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POSTHARVEST CHANGES OF FUNCTIONAL PHYTOCHEMICALS IN BLUEBERRY

Thesis submitted to the Universidade Católica Portuguesa to attain
the degree of PhD in Biotechnology – with specialization in Food Science and
Engineering

By
Daniela de Vasconcelos Teixeira Aguiar da Costa

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Under the supervision of Professor Maria Manuela Estevez Pintado

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Resumo

Os frutos têm sido descritos como essenciais para uma dieta saudável, devido à sua elevada atividade antioxidante, muito correlacionada com os compostos fenólicos totais e com antocianinas. O mirtilo é um dos frutos descritos com uma elevada atividade antioxidante, associado à redução de risco de várias doenças, incluindo a doença cardíaca, algumas formas específicas de cancro (desempenhando um papel importante no tratamento preventivo nutricional anticancro) e outras doenças crónicas. No entanto, o mirtilo apresenta um tempo de prateleira muito curto sendo afetado por diferentes fatores pré- e pós-colheita. O objetivo geral deste programa de doutoramento passa pela compreensão do efeito de diferentes fatores na qualidade comercial e funcional em mirtilos ao longo do armazenamento, por forma a maximizar ou manter a atividade antioxidante ao longo do armazenamento sem comprometer os outros parâmetros de qualidade.

Inicialmente foi realizada a caracterização da qualidade comercial e funcional, assim como do perfil de antocianinas e ácidos hidroxicinâmicos em três cultivares de mirtilo: 'Bluecrop', 'Goldtraube' e 'Ozarkblue' ao longo do armazenamento a 4 ºC, durante 49 dias em 2010 e 56 dias em 2011 e 2012. Foi observado que as antocianinas totais, os compostos fenólicos totais e a atividade antioxidante variam com a cultivar e ano de colheita, sendo que a ação de diferentes características ambientais leva à maior ou menor produção destes compostos, por exemplo em geral, a presença de temperaturas altas pode inibir a biossíntese de flavonoides.

Foi realizado um tratamento pós-colheita dos mirtilos com etileno (1000 $\mu L.L^{-1}$) com posterior armazenamento a 4 ºC, durante 56 dias. O efeito deste tratamento nas antocianinas totais, compostos fenólicos e na atividade antioxidante parece ser dependente da cultivar. O tratamento com etileno aumentou o teor de antocianinas e a atividade antioxidante na 'Bluecrop' e 'Goldtraube', o que faz parecer que possa ser uma boa estratégia para manter estes compostos ao longo do armazenamento, uma vez que estes habitualmente apresentam tendência para diminuírem ao longo do mesmo.

Foi realizado um tratamento com 1-MCP (1 µL.L^{-1}) em frutos de mirtilo das cultivares ‘Bluecrop’, ‘Goldtraube’ e ‘Ozarkblue’ e comparado com a ação de etileno (1000 µL.L^{-1}). Após o tratamento, os frutos foram armazenados a 4º C durante 56 dias. No final do armazenamento o 1-MCP aumentou o conteúdo de antocianinas e ácidos hidroxicinâmicos em relação aos outros tratamentos nas cultivares ‘Goldtraube’ e ‘Ozarkblue’.

Posteriormente testamos o armazenamento em quatro atmosferas controladas diferentes: ar, 2% O_2 + 2% CO_2, 2% O_2 + 15% CO_2, e 21% O_2 + 15% CO_2 em duas cultivares de mirtilo: ‘Goldtraube’ e ‘Ozarkblue’, durante 29 dias a 4 °C. O efeito das diferentes atmosferas foi dependente da cultivar. Enquanto na ‘Goldtraube’ uma baixa concentração de O_2 aumentou o teor em antocianinas, a ‘Ozarkblue’ pareceu beneficiar de uma elevada concentração de CO_2. Elevadas concentrações de CO_2 parecem também ter maior impacto sobre os compostos fenólicos identificados.

Com base nesses trabalhos, dependendo da cultivar, podemos maximizar ou manter a atividade antioxidante, sem comprometer outros parâmetros comerciais de qualidade. O tratamento com etileno para o aumento da atividade antioxidante em algumas cultivares pode ser recomendado, em particular quando os frutos são comercializados em mercados locais ou transportados em pequenas distâncias. Os consumidores irão beneficiar desse aumento dos compostos bioactivos e associada atividade antioxidante. O tratamento com 1-MCP pode ser importante para frutos que são comercializados a longas distâncias, para exportação, pois permite que os frutos mantenham a qualidade funcional durante o armazenamento (maior teor de antocianinas e ácidos hidroxicinâmicos no final do armazenamento).

Abstract

Fruits have been reported as essential for a healthy diet due to their high antioxidant activity, very correlated with total phenolic compounds and total anthocyanins content. Blueberry has been described as a high antioxidant activity fruit, associated with the reduction risk of several diseases, such as heart disease, some specific forms of cancer (playing an important role in the anti-cancer nutritional preventive treatment) and other chronic diseases. However, the blueberry is limited by a very short self-life period and several related properties, including antioxidant activity are affected by different pre- and postharvest factors.

The overall objective of this PhD program was to understand the effect of different factors on commercial and functional quality of blueberries throughout storage, and maximize or maintain the antioxidant activity throughout storage without compromising the other quality parameters.

The characterization of the commercial and functional quality and the profile of anthocyanins and hydroxycinnamic acids in three blueberry cultivars: ‘Bluecrop’, ‘Goldtraube’ and ‘Ozarkblue’ was carried out along storage at 4 ºC, during 49 days in 2010, and 56 days in 2011 and 2012. It was observed that total anthocyanins content, total phenolic compounds and antioxidant activity vary with the cultivar and from year to year, and the action of different environmental characteristics leads to greater or lesser production of these compounds, namely, in general, the presence of too high temperatures was related with inhibition of flavonoid biosynthesis.

The effect of postharvest treatment with ethylene (1000 $\mu L.L^{-1}$) was monitored throughout storage at 4 °C for 56 days. The impact of this treatment on total anthocyanins content, total phenolic compounds content and antioxidant activity seems to be cultivar dependent. The treatment with ethylene increased the anthocyanin content and antioxidant activity in ‘Bluecrop’ and ‘Goldtraube’, which suggests that it may be a good strategy to maintain these compounds throughout the storage, which usually tend to decrease over storage time.

A new treatment with ethylene (1000 $\mu L.L^{-1}$) was compared with 1-MCP (1 $\mu L.L^{-1}$) postharvest treatment of blueberry cultivars ‘Bluecrop’, ‘Goldtraube’ and ‘Ozarkblue’. After the treatments, the fruits were stored at 4 °C, for 56 days. By the end of storage, 1-MCP increased content of anthocyanins and hydroxycinamic acids relative to the other treatments in the ‘Goldtraube’ and ‘Ozarkblue’ cultivars.

Posteriorly storage at four different controlled atmospheres: air, 2% O_2 + 2% CO_2, 2% O_2 + 15% CO_2, and 21% O_2 + 15% CO_2 of two blueberry cultivars (‘Goldtraube’ and ‘Ozarkblue’) were studied during 29 days, at 4 °C. The impact of the different atmospheres was cultivar dependent. While ‘Goldtraube’ at low O_2 concentration improved the anthocyanins content, ‘Ozarkblue’ appeared to benefit from higher CO_2 concentrations. High concentrations of CO_2 seem to have the greatest influence on the identified phenolic compounds.

Based on these works, depending on cultivar, we can maximize or maintain the antioxidant activity without compromising other commercial quality parameters. Treatment with ethylene for increasing antioxidant activity in some cultivars may be recommended when the fruits are marketed in local markets or transported at small distances. The consumers will benefit from this increase of bioactive compounds and associated antioxidant activity. 1-MCP treatment may be important for fruits that are marketed over long distances, for export, because it allows the fruits to maintain functional quality throughout storage (higher content of anthocyanins and hydroxycinamic acids by end of storage).

Aknowledgements

To the Escola Superior de Biotecnologia of the Universidade Católica Portuguesa, for accepting me as PhD student and for providing the necessary conditions to carry out this work.

To my supervisor, Professor Manuela Pintado, for accepting me as a PhD student, for all the dedication and guidance fundamental for the development of this thesis, for all the patience, support, encouraging, and especially for all the knowledge provided. It is a pleasure to work with you! Thank you!

To Professor Isabel Vasconcelos has always solved the problems that have been appearing over the years.

To my daughter and to my husband for all the time that I was not present and for all the support and energy that they transmit. To my parents and the whole family for all the support they have been giving over the years.

To experienced panellists by participation in sensory evaluation.

To Ana Oliveira for the instructions and support throughout all the experimental works.

To Sara Silva and Ana Amaro for the support in the reviews.

To all my ESAV colleagues for the energy, encouragement and interest they have been demonstrating over the years.

To Dália Carvalho, Teresa Deuchande and Helena Gomes, colleagues in the office for their support especially in the most difficult times.

To all the friends that I made at ESB my thank you, always available and good times we spent together, the big THANK YOU!

We thank Mirtilusa for always providing the large quantities of fruit needed for these studies and under the wanted conditions.

The work was financially supported by Agência de Inovação (Portugal) and Quadro de Referência Estratégico Nacional (QREN, Portugal) through project Myrtillus – (QREN-ADI) and National Funds from FCT – Fundação para a Ciência e Tecnologia through project UID/Multi/50016/2013.

This PhD was supported by PhD grant PROFAD to D.V.T.A. Costa by the Polytechnic Institute of Viseu - School of Agriculture.

Keywords

‘Bluecrop’
‘Goldtraube’
‘Ozarkblue’
1-MCP
Anthocyanins profile
Antioxidant activity
Colour
Commercial quality
Conservation
Controlled atmosphere
Cyanidin-3-glucoside
Ethylene
Firmness
Functional quality
Hydroxycinnamic acid
Phytochemicals
Postharvest treatment
Preservation
Sensory profile
Soluble solids content
Storage
Titratable acidity
Total anthocyanins content
Total phenolic
Vaccinium corymbosum
Weight loss

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List of Abbreviations

1-MCP - 1-Methylcyclopropene

a* - Chromaticity coordinate on a green (-a*) to red (+a*) axis

ABTS - 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ADH - Alcohol dehydrogenase

ANR - Anthocyanidin reductase

ANS - Anthocyanidin synthase

b* - Chromaticity coordinate on a blue (-b*) to yellow (+b*) axis

C* - Chroma, $C^* = [(a^*)^2 + (b^*)^2]$ ½

CA - Controlled atmosphere

$CH_3CONa_3H_2O$ - Sodium acetate

CHI - Chalcone isomerase

CHS - Chalcone synthase

CIE - Commision Internationale de L'Eclairage

CO_2 - Carbon dioxide

DFR – Dihydroflavonol 4-reductase

DHK - Dihydrokaempferol

DHM – Dihydromyricetin

DHQ – Dihydroquercetin

F3′5′H - Flavonoid 3’, 5’-hydroxylase

F3′H - Flavonoid 3' hydroxylase

FGT - Flavonoid 3-O-glycosyltransferase

FHT - flavanone 3-hydroxylase

FLS - Flavonol synthase

FRAP - Ferric Reducing Antioxidant Power h^o- Hue angle, h^o = $\tan^{-1}$ (b*)/(a*)

KCl - Potassium chloride

L* - Lightness

LAR - leucoanthocyanidin reductase

LAR - Leucoanthocyanidin reductase

LDOX - Leucoanthocyanidin dioxygenase

LPS - Low pressure storage

MA - Modified atmosphere

MeJa - methyl jasmonate

O_2 – Oxigen

PAL - L-phenylalanine ammonia-lyase

PAs – Proanthocyanidins

PCA - Principal component analysis

PET - polyethylene terephthalate

PPO - polyphenol oxidase

RA - Refrigerated air

RH - Relative humidity

ROS - Reactive oxygen species

TEAC - Trolox Equivalent Antioxidant Capacity

UFGT - UDP glucose-flavonoid 3-O-glucoslyltransferase

UV – Ultraviolet

UV-A - Ultraviolet A

UV-B - Ultraviolet B

UV-C - Ultraviolet C

ΔE* - Colour difference, $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]$ ½)

ΔH^* - Metric-hue difference, $\Delta H^* = [(\Delta a^*)^2 + (\Delta b^*)^2 - (\Delta C^*)^2]$ ½

Scope and outline

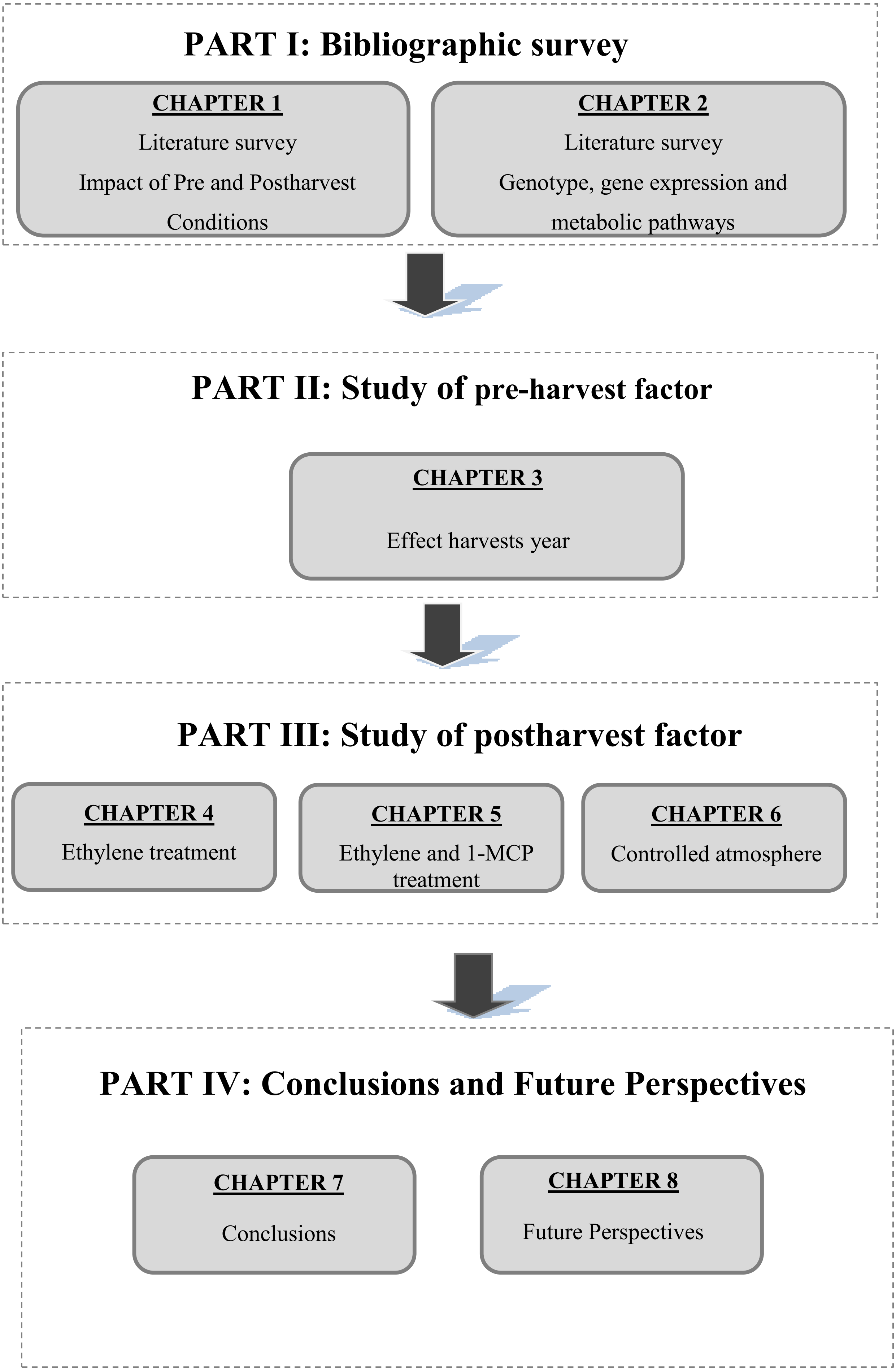
This thesis is organized into four parts, which include 8 chapters. The overall objective of this work was to understand the effect of pre and postharvest factors on commercial and functional quality throughout the storage of different blueberry cultivars. This information was integrated in order to maximize or maintain functional quality throughout the storage without compromising commercial quality.

Part I encompasses Chapters 1 and 2, where a critical review of the literature on pre- and postharvest factors affecting the functional activity of blueberries was performed, and how these factors affect the gene expression of biosynthesis of the blueberry-associated metabolic pathways (*Vaccinium corymbosum*).

Part II includes Chapter 3, which is associated with preharvest factors and describes the effect of different harvests (years) on the evolution of commercial and functional quality throughout the 4 °C storage of three blueberry cultivars: Bluecrop, Goldtraube and Ozarkblue, for 49 or 56 days.

Part III encompasses Chapters 4, 5 and 6. This part describes the effect of different postharvest treatments: ethylene (Chapter 4), ethylene and 1-MCP (Chapter 5) and testing storage at different controlled atmospheres (Chapter 6), in different cultivars of blueberry. These treatments were performed in order to increase / maintain key bioactive compounds and properties as well as quality throughout the storage at 4 ° C for 56 days or 29 days.

In **Part IV**, the conclusions are presented in chapter 7 and future perspectives in chapter 8.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PART I - Bibliographic Survey

CHAPTER 1- Impact of pre and postharvest conditions on the phytochemical profile and antioxidant capacity of *Vaccinium*

This chapter has been submitted to the journal: Food Engineering Reviews

1.1. Abstract

Blueberries are known to be rich in phytochemicals and exhibiting high antioxidant activity. Several factors can influence their phytochemical profile and antioxidant activity. These factors can be classified as pre-harvest and postharvest. The pre-harvest factors can include: cultivar / genotype, maturation and ripening, climate conditions (temperature, light intensity), soil type, compost, mulching, fertilization, increasing carbon dioxide concentration in the atmosphere, and application of naturally occurring compounds, ripness state and harvest date. In the postharvest factors other factors are considered: storage temperature, storage atmosphere, ethylene, 1-MCP, UV-B / UV-C and treatments with: allyl isothiocyanate, methyl jasmonate, abscisic acid, hexanal, carvacrol, anethole, cinnamaldehyde, cinnamic acid, perillaldehyde, linalool and *p*-cymene.

In this work we present a revision with critical analysis about how the different pre-harvest and postharvest factors influence the phytochemical profile and antioxidant capacity of blueberry (*Vaccinium corymbosum*).

1.2. Introduction

Fruit and vegetables quality, both nutritionally and functionally wide, has become one of the most important aspects of horticultural plant’s cultivation. Hence, it is important to not only characterise the beneficial phytonutrients present in fruits and vegetables, but also to understand the factors that influence their content and their bioactive mechanism. A diet rich in plant bioactive compounds has been considered an important element in human health prophylaxis (Łata et al., 2005). Intact berry consumption has become popular among health-conscious consumers due to their high levels of antioxidants (such as anthocyanins) and phenolic compounds (You et al., 2011). Blueberries (*Vaccinium* L. sp.) have been described as one of the best fresh fruit sources of antioxidants (Sellappan et al., 2002), exhibiting higher levels of total phenolic and anthocyanin content than those previously reported for other fruits and vegetables (You et al., 2011). For instance Kalt et al. (1999) reported that blueberries exhibited a higher antioxidant capacity than to strawberries, raspberries and other fruits.

The high antioxidant activity of blueberries has been widely associated with the presence of phenolic (polyphenolic) compounds (Kalt et al., 2001; Koka and Karadeniz, 2009). Overall two major classes of phenolics can be commonly found in small fruit species, namely flavonoids (e.g. anthocyanins) and non-flavonoids (e.g. phenolic acids) both of which have been intrinsically linked with several of the potential health benefits of blueberries and other foods. The phytochemical profiling of blueberries results in a semicomprehensive analysis of the compounds present in the fruits and it may lead to a better understanding of the contribution of each component to the overall beneficial potential of blueberries. Moreover, the diversity of polyphenolic profiles between different berry species may explain the differences in biological activity and biovailability between different fruits, although all have been associated with a reduction of the risk of heart disease, some specific forms of cancer and other chronic diseases (Taruscio et al., 2004).

It is known that the polyphenols composition of blueberries varies widely not only between different plant species (e.g. *V. corymbosum* vs *V. angustifolium*), but even between cultivars, and it is influenced by numerous intrinsic and extrinsic factors. In addition, polyphenols concentrations, and consequently their health-promoting properties may also be affected by harvesting and storage conditions with inappropriate processing resulting in a decrease of these compounds. So, the purpose of this revision is to characterize the impact of various factors on the phenolic compounds composition and antioxidant properties of blueberries in order to provide some insights into possible manipulations (both pre- and postharvest) that may contribute to the production of blueberries with improved phytonutrient profiles; moreover it also aims to establish the best conditions in order to maintain the phytochemical properties of the fruit until it reaches the consumer.

1.3. Factors that affect the functional activity of blueberry

Blueberries contain many antioxidant compounds which in turn, are frequently associated with health promotion and well being. Antioxidants, as oxidative stress coping metabolites are subjected to dynamic concentration fluctuations in plant tissues, which make the identification of which factors influence their content and overall redox ratio difficult (Łata et al., 2005). However, the technological advances in genomics, plant breeding, bioengineering, and biotechnology have allowed for the production of foods with maximal nutritional content, not only through plant breeding and selection (Wang, 2006), but also by the manipulation of growing conditions and/or postharvest processing (Jaakola et al., 2004; Jaakola and Hohtola, 2010; Montalba et al., 2010; Oliveira et al., 2010; Siddiq et al., 2018). In this sense, there are several factors that affect the functional activity of fruits and they can be divided into two groups as detailed in Table 1.1.

Table 1.1. - Pre-harvest and postharvest factors that may affect functional properties.

Functional activity	
Pre-harvest	**Postharvest**
Localization	Storage temperature
Cultivar/Genotype	Storage atmosphere
Part plant	Ethylene
Fruit Size	1-MCP
Environmental Growth Conditions:	UV-B / UV-C
-Temperature	
-Light intensity	Others treatments:
-Soil type	- allyl isothiocyanate,
-Water	- methyl jasmonate,
	- abscisic acid,
Cultivation pratices:	- hexanal,
- compost,	- carvacrol,
- mulching,	- anethole,
- fertilization,	- cinnamaldehyde,
- increasing carbon dioxide concentration in the atmosphere	- cinnamic acid, - perillaldehyde,
- pruning	- linalool,
-exposure sucrose	- *p*-cymene
Ripeness	
Harvest date	

Pre-harvest factors may affect the content and stability of phytochemicals with nutritional value such as: cultivar/genotype, maturation and ripening, climate conditions (Castrejón et al., 2008) (temperature, light intensity), soil type, compost, mulching, fertilization, increasing carbon dioxide concentration in the atmosphere, and application of naturally occurring compounds (Wang, 2006), ripness state and harvest date (Table 1.2.) and postharvest factors like: storage temperature, storage atmosphere, ethylene, 1-MCP, UV-B / UV-C, and treatments with: allyl isothiocyanate, MeJa, abscisic acid, hexanal, carvacrol, anethole, cinnamaldehyde, cinnamic acid, perillaldehyde, linalool, *p*-cymene.

Table 1.2. - Pre-harvest factors affecting the polyphenol composition and antioxidant activity of blueberries.

Factor		**Effect**	**Reference**
Localization		The effect of geographical variation, month-to-month and year-to-year difference on the antioxidant capacity of various fruits is well described and is likely also due to environmental conditions	Wang, 2006
Part Plant		The values of leaf phenolics may be very greater in than the observed fruits on a fresh weight basis.	Ehlenfeldt and Prior, 2001
		The antioxidant activity were mainly derived from peel of the blueberry fruit.	Wang et al., 2012a
Fruit Size		Thus that small-fruited genotypes, which have a larger surface area/volume ratio than large fruits, typically have higher levels of phenolics, antioxidant capacity.	Cho et al., 2005; Connor et al., 2002b; Howard et al., 2003
Environmental Growth Conditions	Temperature	High a temperature can inhibit biosynthesis and cause degradation of flavonoids. A low temperature can increase flavonoid production (although the accumulation of flavonoids in cold temperatures is light dependent).	Jaakola and Hohtola, 2010
	Light	Growing under direct sun exposure increase in the expression of the flavonoid pathway genes and the concentrations of anthocyanins, catechins, flavonols and hydroxycinnamic acids.	Jaakola et al., 2004
	Irrigation	Irrigation resulted in a change in anthocyanin composition in the fruit but did not affect antioxidants or total anthocyanin content	Ehret et al., 2012

Table 1.2 (Continued)

Cultivation Practices	Fertilization	High nitrogen supply delays the quantitative and qualitative biosynthesis and enhances their degradation during the final steps of berry maturation (Merlot vines *Vitis vinifera* L.).	Hilbert et al., 2003
		Organic N increased the leaf antioxidant content, and mycorrhizal colonization relative to conventional nitrogen source (*Vaccinium corymbosum* L. 'Legacy').	Montalba et al., 2010
	Application sucrose	Induce the biosynthesis of anthocyanin. High levels of sucrose act as a signal to the plant that it is actively undergoing photosynthesis.	Kwon et al., 2011
		The application of sugars may be positive in antioxidant activity. The induction of flavonoid /anthocyanin synthesis genes is sugar specific and unlikely to be stress mediated.	Solfanelli et al., 2006
	Pre-harvest treatments with $CaSO_4$	Not alter colour and anthocyanins content during refrigerated storage.	Angeletti et al., 2010
Ripeness state		Anthocyanins increased, but the antioxidant capacity, as well as total phenolic content declined in blueberry fruit ('Bluecrop', 'Reka', 'Puru', and 'Berkeley') during maturation.	Castrejón et al., 2008; Forney et al., 2012a

1.3.1. Pre-harvest factors

1.3.1.1. Location

The location of the production blot can influence the antioxidant activity of blueberries. However, the variations between locations have been reported to be smaller than other factors such as the cultivar (Connor et al., 2002b).

Regardless the effect of geographical variation on the antioxidant capacity of various fruits has been well described and it is likely related to differences in environmental conditions (Wang, 2006). For instance altitude has an effect on the secondary metabolites content of higher plants. The higher solar radiation frequently found at higher altitudes has often been implicated in this variation with authors reporting an increase in phenolic compounds with altitude as a response to an increase of UV radiation (Jaakola and Hohtola, 2010).

Latitude also showed to influence the anthocyanins concentration (Akerström et al., 2010), since appears to exist a latitude related genetic adaption of anthocyanin production by berries (Uleberg et al., 2012).

Different blueberry cultivars (Akerström et al., 2010; Connor et al., 2002b), wild bilberry, and wild bog whortleberry (Häkkinen and Törrönen, 2000) grown in different regions showed significant differences in antioxidant activity, total polyphenols and antochyanins content. This may reflect differences in climate and cultural practices, including differences in ultraviolet radiation, temperatures, osmotic stress, mineral and nutrient availability, while the differences in the antioxidant activity between plants (Blueberries and blackberries) grown in different locations may be more than four times (Sellappan et al., 2002), this variable is not always reported as affecting total phenolics, anthocyanins and antioxidant activity of *Vaccinium corymbosum* L. blueberries (Highbush) (Prior et al., 1998).

1.3.1.2. Cultivar

Different cultivars of blueberries possess varied amounts of total anthocyanins content, total phenolics, and antioxidant capacities in skin and flesh tissues (Häkkinen and Törrönen, 2000). Dragović-Uzelac et al. (2010) found differences between the antioxidant activity and the total phenolic compounds between different cultivars, ‘Sierra’ presenting higher values followed by ‘Elliot’, subsequently ‘Bluecrop’ and ‘Duke’.

If we compare different parts of blueberry plant, leaf assays exhibited the highest antioxidant activity and total phenolics content. The values of total phenolics in leaf may be greater than 30 times in that observed in fruits on a fresh weight basis (Ehlenfeldt and Prior, 2001).

1.3.1.3. Fruit size

In blueberries the skin tissue has higher levels of antioxidant compounds compared to flesh tissue. Skin contributed with a higher percentage of free radical scavenging capacity than flesh tissue even though the fruit contained much higher amounts of flesh than peel in terms of dry weight (Wang et al., 2012a). Phenolic compounds are, in general, concentrated on the epidermal tissues, and small fruits have more epidermal tissue per unit volume than large fruits. Thus, those small-fruited genotypes (which have a larger surface area/volume ratio than large fruits) typically have higher levels of phenolics and, consequently, antioxidant activity (Cho et al., 2005; Connor et al., 2002b; Howard et al., 2003). This relationship between antioxidant activity and surface area-to-volume ratio was observed in *Vaccinium corymbosum* L. (Highbush), *Vaccinium ashei* Reade (Rabbiteye) and *Vaccinium angustifolium* (Lowbush) blueberries as well as and *Vaccinium myrtillus* L. bilberries (Prior et al., 1998).

1.3.1.4. Environmental Growth Conditions

The growing environment strongly affects fruit quality. Factors such as soil fertility and pH, water availability, fruit pollination, pruning, plant age and vigor, and the presence of abiotic and biotic stresses can influence fruit growth, composition, and quality (Forney, 2009) as well as their antioxidant activity and phytonutrient profile. Therefore blueberry genotypes should be screened over multiple seasons in order to identify those with antioxidant-rich germplasm (Łata et al., 2005).

Abiotic conditions (e.g. temperature, moisture, irradiation and soil fertility) that vary markedly from year to year, can affect the phenolic content of fruits (Wang, 2006). In fact, the expression of a number of genes involved in anthocyanin biosynthesis has been reported to be induced by severed abiotic stresses (Guo et al., 2008).

1.3.1.4.1. Temperature

Plants growing in cold climates exhibit higher photosynthetic rates (at lower temperatures) than plants growing in warmer areas. Therefore they can thus increase the amount of fixed carbon available for secondary metabolites. In fact, several studies have reported the effect of temperature (high or low) on the composition concentration of flavonoids (Jaakola and Hohtola, 2010).

Plants can react to elevated and low temperatures by altering flavonoid synthesis in a species-specific way. In general, the presence of too high temperatures can inhibit flavonoid biosynthesis and cause their degradation. On the other hand, lower temperatures can increase flavonoid production, although their accumulation in cold environments is light dependent. Some evidences indicate that cooler temperatures favour the production of flavonoids with a higher hydroxylation levels. Overall, the attributes characteristic to the northern climate (long days with cool night temperatures) have been described as exerting a positive impact on the biosynthesis of flavonoids by plants, although there appears to be a variation in the response between different plant species and within individual flavonoid groups (Jaakola and Hohtola, 2010). On the other hand, Uleberg et al. (2012) reported that, in bilberry, moderate temperature lead to an increase of antocyanin levels (18 ºC). In fact, moderate-low temperatures do not simply enhance the developmental of regulation of anthocyanin biosynthetic gene expression but they act as a specific and separate signal (Shvarts et al., 1997).

1.3.1.4.2. Irradiation

Light is one of the most important environmental factors regulating plant development and gene expression. Ultraviolet (UV) which makes up 7% of sunlight, stimulates distinct responses in plants (Guo et al., 2008). Because of the depletion of the ozone layer, the UV radiation level has increased in the polar areas, including the Northern Hemisphere. Exposure to high amounts of UV radiation can cause damage to macromolecules, such as DNA (Jaakola and Hohtola, 2010). Both UV-A and low influence of UV-B can induce the accumulation of anthocyanin via induction of the expression of anthocyanin biosynthesis genes (Guo et al., 2008).

Growing under direct sun exposure increase the expression of the flavonoid pathway genes in leaves (*Vaccinium myrtillus* L.). The concentrations of anthocyanins, catechins, flavonols and hydroxycinnamic acids were higher in the leaves exposed to direct sunlight. These results have shown the protective role of flavonoids and hydroxy cinnamic acids against high solar radiation in plants (Jaakola et al., 2004).

Anthocyanins contents have been found to be affected by day length, whereas data from others flavonoids are variable. The increase of secondary metabolites levels, in long-day light conditions can be a result of the increase in incident light energies particularly as the contents of flavonols, especially quercetin derivates, appear to correlate more with increased light exposure than temperature, which has a less prominent effect on the total flavonol content (Jaakola and Hohtola, 2010).

Also appears to exist an interaction between light and plant type; for example exposure of Northern bilberry clones to red light resulted in higher anthocyanins and total phenolic's levels as well as in significantly higher levels of antioxidant activity. However, this is dependent on the clones because the Southern bilberry clones showed an inverse relationship in regards to the Northern clones (Uleberg et al., 2012).

The quality of light reaching plants can also affect plant growth and development, with the use of plastic films possibly altering crop quality and production. However, Ordidge et al. (2010) showed that blueberries grown under a polythene film that partially (or completely) blocks UV radiation, were as rich in those health-beneficial phenolics as those crops grown under UV-transparent film.

1.3.1.4.3. Irrigation

One of the inevitable consequences of drought stress is an increase in reactive oxygen species (ROS) production in the different cellular compartments. This enhanced production, is kept under tight control by a versatile and cooperative antioxidant system that modulates intracellular ROS concentration and mantains the redox-status of the cell. When the drought stress is prolonged ROS production will overwhelm the scavenging action of the intrinsic antioxidant system, resulting in extensive cellular damage and death (Carvalho, 2008).

Blueberry plants are highly sensitive to water deficit (Améglio et al., 2000). Non-irrigated plants exhibited lower antioxidant activity in blueberries 'Brigitta' (Lobos et al., 2018) and 'Duke' then the irrigated counterparts (Ehret et al., 2015). However, Cardeñosa et al. (2016) reported no significant differences in antioxidant activity and phenolic content in 'Rocío' blueberries grown with water restriction. In fact, in another work, irrigation treatments did not influence antioxidant levels nor total anthocyanin content of ‘Duke’ blueberries (Ehret et al., 2012), but these differences obtained for ‘Duke’ may be related to the age of the plants, thus plants become more sensitive to soil water deficits with age (Ehret et al., 2015). However, different irrigations resulted in the modification of anthocyanin composition. Moderate irrigation led to lower levels of delphinidin 3-arabinoside and cyanidine 3-arabinoside and an increase of malvidin 3-galactoside and malvidin 3-arabinoside (when compared with no and heavy irrigation). Additionally, moderate irrigation levels also led to a reduction of delphinidin 3-galactoside when compared with heavy irrigation. It is not known how these changes affect the nutritional value of blueberries, but they have demonstrated the potential for irrigation induced control of anthocyanin levels (Ehret et al., 2012).

1.3.1.5. Cultivation practices

Soil types, composts, mulching and fertilization influence the water and nutrient supply to the plant and therefore can affect the nutritional composition and antioxidant activity of the harvested fruit (Wang, 2006). A variety of cultural practices (such as pruning) can alter the plant canopy affecting air movement and solar penetration, which has been reported to influences fruit colour and decay incidence during storage (Forney, 2009).

The organic production seems to influence the levels of sugars (fructose and glucose), malic acid, the total phenolics, the total anthocyanins content and antioxidant activity increasing them when compared with the conventional culture. Blueberries (‘Bluecrop’) produced using an organic system have higher contents of myricetin-3-arabinoside, quercetin-3-glucoside, delphinidin-3-galactoside, delphinidin-3-glucoside, delphinidin-3-arabinoside, petunidin-3-galactoside, petunidin-3-glucoside, and malvidin-3-arabinoside than conventionally grown fruit (Wang et al., 2008). Similar result were observed for chlorogenic acid in ‘Brigitta’ blueberries (Rodriguez-Mateos et al., 2012), and for total phenolic compounds in rabbiteye blueberry ‘Powderblue’ (You et al., 2011).

However, these variations are not consistently reported. Other authors (Sablani et al., 2010, 2011) did not find significant differences in phenolic concentration between the production systems (organic or conventional) of blueberries (‘Duke’ and ‘Reka’), or rabbiteye blueberry ‘Climax’ (You et al., 2011).

Fertilization with organic nitrogen led to higher the leaf antioxidant activity, and mycorrhizal colonization, when comparing to a conventional nitrogen source (*Vaccinium corymbosum* L. ‘Legacy’) (Montalba et al., 2010).

Therefore the application of sugars may exert a positive effect upon the antioxidant activity. Sugars act as signaling molecules, whose signal transduction pathways may lead to the activation or inactivation of gene expression. In fact, whole-genome transcript profiling revealed that flavonoid and anthocyanin biosynthetic pathways were strongly up-regulated following sucrose treatment, resulting in mRNA accumulation, and altering both flavonoid and anthocyanin contents. The mRNA level of several genes increases after the treatment with sucrose with the induction being particularly evident for those genes encoding enzymes that act at the level and downstream of chalcone pathway, namely chalcone synthase, chalcone isomerase, flavanone-3-hydroxylase, flavonoid-3’-hydroxylase, flavonol synthase, dihydrofavonol reductase, leuco anthocyanidin dioxygenase, and flavonoid 3-O-glucosyltransferase. On the other hand, the cinnamate 4-hydroxylase mRNA concentration barely affected by sucrose. The induction of flavonoid /anthocyanin synthesis genes appears to be sugar specific and unlikely to be stress mediated. The sucrose was the most efficient trigger for mRNA accumulation of genes, whose products act downstream along the anthocyanin biosynthetic pathway (e.g. dihydroflavonol reductase, leucoanthocyanindin dioxygenase, flavonoid 3-O-glucosyltransferase), as well as *PAP1*. These results suggested that sugars acted as signaling molecules, activating the *PAP1* gene by means of a sucrose-specific signaling pathway (Solfanelli et al., 2006).

Preharvest treatments with $CaSO_4$ of ‘O’Neal’ and ‘Bluecrop’ blueberries reduced weight loss, softening and deteriorating of highly perishable blueberries, but did not alter sugar, colour and anthocyanins content during refrigerated storage. These treatments might be useful for fruit intended for long distance shipping (Angeletti et al., 2010) but exhibited no significant influence in antioxidant compound levels.

1.3.1.6. Fruit ripeness

Phytonutrients composition varies in parallel with the overall development and maturation of fruits. Therefore, it stands to reason that the antioxidant activity varied considerably throughout the ripening of the fruits (Wang, 2006).

The change in total phenolics during the ripening of blueberry was not extensive, however changes in specific phenolic compounds could have a greater significance on the nutritional value of these fruit. For example, the anthocyanin-related nutritional value may be significantly affected by ripeness stage of the berries as it’s content increases as the fruit ripens (Forney et al., 2012b). Highbush blueberry cultivars presented the same pattern of phenol biosynthesis characterized by decreasing flavonols and hydroxycinnamic acids (procyanidins, quercetin (Jaakola et al., 2002) and kaempferol (Häkkinen et al., 1999) during early maturation stages and throughout ripening, meanwhile accumulation of anthocyanins increased only during the successive harvest. So, during ripening there was a shift in the pool of total phenolics toward anthocyanin synthesis (Castrejón et al., 2008; Prior et al., 1998), with the levels increasing as the colour developed. Blueberries could gained 10% in total anthocyanin content as they went from 100% blue colour to fully ripe, or could lose 2% (Kalt et al., 2003), depending on the cultivar (Perkins-Veazie et al., 2008). The antioxidant activity, as well as total phenolics content, declined in blueberry fruit (*Vaccinium corymbosum* L., 'Bluecrop' and 'Reka'), ('Puru', and 'Berkeley') during maturation (Castrejón et al., 2008; Forney et al., 2012a).

In fact, the antioxidant activity was strongly related to the total phenolic content in all blueberry cultivars. This suggested that anthocyanins had less antioxidant potential than the other phenolic compounds such as flavonols, which among the flavonoids are considered those with highest antioxidant activity (Castrejón et al., 2008).

Regardess of these observations, it was found that antioxidant activity of fruits seemed to be more influenced by genetic differences than physiological ripening changes (Castrejón et al., 2008).

1.3.1.7. Harvest date

The environmental effects, such as the harvesting period, may play an important role in the existence of antioxidant compounds, in particular in less stable anthocyanins such as delphinidin glycosides (Lohachoompol et al., 2008).

The harvest date has been shown to have a significant effect on the level of some phytochemicals. Blueberries (cv. Elliott) harvested when not yet fully mature to extend market-life, the antioxidant activity was still maintained until the commercial distribution (Connor et al., 2002a). Whereas blueberries ('Darrow') harvested at a later date had a lower content of cysteine, glutathione, phenolics, flavonols and anthocyanins. It is possible that some differences in the weather factors could influence the synthesis and accumulation of some tested compounds (Łata et al., 2005).

1.3.2. Postharvest conditions

Fruit quality loss during postharvest handling is mostly the result of decay, physiological breakdown, physical abuse, and dehydration. Fruit must possess a high initial quality in order to maximize storage life, with the initial quality being dependent on many factors including cultivar, cultural practices, growing environment and harvest practices (Forney, 2009).

Postharvest operations could be oriented to minimise weight loss, and to minimise the steps of the supply chain which enhance fruit moisture loss, such as delays in cooling (Paniagua et al., 2013). Postharvest factors such as transport and storage can also influence phytochemical composition of food crops (Wang, 2006).

1.3.2.1. Storage Temperature

Maintaining product quality may be important for domestic storage (where storage may be short-term) but it is critical for internationally shipped products, where sea shipments may take a minimum of 14-20 days (Ehlenfeldt, 2002). Therefore proper temperature management is the most critical factor in the postharvest handling of fruits, with the rapid cooling of blueberries immediately after harvest playing an important role to preserve quality of the fruits and extend market-life. In fact following harvest, blueberries must be cooled and held at temperatures near 0°C for maximum market life (Forney, 2009).

However, storage at temperatures higher than 0 °C may actually enhance antioxidant activity and, therefore, the health functionality of certain small fruit crops (Kalt et al., 1999). In fact, the antioxidant activity increased during storage for six cultivars of blueberries (MSU-58, Brigitta, Legacy, Bluegold, Nelson and Jersey), stored at 5 °C (3 to 7 weeks). Thus, the antioxidant health benefits from these berries can be retained well after harvest, until being consumed (Connor et al., 2002b).

The storage of highbush blueberries, 2-8 days at 10 or 20 ºC, has also been reported to increase the total anthocyanin levels (Kalt et al., 1999, 2003), but this increase was greater when they were stored for 3 weeks at 5 °C.

1.3.2.2. Storage atmosphere

The role of storage conditions is, whenever possible, to decrease, the decay processes of fruits to extend of the quality of fresh fruit for longer periods of time (Borecka and Pliszka, 1985).

Blueberries can be effectively stored in either of three ways, refrigerated air (RA), modified atmosphere (MA), or controlled atmosphere (CA). Decisions on which of these three methods are chosen depends on storage demand. Refrigerated air is the most frequently used for short-term storage (3-4 weeks). It generally refers conditions of 1-2 ºC and 95% relative humidity. Other factors being equal, controlled atmosphere (CA) and modified atmosphere (MA) storage have no advantage over RA in short-term storage. In general, MA and CA rely upon increasing CO_2 levels, to inhibit mold development, and decreasing O_2 levels, to reduce overall respiration rate and ethylene production. Additionally, low O_2 levels can lead to respiratory stress, which is a major factor in producing off-flavors (Ehlenfeldt, 2002).

Different blueberries cultivars respond differently to different atmospheres. Some cultivars such as Duke, Toro, Brigitta, Liberty and Legacy seems to be well suited to extended CA storage, whereas other cultivars such as Elliot can be mantained moderately well and Ozarkblue, Nelson and Jersey are poorly stored (Alsmairat et al., 2011).

Controlled or modified atmospheres have been reported to reduce blueberries decay with optimum concentrations of CO_2 ranging from 10 to 12%. Moreover, reduced O_2 concentrations of 1 to 2% have been recommended but there is little evidence that O_2 reduction is beneficial (Forney, 2009). However, Harb et al. (2010) reported that 18% O_2

and 12 -18% CO_2 as the best commercial conditions widely adopted for the storage of blueberries.

The optimum atmosphere for the storage of 'Burlington' blueberry fruit, at 0 °C, and 15 kPa O_2 was found to be 10 kPa CO_2, allowing for the maintenance of good quality for over 6 weeks of storage (Forney et al., 2003).

Blueberries cv. Duke' can be stored for 5 weeks in cold storage at 0–1 ºC as well as for 7 weeks in CA-storage with CO_2 atmosphere (6–12 %) without O_2 reduction (Harb and Streif, 2004).

'Bluecrop' blueberries stored whith CO_2 levels up to 12%, but with high O_2 level (18%), resulted in a better preservation of ascorbic acid as well as in a reduction of the antioxidant activity of water soluble antioxidants (Harb et al., 2010).

The atmosphere of 2.5 kPa O_2 + 15 kPa CO_2 for two rabbiteye (*Vaccinium ashei* Reade 'Centurion' and 'Maru') blueberries stored at 1.5 ºC did not affect the antioxidant activities and total phenolic content of blueberries. The highest increase in antioxidant activity and total phenolics content occurred during the additional shelf-life time when they were kept at 20 ºC. A positive relationship was observed between antioxidant activity and phenolic content during storage in RA but not in CA (Schotsmans et al., 2007), with similar results were being reported by Gunes et al. (2002) to cranberries.

Nevertheless, Duarte et al. (2009) found that a lower concentration of CO_2 was beneficial to 'Brigitta'. They found that a 5% CO_2 concentration had the most beneficial effect on the quality of 'Brigitta' blueberries after 24 days of storage, at 0 ºC. With the use of controlled atmospheres, the amount of unmarketable fruit after 24 days was reduced and a desirable fruit was obtained in terms of colour, acidity, and higher anthocyanins content. On the other hand, (Hancock et al., 2008), when the storage of eight cultivars ('Elliott', 'Bluegold', 'Brigitta', 'Aurora', 'Draper', 'Toro', 'Bluejay', 'Legacy') with air or with an atmosphere 2 kPa O_2 and 8 kPa CO_2 was studied, no significant enhancement in fruit shelf-life under CA conditions was observed.

High-oxygen (60-100%) treatments may improve the antioxidant capacity of ‘Duke’ blueberries. Furthermore, the antioxidant capacity may be correlated with total phenolic and anthocyanin contents in blueberries. This storage, with atmospheres enriched with O_2 at 60% can therefore improve the health benefits of blueberry fruit, by positively stimulating phenolic metabolism to enhancing the antioxidant activity of the fruits (Zheng et al., 2003).

In Table 1.3. the effect of different atmospheres on blueberries is summarized.

Table 1.3. - The effect of different atmospheres on the storage of blueberries.

Atmophere	**Source**	**Effect**	**Reference**
12-18% CO_2 + 18% O_2	*Vaccinium corymbosum* L.	Extension of the marketing season. Commercial conditions of storage usually widely adopted.	Harb et al., 2010
15 % O_2 + 10 % CO_2	Blueberries cv. 'Burlington'	Fruit maintained good quality for over 6 weeks	Forney et al., 2003
6–12 % without O_2 reduction	Blueberries cv. 'Duke'	If CO_2 upper to 18 kPa or more resulted in an accelerated softening of fruits and coincided with an increased respiratory activity	Harb and Streif, 2004
12% CO_2 + 18% O_2	Blueberries cv. 'Bluecrop'	Better preservation of ascorbic acid. The antioxidant capacity of water soluble antioxidants decreased	Harb et al., 2010
15 kPa CO_2+2.5 kPa O_2	*Vaccinium ashei* Reade 'Centurion' and 'Maru'	The antioxidant activities and total phenolic content of blueberries were not affected adversely by prolonged controlled atmosphere storage.	Schotsmans et al., 2007
5% CO_2+5% CO_2	Blueberries cv. 'Brigitta'	The amount of unmarketable fruit was reduced at 24 days and a desirable fruit was obtained in terms of colour, acidity, and higher anthocyanins content.	Duarte et al., 2009
8 % CO_2+ 2 % O_2	Blueberries cv. 'Elliott', 'Bluegold', 'Brigitta', 'Aurora', 'Draper', 'Toro', 'Bluejay', 'Legacy'	A significant enhancement in storage life of fruit held under controlled atmospheric conditions was observed.	Hancock et al., 2008
Storage at atmospheres enriched with O_2 at 60%	Blueberry cv. 'Duke'	Improve the antioxidant capacity. Furthermore, antioxidant capacity may be correlated with total phenolic and anthocyanin contents in blueberries.	Zheng et al., 2003

The low pressure storage (LPS), particularly under very low levels (e.g. 38 mm Hg), has been reported as good for blueberry preservation exhibiting better results than controlled atmosphere (e.g. 5%CO_2+3%O_2). However, LPS conditions are difficult to use and very expensive. Low temperature, 0 ºC, is very good for blueberry storage as well as much cheaper and easy to use than the others e.g. CA or LPS conditions, so it could be recommended for practice (Borecka and Pliszka, 1985).

Polylactide containers are viable packages in the commercial postharvest for the packaging of small berries because of their capability to enhance fruit shelf life as well as to reduce packaging waste. The effectiveness of polylactide containers in retarding shriveling and loss of plumpness is dependent on temperature (the lower the temperature, the higher the effect of the polylactide container). Oxygen levels inside the polylactide packages decreased as temperature increased, indicating that the respiratory activity of blueberries increased more rapidly than O_2 transmission rate through the container. Gas composition showed temperature dependence although the temperature dependence for blueberries is not as strong as it is for other fruits (Almenar et al., 2008).

Giuggioli et al. (2016) studied blueberries packaged using different films (one commercial polypropylene macroperforated film (6-mm holes) (F1) (Trepack, Italy, 25 µm) and three non-commercial biodegradable and compostable films that were non-perforated (prototypes, Novamont, Italy) (F2 of 25 µm, F3 of 15 µm and F4 of 25 µm)). These authors found that F3 was the only one able to maintain a stable antioxidant activity and the highest anthocyanin content for the entire storage time assayed (18 days).

1.3.2.3. Ethylene treatments

Ethylene, a 2-carbon volatile compound, is produced endogenously by plants. At concentrations as low as 0.1 μL/L, ethylene can induce a wide array of physiological responses, including ripening, senescence, and physiological disorders. These responses can be beneficial or detrimental, depending upon the response and one's need (Watada, 1986). Ethylene affects the genes that encode L-phenylalanine ammonia-lyase (PAL) and 4-coumarate: CoA ligase (enzymes of the phenylpropanoid pathway), the gene encoding chalcone synthase (an enzyme of the flavonoid glycoside pathway), and the genes encoding hydroxyproline-rich glycoprotein (a major component of plant cell walls) (Ecker and Davis, 1987). PAL catalyzes the first step in the biosynthesis of phenylpropanoids, which are further modified into a wide variety of phenolic compounds (Cheng and Breen, 1991).

The UDP glucose-flavonoid 3-O-glucoslyltransferase (UFGT) is a key enzyme for biosynthesis and stability of anthocyanin pigments of red grapes. Sequence analysis revealed seven putative ethylene-responsive cis-elements and others that are related to the three major signals known to induce anthocyanin accumulation in plant tissues: light, sugar, and abscisic acid (Chervin et al., 2009). The ethylene treatment in Grape (*Vitis vinifera* L. cv. Cabernet Sauvignon) led to increased levels of chalcone synthase (CHS) and flavonoid 3' hydroxylase (F3′H) transcription (which persisted over the following 20 days) and enhanced the transcription levels of leucoanthocyanidin dioxygenase (LDOX) and UDP glucose-flavonoid 3-O-glucosyltransferase (UFGT) although to a lesser extent. Elevated levels of CHS, F3′H and UFGT mRNAs were observed in berries during bunch exposure to ethylene, with the alterations of each transcript being observed within the first 6 h of treatment. Also the levels of the anthocyanins (delphinidin, cyanidin, petunidin, peonidin and malvidin) increased during 10 days following the ethylene burst, decreasing thereafter. A stimulation of UFGT activity, following exposure to ethylene, may therefore result in a rapid accumulation of anthocyanins from the pool of precursors that, otherwise, would be directed to the biosynthesis of flavanols and condensed tannins. This would mean an increase in flux through the flavonoid biosynthetic pathway, as observed by the increased transcription of CHS and F3′H, following ethylene treatment (El-Kereamy et al., 2003).

Some authors reported blueberries as climacteric fruits and that respond to ethylene, but they should be harvested nearly fully ripe as the flavor does not improve after harvest and that the removal of ethylene from storage air may reduce disease development (Mitcham et al., 2007). However, blueberry has been claimed as a non-climacteric fruit (Ehlenfeldt, 2002; Owusu-Apenten, 2005; Pallardy, 2008; Zifkin et al., 2012).

Currently, there have been several studies that assess the effect of ethylene upon non-climateric fruits as strawberries and grapes (El-Kereamy et al., 2003, Villarreal et al., 2010). Eyhylene could stimulate anthocyanin accumulation and the degradation of chlorophylls in strawberries. Moreover, the anthocyanin and total sugar content increased, PAL and β-galactosidase activities were upregulated, while chlorophyll production, endo-1,4-β-glucanase and β-xylosidase activities were downregulated by ethylene. While the reducing sugar content, pH, titratable acidity and α-L-arabinofuranosidase activity did not affect significantly (Villarreal et al., 2010).

Watada (1986) indicated that ethephon hastens the ripening of blueberry fruit, but it is used on a very small percentage of the crops to aid the last harvest of the season. In cranberry fruit Craker and Wetherbee (1973) observed that ethylene treatment promoted the synthesis of anthocyanin, when compared to the untreated controls.

1.3.2.4. 1-MCP treatments

Treatment with 1-Methycyclopropene (1-MCP) delayed red colouration (Palapol et al., 2009) and resulted in a significant decrease in alcohol dehydrogenase (ADH) activity and VvADH2 mRNA expression in ripening grapevine berries. The fact that VvADH2 transcription is partially reduced after veraison by the 1-MCP treatment indicates that the *ADH* gene expression is, at least in part, responsive to ethylene (Tesniere et al., 2004). The 1-MCP treatment also resulted in a significant increase in *VvEIN3* gene transcription and to, a lesser extent, the same trend was observed for the *VvMADS4* gene. This result indicated that a blockade of the ethylene receptors had a positive effect on VvEIN3 transcript accumulation (Chervin and Deluc, 2010).

On the other hand, 1-MCP inhibited UFGT mRNA accumulation confirming that the ethylene signal is a likely regulator of grape (a non-climacteric fruit) UFGT expression (Chervin et al., 2009).

