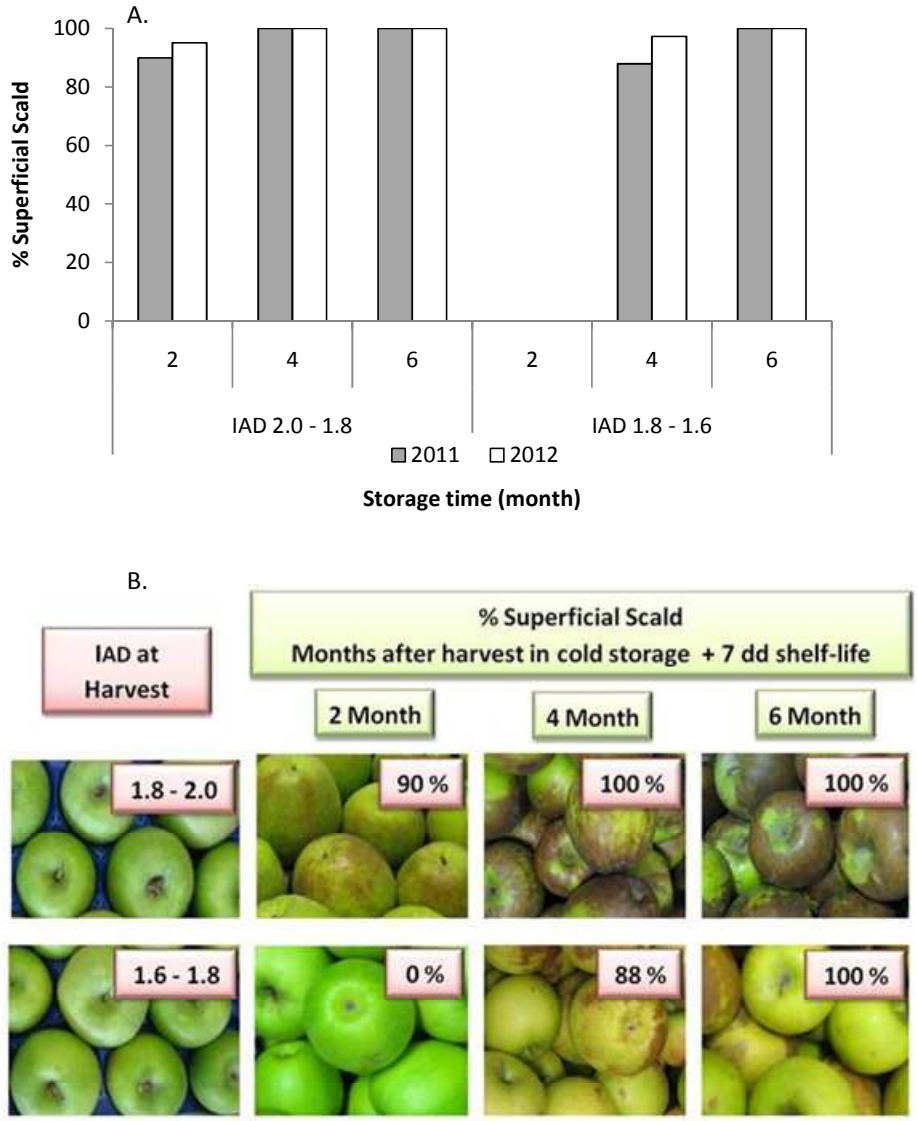

Figure 5. Superficial scald incidence in relation to different I_{AD} classes in 'Granny Smith' expressed as the percentage of injured fruits (A), and scald symptoms (B), after 2, 4 and 6 month of storage. Fruits were removed from the cold room and left for 7 days at room temperature.

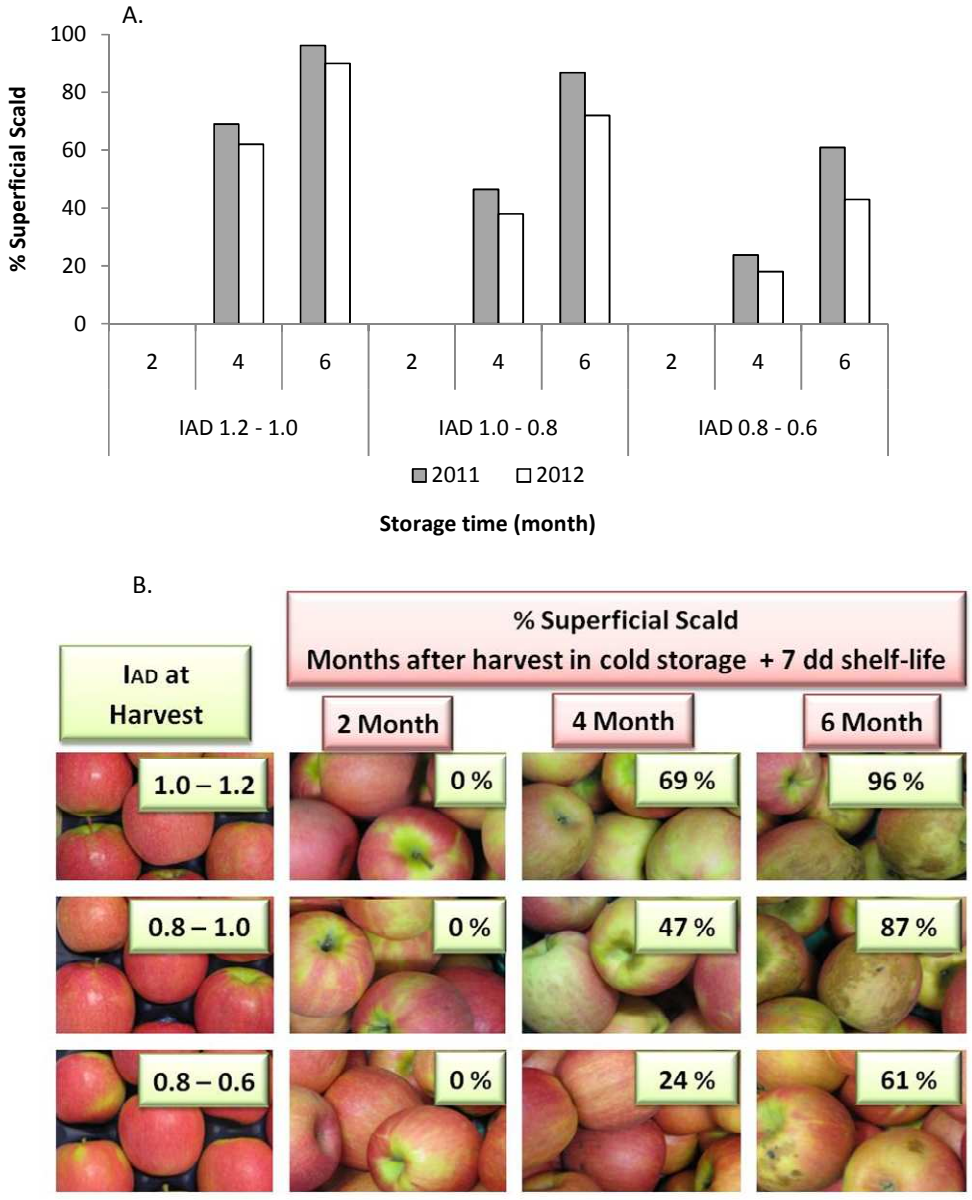

Figure 6. Superficial scald incidence in relation to different I_{AD} classes in 'Pink Lady' expressed as the percentage of injured fruits (A), and scald symptoms (B), after 2, 4 and 6 month of storage. Fruits were removed from the cold room and left for 7 days at room temperature.

6. Use of the index of absorbance difference (I_{AD}) as a tool for tailoring post-harvest 1-MCP application to control apple superficial scald

6.1. Abstract

Maturity stage at harvest is one of the main factors influencing apple susceptibility to superficial scald, one of the major apple storage disorders. The climacteric nature of apple fruit made ethylene considered a key element in the scald induction. An opportunity for scald control is presented by manipulating the ethylene-dependent plant reactions for instance by treating fruit with 1-Methylcyclopropene (1-MCP) before the storage. However, the efficacy of recovery time from 1-MCP is highly dependent on the fruit maturity stage at the time of application. The aim of this research was to better define postharvest strategies to preserve apple fruit quality during storage with particular attention on superficial scald control as the result of the interaction between 1-MCP treatment and fruit ripening stage. Ripening stages were defined by using the "Index of Absorbance Difference" (I_{AD}) measured by the DA-meter which is a portable and non-destructive device based on Visible/Near Infra-Red (Vis/NIR) spectroscopy. Superficial scald incidence and total content of α-farnesene and conjugated trienols (CTols), of the apple cultivars 'Granny Smith' and 'Pink Lady', were assessed at two month intervals, among 6 months of cold storage (1°C). Results demonstrated the reliability of the I_{AD}, not just to assess fruit maturity, but also to predict scald incidence in both apple cultivars as a function of maturity and postharvest control strategy. Consequently, differential post-harvest treatments can be applied to single appropriate apple batches increasing storability and shelf-life, while reducing spoilage.

6.2. Introduction

Scald is a physiological disorder of certain apple varieties associated with chilling injury and oxidative stress acquired during storage. It manifests as a necrosis within the surface layers of the hypodermal cortical tissue cells resulting in a superficial browning of the peel, leaving the pulp unaffected (Whitaker, 2004; Lurie and Watkins, 2012). This disorder still has considerable implications for the marketability and quality of fruits, and together with bitter pit, can be considered one of the major apple storage disorders (Mattheis, 2008). The occurrence of scald seems to be cultivar specific, with some varieties, such as 'Pink Lady', being more tolerant than others such as 'Granny Smith', 'Red Delicious', and 'Fuji', which appear to be predisposed to the onset of the disorder (Little and Holmes, 2000; Tsantili *et al*., 2007).

Effective methods of control have been developed by post-harvest dips in antioxidant solution containing diphenylamine (DPA), which successfully curb and manage oxidative events under a wide range of storage conditions (Whitaker, 2004). Due to the ever increasing concerns about the use of exogenous toxic chemical treatments and the banning of DPA dips, alternative, safer and more sustainable controls are required.

Other control methods, such as the storage under controlled atmosphere or the use of forced ventilation and 1-methylcyclopropane (1-MCP), do exist. However their efficacy is influenced by the maturity stage and uniformity of fruit batch. Indeed, maturity at harvest influences fruit behaviour during storage and responsiveness to post-harvest treatments (Erkan and Pekmezci, 2004; Watkins, 2006; Calvo and Candan, 2010). Furthermore, maturity stage at harvest is one of the major factors influencing fruit susceptibility to scald, with more mature fruits tending to have reduced scald severity than immature fruits (Watkins *et al*.,2000; Lurie and Watkins, 2012). Consequently, an effective treatment to control this storage disorder is difficult in heterogeneous fruit batches which generate a variable response to chemical treatment (Watkins, 2006; Calvo and Candan, 2010). Currently, in apple, starch content is the major indicator used to evaluate the timing of commercial harvest. Due to the small sample size heterogeneity in maturity of large harvest batches often occurs, thus, complicating possible chemical treatments and control.

Studies investigating the causes and possible control of scald were initiated in the 50's and currently, much is known about the physical, physiological and biochemical mechanisms

of scald development (Lurie and Watkins, 2012). Most of the researches have focused on the involvement of a volatile acyclic sesquiterpene, α-farnesene, and the accumulation of its oxidation products, which are conjugated trienols (CTols), as the principal causal agents of scald (Lurie and Watkins, 2012). It is generally posited that α-farnesene, which is induced by ethylene as part of the natural ripening process, accumulates in the apple skin during storage (Whitaker *et al*., 1997; Gong and Tian, 1998; Fan *et al*., 1999; Watkins *et al*., 2000). The oxidative pressure combined with chilling injury promote α-farnesene auto-oxidation, leading to the accumulation of harmful CTols through a free radical process, causing damage to the hypodermal tissue (Whitaker, 2004; Beuning *et al*., 2010). Correlations between scald occurrence, and α-farnesene and CTols quantities are sometimes weak, with susceptible varieties displaying lower α-farnesene accumulation than resistant ones, and with inconsistencies in single varieties in different growing regions (Lurie and Watkins, 2012). Consequently, the possibility of other processes in scald development is highlighted and other factors such as antioxidant capacity, enzyme-mediated oxidation, and associated free radical reactions have been suggested as possible factors affecting the onset (Wang and Dilley, 2000; Whitaker, 2004).

In contrast, the role of ethylene in the onset of scald has been clearly confirmed, with the perception, production and responsiveness of ethylene being key element in the scald induction (Watkins *et al*., 2000; Pechous and Whitaker, 2004; Lurie *et al*., 2005; Whitaker, 2008). Thus, ethylene control could be central in the management of this disorder. Indeed, ethylene does not provide an etiological explanation of scald, but an opportunity for scald control is presented by manipulating the ethylene-dependent plant reactions (Jung and Watkins, 2008).

Among the exogenous compound that can be possibly used to interfere with ethylene biosynthesis and/or perception, 1-MCP is a logical candidate. Applications of 1-MCP have been noted to successfully reduce scald incidence, even in susceptible cultivars (Tsantili *et al*., 2007). 1-MCP acts as an ethylene inhibitor by a long-term competitive binding to ethylene receptors and with a down-regulation of its biosynthesis (Sisler and Serek, 1997; Watkins, 2003; Tassoni *et al*., 2006). In this way, downstream cascades of ethylene-mediated events are temporarily delayed or inhibited. However, the efficacy of recovery time from 1-MCP is highly dependent on the fruit maturity stage at the time of application (Jung and Watkins, 2008). Indeed, as the fruit maturity advances, 1-MCP loses efficacy

(Tsantili *et al*., 2007). Consequently, timely treatment of fruits is essential for effective scald control (Watkins, 2007; Jung and Watkins, 2008). However, as with the control of many other ripening factors, its efficacy varies according to different storage conditions, cultivars, maturities with differing recovery times influenced by these parameters (Watkins *et al*., 2000; Tsantili *et al*., 2007).

Consequently, a decision support-tool that can identify the fruit maturity at harvest is needed. This decision support-tool would enable the division of fruits into homogenous maturity batches which can be subjected to differential post-harvest management regimes. In response to this need, extensive research has been focused on the development of non-destructive Vis/NIR technology for assessing fruit maturity stage (Bobelyn *et al*., 2010; Nicolaï *et al*., 2007). Recently research shows the correlation between the index of absorbance difference (I_{AD}), assessed by using VIS technology, and ripening related attributes in apple; these results show the possible potential for practical implementation in commercial lines (Nyasordzi *et al*., 2013).The implementation of I_{AD} in storage, pack-lines and distribution would provide a significant decision support tool in post-harvest management.

The scope of this study was to investigate the use of I_{AD} as a possible decision-support system to tailor 1-MCP application in order to maximise its efficacy in scald control.

6.3. Materials and Methods

6.3.1. Plant material and growing conditions

'Granny Smith' and 'Pink Lady' apple were selected for this study, with the former being highly susceptible to scald, while the latter more tolerant. Apple trees were grafted on M.9 rootstock. All trees were 4-years-old and were planted at a spacing of 0.8 x 3.3 meters for 'Granny Smith' and 1.0 x 3.3 meters for 'Pink Lady'. The commercial orchard used for the experiments was located in Bagnacavallo (RA), Northern Italy. Standard cultural and disease management strategies were applied.

Both cultivars were harvested at the commercial harvest date assessed by starch content and colouration parameters.

