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Effects of dynamic controlled atmosphere by respiratory quotient on some quality parameters and volatile profile of 'Royal Gala' apple after long-term storage

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ABSTRACT

The effects of dynamic controlled atmosphere (DCA) storage based on chlorophyll fluorescence (DCA-CF) and respiratory quotient (DCA-RQ) on the quality and volatile profile of 'Royal Gala' apple were evaluated. DCA storage reduces ACC (1-aminocyclopropane-1-carboxylate) oxidase activity, ethylene production and respiration rate of apples stored for 9 months at 1.0 °C plus 7 days at 20 °C, resulting in higher flesh firmness, titratable acidity and lesser physiological disorders, and provided a higher proportion of healthy fruit. Storage in a regular controlled atmosphere gave higher levels of key volatiles (butyl acetate, 2-methylbutyl acetate and hexyl acetate), as compared to fruit stored under DCA-CF, but fruit stored under DCA-RQ 1.5 and RQ 2.0 also showed higher amounts of key volatile compounds, with increment in ethanol and ethyl acetate, but far below the odour threshold. Storage in DCA-CF reduces fruit ester production, especially 2-methylbutyl acetate, which is the most important component of 'Royal Gala' apple flavour.

1. Introduction

In many countries, consumers prefer apples that have a red skin colour and there is a tendency to replace traditional apple cultivars by their mutants because of their deeper red skin coloration. Among 'Gala' mutants, 'Royal Gala' shows a significant production area, especially due to its richer red skin colour in comparison to the 'Gala' standard. This 'Gala' mutant ranges from 20 to 25% of the total Brazilian apple production. However, its harvest window is about two weeks, which is short. Therefore, a significant part of the production has to be stored.

Apples are stored under controlled atmosphere (CA) with oxygen partial pressure ranging from 1.0 up to 1.2 kPa and carbon dioxide from 2.0 to 3.0 kPa (Brackmann, Weber, Pinto, Neuwald, & Steffens, 2008). Nevertheless, even under these storage conditions, significant quality losses occur after long-term storage, which are related to the high incidence of physiological disorders (Brackmann et al., 2008), flesh firmness loss (Weber et al., 2015) and a significant reduction of volatile compounds production and emission (Bangerth, Song, & Streif, 2012; Fellmann, Rudell, Mattinson, & Mattheis, 2003; Song & Bangerth, 1996). The storage of apple in lower O_2 conditions, such as 0.3 kPa, may negatively affect the fruit, due to anaerobic metabolism, resulting in higher levels of fermentative volatiles (Lumpkin, Fellman, Rudell, & Mattheis, 2014). Thus, it is necessary to develop a technology that allows fruit storage with low physiological disorders incidence, flesh firmness loss and better volatile compounds maintenance.

During the last few years, a new trend in oxygen partial pressure monitoring has been developed and tested for apple storage under CA. This new technology, called dynamic controlled atmosphere (DCA), is based on the lowest oxygen limit (LOL) tolerated by the fruit in their metabolic stage. When monitoring the LOL, the oxygen partial pressures can be reduced in the storage rooms to the lowest limit tolerated by the fruit and changed according to the LOL throughout the storage. Nowadays, there are three methods to monitor the LOL in real time during apple storage: based on the ethanol production by fruit (Veltman, Verschoor, & Ruijsch van Dugteren, 2003), through chlorophyll fluorescence (Prange et al., 2007; Wright, DeLong, Gunawardena, & Prange, 2012) and by assessing the respiratory quotient of the fruit during storage (Bessemans, Verboven, Verlinden, & Nicolaï, 2016; Weber

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et al., 2015; Wright et al., 2012). The storage of apples under DCA significantly decreases the ACC oxidase enzyme activity, ethylene production and maintains high flesh firmness and lower physiological disorder (Weber et al., 2015). However, there are few reports regarding the DCA effect on volatile profile in comparison to CA. Brackmann, Weber, and Both (2015) found higher flesh firmness and amount of healthy fruit in apples stored under DCA-RQ, compared to those submitted to DCA-CF. Additionally, when evaluating 'Royal Gala' apple, the fruit stored in DCA-RQ maintained a similar quality to the samples submitted to DCA-CF (Weber et al., 2015).