In ‘Lateblue’ blueberries the 1-MCP treatment (0.3 and 0.6 μL) had no significant effect on anthocyanins, phenolics or antioxidant contents of this fruit (Chiabrando and Giacalone, 2011).

1.3.2.5. UV-B / UV-C treatments

Ultraviolet (UV) radiation has been used to extend the shelf life of several fresh fruits and vegetables. It has been applied to fresh produce in longwave (UV-A), medium (UV-B) and short wave (UV-C) wavelengths. UV-A and UV-B radiations are present in atmospheric ultraviolet light of the atmosphere while UV-C is absorved before reaching the earth’s surface (Perkins-Veazie et al., 2008).

UV-B application in blueberry ‘Bluecrop’ during postharvest affected the formation of the phenolic compounds. UV-B exposure resulted in an increase in phenolic compounds

(0.150 Wh/m^2 and considering an adaptation time of 2 h). The formation of these compounds might be regarded as a rapid, physiological response against UV radiation in postharvest (Eichholz et al., 2011).

Postharvest application of UV-C radiation can decrease decay caused by ripe rot in blueberries and enhance the antioxidant levels. Stimulation of antioxidants by UV-C radiation appears to be dependent on cultivar, storage, and light treatment. For instance, in 'Bluecrop' UV-C increased total anthocyanin but not in 'Collins' blueberries (Perkins-Veazie et al., 2008).

1.3.2.6. Others treatments

There are several other postharvest treatments that can be applied: allyl isothiocyanate, MeJa, abscisic acid, hexanal, carvacrol, anethole, cinnamaldehyde, cinnamic acid, perillaldehyde, linalool and *p*-cymene.

The treatment of blueberries with allyl isothiocyanate did not cause an increase of the amounts of phenolic compounds, anthocyanins, antioxidant activity but led to a reduction of decay (Wang et al., 2010).

The postharvest application of MeJa in raspberry fruits (*Rubus* spp) led to an increase in antioxidant activity and total anthocyanins content (Ghasemnezhad and Javaherdashti, 2008). In strawberries it also induced an enhancement of the concentration of total phenolics and anthocyanins as well as antioxidant capacity, colour and a variety of important aroma substances. This could be beneficial if the fruits were consumed close to the treatment. It is important to avoid long storage because, longer storage times (up to 11 days after treatment) resulted in a considerable decline of both anthocyanins and volatile compounds that might be detrimental to fruit quality (Moreno et al., 2010). Methyl jasmonate (MeJa) treatment promoted in 'Blueray' blueberries an increase in pigments as well as in the antioxidant potential, especially in fully ripe berries, but not induced in treated ‘Duke’ (Cocetta et al., 2015).

Abscisic acid treatments resulted in greater red colour development, accumulation of anthocyanin, phenolics, and a softening of strawberry fruit as well as an enhancement in ethylene production and PAL (Jiang and Joyce, 2003). In blueberry, abscisic acid also regulated flavonoid biosynthesis, with a correlation being observed between abscisic acid metabolism and anthocyanin accumulation (Zifkin et al., 2012).

Abscisic acid, in litchi, is suggested to play a crucial role in anthocyanin synthesis, while ethylene is more important in chlorophyll degradation (Wang et al., 2007).

The postharvest application of hexanal vapor in highbush blueberry fruits (*Vaccinium corymbosum* ‘Duke’, ‘Brigitta’ and ‘Burlington’) has been reported to reduce fruit decay, maintain fruit quality and extend storage life. It has potential to be used as an alternative fungicide to reduce postharvest decay in highbush blueberry fruit (Song et al., 2010), although heat treatments using hot water or air also can reduce decay and spoilage during storage by killing pathogens or altering the physiology of the product (Forney, 2009). For example, hot water treatments (60 º C for 15 or 30 s) in ‘Burlington’ blueberries reduced decay incidence, weight loss, induced an increase in ethanol and reduced fruit titratable acidity and soluble solids content, but had no significant effect on fruit firmness, pH, or most flavor volatile concentrations (Fan et al., 2008).

Several naturally occurring essential oils (including carvacrol, anethole and perillaldehyde) showed the capability to promote anthocyanins and phenolics accumulation and enhance antioxidant activity in ‘Duke’ blueberries tissues. More specifically, the essential oils: carvacrol, anethole, cinnamaldehyde, cinnamic acid, perillaldehyde, linalool, and *p*-cymene led to an increased of the levels of chlorogenic acid, malvidin 3-galactoside (except linalool and *p*-cymene), petunidin 3-galactoside, delphinidin 3-galactoside, petunidin 3-glucoside, petunidin 3-arabinoside, delphinidin 3-arabinoside and cyanidin 3-galactoside (Wang et al., 2008).

1.4. Opportunities for modulation of blueberry phytochemicals

There is an increasing interest in developing anthocyanin-rich functional foods. To accomplish this purpose, the identification of transcription factors and a better understanding of the regulatory network controlling anthocyanin biosynthesis in crop plants is a crucial prerequisite (Petroni and Tonelli, 2011).

The increase in antioxidant activity through postharvest phenolic synthesis and metabolism observed in some studies suggested that, commercially feasible technologies may be developed to enhance the health functionality of small fruit crops. Overall, the effect of storage practices upon fruit phytochemicals and antioxidant capacity suggests that storage at ambient or above ambient temperatures will positively affect phenolic metabolism to enhance the antioxidant activity and, therefore, the health promoting potential of some fruit crops, although these storage practices may not be commercially practical. The antioxidant activity of blueberry fruits may increase during storage at low temperatures (5 °C) (Connor et al., 2002b). Other treatments, which stimulate phenolic production (e.g., UV irradiation, ozonation), may be explored for commercial storage when aiming to improve the health quality of fruit (Kalt et al., 1999).

Production at higher altitudes and under increased levels of UV radiation (Jaakola and Hohtola, 2010) promote phenolic compounds. Thus the production under the influence of UV-A or UV-B light can induce the accumulation of anthocyanins (Guo et al., 2008). The postharvest application UV-B (Eichholz et al., 2011) UV-C radiation (Perkins-Veazie et al., 2008) resulted in an increase of antioxidant activity and phenolic compounds. As latitude increases the concentration of anthocyanins (Akerström et al., 2010), with long days and low night temperatures favoring the biosynthesis of flavonoids in plants (Jaakola and Hohtola, 2010). Plants subject to water stress have a lower antioxidant activity (Ehret et al., 2015; Lobos et al., 2018).

Fruit skin has a higher antioxidant activity than flesh tissue (Wang et al., 2012a). In this sense, small fruits with a higher surface area / volume ratio also have higher levels of phenolic compounds (Cho et al., 2005; Connor et al., 2002b; Howard et al., 2003). Moreover, as different cultivars have distinct sizes as well as anthocyanins, total phenolic compounds and antioxidant contents, therefore cultivar selection could also play an important role towards the development of added value blueberries (Ehlenfeldt and Prior, 2001).

Storage under high oxygen (60-100%) atmospheres may also improve the antioxidant activity (Zheng et al., 2003).

Craker and Wetherbee (1973) observed that ethylene treatment promoted the synthesis of anthocyanins; therefore it could also be used to improve the fruit phytonutrient profile.

The postharvest application of MeJa in raspberry fruits (*Rubus* spp) (Ghasemnezhad and Javaherdashti, 2008) and the abscisic acid (Wang et al., 2007) increased antioxidant capacity and total anthocyanins content, so it is possible that their use in blueberries also exhibit a positive impact. Essential oils carvacrol, anethole, and perillaldehyde showed the ability to promote total anthocyanins content and total phenolics and enhance the antioxidant activity of blueberries (Wang et al., 2008).

1.5. Conclusion

Knowledge of the importance of antioxidants in health has increased the relevance of producing fruit and vegetable products rich in these compounds. Blueberry antioxidant activity is strongly correlated with its phenolics and total anthocyanins content. Many factors /treatments can maintain or increase the content of these compounds. The environmental conditions to which the blueberries grow can affect the antioxidant activity. Very high temperatures can inhibit the biosynthesis of flavonoids and promote their degradation, and low temperatures promote the production of flavonoids, for example temperature of 18 ºC pormove the production of anthocyanins. The blueberry exhibits a C3 photosynthetic metabolism, the use of CO_2, can lead to higher photosynthetic rates, which permit the plant to have amount of fixed carbon available for secondary metabolites. A production subject to greater insolation can lead to a higher formation of phenolic compounds and the concentration of anthocyanins.

Although the location of the production influences the antioxidant activity has less effect on this activity than for example the cultivar. Thus different cultivars show a great variation in total anthocyanins content, total phenolic compounds and antioxidant activity. For example one characteristic of the cultivar may be the size of the fruit and the skin tissue has a higher antioxidant activity relative to the flesh tissue. Thus cultivars with small fruits present higher levels of total phenomena and antioxidant activity.

Relatively postharvest factors a storage temperature at 5 ° C may increase antioxidant activity in several cultivars, while storage at controlled atmosphere seems dependent on the cultivar. The application of ethylene increased the anthocyanins in different non-climacteric fruits.

In the future we will be able to study the effect of the application of other plant hormones (brassinosteroids and salicylic acid) or other compounds such as: aloe vera gel, eugenol, thymol, colloidal silver solution, oxalic acid on the antioxidant activity of blueberries or other related fruits such as promegranate, cherries and strawberries and evaluate the positive results.

CHAPTER 2- Genotype, gene expression and metabolic pathways associated with blueberry (*Vaccinium*) phenolic compounds biosynthesis: a review

This chapter is in preparation for submission in the journal: Biologia Plantarum

2.1. Abstract

Consumers are becoming more and more interested in their diet and it's association with health. Antioxidant enriched foods are a good example of this. The high antioxidant activity of fruits and vegetables is frequently correlated with their phenolic compounds content. Blueberries are an example of a fruit rich in these compounds. There are several factors that can affect the pathways that lead to the production and/or to the accumulation of phenolic compounds. The ripness stage, cultivar, application of UV-B, 1-MCP, methyljasmonate and CO_2 concentration, are some of the factors that can affect the genes expression and, consequently, the metabolic pathway of phenolics. With this review we intend to characterize how genetic factors can influence phenolics content and consequently, provide the tools to support selection towards the improvement of blueberries functionally.

2.2. Introduction

Fruits and vegetables contain many different phytonutrients, many of which have antioxidant properties (Prior et al., 1998). Although plants synthesize antioxidants to be used in their own defense against oxidative stress, some are present in sufficient quantities to make a significant contribution to consumer's health (Kalt et al., 2001), particularly as dietary antioxidants. Consumption of berries, and blueberries in particular, has become popular among health-conscious consumers due to the high levels of antioxidants, such as anthocyanins and other phenolic compounds (You et al., 2011). The antioxidant potential of fruits and vegetables is generally attributed to their content of antioxidant constituents such as ascorbate, glutathione, carotenoids, tocopherols and phenolics (Kalt et al., 2001; Łata et al., 2005; Wang, 2006).

These compounds have been reported to play an important role in preventing some chronic diseases (Connor et al., 2002b; Ehlenfeldt and Prior, 2001; Prior et al., 1998), that may result from free radical scavengers, peroxide decomposers, singlet and triplet oxygen quenchers, enzyme inhibitors, and synergists (Wang, 2006) hence protecting lipids, proteins and nucleic acids against oxidative damage. Epidemiological studies and associated meta-analyses strongly suggest that long term consumption of diets rich in plant polyphenols offer protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Pandey and Rizvi, 2009), arthritis and gout-related pain (Seeram et al., 2001).

The two most common classes of phenolics found in blueberries are flavonoids and non-flavonoids. Among the non-flavonoid components, the hydroxycinnamate esters (especially chlorogenic acid) are predominant in many small fruit species, with other non-flavonoid phenolics including hydroxycinnamic acids and simple phenolics. Out of all blueberry flavonoids subclasses, anthocyanins are probably the best known since they are the pigments responsible for red, purple, and blue colour to fruit. Regardeless, other small fruit flavonoids can be found. They include flavonols (and their glycosides), catechins (flavan monomers), and proanthocyanidins (condensed tannins) (Kalt et al., 2001).

Since ancient times, plant preparations have been used to treat common health problems (Sellappan et al., 2002). Phytochemical profiling of berries and other foods provides a semicomprehensive analysis of the antioxidant components contained within the fruits and thus it may allow for a better understanding of their potential nutritional health benefits. Moreover an understanding of it's variation in accordance to several intrinsic and extrinsic factors, like the plant's cultivar may allow for the manipulation of the fruits bioactive potential. Moreover the diversity of polyphenolic profiles between different berry species may explain the differences in biological activity observed and the different relations found with the reduction of the risk of heart disease, specific forms of cancer, and other chronic diseases (Taruscio et al., 2004). The consumption of 1/2 cup of blueberries/day (72.5 g) would increase antioxidant intake by 1-3.2 mmol, depending upon the blueberry variety and maturity. Thus it is important to characterize the beneficial phytonutrients present in these foods and the mechanisms responsible for these effects (Prior et al., 1998).

Considering the above made arguments, the present review aimes to provide some insight into the metabolic pathways and the genetic conditionings associated with the production and accumulation of phenolics and antioxidants in blueberries in an attempt to better understand how those factors can be manipulated to attain fruits with improved phytochemical composition that could possess an increased perceived commercial value.

2.3. Phenolic compounds

These compounds may be classified into different groups according to their number of phenol rings and of the structural elements that bind these rings to one another. Distinctions can thus be made between phenolic acids, flavonoids, stilbenes, and lignans (Figure 2.1.) (Manach et al., 2004).

There are six common hydroxicinnamic acids: cinnamic, *p*-coumaric, caffeic, ferulic, hydroxyferulic and sinapic acids. Chlorogenic acid is an ester of caffeic acid and quinic acid (Vermerris and Nicholson, 2008).

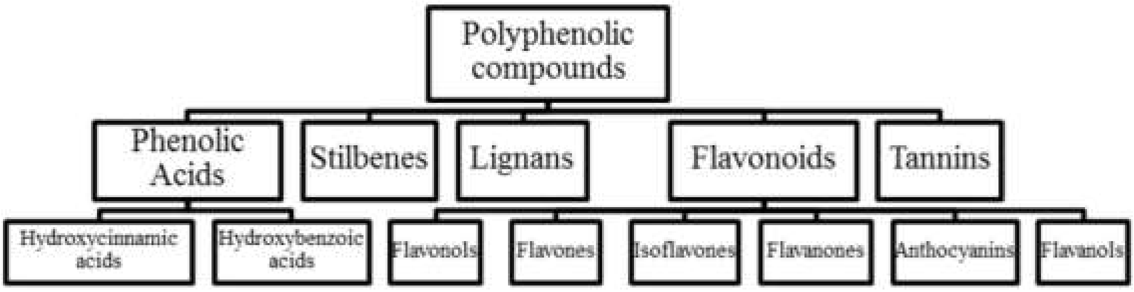

Figure 2.1. – Classification of Polyphenols according to Vermerris and Nicholson (2008).

Flavonoids represent a large class of secondary plant metabolites, of which anthocyanins are one of the most conspicuous class compounds (Table 2.1.), due to their wide range of colours (Holton and Cornish, 1995). In fact anthocyanins represent the major red, purple, violet and blue pigments found in many flowers and fruits (Petroni and Tonelli, 2011), and they play an away of biological functions. Anthocyanins are important to many diverse functions within plants. Synthesis of anthocyanins in petals is undoubtedly intended to attract pollinators, whereas anthocyanin synthesis in seeds and fruits may aid in seed dispersal. Anthocyanins, along with other classes of flavonoids, can function as feeding deterrents, grant protection against damage from UV irradiation (Holton and Cornish, 1995), abiotic and biotic stresses.

The precursors for the synthesis of all flavonoids, including anthocyanins, are malonyl-COA and *p*-coumaroyl-COA. Chalcone synthase (CHS) catalyzes the stepwise condensation of the three acetate units from malonyl-COA with *p*-coumaroyl-COA to yield tetrahydroxychalcone. Chalcone isomerase (CHI) then catalyzes the stereospecific isornerization of the yellow-colored tetrahydroxychalcone to the colorless naringenin, that is converted to dihydrokaempferol (DHK) by flavanone 3-hydroxylase (FHT). DHK can subsequently be hydroxylated by flavonoid 3'-hydroxylase (F3'H) to produce dihydroquercetin (DHQ) or by flavonoid 3;5'-hydroxylase (F3'5'H) to produce dihydromyricetin (DHM). F33'H can also convert DHQ to DHM. At least three enzymatic steps are required for converting the colorless dihydroflavonols (DHK, DHQ, and DHM) to anthocyanins. The first is the reduction of the dihydroflavonols to flavan9,4-cis-diols (leucoanthocyanidins) by dihydroflavonol 4-reductase (DFR). Further oxidation, dehydration, and glycosylation of the different leucoanthocyanidins produce the corresponding brick-red pelargonidin, red cyanidin, and blue delphinidin pigments. Anthocyanidin 3-glucosides may be modified further through glycosylation, methylation, and acylation. There are both species and variety differences in the extent of modification and the types of glycosides and acyl groups attached (Holton and Cornish, 1995).

Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge (Table 2.1.). One of the best studied, naturally occurring stilbene is resveratrol (3,4',5-trihydroxystilbene) (Pandey and Rizvi, 2009).

Lignans are phenylpropanoid dimers, where the phenylpropane units are linked by the central carbon (C8) of their side chains. Lignans vary substantially in oxidation level, substitution pattern, and the chemical structure of their basic carbon framework. Typical lignans can be classified into 12 subgroups based on their basic structures and oxidation levels at C9 (C9') (Umezawa, 2003).

The antioxidant activity of phenolic compounds varies according to their structure, in particularly considering the number and positions of the hydroxyl groups as well as the nature of substitutions of the aromatic rings (Balasundram et al., 2006). Based on the Trolox Equivalent Antioxidant Capacity (TEAC), the Ferric Reducing Antioxidant Power (FRAP) and Hypochlorite scavenging data, the observed antioxidant capacity's order was:

procyanidin dimer > flavanol > flavonol > hydroxycinnamic acids > simple phenolic acids (Soobrattee et al., 2005).

Table 2.1. summarizes the existing polyphenols, their structure, their food source and main identified properties.

Table 2.1. – Phenolic compounds, structure, source and main associated proprieties.

Phenolic Compounds	Subclass	Examples	Struture	Source	Biological Proprieties	References
Phenolic acids	Hydroxybenzoic acid	-Benzoic acid -Gallic acid -Vanillic acid -Salicylic acid -Syringic acid -Protocatechuic acid	C6-C1	*Achillea millefolium* *Acorus calamus* *Archangelica officinalis* *Aristolochia mollissima* *Artemisia vulgaris* *Carum carvi* *Echinacea purpurea* *Glycyrrhiza glabra* *Herniara glebra* *Humulus lupulus* *Hypericum perforatum* *Inula helenium* *Juglans regia* *Levisticum officinale* *Marrubium vulgare* *Melisa officinalis* *Origanum vulgare* *Paeonia suffruticosa* *Petroselinum sativum* *Rhus chinensis* *Rosmarinus officinalis* *Rubus chingii* *Salvia officinalis* *Silybum marianum*	Anticancer Antioxidant	Ghasemzadeh and Ghasemzadeh, 2011 Cai et al., 2004 Wojdyło et al., 2007
					Antimicrobial	Cueva et al., 2010; Kang et al., 2008
					Antibiofilm	Luís et al., 2014; Semiz et al., 2018; Silva et al., 2016
					Anti-inflamatory	Kroes et al., 1992; Liu et al., 2013

Table 2.1. (Continued)

				Tanacetum vulgare *Taraxacum officinale* *Thymus vulgaris*		
	Cinnamic acids	-*p*-Coumaric acid -Caffeic acid -Ferulic acid -Sinapic acid -Hydroxyferulic acid -Chlorogenic acid		*Xanthium sibiricum* *Fagopyrum cymosum* *Cimicifuga dahurica* *Salvia officinalis*	Anticancer Antioxidant	Ghasemzadeh and Ghasemzadeh, 2011 Cai et al., 2004 Wojdyło et al., 2007
				Marrubium vulgare *Silybum marianum* *Taraxacum officinale* *Petroselinum sativum*	Antihyperglycemic Reducing intensity of diabetes	Balasubashini et al., 2004
				Echinacea purpurea *Acorus calamus*	Antimicrobial	Wen et al., 2003
			C6-C3	*Humulus lupulus* *Herniara glebra* *Glycyrrhiza glabra* *Hypericum perforatum* *Juglans regia* *Thymus vulgaris* *Epilobium hirsutum* *Polygonum aviculare* *Valeriana officinalis* *Chelidonium majus* *Curcuma longa*	Antibiofilm	Borges et al., 2012; Luís et al., 2014; Silva et al., 2016

Table 2.1. (Continued)

Flavonoids	Flavonols	-(+)-catechin -(-)-epicatchin -Epigallocatechin gallate -Catechin gallate -Epicatechin gallate -Epigallocatechin -Gallocatechin -Gallocatechin gallate	$C_6{-}C_3{-}C_6$	Apple Blackberries Blueberries Cherries Cranberries Peach Plum Pecans Almonds Onions Kale, Broccoli Japonese green tea (*Camellia sinensis*) Red wine Diplotaxis harra	Anticarcinogenic Cardiovascular Diseases Antioxidant Antibacterial: -Escherichia colli, -Bacillus cereus Antiviral: - A/H1N1, - A/H3N2 - Hepatitis B virus (HBV) Antifungal: - Candida albicans Anti-allergic Anti-inflamatory	Harnly et al., 2006 Hollman and Katan, 1999 Isogai et al., 1998; Friedman et al., 2006 Song et al., 2005 Xu et al., 2008 Betts et al., 2013 Uehara et al., 2001 Nasri et al., 2017
	Flavones	-Chrysin -Apigenin -Rutin -Luteolin -Luteolin glucosides		Grape Kiwi Melons Lettuce red leaf Parsley Thyme	Anticarcinogenic Cardiovascular Diseases Antioxidant	Harnly et al., 2006 Hollman and Katan, 1999
	Isoflavones	-Genistin -Genistein -Daidzin -Daidzein		Soya beans Legumes Soyben derived		
	Flavanones	-Naringin -Naringenin -Taxifolin -Eriodictyol -Hesperidin		Grape Strawberries Citrus		Harnly et al., 2006

Table 2.1. (Continued)

	Anthocyanidins	-Cyanidin -Pelargonidin -Delphinidin -Petunidin -Malvidin -Peonidin		Blueberries Cherries Nectarines Peaches Raspberries Strawberries	Anti-allergic	Harnly et al., 2006 Jin et al., 2012
	Flavanols	-Kaempferol -Quercetin -Myricetin -Tamarixetin		Apple Blackberries Blueberries Cranberries Strawberries Brocolli Tomato Lettuce red leaf	Anti-allergic	Harnly et al., 2006 Joskova et al., 2013
Stilbenes		-Resveratrol -Pterostilbene -Piceatannol	C_6–C_2–C_6	Red wine Itadori tea Lowbush blueberry Sparkleberry Rabbiteye blueberry highbush blueberry Elliott's blueberry Cranberry Bilberry Deerberry Lingonberry Partridgeberry	Anticarcinogenic Antitumor Neuroprotective Anti-aging Anti-angiogenic	Burns et al., 2002 Bastianetto et al., 2009 Kasiotis et al., 2013 Rimando et al., 2004

Table 2.1. (Continued)

Lignans	-Furofuran -Furan -Dibenzylbutane -Dibenzylbutyrolactone -Aryltetralin -Arylnaphthalene -Dibenzocyclooctadiene -Dibenzylbutyrolactol -Secoisolariciresinol -Diglucoside -Pinoresinol -Lariciresinol -Matairesinol -Hydroxymatairesinol	$(C_6–C_3)_2$	Brassica vegetables Broccoli White cabbage Brussels sprout Apricot Strawberry Pear Flax seed Sesame seed Sunflower seed	Anti-inflammatory Anticarcinogenic Cardiovascular Disease	During et al., 2012 Milder et al., 2005
Tanins	-Catechol -Pyrogallol -Tanic acid	$(C_6–C_3–C_6)n$	Almonds *Caryocar brasiliense* *Citrus sinenses* Cherries Grapes Green/Black Tea (*Camellia sinensis*) *Ficus racemosa* Persimmons *Rubus ideaus* *Salix* sp. *Schinopsis brasiliense* Strawberries *Vaccinium myrtillus* Walnuts	Antimicrobial Cardio-protective Anti-cancer Anti-nutritional Antioxidative Antitumor Antibacterial Antiviral Anti-inflammatory	Ribeiro et al., 2018 Smeriglio et al., 2017 Velayutham et al., 2012 Shetty et al., 2016

2.4. Blueberry cultivar

The genetic background of the plant is the main determinant of the content of phenolic compounds in plant tissues (Zoratti et al., 2014a). Genetic factors result in direct differences in antioxidant activity, whereas external factors can only cause qualitative or quantitative changes in the composition of these compounds. Martinussen et al. (2010) showed that, when grown under identical climatic conditions, clones of bilberries originating from northern latitudes were better adapted to lower temperatures and longer days than clones originating from southern regions, therefore confirming that the differences in adaptation and metabolites had a genetic origin. Moreover, in genotypes of different *Vaccinium* species, the variation in antioxidant activity, total phenolics and anthocyanin content was greater than the variation between growing seasons and locations (Connor et al., 2002b; Howard et al., 2003). In fact, the differences in the concentration of the different compounds seemed to have a strong genetic basis with only weak environmental influence. The genetic diversity of plant species may be utilized, when selecting genotypes for cultivation. In this way it will promote the use of genetic variation in crop improvement to select superior genotypes for cultivation (Debnath, 2009).

The quantity and quality of antioxidants present in fruits particularly blueberry could be improved by the selective breeding plants of favourable phenotypes (Connor et al., 2002b; Ehlenfeldt and Prior, 2001; Wang, 2006). The success of breeding depends on identifying the genetic variation for the trait, and the availability of knowledge regarding the impact of environmental parameters on gene expression (Connor et al., 2002b). Several studies have reported the effect of different genotype upon the phenolic content and antioxidant capacity of bilberry (Taruscio et al., 2004), blackberry (Cho et al., 2005) and blueberries (Howard et al., 2003; Łata et al., 2005; Perkins-Veazie et al., 2008).

Different blueberries species have showed differences in scavenging capacity of reactive oxygen species and antioxidant capacity (Cho et al., 2005; Wang, 2006). In fact, genera, species, and genotypes have been reported to exhibited differences in their phenolic content (Kalt et al., 2001) for example, *Vaccinium corymbosum* L (Highbush), *Vaccinium ashei* Reade (Rabbiteye), *Vaccinium angustifolium* (Lowbush), and *Vaccinium myrtillus* L (Bilberry) presented significantly different levels of total phenolics, anthocyanins, and antioxidant capacity (Prior et al., 1998).

Moreover, the potential quality of fruit is dependent on the cultivar, with different cultivars possessing different quality parameters, mainly differences in size, colour, texture, flavor as well as storage potential (Forney, 2009).

The antioxidant capacity and the total anthocyanins content of blueberries have been reported as possessing a significant linear relationship (Prior et al., 1998; Taruscio et al., 2004; You et al., 2011). Some cultivars showed similar anthocyanin profiles, the proportions of each compound were reported to be cultivar dependent (Lohachoompol et al., 2008). In Table 2.2. we can observe different phenolic compounds identified in different cultivars of highbush *Vaccinium corybosum*.

In general, the genetic background determines the content of phenolic compounds in plant whereas external factors, like temperature, light conditions and soil nutrition, can cause qualitative or quantitative changes in the composition of these compounds (Koes et al., 2005).

Table 2.2. - Identified phenolic compounds in different blueberries highbush (*Vaccinium corymbosum*) cultivars.

Cultivar	Phenolic Compounds	References
Augusta	myricetin 3-galactoside, myricetin 3-glucoside, myricetin 3-pentoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, laricitrin 3-galactoside, laricitrin 3-glucoside, quercetin 3-pentoside, laricitrin 3-pentoside, kaempferol 3-rutinoside, kaempferol 3-glucoside, quercetin 3-glucoside acetate, syringetin 3-galactoside, isorhamnetin 3-galactoside, syringetin 3-glucoside, isorhamnetin 3-glucoside, syringetin 3-pentoside, myricetin 3-rhamnoside, quercetin 3-rhamnoside, laricitrin 3-rhamnoside	Vrhovsek et al., 2012
	delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, petunidin 3-galactoside, petunidin 3-glucoside, petunidin 3-arabinoside, malvidin 3-arabinoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside	Cho et al., 2004; Skrede et al., 2000
	myricetin 3-galactoside, myricetin 3-glucoside, myricetin 3-pentoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, laricitrin 3-galactoside, laricitrin 3-glucoside, quercetin 3-pentoside, laricitrin 3-pentoside, kaempferol 3-rutinoside, kaempferol 3-glucoside, quercetin 3-glucoside acetate, syringetin 3-galactoside, isorhamnetin 3-galactoside, syringetin 3-glucoside, isorhamnetin 3-glucoside, syringetin 3-pentoside, myricetin 3-rhamnoside, quercetin 3-rhamnoside, laricitrin 3-rhamnoside	Vrhovsek et al., 2012
Bluecrop	caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, p-hydroxybenzoic, cyanidin, delphinidin, malvidin, peonidin, petunidin, catechin, epicatechin, myricetin, quercetin	Taruscio et al., 2004
	cyanidin 3-glucoside, cyanidin 3-arabinoside, peonidin 3-galactoside, peonidin 3-arabinoside, malvidin 3-arabinoside, delphinidin 3-acetylglucoside, petunidin 3-acetylglucoside, malvidin 3-acetylglucoside, myricetin 3-rhamnoside, quercetin 3-glucoside rutinoside, quercetin 3-acetylrhamnoside	Cho et al., 2004
	myricetin 3-hexoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-methoxyhexoside, quercetin 3-glucoside, quercetin 3-glucuronide, quercetin 3-glucosylpentoside, quercetin 3-caffeoylglucoside, quercetin 3-rhamnoside, quercetin 3-dimethoxyrhamnoside,quercetin 3-acetylgalactoside, quercetin 3-acetylglucoside, quercetin 3-caffeoylgalactoside	Cho et al., 2005
	peonidin 3-glucoside	Giovanelli and Buratti, 2009
Bluegold	delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-glucoside, Petunidin 3-galactoside, cyanidin 3-arabinoside, peonidin 3-galactoside, petunidin 3-arabinoside, malvidin 3-arabinoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside	Bunea et al., 2013
Bluejay	caffeic acid, chlorogenic acid, *p*-coumaricacid, ferulic acid, *p*-hydroxybenzoic acid, cyanidin, delphinidin, malvidin, peonidin, Petunidin,	Taruscio et al., 2004

Table 2.2. (Continued)

	catechin, epicatechin, myricetin, quercetin	
Brigitta blue	myricetin 3-galactoside, myricetin 3-glucoside, myricetin 3-pentoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, laricitrin 3-galactoside, laricitrin 3-glucoside, quercetin 3-pentoside, laricitrin 3-pentoside, kaempferol 3-rutinoside, kaempferol 3-glucoside, quercetin 3-glucoside acetate, syringetin 3-galactoside, isorhamnetin 3-galactoside, syringetin 3-glucoside, isorhamnetin 3-glucoside, syringetin 3-pentoside, myricetin 3-O-rhamnoside, quercetin 3-rhamnoside, laricitrin 3-rhamnoside, syringetin 3-rhamnoside	Vrhovsek et al., 2012
Chandler	myricetin 3-galactoside, myricetin 3-glucoside, myricetin 3-pentoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, laricitrin 3-galactoside, laricitrin 3-glucoside, quercetin 3-pentoside, laricitrin 3-pentoside, kaempferol 3-rutinoside, kaempferol 3-glucoside, quercetin 3-glucoside acetate, syringetin 3-galactoside, isorhamnetin 3-galactoside, syringetin 3-glucoside, isorhamnetin 3-glucoside, syringetin 3-pentoside, myricetin 3-O-rhamnoside, quercetin 3-rhamnoside, laricitrin 3-rhamnoside, syringetin 3-rhamnoside, isorhamnetin 3-rhamnoside	Vrhovsek et al., 2012
Coville	delphinidin 3-monogalactoside, delphinidin 3-monoglucoside, cyanidin 3-monogalactoside, delphinidin 3-monoarabinoside, cyanidin 3-monoglucoside, petunidin 3-monogalactoside, cyanidin 3-monoarabinoside, petunidin 3-monoglucoside, peonidin 3-monogalactoside, petunidin 3-monoarabinoside, peonidin 3-monoglucoside, peonidin 3-monoarabinoside, malvidin 3-monogalactoside, malvidin 3-monoglucoside, malvidin 3-arabinoside	Kader et al., 1996
Crunchie	delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-glucoside, petunidin 3-galactoside, cyanidin 3-arabinoside, petunidin 3-glucoside, peonidin 3-galactoside, petunidin 3-arabinoside, peonidin 3-glucoside, peonidin 3-arabinoside, malvidin 3-arabinoside	Lohachoompol et al., 2008
Darrow	delphinidin 3-galactoside, delphinidin 3-glucoside, delphinidin 3-arabinoside, cyanidin 3-glucoside, petunidin 3-galactoside, cyanidin 3-arabinoside, petunidin 3-arabinoside, malvidin 3-arabinoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside peonidin 3-galactoside, peonidin 3-glucoside, peonidin 3-arabinoside	Bunea et al., 2013 Giovanelli and Buratti, 2009
	delphinidin 3-galactoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, petunidin 3-galactoside, petunidin 3-glucoside, petunidin 3-arabinoside, malvidin 3-arabinoside, malvidin-3-O-galactoside	Wang et al., 2009
Duke	myricetin 3-galactoside, myricetin 3-glucoside, myricetin 3-pentoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, laricitrin 3-galactoside, laricitrin 3-glucoside, quercetin 3-pentoside, laricitrin 3-pentoside, kaempferol 3-rutinoside, kaempferol 3-glucoside, quercetin 3-glucoside acetate, syringetin 3-galactoside,	Vrhovsek et al., 2012

Table 2.2. (Continued)

	isorhamnetin 3-galactoside, syringetin 3-glucoside, isorhamnetin 3-glucoside, syringetin 3-pentoside, myricetin 3-O-rhamnoside, quercetin 3-rhamnoside, laricitrin 3-rhamnoside, syringetin 3-rhamnoside, isorhamnetin 3-rhamnoside	
Elliot	myricetin 3-galactoside, myricetin 3-glucoside, myricetin 3-pentoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, laricitrin 3-galactoside, laricitrin 3-glucoside, quercetin 3-pentoside, laricitrin 3-pentoside, kaempferol 3-rutinoside, kaempferol 3-glucoside, quercetin 3-glucoside acetate, syringetin 3-galactoside, isorhamnetin 3-galactoside, syringetin 3-glucoside, isorhamnetin 3-glucoside, syringetin 3-pentoside, myricetin 3-O-rhamnoside, quercetin 3-rhamnoside, laricitrin 3-rhamnoside, syringetin 3-rhamnoside	Vrhovsek et al., 2012
FL 86-19	gallic acid, *p*-coumaric acid, ferulic acid, catechin, myricetin, quercetin, kaempferol	Sellappan et al., 2002
Goldtraube	delphinidin 3-galactoside, delphinidin 3-glucoside, delphinidin 3-arabinoside, cyanidin 3-galactoside, cyanidin 3-glucoside, petunidin 3-galactoside, cyanidin 3-arabinoside, petunidin 3-glucoside, peonidin 3-galactoside, peonidin 3-glucoside, peonidin 3-arabinoside, malvidin 3-arabinoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside	Giovanelli and Buratti, 2009
Hannah's Choice	delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-glucoside, petunidin 3-galactoside, cyanidin 3-arabinoside, peonidin 3-galactoside, petunidin 3-arabinoside, malvidin 3-arabinoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside	Bunea et al., 2013
Hardyblue	myricetin 3-galactoside, myricetin 3-glucoside, myricetin 3-pentoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, laricitrin 3-galactoside, laricitrin 3-glucoside, quercetin 3-pentoside, laricitrin 3-pentoside, kaempferol 3-rutinoside, kaempferol 3-glucoside, quercetin 3-glucoside acetate, syringetin 3-galactoside, isorhamnetin 3-galactoside, syringetin 3-glucoside, isorhamnetin 3-glucoside, syringetin 3-pentoside, myricetin 3-O-rhamnoside, quercetin 3-rhamnoside, laricitrin 3-rhamnoside, syringetin 3-rhamnoside, isorhamnetin 3-rhamnoside	Vrhovsek et al., 2012
Ivanhoe	cyanidin 3-glucoside, cyanidin 3-rutenoside, pelargonidin-3-glucoside, peonidin 3-glucoside	Koka and Karadeniz, 2009
Jersey	caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, p-hydroxybenzoic acid, cyanidin, delphinidin, malvidin, peonidin, petunidin, catechin, epicatechin, myricetin, quercetin	Taruscio et al., 2004
	delphinidin 3-galactoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, petunidin 3-galactoside, petunidin 3-glucoside, peonidin 3-galactoside, petunidin 3-arabinoside, malvidin 3-galactoside, malvidin 3-glucoside, malvidin 3-arabinoside, peonidin glucoside	Seeram et al., 2001
	cyanidin 3-glucoside, cyanidin 3-rutenoside, pelargonidin-3-glucoside	Koka and Karadeniz, 2009

Table 2.2. (Continued)

Kuopio	quercetin, myricetin, caffeic acid	Häkkinen and Törrönen, 2000
Legacy	delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-glucoside, petunidin 3-galactoside, cyanidin 3-arabinoside, peonidin 3-galactoside, petunidin 3-arabinoside, malvidin 3-arabinoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside	Bunea et al., 2013
	myricetin 3-galactoside, myricetin 3-glucoside, myricetin 3-pentoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, laricitrin 3-galactoside, laricitrin 3-glucoside, quercetin 3-pentoside, laricitrin 3-pentoside, kaempferol 3-rutinoside, kaempferol 3-glucoside, quercetin 3-glucoside acetate, syringetin 3-galactoside, isorhamnetin 3-galactoside, syringetin 3-glucoside, isorhamnetin 3-glucoside, syringetin 3-pentoside, myricetin 3-O-rhamnoside, quercetin 3-rhamnoside, laricitrin 3-rhamnoside, syringetin 3-rhamnoside	Vrhovsek et al., 2012
Nelson	delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-glucoside, petunidin 3-galactoside, cyanidin 3-arabinoside, peonidin 3-galactoside, petunidin 3-arabinoside, malvidin 3-arabinoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside	Bunea et al., 2013
Northland	cyanidin 3-glucoside, cyanidin 3-rutenoside, pelargonidin-3-glucoside, peonidin 3-glucoside	Koka and Karadeniz, 2009
Nui	delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-glucoside, petunidin 3-galactoside, cyanidin 3-arabinoside, peonidin 3-galactoside, petunidin 3-arabinoside, malvidin 3-arabinoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside	Bunea et al., 2013
Ozarkblue	delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-glucoside, petunidin 3-galactoside, cyanidin 3-arabinoside, petunidin 3-glucoside, peonidin 3-galactoside, petunidin 3-arabinoside, malvidin 3-galactoside, malvidin 3-glucoside, peonidin 3-arabinoside, malvidin 3-arabinoside, delphinidin 3-acetylglucoside, myricetin 3-galactoside, myricetin 3-glucoside, myricetin 3-O-rhamnoside, quercetin 3-galactoside, quercetin 3-glucoside rutinoside, quercetin 3-xyloside, quercetin 3-acetylrhamnoside	Cho et al., 2004
	myricetin 3-hexoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-methoxyhexoside, quercetin 3-glucoside, quercetin 3-glucosylpentoside, quercetin 3-caffeoylglucoside, quercetin 3-rhamnoside, quercetin 3-dimethoxyrhamnoside, quercetin 3-acetylgalactoside, quercetin 3-acetylglucoside, quercetin 3-pentoside	Cho et al., 2005
Patriot	cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, delphinidin 3-galactoside, delphinidin 3-glucoside, delphinidin 3-arabinoside, peonidin 3-galactoside, peonidin 3-glucoside, peonidin 3-arabinoside, petunidin 3-galactoside, petunidin 3-glucoside, petunidin 3-arabinoside, malvidin 3-arabinoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside,	Ochmian et al., 2010

Table 2.2. (Continued)

	quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-ramnoside, kaempferol 3-rutinoside	
Piikkiö	quercetin, myricetin, caffeic acid	Häkkinen and Törrönen, 2000
Rekord	cyanidin 3-glucoside, cyanidin 3-rutenoside, pelargonidin-3-glucoside, peonidin 3-glucoside	Koka and Karadeniz, 2009
Sharpblue	gallic acid, caffeic acid, *p*-coumaric acid, ellagic acid, catechin, quercetin, kaempferol	Sellappan et al., 2002
Sharpe	delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, petunidin 3-galactoside, cyanidin 3-arabinoside, petunidin 3-glucoside, peonidin 3-galactoside, petunidin 3-arabinoside, peonidin 3-glucoside, peonidin 3-arabinoside, malvidin 3-arabinoside	Lohachoompol et al., 2008
Sierra	delphinidin 3-galactoside, delphinidin 3-glucoside, delphinidin 3-glucoside, cyanidin 3-galactoside, cyanidin 3-glucoside, petunidin 3-galactoside, petunidin 3-glucoside, petunidin 3-arabinoside, malvidin 3-galactoside, malvidin 3-glucoside, malvidin 3-arabinoside myricetin 3-arabinoside, quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-arabinoside, quercetin derivative, kaempferol 3-glucoside, kaempferol derivative, chlorogenic acid	Zheng and Wang, 2003
Simultan	myricetin 3-galactoside, myricetin 3-glucoside, myricetin 3-pentoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, laricitrin 3-galactoside, laricitrin 3-glucoside, quercetin 3-pentoside, laricitrin 3-pentoside, kaempferol 3-rutinoside, kaempferol 3-glucoside, quercetin 3-glucoside acetate, syringetin 3-galactoside, isorhamnetin 3-galactoside, syringetin 3-glucoside, isorhamnetin 3-glucoside, syringetin 3-pentoside, myricetin 3-O-rhamnoside, quercetin 3-rhamnoside, laricitrin 3-rhamnoside	Vrhovsek et al., 2012
Star	delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, petunidin 3-galactoside, cyanidin 3-arabinoside, peonidin 3-galactoside, petunidin 3-arabinoside, peonidin 3-glucoside, peonidin 3-arabinoside, malvidin 3-arabinoside	Lohachoompol et al., 2008
TH 161	gallic acid, *p*-coumaric acid, ferulic acid, ellagic acid, catechin, myricetin, quercetin, kaempferol	Sellappan et al., 2002
TH 440	gallic acid, *p*-coumaric acid, ferulic acid, ellagic acid, catechin, quercetin, kaempferol	Sellappan et al., 2002
TH 442	gallic acid, caffeic acid, *p*-coumaric acid, ellagic acid, catechin, myricetin, quercetin, kaempferol	Sellappan et al., 2002
Toro	delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-glucoside, petunidin 3-galactoside, cyanidin 3-arabinoside, peonidin 3-galactoside, petunidin 3-arabinoside, malvidin 3-arabinoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside	Bunea et al., 2013

2.5. Genotype and Gene Expression

The biosynthetic pathway encompassing the basis for the accumulation of flavonoids has been elucidated using genetic and biochemical information collected from many plant species namely in fruit crops, bilberry (*Vaccinium myrtillus*), blueberry (*Vaccinium corymbosum* L.), apple (*Malus domestica*), grapevine (*Vitis vinifera*) and nectarine (*Prunus persica*) (Bogs et al., 2005, 2006; Boss et al., 1996; Espley et al., 2007; Jaakola et al., 2002; Ravaglia et al., 2013; Takos et al., 2006; Zifkin et al., 2012).

The synthesis of proanthocyanidins, anthocyanins, and flavonols is known to be regulated by a network of transcription factors that operate as MYB-bHLH-WDR ternary protein complexes (Gupta et al., 2015; Zifkin et al., 2012).

MYBPA1-type R2R3 MYB transcription factor has been shown to play a role in the regulation of anthocyanin biosynthesis; for example in *Vaccinium uliginosum* MYBPA1-type R2R3 MYB transcription factor is down-regulated in white mutant berries, deficient in anthocyanins but not proanthocyanidins (Table 2.3.) (Primetta et al., 2015).

Vaccinium myrtillus has been reported to possess relatively high expression levels of structural genes *CHS*, chalcone isomerase (*CHI)*, *FHT*, *DFR*, leucoanthocyanidin reductase (*LAR)*, anthocyanidin reductase *(ANR)*, anthocyanidin synthase (*ANS)* and flavonoid 3-O-glycosyltransferase (*FGT)* of the flavonoid pathway and of the transcription factors MYBC2 and MYBPA1 (previously described as influencing anthocyanin accumulation in *Vaccinium*) (Zorenc et al., 2017). Zifkin et al. (2012) reported that, in highbush blueberry, the *VcCHS* and *VcFHT* genes encode enzymes at the entry point level of the flavonoid pathway that are necessary for the biosynthesis of all flavonoids, including proanthocyanidins, anthocyanins and flavonols.

A downregulation of the structural genes of the anthocyanin pathway, in *Vaccinium* has been reported to be correlated with a strong decrease in expression of the transcription factors VuMYBPA1 and VuMYBR3 and a moderate, but significantly, decrease in the expression of *VuTDR4* and *VuMYBC2*. VuMYBPA1 is a R2R3 MYB transcription factor with a presumed role in anthocyanin formation in *Vaccinium* species (Primetta et al., 2015).

Jaakola et al. (2010) reported that, in bilberry, the expression pattern and functional analysis of a SQUAMOSA-class MADS box transcription factor (VmTDR4) was associated with anthocyanin biosynthesis. In fact, the levels of *VmTDR4* expression were spatially and temporally linked with colour development and anthocyanin-related gene expression. Moreover, when a virus-induced gene silencing was amplayed to suppress this gene, it resulted in a substantial reduction of the anthocyanin levels of fully ripened fruits. Additionally, in the sectors of fruit tissue with the silenced *VmTDR4* gene, the expression of the R2R3 MYB family transcription factors, related to flavonoid biosynthesis, was also altered. Therefore *VmTDR4* plays an important role in controling anthocyanin biosynthesis, in bilberry, acting directly (or indirectly through MYB transcription factors) to control carbon flux through the phenylpropanoid pathway, possibly through the direct, or indirect, control of transcription factors belonging to the R2R3 MYB family, the known regulators of flavonoid biosynthesis (Jaakola et al., 2010).

There are different factors that may influence the genetic expression of phenolic biosynthesis related genes such as: light, ripening stage, controlled atmosphere with elevation of CO_2, 1-MCP and methyljasmonate.

2.6. Factors influencing gene expression

2.6.1. Light

The flavonoid composition of fruits is strongly affected by surrounding light conditions. In general, high levels of solar radiation tend to cause an increase in the flavonoid content of fruits. Moreover specific wave-lengths can also alter the flavonoid profile of fruit tissues. However, variations in response to solar radiation between and even within species, may vary and are frequently affected by other environmental factors that can also change the flavonoid profile. Zoratti et al. (2014b) reported on the light spectrum upon the flavonoid pathway genes in *Vaccinium myrtillus* leaves. They found that gene expression increased earlier in time under blue and far red light, when compared to red light. Moreover, the R2R3-MYB transcription factors were also expressed earlier than the flavonoid pathway genes, suggesting a regulatory effect of light upon the flavonoid biosynthesis.

In *Vaccinium myrtillus* the expression of *VmCHS*, *VmF3'5'H*, *VmDFR*, *VmANS* and *VmANR* genes from the flavonoid pathway, and the transcription factor VmMYB2 was measured during different monochromatic light treatments of immature berries. The majority of the genes expression was higher during the first 12 hours, in plants treated with monochromatic light than in plants kept in the dark or exposed to white light, even though the variation between samples and times was high. Neverheless, higher expression levels of *VmANS* (after 24 and 48 hours) were observed for plants under monochromatic light when compared to plants grown in the dark. However, white light exposure had no significant effect upon her expression. Monochromatic light continued to up-regulate the expression of *VmANS*, in comparasion will plants grown in the dark. The gene leads increased up to 3-, 2- and 3.5-folds under blue, red and far-red light treatments, respectively in, comparison with dark treated plants. On the other hand, under the light, the expression was only slightly increased (up to 1.3-fold) in comparison to dark grown plants. Overall monochromatic blue, red and far-red lights, when compared to white light or its absence, resulted in significantly higher total anthocyanin contents in ripe berries (Zoratti et al., 2014a).

Vaccinium corymbosum plants have been reported to respond differently to UV-B radiation depending on the UV-B resistance of cultivars (e.g. Legacy is UV-B resistant and Bluegold UV-B sensitive) in accordance with their physiological and biochemical features. Moreover different expression levels of flavonoid biosynthetic genes for both cultivars were transiently induced through UV-B exposure, showing that even after longer UV-B exposures; plants were still adjusting the phenylpropanoids associated transcription levels (Escobar et al., 2017).

The UV-B treatment of *Vaccinium corymbosum* fruits induced the expression of anthocyanin biosynthesis genes *VcPAL*, *VcCHS*, *VcF3'H*, *VcDFR*, and *VcUFGT*, thereby promoting the accumulation of individual anthocyanins, whose levels rise quickly after UV radiation (Nguyen et al., 2017).

2.6.2. Ripening stages

Flavonols and proanthocyanidins (PAs) are the main flavonoids present at the beginning of fruit development, with the accumulation of anthocyanin pigments often being an indicator of ripening (Jaakola, 2013). The ripening process has been reported to affect more the expression of genes related to flavonoid biosynthesis than other factors like tissue or cultivar type (Lin et al., 2018). The presence of proanthocyanidins, anthocyanins, and flavonols in the skin (exocarp) of ripe fruits suggests that this tissue is key for blueberry organoleptic and antioxidant properties as well as their general health-promoting benefits (Zifkin et al., 2012). In fact, anthocyanin synthesis occurs first in epidermal cell layers, after which the berries inner tissues also become pigmented (Jaakola et al., 2002).

The accumulation of polyphenols during fruit maturation and postharvest's correlation with the expression of genes involved in the biosynthetic pathway has been described in different species. Flavonoids biosynthesis is tightly regulated at gene level in developing blueberry fruits. The abundance of flavonoid transcript is followed by a tightly regulated biphasic pattern, with the transcript profiles being consistent with an abundance of the three major classes of flavonoids. Developmental profiles and localization studies emphasize that biosynthetic pathways are controlled in both time and space, suggesting important functions for their end products (Zifkin et al., 2012).

In bilberries, the higher contents of proanthocyanidins and quercetin are found at the early stages of fruit development. At the onset of ripening, the level of proanthocyanidins declines whereas quercetin remains at a constant, but, low level during the ripening phase (Jaakola et al., 2010). Proanthocyanidins (PAs) and corresponding biosynthetic transcripts that encode anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR), were reported to be more concentrated in young fruits and localized predominantly in the inner fruit tissue that encompasses the seeds and placentae. Proanthocyanidins are synthesized early in the fruit development and stored in specific tissues throughout development. Gene expression profiles for *VcDFR* and *VcANS* were elevated early in the development (S1 and S2), decreased to a minimum by mid fruit development (S5), and then increased again as the fruit matured (S6–S8) (Zifkin et al., 2012). Jaakola et al. (2002) also reported variation of CHS and DFR expression throughout ripening and a reduction at stages 2 and 3 compared with the later ripening stages. The expression of flavonoid pathway genes was the highest at stage 5, when the berry was still pale inside but with the skin already red. In ripe bilberries, the expression decreased again.

By contrast, the proanthocyanidins-specific *VcANR* and *VcLAR* genes showed a different behaviour, as their transcripts were detected only in early fruit development, stages S1 to S4. These differences imply that proanthocyanidins synthesis is most active in young fruit. VcMYBPA1 transcription was also high in very young stages, together with VcANR and VcLAR, supporting the idea of their role in proanthocyanidins synthesis.

However, after decreasing to minimal levels at stage 5, VcMYBPA1 transcripts levels increased in the subsequent stages (S6–S8), the period when anthocyanins are synthesized. In stage 7, VcUFGT, VcF3′H, and VcMYBPA1 transcripts were also nearly exclusive in the skin tissue, the site of anthocyanin-based coloration in ripening fruit. Flavonols accumulation and localization patterns were similar to those of the proanthocyanins, and the B-ring hydroxylation pattern of both was correlated with flavonoid-3′-hydroxylase transcript abundance. By contrast, anthocyanins accumulated later in maturation, which coincided with a peak in flavonoid-3-O-glycosyltransferase and flavonoid-3′5′-hydroxylase transcripts. Transcripts of VcMYBPA1, which likely encodes R2R3-MYB, a transcriptional regulator of proanthocyanidins synthesis, was proeminent in both phases of development (Table 2.3.) (Zifkin et al., 2012).

The mRNA levels encoding PAL, CHS, F3′H, DFR, and anthocyanidin synthase (ANS), in developing bilberries, increased proportionally with the accumulation of anthocyanins. In ripe berries, the expression levels started to drop again (Jaakola et al., 2002). Zorenc et al. (2017), when comparing ripe wild albino and blue bilberries (*Vaccinium myrtillus*) found that blue variant had the higher gene expression of flavonoid 3-Oglycosyltransferase (*FGT*), flavanone 3-hydroxylase (*FHT*), *ANS* and *CHS.* Studies conducted on blueberry showed that the *F3'5'H* gene was weakly expressed during the earliest ripening stages and was abundant only during later ripening stages, closely linked with the appearance of anthocyanins (Zifkin et al., 2012).

2.6.3. 1-MCP

In mangosteen fruits treatment with ethylene signaling inhibitor (1-MCP) resulted in a clear delay in red colour development, ethylene production and anthocyanin accumulation (Table 2.3.) (Palapol et al., 2009). 1-MCP application has been associated with the down-regulation of *GmMYB10* gene expression which would then affect transcription of the biosynthetic genes. The ethylene production and *GmMYB10* expression are thought to work closely to control the anthocyanin biosynthesis in mangosteen pigmentation. Moreover the application of 1-MCP to grapes (Chervin et al., 2004) and strawberries (Jiang et al., 2001) has resulted in similar results i.e. a delay in red coloration and anthocyanin content. Ethylene application induced both anthocyanins accumulation and an increase in internal ethylene concentration in grapes (when externally applied) by up-regulating anthocyanin biosynthetic genes (El-Kereamy et al., 2003). Similarly Tira-Umphon et al. (2007) reported that ethylene-induced anthocyanin production in grape berries and cell suspensions was dependent on *UFGT* and independent of *MYBA* expressions.