6.3.2. Maturity assessment

For each cultivar, maturity at harvest was determined using the I_{AD} assessed by the DA-meter which is a portable and non-destructive device based on Vis/NIR spectroscopy (TR, Forli, Italy) (Ziosi *et al*., 2008). I_{AD} usually ranges from 2.2 to 0 where the higher number will indicate a less ripe fruit characterized by a greater amount of chlorophyll present in the apple under skin and flesh. Maturity stage was assessed according to methods reported by Ziosi *et al*. (2008) and Nyasordzi *et al*. (2013). At harvest two maturity classes were identified for 'Granny Smith' (I_{AD} =1.9-1.7 and <1.7) and three maturity classes for 'Pink Lady' (I_{AD} = 1.2-1.0; I_{AD} = <1.0-0.8 and I_{AD}<0.8).

In each maturity class, for each cultivar, 360 apples were selected for post-harvest storage and 1-MCP treatment.

6.3.3. 1-MCP application

1-MCP was applied on the day of harvest to180 fruit for each I_{AD} class. 1-MCP treatment was applied as SmartFresh™ (0.14% active ingredient) according to the manufacturer's instructions (AgroFresh, Rohm and Haas, Philadelphia, Pennsylvania, USA), reaching a final gas concentration of 700 ppb. Fruit were exposed to 1-MCP for 24 hours at 20°C. After exposure, fruit boxes were ventilated and placed in cold storage at +1°C for 6 months in commercial storage conditions. 180non-treated fruits were used as control. The control fruits were processed in the same way of the 1-MCP treated ones.

6.3.4. Superficial scald incidence evaluation

50 fruits per treatment for each I_{AD} class were removed from cold storage every two months and kept at 20°C for 7 days. After this period, scald incidence was visually assessed and recorded as percentage of affected fruit.

6.3.5. Extraction and quantification of α-farnesene, CTols and ethylene emission

At harvest time and at two month intervals during storage, peel tissue, including the epidermis and 1-2 mm of hypodermal cortex, was excised from the equatorial region of 5 fruits per treatment from each maturity class and cultivar and immediately frozen in liquid N_2. Pooled samples of about 20-30 g were stored at -80°C in sealed bags. Extraction of α-farnesene and CTols was performed in three replicates for each pooled sample; extracts were analyzed by a HPLC equipped with a Photodiode Array Detector (Waters 2996), fitted with a 4.6 x 250 mm, Luna C18 column (Phenomenex, Torrence, CA), in accordance with the methodology reported by Whitaker *et al*. (2000). The α-farnesene and CTols identification was carried out through comparison of the retention time values and UV spectra with authentic standards(detected between 210 and 400 nm wavelength). For each compounds, the concentrations, expressed in μg g^{-1} fresh weight (FW), was calculated from curves obtained with known amount of the corresponding external standard.

Ethylene production was measured by placing the whole fruit in a 1 L glass jar sealed with an air-tight lid equipped with a rubber stopper, and left at room temperature for 1 h. An aliquot of 10 ml of the headspace was collected and injected into a Dani HT 86.01 packed-gas chromatograph (Dani, Milan, Italy) as described by Bregoli *et al*. (2002).

6.3.6. Statistical Analysis

Statistical analysis was performed using STATISTICA software Version 5.0 (Statsoft Inc., Tulsa, OK, USA). Analysis of variance and the Student-Newman-Keuls (SNK) test for comparisons between means were performed using the ANOVA procedure in the Stat software at $P = 0.05$. Variability between samples in the graphs was expressed as standard errors (SE) of the means.

6.4. Results

6.4.1. Scald occurrence in relation to I_{AD}

In ‘Granny Smith’, the least mature apples, as classified by the I_{AD} (1.9-1.7), were the most susceptible to scald, with a 100% incidence after just two months in storage (Figure 1). This high scald incidence was maintained throughout the whole storage period (Figure 1). The I_{AD}-classified more mature apple class (<1.7) displayed a maximum scald incidence (100%), but only after four months in storage, and this incidence was also maintained for the rest of the storage period (Figure 1).

In contrast, ‘Pink Lady’ apples displayed a lower scald incidence in all three I_{AD} defined classes of maturity at harvest (Figure 2), comparing with ‘Granny Smith’. In all measured maturity classes, even the least mature fruits were storable for up to two months without developing scald (Figure 2). For periods of storage longer than two months, differences in scald incidence among the maturity classes were observed: most mature fruit (I_{AD}<0.8) resulted the least scald susceptible, while an increasing susceptibility was provoked by the higher immature stages (I_{AD}<1.0-0.8 and 1.2-1.0) (Figure 2). The percentage of scald incidence correlated with I_{AD}-classified maturity stages over four and six months of storage, with more mature fruit displaying less scald incidence than immature fruit (Figure 2). The most mature fruit (I_{AD}<0.8) showed a scald incidence of 17% and 47% after four and 6 months of storage, respectively (Figure 2). Whereas, in the least mature class (I_{AD} 1.2-1.0) 78% and 93% of scald incidence was reported after four and six storage months. Fruit belong to the central ripening class (I_{AD}<1.0-0.8) showed, instead, 57% and 67% of scald incidence, respectively (Figure 2).

6.4.2. Effect of 1-MCP on scald development

In ‘Granny Smith’, 1-MCP treatment effectively reduced to zero the incidence of scald in the most immature apples (I_{AD} 1.9-1.7) after two and four months of storage (Figure 1). At six months scald incidence of 1-MCP-treated apples was recorded as 32%, resulting still significantly lower than the control fruit (100%). In more mature fruits (I_{AD}<1.7), 1-MCP maintained a zero scald incidence after two months of storage, after which incidence increased to 41% and 43% after four and six months storage, respectively. Also in this

case, scald incidence was significantly reduced in comparison to control. However,1-MCP efficacy on most mature apple, as classified by I_{AD}, was less than that observed in immature fruit class (Figure 1).

In 'Pink Lady' apples, 1-MCP application was effective at controlling scald incidence at all maturity stages throughout all storage periods (Figure 2). Even after six months of storage, any symptoms of scald were observed in apples treated with 1-MCPeven in the most immature I_{AD}-classified fruit (I_{AD} 1.2-1.0) (Figure 2).

6.4.3. Trends in accumulation of α-farnesene and CTols in relation to I_{AD}

The concentration of α-farnesene of 'Granny Smith' apples, detected in peel tissue, did not differ between the two maturity classes at harvest and until two months of storage (Figure 3A). In both maturity classes, the α-farnesene values were zero at harvest and increased up to around 100 µg g^{-1} FW after two months of storage. Thereafter, fruit belonging to the most immature class decreased the α-farnesene content till around 40 µg g^{-1} FW after six months of storage. Otherwise more mature fruits maintained the α-farnesene level constant till the end of the storage period (Figure 3A).

Differences in the CTols accumulation between different maturity classes were evident at two months after storage (Figure 3C). In immature fruit (I_{AD} 1.9-1.7) a faster rate of CTols accumulation was observed, when compared to more mature fruit (Figure 3C). Specifically, CTols levels increased to 54ug g^{-1} FW and reached a maximum at four months of storage, after which a progressive decrease was recorded. In more mature fruit CTols concentration after two months of storage was 36 ug g^{-1} FW and remained approximately constant till the end of the storage. At six months, both mature and immature fruits presented similar levels of CTols (Figure 3C).

At harvest, most immature fruit of 'Pink Lady' had lower α-farnesene concentration than mature fruits, (Figure 4A). The α-farnesene concentration increased from harvest reaching a maximum level at around two months of storage when the fruits from the three ripening classes showed comparable α-farnesene concentration (ca. 100 µg g^{-1} FW). Thereafter, α-farnesene level remained approximately stable, with a slight decrease, until the end of storage. In the lower maturity classes this decrease was more consistent (Figure 4A).

CTols levels detected in the skin of the fruit of different maturity classes were similar throughout the storage period with a more evident increasing trend in the two more mature classes (Figure 4C). Immature fruit (I_{AD} 1.2-1.0) exhibited a slight decrease in CTols content between four and six months of storage. CTols levels recorded in 'Pink Lady' were lower than 'Granny Smith' despite the similar α-farnesene levels observed between the two cultivars (Figure 3A and 4A).

6.4.4. Effect of 1-MCP application on α-farnesene and CTols accumulation

'Granny Smith' immature fruits (I_{AD} 1.9-1.7) treated with 1-MCP showed a reduced rate of α-farnesene accumulation during the six months of storage relative to the control (Figure 3A, B).The greatest reduction was observed at two months after storage. The rate of α-farnesene accumulation in the skin of more mature fruit (I_{AD}<1.7) was similar during the six months of storage for both treated and control fruit; for both fruit batches maximum α-farnesene quantities were comparable (Figure 3A, B). Finally, the α-farnesene concentration, regardless of 1-MCP treatment, were higher in more mature fruits than in immature fruits.

CTols accumulation rate was greatly reduced, in immature 'Granny Smith' fruits (I_{AD} 1.9-1.7), when compared to control (Figure 3C, D). In addition, similar to what observed for α-farnesene, CTols content in treated apples constantly increased during the storage while in control apples it started to decrease after four months of storage. On more mature fruits (I_{AD}<1.7), the CTols accumulation rate was reduced by 1-MCP and the effect was more pronounced as for α-farnesene (Figure 3C, D). The maximum accumulation in 1-MCP fruits was approximately half that observed in control fruits. Finally, similar to α-farnesene, also the CTols level, regardless form 1-MCP treatment, was higher in more mature fruits than in immature fruits.

1-MCP application in 'Pink Lady' affected more consistently α-farnesene emission, than CTols accumulation. On the immature fruits (I_{AD} 1.2-1.0) and mature fruits (I_{AD}<1.0-0.8) α-farnesene accumulation rate was delayed by 1-MCP reaching a maximum level only after four months in storage (Figure 4A, B). Whereas, in the most mature fruits (I_{AD}<0.8) no effect of 1-MCP on α-farnesene was found.

1-MCP did not significantly affect CTols concentration as related to the different maturity classes (Figure 4D). Contrary to control immature fruits (I_{AD} 1.2-1.0), reduction of CTols concentration was not observed at 6 months of storage (Figure 4C, D).

6.4.5. Trends in ethylene emission in relation to I_{AD} and 1-MCP

1-MCP treatment strongly reduced the 'Granny Smith' fruit ethylene emission as compared to control. No differences were observed in 1-MCP treated fruit between the two maturity classes (Figure 5A). In control fruits, ethylene emissions were significantly higher in the less mature class throughout the 2, 4 and 6 months storage period (Figure 5A).

In 'Pink Lady', the same trend was observed and 1-MCP significantly reduced ethylene emissions (Figure 5B). No consistent trend in ethylene emission among different classes was observed, but at 6 months storage the least mature class was observed to have significantly higher ethylene emission than the other two maturity classes (Figure 5B).

6.5. Discussion

According to our results less mature fruits of both cultivars, as defined by the I_{AD}, presented an enhanced degree of scald susceptibility either earlier during storage (as with 'Granny Smith') or later in storage (as in 'Pink Lady'). Batches of I_{AD} defined maturity behaved congruently with other studies focused on maturity as key parameter of scald susceptibility (Wang and Dilley, 2000; Lurie and Watkins, 2012). Therefore, I_{AD} showed to be a reliable parameter to assess fruit maturity at harvest and to predict fruit behaviour in storage. Furthermore, the influence of genotype on scald susceptibility was confirmed (Tsantili *et al*., 2007) since 'Granny Smith' fruit had a much higher and sooner incidence of scald during storage than 'Pink Lady'.

Furthermore, it was demonstrated that the use of 1-MCP applied at the correct maturity stage is an efficient method of scald control. 1-MCP treatment of 'Granny Smith' immature fruit was more successful over longer storage periods, whereas the same treatment in mature fruit was not as effective, displaying higher and also sooner scald

incidence during storage. This is congruent with studies advocating earlier 1-MCP treatments results in better scald control (Jung and Watkins, 2008) since delayed applications leads to a reduced efficacy in the control of ripening and ripening related processes (Watkins and Nock, 2005; Tatsuki *et al*., 2007).

In an advanced maturity stage, ethylene biosynthesis has been already initiated, triggering downstream ethylene-mediated physiological events. Thus, even though subsequent ethylene emissions are suppressed, the effects of 1-MCP are negated as maturity signal cascades have been fully or in part initiated (Watkins, 2006; Jung and Watkins, 2008; Calvo and Candan, 2010). Indeed, in our experiments carried on 'Granny Smith', the efficacy of 1-MCP was evidently influenced by the maturity. In this cultivar, 1-MCP only partially controlled scald incidence with a loss of efficacy over time. Whereas, in 'Pink Lady', the use of 1-MCP was effective across all maturity classes in fully suppressing scald even at 6 months of storage. Our results ultimately indicate that if applied at the correct physiological maturity stage 1-MCP can be used effectively to prevent scald, both in 'Granny Smith' and in 'Pink Lady', and thus, conferring a particular value to the non-destructive assessment of the I_{AD} index, that could enable the precise tailoring of 1-MCP treatments.

These results are promising for enhancing the efficacy of scald management; however these do not provide insight on the mechanisms underlying scald development. In order to explain scald incidence in relation to cultivar, maturity, storage duration and 1-MCP application, key substrates, such as α-farnesene and CTols were quantified throughout the storage.

In immature 'Granny Smith' apples, α-farnesene and CTols concentrations seemed to correspond with the traditional reactant-product curve, with a rise, peak and decline in α-farnesene, followed by a similar but delayed trend in CTols. Similarly this trend has been described by Whitaker (2004), stating that 'Granny Smith', along with other susceptible cultivars, show a burst of α-farnesene shortly after being placed in low temperature storage (Whitaker, 2004). However, the traditional reactant-product curve was not observed neither in the other maturity classes, nor in 'Pink Lady', suggesting that the auto-oxidation of α-farnesene in generating CTols may be a too simplified hypothesis (Whitaker, 2008; Lurie and Watkins, 2012). Other factors are likely to be involved in the traditional α-farnesene and CTols hypothesis accounting for anomalies observed between the standard

product-reactant relationships. For example, the decrease in CTols observed towards the end of storage in 'Granny Smith', could be associated with the oxidation of CTols to 6-methyl-5-hepten-2-one (Mir and Beaudry, 1999; Wang and Dilley, 2000). The role of the latter in scald onset is still unclear, with some studies indicating that it is through the production of 6-methyl-5-hepten-2-one that scald symptoms are generated (Mir and Beaudry, 1999; Wang and Dilley, 2000; Whitaker and Saftner, 2000). In addition, the structure of CTols indicated that they could be derived from several naturally occurring ripening volatiles that accumulate and oxidize during storage. Other factors that curb the oxidation of α-farnesene to CTols, such as antioxidants, could be important (Du and Bramlage, 1995). Furthermore, antioxidant systems develop with increasing maturity and therefore their capacity to prevent oxidative stress can also change temporally (Barden and Bramlage, 1994).

According to our results, a quantitative threshold for α-farnesene and CTols concentrations could not be derived for predicting scald onset. For example, both immature and mature fruits of 'Granny Smith' reached similar α-farnesene concentrations in 2 months of storage, but only, the immature fruits displayed severe scald incidence (100%), while the other class did not. Similarly, neither CTols concentrations provided a quantitative limit: for example, in 'Granny Smith' more mature apples, maximum CTols concentrations was reached in the second month of storage, while scald symptoms manifested only two months later.