A complex mixture of organic compounds, such as esters, alcohols, aldehydes, among others, composes the volatile profile of apple. These volatile compounds are very dynamic and can change according to the maturity stage, the cultivar, the application of 1-methylcyclopropene (1-MCP), the storage conditions, besides some other factors. Apples that have a red peel generally show a higher total ester concentration, as compared to those with low red skin coloration, which may be related to the anthocyanin content (Young, Chu, Lu, & Zhu, 2004). 'Royal Gala' apple stored under ultralow oxygen partial pressure (0.5 kPa) significantly reduced the straight-chain ester production, but branched-chain esters were not reduced by lowering oxygen (Both, Brackmann, Thewes, Ferreira, & Wagner, 2014). Another study comparing ultralow oxygen (ULO) with DCA-CF (dynamic controlled atmosphere based on chlorophyll fluorescence) found a significant ester reduction in 'Pinova' apple stored in DCA-CF compared to ULO (1.5 kPa O_2 + 1.3 kPa CO_2), but showed lower reduction in relation to ULO + 1-MCP (Raffo, Kelderer, Paoletti, & Zanella, 2009). However, there are no reports in the literature evaluating and comparing CA, DCA-CF and DCA-RQ (dynamic controlled atmosphere based on respiratory quotient) and their effects on quality and volatile profile of apples.

The aim of this paper was to evaluate the effect of DCA, based on chlorophyll fluorescence and respiratory quotient, on the quality and volatile profile of 'Royal Gala' apples after long-term storage, since there are no results in the literature evaluating the effect of DCA-RQ on the volatile profile of apples.

2. Material and methods

2.1. Fruit harvest and selection process

'Royal Gala' apples were randomly harvested in a commercial orchard in the town of Vacaria-RS, Brazil. Thereafter the fruit were transported to the Postharvest Research Center of the Federal University of Santa Maria, where selection was once again carried out, discarding any damaged fruit. Experimental samples, with 50 fruit each, were put into small experimental chambers (233 L) and different storage conditions were used. Three samples of 50 fruit were used in each treatment.

2.2. Controlled atmosphere and dynamic controlled atmosphere conditions

The experiment was composed of 4 different storage conditions: [1] controlled atmosphere with 1.2 kPa O_2 + 2.0 kPa CO_2 (CA); [2] dynamic controlled atmosphere based on chlorophyll fluorescence (DCA-CF) + 1.2 kPa CO_2; [3] dynamic controlled atmosphere based on respiratory quotient (DCA-RQ), with respiratory quotient 1.5 (DCA-RQ 1.5) + 1.2 kPa CO_2; [4] DCA-RQ 2.0 + 1.2 kPa CO_2. The DCA-CF was performed according to methodology proposed by Prange et al. (2007). DCA-RQ was based on the methodology proposed by Weber et al. (2015). Respiratory quotient (RQ) was daily calculated, by the ratio of CO_2 production through O_2 uptake, and chambers remained closed for 24 h for this evaluation. Thus, the RQ was set at 1.5 (DCA-RQ 1.5) and 2.0 (DCA-RQ 2.0) and the O_2 level was changed daily, in order to keep the RQ at the assigned value for each treatment. The treatments were composed of 3 replicates of 50 fruit each, totalling 150 fruit per treatment.

2.3. Atmosphere establishment

In the first week of storage, fruit were stored at 5 °C and then the temperature was decreased to 1 °C in one week. On the day that the temperature reached 1 °C, the oxygen partial pressure was reduced down to 5 kPa with N_2 flushing and, for one week, the oxygen was decreased to the desired condition by fruit respiration. This gradual temperature and oxygen reduction was undertaken in order to simulate the commercial conditions adopted by the CA stores. Oxygen and carbon dioxide partial pressures were monitored and corrected by an automatic CA and DCA-RQ control system (Valis®, Lajeado, RS, Brazil). The equipment compared the oxygen and carbon dioxide partial pressure at a set point. If the oxygen partial pressure was below the set point, O_2 was injected up to the set point. The same method was used for carbon dioxide correction, but generally the CO_2 was above the desired concentration and excess CO_2 in the chamber was automatically absorbed with a lime scrubber. The O_2 and CO_2 levels during storage and the calculated RQ are shown in Fig. 1. The process of oxygen monitoring and correction in DCA-CF was carried out according to Prange et al. (2007) and in DCA-RQ according to Weber et al. (2015).