2.6.4. CO_2

The expression levels of chalcone synthase (*VcCHS*) and dihydroflavonol-4-reductase (*VcDFR*) were high at harvest time but declined, significantly and drastically, upon storage under very high CO_2 partial pressure levels. For both *VcCHS* and *VcDFR*, the decrease in the expression levels correlated strongly with the increasing partial pressure of CO_2. Therefore, high CO_2 levels seem to have a negative impact on the expression of several enzymes that are involved in flavonoid biosynthesis (Harb et al., 2014).

2.6.5. Methyl jasmonate

Methyl jasmonate is a phytohormone that plays a key role in plant growth and many physiological and biochemical processes (Wasternack and Hause, 2013). Cocetta et al. (2015) evaluated the changes in gene expression, accumulation of phenolic compounds and antioxidant capacity that occurred in response to MeJa (0.1mM) treatment, in the blueberry cultivars ‘Duke’ and ‘Blueray’. After 9 hours, the expression of phenylalanine ammonium lyase, chalcone synthase and anthocyanidin synthase genes was higher in the ‘Blueray’ cultivars coupled with higher antioxidant capacities, epicatechin and anthocyanin concentrations, particularly in ripe fruits. ‘Duke’ is typically richer in anthocyanins than ‘Blueray’, however MeJa treatment had no effect in ‘Duke’ blueberries despite the higher levels of compounds.

MeJa was, in some cases, an effective elicitor of phenolic metabolism and gene expression in blueberry, though with different intensity between cultivars (Cocetta et al., 2015).

Table 2.3. - Different effects of genes associated with flavonoids and anthocyanins biosynthesis

Effect	The transcripts of genes	Plant	Effect	Reference
Fruit Ripness	VcDFR VcANS	*Vaccinium corymbosum* 'Rubel'	Was high early in development (S1 and S2), decreased to a minimum by mid fruit development (S5), and then increased again as the fruit matured (S6–S8)	Zifkin et al., 2012
	VcANR VcLAR	*Vaccinium corymbosum* 'Rubel'	Were detected only early in fruit development, in stages S1 to S4, proanthocyanidins synthesis is most active in young fruit	Zifkin et al., 2012
	CHI DFR F3'H FLS CHS OMT UGT F3'H ANS	*Vaccinium corymbosum* 'Bluecrop' *Vaccinium angustifolium × V. corymbosum* 'Northblue'	Increase in the expression of these genes during fruit ripening is consistent with the dynamic variation in total anthocyanin content	Lin et al., 2018
	VmTDR4	*Vaccinium myrtillus*	Is associated with anthocyanin biosynthesis during ripness	Jaakola et al., 2010
	VcCHS VcFHT	*Vaccinium corymbosum* 'Rubel'	Are necessary for the biosynthesis of all flavonoids, including proanthocyanidins, anthocyanins, and flavonols	Zifkin et al., 2012
	CHS CHI F3'H DFR	*Vaccinium corymbosum* 'Sierra'	Increased with fruit coloring (stage 4) and reached the highest level at red fruit stage (stage 5), then slightly declined with fruit maturation (stage 5 and 6)	Li et al., 2016

Table 2.3. (Continued)

	ANS UFGT		There was a significant correlation between the expression profile of candidate genes and the accumulation of anthocyanins.	
Tissue	FLS1	*Prunus persica*	Correlated with flavonol levels, both temporally and in a tissue specific manner	Ravaglia et al., 2013
Mutation	VuCHS VuDFR VuANS	*Vaccinium uliginosum* L. *V. uliginosum* f. *leucocarpum*	Strong down-regulation of this structural genes in white berries during ripening compared to wild-type berries	Primetta et al., 2015
Monochromatic light	VmCHS, VmF3′5′H, VmDFR, VmANS and VmANR	*Vaccinium myrtillus*	Increase in their expression during the first 12 hours	(Zoratti et al., 2014a)
UV-B	VcPAL VcCHS VcF3′H VcDFR VcUFGT	*Vaccinium corymbosum* ‘Duke’	Increase in gene expression at 3 h after treatment	Nguyen et al., 2017
1-MCP	GmMYB10	*Garcinia mangostana* L.	Delayed red colouration with resulting down-regulation	Palapol et al., 2009
Ethylene	UFGT	*Vitis Vinifera*	Induced anthocyanin	Tira-Umphon et al., 2007

Table 2.3. (Continued)

Methyl jasmonate (0.1mM)	VmPAL VmCHS VmANS	*Vaccinium corymbosum* 'Blueray'	This expression was stimulated and epicatechin and anthocyanin concentrations increased, but in a cultivar dependent way Was, in some cases, an effective elicitor of phenolic metabolism and gene expression in blueberry, though with different intensities between cultivars.	Cocetta et al., 2015
High CO_2 (18 kPa)	VcCHS	*Vaccinium corymbosum* 'Duke'	Decreased this expression	Harb et al., 2014

2.7. Conclusion

Blueberries are fruits with a high antioxidant activity. This activity is correlated with the phenolic compounds content. Several factors can influence the genetic expression of phenolic compunds biosynthesis. These factors may be related to pre-harvest factors such as: cultivar, light or ripening stage, but also to postharvest factors such as the use of controlled atmosphere storage with elevation of CO_2, 1-MCP and MeJa. Consequently, it is important to manipulate these factors in order to increase phenolic compounds biosynthesis, if possible, and with a result in the antioxidant activity of these fruits. Along ripening, the use of UV-B light induced anthocyanin biosynthesis genes as: *VcPAL*, *VcCHS*, *VcF3'H*, *VcDFR* and *VcUFGT*, while a high concentration of CO_2 during storage adversely affected the expression of these genes (Harb et al., 2014).

In the future, it may be interesting to study how different environmental conditions (low or high temperatures), irrigation and different fertilizations (e.g. iron) affect the genetic expression in anthocyanin biosynthesis in blueberries. As well as some postharvest factors (ethylene, abcissic acid) that have been identified as having effect on anthocyanins production. There is the need to understand how these factors influence the gene expression in anthocyanin biosynthesis. By studying the influence of different factors on the expression of anthocyanin biosynthesis genes, it will be easier to assist producers and stockists in promoting and maintaining these compounds.

PART II - Study of pre-harvest factor

CHAPTER 3 - Effect of growing seasons on commercial and phytochemical quality in three blueberry cultivars (*Vaccinium corymbosum*)

This chapter is in preparation for submission in the journal: Journal of Berry Research

3.1. Abstract

Blueberrie fruits (*Vaccinium corymbosum*) of three different cultivars: 'Bluecrop', 'Goldtraube' and 'Ozarkblue', produced in the same locations in three consecutive years, were harvested and stored at 4 ° C, for 49 days in 2010 and 56 days in 2011 and 2012. During storage, commercial quality parameters (weight loss rate, soluble solids content, titratable acidity, firmness and colour), anthocyanins and total phenolic compounds content and antioxidant activity were determined and the identified anthocyanins and hydroxycinnamic acids quantified. Both factors, the cultivar and the production year, influenced the different parameters evaluated. We have verified that different environmental production location can affect the antioxidant activity, the anthocyanins and total phenolic compounds content, as well as the sugar content and titratable acidity. More than 51% of differences were explained by the relation between main component 1 vs. main component 2 (PC1 vs PC2). Compounds such as cyanidin 3-O-galactoside and peonidin 3-O-galactoside appeared linked to antioxidant capacity. Whereas malvidin 3-O-galactoside, dephinidin 3-O-galactoside appeared linked to total anthocyanins content and cultivar. The production year was linked to cyanidin 3-O-glucoside, ferulic acid, chlorogenic acid and total phenolic compounds.

3.2. Introduction

Blueberries consumption reduces degenerative damage caused by free radicals, increases the natural killer cells and inflammatory cytokines, and provides evidence for cellular antioxidant defense against DNA damage, playing also an important role in the anti-cancer nutritional preventive treatment (Pavlidou et al., 2018). At present, there is an increasing concern about the population's diet, with an increase in the demand for foods with high antioxidant activity. Blueberry is a reported as a fruit with a high antioxidant activity (Prior et al., 1998). However, this parameter in blueberry varies with different pre and postharvest conditions.

Wang (2006) reported that pre-harvest conditions such as climate, temperature, light intensity, soil type, compost, mulching, fertilization, increasing CO_2 concentration in the atmosphere, can affect the antioxidant compounds content and antioxidant activity of the harvested fruits. For instance, Kähkönen et al. (2001) reported it is probable that wild berries grown in the cool northern climate, under a short growing season, and without fertilizers, pesticides, or herbicides have high phenolic contents compared to cultivated berries that grow in a warm climate, in fertilized soil, and protected against plant diseases and insects with pesticides and herbicides. Plants growing in cold climates maintain higher photosynthetic rates than plants growing in warmer areas. Therefore they can thus increase the amount of fixed carbon available for secondary metabolites (Jaakola and Hohtola, 2010).

Plants growing under direct sun exposure increase the expression of the flavonoid pathway genes in leaves (*Vaccinium myrtillus* L.). The concentrations of anthocyanins, catechins, flavonols and hydroxycinnamic acids were higher in leaves exposed to direct sunlight (Jaakola et al., 2004). Krüger and Josuttis (2014) also report that besides genotype, which is the main factor determining the concentration of the phytochemicals, diverse growing conditions can influence the amount of the bioactive compounds present in blueberries.

Connor et al. (2002b); Howard et al. (2003); Wang et al. (2012b) found that antioxidant capacity of several highbush, interspecific hybrid blueberry and rabbiteye cultivars grown at three locations varied considerably over two growing seasons.

Howard et al. (2003) found that, in general, genotypes with smaller berries had higher oxygen radical-absorbing capacity values and levels of total phenolic compounds, total anthocyanins content, total hydroxycinnamic acids and total flavonols contents than large-berried genotypes. Over both growing seasons, antioxidant activity correlated highly with phenolic compounds, anthocyanins, hydroxycinnamic acids and total flavonols, while fruit weight correlated inversely with all phenolic compounds measured. Their results indicate that blueberry genotypes should be screened over multiple growing seasons in order to identify antioxidant- and phenolic compounds-rich germplasm. However, Concenço et al. (2014) reported that blueberry is rich in anthocyanins, being its content more affected by genotype than by climatic or environmental factors.

This work aimed to characterize how different environmental conditions can influence overall quality of blueberry fruit. The commercial and phytochemical quality of three blueberry cultivars were characterized over three different years.

3.3. Material and Methods

3.3.1. Fruit storage

Blueberry fruits (*Vaccinium corymbosum*) of cultivars ‘Bluecrop’, ‘Goldtraube’ and ‘Ozarkblue’ were harvested at the commercial maturation stage (full blue) in the Sever do Vouga region in Portugal, cooled to a pulp temperature of 4 °C, packed in ventilated PET (250 g) containers and stored at 4 °C and 70-80% RH, for 49 days in 2010 and 56 days in

2011 and 2012. The fruits were obtained from the same plants in the three years. Texturally the soil was coarse-grained and the organic matter content was 12 %.

3.3.2. Weather characterization

The year 2010, in Portugal, registered an average temperature of 15.42 ºC. It should also be noted that in 2010 the coldest months (January, February, March, October, November and December) registered negative anomalies of maximum, average and minimum air temperature (except the minimum January temperature) and the hottest months (April to September), recorded positive anomalies of maximum, average and minimum air temperature (except the minimum May temperature). In relation to the amount of precipitation in the Continent, the year 2010 was the rainiest of the previous decade (2001-2010). The first and fourth quarters of the year contributed most to the high annual rainfall value, since in the remaining months precipitation values were always lower than the normal values (IMIP, 2011).

With an average annual temperature of 16.02 ºC, the year 2011 in Portugal was among the 7 hottest of the last 80 years, with the maximum annual temperature 21.70 ºC. The months that most contributed to the year 2011 being one of the hottest years in relation to the maximum temperature were April, October, May, June and September. It should also be noted that the months of May and October were the hottest since 1931 in relation to the value of the maximum air temperature and April was the second hottest in the average and maximum air temperature, also since 1931. In 2011, 5 waves of heat that occurred in the months of April, May and October. 2011 was also a year with insolation values above normal. In relation to the total annual precipitation, it is verified that it was inferior to the normal value 1971-2000, with a difference of -132.2mm (IMIP, 2012).

The year 2012 in Portugal was characterized by a meteorological drought situation, which began at the end of 2011 and remained for most of the year. The greatest intensity of the drought situation occurred in late winter and early spring. In the year 2012 a total annual precipitation value of 636 mm was verified. In this year 4 heat waves occurred, in the months of March, May and September, and a cold wave in February (IMIP, 2013).

3.3.3. Weight loss

The average weight loss of fruits was determined by individually weighing 10 fruits with a balance ABJ 120 - 4M, (Kern, Balingen, Germany). The weight loss was registered weekly (0, 7, 14, 21, 28, 35, 42, 49 (2010) and 56 d (2011 and 2012)).

3.3.4. Soluble solids content

The soluble solids content was determined in the juice of 20 fruits, after homogenization (IKA T25, Ultra Turrax, Ika-Labortechnik Staufen, Germany) and centrifugation at 3864 ×g (Universal centrifuge 320 R, Tuttingen, Germany), using a digital manual refractometer (Atago PR 32 Palette, Japan) (Castrejón et al., 2008; Prior et al., 1998).

Three replicates were generated for each treatment and for each of sampling point throughout storage time at 0, 7, 14, 21, 28, 35, 42, 49 (2010) and 56 d (2011 and 2012).

3.3.5. Total acidity

Determination of total acidity was achieved by titration conducted with 0.25 N NaOH until pH 8.1 (Eutech instruments Ecoscan Handheld pH ECPH 502 Plusk) and was expressed as percent of total organic acid on basis of malic acid. For determination of the titratable acidity samples of 20 fruits were used, which were homogenized (IKA T25, Ultra

Turrax, Ika-Labortechnik Staufen, Germany). Three grams of the blueberry homogenate were suspended in 15 mL of distilled water prior to titration (Castrejón et al., 2008).

Three replicates were generated for each treatment and for each of sampling point throughout storage time at 0, 7, 14, 21, 28, 35, 42, 49 (2010) and 56 d (2011 and 2012).

3.3.6. Firmness

Firmness was measured by puncture with a texture analyser (Stable Micro Systems, TA XT plus). The probe diameter was 2 mm with a travel distance of 5 mm and 1 mm s^{-1} test speed. The puncture was conducted in the equatorial region. Thirty replicate measurements were obtained to estimate the mean values. Results were expressed as N mm^{-1}. The firmness was performed weekly (0, 7, 14, 21, 28, 35, 42, 49 (2010) and 56 d (2011 and 2012)).

3.3.7. Skin colour

To determine the colour of the skin, a Minolta CR-300 colorimeter (Osaka, Japan) equipped with a D65 illuminant was used. Data were obtained in the Commision Internationale de L'Eclairage (CIE) L* a* b* space and were converted to hue (h°) and chroma (C*) using the relationships described by McGuire (1992). Chroma ($C^* = [(a^*)^2 + (b^*)^2]$ ½), hue angle [$h^o = \tan^{-1} (b^*)/(a^*)$], metric-hue ($\Delta H^* = [(\Delta a^*)^2 + (\Delta b^*)^2 - (\Delta C^*)^2]$ ½), and the total colour difference ($\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]$ ½) between the initial and subsequent readings were computed and are reported. Colour readings were performed in ten fruit, two measurements in equatorial region of each fruit. Samples were collected weekly during storage (0, 7, 14, 21, 28, 35, 42, 49 (2010) and 56 d (2011 and 2012)).

3.3.8. Extracts preparation

Extracts were obtained by homogenizing 5 g of fruit with an Ultra Turrax (IKA Ultra Turrax T25, Germany) at 24000 rpm for 2 min, in 50 ml of methanol (Merck; Darmstadt, Germany) acidified with 0.01 % (v/v) HCl (Merck; Darmstadt, Germany). The homogenates were stored under stirring at room temperature, in the dark, for 12 h to promote extraction. Later, the extracts were centrifuged (Hettich Zentrifugen 320R Universal; Tuttingen, Germany) at 4000xg for 10 min, at 15 °C. To extract all anthocyanins, at least three extractions with 25 ml of acidified methanol and 0.01 % hydrochloric acid 32 % with agitation were performed, to assure the removal of all anthocyanic related colour. Finally, the extracts were centrifuged at 4000xg, for 10 min at 15 °C. The final extract was stored at -80 °C until analysis. This methodology conforms to that used by Kalt et al. (1999), with minor modifications.

Three replicates were generated for each treatment and for each of sampling points throughout storage time at 0, 28 and 49 (2010) or 56 d (2011 and 2012).

3.3.9. Total anthocyanin contents

The determination of anthocyanins was executed essentially as described by (Giusti and Wrolstad, 2001). A pH differential method was carried out determining the absorbance at 520 and 700 nm in potassium chloride (KCl) buffer (Merck, Darmstadt, Germany) at pH 1.0 and sodium acetate ($CH_3CONa_3H_2O$) (Merck, Darmstadt, Germany) at pH 4.5. Absorbance was measured with a Shimadzu UV-1240 miniature spectrophotometer (UV-mini-1240, Shimadzu, Tokyo, Japan). The total anthocyanin content was calculated using the following equation: $A = [(A520 - A700)_{pH1.0} - (A520 - A700)_{pH4.5}]$ with molar extinction coefficients of cyanidin 3-glucoside ($\varepsilon = 29600$). The results were expressed as milligram equivalents for cyanidin 3-glucoside per kg fruit.

Three replicates were generated for each treatment and for each of sampling points throughout storage time at 0, 28 and 49 (2010) or 56 d (2011 and 2012).

3.3.10. Total phenolic compounds

To determine the total phenolic compounds content, the method reported by Slinkard & Singleton (1977) with the Folin-Ciocalteu reagent was used. Quantification at 750 nm was achieved using a spectrophotometer (UV-mini-1240, Shimadzu, Tokyo, Japan). The results were expressed as milligrams of gallic acid equivalent per kg fruit. All determinations were executed in triplicate. Samples were collected at days 0, 28 and 49 (2010) or 56 (2011 and 2012) of storage.

3.3.11. Antioxidant activity

The antioxidant activity was determined according the methodology described by Gião et al. (2007), the $ABTS\bullet^{+}$ radical cation colourless method. The results were expressed as milligrams of ascorbic acid equivalent per kg of fruit. Ascorbic acid was used as a standard and was used to prepare a calibration curve in the range of 0.02 to 0.50 $mg.ml^{-1}$. All measurements were performed in triplicate. Samples were collected at days 0, 28 and 49 (2010) or 56 d (2011 and 2012) of storage.

3.3.12. HPLC-DAD analysis

Qualitative and quantitative profiles of anthocyanins and hydroxycinnamic acids (namely chlorogenic acid and ferulic acid) were determined by HPLC-DAD (Waters Series 600, Mildford MA, USA). This method was adapted from Silva et al. (2013b). Separation was performed in a reverse phase Symmetry® C18 column (250 x 4.6 mm i.d., 5 µm particle size and 125 Å pore size). Methanolic extracts were injected (40 µl) and chromatographic separation of phenolic compounds was carried out with solvent A –

water, formic acid and methanol (92.5:2.5:5 v/v/v) (pH1.9) and solvent B – water, formic acid and methanol (50:25:25 v/v/v) (pH1) with the following profile: 0 min, 100% A and 0% B; 60 min, 40% A and 60% B; 65 min, 90% A and 10%B; and 70 min, 100% A and 0%B. The flow rate was 0.65 ml.min^{-1} and detection was achieved by a diode array detector (Waters, Milford, MA, USA) at wavelengths ranging from 200 to 600 nm. Absorbance was measured at 320 nm (hydroxycinnamic acid) and 510 nm (anthocyanins). Analysis was carried out in triplicate on each extract and performed for each condition analysed.

Pure standards used for quantification of cyanidin-3-arabinoside, cyanidin-3-galactoside, delphinidin-3-glucoside, malvidin-3-galactoside and malvidin-3-glucoside were obtained from Extrasynthese (Lyon, France). Cyanidin-3-glucoside, peonidin-3-galactoside, ferulic acid and chlorogenic acid were obtained from Sigma-Aldrich Chemic GmbH (Buchs, Switzerland). Delphinidin-3-galactoside was obtained from AppliChem GmbH (Darmstadt, Germany). Petunidin-3-glucoside was obtained from (Poliphenols Laboratories (Sandnes, Norway). They were expressed as g.kg^{-1} of fruit. The identification of the compounds was established by comparison of retention times and absorption spectra of compounds with pure standards. Quantification was carried out using calibration curves (Annex 1.) prepared in a standard solution of pure compounds considering a detection limit of 2.5 mg.100 g^{-1} of fresh blueberries.

3.3.13. Statistical analysis

The data were subjected to analysis of variance according to a randomized block design with three replicates. The Tukey's test was used to test differences between storage treatments and storage days. Differences at $p \leq 0.05$ were considered to be significant. The association between colour measurement and qualitative or quantitative profiles was calculated using an analysis of factorial components (PCA). All analyses were performed with the statistical software SPSS 21.0 for Windows (SPSS, Chicago, USA).

3.4. Results and Discussion

3.4.1. Effect of growing season on weight loss rate, skin colour, firmness, total acidity and soluble solids content.

The rate of water loss was only influenced by the year of production. In specific days for each cultivar. On day 7 of storage for ‘Bluecrop’ (3.1.a.). Day 7 and 42 (Figure 3.1.b.) for ‘Goldtraube’. And day 14 (Figure 3.1.c.) on ‘Ozarkblue’. For the cultivar Bluecrop, the years 2010 and 2012 led to lower rate of water loss. While at ‘Goldtraube’ the rate of water loss was lower on day 7 in 2010 and day 42 in 2010 and 2011. While the lowest rate of weight loss for ‘Ozarkblue’ was in the year 2012.

In ‘Bluecrop’ the average rate of weight loss was 0,216; 0,197 and 0,173 % day^{-1} for 2010, 2011 and 2012, respectively. In ‘Goldtraube’ the average rate of weight loss was 0,214, 0,261 and 0,378 % day^{-1} for 2010, 2011 and 2012 respectively. In ‘Ozarkblue’ the average rate of weight loss was 0,160; 0,150 % and 0,210 day^{-1} for 2010, 2011 and 2012 respectively. The maximum limit of loss of water for commercialization is 5 to 8% (Nunes et al., 2004) and in this study, this value was only reached around 28 days of storage.

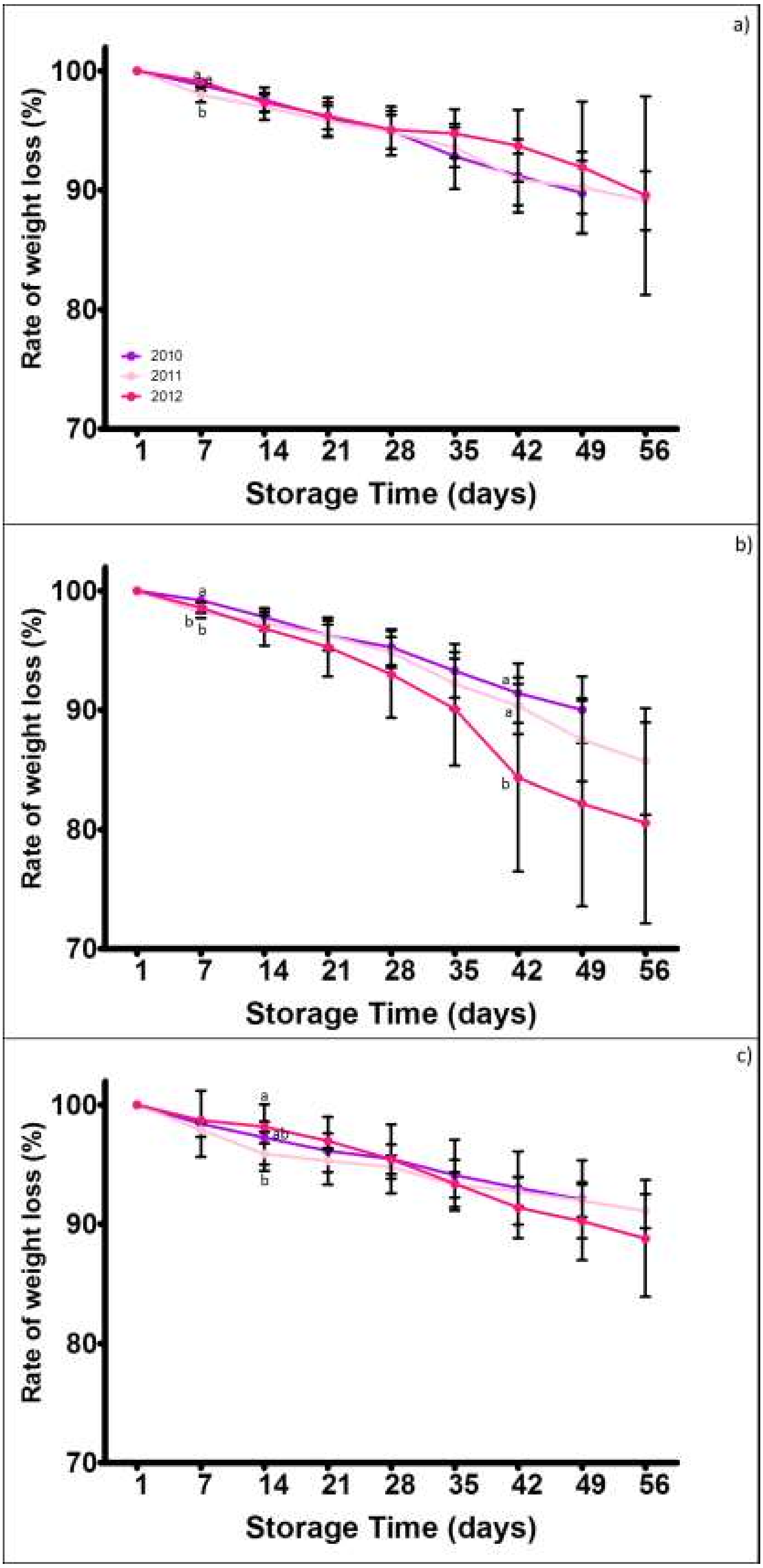

Figure 3.1. - Effect of year on the weight loss rate during storage at 4 ºC, on cultivar: a) Bluecrop, b) Goldtraube c) Ozarkblue. Results are expressed as mean ± SD (n=10). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

The colour parameters (L *, C *, h °) were significantly influenced by the year of production and by cultivar. During storage, the luminosity (L *) (Table 3.1.), tonality (h º) (Table 3.2.) and chroma parameters (Table 3.3.) remained relatively constant, with the 'Goldtraube' at 2010 presenting the lower values at the beginning of storage. In contrast to these results, Zheng et al. (2003) found a decrease in L * after harvest and an increase in the hue angle during storage of blueberries. The parameters L * and C * of the cultivars 'Goldtraube' and 'Ozarkblue' presented the highest values in the year of 2012 and the 'Bluecrop' in the year 2011. The parameter h º of the cultivars 'Bluecrop' and 'Goldtraube' obtained higher values in the year 2011 and 'Ozarkblue' in the year 2010.

Table 3.1. - Effect of year and cultivar in the colour parameter L*.

Day	**Cultivar**	**Year**		
		2010	**2011**	**2012**
1	**Bluecrop**	27,25±2,64^{cd}	29,60±1,63^{a}	29,27±1,82^{ab}
	Goldtraube	26,43±1,39^{d}	27,51±2,26^{bcd}	28,70±1,13^{abc}
	Ozarkblue	25,54±2,56^{d}	30,00±1,90^{a}	30,29±2,03^{a}
7	**Bluecrop**	29,27±1,52^{bc}	29,67±1,61^{ab}	31,49±1,67^{a}
	Goldtraube	27,09±1,53^{d}	27,58±2,50^{cd}	26,55±1,47^{d}
	Ozarkblue	30,56±2,70^{ab}	29,16±1,72^{bc}	29,70±1,33^{ab}
14	**Bluecrop**	27,70±1,53^{cd}	30,08±1,63^{ab}	31,37±1,36^{a}
	Goldtraube	25,74±1,31^{e}	29,37±2,07^{bc}	26,56±1,88^{de}
	Ozarkblue	27,76±2,91^{cd}	30,65±1,66^{ab}	30,40±1,25^{ab}
21	**Bluecrop**	25,94±1,67^{c}	30,41±1,80^{ab}	31,24±1,47^{a}
	Goldtraube	24,20±1,35^{d}	29, 90±2,34^{ab}	29,37±1,56^{b}
	Ozarkblue	27,35±1,67^{c}	29,50±1,97^{b}	30,04±1,47^{ab}
28	**Bluecrop**	27,15±1,95^{de}	30,21±1,11^{ab}	30,36±0,84^{ab}
	Goldtraube	26,37±1,16^{e}	29,50±2,16^{bc}	26,75±1,74^{e}
	Ozarkblue	28,50±1,87^{cd}	29,70±1,74^{bc}	31,55±1,27^{a}
35	**Bluecrop**	31,96±1,84^{a}	29,68±2,44^{bcd}	31,10±1,50^{ab}
	Goldtraube	28,70±2,42^{cd}	26,39±3,09^{e}	30,17±1,46^{abc}
	Ozarkblue	31,33±2,02^{ab}	27,94±1,73^{de}	31,10±0,62^{ab}
42	**Bluecrop**	31,63±1,49^{a}	29,23±1,38^{bc}	31,15±1,26^{ab}
	Goldtraube	29,34±1,88^{bc}	28,17±3,81^{c}	29,15±1,53^{c}
	Ozarkblue	31,65±1,61^{a}	29,24±2,04^{bc}	31,97±1,15^{a}
49	**Bluecrop**	31,44±1,83^{abc}	29,41±2,21^{cd}	32,46±1,61^{a}
	Goldtraube	28,02±1,11^{de}	27,00±3,66^{e}	31,69±1,47^{ab}
	Ozarkblue	31,13±2,91^{abc}	30,09±2,03^{bcd}	32,61±0,88^{a}
56	**Bluecrop**	-	29,25±1,65^{a}	30,19±0,90^{a}
	Goldtraube	-	26,23±2,57^{b}	29,76±1,78^{a}
	Ozarkblue	-	28,79±1,70^{a}	29,96±0,77^{a}

Results are expressed as mean ± SD (n=20). Different letters represent significant differences ($P < 0.05$) between year and cultivar for each storage day, by the Tukey test.

Table 3.2. - Effect of year and cultivar in the colour parameter h^{o}.

Day	Cultivar	Year		
		2010	2011	2012
1	**Bluecrop**	272,79±9,72^{bc}	286,15±14,35^{ab}	284,60±23,18^{ab}
	Goldtraube	269,25±15,42^{bc}	300,34±29,64^{a}	263,63±7,87^{c}
	Ozarkblue	298,45±26,98^{a}	280,45±7,94^{bc}	270,42±5,37^{bc}
7	**Bluecrop**	279,97±14,23^{bc}	285,66±11,95^{abc}	271,81±7,52^{c}
	Goldtraube	278,95±12,87^{bc}	299,89±30,16^{a}	275,05±12,03^{bc}
	Ozarkblue	275,86±10,07^{bc}	287,47±12,36^{ab}	272,52±10,74^{c}
14	**Bluecrop**	280,97±11,91^{a}	288,32±17,50^{a}	271,81±7,52^{ab}
	Goldtraube	276,23±9,29^{ab}	297,07±25,27^{a}	270,81±6,92^{b}
	Ozarkblue	285,72±19,95^{a}	277,97±9,78^{a}	279,01±9,48^{a}
21	**Bluecrop**	281,64±15,32^{a}	282,93±12,92^{a}	271,88±5,74^{ab}
	Goldtraube	273,88±9,86^{ab}	281,79±18,69^{a}	268,43±3,45^{b}
	Ozarkblue	282,21±18,39^{a}	282,66±13,21^{a}	281,55±9,36^{a}
28	**Bluecrop**	274,87±11,88^{ab}	283,26±9,90^{ab}	274,03±6,18^{ab}
	Goldtraube	271,61±11,52^{b}	287,34±24,98^{a}	276,74±11,09^{ab}
	Ozarkblue	276,83±16,79^{ab}	278,79±7,11^{ab}	277,65±11,48^{ab}
35	**Bluecrop**	279,55±11,09^{bc}	288,58±20,97^{ab}	277,50±16,71^{bc}
	Goldtraube	271,53±8,76^{c}	302,26±32,18^{a}	269,48±6,00^{c}
	Ozarkblue	270,99±6,84^{c}	287,69±11,82^{ab}	273,45±6,96^{bc}
42	**Bluecrop**	268,33±1,69^{b}	285,21±11,36^{ab}	273,18±6,85^{b}
	Goldtraube	271,24±6,92^{b}	299,17±33,03^{a}	280,36±13,55^{b}
	Ozarkblue	276,38±13,01^{b}	280,63±11,67^{b}	298,81±29,90^{a}
49	**Bluecrop**	269,58±2,72^{d}	287,39±15,90^{bc}	269,722±4,92^{d}
	Goldtraube	271,66±7,27^{d}	304,73±32,48^{a}	270,400±7,28^{d}
	Ozarkblue	274,53±8,65^{cd}	277,31±7,07^{cd}	296,414±25,23^{ab}
56	**Bluecrop**	-	282,67±11,51^{b}	281,03±12,44^{b}
	Goldtraube	-	319,64±28,95^{a}	277,99±12,99^{b}
	Ozarkblue	-	279,95±7,67^{b}	287,31±21,21^{b}

Results are expressed as mean ± SD (n=20). Different letters represent significant differences ($P < 0.05$) between year and cultivar for each storage day, by the Tukey test.

Table 3.3. - Effect of year and cultivar in the colour parameter C*.

Day	Cultivar	Year		
		2010	2011	2012
1	**Bluecrop**	3,44±1,10^{ab}	4,53±1,38^{a}	3,68±0,93^{ab}
	Goldtraube	1,72±0,81^{c}	3,07±1,68^{b}	3,13±0,59^{b}
	Ozarkblue	2,64±2,26^{bc}	4,46±1,36^{a}	4,48±0,75^{a}
7	**Bluecrop**	3,86±0,95^{abc}	4,18±1,41^{a}	4,53±0,72^{a}
	Goldtraube	2,62±0,75^{d}	3,01±1,52^{bcd}	2,85±0,65^{cd}
	Ozarkblue	3,93±1,34^{ab}	4,27±1,28^{a}	4,690±0,53^{a}
14	**Bluecrop**	3,05±0,92^{c}	4,33±1,34^{ab}	4,53±0,72^{a}
	Goldtraube	2,36±0,62^{c}	3,27±1,81^{bc}	3,06±0,75^{c}
	Ozarkblue	2,73±1,34^{c}	4,85±1,05^{a}	4,73±0,70^{a}
21	**Bluecrop**	2,32±0,90^{b}	4,46±1,40^{a}	4,90±0,67^{a}
	Goldtraube	1,43±0,59^{b}	4,17±2,12^{a}	3,91±0,64^{a}
	Ozarkblue	2,12±0,92^{b}	4,57±1,54^{a}	4,62±0,78^{a}
28	**Bluecrop**	2,59±0,95^{c}	4,33±1,19^{ab}	4,47±0,37^{ab}
	Goldtraube	1,54±0,65^{d}	4,08±1,87^{b}	2,94±0,60^{c}
	Ozarkblue	2,37±0,98^{cd}	4,46±1,25^{ab}	5,26±0,61^{a}
35	**Bluecrop**	4,19±1,22^{ab}	3,97±1,48^{ab}	4,71±0,62^{a}
	Goldtraube	2,46±1,31^{c}	3,41±1,53^{bc}	3,36±1,24^{bc}
	Ozarkblue	3,77±1,12^{ab}	3,82±1,34^{ab}	4,33±0,54^{ab}
42	**Bluecrop**	4,65±0,97^{ab}	4,20±1,08^{bc}	5,00±0,48^{ab}
	Goldtraube	2,59±0,97^{d}	4,89±1,92^{ab}	3,23±0,80^{cd}
	Ozarkblue	4,23±0,80^{bc}	4,64±1,03^{ab}	5,63±1,11^{a}
49	**Bluecrop**	4,27±1,07^{ab}	4,65±1,31^{ab}	4,72±0,79^{ab}
	Goldtraube	2,45±0,64^{b}	3,65±1,79^{b}	4,05±1,15^{ab}
	Ozarkblue	3,74±1,11^{ab}	4,76±1,19^{ab}	4,83±0,96^{a}
56	**Bluecrop**	-	4,58±1,18^{a}	4,01±0,43^{ab}
	Goldtraube	-	2,87±1,11^{c}	3,78±0,66^{ab}
	Ozarkblue	-	4,23±1,34^{a}	3,36±0,54^{bc}

Results are expressed as mean ± SD (n=20). Different letters represent significant differences ($P < 0.05$) between year and cultivar for each storage day, by the Tukey test.

The firmness (Table 3.4.) of the fruits was significantly influenced by the year of production and by the cultivar. It remained relatively constant throughout the storage in the three years. Overall, ‘Goldtraube’ in 2011, presented higher firmness values. The lower firmness values were obtained in 2012 which could be related to the drought conditions that characterized this year (IMIP, 2013).

Table 3.4. - Effect of year and cultivar in the firmness (N).

Day	Cultivar	Year		
		2010	2011	2012
1	**Bluecrop**	1,17±0,16^{c}	1,46±0,25^{ab}	1,17±0,20^{c}
	Goldtraube	1,49±0,28^{ab}	1,55±0,27^{a}	1,38±0,25^{abc}
	Ozarkblue	1,31±0,23^{bc}	1,33±0,37^{bc}	1,19±0,34^{c}
7	**Bluecrop**	1,22±0,20^{d}	1,60±0,24^{ab}	1,20±0,25^{d}
	Goldtraube	1,48±0,18^{bc}	1,78±0,25^{a}	1,38±0,29^{bcd}
	Ozarkblue	1,34±0,30^{cd}	1,17±0,45^{d}	1,27±0,34^{cd}
14	**Bluecrop**	1,35±0,23^{de}	1,85±0,28^{ab}	1,162±0,226^{e}
	Goldtraube	1,60±0,23^{bcd}	1,93±0,43^{a}	1,696±0,369^{abc}
	Ozarkblue	1,49±0,36^{cd}	1,53±0,46^{cd}	1,426±0,316^{de}
21	**Bluecrop**	1,46±0,24^{cd}	1,67±0,33^{abc}	1,16±0,25^{e}
	Goldtraube	1,60±0,19^{bcd}	1,80±0,41^{ab}	1,89±0,36^{a}
	Ozarkblue	1,51±0,39^{cd}	1,39±0,42^{de}	1,45±0,30^{cd}
28	**Bluecrop**	1,35±0,21^{c}	1,72±0,34^{ab}	1,36±0,31^{c}
	Goldtraube	1,69±0,29^{b}	1,95±0,37^{a}	1,98±0,40^{a}
	Ozarkblue	1,42±0,34^{c}	1,48±0,32^{bc}	1,40±0,26^{c}
35	**Bluecrop**	1,48±0,18^{bcd}	1,70±0,37^{ab}	1,26±0,20^{d}
	Goldtraube	1,65±0,27^{abc}	1,87±0,51^{a}	1,92±0,58^{a}
	Ozarkblue	1,40±0,32^{cd}	1,57±0,41^{bc}	1,38±0,24^{cd}
42	**Bluecrop**	1,35±0,21^{cd}	1,61±0,22^{b}	1,32±0,32^{cd}
	Goldtraube	1,58±0,22^{bc}	1,66±0,55^{b}	2,15±0,51^{a}
	Ozarkblue	1,25±0,31^{d}	1,23±0,32^{d}	0,92±0,32^{e}
49	**Bluecrop**	1,29±0,19^{cd}	1,64±0,26^{b}	1,30±0,25^{cd}
	Goldtraube	1,57±0,22^{bc}	1,74±0,77^{b}	2,36±0,61^{a}
	Ozarkblue	1,22±0,37^{d}	1,24±0,33^{cd}	1,07±0,32^{d}
56	**Bluecrop**	-	1,48±0,34^{bc}	1,27±0,17^{c}
	Goldtraube	-	1,73±0,60^{ab}	1,90±0,69^{a}
	Ozarkblue	-	1,25±0,26^{cd}	0,92±0,48^{d}

Results are expressed as mean ± SD (n=30). Different letters represent significant differences ($P < 0.05$) between year and cultivar for each storage day, by the Tukey test.

Titratable acidity (Table 3.5.) and soluble solids content (Table 3.6.) were significantly influenced by the year of production and cultivar. While titratable acidity decreased over the course of storage for all cultivars and production years with the lowest acidity being obtained in 2012, soluble solids content remained relatively constant throughout the storage, with 'Bluecrop' and 2010 presenting the higher values. Wang et al. (2012b) also reported significant differences in fruit soluble solids content and titratable acidity were found among cultivars within years and also between years for many cultivars. They refer that sugars and organic acids have an important impact on the sensory quality of fruit. The general flavour selection criteria for fruits are a combination of high sweetness and high acidity.

Table 3.5. - Effect of year and cultivar in the tritatable acidity (% malic acid).

Day	Cultivar	Year		
		2010	2011	2012
1	Bluecrop	1,57±0,09^{a}	1,57±0,29^{a}	0,84±0,05^{c}
	Goldtraube	1,27±0,06^{abc}	1,46±0,39^{ab}	0,94±0,14^{bc}
	Ozarkblue	1,29±0,34^{abc}	1,12±0,02^{abc}	1,09±0,09^{abc}
7	Bluecrop	1,89±0,39^{b}	1,20±0,01^{cd}	0,93±0,04^{d}
	Goldtraube	2,75±0,45^{a}	1,10±0,06^{cd}	0,86±0,12^{d}
	Ozarkblue	1,70±0,25^{bc}	1,13±0,01^{cd}	0,97±0,10^{d}
14	Bluecrop	2,06±0,23^{b}	1,21±0,01^{cd}	0,83±0,08^{e}
	Goldtraube	3,05±0,04^{a}	1,10±0,03^{de}	0,85±0,02^{e}
	Ozarkblue	1,54±0,26^{c}	0,95±0,05^{de}	1,23±0,09^{cd}
21	Bluecrop	1,77±0,24^{b}	1,13±0,03^{cd}	0,81±0,02^{d}
	Goldtraube	2,26±0,38^{a}	1,09±0,04^{cd}	1,02±0,16^{cd}
	Ozarkblue	1,34±0,13^{bc}	0,99±0,05^{cd}	0,84±0,11^{d}
28	Bluecrop	1,42±0,20^{b}	1,16±0,01^{bc}	0,81±0,06^{d}
	Goldtraube	2,07±0,04^{a}	1,08±0,06^{c}	0,97±0,10^{cd}
	Ozarkblue	1,37±0,11^{b}	0,97±0,06^{cd}	0,99±0,07^{cd}
35	Bluecrop	1,37±0,15^{a}	1,13±0,02^{b}	0,86±0,05^{cd}
	Goldtraube	1,55±0,06^{a}	1,07±0,05^{bc}	0,83±0,07^{d}
	Ozarkblue	0,99±0,05^{bcd}	0,96±0,13^{bcd}	0,90±0,07^{bcd}
42	Bluecrop	1,13±0,07^{b}	1,12±0,03^{bc}	0,87±0,08^{d}
	Goldtraube	1,49±0,06^{a}	1,07±0,04^{bc}	1,11±0,06^{bc}
	Ozarkblue	0,92±0,10^{cd}	0,94±0,10^{bcd}	0,97±0,03^{bcd}
49	Bluecrop	1,13±0,05^{b}	1,07±0,02^{bc}	0,89±0,08^{cd}
	Goldtraube	1,49±0,02^{a}	1,07±0,13^{bc}	1,11±0,12^{bc}
	Ozarkblue	0,92±0,01^{bcd}	0,82±0,05^{d}	1,04±0,13^{bcd}
56	Bluecrop	-	1,04±0,04^{ab}	0,78±0,07^{c}
	Goldtraube	-	1,03±0,18^{ab}	1,11±0,11^{a}
	Ozarkblue	-	0,82±0,04^{bc}	1,04±0,19^{ab}

Results are expressed as mean ± SD (n=3). Different letters represent significant differences ($P < 0.05$) between year and cultivar for each storage day, by the Tukey test.

Table 3.6. - Effect of year and cultivar in the soluble solids content (º Brix).

Day	**Cultivar**	**Year**		
		2010	**2011**	**2012**
1	**Bluecrop**	14,31±0,13^{a}	11,36±0,22^{ef}	10,60±0,17^{g}
	Goldtraube	11,99±0,09^{cd}	11,91±1,05^{cde}	12,76±0,11^{b}
	Ozarkblue	12,42±0,31^{bc}	11,100±0,18^{fg}	11,58±0,25^{def}
7	**Bluecrop**	14,43±0,35^{a}	11,46±0,38^{d}	9,47±0,30^{f}
	Goldtraube	12,17±0,53^{bc}	11,30±0,15^{de}	12,50±0,09^{b}
	Ozarkblue	11,80±0,19^{cd}	10,86±0,77^{e}	11,60±0,11^{cd}
14	**Bluecrop**	13,94±0,31^{a}	11,10±0,53^{d}	9,77±0,20^{e}
	Goldtraube	11,61±0,61^{cd}	11,13±0,38^{d}	12,94±0,19^{b}
	Ozarkblue	11,81±0,76^{c}	11,33±0,35^{cd}	11,07±0,40^{d}
21	**Bluecrop**	13,94±0,70^{a}	11,04±0,13^{d}	10,00±0,31^{e}
	Goldtraube	11,19±0,25^{cd}	11,00±0,54^{d}	12,63±0,10^{b}
	Ozarkblue	11,76±0,30^{c}	11,57±0,35^{cd}	11,76±0,41^{c}
28	**Bluecrop**	14,16±0,10^{a}	11,08±0,04^{de}	10,51±0,47^{f}
	Goldtraube	11,83±0,58^{c}	10,92±0,30^{ef}	12,66±0,47^{b}
	Ozarkblue	11,78±0,22^{c}	11,49±0,25^{cd}	11,56±0,35^{cd}
35	**Bluecrop**	13,49±0,16^{a}	11,06±0,15^{ef}	9,52±0,24^{g}
	Goldtraube	12,11±0,06^{c}	10,91±0,13^{f}	12,63±0,13^{b}
	Ozarkblue	11,68±0,16^{d}	11,33±0,22^{de}	11,33±0,51^{de}
42	**Bluecrop**	13,93±0,21^{a}	10,81±0,09^{f}	9,63±0,46^{g}
	Goldtraube	11,46±0,12^{d}	11,03±0,10^{ef}	12,39±0,24^{b}
	Ozarkblue	11,92±0,39^{c}	11,26±0,19^{de}	10,90±0,15^{ef}
49	**Bluecrop**	13,93±0,26^{a}	11,08±0,04^{de}	9,74±0,19^{f}
	Goldtraube	11,56±0,25^{c}	11,00±0,15^{de}	12,29±0,25^{b}
	Ozarkblue	12,39±0,19^{b}	11,20±0,17^{d}	10,83±0,13^{e}
56	**Bluecrop**	-	10,96±0,34^{b}	9,73±0,43^{c}
	Goldtraube	-	11,13±0,21^{b}	11,53±0,21^{a}
	Ozarkblue	-	11,19±0,12^{ab}	11,16±0,27^{ab}

Results are expressed as mean ± SD (n=3). Different letters represent significant differences ($P < 0.05$) between year and cultivar for each storage day, by the Tukey test.

3.4.2. Effect of growing season on total anthocyanins content, total phenolic compounds and antioxidant activity.

Total anthocyanins content were significantly influenced by the year of production and cultivar (Figure 3.2.). The year 2011 and cultivar 'Goldtraube' were those that presented a higher content of total anthocyanins. Between 2010 and 2011 there was an increase in total anthocyanins content of over 43%, 40% and 52% for the cultivars Bluecrop, Goldtraube and Ozarkblue respectively. Kalt and McDonald (1996) also found that seasonal variation in anthocyanin content among lowbush blueberry cultivars over seven seasons was quite marked in fruit harvested from the same site. There was a 30% difference in the level of anthocyanins between the 2 years of the study.

Total anthocyanins content (Figure 3.2.a., 3.2.b. and 3.2.c.) showed the same trend for the three cultivars during storage in 2010, thus exhibited a large decrease at 28 days of storage and then remained relatively constant until the end of storage. In 2011, ‘Bluecrop’ and ‘Ozarkblue’ total anthocyanins content remained relatively constant during storage, while ‘Goldtraube’ behaviored similarly to 2010. In 2012, total anthocyanins content increased by up to 28 days of storage in ‘Bluecrop’ and in ‘Ozarkblue’. After this period, in ‘Bluecrop’, they remained relatively stable and in ‘Ozarkblue’ decreased until the end of storage. In ‘Goldtraube’ the total anthocyanins content decreased during the storage. The anthocyanins can be rapidly degraded by polyphenol oxidase (PPO) in blueberries (Kader et al., 1997). In strawberries anthocyanin degradation and oxidation of soluble phenolic compounds was possibly caused by increased PPO activity as a result of water loss which, contributed to the development of surface browning during storage (Nunes et al., 2005).

The year 2011 was characterized for being a high temperatures throught the year and with sunshine values above normal. Although Jaakola and Hohtola (2010) report that in general, the presence of too high temperatures can inhibit flavonoid biosynthesis and cause their degradation, Jaakola et al. (2004) report that plants that grow under direct sun exposure increase in the expression of the flavonoid pathway genes in leaves (*Vaccinium myrtillus* L.). The concentrations of anthocyanins, catechins, flavonols and hydroxycinnamic acids were higher in the leaves exposed to direct sunlight. These results have shown the protective role of flavonoids and hydroxycinnamic acids against high solar radiation in plants. Increase in solar radiation generally yields higher contents of phenolics, especially anthocyanins, in fruits (Macheix et al., 2017).

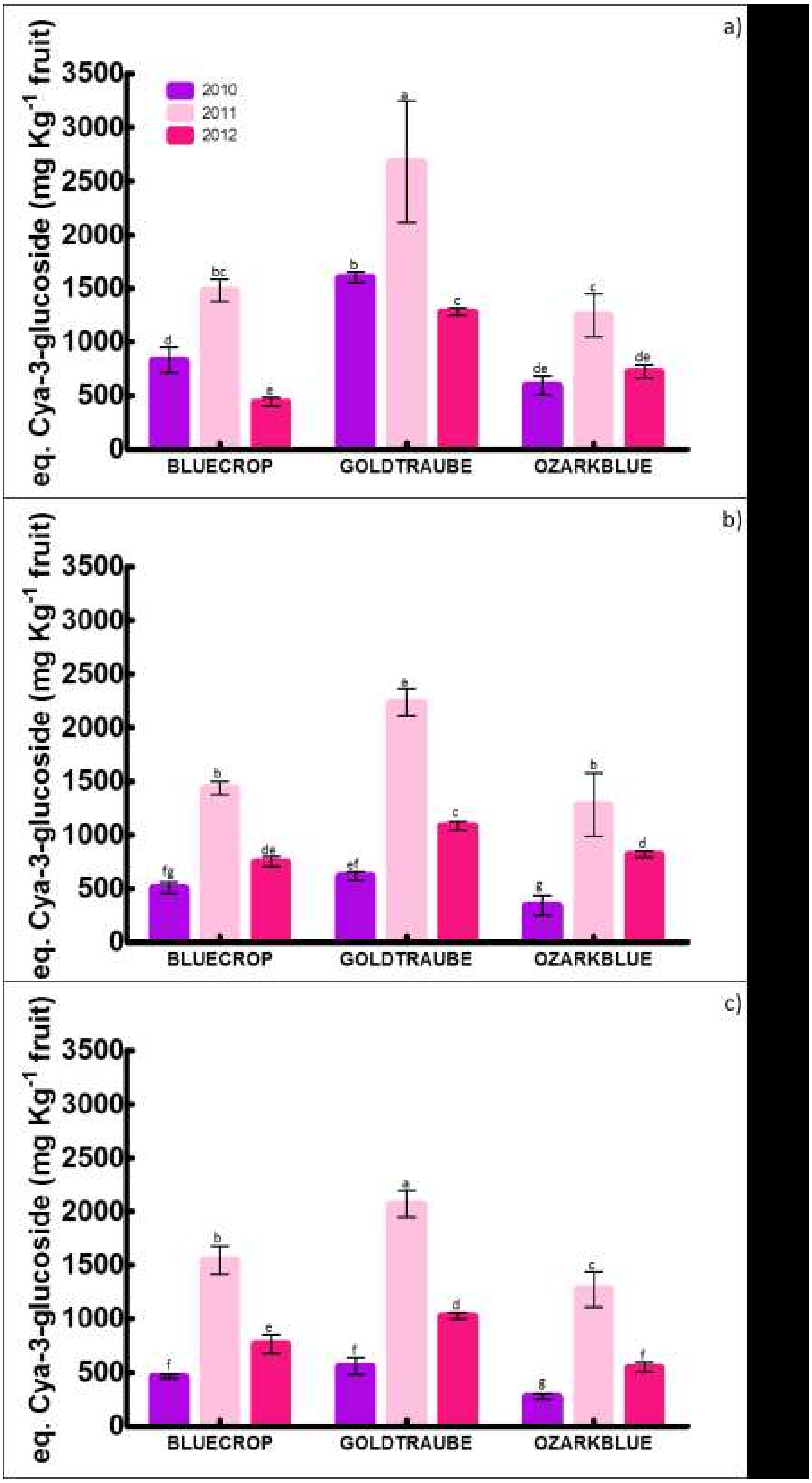

Figure 3.2. - Effect of year and blueberry cultivar on the total anthocyanins content on day: a) 1, b) 28 c) 49 (2010) or 56 (2011 and 2012) in fruit during storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

The total phenolic compounds and antioxidant activity were significantly affected by year of production and cultivar (Figure 3.3. and Figure 3.4.); however cultivars were affected differently. For example, in 2012 ‘Goldtraube’ and ‘Ozarkblue’ were highest in total phenolic compounds, but in ‘Bluecrop’ there weren’t significant differences between 2011 and 2012.