The effect of 1-MCP on these compounds is interesting, as it further highlights the inconclusiveness of the role of α-farnesene and CTols in scald incidence. For example, in 'Granny Smith' immature fruits, the application of 1-MCP delays and reduces the production of α-farnesene, reaching a maximum at month 4 and remaining constant thereafter, but scald occurring only in month 6. Similarly, 1-MCP applied in more mature fruits, delays the rate of α-farnesene accumulation, but ultimately the same quantities as the control fruit of the same class are reached. In addition, in 'Pink Lady' even though the concentration of α-farnesene was not reduced by 1-MCP application, a complete control of scald was observed in treated fruits. Our results indicate that the absolute concentration of these compounds may not be as important as their rate of accumulation in scald development, in support on the results obtained by Pechous *et al*. (2005) that reported a faster rate of α-farnesene and CTols accumulation is associated with more susceptible

varieties. Interestingly, also in ‘Pink Lady’, the immature class had a faster rate of α-farnesene accumulation than more mature classes, again highlighting the possibility that the rate of accumulation is more important in generating a scald than absolute values of α-farnesene. For example, in this cultivar, treatments with 1-MCP delayed the rate of α-farnesene and CTols biosynthesis, with the most mature fruits being the least responsive to 1-MCP treatment. However, in these fruits, α-farnesene and CTols concentrations reached levels similar to the control ones, but the scald was completely suppressed.

The role of α-farnesene and CTols in the onset of scald in ‘Pink Lady’ fruit is unclear, with more mature classes having higher concentrations throughout storage than immature fruit that, instead, displayed the highest scald incidence. In addition, relatively low levels of CTols were recorded indicating that something must prevent or limit the oxidation of α-farnesene to generate CTols present in ‘Pink Lady’ to confer a type of resistance.

The treatment with 1-MCP, as expected, completely suppressed ethylene emission in both cultivar and in all the maturity classes. Nonetheless, the ethylene suppression did not result in a subsequent robust reduction also in α-farnesene which is supposed to be under ethylene regulation. This also highlight the role of ethylene in possibly coordinating other processes associated with the onset of scald and not just in the regulation of α-farnesene (Pechous *et al*., 2005). In our experiment, also the role of ethylene in influencing scald incidence is not fully clarified. In ‘Granny Smith’ less mature apples, which are more prone to scald development, consistently displayed significantly higher ethylene emission when compared to more mature apples. Indeed, other studies have indicated anomalous ethylene evolution in ‘Granny Smith’, when compared to other cultivars. In fact, ‘Granny Smith’ has been found to have lower ethylene and ACC levels at room temperature compared to other cultivars at commercial harvest (Larrigaudiere *et al*., 1997). In addition, also the basal ethylene emission was lower during storage (Larrigaudiere *et al*., 1997). Furthermore, in other cultivars it was observed, that low storage simply postpones the ethylene-mediated fruit behavior, while in ‘Granny Smith’ this is modified by a cold storage period. Also ACC levels in apple are usually inhibited by low temperatures, whereas in ‘Granny Smith’ they are stimulated, with also a *de nuovo* synthesis of ACC oxidase (Larrigaudiere *et al*., 1997). This anomalous behaviors may be attributed, at least partially, to a different physiological status as commercial harvest when ‘Granny Smith’ may be in a pre-climateric stage. It has been noted that ethylene biosynthesis in the peel

and pulp of ‘Granny Smith’ is differentially during maturation and ripening (Lara and Vendrell, 2000a,b). These unusual aspects of ethylene metabolism in ‘Granny Smith’, possibly may generate the differences in metabolic events leading to scald susceptibility.

6.6. Conclusions

In conclusion, fruit batches with differing maturity stages, as defined by I_{AD}, showed differential incidence of scald, with a trend congruent to that revised in the literature: more immature fruit demonstrating a higher scald incidence than mature once. This demonstrates the reliability of the I_{AD}, not only to assess fruit maturity non-destructively, but also to predict scald incidence in both ‘Granny Smith’ and ‘Pink Lady’ as a function of this parameter. Furthermore these results support the use of 1-MCP in the control of this postharvest disorder, with its efficacy strictly dependent on the cultivar and maturity stage at harvest.The integration of the I_{AD} assessment into pack-line could contribute to an overall optimisation of the post-harvest management by a selective application of chemicals, and a reduction of input costs.

6.7. References

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6.8. Figures

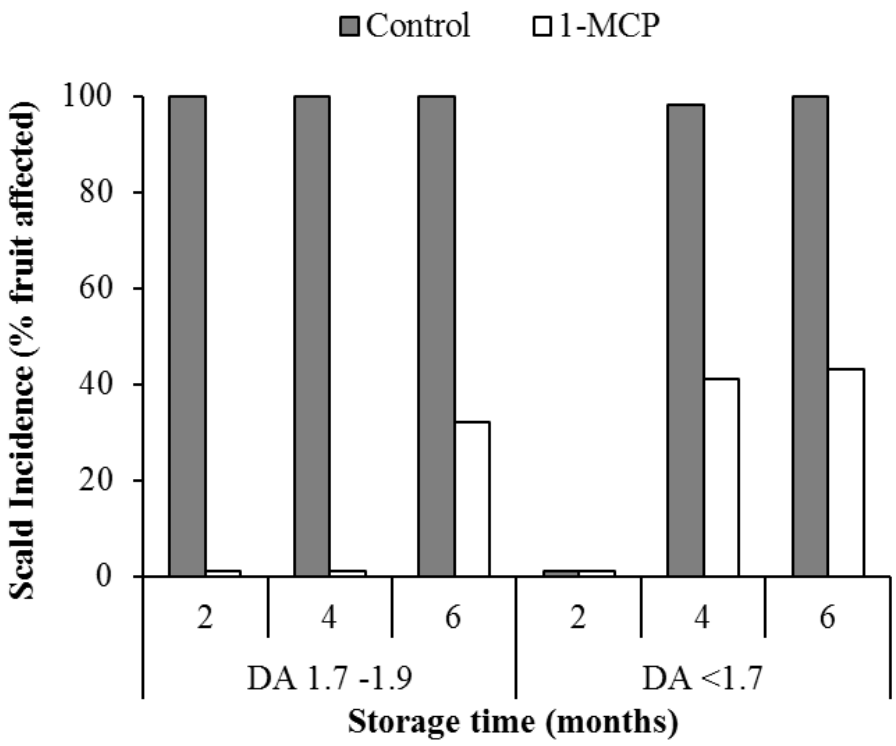

Figure 1. Scald incidence (%) in Granny Smith apples in relation to 1-MCP treatment performed at two different ripening stages determined by I_{AD}. The scald incidence was evaluated on fruit left at room at temperature for 1 week after 2, 4 and 6 months of commercial storage.

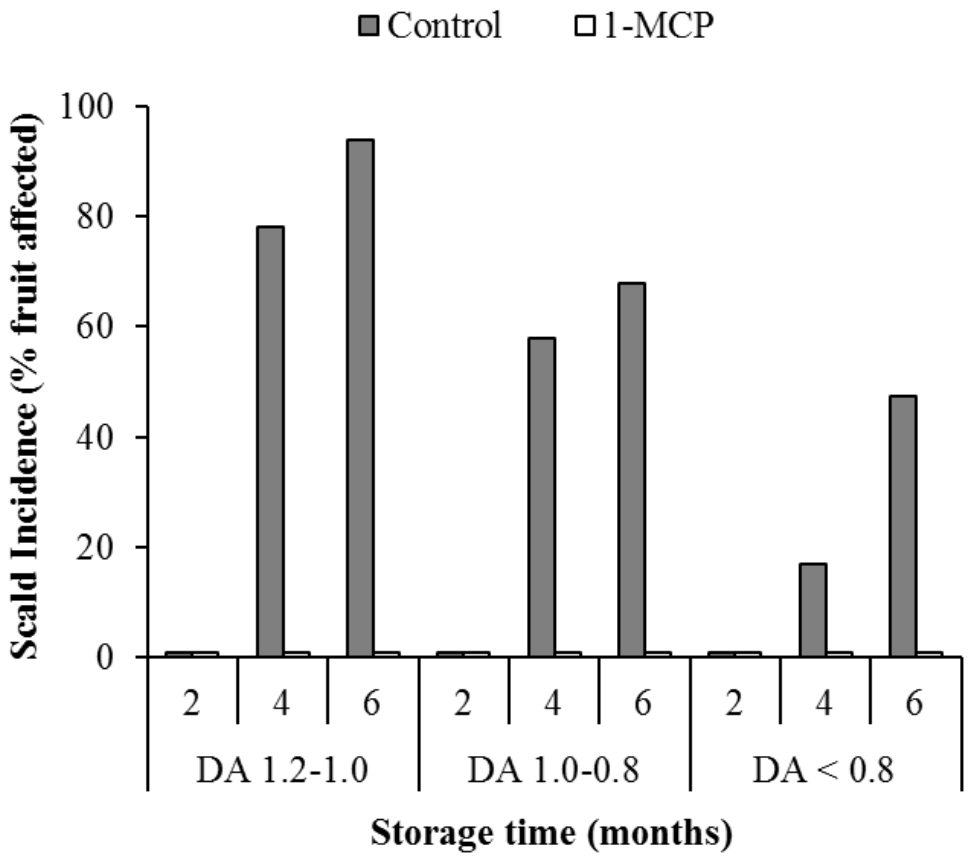

Figure 2. Scald incidence (%) in Pink Lady apples in relation to 1-MCP treatment performed at three different ripening stages determined by I_{AD}. The scald incidence was evaluated on fruit left at room at temperature for 1 week after 2, 4 and 6 months of commercial storage.

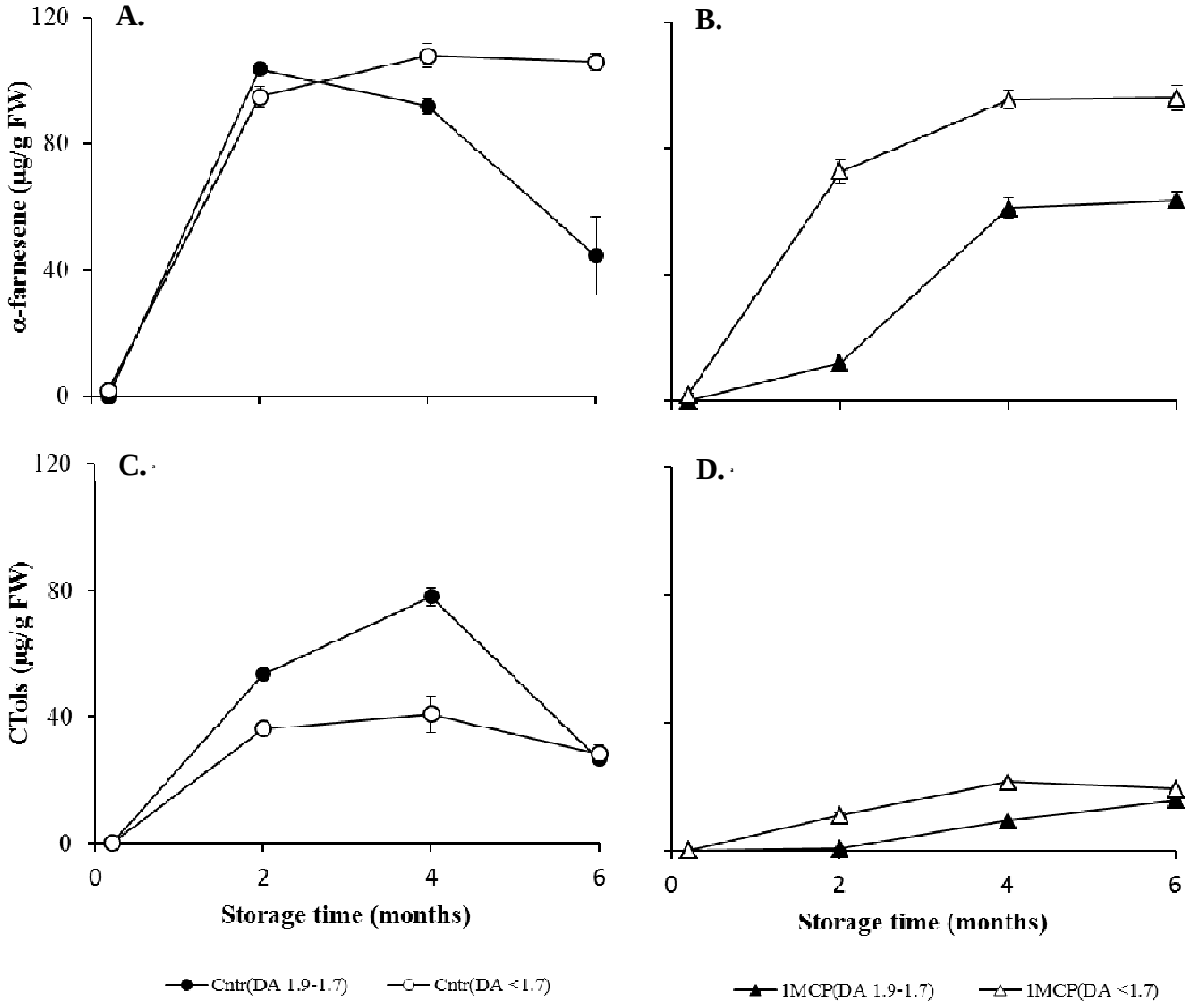

Figure 3. Storage evolution of α-farnesene (a,b) and CTols (c,d) content of "Granny Smith" apple harvested at two ripening stages, determined by I_{AD} and treated. Measurements were assessed on skin tissue of fruit left at room at temperature for 1 week after 2, 4 and 6 months of commercial storage. Half fruit batch was treated with 1-MCP (right hand side graphs) immediately after harvest. Standard error is shown.

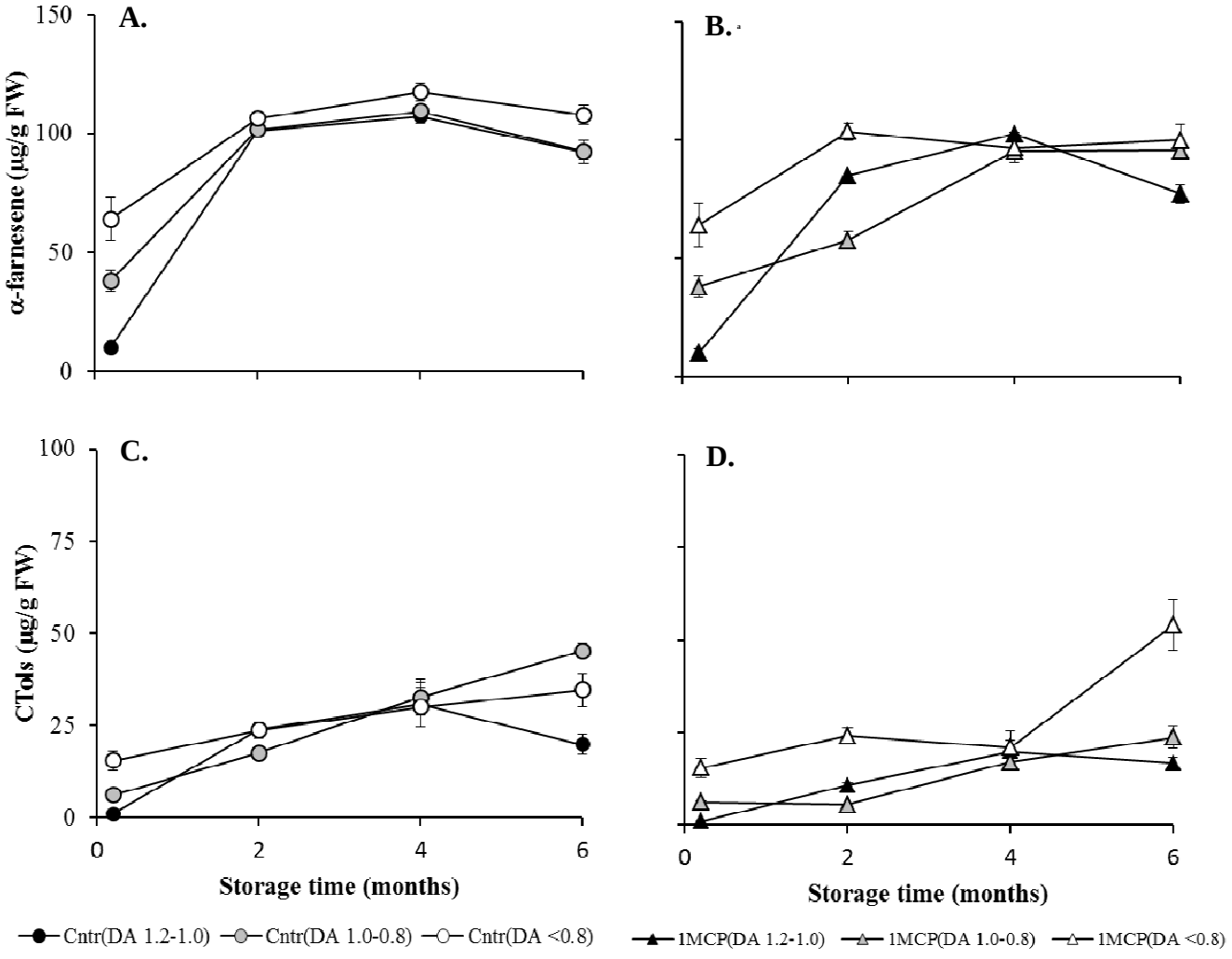

Figure 4. Storage evolution of α-farnesene (a,b) and CTols (c,d) content of "Pink Lady" apple harvested at two ripening stages, determined by I_{AD} and treated. Measurements were assessed on skin tissue of fruit left at room at temperature for 1 week after 2, 4 and 6 months of commercial storage. Half fruit batch was treated with 1-MCP (right hand side graphs) immediately after harvest. Standard error is shown.