2.4. Temperature and relative humidity

The storage temperature was 1.0 ± 0.1 °C. Mercury thermometers, with 0.1 °C resolution, were inserted in the apple flesh (3 apples), in order to monitor the temperature. The apple with the thermometer was allocated inside the refrigeration chamber.

Relative humidity was set at 94 ± 1% throughout the storage and monitored weekly with a psychrometer. In order to absorb the humidity, calcium chloride was placed in the experimental chamber (7.5 g kg^{-1} of fruit).

2.5. Biochemical analyses

After 9 months of storage plus 7 days of shelf life (20 ± 1 °C and relative humidity 80 ± 5%), aiming to simulate commercial practice, the fruit were submitted to quality analyses.

2.5.1. ACC oxidase enzyme activity

ACC oxidase enzyme activity was evaluated according to methodology developed by Bufler (1986). Thus, 3 g skin samples extracted from fruit equatorial region were immediately dipped into a solution containing 0.1 mol L^{-1} ACC and 10 mmol L^{-1} MES (2-(*N*-morpholino)ethanesulfonic acid) buffer at pH 6.0; after 30 min, samples were transferred to hermetic 50-mL syringes, to which 1 mL CO_2 was added. The ethylene concentration in the syringes was measured by gas chromatography (as described for ethylene in the next section) after 30 min, and the results were expressed in ng C_2H_4 kg^{-1} s^{-1}.

2.5.2. Ethylene production and respiration rate

Ethylene production was evaluated by gas chromatography. About 1.5 kg of fruit were put into a 5-L glass container and hermetically closed for 2 h. Thereafter, 2 aliquots of 1 mL were taken from the container and injected in a Varian® gas chromatograph model Star 3400CX (Varian, Palo Alto, CA) equipped with a flame ionisation detector (FID) and a Porapak N80/100 column (2 m length × ⅛″ diameter). The temperatures of the injector, column and detector were 140, 90 and 200 °C respectively. The ethylene