Total phenolic compounds content (Figure 3.3.a., 3.3.b. and 3.3.c.) showed a similar trend for ‘Bluecrop’ and ‘Goldtraube’ cultivars during storage. In 2010, the total phenolic compounds content decreased in the first 28 days of storage and then remained relatively constant until the end of storage, while in 2011 total phenolic compounds content increased until the 28th day of storage and then remained relatively constant. In 2012, total phenolic compounds content increased during storage. In 2010 this parameter in ‘Ozarkblue’ decreased during storage. In 2011 and 2012, the total phenolic compounds showed a similar trend in this cultivar, showing a decrease in the first 28 days of storage and then remaining relatively stable during the remaining storage.

The antioxidant activity (Figure 3.4.a., 3.4.b. and 3.4.c.) was significantly affected by the year of production and cultivar. In general, the year 2010 was when the highest antioxidant activity was obtained for the three cultivars, however for ‘Goldtraube’ there were no significant differences between 2010 and 2012. Howard et al. (2003) also did not obtain consistent change among all cultivars in a given year; some cultivars had higher contents whereas others had lower.

Gonçalves et al. (2004) found that the level of phenolic acids were higher in 2001 and anthocyanin levels were higher in 2002 in cherry fruit, which suggest a significant influence of climatic conditions on these compounds.

Wang et al. (2012b) also reported significant differences in total phenolic content and antioxidant activity were found among in various cultivars within years, but also between the 2 years for many blueberries cultivars. Gonçalves et al. (2004) also reported for cherries harvested in two different years indicate that the influence of weather conditions during cherry growth may have a profound influence on the phenolic compounds levels.

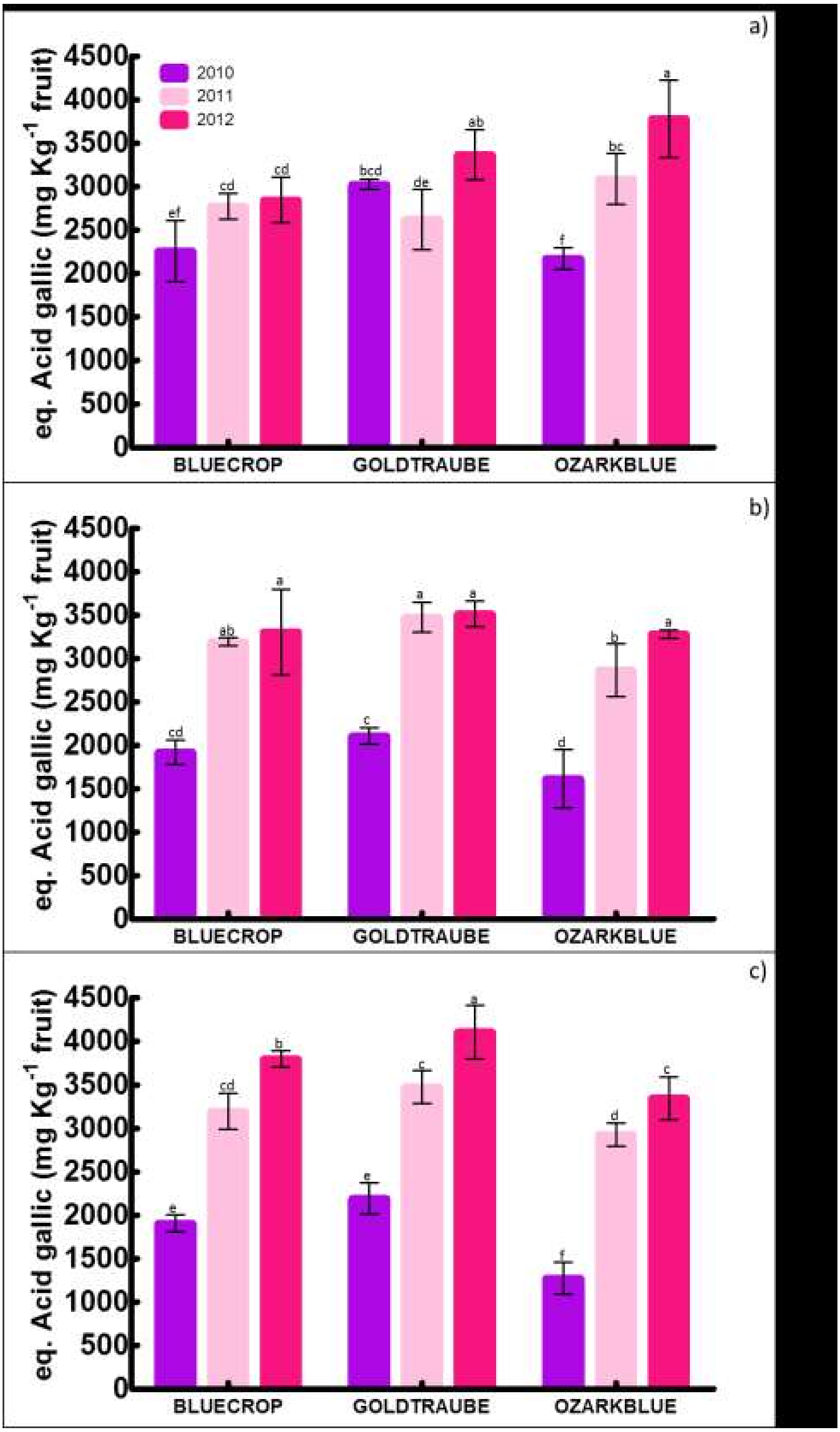

Figure 3.3. - Effect of year and blueberry cultivar on the total phenolic compounds on day: a) 1, b) 28 c) 49 (2010) or 56 (2011 and 2012) in fruit during storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

In ‘Bluecrop’ antioxidant activity increased during storage in 2010 and 2012. While in 2011 the antioxidant activity had a slight increase (2%) in the 28 day of storage decreasing (17%) at the end of storage. In ‘Goldtraube’ the antioxidant activity was relatively stable throughout the storage (2010 and 2012), presenting a decrease to the 28 day of storage and increasing again in the remaining period of storage; however in 2011 the activity decreased during storage. In ‘Ozarkblue’ in 2010 and 2011, the antioxidant activity decreased during the 56 days storage. However, after 28 days of storage there was an increase in antioxidant activity. In the year 2012 the antioxidant activity increased in the 56 days of storage, however from day 28 on, there was a decrease. Connor et al. (2002a) found that antioxidant activity increased during storage for six cultivars of blueberries (MSU-58, Brigitta, Legacy, Bluegold, Nelson and Jersey), stored at 5 °C for 3 to 7 weeks.

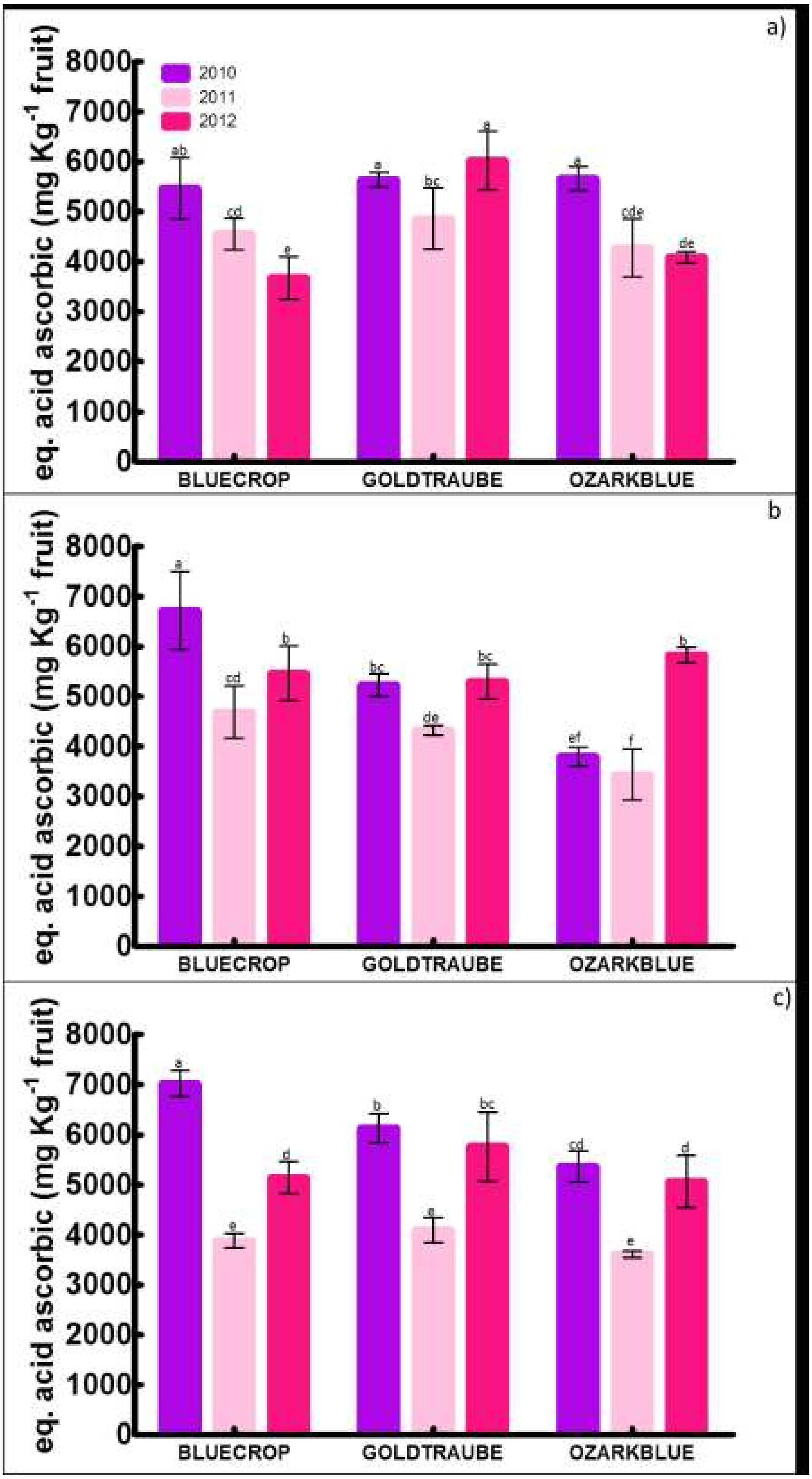

Figure 3.4. - Effect of year and blueberry cultivar on antioxidant activity on day: a) 1, b) 28 c) 49 (2010) or 56 (2011 and 2012) in fruit during storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

3.4.3. Effect of growing season on the profile of anthocyanins and other polyphenols.

In ‘Bluecrop’ and ‘Goldtraube’, nine anthocyanins: delphinidin 3-O-galactoside, delphinidin 3-O-glucoside, cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, cyanidin 3-O-arabinoside, petunidin 3-O-glucoside, malvidin 3-O-galactoside, malvidin 3-O-glucoside and peonidin-3-galactoside, and two hydroxycinnamic acids: ferulic and chlorogenic acids were identified and quantitated in the three years. In ‘Ozarkblue’, seven anthocyanins were quantified: delphinidin 3-O-galactoside, cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, cyanidin 3-O-arabinoside, malvidin 3-O-galactoside, malvidin 3-O-glucoside, peonidin-3-O-galactoside and the same two hydroxycinnamic acids.

The anthocyanins (Figure 3.5., 3.6. and 3.7.) and hydroxynamic acids (Table 3.7.) identified were affected by year of production and cultivar, however not all of them behaved in a similar way in different years. Only delphinidin 3-O-galactoside and malvidin 3-O-galactoside were obtained in higher values in 2011 in the three cultivars. Regarding hydroxynamic acids (Table 3.7.): chlorogenic acid showed a higher value in 2011 for ‘Bluecrop’, while ferulic acid was higher in 2012 for ‘Bluecrop’ and ‘Ozarkblue’. These compounds did not show a pattern during storage over the three years. Thus, chlorogenic acid increased throughout storage in 2010 and decreased in 2011 during storage, for all three cultivars. In the year 2012 this compound increased during the storage for ‘Bluecrop’, but for ‘Goldtraube’ and ‘Ozarkblue’ it increased in the 28 days of storage and decreased over the remaining storage period. Ferulic acid increased during storage in 2010 for the three cultivars, in 2011 for ‘Ozarkblue’ and in 2012 for ‘Bluecrop’ and ‘Goldtraube’.

Different production conditions may lead to changes in blueberries different anthocyanins contents. For example, different irrigation system resulted in a change in anthocyanin composition. Moderate irrigation led to lower levels of delphinidin 3-arabinoside and cyanidine 3-arabinoside and an increase of malvidin 3-galactoside and malvidin 3-arabinoside (when compared with no and heavy irrigation). Additionally, moderate irrigation levels also led to a reduction of delphinidin 3-galactoside when compared with heavy irrigation. It is not known how these changes affect the nutritional value of blueberries, but they demonstrate the potential for irrigation induced control of anthocyanin levels (Ehret et al., 2012).

Table 3.7. - Effect of year and cultivar on the composition of relevant polyphenolics (chlorogenic acid and ferulic acid) (g kg^{-1} fruit).

Day	Compound g kg^{-1} fresh weight	Cultivar	Year		
			2010	**2011**	**2012**
1	**Chlorogenic Acid**	**Bluecrop**	0,14±0,02^{c}	0,35±0,04^{a}	0,04±0,01^{d}
		Goldtraube	0,15±0,05^{c}	0,16±0,04^{c}	0,01±0,01^{d}
		Ozarkblue	0,11±0,04^{c}	0,21±0,07^{b}	0,02±0,01^{d}
	Ferulic Acid	**Bluecrop**	0,09±0,01^{e}	1,39±0,05^{b}	1,62±0,16^{a}
		Goldtraube	0,29±0,05^{e}	0,68±0,14^{cd}	0,79±0,10^{c}
		Ozarkblue	0,26±0,01^{e}	0,57±0,19^{d}	1,55±0,26^{ab}
28	**Chlorogenic Acid**	**Bluecrop**	0,16±0,00^{bc}	0,24±0,01^{a}	0,05±0,02^{de}
		Goldtraube	0,19±0,13^{ab}	0,11±0,05^{cd}	0,04±0,01^{de}
		Ozarkblue	0,18±0,04^{ab}	0,18±0,01^{ab}	0,04±0,00^{e}
	Ferulic Acid	**Bluecrop**	0,11±0,01^{e}	1,18±0,14^{b}	1,83±0,12^{a}
		Goldtraube	0,30±0,11^{d}	0,58±0,18^{c}	0,66±0,10^{c}
		Ozarkblue	0,30±0,04^{d}	0,73±0,10^{c}	1,69±0,16^{a}
49 (2010) 56 (2011 and 2012)	**Chlorogenic Acid**	**Bluecrop**	0,37±0,02^{a}	0,23±0,04^{b}	0,07±0,01^{c}
		Goldtraube	0,23±0,06^{b}	0,11±0,04^{c}	0,01±0,00^{d}
		Ozarkblue	0,24±0,02^{b}	0,11±0,03^{c}	0,02±0,00^{d}
	Ferulic Acid	**Bluecrop**	0,13±0,03^{c}	0,86±0,12^{b}	2,08±0,39^{a}
		Goldtraube	0,31±0,06^{c}	0,31±0,07^{c}	0,85±0,34^{b}
		Ozarkblue	0,36±0,06^{c}	0,72±0,07^{b}	0,95±0,12^{b}

Results are expressed as mean ± SD (n=3). Different letters represent significant differences ($P < 0.05$) between year and cultivar for each compound and storage day, by the Tukey test.

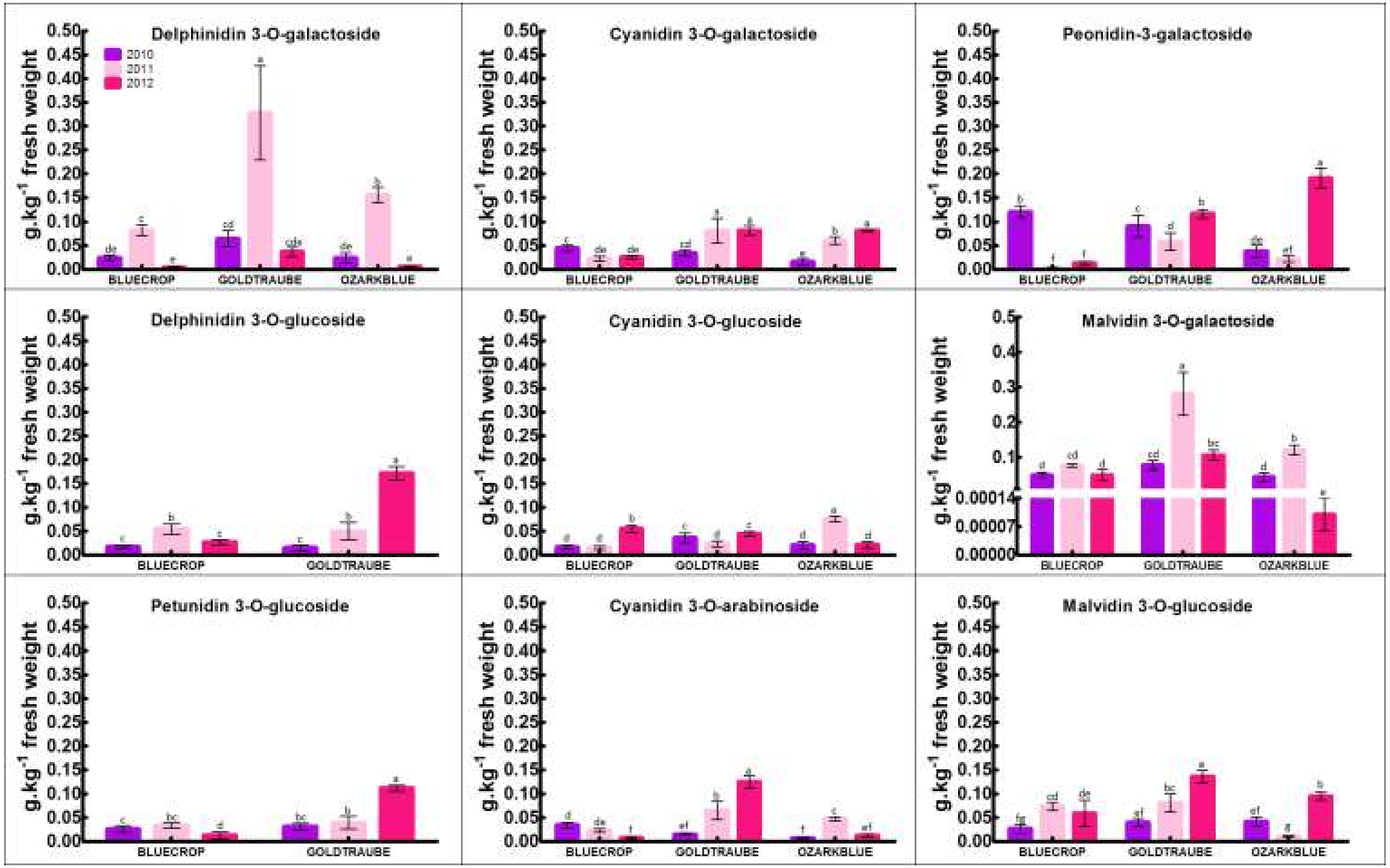

Figure 3.5. – Effect of year and cultivar on the composition of anthocyanins in blueberry fruit during storage at 4 ºC on day 1. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

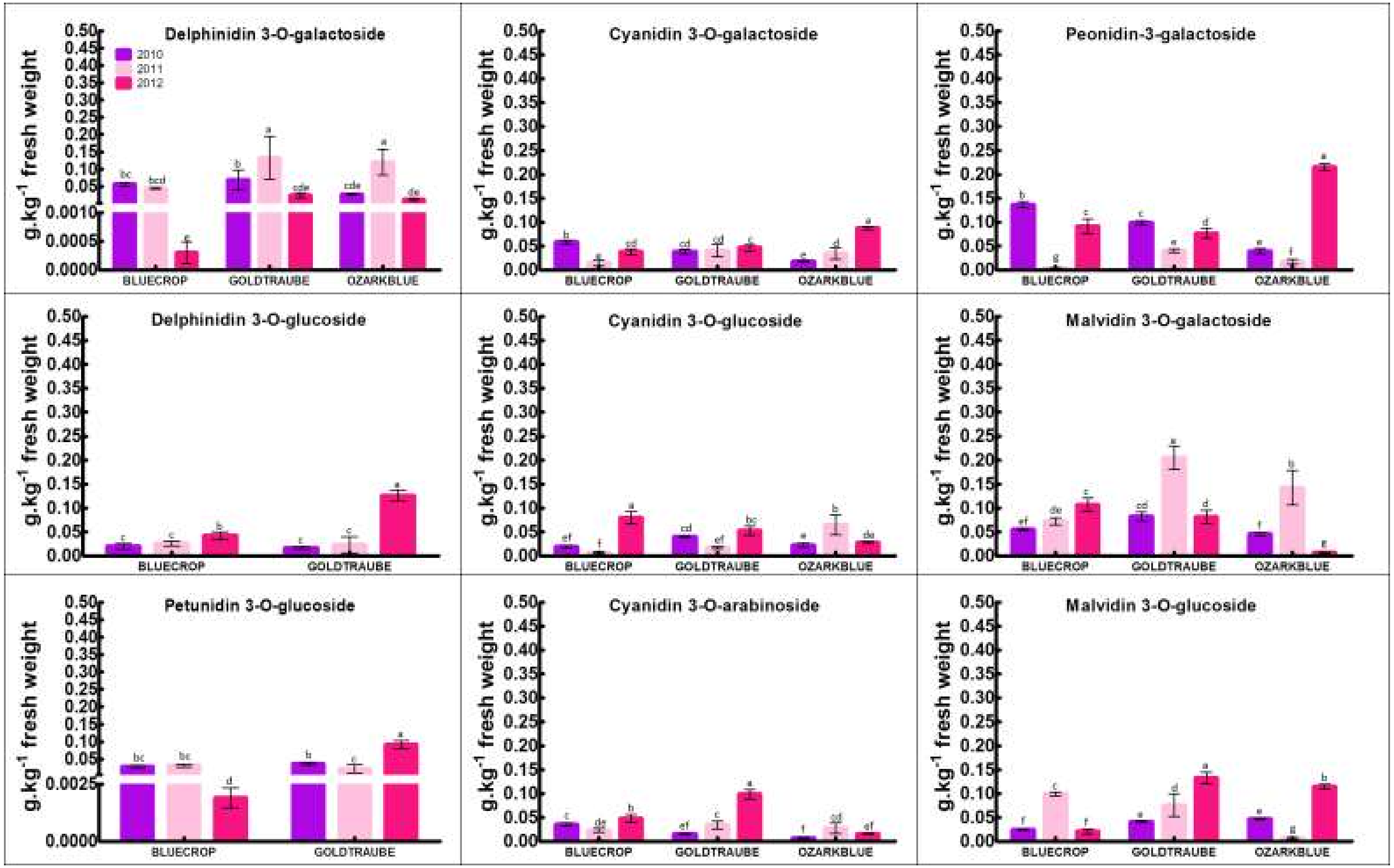

Figure 3.6. – Effect of year and cultivar on the composition of anthocyanins in blueberry fruit during storage at 4 ºC on day 28. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

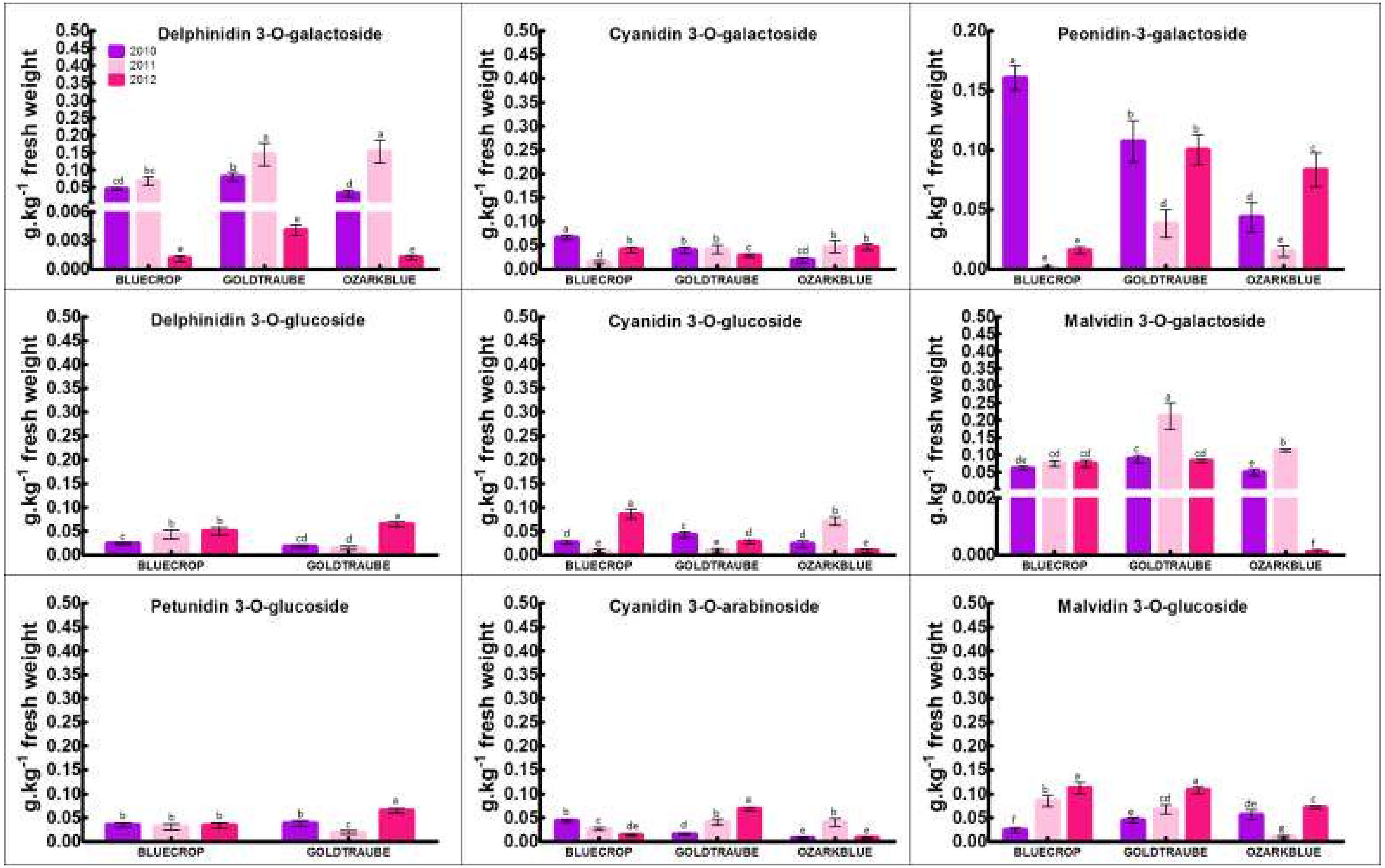

Figure 3.7. – Effect of year and cultivar on the composition of anthocyanins in blueberry fruit during storage at 4 ºC on day 49 (2010) or 56 (2011 and 2012). Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

3.4.4. Relationship between colour and quantitative / qualitative anthocyanins and other polyphenols.

The complete dataset was analyzed by means of principal component analysis (PCA) (Figure 3.8.) based on the correlation matrix. Principle component analysis (PCA) yielded five interpretable components that together explain 90.989% of the total variance of the variables. The first two principal components (PC1 and PC2) explain 51.087% of the variability in the data set.

The most important component (PC1) explained 28.949% of the total variance. PC1 is related to petunidin 3-O-glucoside, delphinidin 3-O-glucoside, cyanidin 3-O-arabinoside and malvidin 3-O-glucoside. PC2 explained 22.138% of the total variance and mainly correlates peonidin 3-O-galactoside, cyanidin 3-O-galactoside and antioxidant activity. The antioxidant activity is positively related to these compounds.

PC3 explained 19.388% of variance, and this variation was mostly described by malvidin 3-O-galactoside, delphinidin 3-O-galactoside, total anthocyanins content, and cultivars. The total antocyanins and cultivars are positively related to these compounds.

Finally, PC4 explained 7.850% of variance, and this variation was mostly described by cyanidin 3-O-glucoside, ferulic acid, chlorogenic acid, total phenolic compounds and year of production. The year of production and total phenolic compounds are positively related to cyanidin 3-O-glucoside, ferulic acid and negatively related chlorogenic acid. Wang et al. (2012b) also reported that the effect of seasonal differences had a large impact on the accumulations of phenolics.

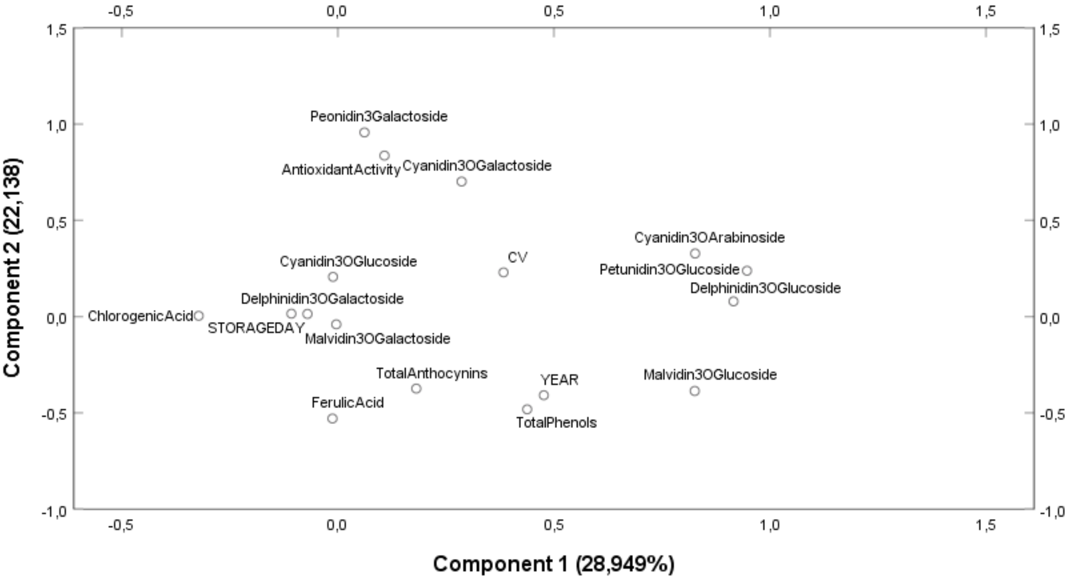

Figure 3.8. – Positions of PCA scores for the composition of anthocyanins, hidroxinamic acid, total anthocyanins content, total phenolic compounds, antioxidant activity, cultivar and year of production, based on PC1 and PC2. The percentage represents the variance of each principal component.

3.5. Conclusions

Although blueberries are fruits characterized with a high antioxidant activity, this work suggests that cultivar and year of production conditions have a major influence on the commercial and functional parameters in blueberries.

Parameters of commercial quality such as titratable acidity, soluble solids content and firmness and functional quality were influenced by the years of production. Although total phenolic compounds and antioxidant activity were significantly influenced by year of production and cultivar, they were not consistently influenced in the three cultivars. In general the 'Goldtraube' cultivar presented a higher antioxidant activity, but the environmental conditions of production influenced this parameter a lot.

We found that high insolation positively influenced total anthocyanins content, suggesting the protective role of flavonoids and hydroxycinnamic acids against high solar radiation in plants. The total antocyanins and cultivars are positively related to malvidin 3-

O-galactoside, delphinidin 3-O-galactoside, and the year of production and total phenolic compounds are positively related to cyanidin 3-O-glucoside, ferulic acid.

PART III - Study of postharvest factor

CHAPTER 4 – The effect of postharvest application of ethylene on commercial and phytochemical quality in three Northern Highbush Blueberry Cultivars

This chapter has been published:

Journal Science of Food and Agriculture. (2018). doi: 10.1002/jsfa.9042.

Acta Horticulturae. (2014). doi: 10.17660/ActaHortic.2014.1017.65

4.1. Abstract

Blueberry is a fruit that has been extensively studied for its health benefits, mainly due to its high antioxidant activity. There is a high correlation between antioxidant activity and total anthocyanins content and phenolic compounds. The postharvest treatment using ethylene may be a factor affecting the anthocyanins content. The objective of this work was to analyze at postharvest treatment using ethylene on the anthocyanins profile during the storage of blueberries and phytochemical composition of ‘Bluecrop’, ‘Goldtraube’ and ‘Ozarkblue’. Fruits were harvested at commercial maturity; the treatment was carried out with 1000 µL L^{-1} of ethylene, for 24 h followed by storage at 4 °C under normal atmosphere for 56 days. The rate of weight loss and titratable acidity were not affected by the ethylene treatment in ‘Bluecrop’ and ‘Ozarkblue’, but the acidity in ‘Goldtraube’ treated ethylene is higher. The soluble solids content was higher in ethylene-treated ‘Bluecrop’ and ‘Goldtraube’, but was not in affected ‘Ozarkblue’. Colour and firmness were not affected by ethylene.

One day after treatment with ethylene, an increase in seven (more than 45%) and four (more than 65%) of the nine anthocyanins identified was observed in the cultivars ‘Bluecrop’, ‘Goldtraube’ respectively, and a decrease in five of the seven anthocyanins identified in ‘Ozarkblue’. For ‘Bluecrop’ however this increase was reduced until the end of storage while in ‘Goldtraube’ seven anthocyanins increased. The effect of ethylene on anthocyanin composition appeared to depend on the cultivar. ‘Bluecrop’ and ‘Goldtraube’ responded positively with increase on total anthocyanins content.

4.2. Introduction

Ethylene, a 2-carbon volatile, is produced endogenously by all plants and their organs. At concentrations as low as 0.1 µL L^{-1}, ethylene can induce a wide array of physiological responses, including ripening, senescence, and physiological disorders (Watada, 1986).

Ethylene influences the growth and development of many plants (Chae and Kieber, 2005). Ethylene plays a key regulatory role in the ripening of many fruits, including several that represent important sources of nutrients to the human diet (Barry and Giovannoni, 2007). Therefore, the mechanisms of fruit ripening and/or maturation could involve both ethylene-dependent and ethylene-independent processes, which may differ in extent and/or manner depending on the species (Wang et al., 2007). Fruits are categorized as either climacteric or non-climacteric, based on differences in their physiological ripening patterns (Symons et al., 2012). Blueberries have been classified as non-climacteric fruits (Owusu-Apenten, 2005; Pallardy, 2008; Zifkin et al., 2012).

However, there have been several studies showing that ethylene could play a role in regulating some ripening aspects of non-climacteric fruit such as strawberries (Villarreal et al., 2010), grapes (Bellincontro et al., 2006; Chervin et al., 2009; El-Kereamy et al., 2003) and litchis (Wang et al., 2007).

Among the many effects of ethylene, we can mention the following: a decrease in berry acidity in the ripening of grape berries (Chervin et al., 2004), accelerated chlorophyll degradation and anthocyanin synthesis in litchi (Wang et al., 2007), as well as in *Capsicum annuum* L. 'Robusta' (Fox et al., 2005). Ethylene also accelerates anthocyanin accumulation, total sugar content and causes an increment in the activity of phenylalanine ammonia-lyase (PAL; EC 4.3.1.24) and β-galactosidase (EC 3.2.1.23) in strawberries (Villarreal et al., 2010). In Aleatico grape berries, it was reported to increase total phenolic compounds and delay anthocyanin loss (Bellincontro et al., 2006).

Exogenous ethylene can elicit the synthesis of secondary metabolites (Heredia and Cisneros-Zevallos, 2009). In general, ethylene promotes PAL activity. PAL catalyses the first step in the biosynthesis of phenylpropanoids, which are further modified into a wide variety of phenolic compounds (Cheng and Breen, 1991).

Ethylene can enhance transcription levels of chalcone synthase (CHS), flavanone 3-hydroxylase (FHT), leucoanthocyanidin dioxygenase (LDOX) and UDP glucose-flavonoid 3-O-glucosyl transferase (UFGT) in grapes. The increase of these transcript variants may result in a rapid accumulation of anthocyanins (El-Kereamy et al., 2003). Chervin et al. (Chervin et al., 2009) also reported that ethylene is involved in the signal mix leading to UFGT expression in grape cells, and particularly in berry skin tissues that accumulate high concentrations of anthocyanins.

Blueberry fruits are rich in phenolic compounds with high antioxidant activity (Vicente et al., 2009). Consumption of berries has become popular among health-conscious consumers due to the high levels of valuable antioxidants, such as phenolic compounds (namely caffeic acid, chlorogenic acid, *p*-coumaric acid, 4-O-feruloylquinic acid, 5-O-feruloylquinic acid, trans-ferulic acid, quercetin, isoferulic acid) and anthocyanins (namely delphinidin-3-galactoside, delphinidin-3-glucoside, cyanidin-3-galactoside, delphinidin-3-arabinoside, cyanidin-3-glucoside, petunidin-3-glucoside, cyanidin-3-arabinoside, peonidin-3-galactoside, petunidin-3-arabinoside, peonidin-3-glucoside, malvidin-3-galactoside, malvidin-3-glucoside, malvidin-3-arabinoside) present in berries (You et al., 2011). Sellappan et al. (2002) reported that phenolic acids ranged from 0.75 to 29.28 mg.100 g^{-1} fresh weight in Southern highbush blueberries (Sellappan et al., 2002). The mode of production influences the phenolic compounds. So 'Climax' fruits organically grown showed a lower chlorogenic acid (13.84 ± 2.12 mg.100 g^{-1} fresh weight) than the same cultivar conventionally grown (17.81 ± 0.31 mg.100 g^{-1} fresh weight) (You et al., 2011). Several authors observed remarkable differences in individual anthocyanin contents among different blueberries cultivars and products (Li et al., 2017; Müller et al., 2012; Pertuzatti et al., 2016; Timmers et al., 2017; Yousef et al., 2013). For example, in Rabbiteye genotypes (Ira, Montgomery and Onslow) contained higher percentages of cyanidin glycosides than the southern highbush genotypes (Legacy, Sampson and SHF2B1-21:3), while the reverse was found for the levels of delphinidin glycosides present (Timmers et al., 2017). Müller et al. (2012) also found that the anthocyanin profiles of blueberry fruits and juice or nectar were similar, however the amount was lower due to different causes such as: the conditions of fruit production, the state of maturation to the harvest or methodology used to make the juice.

Some authors have reported, on the effects of storage, 10–25% increase in total anthocyanins content for *Vaccinium corymbosum* (highbush blueberries stored 2–8 d at 10 or 20 ºC (Kalt et al., 1999, 2003), and 0–28% stored for 3 weeks at 5 ºC. Kalt et al. (2003) found that blueberries could gain 10% in total anthocyanin content as they went from a 100% blue colour to fully ripe, or could lose 2%, depending on the cultivar. Perkins-

Veazie et al. (2008) highlighted that it is important to try to find strategies to maximize these compounds during storage. However, only a few studies have put to the test strategies with this aim in mind.

Recently, several studies have investigated the effect of ethylene on non-climacteric fruits such as grapes and strawberries, but no study on blueberries has been performed. So, the aim of this study was to evaluate the effect of postharvest ethylene treatment on the nutritional properties, namely profile of anthocyanins and others polyphenols and related antioxidant activity, as well as sensory profile throughout the storage period of three highbush blueberry cultivars.

The production of blueberries in Portugal has recently increased. According to FAO ("FAOSTAT," n.d.), the production of blueberries increased from 700 tonnes in 2011 to 6572 tonnes in 2016. The largest proportion of blueberries installed in Portugal is essentially *Vaccinium corymbosum*, with which we have studied the effect of ethylene, since they are among the most produced in the Sever do Vouga region. *Vaccinium myrtillus*, although being richer in anthocyanins, has no expression in Portugal.

4.3. Material and Methods

4.3.1. Fruit treatment and storage

The Northern highbush blueberry (*Vaccinium corymbosum*) from 'Bluecrop', 'Goldtraube', and 'Ozarkblue' cultivar plants had been planted at sixteen, ten and twelve years before, respectively. The soil is characterized by a coarse-grained and organic matter content of 12%. The fruit were harvested at commercial maturity (full blue) in Sever do Vouga, Portugal, cooled to 4 ºC, and treated with ethylene (Sigma-Aldrich) at 1000 $\mu L\ L^{-1}$ for 24 h, the same amount used by Botondi *et al.* (Botondi et al., 2011) for grapes. Treatments were performed in 60 L buckets (to 16 kg of fruit), which were sealed and ethylene was injected with a syringe via the rubber cap. Treatment was performed on each cultivar. Fruit were subsequently packaged in ventilated polyethylene terephthalate (PET) clamshells (0,250 kg each) and stored at 4 °C, 70-80% RH, for 56 days. Untreated control fruit were handled in the same way but not exposed to exogenous ethylene.

4.3.2. Weight loss

The average weight loss of fruits was determined by individually weighing 10 fruits with a model ABJ 120 - 4M balance (Kern, Balingen, Germany).

4.3.3. Soluble solids content

The soluble solids content was determined in juice using the method according by Castrejón et al. (2008) and Prior et al. (1998) as described on section 3.3.4.

Three replicates were generated for each treatment and for each of sampling points throughout storage time at 0, 7, 14, 21, 28, 35, 42, 49 and 56 days.

4.3.4. Total acidity

Total acidity was measured in an homogenate of 20 fruit using the method according by Castrejón et al. (2008) as described on section 3.3.5.

The total acidity was performed weekly during 56 days of storage.

4.3.5. Firmness

The firmness was measured by puncture using a TA XT Plus texture analyzer (Texture Technologies Corp., London, UK). The test was made with a probe with 2 mm diameter with a travel distance of 5 mm at 1 mm s^{-1}. The puncture was conducted in the equatorial region. Thirty replicates were obtained and the maximum firmness expressed as N mm^{-1}.

4.3.6. Skin colour measurement

Skin colour was determined in the CIE L* a* b* space with a Minolta CR-300 colorimeter (Osaka, Japan) equipped with a D65 illuminant. The data was converted to hue (h °) and chroma (C *) using the relationships described by McGuire (1992). The method as described on section 3.3.7.

Samples were taken weekly during storage.

4.3.7. Extracts preparation

Extraction of phenolic compounds was performed according to Kalt et al. (1999) with minor modifications, as described on section 3.3.8.

Three replicates were made for each treatment and for each of sampling points throughout storage time at 0, 28, and 56 days.

4.3.8. Total anthocyanins content

Evaluation of anthocyanins was performed by the differential pH method (Giusti and Wrolstad, 2001) as described on section 3.3.9. Three replicates were made for each sample and for each of sampling points throughout storage time at 0, 28, and 56 days.

4.3.9. Total phenolic compounds

The content of phenolic compounds was determined by the Folin-Ciocalteu reagent (Slinkard and Singleton, 1977) as described on section 3.3.10.

All measurements were performed in triplicate, for each of sampling points throughout storage time at 0, 28, and 56 days.

4.3.10. Antioxidant activity

Antioxidant activity was evaluated by the ABTS$\cdot^+$ radical cation decolourization assay (Gião et al., 2007) as described on section 3.3.11.

All measurements were performed in triplicate. Samples were collected at days 0, 28 and 56 of storage.

4.3.11. HPLC-DAD analysis

Qualitative and quantitative profiles of anthocyanins and hydroxycinnamic acids (namely chlorogenic and ferulic acids) present in the extracts were determined by HPLC-DAD (Waters Series 600, Mildford MA, USA). This method was adapted from Silva et al. (2013b), as described on section 3.3.12.

4.3.12. Sensory evaluation

The Attribute Intensity Ranking was used to assess the sensory properties. The sensory evaluation sessions took place in the ISO 8589:2007 compliant sensory testing facilities of Escola Superior de Biotecnologia - Universidade Católica Portuguesa (ESB-UCP). Ten experienced panellists participated. The descriptors evaluated were intensity of exterior colour, off odors, resistance to shell breaking, fruit total hardness, mealy, sweetness, acidity, adstringency, flavor, off flavour (Annex 2.). The panellists ranked the samples according to the relative intensity of each selected descriptor. Responses were given on a horizontal, 10-cm long, line scale, anchored with verbal labels at the left ('less intense') and the right ('more intense') ends. Fruits were sampled after 7 and 21 days of storage at 4 ºC. Fruits were presented to the panellists on coded plates in a sensory testing room equipped with individual booths, white tables, and controlled cool white fluorescent light. Samples were coded with random, three-digit numbers. Water was provided to clean the palate between tastings.

4.3.13. Statistical analysis

The experiments designed to evaluate the effect of ethylene per se were conducted in a completely randomized block design with three replicates per treatment. Data was analysed by one-way ANOVA with ethylene as a fixed factor.

Data from qualitative and quantitative profiles of anthocyanins and hydroxycinnamic acid (chlorogenic and ferulic acids) were analysed by two-factor ANOVA with ethylene. Tukey's test ($P < 0.05$) was used to highlight significant differences. The association between colour measurement and quantitative / qualitative profiles was estimated using an analysis of factorial components (PCA). All ANOVA and correlation analyses were performed with the statistical software SPSS 25.0 for Windows (SPSS, Chicago, USA).

4.4. Results and Discussion

4.4.1. Effect of ethylene treatment on weight loss rate, soluble solids content, titratable acidity and firmness

The average rate of weight loss was 0.197, 0.261, and 0.150 % day^{-1} (Table 4.1.) for 'Bluecrop', 'Goldtraube' and 'Ozarkblue', respectively. The ethylene treatment decreased the average rate of weight loss of 'Goldtraube' to 0.194 % day^{-1}, but did not affect weight loss from 'Bluecrop' or 'Ozarkblue' (Figure 4.1.).

Table 4.1. - Effect of 1-MCP and ethylene on water loss throughout the store. Y = a+ bx, y being the loss of water and x the storage time (days).

		Water loss			
Cultivar	**Treatment**	**a±sd**	**b±sd**	**r^2**	**P**
Bluecrop	**Control**	99.980±0.276	-0.197±0.008	0.988	0.000
	Ethylene	100.390±0.141	-0.206±0.004	0.997	0.000
Goldtraube	**Control**	100.993±0.478	-0.261±0.014	0.979	0.000
	Ethylene	100.661±0.162	-0.194±0.005	0.996	0.000
Ozarkblue	**Control**	99.014±0.413	-0.150±0.012	0.954	0.000
	Ethylene	99.328±0.328	-0.159±0.010	0.972	0.000

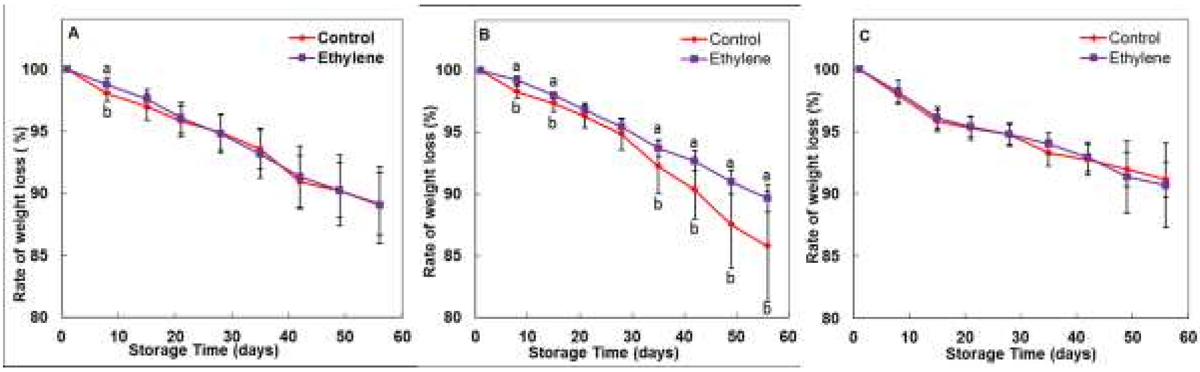

Figure 4.1. – Effect of ethylene on weight loss during storage at 4 ºC of 'Bluecrop' (A), 'Goldtraube' (B) and 'Ozarkblue' (C) blueberry fruit. Results are expressed as mean ± SD (n=10). Different letters represent significant differences ($P < 0.05$), by the Tukey test.

Soluble solids content was affected by the ethylene treatment in 'Bluecrop' and 'Goldtraube' but unaffected in 'Ozarkblue' and remained relatively constant throughout the storage period (Figure 4.2.). Titratable acidity was enhanced by ethylene in

‘Goldtraube’ but in general unaffected in ‘Bluecrop’ and ‘Ozarkblue’ (Figure 4.3.). Other authors also not found significant differences with the application of ethylene in soluble solids content in grapes (Bellincontro et al., 2006) and titratable acidity in strawberry (Villarreal et al., 2010), as would be expected in non-climacteric fruits.

Firmness (Figure 4.4.) was not affected by the ethylene treatment and remained relatively constant during storage.

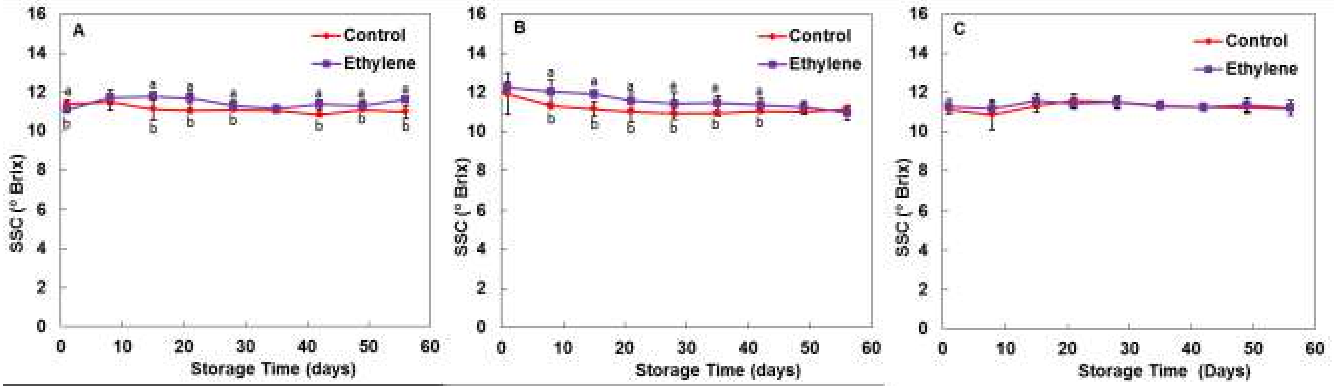

Figure 4.2. - Effect of ethylene treatment on soluble solids content during storage of ‘Bluecrop’ (A), ‘Goldtraube’ (B) and ‘Ozarkblue’ (C), blueberry fruit at 4 ºC. Results are expressed as mean ± SD (n=3). Different letters represent significant differences (P < 0.05), by the Tukey test.

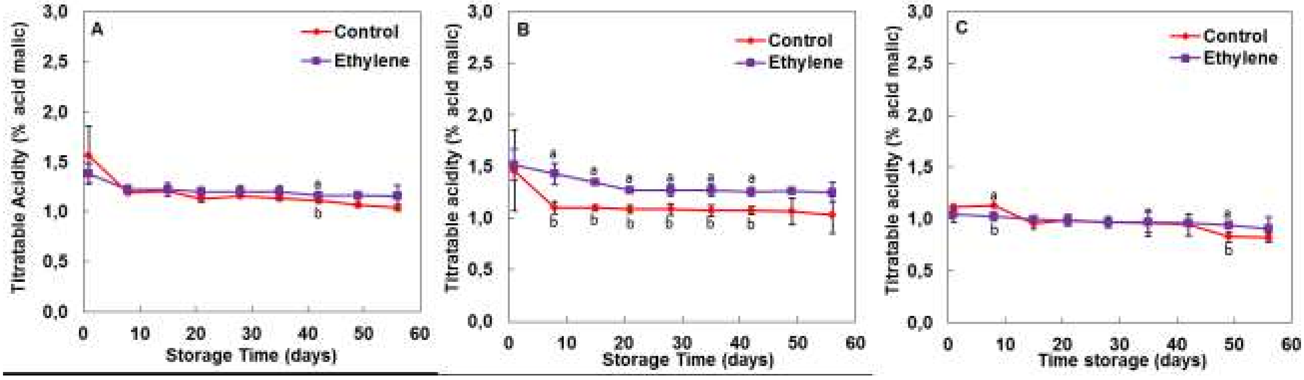

Figure 4.3. - Effect of ethylene treatment on titratable acidity during storage of ‘Bluecrop’ (A), ‘Goldtraube’ (B) and ‘Ozarkblue’ (C), blueberry fruit at 4 ºC. Results are expressed as mean ± SD (n=3). Different letters represent significant differences (P < 0.05), by the Tukey test.