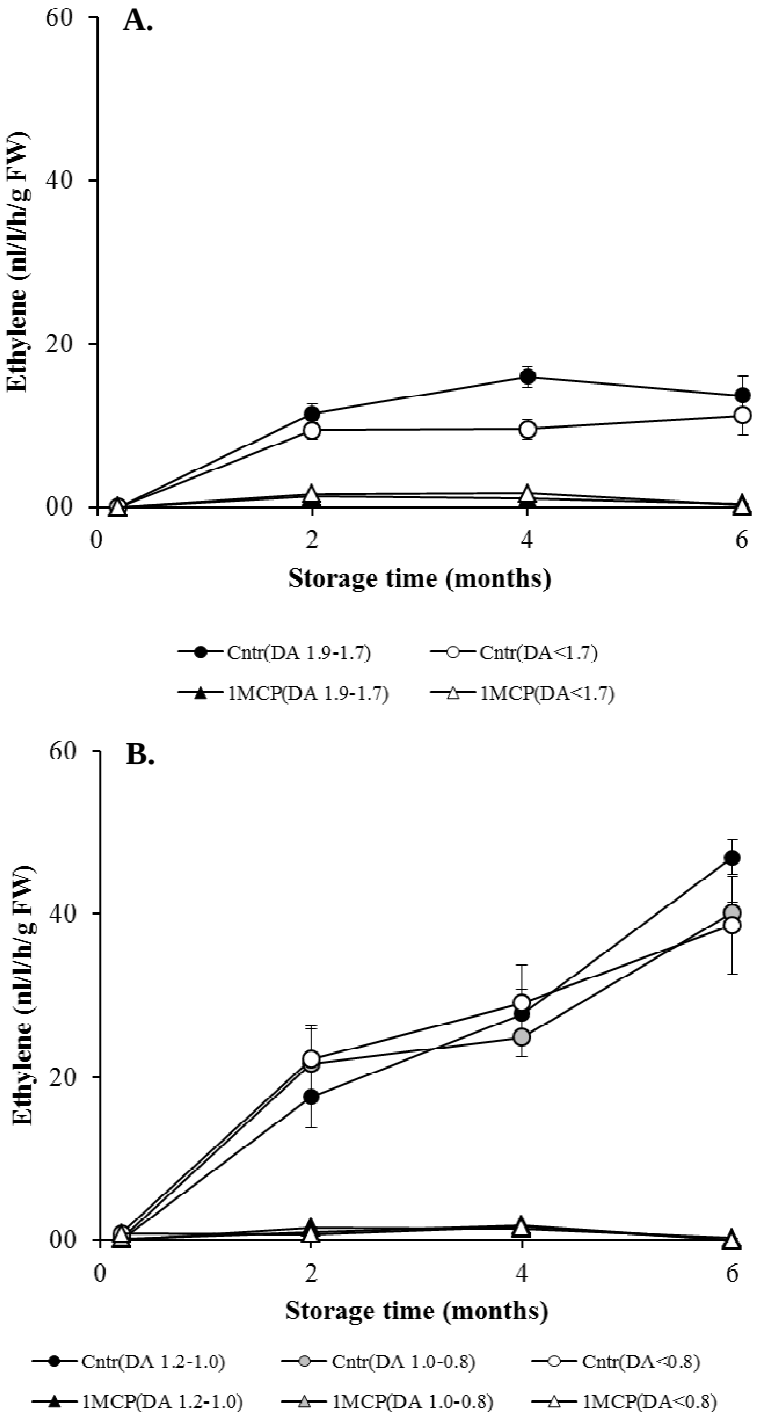

Figure 5. Ethylene emission (nl L/h/g/FW) in Granny Smith (a) and Pink Lady (b) apples in control fruit and fruit treated with 1-MCP harvested at different ripening stages determined by I_{AD}. Measures were assessed from whole apples left at room temperature for 1 h after 2, 4 and 6 months of commercial storage. Standard error is shown.

7. Use of 1-methylcyclopropene (1-MCP) to control superficial scald in apple

7.1. Abstract

Superficial scald is one of the major post harvest disorders of apple fruits and it causes severe losses during long term cold storage. Scald incidence is highly dependent on maturity stage at harvest and differs between cultivars. In this study, the fruit maturity was assesses at harvest by using a the DA-meter, a non-destructive device. 1-methylcyclopropene (1-MCP) was evaluated to reduce scald incidence in the different maturity classes both in a susceptible variety 'Granny Smith' and in a tolerant one, 'Pink Lady'. 1-MCP treatments clearly inhibits superficial scald, and its efficacy was cultivar dependent and influenced by the maturity stage at harvest assessed by the DA-meter. The mechanism underlying 1-MCP efficacy were also studied. 1-MCP treatments inhibited ethylene emission in both cultivars for all maturity classes, however the ethylene inhibition did not correlates with a reduction of α-farnesene which has been traditionally associated with scald development. 1-MCP effect on other compounds, such as phenol conjugated trienols and phenolic compounds such chlorogenic acid, which related with oxidative stress and scald development, were also investigated.

7.2. Introduction

Apple fruits are stored for several months at low temperatures until consumption. These conditions increase the occurrence of scald, a physiological post-harvest disorder, which causes major losses in fruit storage. Superficial scald is a chilling injury characterized by the progressive appearance of brown discolorations on fruit peel, which also involve the necrosis of hypodermal cells (Bain and Mercer, 1963; Watkins *et al*., 1995; Lurie and Watkins, 2012). Genetic and physiological factors influence scald occurrence (Ingle and

D'Souza, 1989; Watkins *et al*., 1995; Eccher Zerbini *et al.*, 1997; Watkins *et al*., 2000; Erkan and Pekmezci, 2004; Whitaker, 2004; Lurie and Watkins, 2012). Among the latter, fruit maturity at harvest plays a key role: the most mature fruits are generally less affected by scald incidence (Eccher Zerbini, 1997; Watkins *et al*., 2000; Erkan and Pekmezci, 2004; Calvo and Candan, 2010).

Different cultivars are also differentially prone to this disease being 'Pink Lady', 'Gala', 'Empire', 'Golden Delicious', 'Fuji', 'Braeburn' low susceptible, whereas 'Granny Smith', 'Delicious', 'Cortland', 'Low Rome'are highly sensitive (Ingle and D'Souza, 1989; Little and Holmes, 2000; Whitaker, 2004; Tsantili, *et al*., 2007; Trivedi, 2010, Lurie and Watkins, 2012). The development of scald symptoms is associated with the oxidation of the sesquiterpene α-farnesene and its oxidative products, the conjugated trienols (CTols). However, α-farnesene and CTols are not essential for scald development, while their oxidation products, such as 6-methyl-5-hepten-2-one (MHO), can intensify the symptoms (Watkins *et al*., 1995; Mir and Beaudry, 1999; Whitaker and Saftner, 2000; Whitaker *et al*., 2000, Whitaker, 2004, 2008, 2013; Lurie and Watkins, 2012). Since scald seems related with the oxidative processes, also its relation with phenolic compounds was studied. On one hand, phenols may reduce scald incidence due to their well known antioxidant activity, on the other hand, phenols may contribute to scald development by their oxidation via polyphenol oxidase (Ju *et al*., 1996; Abdallah *et al*., 1997; Golding *et al*., 2001; Treutter, 2001; Lurie and Watkins, 2012, Whitaker, 2013).

Ethylene plays an important role in scald development since it promotes α-farnesene synthesis (Du and Bramlage, 1994; Fan *et al*., 1999; Ju and Curry, 2002; Watkins *et al*., 2000; Whitaker, 2008; Rupasinghe *et al*., 2000; Lurie and Watkins, 2012; Whitaker 2013). Therefore, ethylene inhibitors and antioxidant treatments, such as the application of diphenylamine (DPA), may reduce scald development (Du and Bramlage, 1994; Mir and Beaudry, 1999; Whitaker, 2004). Among the ethylene inhibitors, 1-methylcyclopropene (1-MCP) is the most effective and widely studied compound to control scald (Fan *et al*., 1999; Watkins *et al*., 2000; Watkins and Nock, 2005; Tsantili *et al*., 2007; Jung and Watkins, 2008; Trivedi *et al*., 2010). 1-MCP efficacy is influenced by the fruit maturity stage at the time of application, storage conditions and the time-span between harvest and treatment (Watkins *et al*., 2000; Watkins and Nock 2005; Watkins, 2006; Jung and Watkins, 2008; Calvo and Candan, 2010). Based on these considerations, a precise and reliable assessment

of fruit maturity at harvest and before the treatment is essential. To achieve this result, non destructive technologies, such as Vis/NIR, can be applied (Peirs *et al*., 2000, 2005; Zude-Sasse *et al*., 2002; Zude *et al*., 2006; Bertone *et al*., 2012). The DA-meter (TR Turoni, Forli, Italy) is Vis/NIR-based instrument able to assess fruit maturity stage by evaluating the changes in chlorophyll content, during the natural maturity process, (Ziosi *et al*., 2008; Costa *et al*., 2009). The changes in chlorophyll contents are expressed as an Index of Absorbance Difference (I_{AD}) (Ziosi *et al*., 2008; Costa *et al*., 2009). Previous studies has demonstrated that the I_{AD} is a maturity index, which correlates with ethylene production, fruit quality traits and transcription of ripening-related genes related to the progression of the ripening process (Ziosi *et al*., 2008; Costa *et al*., 2009; Infante *et al*., 2011; Bonora *et al*., 2013a,b; Shinya *et al*., 2013). On apple fruits, a previous research proposed the use of the I_{AD} to predict harvest date and fruit quality during storage (Nyasordzi *et al*., 2013). The main objective of this study was to evaluate the effect on scald incidence of the maturity stage at harvest, asses by the I_{AD}, and of 1-MCP treatment. The effect of 1-MCP on ethylene emission, α-farnesene, CTols and phenolic compounds was also evaluated. The experiments were performed on a highly susceptible cultivar, 'Granny Smith', and on a tolerant one, 'Pink Lady'.

7.3. Materials and Methods

7.3.1. Plant material and fruit maturity assessment

Trials were carried out in 'Granny Smith' and 'Pink Lady orchards located in Emilia Romagna, Italy. At harvest, 600 fruits were divided in homogeneous ripening classes by the Index of Absorbance Difference (I_{AD}) assessed with a DA-meter. The I_{AD} was measured on the opposite sides of the fruits in the equatorial region and the mean of both lectures was averaged. 'Granny Smith' fruits were divided in two maturity classes I_{AD} = 2.0-1.8 and < 1.8-1.6, while 'Pink Lady' fruits were grouped in three classes I_{AD} =1.2-1.0; I_{AD}< 1.0-0.8 and I_{AD}<0.8-0.6.

7.3.2. 1-MCP application

Each ripening class for both cultivars was divided in two halves and one was sprayed with 1-MCP, whereas, the other half was kept as untreated control. 1-MCP was applied at 1 ppm as the commercial formulate (0.14% active ingredient, SmartFresh^{TM}), according to the manufactures instructions (AgroFresh, Rhom& Haas, Philadelphia, Pennsylvannia, USA). After 24hs, the fruits were removed to the cold storage chamber atmosphere conditions at 0.5 °C and 95% RH. Fruit were stored up to six months.

7.3.3. Fruit sampling

From harvest (T0) up to 6 months, control and treated fruits were sampled every two months (T2, T4 and T6). At each time point, a sample of 60 fruits for both control and 1-MCP treatment per was kept at room temperature for each maturity class. A sub-sample of 10 fruits was used for ethylene analysis and tissue sampling. The ethylene emission was measured when the fruit temperature was approximately 20°C. For the tissue sampling, the first layer of the apple skin was removed from the equatorial region of the fruit, and immediately frozen in liquid nitrogen. Sample were stored at -80°C till the analysis. The rest of the fruits were kept at room temperature in shelf life for seven days (T2+7, T4+7, T6+7).

7.3.4. Superficial scald incidence assessment

Superficial scald incidence was detected on 50 fruits per class that were removed from storage after 2, 4 and 6 months. The symptoms were visually evaluated after 7 days of shelf life at 20°C. Superficial scald incidence was expressed as the percentage of injured fruits.

7.3.5. Ethylene determination, extraction and measurement of α-farnesene and Ctols

Ethylene production was determined by placing the whole fruit in a 0.8 L jar tightly sealed with a lid equipped with a rubber stopper, and left at room temperature for 1 h. A 10 ml gas sample of the head-space was taken and was injected in a Dani HT 86.01 (Dani, Milan, Italy) packed-gas chromatograph fitted with FID and a Porapak Q column (Supelco,

Bellefonte, PA, USA). Oven temperature was set at 80°C, and for the injector and detector at 180°C. $N_2$was used as the carrier gas at16 mL min^{-1} flow rate. Ethylene concentration was calculated and expressed as nanoliter/gFW/h.

Extraction of α-farnesene and CTols was performed on three replicates for each pooled tissue sample, following the methodology described by Whitaker *et al.* (2000). The extracts were analyzed by a Waters HPLC system with a Photodiode Array Detector (Waters 2996), fitted with a 4.6 x 250 mm, Luna C18 column (Phenomenex, Torrence, CA). The α-farnesene and CTols identification was carried out through comparison of the retention time values and UV spectra (detected between 210 and 400 nm wavelength) with authentic standards. Concentrations, expressed in μg g-1 fresh weight (FW), were calculated from curves obtained with the corresponding external standards.

7.3.6. Extraction and determination of total phenols and chlorogenic acid

Apples tissue samples were ground under liquid nitrogen to obtain a frozen powder, and the extraction was made following a protocol adapted from Theodoridis *et al*. (2012). HPLC analyses (Waters Acquity UPLC system; Milford, MA)were performed. Separation of the phenolic compounds was achieved on a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm (Milford, MA, USA). Mass spectrometry detection was performed on a Waters Xevo TQMS (Milford, MA, USA) instrument equipped with an electrospray (ESI) source. Data processing was done using Waters MassLynx4.1 and TargetLynx software.