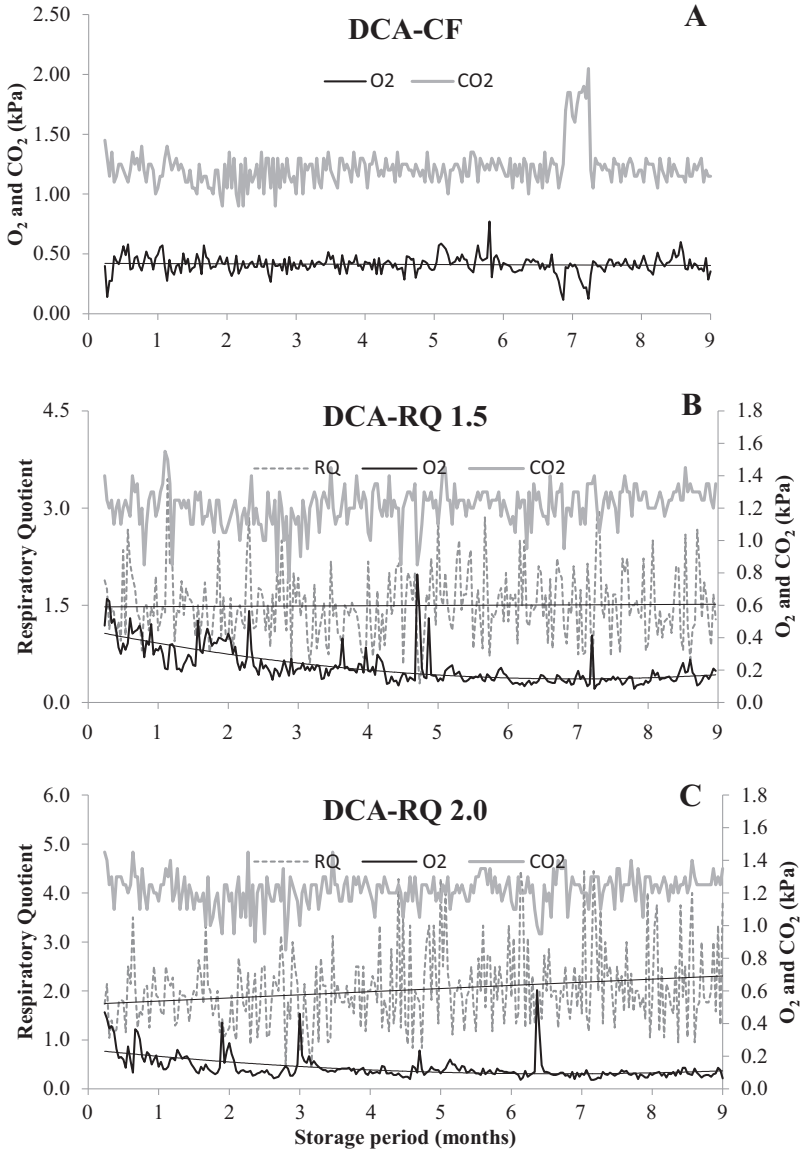

Fig. 1. Respiratory quotient, O_2 and CO_2 levels of 'Royal Gala' apple during 9 months storage in DCA monitoring by chlorophyll fluorescence (A) and respiratory quotient of 1.5 (B) and 2.0 (C).

production rate was calculated taking into account the volume of the container, fruit mass, time of closure and ethylene concentration inside the container; the results were expressed in ng C_2H_4 kg^{-1} s^{-1}. The respiration rate was evaluated by fruit CO_2 production. The air of the same container used to evaluate ethylene production was circulated throughout a gas analyzer (Schele®, model KB7), which quantified the CO_2 concentration. The respiration rate was calculated taking into account the container's volume, fruit mass, time of closure and CO_2 concentration inside the container, the results were expressed in μg CO_2 kg^{-1} s^{-1}.

2.6. Fruit quality evaluation

2.6.1. Flesh firmness

Flesh firmness was determined by the insertion of an 11-mm tip of the penetrometer (Effegi Systems, Milan, Italy) into two opposite sides of the equatorial region of 25 fruit per replicate, where the skin had been previously removed. Results were expressed in newton (N).

2.6.2. Titratable acidity

Titratable acidity was performed by the titration of 10 mL apple juice diluted in 100 mL of distilled water, with 0.1 N NaOH solution up to pH 8.1. The results were expressed as % malic acid.

2.6.3. Soluble solids

Soluble solids were determined by refractometry with a 1-mL sample of juice, obtained from 50 apples; the results were expressed as percentages.

2.6.4. Flesh breakdown, mealiness and healthy fruit amount

These aspects were evaluated by counting the fruit that presented the disorders in relation to the total number of fruit per replicate (50 fruit). A detailed description is presented by Thewes, Both, Brackmann, Weber, and Anese (2015). Data were presented as percentages.

2.6.5. Volatile compounds analysis

The volatile compounds were identified and quantified according to methodology proposed by Both et al. (2014). Thus, 25 apples

were cooled to 0 °C (pulp temperature). Immediately after pulp cooling, horizontal slices of the equatorial region of fruit were taken, the seeds discharged, and centrifuged at low temperature, to avoid chemical and enzymatic oxidation of samples (the maximum juice temperature during sample preparation was 5.0 °C). Thereafter, the juice was put into 100-mL amber flasks and frozen at −30 ± 1.0 °C.

One day before the volatile compounds analysis, the juice was thawed for 24 h in a refrigerator at 5.0 °C. Thereafter, an aliquot of 10 mL juice was put in a 20-mL amber vial, with 3 g of NaCl and 10 μL of 3-octanol standard solution (82.2 μg mL^{-1}), and immediately hermetically closed with a PTFE-coated silicone cap. The vial was then put in a water bath at 35 °C for 5 min; thereafter a divinylbenzene/Carboxen/polydimethylsiloxane (DVB/Car/PDMS) fibre (50/30 μm film thickness × 20 mm; Supelco, Bellefonte, PA) was exposed to the vial headspace for 60 min, under constant stirring (400 rpm), to adsorb the volatile compounds.