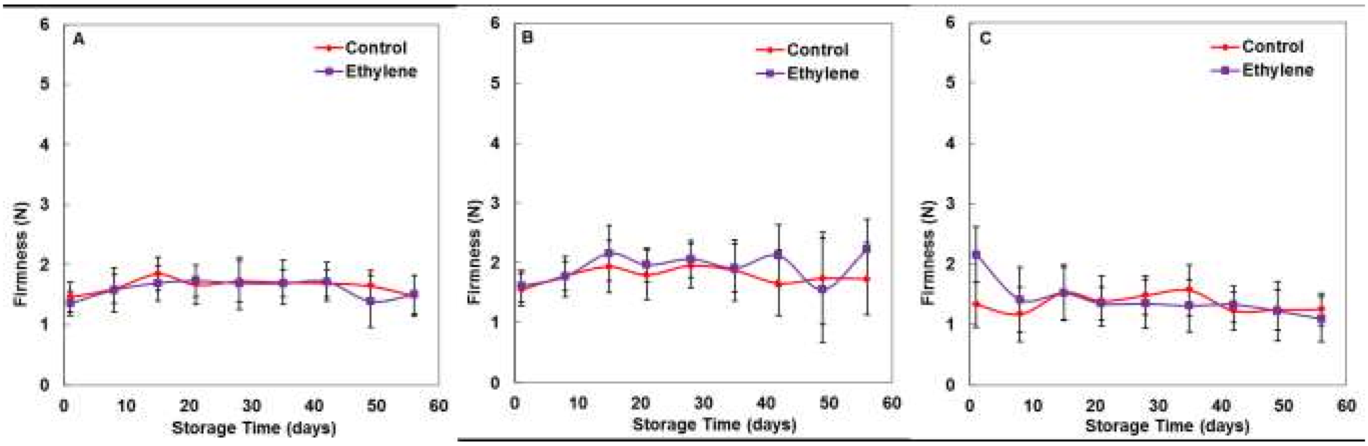

Figure 4.4.– Effect of ethylene treatment on firmness during storage of ‘Bluecrop’ (A), ‘Goldtraube’ (B) and ‘Ozarkblue’ (C), blueberry fruit at 4 ºC. Results are expressed as mean ± SD (n=30).

4.4.2. Effect of ethylene treatment on skin colour, total phenolic compounds and sensorial evaluation

The effect of ethylene on anthocyanin content was influenced by the cultivar. Thus, ethylene induced an increase in anthocyanin content within 24 h in ‘Bluecrop’ and ‘Goldtraube’ (Figure 4.5.a.). In the case of ‘Bluecrop’, ethylene led to an initial increase in anthocyanin content, however these differences were no longer noticeable after 28 d of storage. In ‘Goldtraube’, the fruit treated with ethylene showed higher anthocyanin content throughout the entire storage period (56 d). In ‘Ozarkblue’, this parameter remain relatively constant throughout the storage (Figure 4.5.a).

When comparing the total anthocyanin values with the anthocyanins identified by HPLC-DAD (Table 4.2.) we also observed that the ethylene treatment positively influenced these compounds 24 h after the treatment in ‘Bluecrop’ and ‘Goldtraube’. The response of the sum of the identified anthocyanins was equal to the total anthocyanins content for the three cultivars throughout the storage. At ‘Bluecrop’, ethylene led to an initial increase in anthocyanin content, however, these differences were no longer noticeable after 28 days of storage. At ‘Goldtraube’, this effect was visible throughout the storage. In ‘Ozarkblue’ the identified anthocyanins remained relatively constant throughout the storage.

Table 4.2. – Effect of ethylene treatment in total anthocyanins (g kg^{-1} fruit) identified by HPLC-DAD in selected cultivars in ‘Bluecrop’, ‘Goldtraube’ and ‘Ozarkblue’ during storage at 4 ºC.

Cultivar	Treatment	Storage times (days)		
		1	28	56
Bluecrop	Control	0.38 ± 0.06^{ghi}	0.32 ± 0.04^{hi}	0.35 ± 0.06^{ghi}
	Ethylene	0.65 ± 0.16^{cde}	0.23 ± 0.03^{i}	0.41 ± 0.03^{gh}
Goldtraube	Control	1.01 ± 0.28^{b}	0.59 ± 0.17^{def}	0.59 ± 0.12^{def}
	Ethylene	1.19 ± 0.15^{a}	0.77 ± 0.11^{c}	0.73 ± 0.11^{cd}
Ozarkblue	Control	0.49 ± 0.06^{efg}	0.41 ± 0.13^{gh}	0.45 ± 0.07^{fgh}
	Ethylene	0.36 ± 0.08^{ghi}	0.39 ± 0.05^{ghi}	0.45 ± 0.14^{fgh}

Results are expressed as mean ± SD (n=3). Different letters represent significant differences ($P < 0.05$) between storage day and treatment for each cultivar, by the Tukey test.

Total anthocyanins content decreased during storage in ‘Bluecrop’ and ‘Goldtraube’. Some authors found that anthocyanins can be rapidly degraded by polyphenol oxidase (PPO) in blueberries (Kader et al., 1997) and in litchi (Zhang et al., 2001). Nunes et al. (Nunes et al., 2005) found in strawberries that anthocyanin degradation and oxidation of soluble phenolic compounds, possibly caused by increased PPO activity as a result of water loss, contributing to the development of surface browning during storage. Although the sample control exhibited a higher rate of weight loss there was no impact on anthocyanins loss.

The ethylene led to a delay in ‘Aleatico’ grape berries anthocyanin loss (Bellincontro et al., 2006). Wicks and Kliewer (Wicks and Kliewer, 1983) also observed in table grapes that ethylene effect is cultivar dependent.

Treatment of blueberries with ethylene increased total phenolic content only in ‘Goldtraube’ throughout storage (28 days and 56 days). In ‘Bluecrop’ and ‘Ozarkblue’, this parameter remained relatively constant throughout the storage period (Figure 4.5.b.). If we compare the total phenolic compounds levels with the hydroxycinnamic acids identified by HPLC-DAD (Table 4.3.) we also observed that the treatment with ethylene positively influenced these values 1 day after treatment and at 28 days of storage in ‘Goldtraube’. In ‘Bluecrop’ and ‘Ozarkblue’ the identified hydroxycinnamic acids also remained relatively constant throughout the storage.

The blueberries antioxidant activity was affected by ethylene in the same way as the anthocyanin content (Figure 4.5.c.). Thus, ethylene increased antioxidant activity in ‘Bluecrop’ and ‘Goldtraube’. In ‘Goldtraube’, antioxidant activity of ethylene treated fruit remained higher than that of untreated controls throughout the 56 days storage period. Connor et al. (2002b) and Cho et al. (2005) also observed differences in antioxidant activity, total phenolic compounds and anthocyanins in different blueberry cultivars. The same authors reported that anthocyanins are an important contributor to antioxidant activity, and because they are mainly present in the skin in blueberries, a smaller sized fruit has a much larger surface area of skin relative to pulp, which may be reflected in higher antioxidant activity. In the present study results also showed that the cultivar with the smallest sized fruits, ‘Goldtraube’, had the highest antioxidant activity (Figure 4.5.c.).

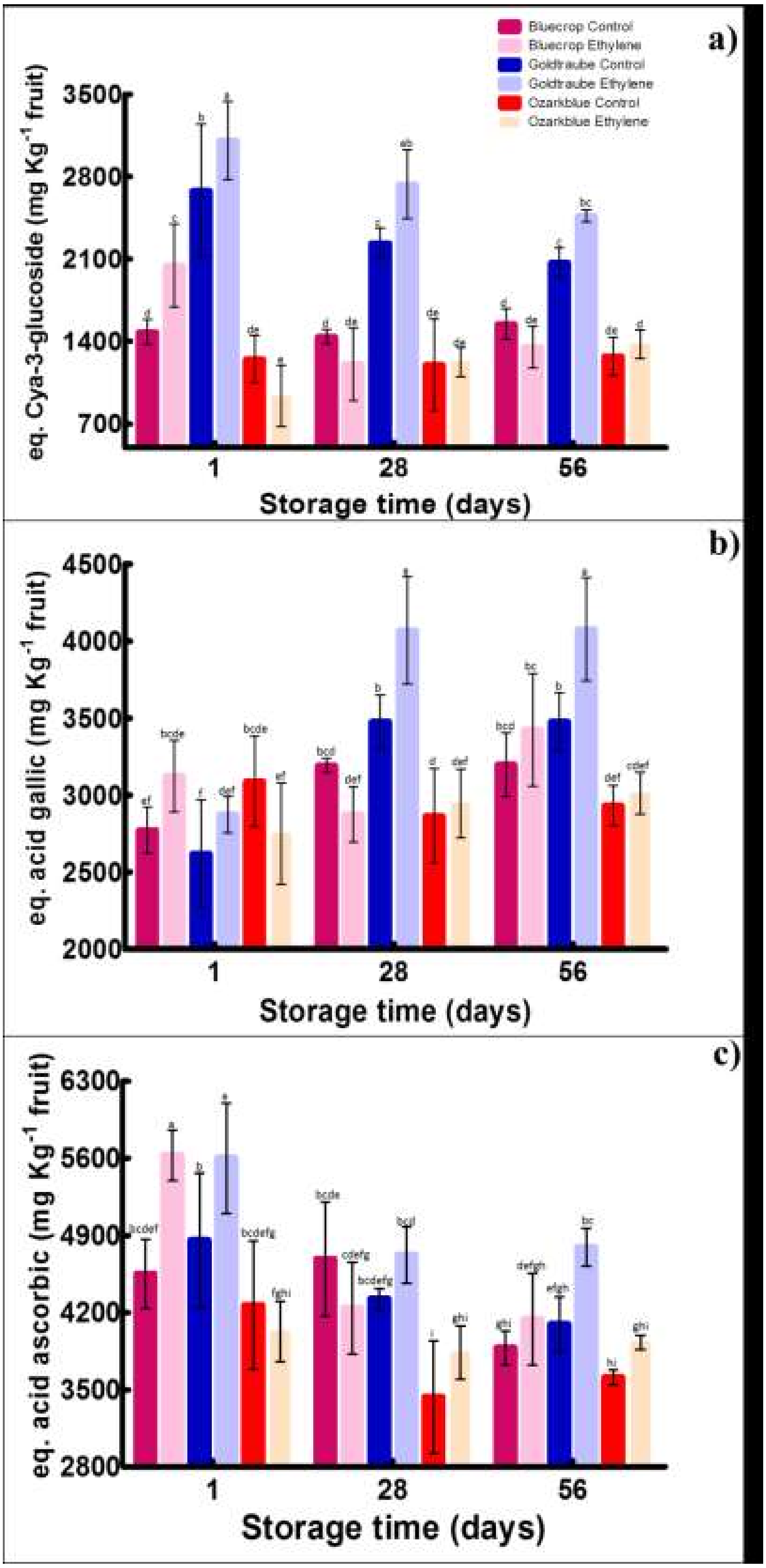

Figure 4.5. - Effect of ethylene treatment on the: a) total anthocyanins content, b) total phenolic compounds and c) antioxidant activity in blueberry fruit during storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

The mechanism that is possibly involved was also described in strawberries by (Cheng and Breen, 1991), who reported that ethylene application may have promoted PAL activity, as well as the transcription levels of CHS and UFGT, described by El-Kereamy et al. (2003) and Chervin et al. (2009). These sequential phenomena led to an increase in the content of anthocyanins, total phenolic compounds and in antioxidant activity, since they are precursors of these compounds. Though ethylene sensitivity is considered to be low in blueberries (Cantwell, 2002), the different cultivars may possibly have responded differently due to differences in ethylene sensitivity, also observed by Wang et al. (2007) for different lychee cultivars.

On the other hand ethylene treatment applied to the three cultivars did not affect the colour parameters L * (Figure 4.6.), h ° (Figure 4.7.) and C * (Figure 4.8.), and in general these remained relatively constant during storage for all the studied cultivars. The absence of changes in the progression of colour after the application of ethylene has already been described, in strawberries and cherries (Gong et al., 2002; Li et al., 1994; Tian et al., 1997). Gong et al. (2002) have suggested that the regulation of colour changes during sweet cherry ripening occurs independently of ethylene's activity or the lack thereof.

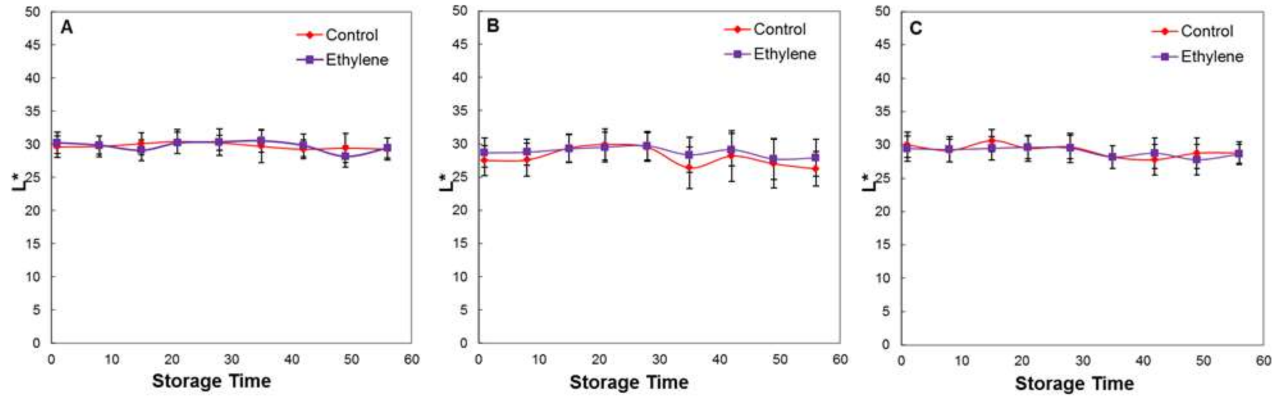

Figure 4.6.– Effect of ethylene treatment on the colour parameters L * during storage of 'Bluecrop' (A), 'Goldtraube' (B) and 'Ozarkblue' (C), blueberry fruit at 4 ºC. Results are expressed as mean ± SD (n=20).

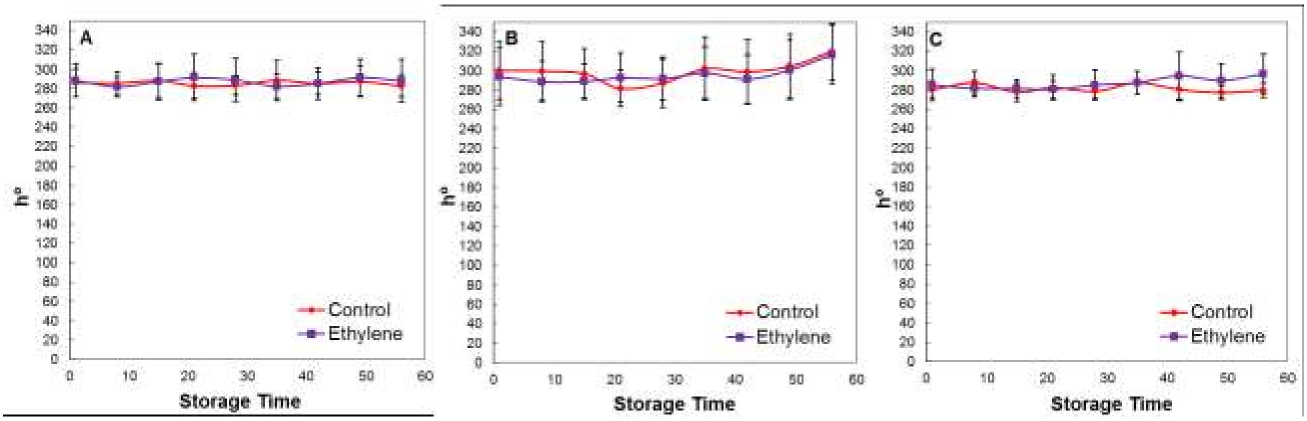

Figure 4.7.– Effect of ethylene treatment on the colour parameters h° during storage of ‘Bluecrop’ (A), ‘Goldtraube’ (B) and ‘Ozarkblue’ (C), blueberry fruit at 4 ºC. Results are expressed as mean ± SD (n=20).

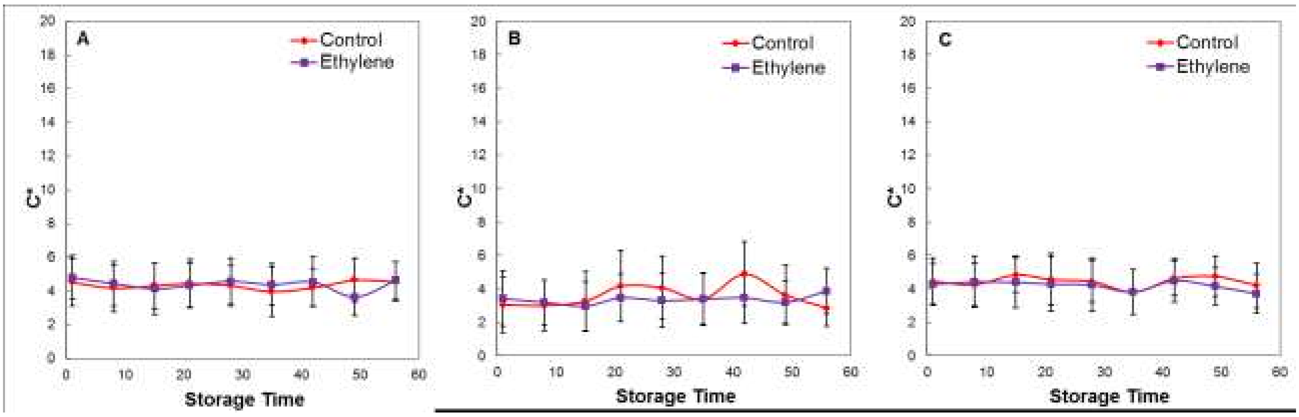

Figure 4.8.– Effect of ethylene treatment on the colour parameters C * during storage of ‘Bluecrop’ (A), ‘Goldtraube’ (B) and ‘Ozarkblue’ (C), blueberry fruit at 4 ºC. Results are expressed as mean ± SD (n=20).

The ethylene treatment induced an increase in anthocyanin content and antioxidant activity of ‘Bluecrop’ and ‘Goldtraube’ blueberries. Thus improve the functional value, without causing changes in colour/appearance. Sensory panel also did not identify significant differences in ethylene treated fruits compared to untreated ones, for the different qualitative descriptors as exterior colour, off odors, flavor, off flavor, resistance to breaking the cuticle, fruit total hardness, sweetness and adstringency (figure 4.9.a., 4.9.b. and 4.9.c.).

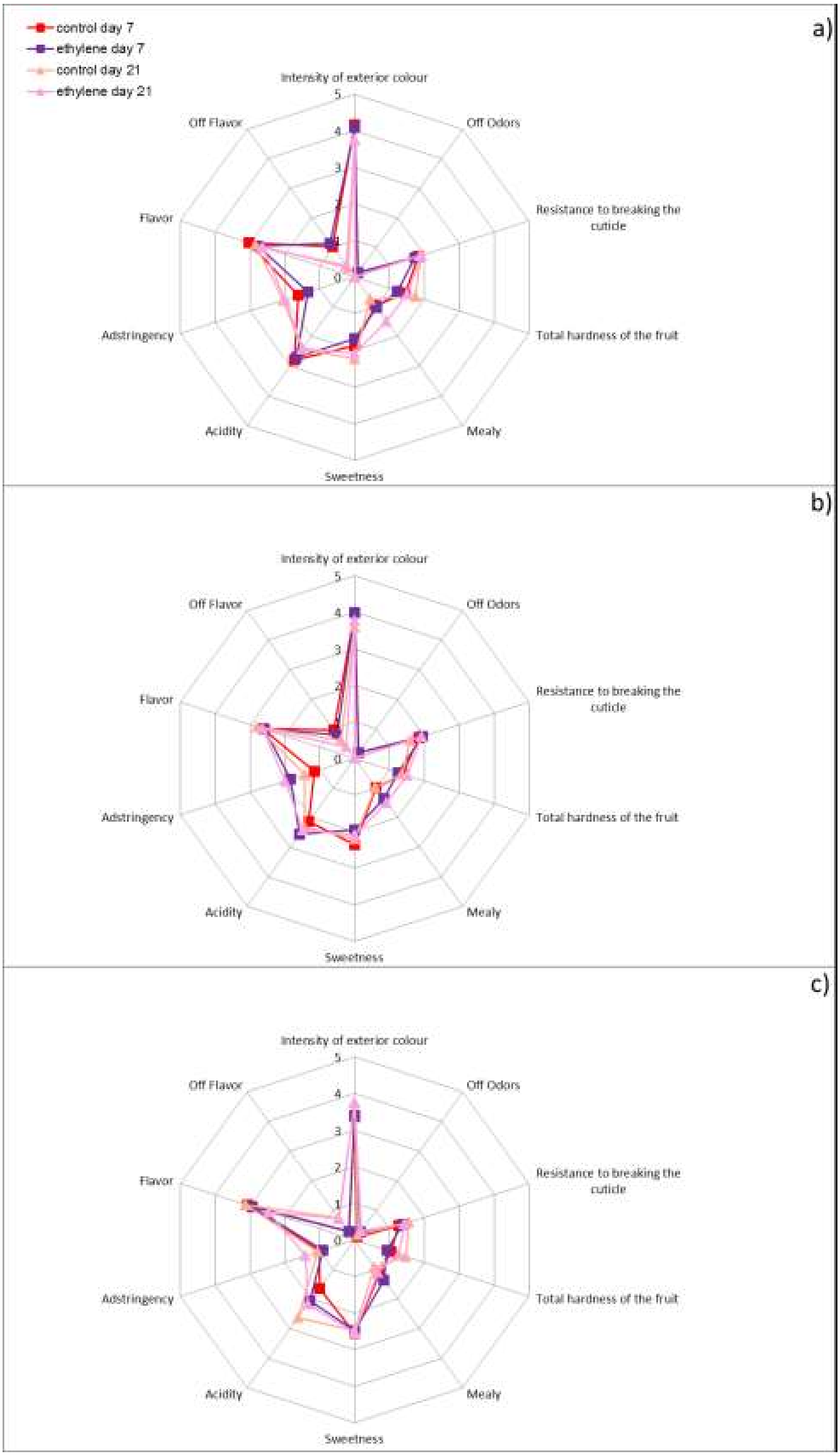

Figure 4.9. - Effect of ethylene treatment on the sensory profile after 7 and 21 days of storage of: a) 'Bluecrop', b) 'Goldtraube', c) 'Ozarkblue'.

4.4.3. Effect of ethylene treatment on the profile of anthocyanins and other polyphenols

In ‘Bluecrop’ and ‘Goldtraube’, nine anthocyanins: delphinidin 3-O-galactoside, delphinidin 3-O-glucoside, cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, cyanidin 3-O-arabinoside, petunidin 3-O-glucoside, malvidin 3-O-galactoside, malvidin 3-O-glucoside and peonidin-3-galactoside, and two hydroxycinnamic acids: ferulic and chlorogenic acids were identified and quantified. In ‘Ozarkblue’, seven anthocyanins (delphinidin 3-O-galactoside, cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, cyanidin 3-O-arabinoside, malvidin 3-O-galactoside, malvidin 3-O-glucoside, peonidin-3-O-galactoside) and the same two hydroxycinnamic acids were identified and quantified. Timmers et al. (2017), Pertuzatti et al. (2016) and Bunea et al. (2013) also observed that anthocyanins were derived from five anthocyanidins: delphinidin, cyanidin, petunidin, peonidin, and malvidin and different anthocyanin chromatographic profiles in different blueberry cultivars.

Genetic factors predispose certain cultivars to accumulate higher levels of anthocyanins, thus Bunea et al. (2013); Pertuzatti et al. (2016); Timmers et al. (2017) also found that different cultivars of blueberries showed different anthocyanins profiles. However, Lohachoompol et al. (2008), when studying three blueberry cultivars, found that the anthocyanin profile was similar in all cultivars, but the proportions of each compound were cultivar-dependent.

The application of ethylene influenced the quantitative profile of anthocyanin in ‘Bluecrop’ and ‘Goldtraube’. In ‘Bluecrop’ on day 1 of storage after treatment, all anthocyanins except cyanidin 3-O-glucoside and malvidin 3-O-glucoside had been influenced by ethylene, (Figure 4.10., 4.11. and 4.12.). By the end of the study, no ethylene effect was observed in these anthocyanins. In terms of chlorogenic acid levels in

‘Bluecrop’, this compound was influenced by ethylene only after 28 days after treatment (table 4.3.). Ferulic acid was affected after 1 and 56 days after treatment.

In ‘Goldtraube’ one day after treatment an increase in the following anthocyanins was identified: delphinidin 3-O-glucoside, cyanidin 3-O-galactoside cyanidin 3-O-glucoside, malvidin 3-O-glucoside and petunidin 3-O-glucoside, though malvidin 3-O-galactoside decreased. By the end of the study, with this treatment, the identified anthocyanins delphinidin 3-O-glucoside, malvidin 3-O-glucoside and petunidin 3-O-glucoside were affected by ethylene, and treated fruit showed a higher content of this compounds (Figure 4.10. and 4.12.). The levels of chlorogenic acid was influenced by ethylene only after one day and ferulic acid was influenced by ethylene after one day and 28 days after the treatment (table 4.3.).

In ‘Ozarkblue’ some of the identified anthocyanins were influenced ($p<0,005$) negatively by ethylene, namely cyanidin 3-O-galactoside and cyanidin 3-O-arabinoside (Figure 4.11.). The content of these anthocyanins decreased one day after the treatment. By the end of storage only malvidin 3-O-galactoside showed significant impact. Chlorogenic acid levels were influenced by ethylene during storage in a negative way (table 4.3.). The level of ferulic acid was not influenced by treatment during the storage period.

For ‘Bluecrop’ and ‘Goldtraube’, the treatment proved to be valuable in that it caused an increase in the content of the identified anthocyanins and hydroxycinnamic acids. However, ‘Ozarkblue’ did not respond positively to treatment. Most of the compounds identified in ‘Bluecrop’ and ‘Goldtraube’ decreased during storage. However, treatment with ethylene compared with control showed a positive smaller decrease. Wicks and Kliewer (1983) found in grapes that the response of anthocyanin accumulation to ethephon application is highly cultivar-specific.

Table 4.3. - Effect of ethylene treatment on the composition of relevant polyphenolics (chlorogenic acid and ferulic acid) (g kg^{-1} fruit) in 'Bluecrop', 'Goldtraube' and 'Ozarkblue' blueberry fruit during storage at 4 ºC.

Cultivar	Compound g kg^{-1} fresh weight	Treatment	Storage times (days)		
			1	28	56
Bluecrop	**Chlorogenic Acid**	**Control**	0.349±0.040^{a}	0.237±0.013^{b}	0.234±0.035^{b}
		Ethylene	0.361±0.085^{a}	0.286±0.047^{ab}	0.228±0.071^{b}
	Ferulic Acid	**Control**	1.317±0.010^{ab}	1.177±0.136^{bc}	0.863±0.118^{d}
		Ethylene	1.490±0.356^{a}	1.128±0.248^{bcd}	1.025±0.071^{cd}
TOTAL		**Control**	1.666±0.050^{a}	1.414±0.149^{b}	1.097±0.153^{cde}
		Ethylene	1.851±0.441^{a}	1.414±0.295^{b}	1.253±0.142^{bc}
Goldtraube	**Chlorogenic Acid**	**Control**	0.156±0.036^{b}	0.110±0.045^{b}	0.105±0.044^{b}
		Ethylene	0.220±0.019^{a}	0.116±0.046^{b}	0.108±0.043^{b}
	Ferulic Acid	**Control**	0.677±0.137^{bc}	0.576±0.179^{c}	0.309±0.066^{d}
		Ethylene	0.805±0.066^{b}	1.015±0.111^{a}	0.317±0.010^{d}
TOTAL		**Control**	0.833±0.173^{efg}	0.686±0.224^{gh}	0.414±0.110^{i}
		Ethylene	1.025±0.085^{cdef}	1.131±0.157^{cd}	0.425±0.053^{hi}
Ozarkblue	**Chlorogenic Acid**	**Control**	0.214±0,070^{a}	0.181±0.007^{ab}	0.111±0.034^{cd}
		Ethylene	0.155±0.002^{bc}	0.098±0.010^{d}	0.077±0.011^{d}
	Ferulic Acid	**Control**	0.573±0.185^{bc}	0.732±0.095^{ab}	0.720±0.069^{ab}
		Ethylene	0.545±0.170^{c}	0.809±0.061^{a}	0.649±0.104^{abc}
TOTAL		**Control**	0.787±0.255^{fg}	0.913±0.102^{defg}	0.831±0.103^{efg}
		Ethylene	0.700±0.172^{g}	0.907±0.071^{defg}	0.726±0.115^{g}

Results are expressed as mean ± SD (n=3). Different letters represent significant differences ($P < 0.05$) between storage day and treatment for each compound and cultivar, by the Tukey test.

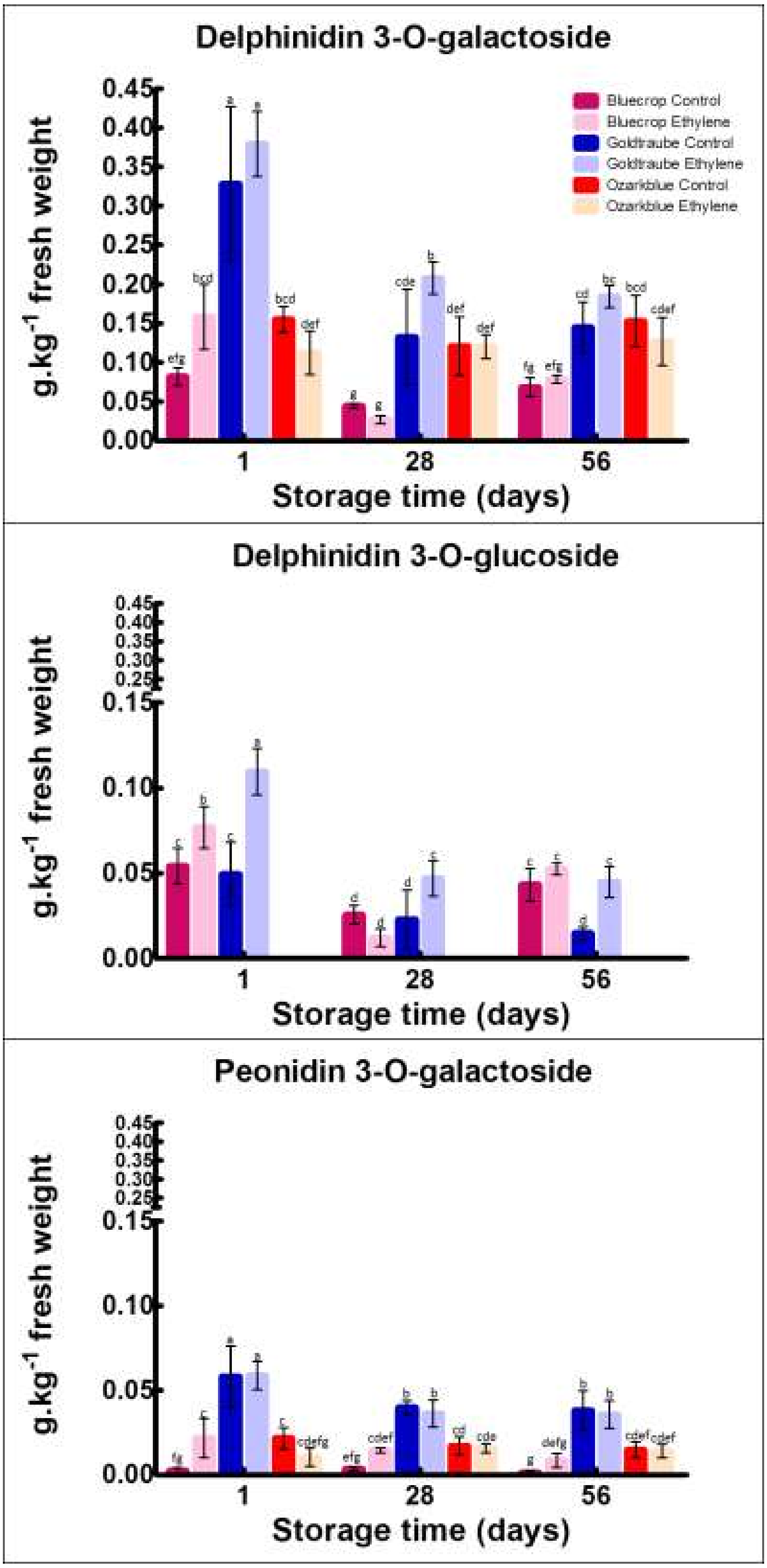

Figure 4.10. – Effect of ethylene treatment on Delphinidin 3-O-galactoside, Delphinidin 3-O-glucoside and Peonidin 3-O-galactoside in blueberry fruit during storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

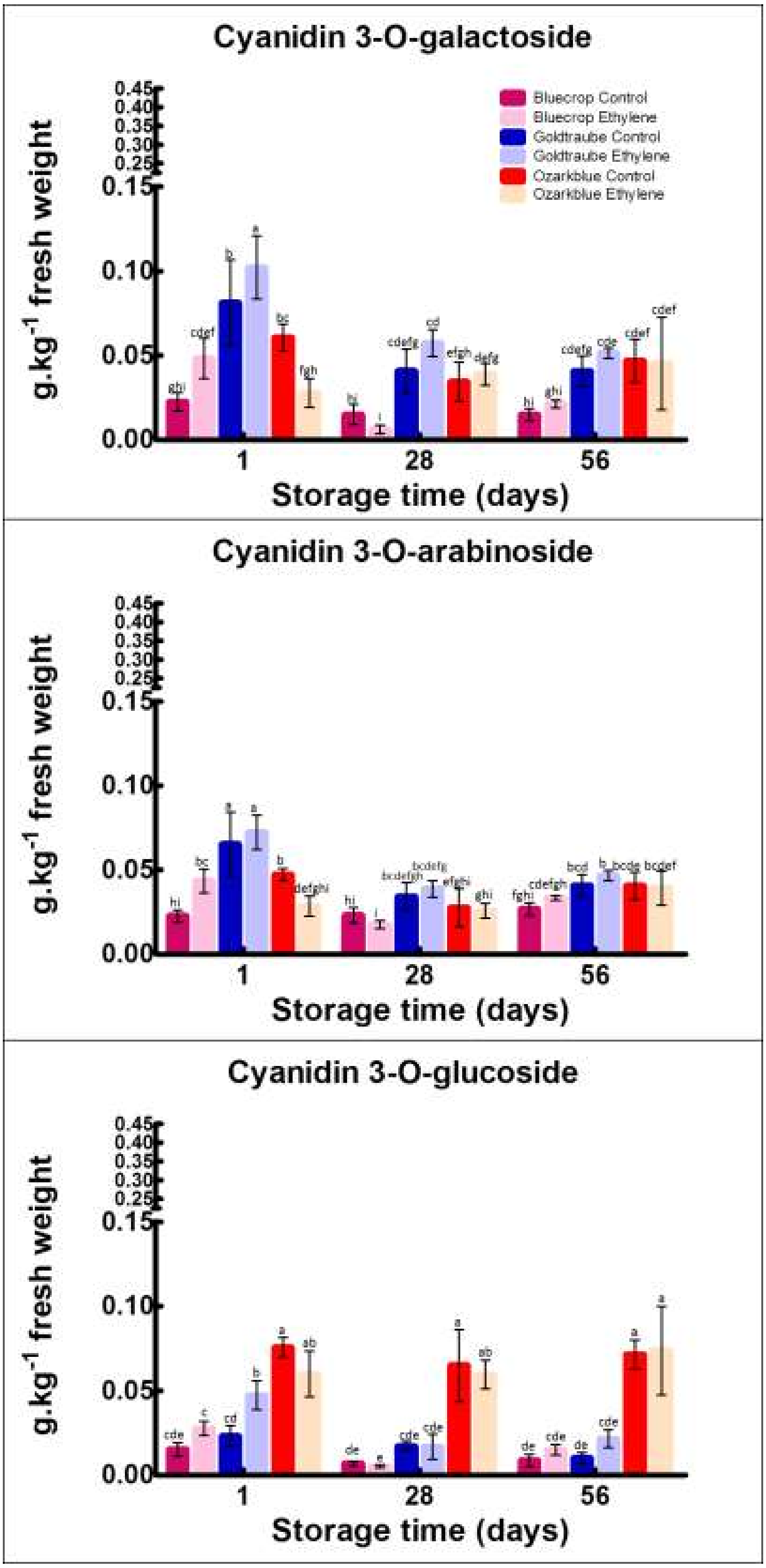

Figure 4.11. – Effect of ethylene treatment on Cyanidin 3-O-galactoside, Cyanidin 3-O-arabinoside and Cyanidin 3-O-glucoside in blueberry fruit during storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

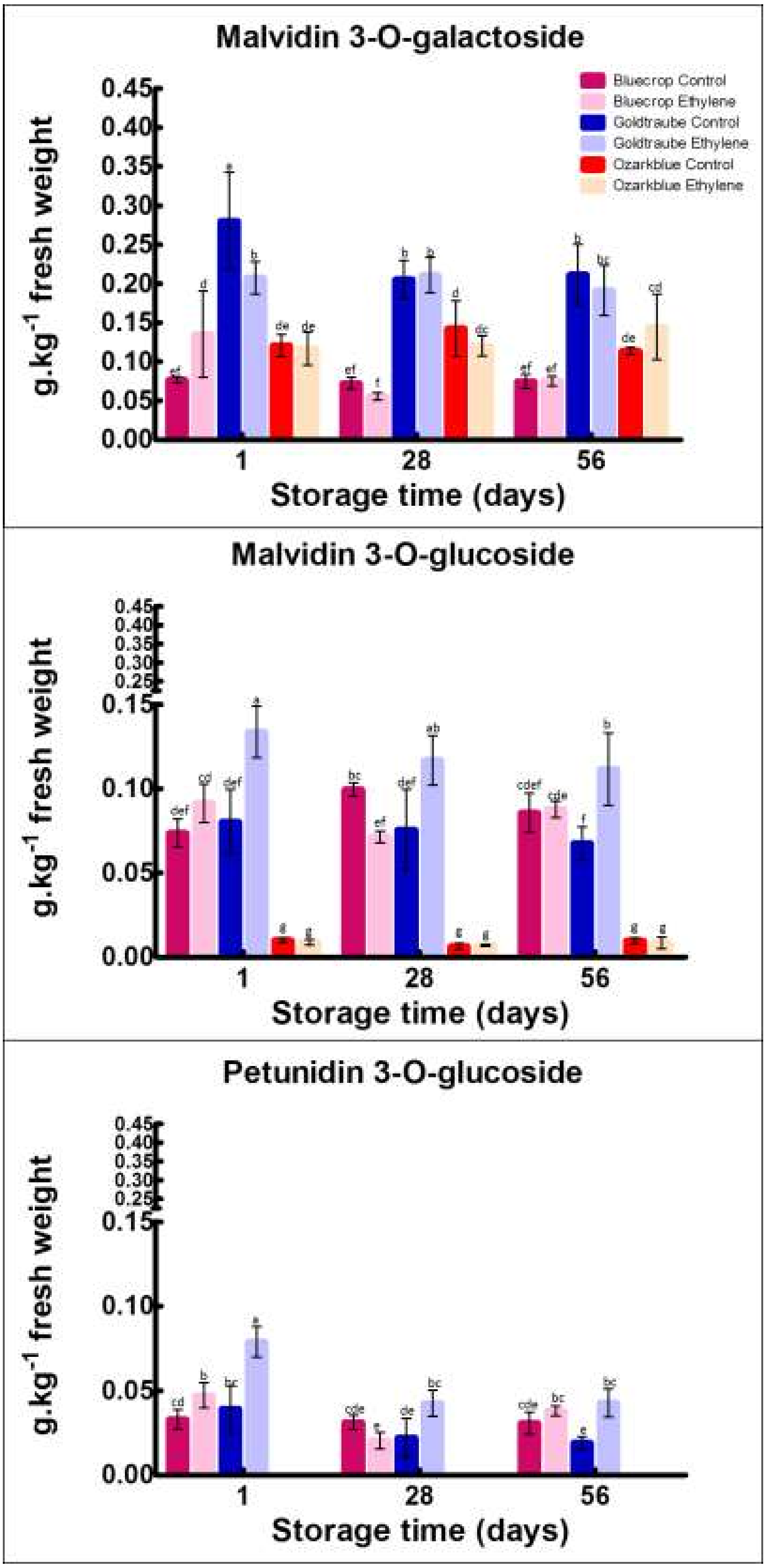

Figure 4.12. – Effect of ethylene treatment on Malvidin 3-O-galactoside, Malvidin 3-O-glucoside, Petunidin 3-O-glucoside in blueberry fruit during storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

He et al. (2010) also found that the application of exogenous phytohormones can greatly modify the expression of the genes involved in anthocyanin biosynthesis, as well as the production and accumulation of anthocyanins in grape berries. Additionally, in cranberry fruit, Craker and Wetherbee (1973) observed that ethylene treatment promoted the synthesis of anthocyanins. Villarreal et al. (2010) also observed that ethylene could play a role in some ripening regulation aspects in non-climacteric fruit such as the strawberry. Specifically, ethylene could stimulate anthocyanins accumulation and the degradation of chlorophylls.

El-Kereamy et al. (2003) also found an increase in the levels of each of the anthocyanins analysed in grape berries over the ten days following treatment with ethylene, though the levels then decreased. Ethylene treatment has led to increased levels of chalcone synthase (CHS) and flavanone 3- hydroxylase (FHT).

Ethephon increased PAL activity in table grapes (Steenkamp et al., 1977). PAL catalyses the first step in the biosynthesis of phenylpropanoids, which are then modified in a variety of phenolic compounds (Cheng and Breen, 1991).

Exogenous ethylene can elicit the synthesis of secondary metabolites (Heredia and Cisneros-Zevallos, 2009). In your study, treatment with ethylene also showed to promote PAL activity as it catalyses the first step in the biosynthesis of phenylpropanoids and thus increasing overall anthocyanins, total phenolic compounds and antioxidant activity in fruit. So, it seems that ethylene can induce PAL activity in blueberries and reinforce phenylpropanoid accumulation as phenolic acids. It appears that it may have also increased UFGT activity, which led to increased production of cyanidin and delphinidin, and of methyltransferase - that may have led to the transformation of these compounds into peonidin, malvidin and petunidin. However, it seems necessary to further study the processes involved in ethylene treatment on blueberries.

4.4.4. Relationship between colour and quantitative / qualitative anthocyanins and other polyphenols

An analysis of factorial principal components (PCA) was carried out to examine the relationship between colour parameters, content of total and individual anthocyanins, and other polyphenols, and antioxidant activity.

Principal components analysis (PCA), which attempts to link the different variables analysed for each cultivar, was established. Based on their contribution by percentage, two main components, PC1 and PC2, were determined. In ‘Bluecrop’ (KMO=0,688), PC1 was largely decided by the identified anthocyanins, PC2 was associated with the colour parameters (Figure 4.10.). The parameters can be associated into four groups (A, B, C and D). Group A combined the identified anthocyanins, the total anthocyanins content and antioxidant activity, group B associated the colour parameters, group C associated chlorogenic and ferulic acid and group D included the total phenolic compounds.

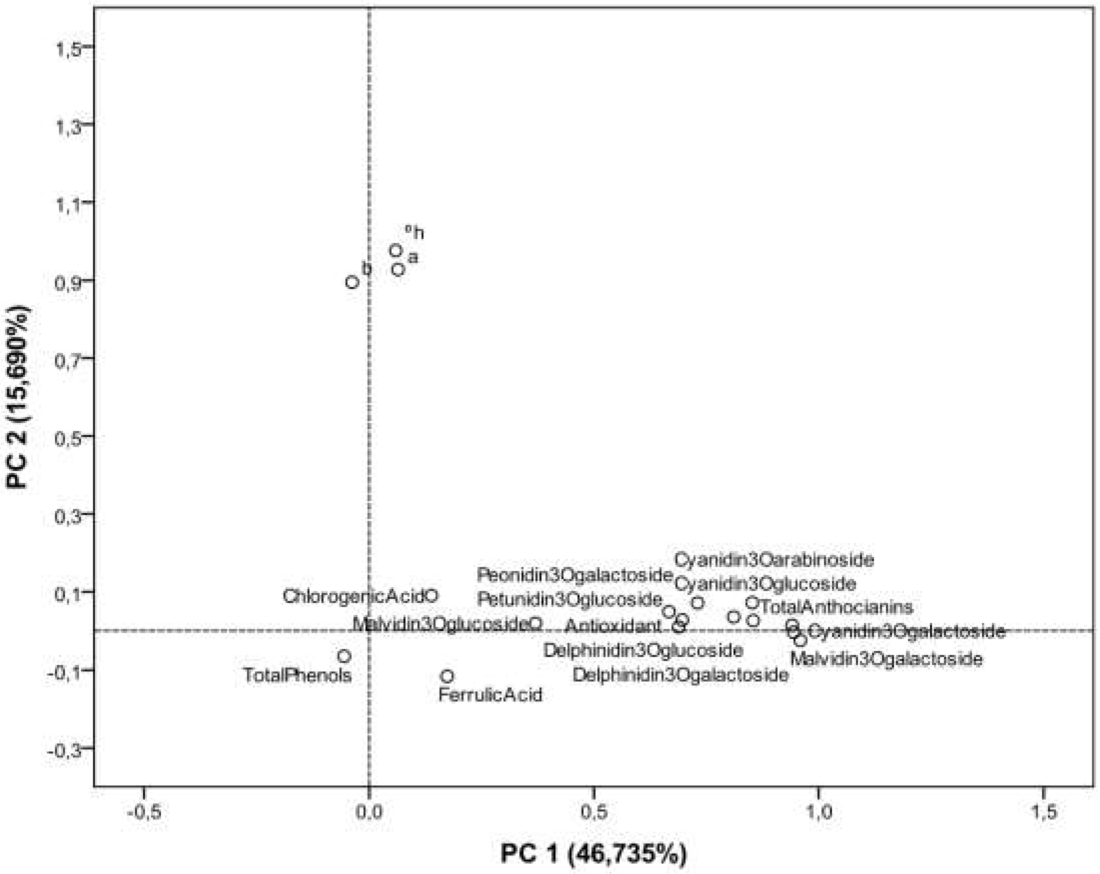

Figure 4.10. – Positions of PCA scores for the composition of anthocyanins, total anthocyanins content, total phenolic compounds, antioxidant activity and some colour parameters in 'Bluecrop', based on PC1 and PC2. The percentage represents the variance of each principal component.

In 'Goldtraube', PC1 was decided largely by the identified anthocyanins, PC2 was associated with the colour parameters (Figure 4.11.). The parameters were associated into four groups (A, B, C and D) (KMO = 0.813). Group A associated the identified anthocyanins, two hydroxycinnamic acids, total anthocyanins content and antioxidant activity, group B associated the colour parameters, group C included the total phenolic compounds and group D only included Malvidin 3-O-galactoside.

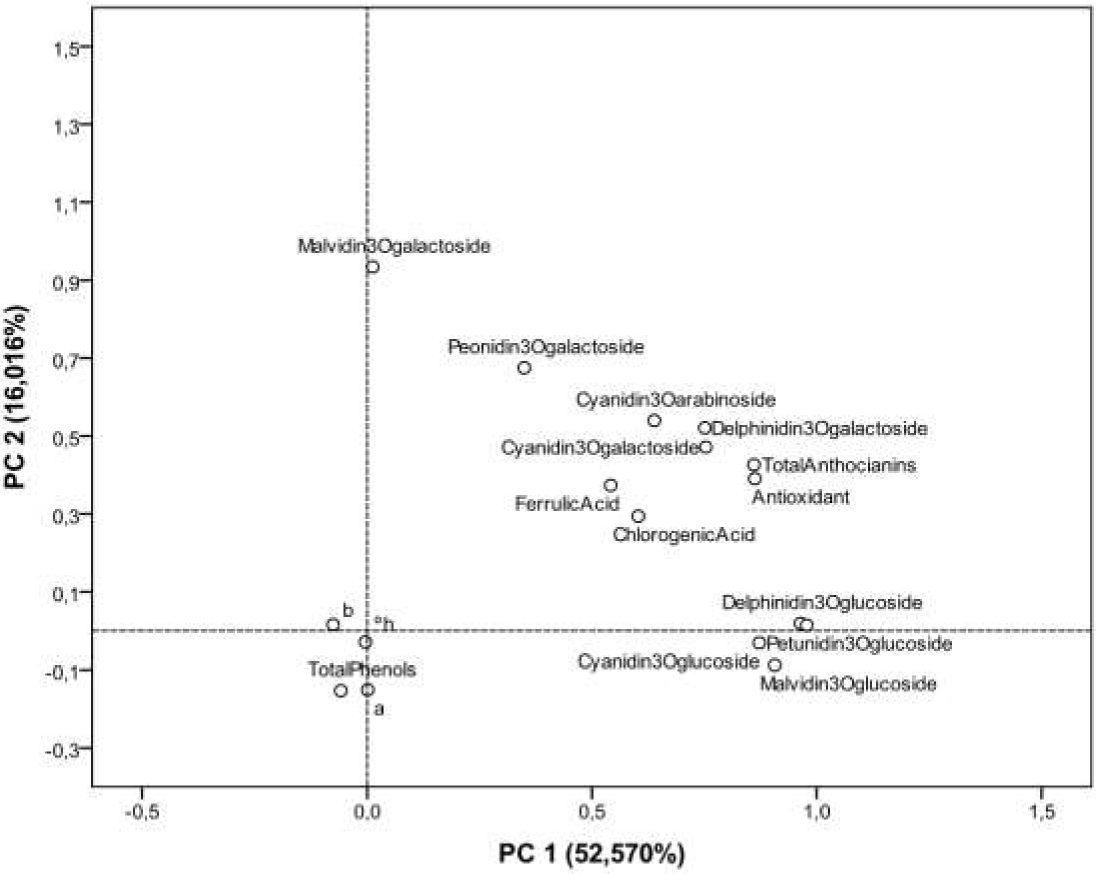

Figure 4.11. – Positions of PCA scores for the composition of anthocyanins, total anthocyanins content, total phenolic compounds, antioxidant activity and some colour parameters in 'Goldtraube', based on PC1 and PC2. The percentage represents the variance of each principal component.

In 'Ozarkblue', PC1 was decided largely by the identified anthocyanins, PC2 was associated with colour parameters (Figure 4.12.). The parameters were also associated into four groups (A, B, C and D) (KMO = 0.655). Group A associated the identified anthocyanins, total anthocyanins content, total phenol, antioxidant activity and group B associated the colour parameters.

In all cultivars, a relationship can be observed between the total antioxidant activity and anthocyanin values, as expected. However, (Kalt et al., 2001) and (Koka and Karadeniz, 2009) related the high blueberries antioxidant activity to the presence of phenolic compounds.

Moreover, an increase in anthocyanins was not correlated with colour parameters, possibly because these fruits have reached luminous saturation.

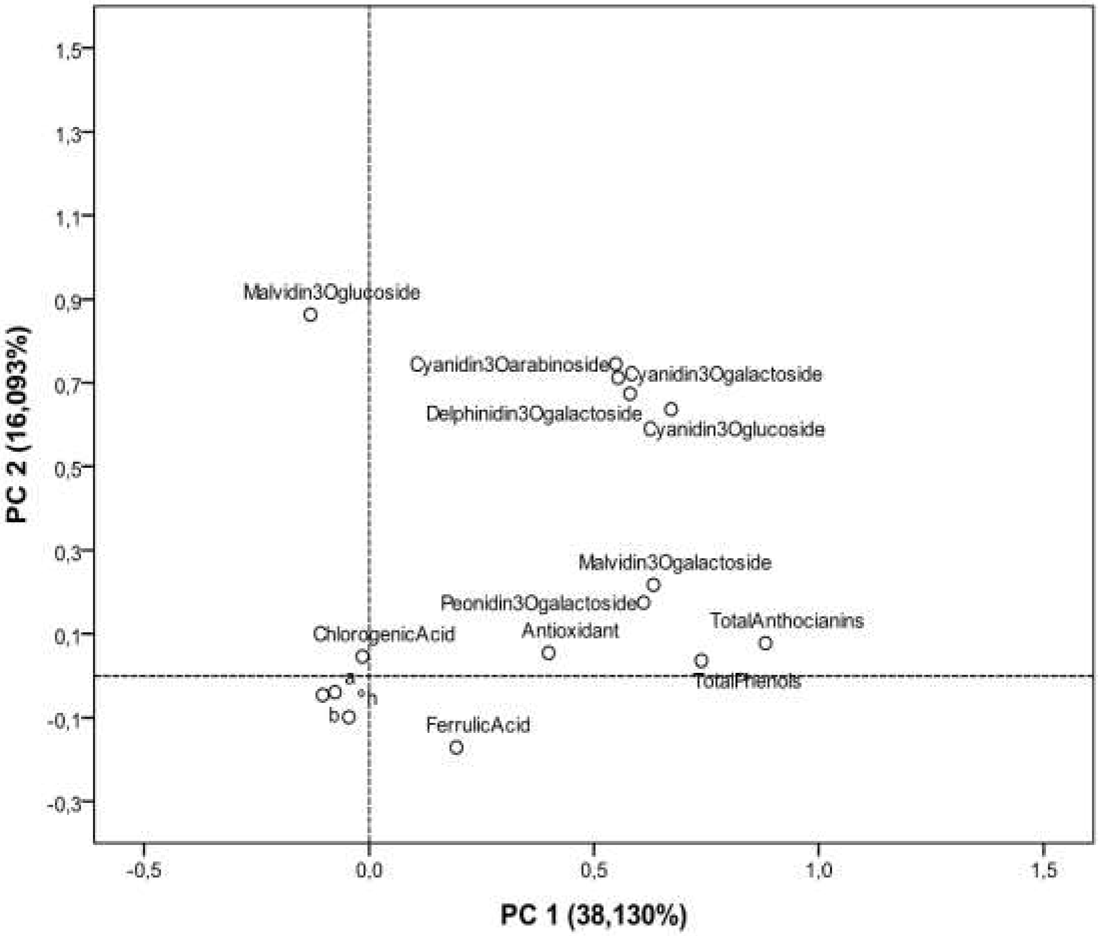

Figure 4.12. – Positions of PCA scores for the composition of anthocyanins, total anthocyanins content, total phenolic compounds, antioxidant activity and some colour parameters in 'Ozarkblue', based on PC1 and PC2. The percentage represents the variance of each principal component.

4.5. Conclusions

Postharvest ethylene treatment enhanced anthocyanin content and antioxidant activity of Northern highbush blueberry cultivars 'Bluecrop', and 'Goldtraube', but not 'Ozarkblue', without apparent detrimental effects on other quality attributes.