7.3.7. Statistical Analysis

Data were analyzed by ANOVA and the means were compared by the Tukey's HSD test at significant level of 0.05.

7.4. Results and Discussion

7.4.1. Scald incidence in response to maturity stage at harvest and 1-MCP treatment

The incidence of superficial scald is reported on Tables 1 and 2 for 'Granny Smith' and 'Pink Lady' apple fruits, respectively. It is clearly shown that the cultivar plays a very important role, 'Granny Smith' showed higher susceptibility and early during the storage and shelf life than 'Pink Lady'. In 'Granny Smith' first symptoms of scald appear during the shelf life after 2 month under cold storage (T2+7) in the less mature fruits (I_{AD} 2.0-1.8), even when no symptoms were observed for the more mature fruits (I_{AD}< 1.8-1.6) (Table 1). After four and six month, immediately after the removal from cold storage differences on scald susceptibility between I_{AD} classes were observed, while after the shelf life the totality of the fruits was affected by scald. In 'Pink Lady', symptoms of scald were observed after the shelf life after 4 and 6 months under cold storage condition (T4+7 and T6+7), with a percentage of affected fruits, in the three maturity classes, generally less than 50% (Table 2).

Scald incidence resulted also correlated with the length of the storage: longer the storage resulted in more severe the scald incidence.

Also the ripening stage at harvest plaid a key role in determining scald incidence. As expected, the more immature fruits were more prone to this disorder (Tables 1 and 2). This is in accord with the literature where several authors stated that the ripening stage reached at harvest by the fruits influencing scald incidence (Eccher Zerbini, 1997; Watkins *et al*., 2000; Erkan and Pekmezci, 2004; Calvo and Candan, 2010).

The 1-MCP application clearly inhibited superficial scald, and its effect was related to cultivar and ripening stage at harvest. In the more mature fruits (lower I_{AD} value), 1-MCP was less effective both at 4 and 6 months of cold storage in 'Granny Smith' and after 6 months in 'Pink Lady' (Table 1 and 2). Our data confirm what has been observed in previous studies where 1-MCP treatments were shown to be cultivar dependent and its effect varied in relation to the harvesting date and the storage length (Fan *et al*., 1999; Watkins *et al*., 2000; Zanella, 2003; Bai *et al*., 2005; Watkins and Nock 2005; Watkins 2006; Jung and Watkins, 2008; Calvo and Candan, 2010).

7.4.2. Effect of fruit ripening stage and 1-MCP treatment on ethylene, α-farnesene and CTols biosynthesis

Ethylene, α-farnesene and CTols were analyzed at harvest and after two, four and six months of cold storage.

Ethylene

Ethylene emission differed in relation to the cultivar being 'Granny Smith' a cultivar with a low emission and 'Pink Lady' one with a average emission. In 'Granny Smith', the ethylene production was similar for both maturity classes. The emission was very low at harvest and it increased after two months under cold storage, and this increment remained constant (around 15-20 nl/LhgFW) along the remaining months of storage. The shelf life period increased the ethylene production (about 30-50 nl/LhgFW) (Figure 1).

In 'Pink Lady', ethylene emission differed in the different I_{AD} classes at harvest. I_{AD} class 1.0-0.8 presented the lowest ethylene emission (Figure 2). Along storage 'Pink Lady' ethylene emission showed a trend similar to the one observed for 'Granny Smith', but with higher emission (about 40-50 nl/LhgFW after removal and 60-80 nl/LhgFW after shelf life) (Figure 2). The application of 1-MCP on 'Granny Smith' and 'Pink Lady' reduced ethylene emission independently of the ripening stage reached by the fruit at harvest (Figures 1, 2). These results confirmed previous studies (Fan *et al*., 1999; Watkins *et al*., 2000; Watkins and Nock, 2005, Gapper *et al*., 2006, Watkins, 2006; Jung and Watkins, 2008).

α-Farnesene

Concentration of α-farnesene in apple skin at harvest was very low in 'Granny Smith'. In both cultivars, as the I_{AD} at harvest decrease, the α-farnesene content increased (Figures 3 and 4). In both cultivars, and regardless for the maturity stage at harvest, the highest α-farnesene content was recorded after 2 months of storage. The α-farnesene concentration after 2 months was higher in 'Granny Smith' than 'Pink lady', showing the higher

perceptual increment in the first cultivar. In both cultivars, α-farnesene content generally decrease from the 2nd month of storage till the 6thmonth. This pattern could be explained considering that α-farnesene assuming a reduction in its content along shelf life it is a consequence of its oxidation (Whitaker *et al*., 1997; Shaham *et al*., 2003; Whitaker, 2004; Gapper *et al*., 2006; Tsantili *et al*., 2007; Jung and Watkins, 2008; Trivedi *et al*., 2010). The α-farnesene did not show any clear trend in relation to the maturity stage at harvest in any of the two cultivars.

1-MCP treatment had a different effect on α-farnesene content in relation to the cultivar. In, 'Granny Smith' 1-MCP treated fruits presented a general reduction in α-farnesene content, with a highest effect on less mature fruit class (I_{AD} 2.0-1.8) (Figure 3). In more mature fruits, 1-MCP reduced α-farnesene content during the storage, while on least mature fruits the treatment slightly increase α-farnesene content along the storage. Interestingly, the treatments significantly reduced, in comparison to control, the α-farnesene emission after the 7 days of shelf life for fruit stored for 4 and 6 months in the least mature class and in all the time points (2, 4, and 6 months) for the most mature fruits (Figure 3).

In 'Pink Lady', 1-MCP treatment clearly reduced α-farnesene content, in comparison to control, in fruits stored for 4 and 6 months. This effect was more pronounce after 7 days of shelf-life (Figure 4). The treatment did not shown any clear trend in relation to the maturity classes.

It is generally accepted that α-farnesene production is under ethylene regulation (Du and Bramlage, 1994; Fan *et al*., 1999; Ju and Curry, 2002; Watkins *et al*., 2000; Whitaker, 2008; Rupasinghe *et al*., 2000; Lurie and Watkins, 2012). However, the relation between α-farnesene and ethylene has not yet been fully elucidated. In our experiments, a complete inhibition of ethylene emission by 1-MCP did not correlate with the reduction of α-farnesene content. In addition, differences were observed in the different I_{AD} classes and between cultivars. Higher content of α-farnesene in 1-MCP treated fruits could be explained supposing that its synthesis was mediated by residual ethylene or induced by other regulatory elements (Lurie and Watkins, 2012).

CTols

The CTols content was different in the two cultivars also in relation to maturity stage. In 'Granny Smith', fruits belong to the I_{AD} 2.0-1.8 class, which are less mature and more susceptible, CTols content was generally high and it moderately decreased along storage (Figura 5). In more mature fruits, its content was lower and it almost remained constant during time. Shelf life clearly decreased CTols content on less mature fruits, while this effect was not so pronounced on more mature fruits. Previous studies explained the reduction of CTols content after shelf life, and at the end of the storage, with their oxidation to 6-methyl-5-hepten-2-one (MHO) (Mir and Beaudry, 1999; Wang and Dilley, 2000; Whitaker and Saftner, 2000; Whitaker, 2004; Lurie and Watkins, 2012). In 'Pink Lady' lower concentrations of CTols were observed and the changes along the storage and during shelf life were not significant (Figures 6).

In 'Granny Smith', 1-MCP substantially reduced CTols content, in both maturity classes. However, this effects was reduced after 6 months of storage in the less mature fruits (Figure 5). In 'Pink Lady', 1-MCP treatment was not effective in any of the maturity classes. In addition, 1-MCP did not affect CTols content in fruit neither along the storage, nor after the shelf life (figure 6).

The CTols content correlated with scald susceptibility. Indeed, 'Pink Lady' that is less susceptible, also showed a lower content of CTols in comparison to 'Granny Smith'. In 'Granny Smith', the most mature fruits, which are less prone to scald, presented a significantly lower content of CTols in comparison with immature fruits. In addition, the CTols level was comparable with the one observed in 'Pink Lady'.

However, the CTols content cannot be considered a reliable marker to predict scald incidence. In fact, though most mature 'Granny Smith' fruits and the ones of 'Pink Lady' presented similar level of these compounds, the latter showed a much lower scald incidence (Table 1, 2). Similarly, 'Granny Smith' mature and immature fruits showed, at 6 months of storage, very different level of CTols, but the scald incidence did not differ. Some researchers have been propose that scald is a consequence of a general oxidative process, and that the autoxidation of α-farnesene to CTols is just a secondary response of a free radical reactions (Whitaker, 2004; Lurie and Watkins, 2012).

7.4.3. Effect of fruit ripening stage and 1-MCP treatment on total phenols and chlorogenic acid

To better understand the possible metabolites involved on scald development, total phenols and chlorogenic acid were measured on 'Granny Smith' and 'Pink Lady' for two maturity classes which presented a different trend on α-farnesene and CTols content during storage and showed different scald susceptibility.

At harvest, in 'Granny Smith' the total phenols did not differ in relation to maturation. Total phenols content showed a moderate increase at 2 months of storage and, thereafter, their content generally decreased. Shelf-life slightly increased the total phenols contents (Figure 7, A-B). 1-MCP did not affect total phenols content in 'Granny Smith'.

In 'Pink Lady', total phenols content was not influenced by the maturation stage. In addition, 1-MCP did not show any significant effect in relation to the duration of storage. After 4 months of storage and 7 days of shelf life, control fruits showed an increase total phenols, while in 1-MCP treated fruit their content remained constant along all the storage and shelf life (Figure 8, A-B).

Regarding chlorogenic acid, in less mature 'Granny Smith' fruits, its content was increased by shelf life, with the highest amount observed after 4 months of cold storage and 7 days of room temperature. In more mature fruits, the only increase was observed after 4 months of cold storage and 7 days of room temperature, while its content was constants in all the other time points (Figure 7, C-D). 1-MCP kept constant the level of chlorogenic acid regardless form the duration of storage and shelf life (Figure 7, C-D). Therefore, the level of chlorogenic acid in control and treated fruits was significantly different at 2 and 4 months plus shelf life in most immature fruits and, at 4 months, in the most mature ones.

In 'Pink Lady', chlorogenic acid content was not influenced by the maturation stage and length of storage. In more mature fruits, 1-MCP did not show any significant effect in relation to the duration of storage and shelf life (Figure 8, C-D). In less mature fruits, control and 1-MCP treated fruits showed different contents of chlorogenic acid after shelf life. On these fruits, 1-MCP kept constant chlorogenic acid level, while this acid increased in untreated fruits.

The significant role that phenolic compounds play on scald development reside on their dual function during superficial scald formation. In fact, phenolic compounds present an

antioxidant activity that may contribute to resistance scald development. On the other hand, the oxidation of phenolic compounds via polyphenol oxidase (PPO) may contribute to the development of scald symptoms. High levels of phenolic acids as chlorogenic acid, which is a potent cofactor of apple PPO, may unbalance this equilibrium anti-oxidant/oxidant in favor to latter one, causing the higher susceptibly to scald of immature apple fruits (Du and Bramlage, 1995; Ju *et al*., 1996, Treutter, 2001, Lurie and Watkins, 2012). On this study, the trend of chlorogenic acid development in 'Granny Smith' shows its possible role in the induction of scald development. Chlorogenic acid shows lower content immediately after cold storage in all the removals and a high increment during shelf life on fruits that manifest scald symptoms (Figure 7, C-D). Our findings are in agreement with previous studies where high levels of chlorogenic acid were observed on more susceptible tissue supporting the phenol oxidation process via PPO (Du and Bramlage, 1995; Ju *et al*., 1996, Treutter, 2001, Lurie and Watkins, 2012).

On the other hand, in 'Pink Lady' the chlorogenic acid development does not show any specific trend during storage and shelf life. In addition, any relation with the content of chlorogenic acid and scald incidence was observed (Figure 8, C-D). These results stress how the different cultivars react to cold injuries and oxidative stresses, and they highly that scald development is regulated by a extremely complex network of reactions that may differ from cultivar to cultivars. Therefore the identification of a single or multiple markers (i.e. a-farnesene, Ctols, phenolic compounds) for predicting scald incidence is not possible.

7.5. Conclusions

The results obtained in this research pointed out that 1-MCP application is extremely effective in reducing superficial scald. It has been confirmed that the efficacy of 1-MCP is dependent on fruit ripening stage. 1-MCP efficacy is also related to the cultivar. However, in both in the very susceptible cultivar ('Granny Smith') and in a tolerant one ('Pink Lady'), 1-MCP was effective in controlling scald, up to 4 months.

1-MCP strongly inhibits ethylene emission in both cultivars for all the I_{AD} classes. Surprisingly, ethylene inhibition did not correlate with the inhibition in α-farnesene

content, underlining that the relation between ethylene and α-farnesene is not entirely elucidate. This highlight that ethylene can be involved in other processes associated with scald and not only with α-farnesene regulation. Superficial scald is a complex phenomenon and is a consequence of a general oxidative process; the autoxidation of α-farnesene could be just a secondary response of a free radical reactions.

Phenolic compounds, such as chlorogenic acid, are involved in scald development as previously reported by other researches carried on in 'Granny Smith'. In our study, the high level of chlorogenic acid in this cultivar, and its trend during storage and shelf life, support its role in the increase of scald incidence in a susceptible cultivar. Further research study should be carry on related to possible alternative processes involved in scald development.

7.6. References

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7.7. Figures and Tables

Table 1. Superficial scald incidence on ‘Granny Smith’ apples on control and 1-MCP treated fruits performed at two different maturity classes assed by I_{AD}. Percentage of affected fruit was performed after removal from cold storage at two, four and six month (T2, T4, T6) and after their respective shelf life at seven days under room temperature (T2+7d, T4+7d, T6+7d).

Maturity Stage at Harvest		Time after storage + Shelf Life					
I_{AD}		T2	T2 + 7d	T4	T4 + 7d	T6	T6 + 7d
2.0 - 1.8	1-MCP	0	0	0	0	0	0
	Control	0	95	96	100	100	100
< 1.8 - 1.6	1-MCP	0	0	0	15	0	15
	Control	0	0	9	97	63	100

Table 2. Superficial scald incidence on ‘Pink Lady’ apples on control and 1-MCP treated fruits performed at two different maturity classes assed by I_{AD}. Percentage of affected fruit was performed after removal from cold storage at two, four and six month (T2, T4, T6) and after their respective shelf life at seven days under room temperature (T2+7d, T4+7d, T6+7d).