The effect of treatment with ethylene on the total anthocyanin, the total antioxidant activity and the different anthocyanins appears to be cultivar dependent. In general, the values of these compounds decreased during storage, showing an extensive decrease over the first 15 days. However, as ethylene treatment increased antioxidant activity due to an increase in most specific anthocyanins, this strategy can prove beneficial towards overcoming the natural decrease observed during storage, assuring a higher level of these compounds throughout this period, and consequently maintaining the fruit's health benefits until the moment of consumption.

Although the blueberry fruit is not considered climacteric, depending on the cultivar it appears that ethylene can affect its physiological processes after harvest, thereby increasing its content in anthocyanins and antioxidant activity.

CHAPTER 5- The effect of postharvest application of ethylene and 1-MCP on commercial and phytochemical quality in three Northern Highbush Blueberry Cultivars

This chapter has been submitted to the journal: Plant Foods for Human Nutrition

5.1. Abstract

Blueberries are characterized by a high antioxidant capacity but this quality parameter levels tend to decrease during storage constraining their functional advantages when they aren't consumed soon after harvest. In this experiment the effect of 1-MCP (1 μL L^{-1}) and ethylene (1000 μL L^{-1}) on some parameters of commercial and functional quality of three blueberry cultivars: Bluecrop, Goldtraube and Ozarkblue were assessed during 56 days of storage. Ethylene treatment led to an initial increase in total anthocyanins content, phenolic compounds and antioxidant activity in 'Goldtraube'. In general, 1-MCP treatment increase these levels at the end of storage period. Concerning the qualitative profiles of individual anthocyanins and hydroxycinnamic acids, the effect of ethylene seems to be cultivar dependent, increasing the content of these compounds in 'Bluecrop' and 'Goldtraube', but not in 'Ozarkblue'. The 1-MCP treated fruits, at the end of storage, showed higher content of anthocyanins compounds and hydroxycinnamic acids in 'Goldtraube' and 'Ozarkblue'.

4.2. Introduction

Blueberry is a fruit characterized by high antioxidant activity (Wolfe et al., 2008; Wu et al., 2004) associated to its high total phenolic content (Prior et al., 1998). Although these properties are highly valorized by consumers, blueberry antioxidant activity, as well as total phenolic compounds content tend to decrease during ripening (Castrejón et al., 2008). However, the antioxidant activity may increase or be maintained during blueberries storage (Connor et al., 2002a; Kalt et al., 1999). Thus, postharvest technologies can be developed to enhance or retain these compounds in blueberries, such as the application of ethylene or 1-Methylcyclopropene (1-MCP). 1-MCP has been used as a postharvest tool to extend shelf life in numerous nutraceutical-producing crops (MacLean et al., 2007) but scarce studies has been developed using this postharvest approach.

For products such as most vegetables and non-climacteric fruit, where further senescence (e.g., yellowing) decreases product value, 1-MCP applications delaying the process may prevent changes and extent shelf-life of these products (Watkins, 2006).

1-MCP is an inhibitor of ethylene action that links at the receptor level (Villarreal et al., 2010). Nevertheless, in non-climacteric fruit the 1-MCP treatment stimulated the ethylene production in ‘Austin’, ‘Brightwell’, ‘Premier’ rabbiteye blueberry (MacLean and NeSmith, 2011), and in sweet cherry ‘Bing’ and ‘Rainier’ (Gong et al., 2002). In strawberry fruit this stimulation was mainly due to an increase in 1-aminocyclopropane-1-carboxylic acid (ACC) content in the tissue without significantly changing ACC oxidase activity (Tian et al., 1997).

Treatment with 1-MCP did not alter the shelf-life quality of the highbush blueberry ‘Burlington’ and ‘Coville’ stored at 10-15 KPa O_2 and 10 KPa CO_2 at -1 to 1 º C for 4, 8 and 12 weeks, followed by a 20 º C shelf-life of up to 20 days (DeLong et al., 2003). 1-MCP reduced weight loss during storage, led to lower total soluble solid content and delayd fruit firmness increase of blueberries ‘Lateblue’ (Chiabrando and Giacalone, 2011). In blueberry ‘O`Neal’ and ‘North Road’ 1-MCP reduced the rotting rate, while keeping high-leveled stability in soluble sugar content and titratable acid content (Tao et al., 2017).

The effect of 1-MCP maintained anthocyanins and phenolic compounds and antioxidant activities of blueberry ‘Lateblue’ (Chiabrando and Giacalone, 2011), and similarly for blueberry ‘O`Neal’ and ‘North Road’ which antioxidant enzyme activities were maintained (Tao et al., 2017) and the high antioxidant capacity of goldenberry fruit during storage was preserved (Valdenegro et al., 2012).

Plants to protect themselves against oxidative damage produce a variety of antioxidant enzymes. Yang et al. (2011) reported that postharvest exposure to 1-MCP enhances oxidative stress tolerance and maintains cell membrane integrity during storage of cherry fruits.

However, higher applications of 1-MCP can lead to an acceleration of the deterioration, for example strawberry fumigation with 500 nL L^{-1} 1-MCP reduced the storage shelf-life by ≈40% compared with non-treated fruit (Ku et al., 1999). Jiang et al. (2001) associated decreased disease resistance of fruit with low levels of phenolic compounds in fruit treated at the highest 1-MCP concentration.

This differential response suggests that 1-MCP may have at least partially independent effects on the regulation of ripening processes in contrast with systems of natural defense against pathogens (Jiang et al., 2001).

Recently, some studies have been carried out with the 1-MCP application in blueberry (Blaker and Olmstead, 2014; Chiabrando and Giacalone, 2011; DeLong et al., 2003; Ji et al., 2014; Tao et al., 2017; Xu and Liu, 2017), all of them considering the physicochemical and sensory quality. Nevertheless, there is no characterization of the profile of anthocyanins and other polyphenols of blueberries throughout the storage period with 1-MCP application. Thus, this work aims to maintain or increase the phytochemicals of three highbush blueberry cultivars during storage by postharvest treatment with 1-MCP and compare with ethylene application, assessing the impact on anthocyanins and other polyphenols content and profile, antioxidant activity and also on quality parameters such as weight loss, firmness and colour.

5.3. Material and Methods

5.3.1. Fruit treatment and storage

Northern highbush blueberry (*Vaccinium corymbosum*) fruit from 'Bluecrop', 'Goldtraube' and 'Ozarkblue' cultivars were harvested at commercial maturity (full blue) in Sever do Vouga, Portugal and cooled to 4 ºC. The 'Bluecrop', 'Goldtraube' and 'Ozarkblue' blueberry plants had seventeen, eleven and thirteen years respectively. Sever do Vouga has temperate climate characteristics of a micro-climate, not subject to excessive temperatures in the hottest month, but whose average minimum temperatures vary between 2 and 4 degrees.

According to the soils charter, the region Sever do Vouga soils are the cambisols humic type (eruptive rocks, schists and shales associated with Luvisols). Blueberries of 'Bluecrop' and 'Goldtraube' were produced in a soil of coarse texture, with a pH of 5.1 and an organic matter content of 12%. The 'Ozarkblue' was produced in soil also with coarse texture, pH 4.6 and organic matter content of 11%.

In this work the fruits were divided into three groups (20 kg of each). One group was subjected to treatment with ethylene, another to treatment with 1-MCP, and a third was stored under normal conditions, without treatment (Control).

Blueberries were treated with ethylene (Sigma-Aldrich) at 1000 $\mu L\ L^{-1}$ for 12 h (4 ºC). Treatments were performed in a 60 L polypropylene container, where 20 kg of fruit were sealed and ethylene was injected with a syringe via the rubber cap. Treatment was performed on each cultivar. Calculations of ethylene concentration were based on the free space volume of the sealed treatment container.

Blueberries were treated with 1-MCP (SmartFresh 0.14%, AgroFresh, Philadelphia, PA, USA) at 1 μL L^{-1} for 12 h (4 ºC). Treatments were performed in a 60 L polypropylene container (20 kg of fruit). 1-MCP was generated inside a volumetric flask by injecting water via a rubber stopper to the SmartFresh powder located in the flask. The 1-MCP gas was generated inside the flask for 10 min and the volume required to yield 1 μL L^{-1} in the treatment container was extracted from the headspace with a syringe and injected into the sealed polypropylene container. Calculation of 1-MCP concentration was based on the free space volume of the sealed treatment container.

Untreated control fruit was handled in the same way (4 ºC) but not exposed to exogenous ethylene neither 1-MCP.

After treatments the fruit were subsequently packaged in ventilated polyethylene terephthalate (PET) clamshells (0,250 kg each) and stored at 4 °C, 70-80% RH, for 56 days. Samples were taken weekly during storage period (0, 7, 14, 21, 28, 35, 42, 49 e 56 days).

5.3.2. Skin colour measurement

Skin colour was determined in the CIE L* a* b* space with a Minolta CR-300 colorimeter (Osaka, Japan) equipped with a D65 illuminant. The data was converted to hue (h °) and chroma (C *) using the relationships described by McGuire (1992). The method as described on section 3.3.7.

Samples were taken weekly during storage.

5.3.3. Weight loss

The average weight loss of fruits was determined by individually weighing 10 fruits with a model ABJ 120 - 4M balance (Kern, Balingen, Germany). Samples were taken weekly during storage.

5.3.4. Firmness

The firmness was measured by puncture using a TA XT Plus texture analyzer (Texture Technologies Corp., London, UK). The test was made with a probe with 2 mm diameter with a travel distance of 5 mm at 1 mm s^{-1}. The puncture was conducted in the equatorial region. Thirty replicates were obtained and the maximum firmness expressed as N mm^{-1}. Samples were taken weekly during storage period (0, 7, 14, 21, 28, 35, 42, 49 e 56 days).

5.3.5. Respiration Rate

Ethylene, 1-MCP-treated and control fruits were weighed (~100 g) and placed in 1000 mL sealed glass jars at 20 °C. The glass jars were sealed for 2 h before analyses of the headspace. Carbon dioxide production rate (mg L h^{-1}) was determined inserting a small needle of CheckMate II, (PBI Dansensor, Ringsted, Denmark) into the glass jar headspace through a rubber septum. Three replicates were made for each treatment. Samples were taken weekly during storage period (0, 7, 14, 21, 28, 35, 42, 49 e 56 days).

5.3.6. Soluble solids content

The soluble solids content was determined in juice using the method according by Castrejón et al. (2008) and Prior et al. (1998) as described on section 3.3.4.

Three replicates were generated for each treatment and for each of sampling points throughout storage time at 0, 7, 14, 21, 28, 35, 42, 49 and 56 days.

5.3.7. Total acidity

Total acidity was measured in an homogenate of 20 fruit using the method according by Castrejón et al. (2008) as described on section 3.3.5.

The total acidity was performed weekly during 56 days of storage.

5.3.8. Sensory evaluation

The Attribute Intensity Ranking was used to assess the sensory properties. The sensory evaluation sessions took place in the ISO 8589:2007 compliant sensory testing facilities of Escola Superior de Biotecnologia - Universidade Católica Portuguesa (ESB-UCP). This method as described on section 4.3.12..

'Bluecrop' and 'Goldtraube' fruits were sampled after 7 and 21 days of storage at 4 ºC. 'Ozarkblue' fruits were sampled after 7 days of storage at 4 ºC.

5.3.9. Extracts preparation

Extraction of phenolic compounds was performed according to Kalt et al. (1999) with minor modifications, as described on section 3.3.8.

Three replicates were made for each treatment and for each of sampling points throughout storage time at 0, 14, 28, 42, and 56 days.

5.3.10. Total anthocyanins content

Evaluation of anthocyanins was performed by the differential pH method (Giusti and Wrolstad, 2001) as described on section 3.3.9.

Three replicates were made for each sample and for each of sampling points throughout storage time at at 0, 14, 28, 42, and 56 days.

5.3.11. Total phenolic compounds

The content of phenolic compounds was determined by the Folin-Ciocalteu reagent (Slinkard and Singleton, 1977) this method as described on section 3.3.10.

All measurements were performed in triplicate, for each of sampling points throughout storage time at 0, 14, 28, 42, and 56 days.

5.3.12. Antioxidant activity

Antioxidant activity was evaluated by the ABTS$\cdot^{+}$ radical cation decolourization assay (Gião et al., 2007) as described on section 3.3.11.

All measurements were performed in triplicate, for each of sampling points throughout storage time at 0, 14, 28, 42, and 56 days.

5.3.13. HPLC-DAD analysis

Qualitative and quantitative profiles of anthocyanins and hydroxycinnamic acid (namely chlorogenic and ferulic acids) present in the extracts were determined by HPLC-DAD (Waters Series 600, Mildford MA, USA). This method was adapted from Silva et al. (2013b), as described on section 3.3.12.

5.3.14. Statistical analysis

The experiments designed to evaluate the effect of treatment (ethylene, 1-MCP and control) *per se* were conducted in a completely randomized block design with three replicates per treatment. Data was analysed by two-factor ANOVA with treatment (ethylene, 1-MCP and control). Mean separation was performed with the Least Significant Difference (Tukey) at $\alpha = 0.05$. The effect of the treatments (ethylene, 1-MCP and control) in weight loss rate was analysed by linear regression. The association between treatment, firmness, colour measurement, respiration rate, antioxidant activity, anthocyanins and polyphenols profiles was estimated using an analysis of factorial components (PCA).

All ANOVA and correlation analyses were performed with the statistical software SPSS 25.0 for Windows (SPSS, Chicago, USA).

5.4. Results and Discussion

5.4.1. Effect of ethylene and 1-MCP treatment on skin colour, firmness, weight loss rate, respiration rate, soluble solids content, total acidity and sensory evaluation

The colour parameters L *, C * and h° depicted in table 5.1., showed to be relatively constant during storage. The colour parameter L* was significantly ($p < 0.01$) influenced by the treatment at 1, 7, 28, 42 and 56 days of storage. At the end of storage 'Bluecrop' and 'Ozarkblue' (Table 5.1.) showed the highest L* value with 1-MCP treatment, but in the case of 'Goldtraube' (Table 5.1.) the highest L* value was attained with ethylene or even control. The parameter C* was influenced by the treatment, but not in a consistent way for all three cultivars. 'Bluecrop' and 'Ozarkblue' fruits, at the end of storage after ethylene treatment, presented the highest values of C *. Also at the end of storage, the parameter h° was influenced by the treatment in 'Goldtraube' and 'Ozarkblue'. The highest value was generally attained in the 1-MCP treated fruit.

Table 5.1. - The effect of ethylene and 1-MCP on the colour parameters: L*, C* and °h in 'Bluecrop', 'Goldtraube' and 'Ozarkblue' blueberry fruit during storage at 4 ºC.

CULTIVAR	TREATMENT		DAC 1	DAC7	DAC14	DAC21	DAC28	DAC35	DAC 42	DAC49	DAC56
BLUECROP	CONTROL	L*	29,27^{b}	31,49^{a}	31,37	31,24	30,36^{b}	31,10	31,15	32,46^{a}	30,19^{b}
	1-MCP		31,66^{a}	30,46^{ab}	31,60	31,32	32,44^{a}	30,96	31,13	31,22^{b}	32,07^{a}
	ETHYLENE		32,45^{a}	29,78^{b}	30,83	30,90	31,27^{b}	30,44	31,14	32,52^{a}	30,99^{b}
GOLDTRAUBE	CONTROL		28,70	26,55^{b}	26,56^{b}	29,37^{a}	26,75	30,17	29,15	31,69	29,76^{a}
	1-MCP		27,84	28,24^{a}	27,82^{a}	27,89^{b}	27,72	30,65	28,47	31,03	28,42^{b}
	ETHYLENE		28,72	28,69^{a}	28,07^{a}	26,96^{b}	27,85	29,45	28,94	30,89	30,35^{a}
OZARKBLUE	CONTROL		30,29	29,69	30,40	30,04	31,55^{a}	31,10	31,97	32,61	29,96^{b}
	1-MCP		30,01	29,85	30,75	30,84	30,47^{b}	31,38	32,03	32,02	31,74^{a}
	ETHYLENE		30,76	30,08	31,03	30,88	31,06^{ab}	31,42	31,35	31,88	30,29^{b}
BLUECROP	CONTROL	C*	3,68^{b}	4,53^{a}	4,53	4,90	4,47^{b}	4,71	5,00	4,72^{a}	4,01^{b}
	1-MCP		5,06^{a}	4,56^{a}	4,42	4,93	5,50^{a}	4,74	4,75	3,97^{b}	4,12^{b}
	ETHYLENE		5,23^{a}	3,85^{b}	4,32	4,65	4,93^{b}	4,54	4,98	4,80^{a}	4,48^{a}
GOLDTRAUBE	CONTROL		3,13	2,85^{b}	3,06^{b}	3,91^{a}	2,94	3,36	3,23	4,05	3,78
	1-MCP		2,82	3,61^{a}	3,42^{ab}	3,28^{b}	3,32	3,79	3,15	4,26	3,69
	ETHYLENE		3,24	3,65^{a}	3,59^{a}	3,26^{b}	3,41	2,90	3,69	3,96	4,24
OZARKBLUE	CONTROL		4,48	4,69	4,738	4,62	5,26	4,33	5,63^{a}	4,83	3,36^{b}
	1-MCP		4,70	4,80	4,95	5,10	5,18	4,64	4,95^{b}	4,25	4,29^{ab}
	ETHYLENE		4,90	5,02	5,17	4,92	5,15	4,54	5,04^{ab}	4,41	4,80^{a}
BLUECROP	CONTROL	h^{o}	284,60	271,81^{b}	271,81^{b}	271,88^{b}	274,03	277,50	273,18^{b}	269,72^{b}	281,03
	1-MCP		282,82	294,35^{a}	280,04^{a}	284,07^{a}	281,75	283,91	284,94^{a}	282,54^{a}	278,38
	ETHYLENE		275,17	283,90^{a}	276,11^{ab}	281,63^{a}	281,35	279,98	278,78^{ab}	273,76^{b}	277,31
GOLDTRAUBE	CONTROL		263,63^{b}	275,05^{a}	270,81	268,43^{b}	276,74	269,48^{b}	280,36^{b}	270,40^{b}	277,99^{b}
	1-MCP		261,97^{b}	266,34^{b}	267,90	273,16^{ab}	275,42	277,17^{b}	298,12^{a}	289,95^{a}	315,40^{a}
	ETHYLENE		271,97^{a}	265,19^{b}	270,24	278,80^{a}	274,31	293,84^{a}	300,40^{a}	292,03^{a}	291,45^{b}
OZARKBLUE	CONTROL		270,42	272,52	279,02^{a}	281,55^{a}	277,65^{b}	273,46^{b}	298,81^{a}	296,42^{a}	287,31^{b}
	1-MCP		269,37	273,74	276,74^{ab}	275,21^{b}	285,49^{a}	285,28^{a}	272,05^{b}	277,46^{b}	289,43^{ab}
	ETHYLENE		269,79	276,79	272,30^{b}	274,92^{b}	272,07^{b}	282,28^{ab}	289,55^{a}	292,39^{ab}	304,84^{a}

Different letters represent significant differences (*P*<0.05) between columns, by the Tukey test.

Xu and Liu (2017) reported no significant difference in L* value between 1-MCP treated compared to untreated blueberries 'Berkeley' blueberries, during storage period at 4 ± 1 °C, just lasted 8 days. Gong et al. (2002) also did not observe any effects of 1-MCP (0,1; 1 or 10 µL.L^{-1}) application on colour change of 'Bing' cherries (the fruit were stored at 20 ºC for 7 days). However, Sivakumar and Korsten (2010) also observed an increase in the L* value with 1-MCP treatment in litchi stored 3% O_2 + 7% CO_2 for 21 days at 2 °C and 90% RH. In this study, at the end of storage the treatment with 1-MCP induced a general lower lightness and a more intense blue hue colour in ‘Goldtraube’.

Fruit firmness has been influenced by cultivar and treatment (Figure 5.1.). The ‘Bluecrop’ fruits showed the hogher firmness after the treatment with 1-MCP. At the end of storage, the ‘Goldtraube’ fruits showed lower firmness after treatment with ethylene and 1-MCP, relatively to the control treatment. The 1-MCP treated ‘Goldtraube’ fruits firmness throughout storage was only significantly affected after 49 and 56 days, with the control treated fruit presenting the greatest firmness values. MacLean and NeSmith (2011) also observed decreased firmness in Rabbiteye blueberry fruit treated with a postharvest application of 1-MCP (1 µL.L^{-1}, fruit stored 2 w at 0 - 1 º C and 90 a 95% de HR). Probably the concentration 1 µL.L^{-1} was not the most effective in retention of firmness. In ‘Bluecrop’, treatment with 1-MCP led to firmer fruits throughout storage with the exception of the first day after the treatment, where no significant differences between treatments were found. Softening was clearly delayed for treatment with 1-MCP in ‘Lateblue’ blueberry (0,3; and 0,6 µL.L^{-1}) storage during 35 d at 0 ºC (Chiabrando and Giacalone, 2011); and in ‘Isabel’ grape (2 µL.L^{-1}), from the 8 to 12 days storage (25±2°C and 75±2% RH) (Silva et al., 2013a). Thus, 1-MCP-induced firmness retention can be a result of the suppression of activity of the enzymes polygalactoronase, glucanase, and pectinmethylesterase in 1-MCP-treated berries (Silva et al., 2013a).

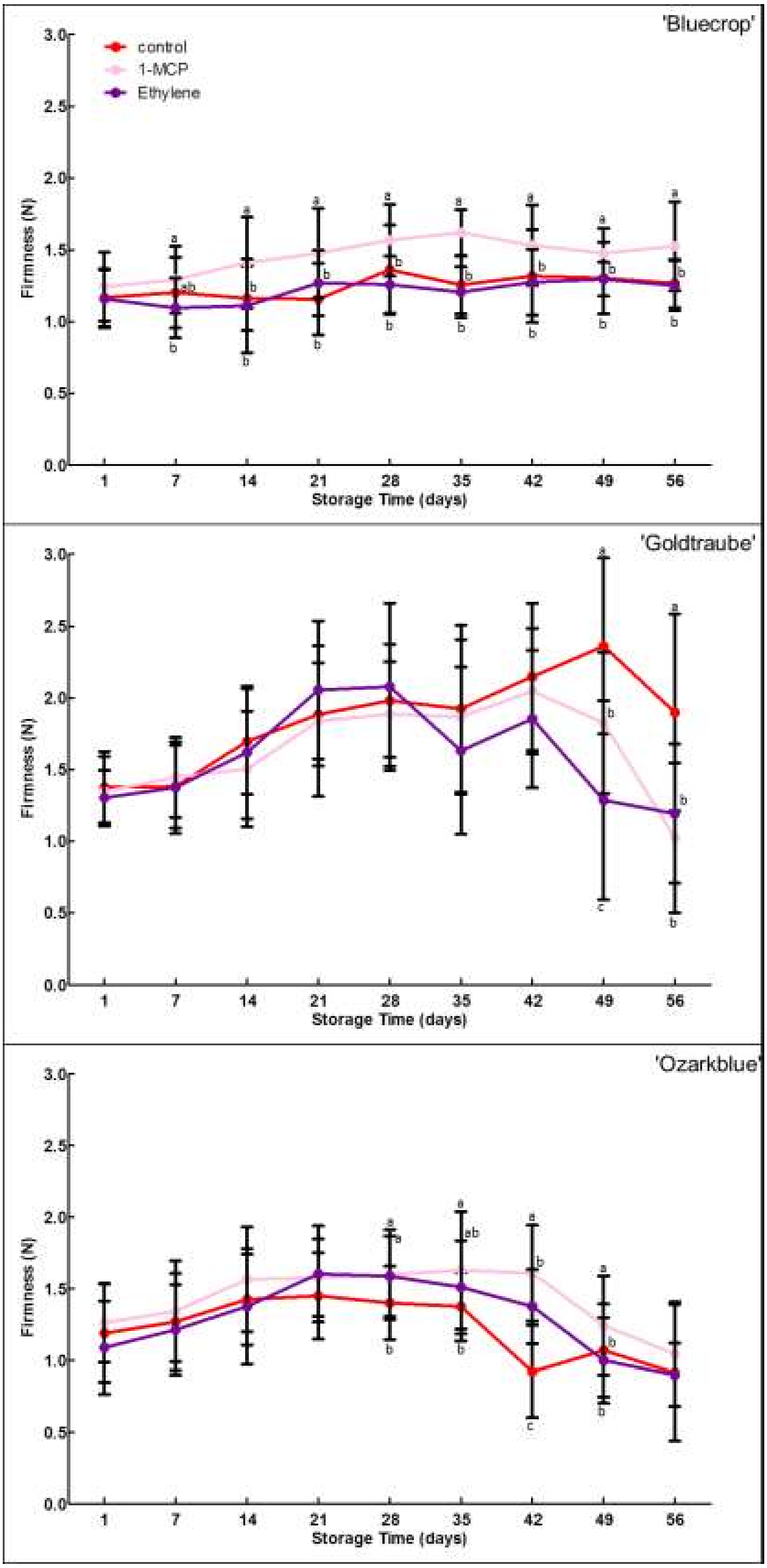

Figure 5.1. – Effect of ethylene and 1-MCP on firmness (n=30), of blueberries during storage at 4 ºC. Results are expressed as mean ± SD. Different letters represent significant differences (P < 0.05), by the Tukey test.

The ‘Ozarkblue’ firmness was affected by 1-MCP on day 28, 35, 42 and 49, showing higher firmness then the control fruits. The decrease of firmness during storage can be associated with the rate of water loss (Paniagua et al., 2013) or to be a result of hemicellulosic depolymerization (Vicente et al., 2007). The treatment with 1-MCP induced firmness retention in ‘Bluecrop’ and ‘Oarkblue’.

Fruit weight loss was similar to that obtained by (Grozeff et al., 2017) in ‘Misty’ and ‘Blue Cuinex’ blueberries stored at 4 ° C for 14 days. Treatment with 1-MCP compared to control, allowed to extend one week the limit of acceptance by the consumers (8%) (Paniagua et al., 2013). In this way, the fruits presented an acceptable value at 49 days of storage in ‘Bluecrop’, 42 days in ‘Ozarkblue’ and 28 days in ‘Goldtraube’ cultivar. The weight loss rate was influenced by cultivar (Figure 5.2. and Table 5.2.). In general the ‘Goldtraube’ was the one with the highest rate of weight loss and ‘Ozarkblue’ the least. The treatment with 1-MCP did not affect the weight loss rate in ‘Bluecrop’ and ‘Goldtraube’. However, treatment influenced the weight loss rate in ‘Ozarkblue’ on days 28, 35 and 42 of storage. The control treatment showed the greatest weight loss. Some authors also showed that treatment with 1-MCP reduced weight loss during storage in ‘Lateblue’ blueberries (0,3 or 0,6 $\mu L.L^{-1}$, 35 days at 0 ºC) (Chiabrando and Giacalone, 2011), ‘O’Neal’ and ‘North Land’ blueberries (10, 20 and 30 $\mu L.L^{-1}$, 30 d at 4 ºC) (Tao et al., 2017).

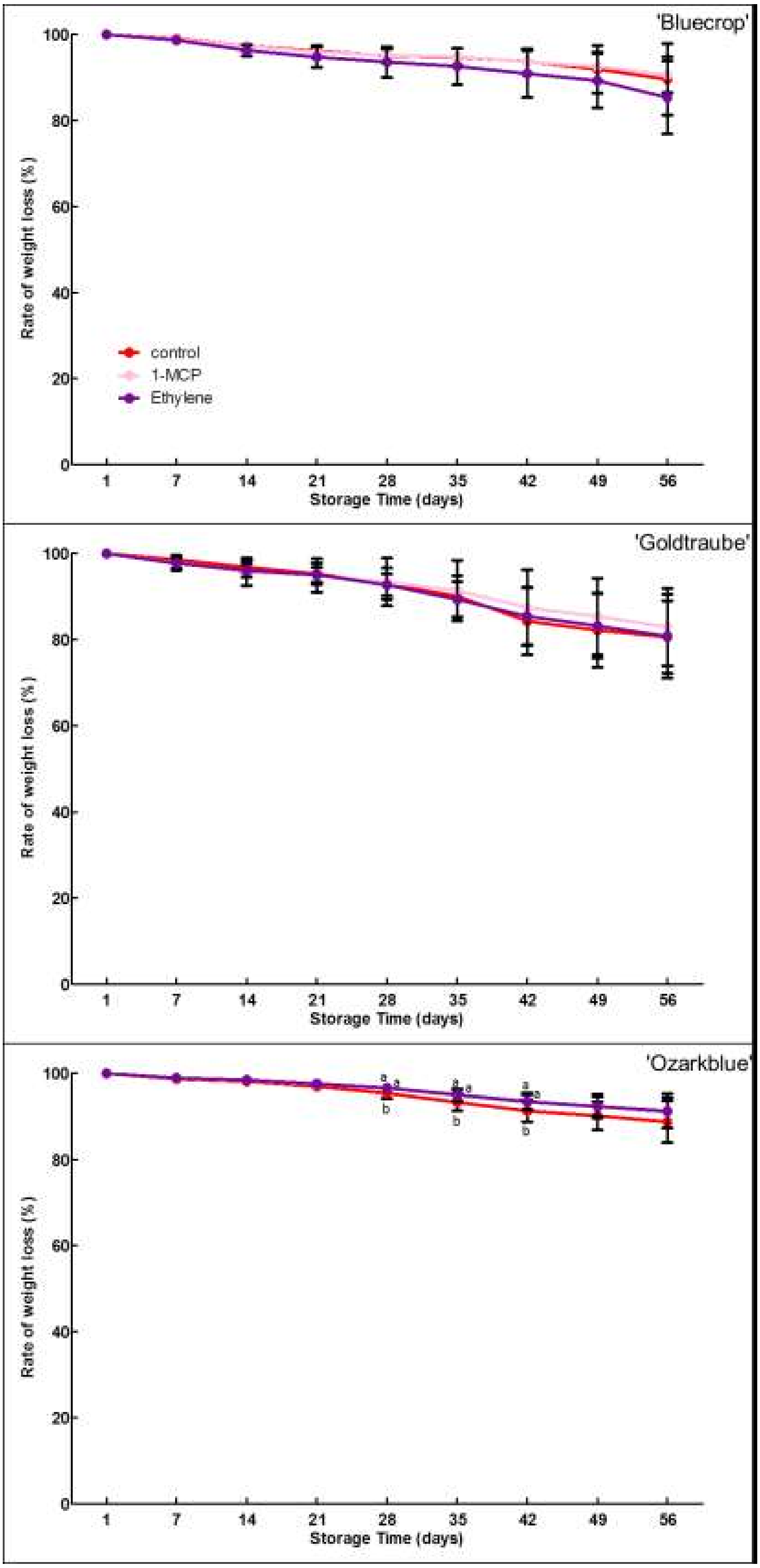

Figure 5.2. – Effect of ethylene and 1-MCP on on the weight loss rate (n=10) of blueberries during storage at 4 ºC. Results are expressed as mean ± SD. Different letters represent significant differences (P < 0.05), by the Tukey test.

Table 5.2. - Effect of 1-MCP and ethylene on water loss throughout the store. Y = a+ bx, y being the loss of water and x the storage time (days).

		Water loss			
Cultivar	**Treatment**	**a±sd**	**b±sd**	**r^2**	**P**
Bluecrop	**Control**	100.167±0.350	-0.173±0.011	0.975	0.000
	1-MCP	99.863±0.278	-0.156±0.008	0.980	0.000
	Ethylene	100.263±0.496	-0.240±0.015	0.974	0.000
Goldtraube	**Control**	101.838±0.869	-0.378±0.026	0.968	0.000
	1-MCP	100.626±0.562	-0.303±0.017	0.979	0.000
	Ethylene	101.122±0.631	-0.354±0.019	0.980	0.000
Ozarkblue	**Control**	100.694±0.318	-0.210±0.010	0.986	0.000
	1-MCP	100.543±0.272	-0.165±0.008	0.983	0.000
	Ethylene	100.501±0.249	-0.160±0.007	0.983	0.000

Respiration rate was significantly influenced by treatment and storage time (Figure 5.3.). At the end of storage, respiration rate was higher in the three cultivars. The treatment with ethylene increased the respiration rate in ‘Goldtraube’ and ‘Ozarkblue’, whereas in ‘Bluecrop’ this increase was more evident by the end of storage. Treatment with 1-MCP led to lower respiration rate in the three cultivars studied. Xu and Liu (2017) also observed a gradual increase of respiration in blueberry ‘Berkeley’ during 8 days of storage at 4 ± 1 °C. Thus 1-MCP treatment can inhibit the increase of respiration rate. This respiration rate inhibition by 1-MCP may be related to inhibition effect on the blueberries ethylene production. DeLong et al. (2003) found that blueberry fruits treated with 1-MCP, although inhibiting ethylene action, did not have any effect on blueberry fruit quality or storage life. Bower et al. (2003) found no effects of 1-MCP on respiration rate in strawberry. Tian et al. (2000) report that, in strawberry, it may/may not affect the respiratory rise induced by exogenous ethylene dependent on fruit maturity. Although blueberries have been classified as non-climacteric fruits (Owusu-Apenten, 2005; Pallardy, 2008; Zifkin et al., 2012) the 1-MCP treated fruits showed a lower respiration rate, in the three cultivars, and ethylene application increased respiration rate in ‘Goldtraube’ and ‘Ozarkblue’ and retarded the reduction in hardness in ‘Bluecrop’ and ‘Ozarkblue’. Thus the 1-MCP presents potential for commercial application in blueberries.

In general the titratable acidity (Table 5.3.) was not influenced by the treatment with ethylene nor 1-MCP in cultivars ‘Bluecrop’ and ‘Ozarkblue’. However on ‘Goldtraube’ the titratable acidity was influenced and in general the control fruits or those treated with 1-MCP showed a higher titratable acidity. Grozeff et al. (2017) also found that 1-MCP treatment maintained better the malic acid than the untreated fruit at the same time.

The content of soluble solids was influenced by treatment with ethylene and 1-MCP, and the fruits that were treated with 1-MCP presented generally less ºBrix. The treatment with ethylene in ‘Goldtraube’ led to a decrease in the titratable acidity and the increase of º Brix. Castrejón et al. (2008) also reported the decrease in titratable acidity and the increase in total sugars content found in other *V. corymbosum* cultivars. This decrease in titratable acidity may be related to increased respiratory rate with ethylene treatment.

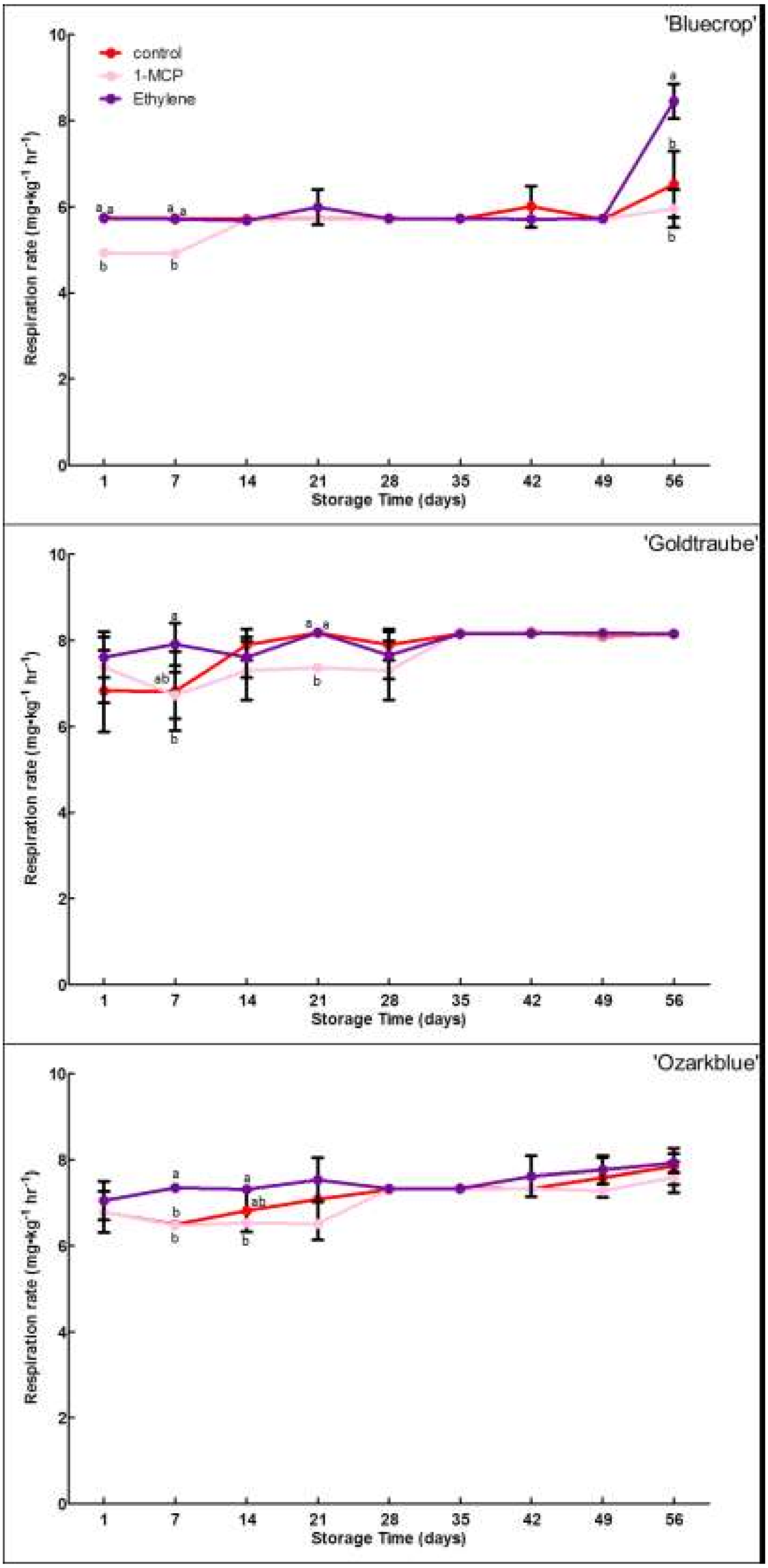

Figure 5.3. – Effect of ethylene and 1-MCP on on the respiration rate (n=3) of blueberries during storage at 4 ºC. Results are expressed as mean ± SD. Different letters represent significant differences (P < 0.05), by the Tukey test.

Table 5.3. - Effect of 1-MCP and ethylene applications on titratable acidity (TA) and soluble solids content (SSC).

Storage days	Treatment	Bluecrop		Goldtraube		Ozarkblue	
		TA (% malic acid)	SSC (ºBrix)	TA (% malic acid)	SSC (ºBrix)	TA (% malic acid)	SSC (ºBrix)
1	Control	0.84±0.04	9.94±0.55^{b}	0.94±0.14	12.76±0.11^{a}	1.08±0.10	11.59±0.24^{ab}
	1-MCP	0.85±0.20	9.31±0.53^{c}	1.00±0.15	12.31±0.32^{b}	1.00±0.12	11.44±0.28^{b}
	Ethylene	0.92±0.04	10.60±0.17^{a}	0.75±0.04	12.92±0.56^{a}	1.05±0.15	11.83±0.26^{a}
7	Control	0.93±0.04	9.47±0.30^{a}	0.85±0.12^{a}	13.13±0.43^{a}	0.97±0.10	11.60±0.11^{a}
	1-MCP	0.80±0.20	8.57±0.28^{b}	0.76±0.03^{ab}	12.50±0.09^{b}	1.15±0.03	11.13±0.10^{b}
	Ethylene	0.95±0.22	9.89±0.89^{a}	0.60±0.05^{b}	12.93±0.15^{a}	1.13±0.15	11.23±0.49^{b}
14	Control	0.83±0.08	9.78±0.19^{a}	0.85±0.02^{ab}	12.94±0.19^{0}	1.23±0.09	11.07±0.39^{b}
	1-MCP	0.70±0.03	8.94±0.27^{b}	0.99±0.08^{a}	12.49±0.30^{b}	0.89±0.24	11.20±0.33^{b}
	Ethylene	0.81±0.12	10.00±0.71^{a}	0.71±0.11^{b}	12.77±0.32^{ab}	0.93±0.09	12.06±0.79^{a}
21	Control	0.81±0.02^{a}	10.00±0.19^{b}	1.02±0.16^{a}	12.63±0.10	0.84±0.11	11.76±0.41
	1-MCP	0.78±0.05^{ab}	9.84±0.19^{b}	0.85±0.12^{ab}	12.57±0.19	0.80±0.03	12.01±0.43
	Ethylene	0.71±0.03^{b}	11.14±0.22^{a}	0.68±0.06^{b}	12.63±0.17	0.89±0.20	11.57±0.44
28	Control	0.81±0.06	10.51±0.46^{a}	0.96±0.10^{a}	12.66±0.47^{a}	0.99±0.07	11.10±0.28^{b}
	1-MCP	0.79±0.06	9.36±0.46^{b}	1.08±0.06^{a}	11.90±0.23^{b}	1.06±0.09	11.22±0.12^{b}
	Ethylene	0.77±0.09	10.23±0.44^{a}	0.68±0.03^{b}	12.37±0.35^{a}	0.91±0.09	11.56±0.35^{a}
35	Control	0.86±0.05	9.52±0.24	0.83±0.07	12.63±0.13^{b}	0.90±0.07	11.33±0.51^{a}
	1-MCP	0.82±0.08	9.39±0.47	0.89±0.15	12.07±0.10^{c}	0.88±0.05	10.66±0.15^{b}
	Ethylene	0.90±0.07	9.77±0.52	0.83±0.10	12.83±0.21^{a}	0.96±0.12	11.50±0.11^{a}
42	Control	0.87±0.08^{ab}	9.63±0.46	1.11±0.06^{ab}	12.39±0.24^{a}	0.97±0.03	10.90±0.15^{b}
	1-MCP	1.00±0.08^{a}	9.47±0.39	1.29±0.09^{a}	11.74±0.27^{b}	0.85±0.10	10.60±0.53^{b}
	Ethylene	0.77±0.04^{b}	9.31±0.27	0.93±0.10^{b}	12.10±0.28^{a}	1.03±0.17	11.43±0.38^{a}
49	Control	0.89±0.08	9.74±0.19^{a}	1.11±0.12	12.29±0.25^{a}	1.04±0.13	10.83±0.13
	1-MCP	0.93±0.14	9.33±0.23^{b}	1.08±0.14	11.14±0.17^{c}	0.98±0.15	10.99±0.16
	Ethylene	0.78±0.04	9.31±0.15^{b}	0.95±0.18	11.47±0.18^{b}	0.98±0.17	10.81±0.44
56	Control	0.78±0.07	9.73±0.43	1.09±0.08^{ab}	11.53±0.21^{a}	1.04±0.18^{ab}	11.16±0.27
	1-MCP	0.78±0.07	9.66±0.19	1.14±0.01^{a}	11.30±0.16^{b}	1.26±0.07^{a}	10.86±0.57
	Ethylene	0.73±0.08	9.58±0.22	0.97±0.03^{b}	11.67±0.21^{a}	0.96±0.01^{b}	10.91±0.23

Results are expressed as mean ± SD (n=3). Different letters represent significant differences (*P*<0.05) between columns, by the Tukey test.

In general, the sensory evaluation of blueberries was similar in control fruits and treated with ethylene or 1-MCP, with the exception of the ‘Goldtraube’ treated with ethylene at 21 days of storage, which were indicated as more mealy (Figure 5.4., Figure 5.5. and Figure 5.6.). The 1-MCP and the ethylene influenced total phenolic compounds and antioxidant activity, thus improving the functional value, without causing changes in colour/appearance. In general sensory panel also did not identify significant differences in fruits treated with ethylene or 1-MCP.

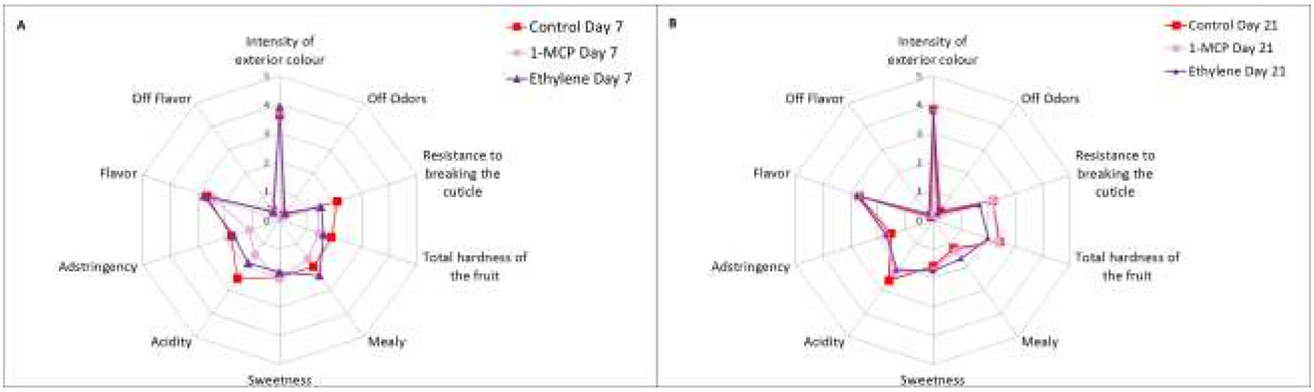

Figure 5.4. – Effect of ethylene and 1-MCP treatment on the sensory profile after 7 (A) and 21 (B) days of storage at 4 ºC in 'Bluecrop' blueberry fruit.

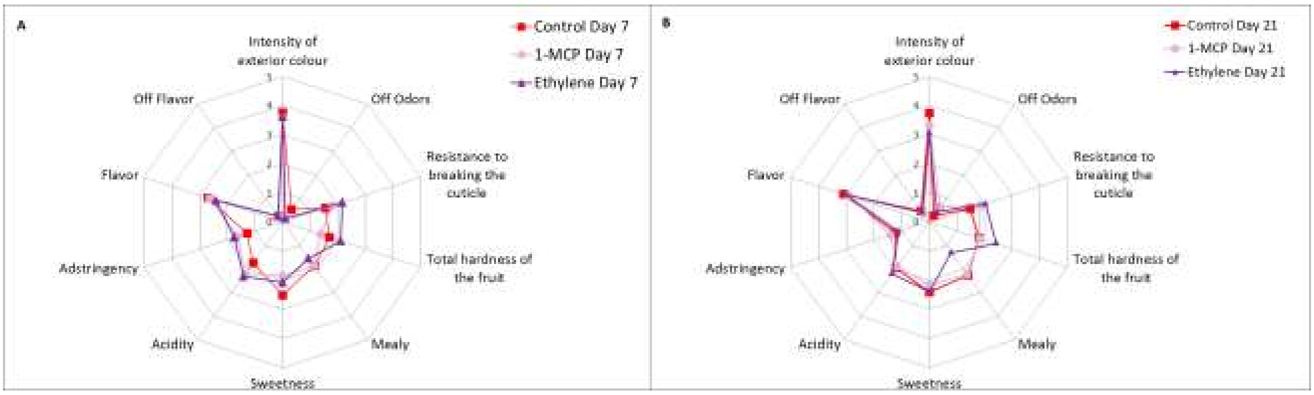

Figure 5.5. – Effect of ethylene and 1-MCP treatment on the sensory profile after 7 (A) and 21 (B) days of storage at 4 ºC in 'Goldtraube' blueberry fruit.

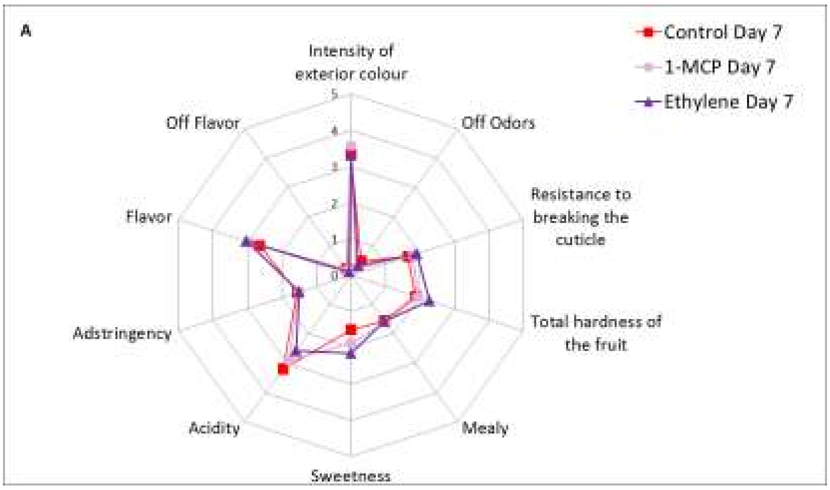

Figure 5.6. – Effect of ethylene and 1-MCP treatment on the sensory profile after 7 days of storage at 4 ºC in 'Ozarkblue' blueberry fruit.

5.4.2. Effect of ethylene and 1-MCP treatment on anthocyanins and total phenolic compounds content and antioxidant activity.

The content of anthocyanins (Figure 5.7.), total phenolic compounds (Figure 5.8.) and antioxidant activity (Figure 5.9.) were significantly influenced by the treatments with ethylene and 1-MCP, in the three cultivars.

In ‘Bluecrop’, a day after the ethylene treatment, fruits showed the highest content of anthocyanins. At the end of storage, the control treatment presented the highest anthocyanin content. In ‘Goldtraube’ fruits, ethylene treatment showed the highest anthocyanin content after ethylene treatment and at the end of storage was the 1-MCP treated ones. In ‘Ozarkblue’ at the end of storage both treatments with 1-MCP and ethylene showed the highest anthocyanin content.

Concerning the total phenolic compounds, in ‘Bluecrop’, the treatment with 1-MCP and the control showed the greatest value by end of the storage. In ‘Goldtraube’ and ‘Ozarkblue’ the treatment with ethylene in the first stage of storage showed the highest phenolic content, but after 56 days of storage 1-MCP treatment and control showed the highest values. However, Chiabrando & Giacalone (2011) showed that 1-MCP (0.3 and 0.6 $\mu L.L^{-1}$) had no significant effects on anthocyanin in ‘Lateblue’ blueberries.

At initial stage, ‘Bluecrop’ and ‘Goldtraube’ showed the highest antioxidant activity without treatment (control) and when treated with ethylene and, however at the end of storage the control treatments and 1-MCP showed the highest activity. In ‘Ozarkblue’ the antioxidant activity in the first 28 days of storage increased in fruits treated with ethylene that showed the highest activity.

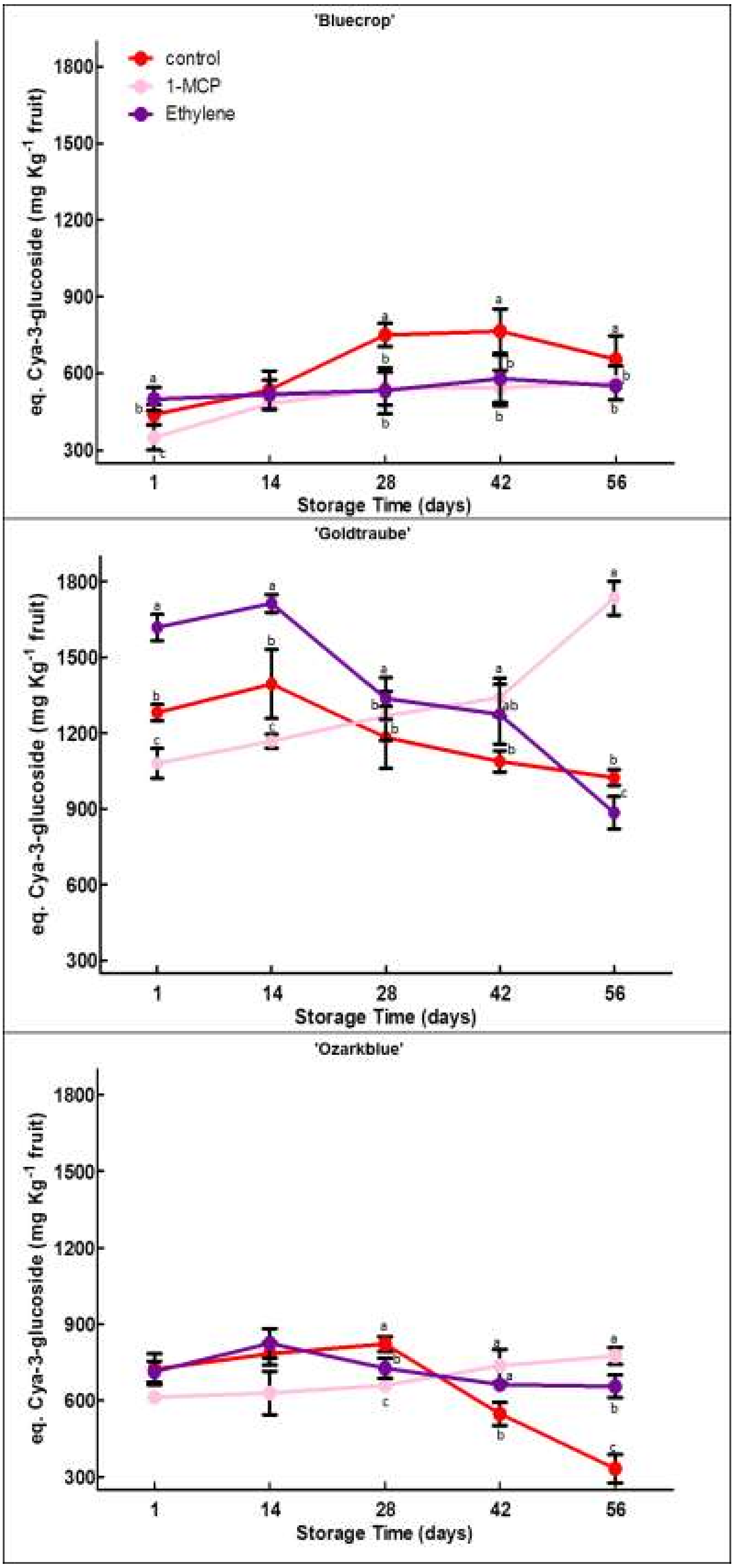

Figure 5.7. – Effect of ethylene and 1-MCP treatment on the total anthocyanins content in blueberry fruit during storage at 4 ºC. Results are expressed as mean ± SD (n=3). Different letters represent significant differences (P < 0.05), by the Tukey test.