Maturity Stage at Harvest		Time after storage + Shelf Life					
I_{AD}		T2	T2 + 7d	T4	T4 + 7d	T6	T6 + 7d
1.0 - 0.8	1-MCP	0	0	0	0	0	5
	Control	0	0	0	38	2	56
< 0.8 - 0.6	1-MCP	0	0	0	0	0	6
	Control	0	0	0	18	1	43
< 0.6 – 0.4	1-MCP	0	0	0	0	0	9
	Control	0	0	0	9	1	21

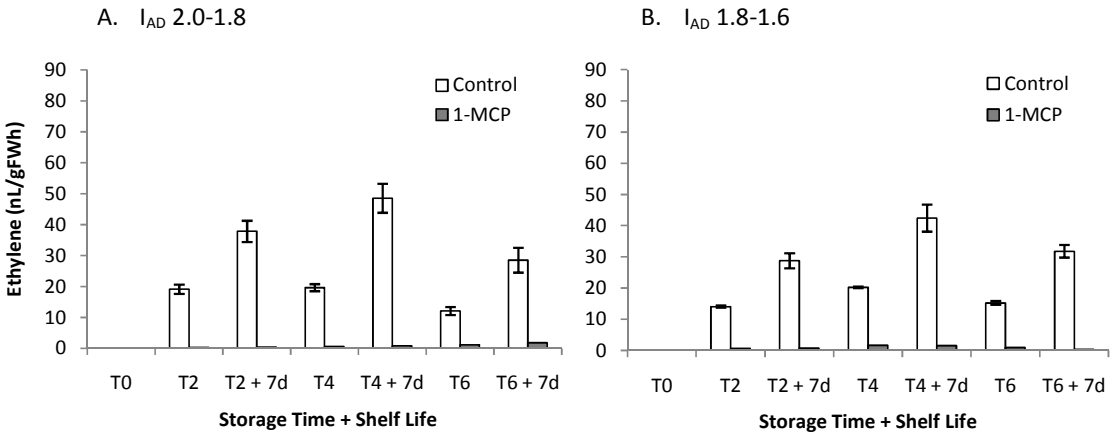

Figure 1. Ethylene emission in 'Granny Smith' apples on control and 1-MCP treated fruits for two different maturity classes assed by I_{AD} (less mature A: I_{AD} 2.0-1.8 and more mature B: I_{AD} 1.8-1.6). Measures were performed after removal from cold storage at two, four and six month (T2, T4, T6) and after their respective shelf life at seven days under room temperature (T2+7d, T4+7d, T6+7d). Standard errors are represented by bars on the figure.

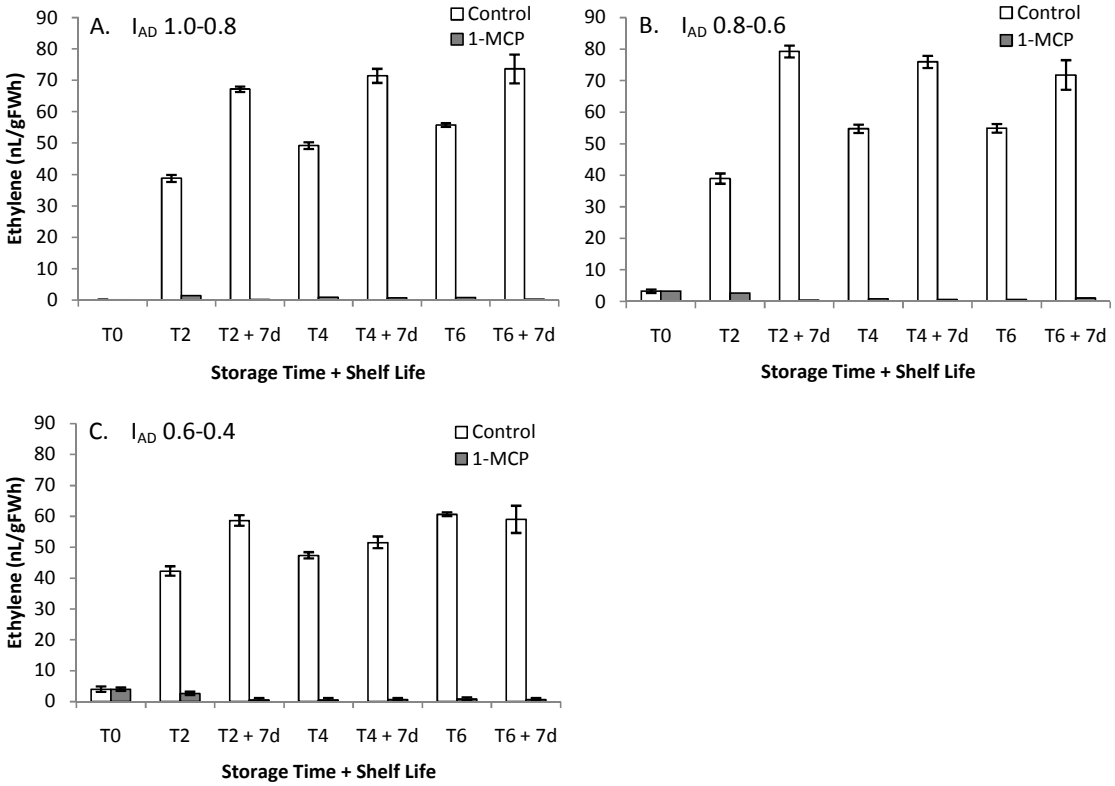

Figure 2. Ethylene emission in 'Pink Lady' apples on control and 1-MCP treated fruits for three different maturity classes assed by I_{AD} (A: I_{AD} 1.0-0.8, B: I_{AD} 0.8-0.6 and C: I_{AD} 0.6-0.4 from less to more mature respectively). Measures were performed after removal from cold storage at two, four and six month (T2, T4, T6) and after their respective shelf life at seven days under room temperature (T2+7d, T4+7d, T6+7d). Standard errors are represented by bars on the figure.

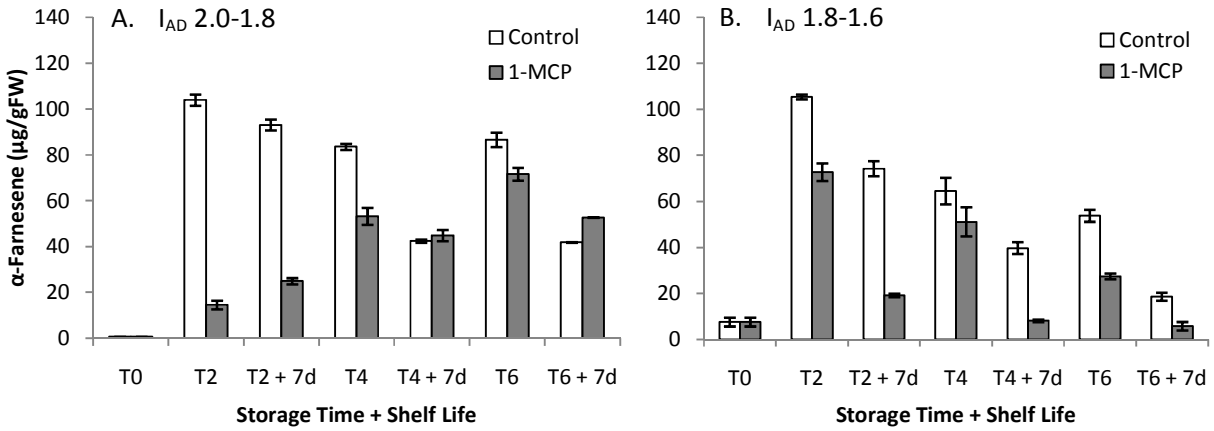

Figure 3. α-Farnesene concentrations in peel tissue in 'Granny Smith' apples on control and 1-MCP treated fruits for two different maturity classes assed by I_{AD} (less mature A: I_{AD} 2.0-1.8 and more mature B: I_{AD} 1.8-1.6). Measures were performed after removal from cold storage at two, four and six month (T2, T4, T6) and after their respective shelf life at seven days under room temperature (T2+7d, T4+7d, T6+7d). Standard errors are represented by bars on the figure.

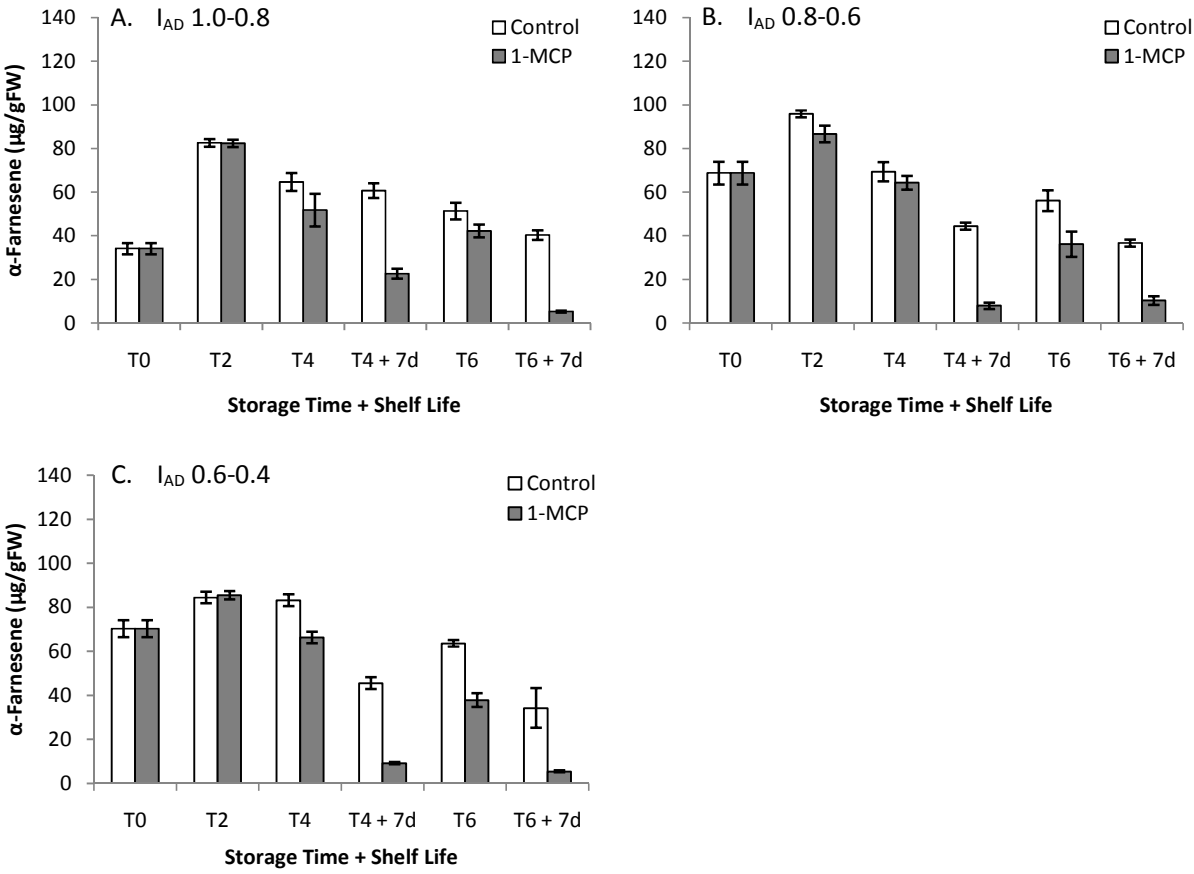

Figure 4. α-Farnesene concentrations in peel tissue in 'Pink Lady' apples on control and 1-MCP treated fruits for three different maturity classes assed by I_{AD} (A: I_{AD} 1.0-0.8, B: I_{AD} 0.8-0.6 and C: I_{AD} 0.6-0.4 from less to more mature respectively). Measures were performed after removal from cold storage at two, four and six month (T2, T4, T6) and after their respective shelf life at seven days under room temperature (T4+7d, T6+7d). Standard errors are represented by bars on the figure.

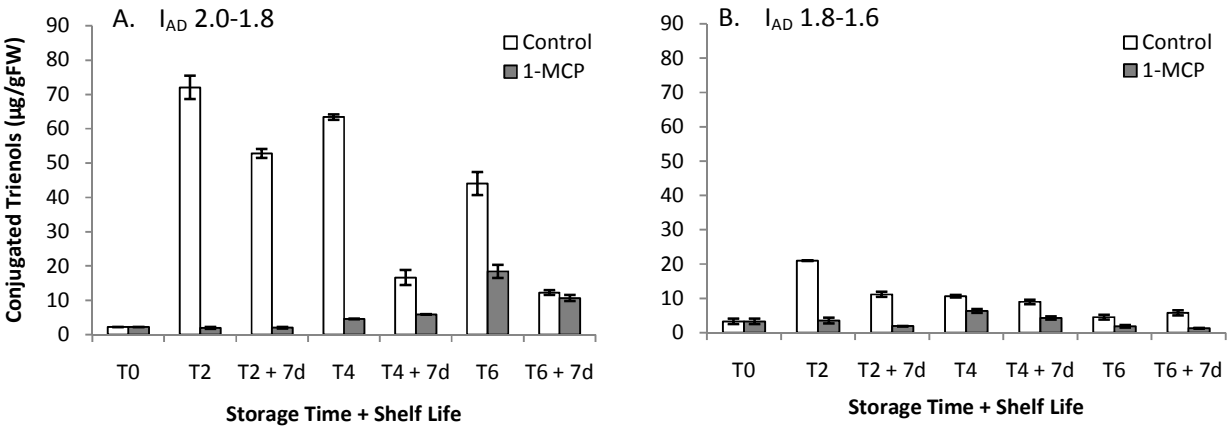

Figure 5. Conjugated trienols concentrations in peel tissue in 'Granny Smith' apples on control and 1-MCP treated fruits for two different maturity classes assed by I_{AD} (less mature A: I_{AD} 2.0-1.8 and more mature B: I_{AD} 1.8-1.6). Measures were performed after removal from cold storage at two, four and six month (T2, T4, T6) and after their respective shelf life at seven days under room temperature (T2+7d, T4+7d, T6+7d). Standard errors are represented by bars on the figure.

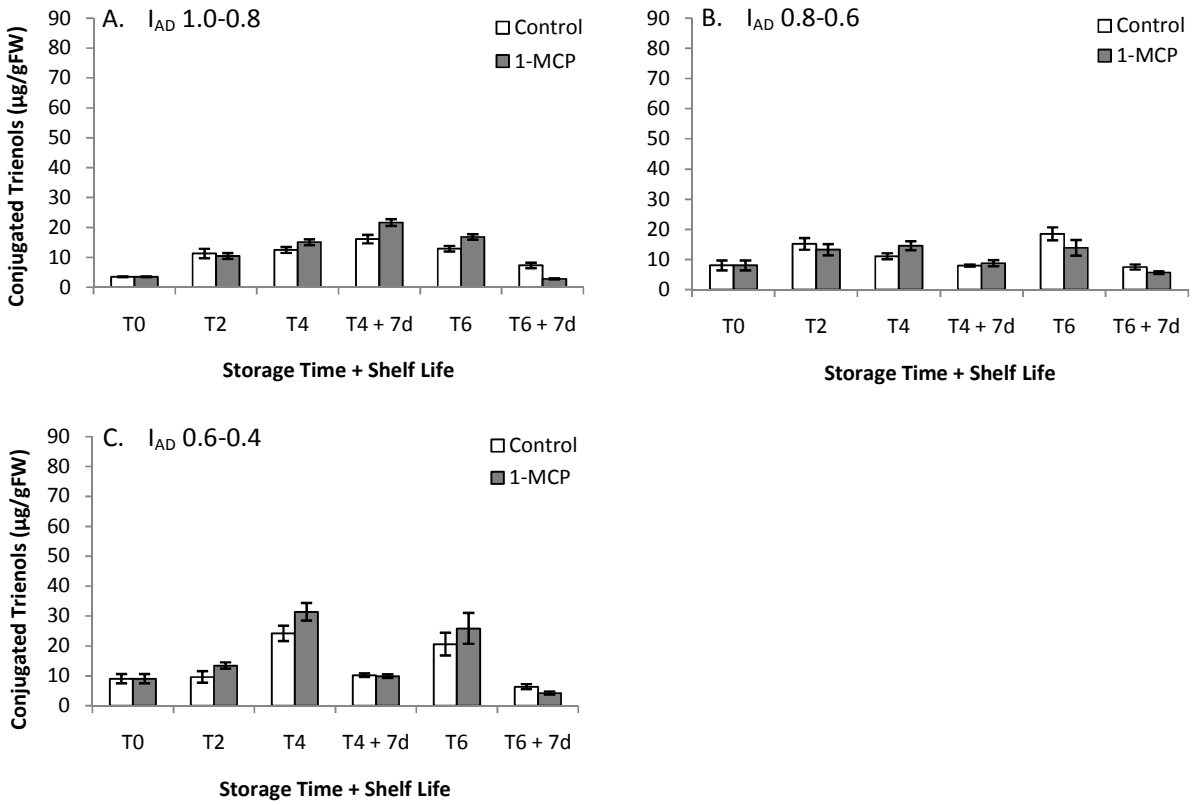

Figure 6. Conjugated trienols concentrations in peel tissue in 'Pink Lady' apples on control and 1-MCP treated fruits for three different maturity classes assed by I_{AD} (A: I_{AD} 1.0-0.8, B: I_{AD} 0.8-0.6 and C: I_{AD} 0.6-0.4 from less to more mature respectively). Measures were performed after removal from cold storage at two, four and six month (T2, T4, T6) and after their respective shelf life at seven days under room temperature (T4+7d, T6+7d). Standard errors are represented by bars on the figure.

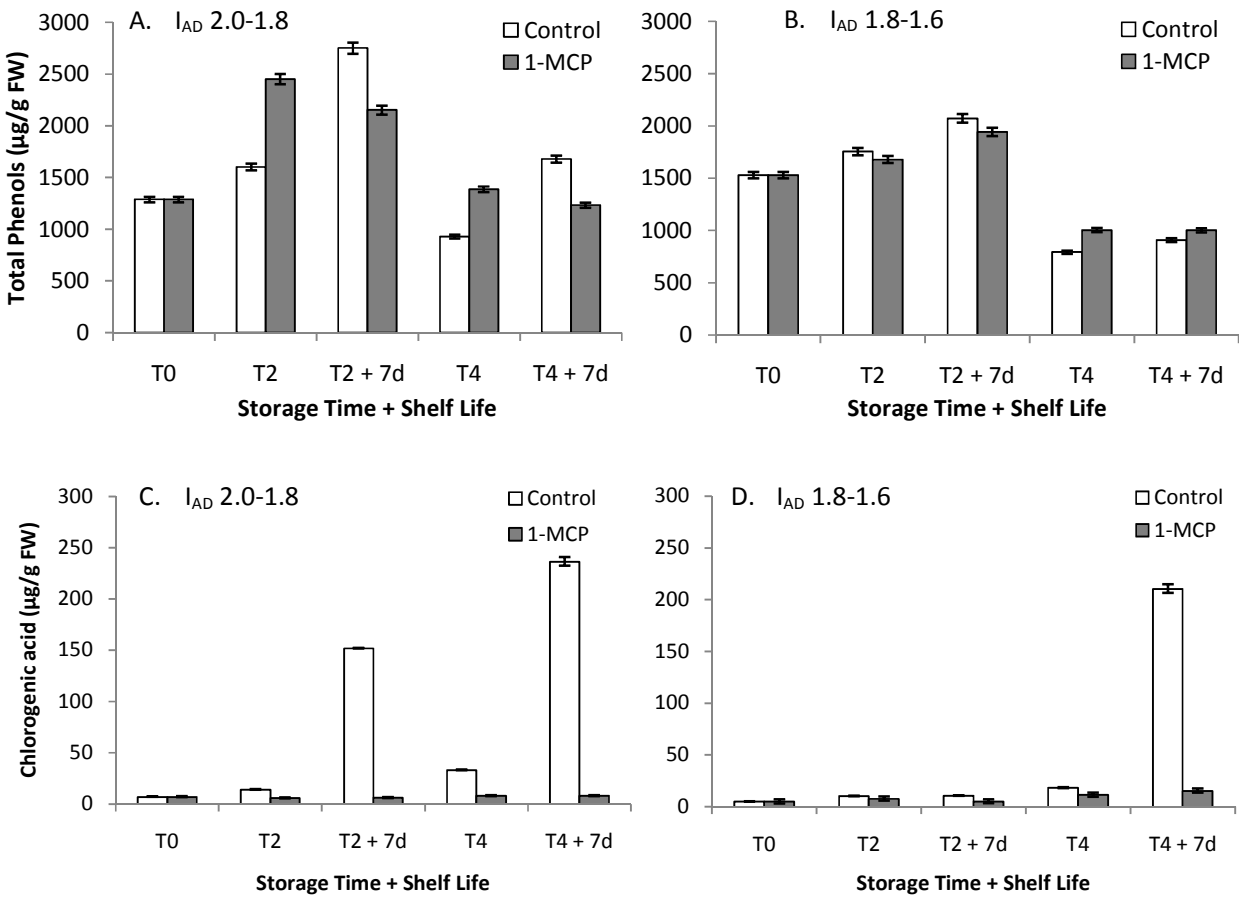

Figure 7. Total phenols (A,B) and chlorogenic acid (C,D) concentrations in peel tissue in 'Granny Smith' apples on control and 1-MCP treated fruits for two different maturity classes assed by I_{AD} (less mature A and C: I_{AD} 2.0-1.8 and more mature B and D: I_{AD} 1.8-1.6). Measures were performed after removal from cold storage at two and four (T2, T4) and after their respective shelf life at seven days under room temperature (T2+7d, T4+7d). Standard errors are represented by bars on the figure.

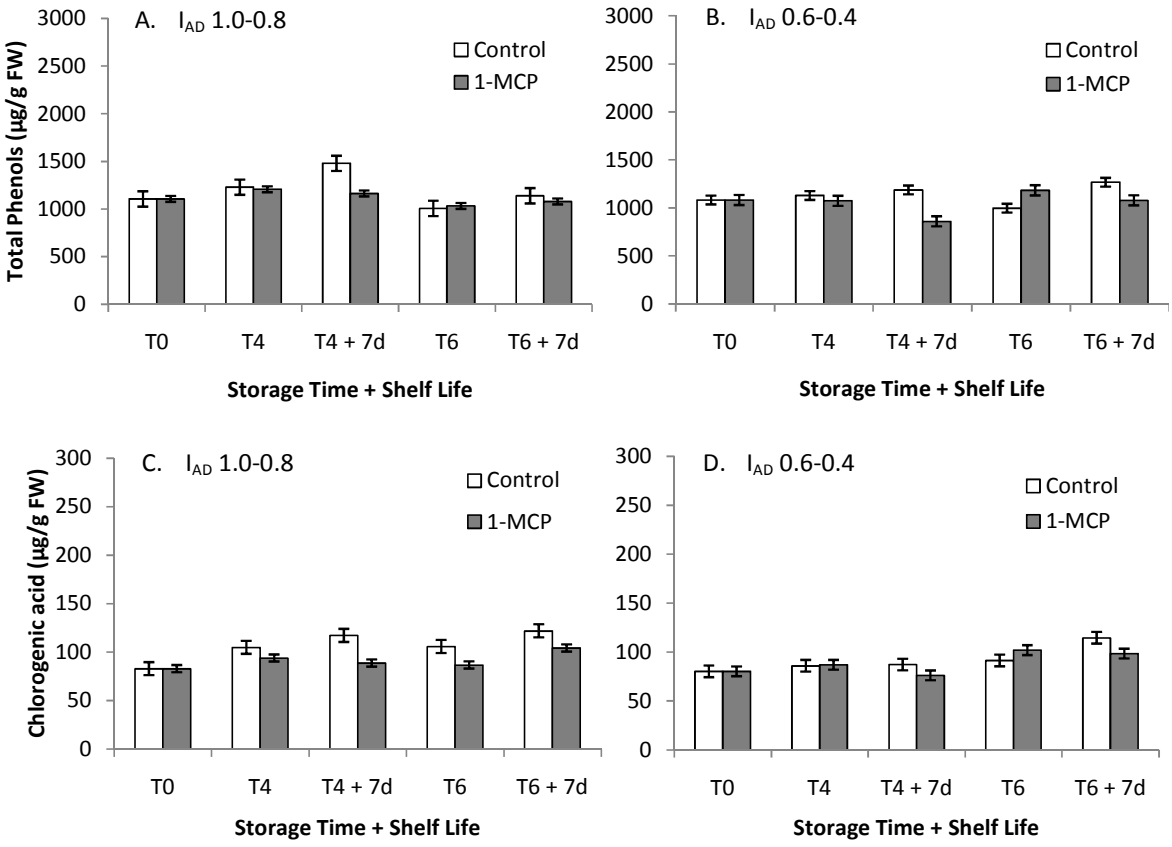

Figure 8. Total phenols (A,B) and chlorogenic acid (C,D) concentrations in peel tissue in 'Pink Lady' apples on control and 1-MCP treated fruits for two different maturity classes assed by I_{AD} (less mature A and C: I_{AD} 1.0-0.8 and more mature B and D: I_{AD} 0.6-0.4). Measures were performed after removal from cold storage at four and six month (T4, T6) and after their respective shelf life at seven days under room temperature (T4+7d, T6+7d). Standard errors are represented by bars on the figure.

8. Detection of α-farnesene and 6-methyl-5-hepten-2-one involved in the development of apple superficial scald by PTR-ToF-MS

8.1. Abstract

To date, the accepted hypothesis about the development of superficial scald in apple fruit is related to the accumulation of harmful α-farnesene autoxidation products, such as conjugated trienols or 6-methyl-5-hepten-2-one (MHO). The aim of this research was the implementation of an alternative rapid and reliable analytical technique based on PTR-ToF-MS (proton transfer reaction – time of flight – mass spectrometry), to measure the volatile organic compounds released during the progression of this disorder in apple. This assessment was performed taking into consideration two specific tissues (skin and pulp), as well as the comparison between control and treated samples with 1-MCP applied before storage. The results described here suggest the use of MHO as a novel biochemical marker to monitor the oxidative stress of apple fruit, since its concentration is significantly correlated with the early development of superficial scald visible symptoms.

8.2. Introduction

Superficial scald is a post-harvest physiological disorder affecting pome fruits, and it is normally associated with chilling injury and oxidative stress occurring during storage (Watkins *et al*., 1995). This physiopathy has a considerable implication in fruit marketability and quality and, together with bitter pit, can be considered one of the major apple storage disorders (Mattheis, 2008). Superficial scald symptoms show necrotic areas on the surface layers of the hypodermal cortical tissue cells, resulting in a browning coloration of the fruit skin, without impacting the inner flesh tissues (Lurie and Watkins, 2012). The development of scald seems to be cultivar specific, since specific varieties, such as ‘Granny Smith’, ‘Delicious’, ‘Cortland’, ‘Low Rome’ are more susceptible to the onset of this disorder (Whitaker *et al*., 2000; Tsantili *et al*., 2007).

The effect of superficial scald is still highly discussed in apple, since the real aetiology of this phenomenon is not yet completely elucidated (Lurie and Watkins, 2012). To date, the most investigated and accepted hypothesis about scald development is related to the accumulation of products derived by the autoxidation of α-farnesene, such as conjugated trienols (Whitaker *et al*., 1997; Whitaker *et al*., 2000; Rowan *et al*., 2001) or 6-methyl-5-hepten-2-one (MHO) (Mir *et al*., 1999; Wang and Dilley, 2000; Rudell *et al*., 2009) which cause serious damage to the hypodermal tissue. The browning coloration resulting in the fruit skin with the ongoing of the superficial scald phenomenon can be ascribed to an oxidation process of polyphenols, accumulated in the cell vacuole as secondary metabolites (Du and Bramlage, 1995; Abbasi *et al*., 2008).

To date, the majority of the studies focused on the basic physiology of superficial scald, were focused on the analysis of α-farnesene and its autoxidation products, by using GC-MS, HPLC-UV/vis-APCI-MS or spectrophotometric analysis of hexane extraction by apple skin (Lurie and Watkins, 2012). Technological improvements towards the assembling of advanced equipments and the release of data processing algorithms, are fundamental requirements to employ untargeted metabolomics approaches in order to have a more comprehensive characterization of this phenomenon. This knowledge would allow the definition of valuable biochemical markers for an on-line and rapid monitoring of the scald disorder development. Several methods for the prevention of this phenomenon have been already proposed, such as low-oxygen controlled atmosphere storage, forced ventilation and 1-methylcyclopropene (1-MCP) treatment (Lurie and Watkins, 2012). However, their efficacy is influenced by the maturity stage and uniformity of fruit batch. Indeed, maturity stage at harvest is one of the major factors influencing the fruit susceptibility to scald, mature fruits have in fact a reduced scald sensitivity with respect to immature ones (Wilkinson and Fidler, 1973; Wang and Dilley, 1999; Whitaker *et al*., 1997). However, all these methodologies lead to higher input and managing costs.

The aim of this research was to develop an alternative rapid and reliable analytical technique based on PTR-ToF-MS (proton transfer reaction – time of flight – mass spectrometry) to assess the volatile compounds (VOCs) involved in superficial scald disorder in different apple tissue during storage, and 1-MCP treatment.

8.3. Materials and Methods

8.3.1. Plant Material and growing conditions

The apples used in this study have been collected from 'Granny Smith' apple trees of fiver years old and grafted on M9 rootstocks. The orchard was realized following a planting scheme of 3.3m x 0.8m, and located in Bagnacavallo (Ravenna), in the Northern Italy. Standard cultural practice and disease management strategies were applied.

8.3.2. Fruit selection and storage condition

Apples were harvested at maturity stage assessed according to method reported by Ziosi *et al*. (2008) and Nyasordzi *et al*. (2013), which is represented by the I_{AD} index generated by the DA-meter, a portable non-destructive device based on Visible/Near Infra Red (Vis/NIR) spectroscopy (TR, Forli, Italy). I_{AD} usually ranges from 2.2 to 0, indicating the less ripe (thus characterized by a greater amount of chlorophyll) and the full ripening apple fruit, respectively. For this investigation only fruits belonging to the I_{AD} class 1.8-2.0 were selected.

Homogeneous fruits (in both ripening stage and shape) were sampled immediately after harvest. Two apple batches, of about 80 fruits each, were distinguished: the first was used as control while the second was treated with 1ppm of 1-MCP. Treatment was applied for 24 hours as SmartFresh™ (0.14% active ingredient) according to the manufacturer's instructions (AgroFresh, Rohm and Haas, Philadelphia, Pennsylvania, USA).

Both apple batches were stored under normal atmosphere condition at +0.5°C and 95% of relative humidity. Samples from the two batches were then removed after one and two months of cold storage, respectively. For both storage periods, additional sampling and scald incidence evaluation were performed after 1, 4 and 8 days of shelf-life at room temperature (around 20 °C), in order to promote the incidence of the superficial scald on the apple fruit surface.

At each evaluation day, apple skin and pulp tissues were assessed separately for each sample. Samples were represented by 10 randomly picked apples per treatment, and immediately frozen in liquid nitrogen and stored at -80°C till the analysis.

8.3.3. Sample preparation

2.5 grams of powdered frozen sample were immediately inserted into a 20 ml glass vial equipped with PTFE/silicone septa (Agilent, Cernuscosul Naviglio, Italy) and mixed with 2.5 mL of deionized water, 1 g of sodium chloride, 12.5 mg of ascorbic acid, and 12.5 mg of citric acid (for more details see Aprea *et al*., 2011). Samples were preserved at 4°C till the analysis.

8.3.4. PTR-ToF-MS analysis

Measurements of VOCs in peel tissues were performed in three replicates with a commercial PTR-ToF-MS 8000 apparatus (Ionic on Analytik GmbH, Innsbruck, Austria; Soukoulis *et al*., 2013). The conditions in the drift tube were the following: 110 °C drift tube temperature, 2.25 mbar drift pressure, 550 V drift voltage. This leads to an E/N ratio of about 140 Townsend (Td) (E corresponding to the electric field strength, and N to the gas number density; 1 Td = 10−17 Vcm^2). The sampling time per channel of ToF acquisition was 0.1 ns, amounting to 350,000 channels for a mass spectrum ranging up to m/z = 400. Every single spectrum is the sum of about 28.600 acquisitions lasting 35 μs each, resulting in a time resolution of 1 s. Sampling measurement was performed in 60 cycles resulting in an analysis time of 60 s/sample.