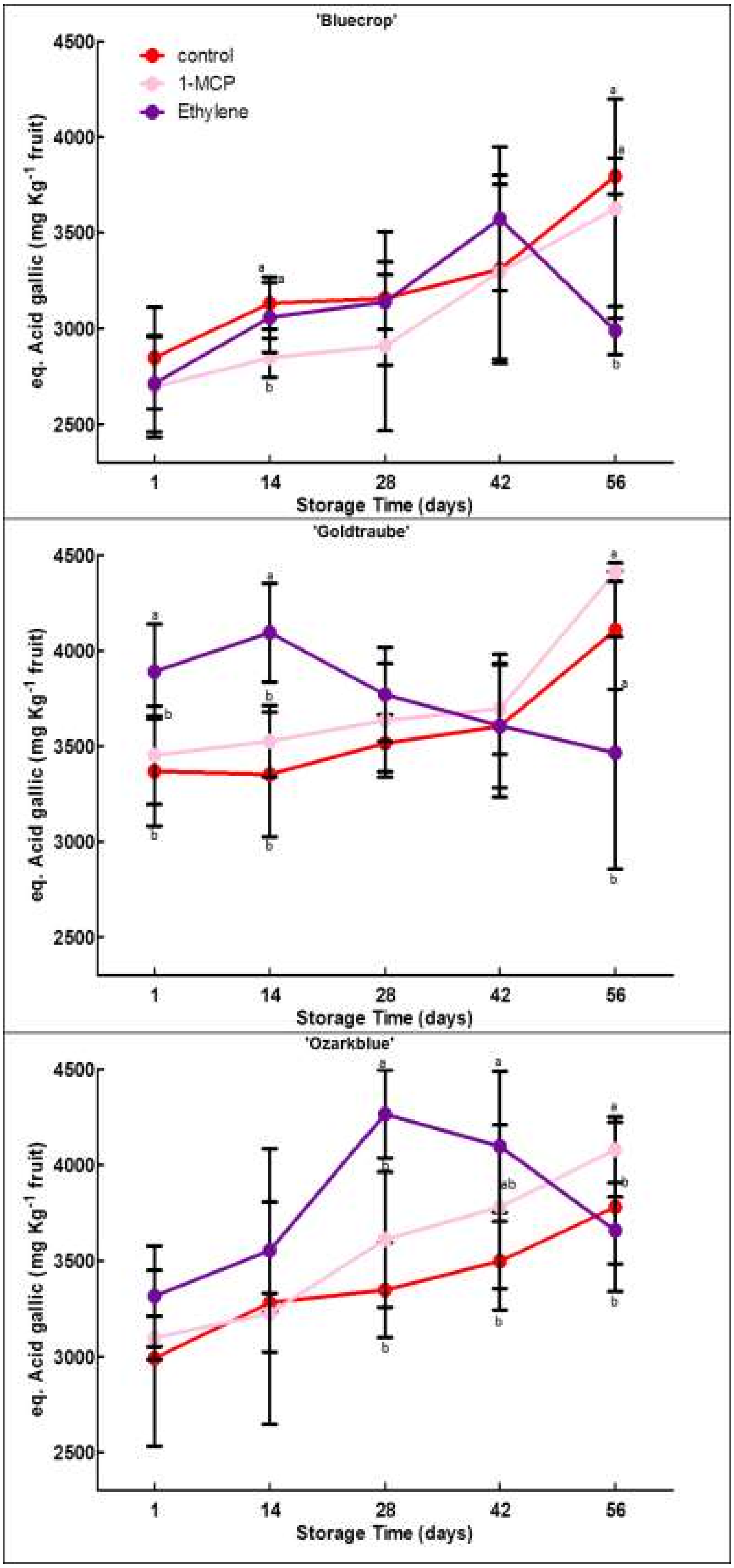

Figure 5.8. – Effect of ethylene and 1-MCP treatment on the total phenolic compounds in blueberry fruit during storage at 4 ºC. Results are expressed as mean ± SD (n=3). Different letters represent significant differences ($P < 0.05$), by the Tukey test.

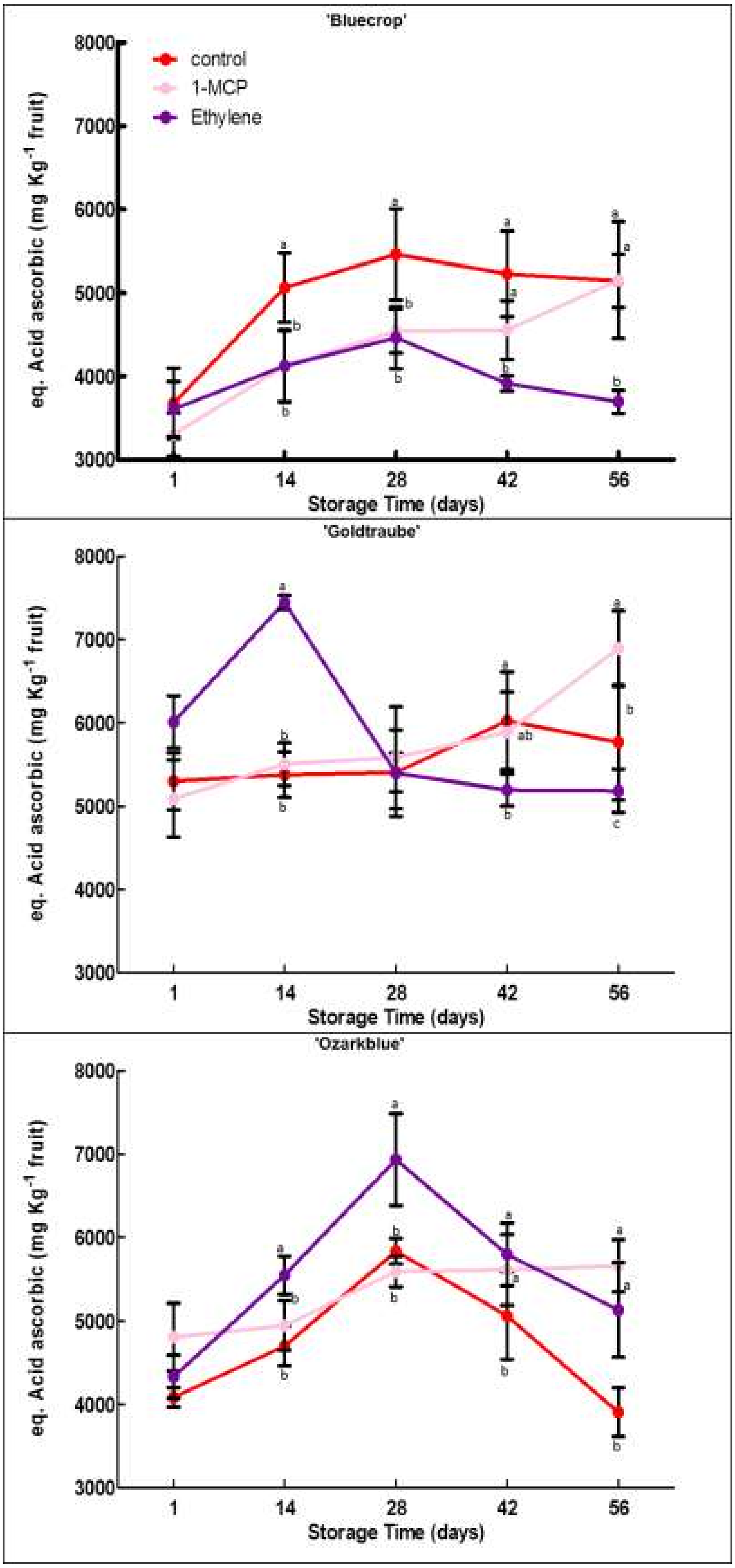

Figure 5.9. – Effect of ethylene and 1-MCP treatment on the antioxidant activity in blueberry fruit during storage at 4 ºC. Results are expressed as mean ± SD (n=3). Different letters represent significant differences ($P < 0.05$), by the Tukey test.

Ethylene is known to induce cell wall degradation by acting on cell wall enzymes in grape 'Aleatico' (1000 μL.L^{-1} ethylene) (Botondi et al., 2011). This can facilitate the extraction of these bioactive compounds from the fruits, so the fruit treated with ethylene generally initially have higher anthocyanins and total phenolics contents and consequently antioxidant activity. Villarreal et al. (2010) reported with ethylene treatment in strawberries an anthocyanin accumulation, total sugar content and increment of phenylalanine ammonia-lyase (PAL) and β-galactosidase activities.

Contrarily, the treatment with 1-MCP (0,5 μL.L^{-1}) retained membrane integrity and anthocyanin content during storage in litchi fruit 'McLean's Red'(Sivakumar and Korsten, 2010). This treatment may have inhibited PAL activity, but during storage the effect of 1-MCP may have decreased leading to an increase in PAL and β-galactosidase activities, and consequently an increase of the total anthocyanins content and total phenolic compounds (Villarreal et al., 2010) as well as the antioxidant activity. The lower initial values exhibited by 1-MCP treatment can be justified by the inhibition of enzymes activity that catalyzes the first step in biosynthesis of phenylpropanoids. For example Jiang et al. (2001); MacLean et al. (2006, 2007) found the expression of PAL, CHS and ERS1 transcripts were all inhibited by 1-MCP.

5.4.3. Effect of ethylene treatment on the profile of anthocyanins and other polyphenols.

The cultivars Bluecrop and Goldtraube (Figure 5.10., 5.11., 5.13. and 5.14.) presented more compounds than 'Ozarkblue' (Figure 5.12., 5.15.). In 'Bluecrop' and 'Goldtraube' nine anthocyanins: delphinidin 3-O-galactoside, delphinidin 3-O-glucoside, cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, cyanidin 3-O-arabinoside, petunidin 3-O-glucoside, malvidin 3-O-galactoside, malvidin 3-O-glucoside and peonidin-3-

galactoside, and two hydroxycinnamic acids: ferulic acid and chlorogenic acid were identified. In ‘Ozarkblue’ blueberries seven anthocyanins (delphinidin 3-O-galactoside, cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, cyanidin 3-O-arabinoside, malvidin 3-O-galactoside, malvidin 3-O-glucoside and peonidin-3-O-galactoside) were identified as well as the same two hydroxycinnamic acids chlorogenic acid and ferulic acid.

In ‘Bluecrop’ ethylene treatment influenced positively most of the compounds (delphinidin 3-O-glucoside, cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, petunidin 3-O-glucoside, peonidin-3-O-galactoside, malvidin 3-O-glucoside, chlorogenic acid and ferulic acid). However, oppositely at the end of storage, in general, the control treatment showed higher contents of those compounds.

In ‘Goldtraube’ different compounds generally exhibit higher levels in ethylene treatment one day after treatment. At the end of storage in general, the 1-MCP treatment showed higher content of these compounds.

Contrarily in ‘Ozarkblue’ the fruits treated with ethylene showed lower levels of the compounds on day after treatment. At the end of storage in the general, the control treatment showed lower contents of those compounds.

The effect of postharvest treatment with ethylene seems to be dependent on the cultivar. Villarreal et al. (2010) also reported that different strawberry cultivars showed differences in their metabolism. Thus differences in the effects caused by ethylene treatments could be due to differences in responses among cultivars. The ethylene treatment induces cell wall degradation by acting on cell wall enzymes in grapes (Botondi et al., 2011) and favors PAL activity. This increase in PAL activity led to an increase in the levels of each of the anthocyanins in grapes (El-Kereamy et al., 2003; He et al., 2010; Wicks and Kliewer, 1983). This may have led to a higher content of anthocyanins and two hydroxycinnamic acids at the beginning of storage in ‘Bluecrop’ and ‘Goldtraube’.

Generally, at the end of storage 1-MCP treatment showed higher values in several identified compounds in ‘Goldtraube’ and ‘Ozarkblue’. Total anthocyanins content exhibited this trend at the end of storage in these two cultivars. Bellincontro et al. (2006) reported in Aleatico grape berries that 1-MCP treatment delayed the loss of phenolic compounds and anthocyanins content. MacLean et al. (2006) also found that 1-MCP on apples may inhibit the activity of phenylalanine ammonia-lyase and subsequent biosynthesis of flavonoid compounds. It appears that the initial effect of 1-MCP was probably due to lower PAL enzyme activity (Jiang et al., 2001), and down-regulated the GmMYB10 expression and this GmMYB10 expression to control the anthocyanin biosynthesis in mangosteen pigmentation (Chervin et al., 2004). 1-MCP treatment also retained membrane integrity and anthocyanin content during storage (Sivakumar and Korsten, 2010). However, during storage there may be cell wall degradation and facilitate the extraction of the compounds.

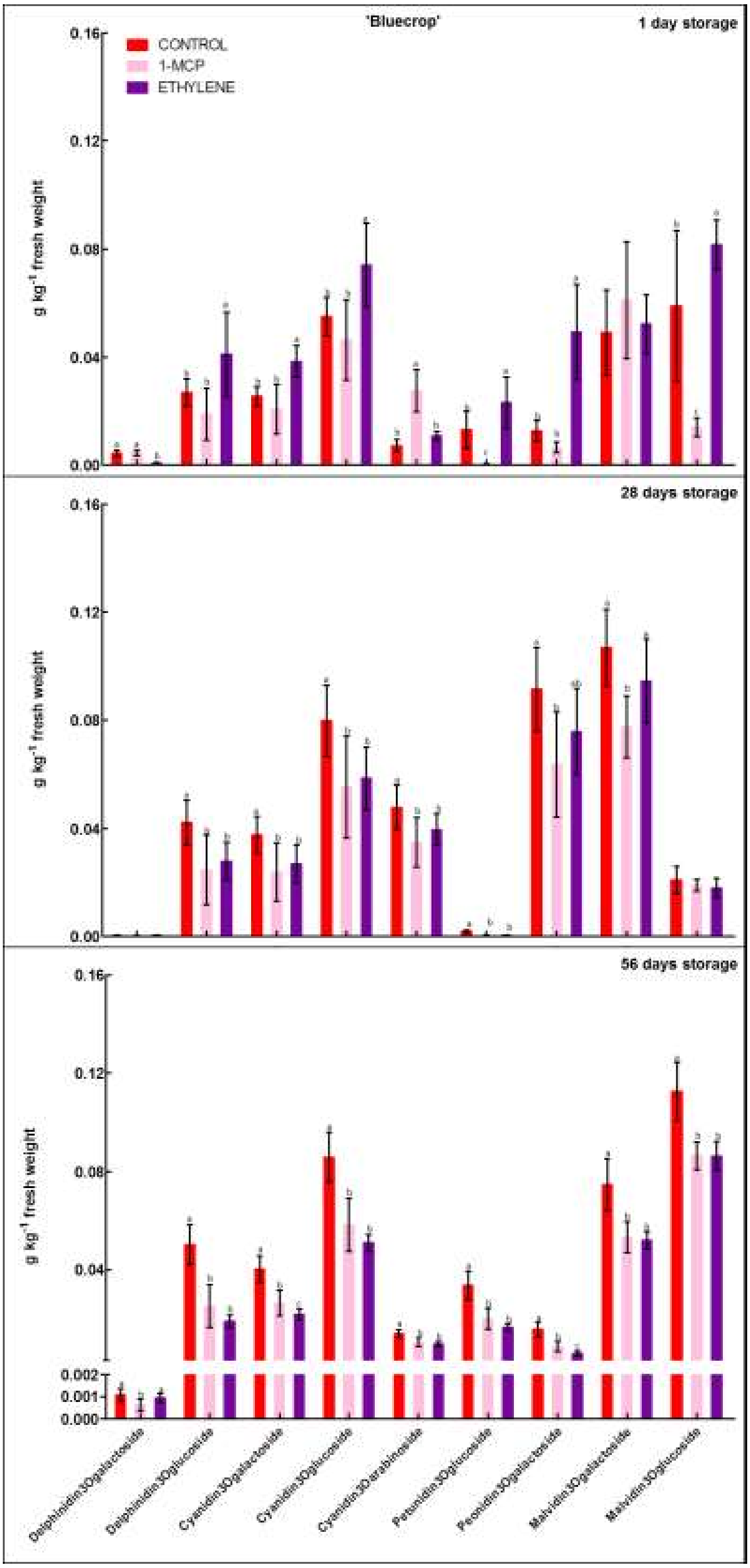

Figure 5.10. – Effect of ethylene and 1-MCP treatment on the composition of anthocyanins in 'Bluecrop' blueberry fruit 1, 28 and 56 days after storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey test.

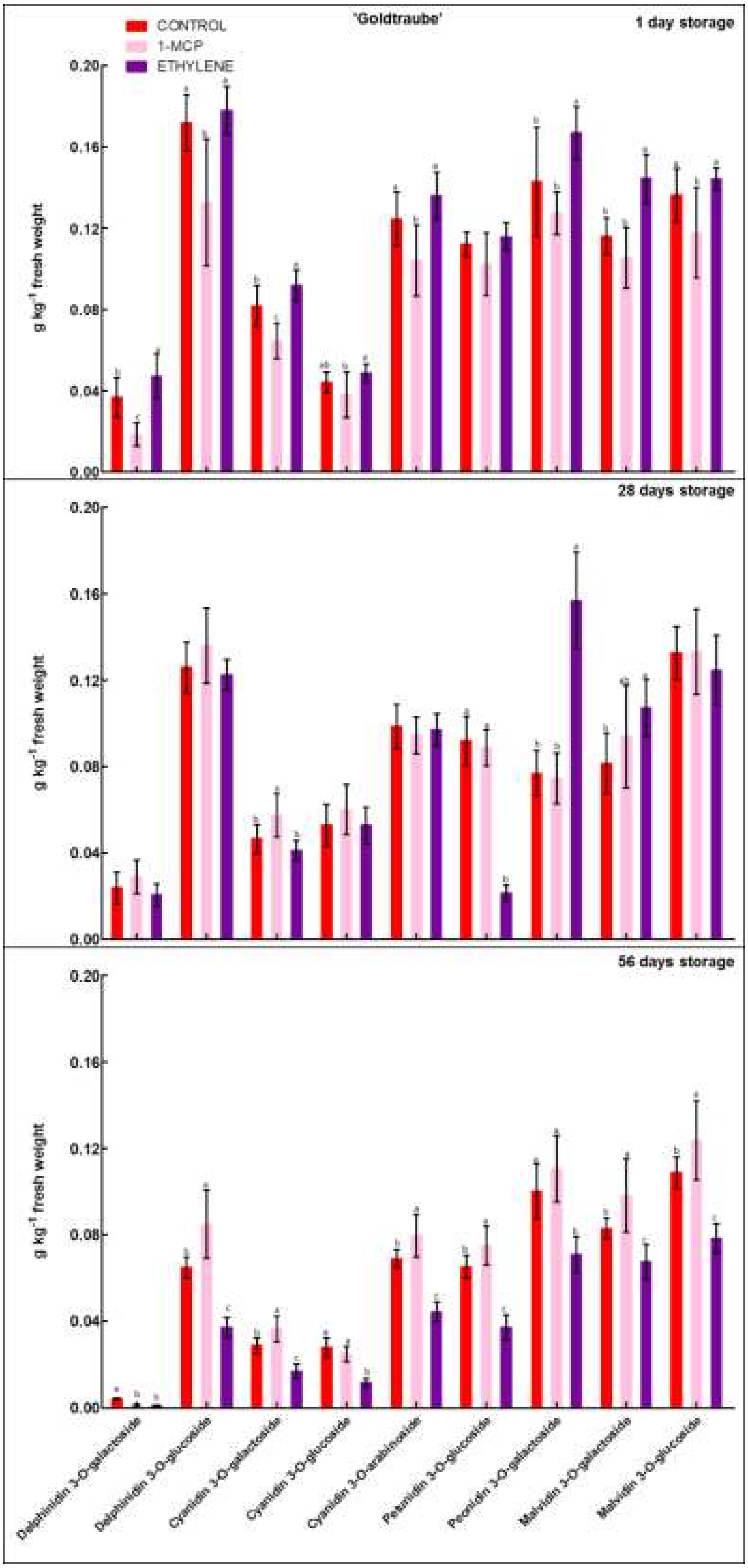

Figure 5.11. – Effect of ethylene and 1-MCP treatment on the composition of anthocyanins in 'Goldtraube' blueberry fruit 1, 28 and 56 days after storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey test.

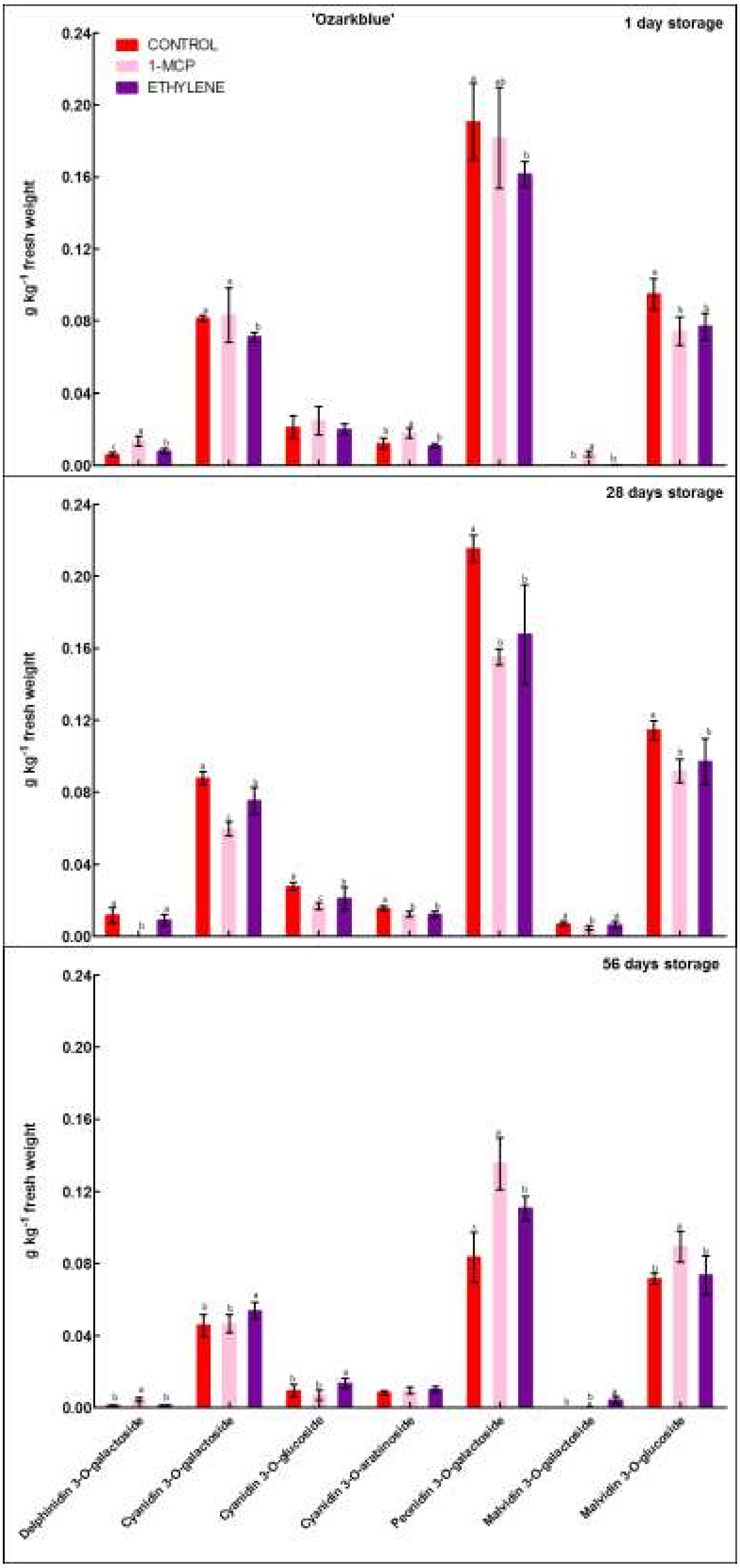

Figure 5.12. – Effect of ethylene and 1-MCP treatment on the composition of anthocyanins in 'Ozarkblue' blueberry fruit 1, 28 and 56 days after storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey test.

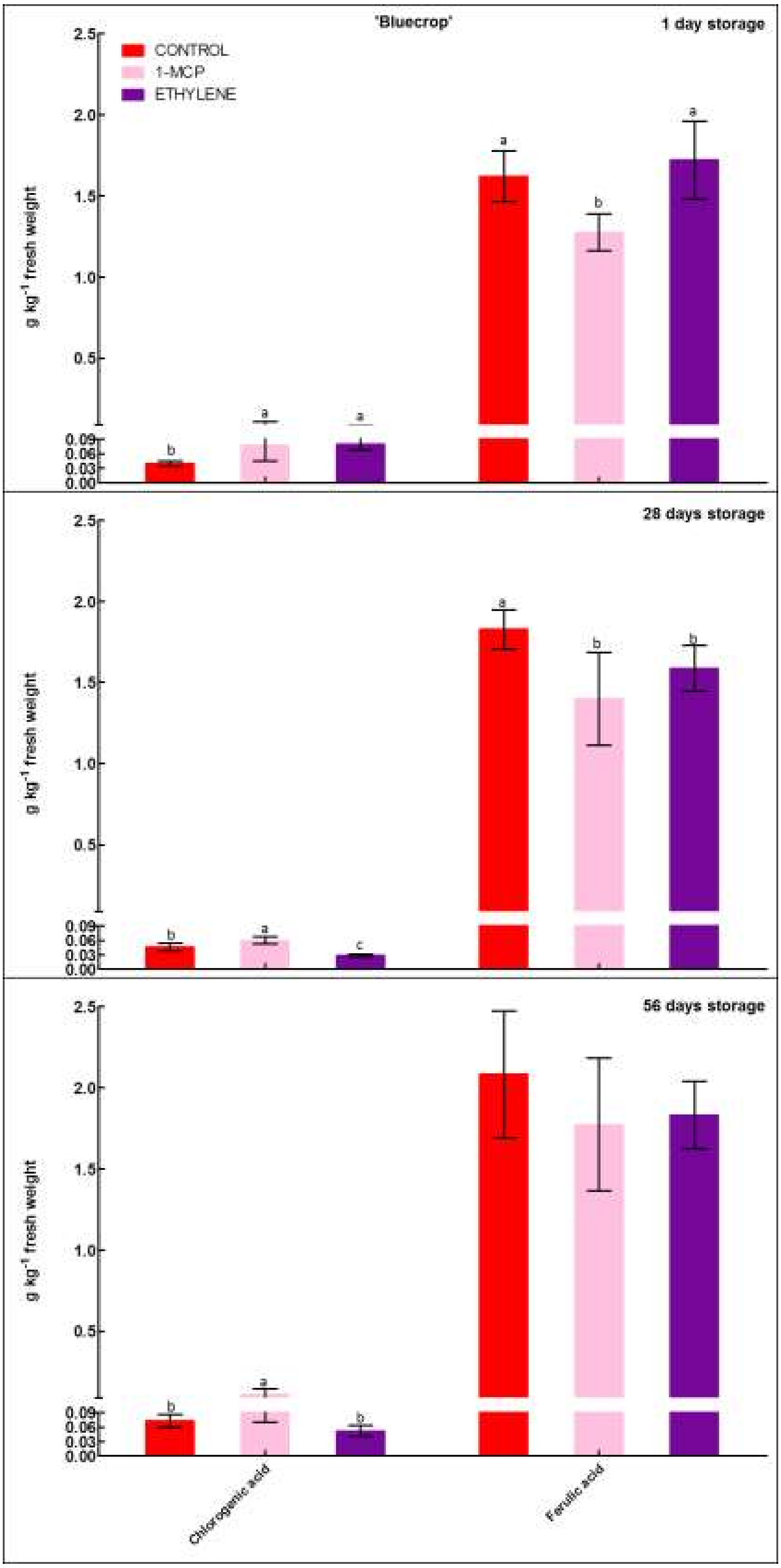

Figure 5.13. – Effect of ethylene and 1-MCP treatment on the composition of hydroxycinnamic acids in 'Bluecrop' blueberry fruit 1, 28 and 56 days after storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey test.

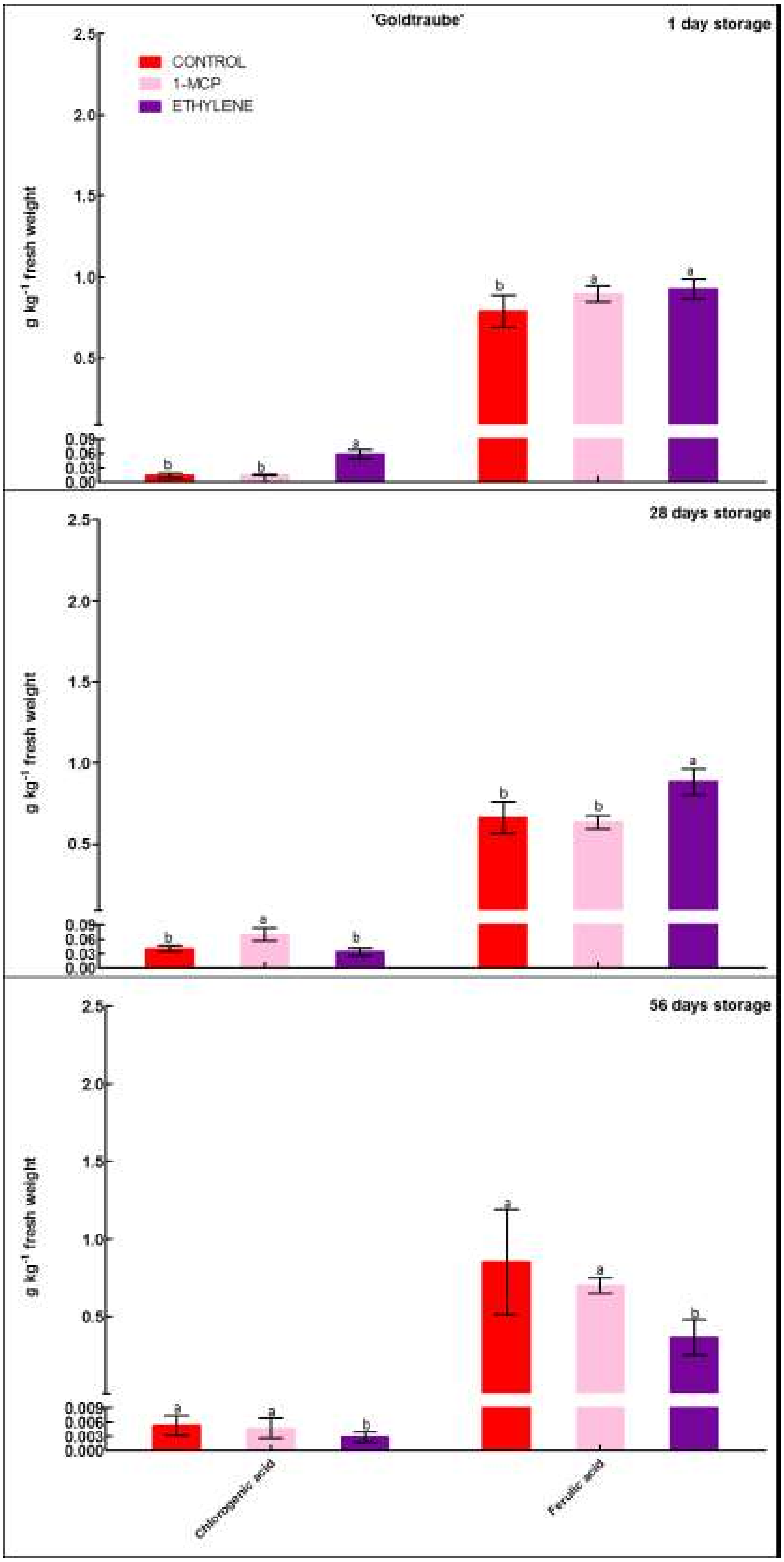

Figure 5.14. – Effect of ethylene and 1-MCP treatment on the composition of hydroxycinnamic acids in 'Goldtraube' blueberry fruit 1, 28 and 56 days after storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey test.

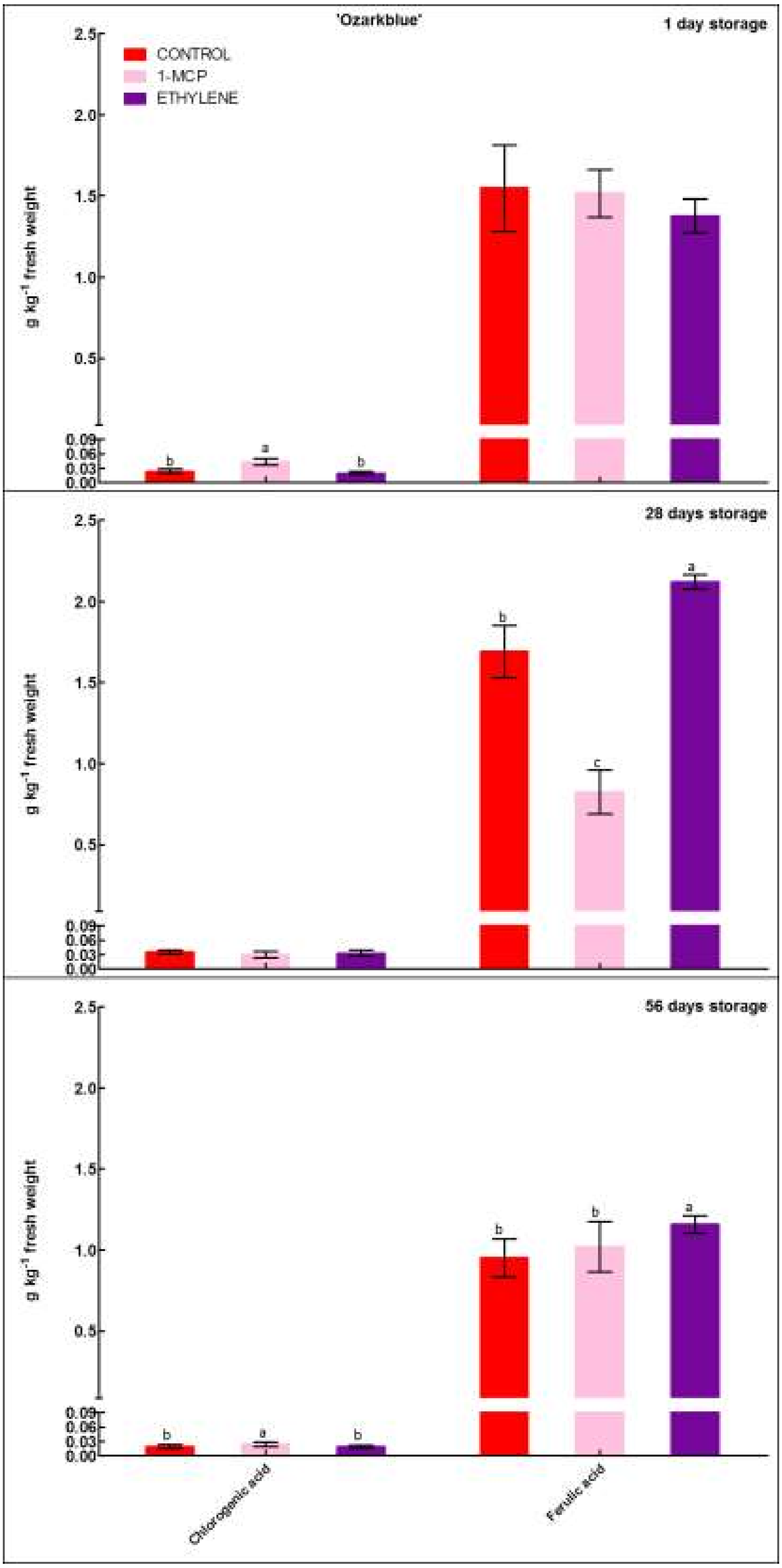

Figure 5.15. – Effect of ethylene and 1-MCP treatment on the composition of hydroxycinnamic acids in 'Ozarkblue' blueberry fruit 1, 28 and 56 days after storage at 4 ºC. Results are expressed as mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey test.

5.4.4. Relationship between treatment, firmness, colour, respiration rate, antioxidant activity, anthocyanins and polyphenols.

The complete dataset was analyzed by means of principal component analysis (PCA) (Figure 5.16.) based on the correlation matrix. Principle component analysis (PCA) yielded six interpretable components that together explain 86.554% of the total variance of the variables. The first two principal components (PC1 and PC2) explain 63.501% of the variability in the data set.

The most important component (PC1) explained 49% of the total variance. PC1 is related to cultivar, respiration rate, antioxidant activity; total anthocyanins content the colour parameters (L*and C*), cyanidin 3-O-arabinoside; cyanidin 3-O-galactoside, delphinidin 3-O-glucoside; delphinidin 3-O-galactoside, petunidin 3-O-glucoside; peonidin 3-O-galactoside, malvidin 3-O-glucoside, malvidin 3-O-galactoside, and ferulic acid. The antioxidant activity and the total anthocyanins content are positively related to these compounds. This relationship as expected, Kalt et al. (2001); Koka and Karadeniz (2009) also reported the high antioxidant activity of blueberries to the presence of phenolic compounds. On the other hand negative correlations were observed for this compounds antioxidant activity, total anthocyanins content, respiration rate, cultivar and the colour parameters (C* and L*) and ferulic acid. Márkus et al. (1999) reported a relationship between antioxidant activity and respiratory rate during ripening.

PC2 explained 15% of the total variance and mainly correlates storage time, total phenolic compounds, cyanidin 3-O-glucoside and h°. The storage time was highly correlated with total phenolic compounds and h°, where the increase in storage time promoted a total phenolic compounds and h°. The relationship between storage time and h° shows a slight increase in blue colour throughout the storage.

Finally, PC3 explained 7% of variance, and this variation was mostly described by chlorogenic acid. Chlorogenic acid, treatment and firmness were not associated with any factor.

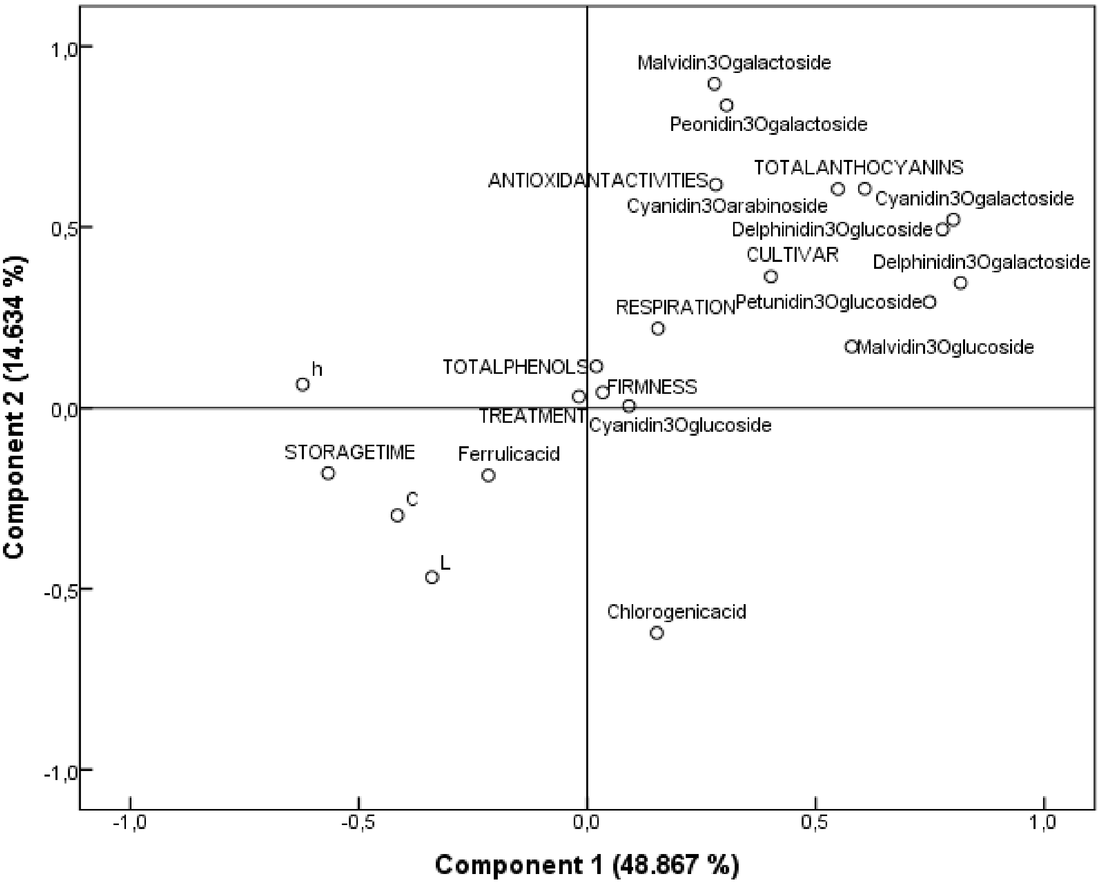

Figure 5.16. – Positions of PCA scores for the total anthocyanins content, total phenolic compounds, antioxidant activity, firmness, respiration rate, the colour parameters hº, L* and C*, storage time and cultivar based on PC1 and PC2. The percentage represents the variance of each principal component.

5.5. Conclusions

Treatment with 1-MCP and ethylene did not influence some quality parameters such as weight loss rate and the respiration rate. However, they have influenced the content of anthocyanins, total phenolic compounds and antioxidant activity. In general, treatment with ethylene induced the increase of the levels of those parameters until a certain storage period depending on the cultivar, after which they decreased. However, treatment with 1-MCP initially retained the level of anthocyanins, total phenolic compounds and antioxidant activity, but in general, an increase of those levels at the end of storage was positively observed. Thus, the application of 1-MCP to preserve blueberries may be an efficient strategy in the case of long term distances, so as to present the functional advantages when it reaches consumers.

The treatment effect (ethylene and 1-MCP) in the identified bioactive compounds (anthocyanins and hydroxycinnamic acids) appear to depend on the cultivar. So, in ‘Goldtraube’ and ‘Bluecrop’ ethylene increased these compounds, thus securing the benefits of these compounds in health. At the end of storage the 1-MCP treatment showed a higher content of anthocyanins and hydroxynamic acids relative to the other treatments in the Goldtraube and Ozarkblue cultivars. However, future experiments will be needed to better understand the mechanisms that conduce to these effects and in particular the variability observed among cultivars.

CHAPTER 6- The effect of different controlled atmosphere on commercial and phytochemical quality in three Northern Highbush Blueberry Cultivars

This chapter has been submitted to the journal: Journal of Agricultural Science

Part this chapter has been published:

Acta Horticulturae. (2015). doi: 10.17660/ActaHortic.2015.1071.89

6.1. Abstract

The blueberry is consumed for health benefits associated with a high antioxidant activity, which is attributed to high anthocyanin content. In this work, the effect of different controlled atmospheres (air, 2% O_2 + 2% CO_2, 2% O_2 + 15% CO_2, and 21% O_2 + 15% CO_2) for 29 days at 4° C on the anthocyanins profile, phytochemical composition and colour of two blueberry (*Vaccinium corymbosum*) cultivars 'Goldtraube' and 'Ozarkblue' has been studied.

Soluble solids content was affected by atmosphere composition on 29 day in 'Goldtraube' but not in 'Ozarkblue'. Acidity was not significantly affected by the atmosphere composition and remained relatively constant during storage. Fruit firmness of both cultivars was also significantly affected by the atmosphere composition. At the end of the storage, firmness was lower in 'Goldtraube' stored in air and in 21% O_2+15% CO_2 (1.86 N and 1.85 N, respectively), whereas 'Ozarkblue' showed lower firmness in air (1.19 N).

In 'Goldtraube' a low O_2 concentration improves the preservation of anthocyanins. However, 'Ozarkblue' appeared to benefit from higher CO_2 concentrations. Compounds such as cyanidin 3-o-arabinoside, cyanidin 3-o-galactoside, cyanidin 3-o-glucoside, dephinidin 3-o-galactoside and malvidin 3-o-glucoside appeared to be linked to the total phenol content, total anthocyanin content, antioxidant activity and cultivar at 0 and 14 storage days. Furthermore, the different storage atmospheres appeared to affect the ferulic acid content, where the high CO_2 concentration favoured this compound.

Sensory profile of blueberries stored under the different atmospheres was similar by 7 and 21 days of storage with one exception: berries from 'Ozarkblue' stored for 7 days were classified as less sweet when stored under 2% O_2+15% CO_2 than for other conditions.

6.2. Introduction

During the last two decades consumption of fruit has been strongly encouraged by medical professionals, due to the increase in obesity, diabetes and many other health issues. The consumption of fruit is beneficial not only for their high content of vitamins, minerals, and fibre but also for their antioxidant compounds, particularly polyphenols (Khorshidi et al., 2011).

The importance of the function of antioxidants in health has driven research to evaluate fruit and vegetable antioxidants compounds and related bioactivity as well as to define how they can be maintained or enhanced through crop breeding, horticultural practices, postharvest processing and storage (Ayala-Zavala et al., 2007).

One postharvest technology frequently used to extend fruit shelf-life is controlled atmosphere (CA) storage. This technology can extend the shelf life of fruit and vegetables by reducing metabolism and postharvest decay (Gunes et al., 2002). The benefits of CA storage have been known for many years and include a decreased rate of ripening (i.e., a lower respiration rate, regulated ethylene production, and a reduce in ethylene sensitivity) and a reduction in postharvest chemicals (i.e., the inhibition of microorganisms such as bacteria and mould, insect control, and the elimination of birds and rodents) (Bower, 2007). However, fruit species differ in their tolerance to an elevated CO_2 level and a decrease in O_2 in the storage atmosphere. Acceptable thresholds depend of several factors, including fruit type, maturity stage, and storage time in CA (Harb and Streif, 2004). Furthermore, storage under a controlled or modified atmosphere result in loss of other nutrients such as ascorbic acid (Mitcham et al., 1998). CA storage in conjunction with low temperature was proposed to extend the storage life of blueberries by slowing physiological and metabolic processes so as to retard senescence and prevent or delay physiological disorders, and reduce postharvest decay (Rodriguez and Zoffoli, 2016).

Fruit that have higher respiratory rates have a lower shelf life, when controlling the concentration of O_2 and CO_2 in storage, the respiratory rate is reduced, consequently slow down senescence and increase the shelf life. Low concentration of O_2 inhibits the enzymes of ethylene production; high concentration of CO_2 may hinder the enzymatic activity, lipoxygenase pathway, which is implicated in the formation of volatile aromatic compounds (Bower, 2007).

Blueberries are highly perishable fruit, and consequently, storage under high CO_2 and low O_2 levels is recommended to preserve the highly valued polyphenols (Paniagua et al., 2014). In 'Sunrise' blueberries the CA was favourable for maintaining the high content of polyphenols and also led to lower losses of anthocyanins and chlorogenic acid content (Ochmian et al., 2015). However, for some cultivars high concentration of CO_2 can be prejudicial (Harb et al., 2014).

In blueberries, CA storage at 15% O_2+10% CO_2 (Forney et al., 2003) or with 2.5% O_2+15% CO_2 (Schotsmans et al., 2007) or with CO_2 (6-12%) without O_2 (Harb and Streif,

2004) is clearly beneficial for long-term blueberry storage. However, controlled atmosphere did not influence the shelf life of seven blueberry cultivars (Bluecrop, Bluegold, Brigitta, Elliott, Jersey, Legacy, and Liberty) (Hancock et al., 2008).

A high CO_2 (18 %) concentration may have an adversely effect on the expression of some enzymes (e.g., chalcone synthase (VcCHS)) that are implicated in flavonoid biosynthesis in blueberries (Harb et al., 2014). In 'Coville' and 'Chandler' blueberries a high CO_2 (15%) led to a slight increase in antioxidant activity at the end of storage (4 months) (Cătuneanu et al., 2017), and inhibited decay (Forney et al., 2003). Suggesting that metabolic processes in fruit were slowed down due to CO_2 (Cătuneanu et al., 2017).

The impact of high oxygen concentrations on antioxidant activity and phenolic content and may vary depending on the product, oxygen level, storage duration and temperature, (Ayala-Zavala et al., 2007). For blueberries, high oxygen levels 60 and 100 % O_2 promoted increases in total phenolic and total anthocyanin content as well as individual phenolic compounds (Zheng et al., 2003, 2007). Whereas storage of blueberries 'Brigitta' at 1% O_2 resulted in a higher softening (Rodriguez and Zoffoli, 2016).

Increasingly, the quality of fruit and vegetables is related to the antioxidant content, and changes in antioxidant status is a parameter of great interest to evaluate postharvest storage in vegetables and fruit (Ayala-Zavala et al., 2007). Some studies have shown that blueberries (*Vaccinium corymbosum*) exhibit high levels of total anthocyanins content and phenolics and have a high antioxidant activity (Kalt et al., 1999; Sellappan et al., 2002; You et al., 2011). However, few data exist describing changes in these levels throughout storage and with relation to postharvest technologies, in particular related with controlled atmosphere (CA) storage. Thus, this study aims was to evaluate the effect of different controlled atmospheres on the changes in the commercial quality and in the levels of phenolic phytochemicals, antioxidant activity and colour throughout the storage period of two cultivars of highbush blueberry.

6.3. Material and Methods

6.3.1. Fruit treatment and storage

Northern Highbush blueberry (*Vaccinium corymbosum*) from the Goldtraube and Ozarkblue cultivars plants had been established at eleven and thirteen years, respectively. Texturally the soil is coarse-grained and the organic matter content is 12%.

The fruit were harvested in the region of Sever do Vouga, Portugal, at commercial maturity (full blue), and stored at 4 ºC for 29 d under the following atmospheres: air, 2% O_2 + 2% CO_2, 2% O_2 + 15% CO_2, and 21% O_2 + 15% CO_2.

The blends of compressed air and gases were acquired to Gasin – Gases industriais (Group air products). The blends were placed outside the chamber and were humidified inside the chamber.

The fruits were placed in 12 (4 treatments with 3 replicates each) 20 L plastic buckets, each bucket had 3.5 kg of each cultivar in mesh bags, with a continuous flow system. The gas mixture was checked daily at the chamber exit with CheckMate II, (PBI Dansensor, Ringsted, Denmark).

Samples were collected at days 1, 14 and 29 of storage.

6.3.2. Soluble solids content

The soluble solids content was determined in the 20 fruit juice, using the method according by Castrejón et al. (2008) and Prior et al. (1998) as described on section 3.3.4..

Three replicates were generated for each treatment and for each of sampling point throughout storage time at 1, 14 and 29.

6.3.3. Total acidity

Total acidity was measured in an homogenate of 20 fruit using the method according by Castrejón et al. (2008) as described on section 3.3.5..

The total acidity was performed at 1, 14 and 29 days storage.

6.3.4. Firmness

Firmness was measured by puncture with a texture analyzer (Stable Micro Systems, TA XT plus). The probe diameter was 2 mm with a travel distance of 5 mm and 1 mm s^{-1} test speed. The puncture was conducted in the equatorial region. Thirty replicates were obtained to estimate the mean values. Results were expressed as N mm^{-1}.

6.3.5. Skin colour

Skin colour was determined in the CIE L* a* b* space with a Minolta CR-300 colorimeter (Osaka, Japan) equipped with a D65 illuminant. The data was converted to hue (h °) and chroma (C *) using the relationships described by McGuire (1992). The method as described on section 3.3.7.

Samples were collected biweekly at days 1, 14 and 29 of storage.

6.3.6. Sensory evaluation

The Attribute Intensity Ranking was used to assess the sensory properties. The sensory evaluation sessions took place in the ISO 8589:2007 compliant sensory testing facilities of Escola Superior de Biotecnologia - Universidade Católica Portuguesa (ESB-UCP). This method as described on section 4.3.12.

‘Goldtraube’ and ‘Ozarkblue’ fruits were sampled after 7 and 21 d of storage at 4 ºC.

6.3.7. Extracts preparation

Extraction of phenolic compounds was performed according to Kalt et al. (1999) with minor modifications, as described on section 3.3.8.

Three replicates were generated for each treatment and for each of sampling point throughout storage time at 1, 14 and 29 d.

6.3.8. Total anthocyanins content

Evaluation of anthocyanins was performed by the differential pH method (Giusti and Wrolstad, 2001) as described on section 3.3.9.

Three replicates were performed for each sample at 1, 14 and 29 d of storage.

6.3.9. Total phenolic compounds

The content of phenolic compounds was determined by the Folin-Ciocalteu reagent (Slinkard and Singleton, 1977) this method as described on section 3.3.10.

All determinations were executed in triplicate. Samples were collected at days 1, 14 and 29 of storage.

6.3.10. Antioxidant activity

Antioxidant activity was evaluated by the $ABTS\cdot^{+}$ radical cation decolourization assay (Gião et al., 2007) as described on section 3.3.11.

All measurements were performed in triplicate. Samples were collected at days 1, 14 and 29 of storage.

6.3.11. HPLC-DAD analysis

Qualitative and quantitative profiles of anthocyanins and hydroxycinnamic acid (namely chlorogenic acid and ferulic acid) present in the extracts were determined by HPLC-DAD (Waters Series 600, Mildford MA, USA). This method was adapted from Silva et al. (2013b), as described on section 3.3.12.

6.3.12. Statistical analysis

The data were subjected to analysis of variance according to a randomized block design with three replicates. The Tukey's test was used to test differences between storage treatments and storage days. Differences at $p \leq 0.05$ were considered to be significant. The association between colour measurement and qualitative or quantitative profiles was calculated using an analysis of factorial components (PCA). Correlations were determined by Pearson correlation coefficient for bivariate correlations. Differences between means at the 5 % ($P<0.05$) level were considered significant. All analyses were performed with the statistical software SPSS 21.0 for Windows (SPSS, Chicago, USA).