Each measurement was conducted automatically after 20 minutes of sample incubation at 40°C by using an adapted GC autosampler (MPS Multipurpose Sampler, GERSTEL) and it lasted for around 2 minutes. During measurements 100 sccm of zero air was continuously injected into the vial, through a needle heated to 40 °C, and the outflow going through a second heated needle was delivered via Teflon fittings to the PTR-Tof-MS.

α-farnesene and 6-methyl-5-hepten-2-one identification was carried out through comparison of the PTR-ToF-MS fragmentation masses with pure standards.

8.3.5. Statistical Analysis

Multivariate statistical analysis have been performed employing R package “PCA” on Log transformed data.

8.4. Results and Discussion

Apple of cv. 'Granny Smith' harvested at the ripening stage I_{AD} 1.8-2.0, resulted highly susceptible to superficial scald after two months of cold storage, showing from 35 to 95 % of fruit with visible scald symptoms after 4 and 8 days of shelf life at room temperature, respectively. Treatment with 1-MCP effectively reduced to zero the incidence of scald, even after two months of cold storage. These data are in agreement with the previous studies carried out on the same apple cultivar by Lu *et al*. (2013), showing also that early harvested apple are generally more susceptible to scald (Bordonaba *et al*., 2013).

The majority of VOCs detected in the apple tissues collected here, showed a different accumulation upon storage condition and ethylene effect, as suggested by Schaffer *et al*. (2007). This fact was experimentally validated in this work by the application of the ethylene competitor 1-MCP. Only a limited and specific subset of VOCs resulted associated with the occurring of the superficial scald, as suggested by Lurie and Watkins (2012). The entire VOCs variability among the samples were analysed by the means of a Principal Component Analysis (Fig. 1). The distribution depicted on the PCA 2D-plotclearly highlights the difference between control and 1-MCP treated samples as well as between the samples collected at different stages. These differences were mainly attributed to known VOCs that have a direct influence on fruit quality such as esters aldehydes, alcohols, and ketones (Soukoulis *et al*., 2013). In the control skin samples, the separation between T1 and T2 stages, characterized by the presence of scald symptoms (in the latter stage) was mainly due to high concentration of α-farnesene and 6-methyl-5-hepten-2-one (MHO), also supported by the PCA loadings (data not shown).

The main fragment masses of α-farnesene (m/z 205.195 and m/z 149.114) and MHO (m/z 127.089 and m/z 109.076) detected by PTR-Tof-MS analysis were also confirmed by using pure standards, showing a R^2 value of 0.99 and 0.97, respectively.

To verify the effect of α-farnesene and 6-methyl-5-hepten-2-one (MHO) during the development of superficial scald, the accumulation of these two compounds over the samples collected during storage was assessed in apple fruit skin and pulp (Figure 2). In the figure only the data obtained from the control batch are reported, since the ones treated

with 1-MCP were not affected by scald, and consistently did not show any accumulation of either compounds.

It is interesting to note the distinct behaviour between the two compounds assessed by PTR-ToF-MS. The concentration of α-farnesene (Figure 2A) did not considerably differ throughout the time course. The accumulation of this compound, in fact, started already at the first day of shelf-life after 1 month of storage, resulting in a two-fold increasing after only three days of shelf-life. From this point, the content of α-farnesene remained basically unchanged, showing only a slight increase in the stages assessed after two months of cold storage. Different was instead the physiological dynamics of MHO. The concentration of this volatile ketone, originated by the oxidation of α-farnesene, showed a marked burst only in the samples collected after two months of storage and anticipated by couple of days the visual appearing of scald symptoms. During the postharvest shelf-life after one month of cold storage, the accumulation of MHO was consistent with the concentration detected at harvest, while after two months it increased, reaching its maximum 8 days after cold storage, showing aneight-fold increase with regards to harvest (Figure 2B). It is worth noting that the accumulation of MHO anticipates the appearance of superficial scald symptoms, since MHO significantly changes in T2_1 samples, while scald impacted 35% of the samples in T2_4 stage. The different accumulation trend observed between these two compounds is consistent with the physiological pathway, the low concentration of MHO in the first month can be in fact assigned to the oxidation of the α-farnesene. This process might be in fact the reason of the shift observed between the two compounds and depicted in Figure 2.

In the light of these results α-farnesene cannot be considered as a reliable biological marker for superficial scald detection since its concentration rapidly increases with postharvest ripening, rather than with the occurrence of the scald symptoms. On the contrary, the accumulation trend of MHO resulted to be more specifically coincident with the ongoing of scald (as already evidenced by Mir *et al*., 1999 and Wang and Dilley, 2000), suggesting this as a more reliable compound for an early detection of the scald development. To further validate the efficacy of PTR-ToF-MS in predicting the scald development in a real and competitive postharvest management, both α-farnesene and MHO were successfully detected also on the headspace of whole ‘Granny Smith’ fruits after two months of cold storage (Figure 3).

8.5. Conclusions

In conclusion, we confirm the efficiency of 1-MCP treatment in the control of superficial scald development in 'Granny Smith' fruits by reducing the synthesis of ripening related VOCs in the skin, especially of α–farnesene and, consequently, its autoxidation products MHO. Results of this investigation also pointed out i) the possibility to monitor VOCs involved in the superficial scald disorder by PTR-ToF-MS without the necessity to pre-extract samples with hexane or even in a non-destructively approach and ii) the opportunity to use MHO as a VOC marker to monitor oxidative stress processes of apple during storage and in particular to identify scald before the appearance of visible symptoms.

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8.7. Figures

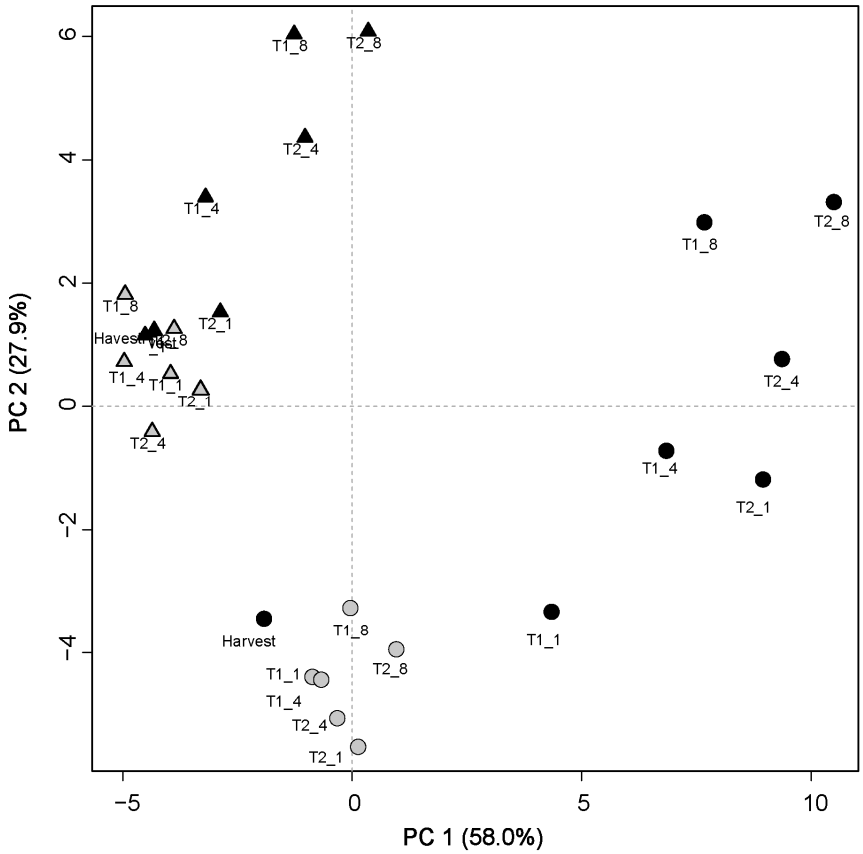

Figure 1. PCA distribution of VOCs assessed by PTR-Tof-MS analysis on 'Granny Smith' apples. Measurements were performed on skin (circle), and pulp (triangle) tissue of fruit stored for 1 (T1) and 2 (T2) months of cold storage at +0.5 °C, and then maintained for 1, 4 and 8 days of shelf-life at room temperature (~20°C). Black points indicate control samples, while in grey are the ones treated with 1-MCP. Each point is the average of 3 single measurements.

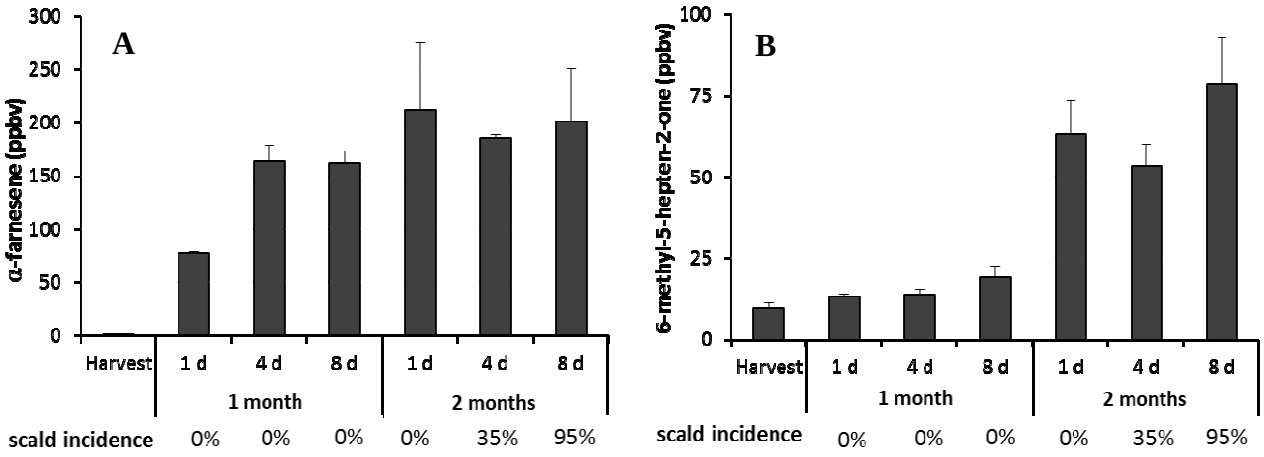

Figure 2. Content of (A) α-farnesene (*m/z* 205.195) and (B) 6-methyl-5-hepten-2-one (*m/z* 127.089) in fruit of 'Granny Smith' during storage and shelf-life. Measurements were assessed by PTR-ToF-MS on skin tissue of fruit left at room at temperature for 1, 4, and 8 days after 1 and 2 months of commercial storage at +0.5 °C, respectively. Percentage of scald incidence (number of fruits with scald symptoms) and standard deviation (3 replicates) are shown.

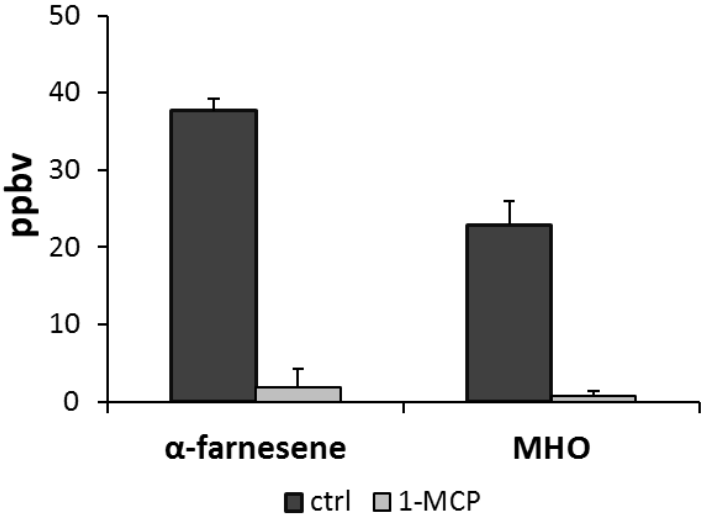

Figure 3. Quantification of α-farnesene(m/z 205.195) and 6-methyl-5-hepten-2-one (m/z 127.089) content in 'Granny Smith' apple fruit (control and 1-MCP treated) assessed by PTR-Tof-MS after 2 month of cold storage (+0.5 °C). Measurements were done on headspace of intact fruit incubated for 30 minutes into a 5L glass jar. Each data point is the average of 5 fruits. Bars indicate standard deviation.

9. General Conclusions

The present research work highlights the importance of the definition of the proper harvesting time and the maturity stage reached at harvest in apple fruits in order to aid post-harvest management.

With regard to the used technology, it was demonstrated that I_{AD} could represent a reliable indicator for monitoring fruit ripening evolution both *in planta* during the growing season and at harvest. Our data confirmed that chlorophyll degradation can be taken as a reliable index to define apple fruit maturity development by the use of non-destructive, easy to use and fast device, the DA-meter.

The results presented in this work confirm the cultivar-specific behaviour of the I_{AD} before harvest and the consistency over growing seasons. Similar trends were observed in the maturation of fruits of the two studied apple cultivars, which could be represented as linear regression over time. These information were used to create a model for forecasting of harvesting time, and furthermore to predict the variability of the ripening stages at harvest. In this sense, the adoption of these methodology could represent a decisional support tool for pre-harvest and post-harvest management.

From the results of this work, the use the I_{AD} to segregate apple fruits on different maturity classes, which behave differently during storage, also emerged as a possible application. Indeed, I_{AD} correlated with FF reduction during cold storage and with scald incidence, being the less mature fruits more susceptible. In addition, I_{AD} trend remained similar or equal in the different seasons. Concerning with the relation between the I_{AD} and the reduction of flesh firmness, the DA-meter technology by real time monitoring could be useful since the I_{AD} may represent a predictor index for long term storage. Based on these considerations, the development of an on-line DA-meter device to be placed in the sorting machine may be seen as a promising strategy to improve and optimise the post-storage procedure and management.

As regards superficial scald metabolism, the results obtained in this research highlight the influence of the maturity stage at harvest on scald development. Moreover, it was pointed out that 1-MCP application is extremely effective in reducing superficial scald. The efficacy of 1-MCP was confirmed to be dependent on fruit ripening stage reached at

harvest. 1-MCP strongly inhibits ethylene emission in both cultivars for all the I_{AD} classes. Surprisingly, ethylene inhibition did not correlate with the inhibition in α-farnesene content, therefore leaving the relation between ethylene and α-farnesene not entirely elucidated. This highlights that ethylene can be involved in other processes associated with scald and not only with α-farnesene regulation. Superficial scald is a complex phenomenon and is a consequence of a general oxidative process; the autoxidation of α-farnesene could be just a secondary effect of free radical reactions. Results of this investigation also pointed out the possibility to monitor VOCs involved in the superficial scald disorder by PTR-ToF-MS. In fact, MHO emerged as a VOC marker potentially allowing the monitoring of oxidative stress processes of apples during storage, and in particular to identify scald before the appearance of visible symptoms. Phenolic compounds, such as chlorogenic acid, are involved in scald development as previously reported by other researches carried on in 'Granny Smith'. In our study, the high level of chlorogenic acid in this cultivar, and its trend during storage and shelf life, support its role in the increase of scald incidence in a susceptible cultivar. Alternative processes involved in scald development should be considered, such as free radical-mediated oxidation or the possible involvement of the cuticular structure and changes in wax composition, for a full metabolic understanding of the phenomenon.

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