6.4. Results and Discussion

6.4.1. Soluble solids content, Acidity, Firmness

Soluble solids content was affected by atmosphere composition in ‘Goldtraube’ on the 29 day with lower levels under normal atmosphere. Soluble solids content was not affected in ‘Ozarkblue’ (Table 6.1.). During storage the soluble solids content decreased in all storage atmospheres for the two cultivars. Eccher et al. (2010) also found generally soluble solids content decreased after long storage. Acidity was not significantly influenced by the different atmospheres and remained relatively constant during storage (Table 6.1.). Other authors when studying the effects of controlled atmosphere on fruit ‘Burlington’ (Forney et al., 2003) or ‘Sunrise’ (Ochmian et al., 2015) blueberries also did not observe significant differences in soluble solids and titratable acids for the different storage atmospheres.

Fruit firmness of both cultivars was significantly affected by the atmosphere composition. At the end of the storage, firmness was lower in ‘Goldtraube’ stored in air and in 21% O_2+15% CO_2, whereas ‘Ozarkblue’ showed lower firmness in air (Table 6.1.). At the end of the storage the fruits showed a greater firmness, this enhanced resistance of the fruit to the penetration can be interpreted as excessive elasticity or gumminess, due to a strong loss of internal water turgor pressure (Giongo et al., 2013).

Table 6.1. – Effect of atmosphere composition on soluble solids, titratable acidity, firmness and anthocyanins content of blueberries during storage at 4 ºC.

Cultivar	**Atmosphere**	**Soluble solids (º Brix)**			**Acidity (% malic acid)**			**Firmness (N)**		
		1 d	**15 d**	**29 d**	**1 d**	**15 d**	**29 d**	**1 d**	**15 d**	**29 d**
Goldtraube	**air**	12.69±0.03	11.80±0.17	11.24±0.11^{b}	1.45±0.03	1.44±0.15	1.44±0.09	1.54±0.24	1.88±0.43^{a}	1.86±0.64^{b}
	2% O_2 + 2% CO_2	12.63±0.13	11.78±0.14	11.37±0.10^{a}	1.42±0.14	1.36±0.18	1.28±0.13	1.51±0.25	1.75±0.49^{ab}	2.21±0.49^{a}
	2% O_2 + 15% CO_2	12.70±0.31	11.63±0.56	11.66±0.28^{a}	1.56±0.07	1.53±0.10	1.53±0.10	1.52±0.21	1.70±0.33^{b}	2.06±0.48^{a}
	21% O_2 + 15%CO_2	12.77±0.22	11.83±0.18	11.47±0.30^{a}	1.48±0.08	1.26±0.04	1.20±0.10	1.52±0.20	1.59±0.42^{c}	1.85±0.47^{b}
Ozarkblue	**air**	11.87±0.05	11.59±0.41	11.09±0.28	1.04±0.26	0.99±0.08	0.98±0.14	1.07±0.27	1.18±0.50	1.19±0.51^{b}
	2% O_2 + 2% CO_2	11.90±0.09	11.47±0.30	11.10±0.35	1.08±0.03	0.89±0.05	0.84±0.08	1.15±0.30	1.29±0.54	1.49±0.47^{a}
	2% O_2 + 15% CO_2	11.83±0.23	11.64±0.07	11.17±0.13	1.03±0.08	0.95±0.17	0.94±0.11	1.16±0.26	1.13±0.43	1.45±0.43^{a}
	21% O_2 + 15%CO_2	11.73±0.22	11.37±0.10	11.37±0.53	1.00±0.08	0.89±0.11	0.87±0.11	1.15±0.24	1.14±0.42	1.37±0.41^{a}

Different letters represent significant differences (*P*<0.05) between columns, by the Tukey test.

6.4.2. Effect of atmosphere and storage duration on skin colour.

One day after storage, the colour parameter C* was 2.79 and 4.15, h^o was 296.7 and 288.4, and L* was 26.33 and 28.35 for the 'Goldtraube' and 'Ozarkblue' respectively. These values were slightly lower than those reported by (Zheng et al., 2003) for the 'Duke' (C* 4.71, h^o 305.7 and L* 32.0).

Relatively the parameter of colour C*, there were significant differences between the two cultivars throughout the storage, and 'Ozarkblue' presented the highest values for this parameter (Figure 6.1.a). In 'Goldtraube' and 'Ozarkblue' the colour parameters (i.e., C*, L* and h^o) (Figure 6.1.a., 6.1.b. and 6.1.c.) were significantly influenced by the atmosphere throughout storage time. However Haffner et al. (2002) not found effect of a 10% O_2 + 15% CO_2 atmosphere on any of the colour parameters during raspberry storage. Similarly, Holcroft et al. (1998) did not observe changes in the chroma (C*) and hue angle (h^o) of pomegranates stored under air or in air enriched with 10 or 20% CO_2 at 10 ºC for 6 weeks.

For the Goldtraube cultivar, the h^o and L* colour parameters (Figure 6.1.b. and 6.1.c.) were significantly influenced by the duration of storage. Thus, h^o tended to increase slightly after 14 days of storage. In 'Ozarkblue' the C*, h^o and L* colour parameters (Figure 6.1.b. and 6.1.c.) were significantly influenced by the duration of storage. Thus, the C* and L* parameters showed the highest values at the beginning of storage, indicating that fruit has its highest colour intensity and brightness at the beginning of storage. The h^o parameter tended to increase slightly during storage.

Sanford et al. (1991) also reported that the hue angle showed a slight increasing trend after 15 days of storage in lowbush blueberries, with the skin acquiring a reddish hue. Schotsmans et al., (2007) also observed an increase in hue angle under controlled atmosphere storage in rabbiteye blueberries, although the colour parameter L* presented a slight tendency to decrease after 14 days.

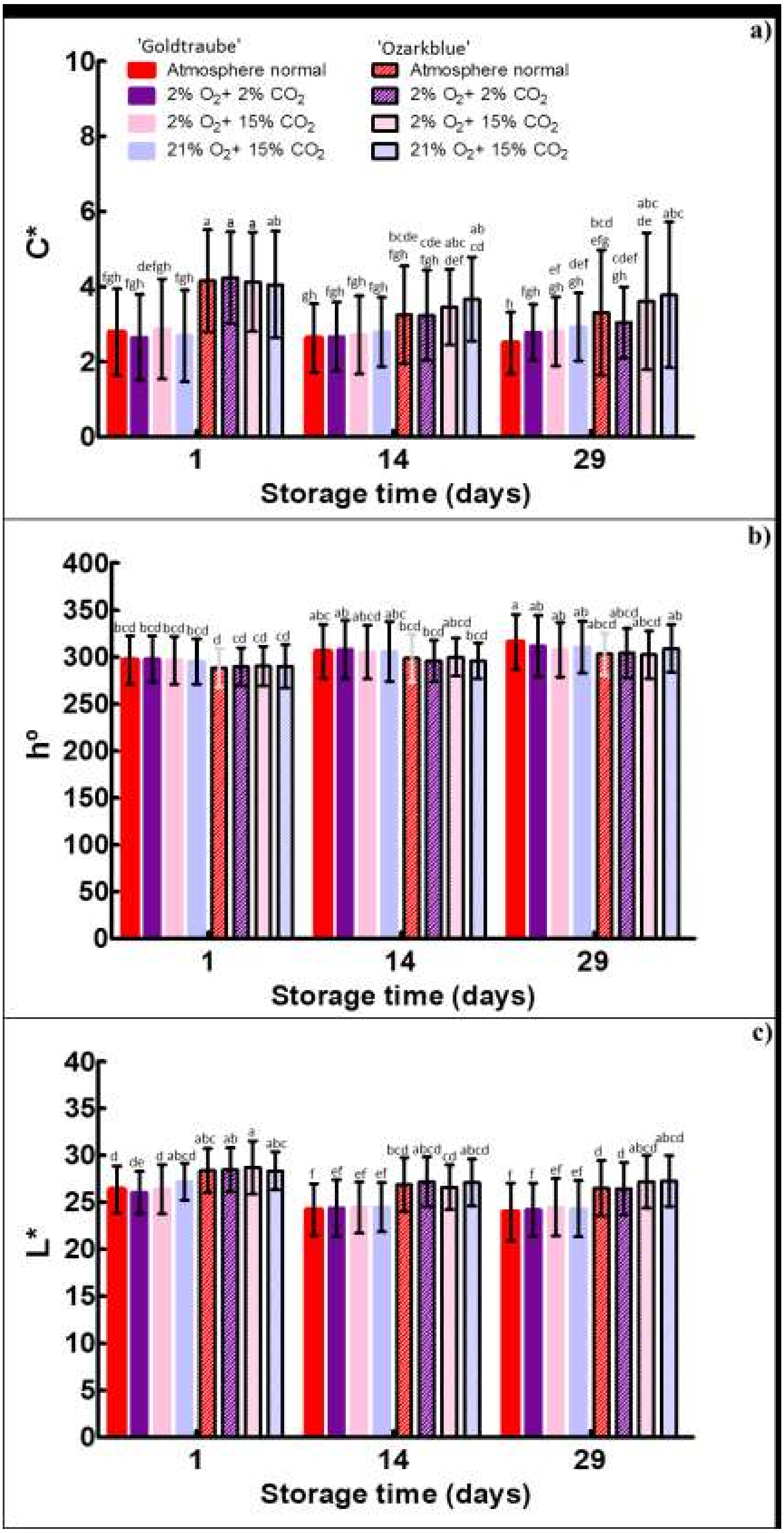

Figure 6.1. - The effect of different atmospheres throughout storage time on the colour parameters of fruit: a) C*, b) hº and c) L* and cultivar (Goldtraube and Ozarkblue). Results are expressed as the mean ± SD (n=60). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

6.4.3. Effect of atmosphere storage and storage duration on total anthocyanins content, total phenolic compounds and antioxidant activity.

Total anthocyanins content, total phenolic compounds and antioxidant activity were influenced by the cultivar, so ‘Goldtraube’ showed the highest values of those compounds (Figure 6.2.a., 6.2.b. and 6.2.c.). In ‘Goldtraube’ and ‘Ozarkblue’, total anthocyanins content (Figure 6.2.a.) were significantly influenced by storage atmosphere and by the duration of storage. The highest total anthocyanin content was obtained one day after storage. In both cultivars, the total anthocyanin content decreased with storage. At the end of storage in ‘Goldtraube’, the total anthocyanins content is higher on atmosphere normal and 2% O_2 + 2% CO_2.

Harb et al. (2014) found that during storage with high CO_2 concentration the expression levels of chalcone synthase (VcCHS) and dihydroflavonol-4-reductase (VcDFR) decreased significantly and dramatically. The decrease in expression level of these two enzymes was well correlated with the increase in CO_2 concentration especially when combined with 3 kPa O_2. Holcroft and Kader (1999a) also reported that ‘Selva’ strawberries exhibited changes in both phenylalanine ammonia lyase (PAL) and UDP glucose attributed to flavonoid glucosyltransferase (FGT) activity (two important enzymes involved in anthocyanin biosynthesis decreased under CO_2-enriched atmospheres).

Holcroft and Kader (1999b) found increased anthocyanin concentrations in strawberries stored in air and 2% O_2 after 5 and 10 days. Storage with low concentrations of O_2 (0.5%) tended to have slightly higher anthocyanin concentrations than those kept in CO_2-enriched storage. Zheng et al. (2003) also found that anthocyanins can be increased during storage of blueberry fruit, but with the application a higher O_2 concentration than that used in our study (i.e., between 60 and 100 %).

In ‘Goldtraube’ berries, the total phenol content (Figure 6.2.b.) was significantly influenced by the storage atmosphere and the duration of storage. Thus, storage at 2% O_2 + 15% CO_2 was the most advantageous 14 days after storage. In the cultivar Ozarkblue, the total phenol content was not significantly influenced by the storage atmosphere or by the duration of storage.

In both the Goldtraube and Ozarkblue cultivars, the antioxidant activity (Figure 6.2.c.) was significantly influenced by storage atmosphere and by the duration of storage. In ‘Goldtraube’ the storage atmospheres that showed the highest antioxidant activity 1 d after storage were 2% O_2 + 15% CO_2 and 21% O_2 + 15% CO_2. In ‘Ozarkblue’ berries, the storage atmospheres that resulted in the highest antioxidant activities 14 d after storage were a normal atmosphere and 21% O_2 + 15% CO_2.

In ‘Goltraube’ a CO_2 rich storage atmosphere appeared to influence antioxidant activity more than oxygen. While in ‘Ozarkblue’ O_2 rich storage atmosphere appeared to influence antioxidant activity more than carbon dioxide. Zheng et al. (2003) found that storage of blueberry fruit at elevated O_2 atmosphere (i.e., between 60 and 100%) improved the total phenolics, individual phenolic compounds and the antioxidant activity. Ayala-Zavala et al. (2007) also reported a higher antioxidant activity and total phenolic content in strawberries stored under high-oxygen atmospheres (>40%) than in those stored in air. González-Roncero and Day (1998) suggested that the improved in total phenolic compounds could be a reaction to the oxidative stress originated by elevated O_2 levels.

However, in cranberries, stored in air the total antioxidant activity was 45% higher. This increment was limited by storage in 21% oxygen and 30% carbon dioxide (Gunes et al., 2002).

In both cultivars, the highest antioxidant activity was obtained at the end of the storage period. Independent of cultivar, the antioxidant activity increased over the storage period. Schotsmans et al. (2007) suggested that extended storage may conduce to the oxidation of polyphenolic compounds. However, these oxidized polyphenols still can possess antioxidant activity, which can justify the increment in the antioxidant activity.

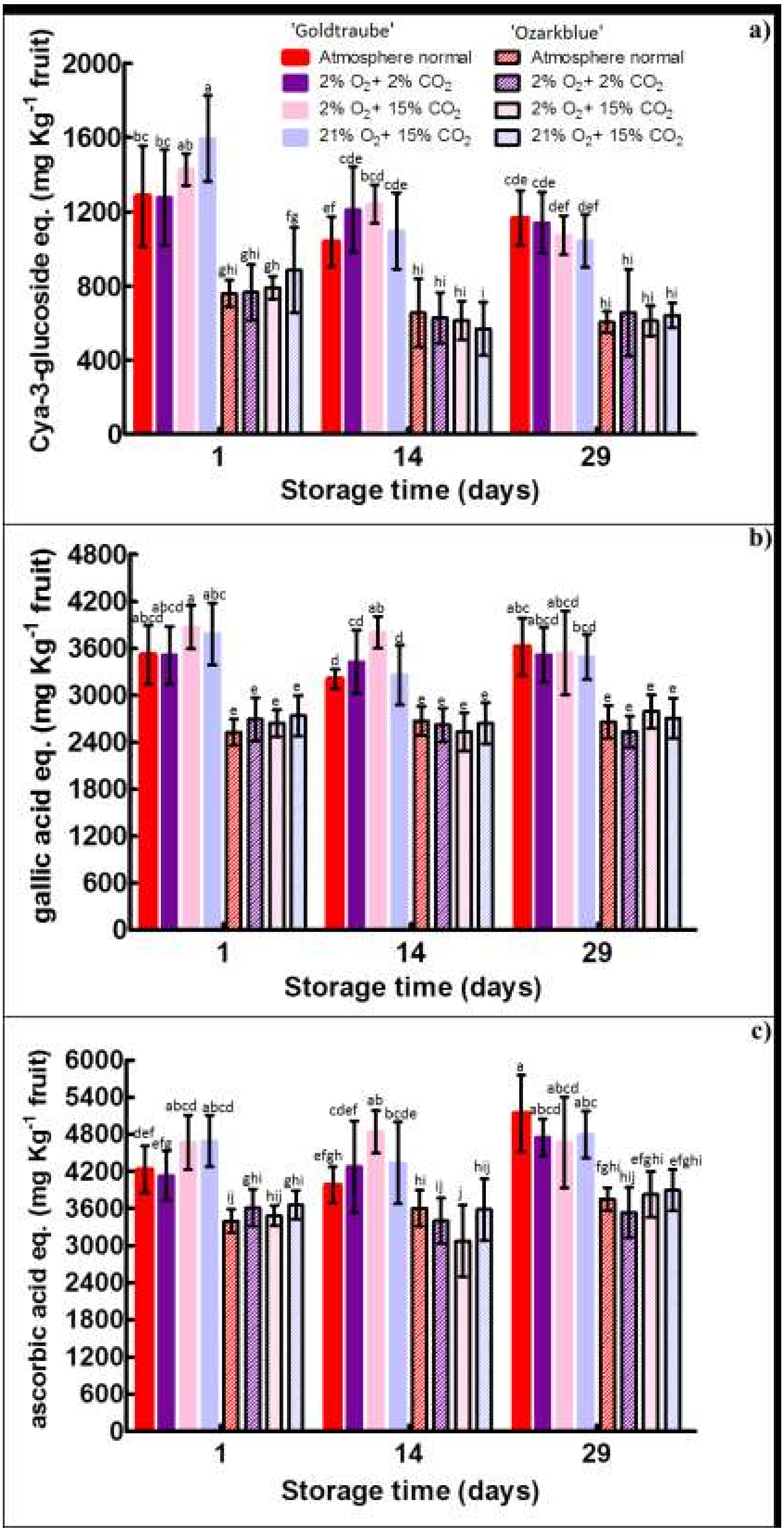

Figure 6.2. – The effect of different atmospheres throughout storage time on the a) total anthocyanin content, b) total phenolic compound content, and c) antioxidant activity in blueberry fruit during storage at 4 ºC. Results are expressed as the mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

6.4.4. Effect of atmosphere storage on sensory evaluation.

Sensory evaluation of blueberries stored under the different atmospheres was similar by 7 and 21 days of storage with one exception: berries from ‘Ozarkblue’ stored for 7 days were classified as less sweet when stored under 2% O_2+15% CO_2 than for other conditions (Figure 6.3. and 6.4.). The controlled atmospheres influenced total phenolics and antioxidant activity. But thus improve the functional value, without causing changes perceptible by sight. In general sensory panel also did not identify significant differences in fruits stored under the different atmosphere.

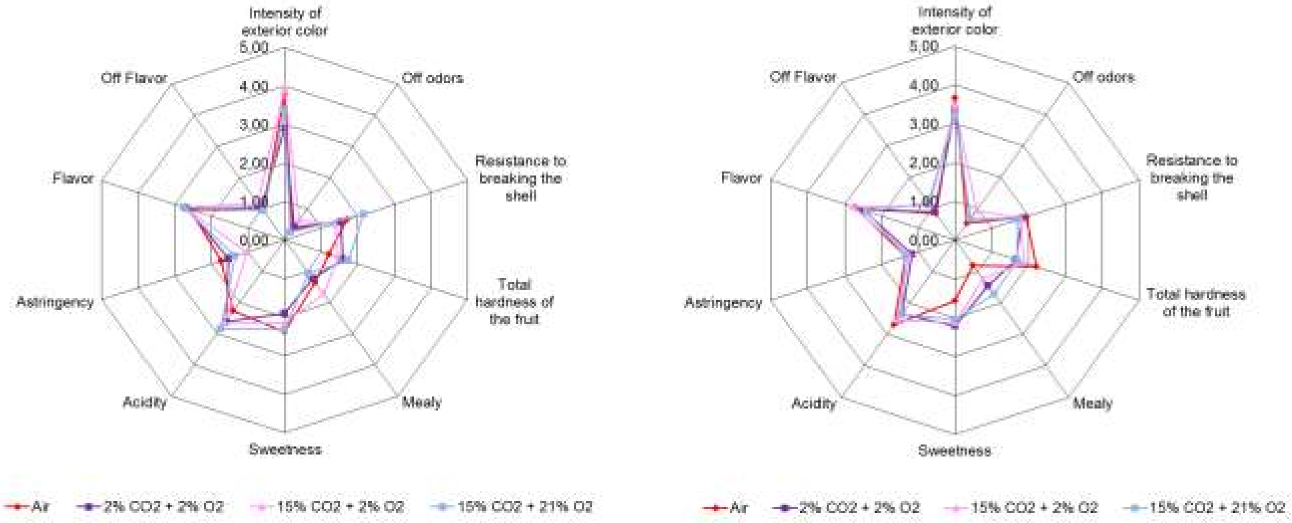

Figure 6.3. – Effect of atmosphere composition on the sensory profile after 7 and 21 days of storage of ‘Goldtraube’.

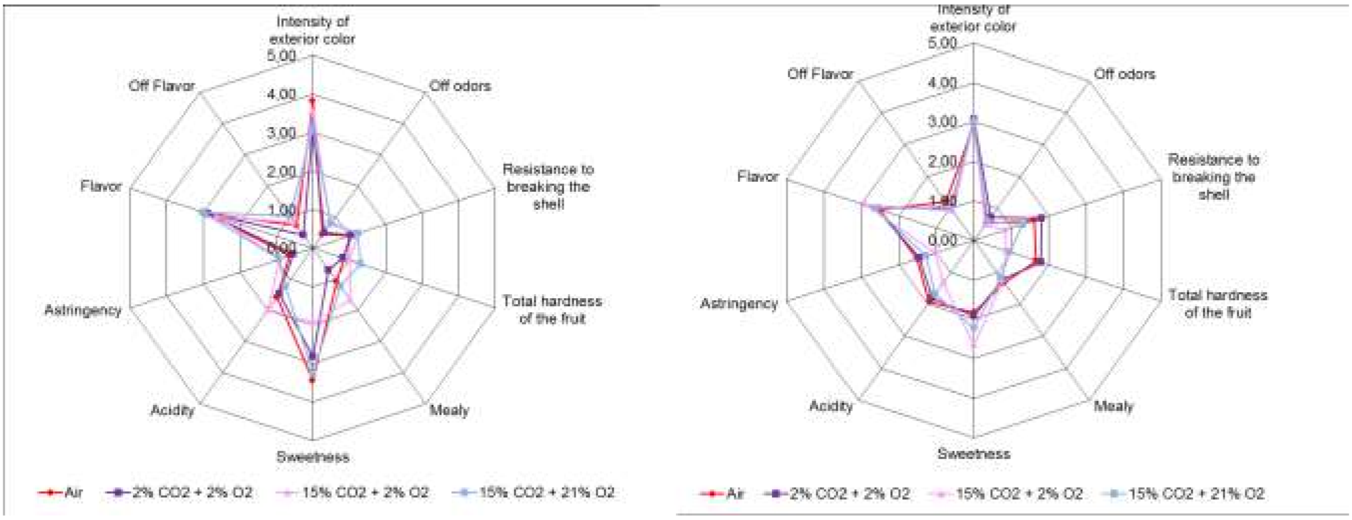

Figure 6.4. – Effect of atmosphere composition on the sensory profile after 7 and 21 days of storage of ‘Ozarkblue’.

6.4.5. Effect of atmosphere and storage time on anthocyanin and polyphenol profiles.

In this study, 11 phenolic compounds were identified in the ‘Goldtraube’ under all storage atmospheres (Figure 6.5., 6.6.) (malvidin 3-O-galactoside, malvidin 3-O-glucoside, petunidin 3-O-glucoside, cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, cyanidin 3-O-arabinoside, dephinidin 3-O-galactoside, delphinidin 3-O-glucoside, peonidin 3-O-galactoside, ferulic acid and chlorogenic acid) and nine in ‘Ozarkblue’ (Figure 6.7., 6.8.) (malvidin 3-O-galactoside, malvidin 3-O-glucoside, dephinidin 3-O-galactoside, cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, cyanidin 3-O-arabinoside, peonidin 3-O-galactoside, ferulic acid and chlorogenic acid). Just like us Zheng et al. (2003) identified nine anthocyanins in ‘Duke’ blueberries: cyanidin 3-galactoside, cyanidin 3-glucoside, delphinidin 3-galactoside, delphinidin 3-glucoside, malvidin 3-arabinoside, malvidin 3-galactoside, malvidin 3-glucoside, petunidin 3-arabinoside, and petunidin 3-galactoside.

In both cultivars, dephinidin 3-O-galactoside and malvidin 3-O-galactoside were the predominant anthocyanins in all atmospheres throughout storage.

In ‘Goldtraube’ the compounds delphinidin 3-O-galacoside, cyanidin 3-O-galactoside, cyanidin 3-O-arabinoside and ferulic acid were affected by storage atmosphere. The atmospheres resulting in higher concentrations of these compounds were 2% O_2+2% CO_2 and 2% O_2+15% CO_2, with the exception of ferulic acid (2% O_2+15% CO_2 and 21% O_2+15% CO_2). The storage duration also influenced the concentration of all compounds; all decreased during storage, with the exception of chlorogenic acid.

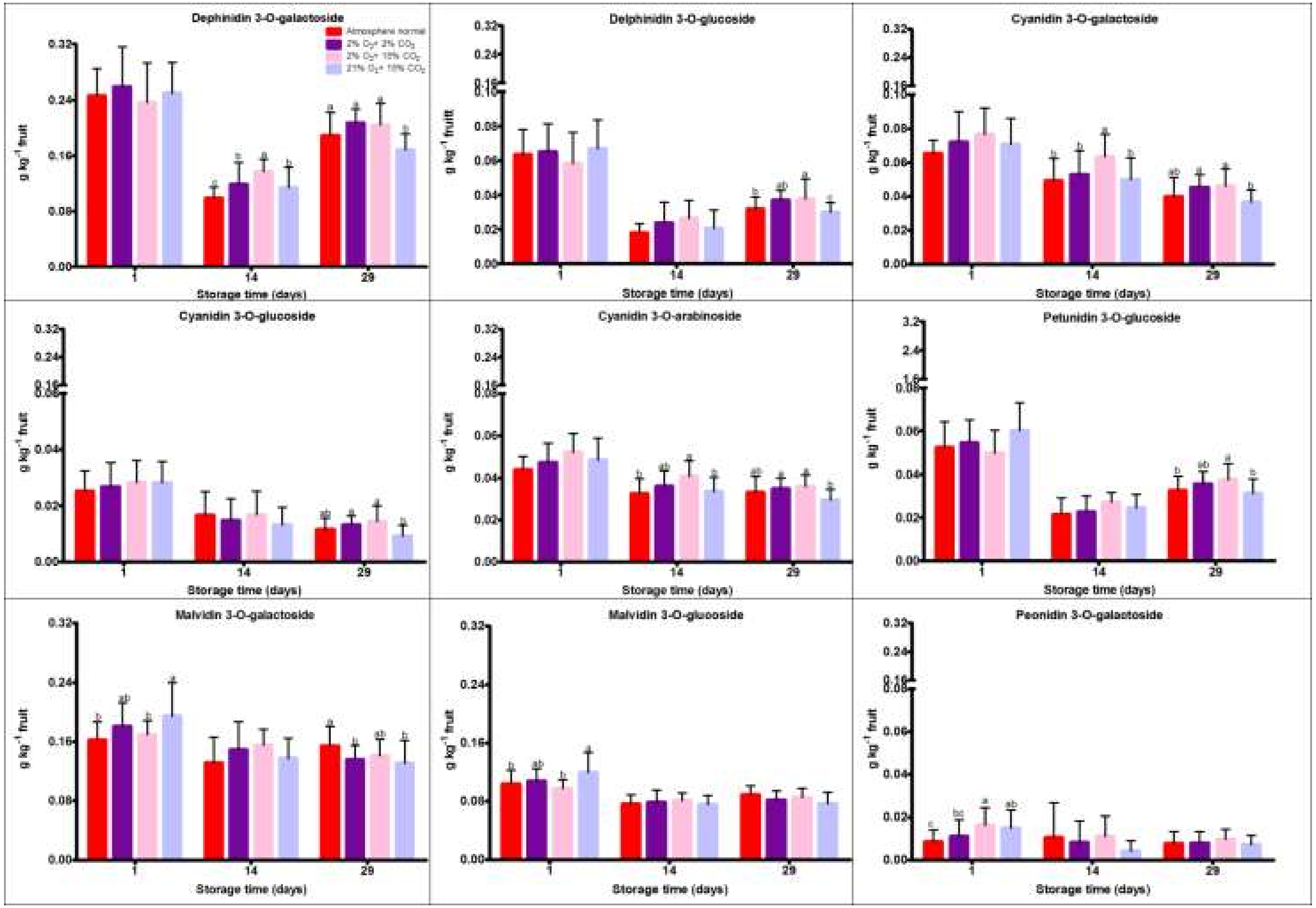

Figure 6.5. – The effect of different atmospheres throughout storage time on anthocyanin composition in the 'Goldtraube' blueberry fruit during storage at 4ºC. Results are expressed as the mean ± SD (n=18). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

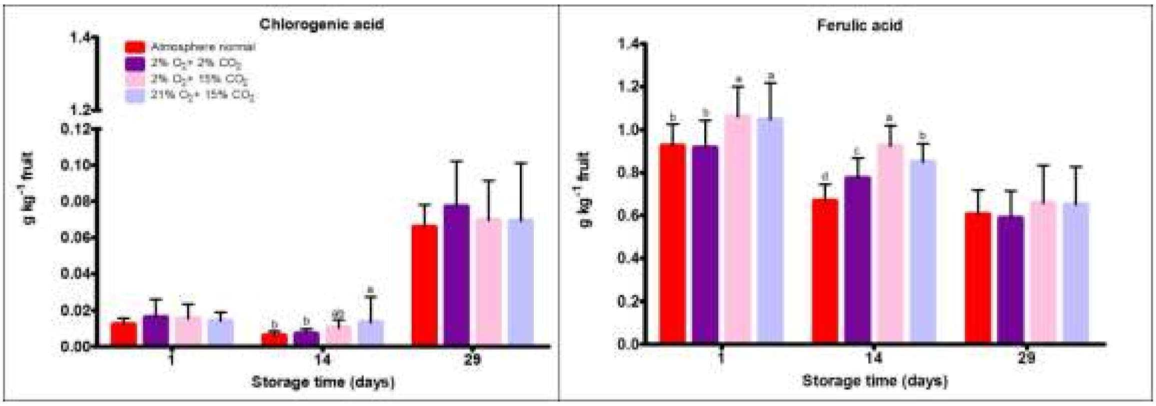

Figure 6.6. – The effect of different atmospheres throughout storage time on hydroxycinnamic acids (chlorogenic acid and ferulic acid) in the ‘Goldtraube’ blueberry fruit during storage at 4ºC. Results are expressed as the mean ± SD (n=18). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

In 'Ozarkblue' the compounds delphinidin 3-O-galacoside, cyanidin 3-O-galactoside, cyanidin 3-O-arabinoside, malvidin 3-O-galactoside and ferulic acid were affected by the storage atmosphere. The 2% O_2+15% CO_2 and 21% O_2+15% CO_2 atmospheres resulted in higher concentrations of these compounds, with the exception of malvidin 3-O-galactoside (2% O_2+2% CO_2). The storage duration also influenced the concentration of all compounds; all decreased during storage, with the exception of chlorogenic acid. The compounds cyanidin 3-O-glucoside, malvidin-3-O-glucoside, malvidin-3-O-glucoside, peonidin-3-galactoside and ferulic acid decreased throughout storage. The concentrations of dephinidin 3-O-galactoside and cyanidin 3-O-galactoside did not differ at the beginning and the end of storage. Furthermore, the concentrations of cyanidin 3-O-arabinoside and chlorogenic acid increased during storage.

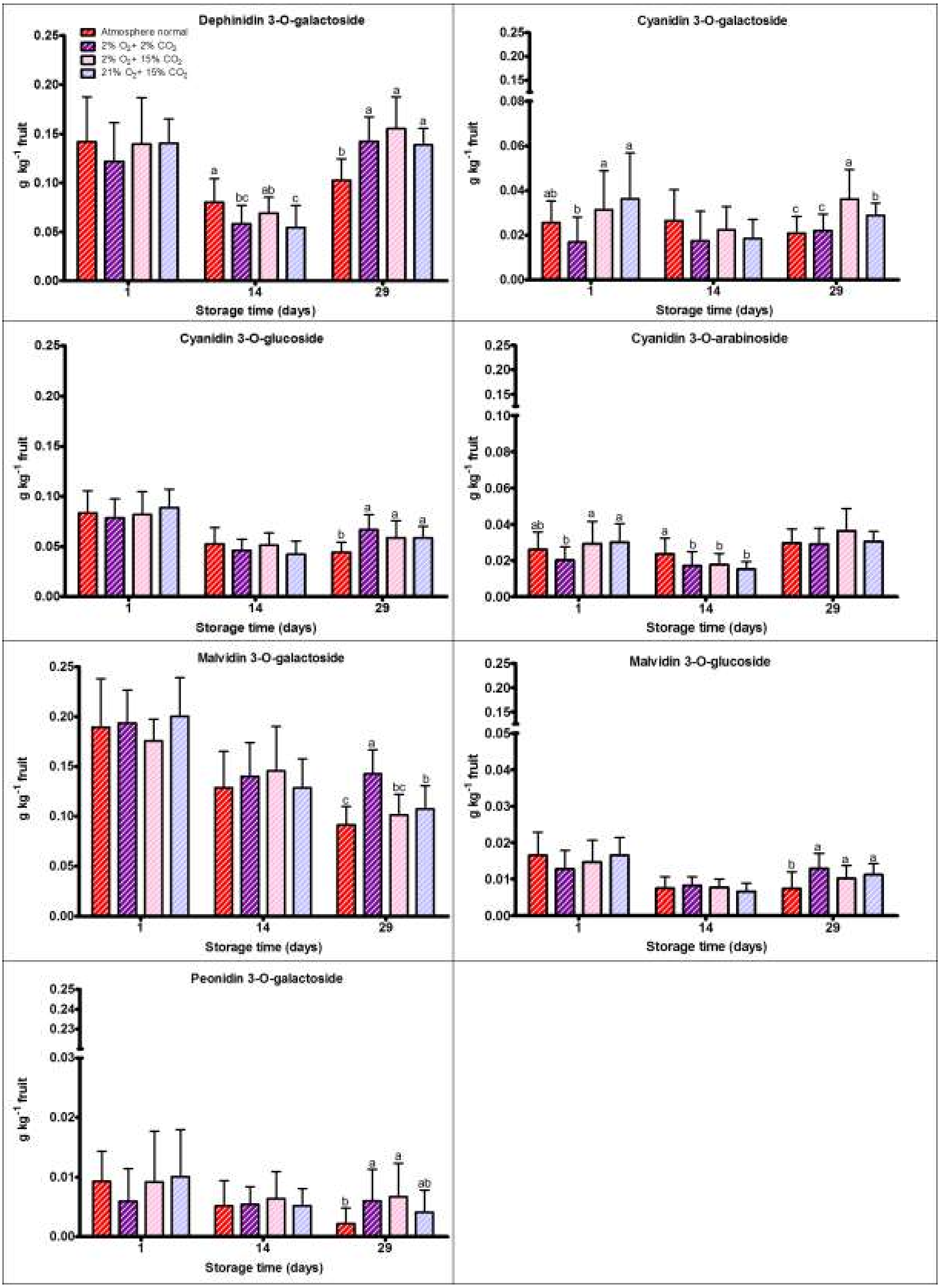

Figure 6.7. - The effect of different atmospheres throughout storage time on anthocyanin composition in the Ozarkblue blueberry fruit during storage at 4ºC. Results are expressed as the mean ± SD (n=18). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

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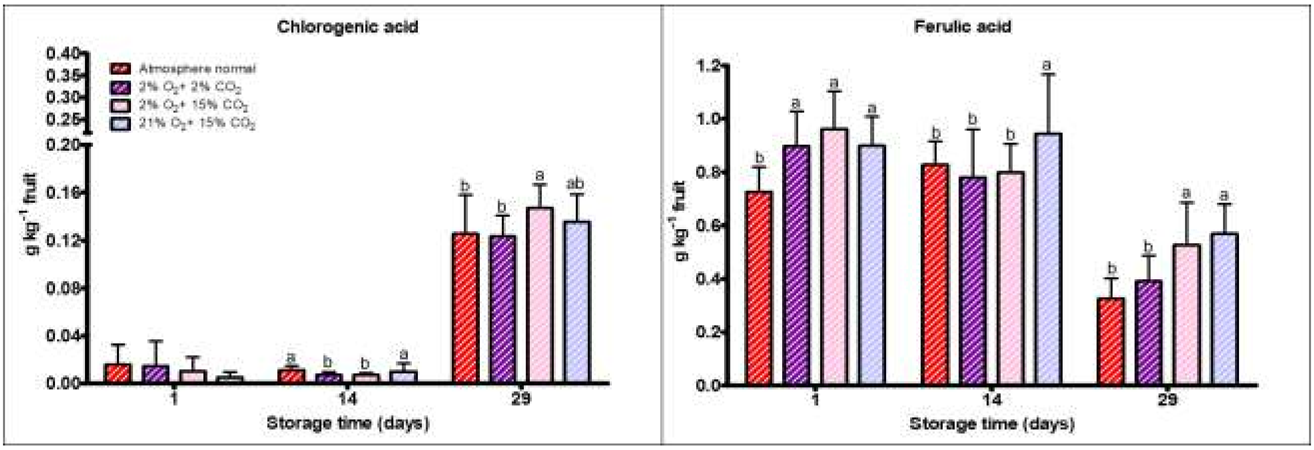

Figure 6.8. - The effect of different atmospheres throughout storage time on hydroxycinnamic acids in the Ozarkblue blueberry fruit during storage at 4ºC. Results are expressed as the mean ± SD (n=3). Means followed by the same letter, by day, are not significantly different at α=0.05 by the Tukey's test.

In 'Goldtraube' a low O_2 concentration seemed to have the greatest influence on those compounds. However, Ozarkblue appeared to benefit from higher concentrations of CO_2. Rodriguez and Zoffoli (2016) also observed that different blueberry cultivars presented different sensitivities to CO_2 and Zheng et al. (2003) found a negative effect associated with high O_2 concentration.

Storage time also negatively influenced anthocyanin concentrations. Zheng et al. (2003) found that all anthocyanins identified, except malvidin 3-arabinoside, reduced constantly, presented much lower levels by the end of the storage compared to the initial content.

6.4.6. Relationship between colour, anthocyanins and polyphenols.

Principle component analysis (PCA) yielded four interpretable components that together explain 76.8%, 74.1 % and 75.4% of the total variance of the variables at days 0 (Figure 6.9.a.), 14 (Figure 6.9.b.) and 29 (Figure 6.9.c.) after storage, respectively.

On days 1 and 14, the analysed data by principal components 1 (PC1) explains for 47.5 and 42.9% of the total variation on days 0 and 14, respectively. PC1 is related to:

malvidin 3-O-glucoside, dephinidin 3-O-galactoside, cyanidin 3-O-galactoside, cyanidin 3-O-arabinoside, total phenolic compounds, total anthocyanins content, antioxidant activity, cultivar, cyanidin 3-O-glucoside (PC1). The antioxidant activity, total phenolic compounds and the total anthocyanins content are positively related to malvidin 3-O-glucoside, dephinidin 3-O-galactoside, cyanidin 3-O-galactoside, cyanidin 3-O-arabinoside. Other authors have reported a correlation between antioxidant activity and total phenolic compounds and anthocyanins content in blueberries (Zheng et al., 2003), highbush blueberries, lowbush blueberries, raspberries (Kalt et al., 1999) and strawberries. Kalt et al. (1999); Prior et al., (1998); Zheng et al., (2007) found that different blueberries exhibited the highest correlation between total phenolics and antioxidant activity and the lowest correlation between total anthocyanins content and antioxidant activity, although both correlations were significant. Gil et al. (2002) also found a correlation between phenolic compounds and antioxidant activity in stone fruit (i.e., nectarine, peach and plum). Cordenunsi et al. (2005) found that the antioxidant activity of strawberries is correlated with total phenolic compounds, but an improve in anthocyanins during storage was not correlated with total phenolic content.

PC2 explains for 11.6 and 11.9% (PC2) of the total variation on days 1 and 14, respectively. PC2 is related to: h°, C* and L*. The parameter h° is negatively correlated to C* and L*. This work did not demonstrate an evident correlation between colour parameters (C*, L* and h°) and total phenolic compounds, antioxidant activity, or total anthocyanin content in either cultivar under different controlled atmospheres and storage periods. Haffner et al. (2002) also reported in *Rubus* that the skin colour measurements were not good indicators of anthocyanin contents.

On day 1, the storage treatments are positively related to ferulic acid and peonidin 3-O-galactoside and negatively to chlorogenic acid. On day 14, the storage treatments are positively related to ferulic acid and chlorogenic acid.

On day 29, the main variation represented by principal component 1 and 2 (PC1 and 2), was explained by malvidin 3-O-glucoside, total anthocyanins content, antioxidant activity, total phenolic compounds, cultivar, cyanidin 3-O-glucoside and chlorogenic acid (PC1); and cyanidin 3-O-arabinoside, cyanidin 3-O-galactoside, delphinidin 3-O-galactoside, peonidin 3-O-galactoside, and malvidin 3-O-galactoside (PC2). The two components account for 45.5% (PC1) and 13.1% (PC2) of the total variation. The antioxidant activity, total phenolics compounds and the total anthocyanins content are positively related to malvidin 3-O-glucoside, and negatively related to cultivar, cyanidin 3-O-glucoside and chlorogenic acid.

On day 29, the differences between the storage treatments and in ferulic acid content explained 7.6 % of the total variation. On day 29, the storage treatments are positively related to ferulic acid.

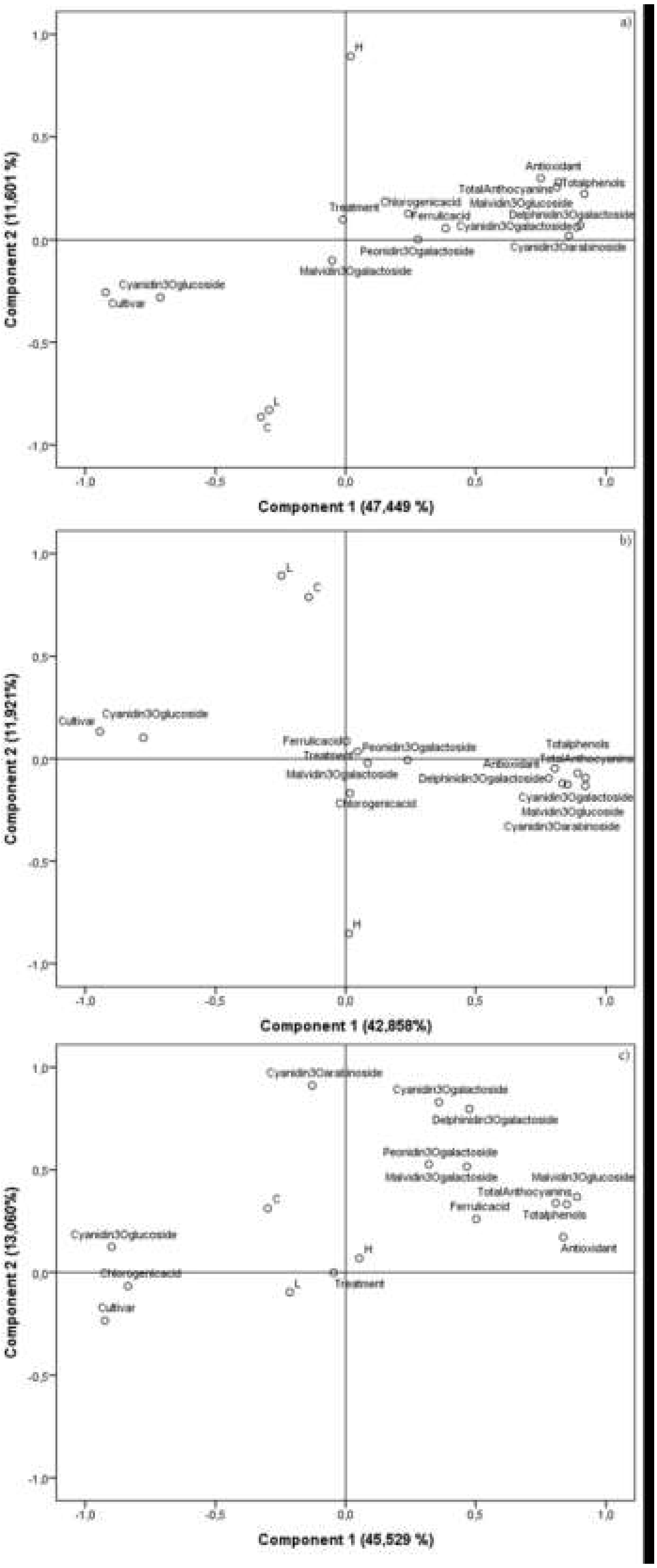

Figure 6.9. – Positions of PC scores for anthocyanin composition, total anthocyanins content, total phenolic compounds, antioxidant activity, colour parameters (hº, C* and L*), cultivar and atmosphere based on PC1 and PC2. Each percentage represents the variance of each principal component. a) day 1, b) day 14 and c) day 29.

6.5. Conclusions

The effects of the atmospheres studied on the commercial, phytochemical, and sensory quality attributes were generally minor in the blueberry cultivars ‘Goldtraube’ ‘Ozarkblue’. In general, controlled atmosphere improved firmness retention in relation to fruit stored in air. Sensory differences could not be perceived by a trained panel.

The effect of the atmosphere on the total anthocyanin content, total antioxidant activity and anthocyanin profile appears to be cultivar-dependent. The colour and total anthocyanin content of blueberries were not affected by the storage atmosphere for either cultivar.

For the ‘Goldtraube’ we observed a greater positive effect on total phenol content and antioxidant activity under a high concentration of CO_2. However, in the ‘Ozarkblue’ the total phenol content was not influenced by storage atmosphere, although the antioxidant activity was affected, seemingly by the concentration of O_2.

The total anthocyanin and phenol contents of the blueberries decreased during storage. However, their antioxidant activity increased over time. This increase was linked to an increase in specific anthocyanins, for example, chlorogenic acid in ‘Goldtraube’ and dephinidin 3-O-galactoside, cyanidin 3-O-galactoside, cyanidin 3-O-arabinoside and chlorogenic acid in ‘Ozarkblue’ fruit. In Goldtraube’, the factor with the greatest influence was O_2 concentration. However, ‘Ozarkblue’ appeared to be more affected by high concentrations of CO_2.

We also found that to preserve anthocyanin, a low concentration of O_2 (2%) is favourable in the ‘Goldtraube’, while in ‘Ozarkblue’, a high concentration of CO_2 (15%) has the greatest effect. Furthermore, high concentrations of CO_2 seem to have the greatest influence on the identified phenolic compounds.

Because the response to different storage atmospheres appears to be cultivar dependent, we believe that further studies involving additional cultivars are necessary.

PART IV - Conclusions and Future Perspectives

CHAPTER 7-Conclusions

Blueberry production in Portugal has increased significantly in recent years (according to FAO data it has increased from 510 tonnes in 2010 to 6572 in 2016). This increase in production is associated with community support for agriculture, promoting the establishment of young farmers who have chosen the production of this crop. Blueberry is a fruit with a high antioxidant activity and has been reported with many preventive effects of various diseases such as heart disease and cancer. In this work we intended to study the effect of different factors on the functional and commercial quality of blueberries of three cultivars: Bluecrop, Goldtraube and Ozarkblue throughout the storage at 4 º C.

Initially, we performed a characterization of the parameters of commercial and functional quality, anthocyanins and hydrodynamic acids present in each cultivar, its evolution along the storage at 4 ºC was also studied.

We found that the cultivar Ozarkblue had the lowest water loss rate (0.160% day^{-1}), the Goldtraube (0.214% dia^{-1}) and the Bluecrop (0.216% day^{-1}) cultivars had the highest water loss rate. The ‘Goldtraube’ was the one with the highest content of total anthocyanins content. We identified nine anthocyanins in ‘Bluecrop’ and ‘Goldtraube’ (delphinidin 3-O-galactoside, delphinidin 3-O-glucoside, cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, cyanidin 3-O-arabinoside, petunidin 3 -O-glucoside, malvidin 3-O-galactoside, malvidin 3-O-glucoside and peonidin-3-galactoside), and in ‘Ozarkblue’ seven (delphinidin 3-O-galactoside, cyanidin 3-O-galactoside, cyanidin 3- O-glucoside, cyanidin 3-O-arabinoside, malvidin 3-O-galactoside, malvidin 3-O-glucoside, peonidin-3-O-galactoside) and two hydroxycinnamic acids: ferulic and chlorogenic acids in the three cultivars.

The effect of ethylene treatment on total anthocyanin, antioxidant activity and different anthocyanins seems to depend on the cultivar, with increased antioxidant activity, but without detrimental apparent effects on other quality attributes such as firmness. In this way the application of ethylene seems to be a beneficial strategy to overcome the natural decrease observed during the storage, guaranteeing a greater activity during the storage and, consequently, guaranteeing the benefits of the fruit until the moment of consumption.

The effect of 1-MCP did not influence some quality parameters, such as weight loss rate and respiration rate. However, it influenced the parameters of functional quality as the content of anthocyanins, total phenolic compounds and antioxidant activity. 1-MCP treatment initially retained the level of anthocyanins, total phenolic compounds and antioxidant activity, but generally led to an increase in these levels at the end of storage. The application of 1-MCP was also dependent on the cultivar. Thus, the application of 1-MCP in the preservation of blueberries can be an efficient strategy in the case of transport at great distances, in order to present the functional advantages when they reach consumers.

When we studied different controlled atmospheres (air, 2% O_2 + 2% CO_2, 2% O_2 + 15% CO_2, and 21% O_2 + 15% CO_2) in the fruits storage of the ‘Goldtraube’ and ‘Ozarkblue’ cultivars at 4 ºC (L * and hº) and the titratable acidity were not influenced by the different atmospheres. However the controlled atmosphere improved the retention of firmness in relation to the fruits stored in the air.

The effect of the atmosphere on total anthocyanins content, antioxidant activity and anthocyanin profile also appeared to be dependent on cultivar. Total anthocyanins content and phenolic compounds decreased during storage for all studied atmospheres. However, to preserve the anthocyanin content, a low concentration of O_2 (2%) in the ‘Goldtraube’ is favored, while in ‘Ozarkblue’ a high concentration of CO_2 (15%) has the greatest effect. In addition, high concentrations of CO_2 seem to have the greatest influence on the identified phenolic compounds.

In general, the panel trained for sensory analysis did not distinguish sensory differences in blueberries stored at different atmospheres after 7 and 21 days of storage.

CHAPTER 8-Future Perspectives

The present work showed some postharvest techniques that can be used to improve the functional activity of blueberry fruits of three cultivars: ‘Bluecrop’, ‘Goldtraube’ and ‘Ozarkblue’, without changing commercial quality parameters. Thus, we intend to suggest some work that may be carried out with the final aim of improving functional quality without compromising parameters such as soluble solids content, titratable acidity, firmness and colour.

In the treatment with ethylene we verified that it has a cultivar-dependent effect, so it seems to us important to remake these tests but with other cultivars, since at present in our country a great diversity of cultivars are produced. Also in relation to the effect of ethylene, it is important in future work’s to measure ethylene production during the storage of blueberries, as well as to measure PAL and Pal mRNA, so that we can better explain the process that is behind the increase of anthocyanins after the treatment with ethylene and the delay in this increase with 1-MCP.

In relation to pre-harvest treatments the strategy was to apply other exogenous growth regulators such as abscisic acid, brassinosteroids and then observe its effect on the accumulation of anthocyanins and gene expression of anthocyanin biosynthesis in different blueberry cultivars.

In recent years because of climate change we have been facing periods of extreme drought and high temperatures that greatly influence the production of blueberry. The application of kaolinite can reduce these stress situations in the plant. Another study that may be interesting is the application of kaolinite in the plants to observe its subsequent effect on fruit quality.

One of the problems of the blueberry plant is that it does not have root hair which makes it difficult to absorb water; recently studies have been carried out with the inoculation of mycorrhizae which may improve the absorption of water by the plants. In this way it may be interesting to also evaluate the effect of mycorrhization in the functional quality of fruit throughout the storage.

Even in the production, nutrient deficiencies may lead to an increase in the production of anthocyanins, so it seems important to first study, for example, the effect of the lack of inorganic phosphate and nitrogen on blueberries of different cultivars. Later verify what can make this lack lead to the increase of anthocyanins, and how these fruits behave throughout the storage, since many cultural techniques may negatively influence storage.

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ANNEX 1 - Standards and chromatographic information regarding the calibration curves

Annex 1. Standards and chromatographic information regarding the calibration curves

Standard	Retention time	Maximum absorbance (λ)	Calibration curves		
			Slope	y-Intercept	R^2
Delphinidin 3-O-galactoside	34.21	526.9	167267	150183	0,979
Delphinidin 3-O-glucoside	36.32	524.7	188831	144981	0,978
Cyanidin 3-O-galactoside	38.06	515.0	184295	103285	0,992
Cyanidin 3-O-glucoside	40.57	516.2	162294	93261	0,994
Cyanidin 3-O-arabinoside	42.75	517.4	183876	5962	0,981
Petunidin 3-O-glucoside	43.57	524.7	197635	78029	0,981
Peonidin-3-galactoside	44.34	527.2	69860	63978	0,977
Malvidin 3-O-galactoside	48.15	527.2	158006	75292	0,975
Malvidin 3-O-glucoside	50.11	528.4	166282	13416	0,981
Chlorogenic acid	32.99	324.9	244313	33315	0,983
Ferulic acid	46.48	322.5	85666	66924	0,989

ANNEX 2 –Sensory evaluation - Prove sheet

Prove sheet

Name ____ Date ________________

Sample type ____

Intensity of exterior colour (Evaluate the whole sample)

|____________|____________|____________|____________|____________|

Light
Dark

PLACE 4 FRUITS IN THE MOUTH AND CHECK THE FOLLOWING PARAMETERS:

Resistance to breaking the cuticle

|____________|____________|____________|____________|____________|

Soft
Hard

Total hardness of the fruit

|____________|____________|____________|____________|____________|

Soft
Hard

Mealy

|____________|____________|____________|____________|____________|

No Mealy
Very Mealy

Sweetness

|____________|____________|____________|____________|____________|

Not sweet
Very sweet

Acidity

|____________|____________|____________|____________|____________|

No acid
Very acid

Adstringency

|____________|____________|____________|____________|____________|

No Astringent
Very Astringent

Flavor

|____________|____________|____________|____________|____________|

Does not taste blueberry
Taste blueberry

Off Flavor (presence of strange aromas)

|____________|____________|____________|____________|____________|

Absence
Presence

Off Odors (presence of strange odors)

|____________|____________|____________|____________|____________|

Absence
Presence