In order to identify and quantify the volatile compounds, a Shimadzu QP2010 Plus gas chromatograph coupled to a mass spectrometer (GC/MS; Shimadzu Corporation, Kyoto, Japan) was used, and the main compounds are shown in the total ion chromatogram (Supplementary Fig. S1). The volatile compounds adsorbed on the fibre were thermally desorbed in the injection port at 250 °C for 10 min and in splitless mode for 2 min. The chromatograph was equipped with a polar CP-WAX 52 CB (Chrompack; 60 m × 0.25 mm × 0.25 μm) column. The temperature of the column was set at 35 °C for 3 min, and then increased up to 80 °C at a rate of 2 °C min^{-1} followed by another ramp up to 230 °C at a rate of 5 °C min^{-1} and maintained at 230 °C for 5 min. The detector operated in electron ionisation mode with ionisation energy of +70 eV, a scan range from m/z 35 to 350 and a temperature of 250 °C. The identification of compounds was based on the National Institute of Standards and Technology (NIST) library (tentative identification) and by comparing the calculated Kovats indices with values in the literature. The semi-quantitative determination of volatile compounds concentration was obtained relative to the concentration of the internal standard 3-octanol, according to the guidelines proposed by Both et al. (2014).

2.7. Statistical analysis

Results were subjected to analysis of variance (ANOVA). Data that showed significant difference ($p < 0.05$) by ANOVA were subjected to principal component analysis (PCA). The data matrix was auto scaled for each variable, before the PCA, in order to obtain the same weight for all variables (mean = 0 and variance = 1). When the ANOVA was significant ($p < 0.05$), means were also compared by Tukey's test at 5% of error probability. Data expressed as percentages were transformed by the formula $\text{arc.sin}\sqrt{x/100}$ before ANOVA.

3. Results and discussion

3.1. Principal component analysis

Fruit were submitted to quality and volatile compounds analysis after 9 months of storage plus 7 days of shelf life. The two major components (PC1 and PC2) together represented 72.57% of the overall variability. PC1 (49.59% of data variance) showed the separation of the fruit stored under CA from the ones stored under DCA, regardless of the DCA treatment used (Fig. 2A). This result showed that fruit stored under CA show a completely different response on quality maintenance and volatile compounds production compared to the ones stored under DCA. Perhaps, this is an effect of the different oxygen partial pressure adopted in CA (1.2 kPa), DCA-CF (0.35–0.45 kPa) and DCA-RQ (<0.25 kPa).

PC2 (22.98% percentage of variance) discriminated the different DCA storage conditions, showing an opposite response of the storage under DCA-RQ 2.0 when compared to DCA-CF, with an intermediate behaviour under DCA-RQ1.5 (Fig. 2A). Most branched-chain volatile compounds were related to fruit stored under DCA-RQ, either under 1.5 or 2.0 (Fig. 2B). Both et al. (2014) found higher branched-chain esters in 'Royal Gala' apples stored under ULO (0.5 and 0.7 kPa O_2) in relation to CA (1.0 kPa O_2). This result shows that branched-chain esters are not affected by oxygen lowering. DCA-RQ 2.0 storage resulted in higher acetaldehyde, ethanol and ethyl acetate accumulation, which are compounds related to the off-flavour of apple (Wright, Delong, Arul, & Prange, 2015).

3.2. Biochemical analyses

ACC oxidase is a key enzyme in the ethylene production pathway, consequently in fruit ripening and volatile compound synthesis. Fruit stored under CA showed the highest ACC oxidase enzyme activity; an intermediate level of activity was observed in fruit submitted to DCA-CF and the lowest level was found in fruit stored under DCA-RQ, independently of the RQ level (Fig. 3A). The high level of ACC oxidase enzyme activity found in fruits stored under CA is a result of the high oxygen partial pressure during storage (1.2 kPa O_2). Previous studies have found high ACC oxidase enzyme activity in CA storage as compared to DCA-CF (Thewes et al., 2015; Weber et al., 2015) and DCA-RQ (Weber et al., 2015). Nevertheless, the low ACC oxidase enzyme activity observed in fruit stored under DCA, may be a result of high ethanol production, since ethanol reduces ACC oxidase enzyme activity (Thewes et al., 2015). The ACC oxidase enzyme activity is inhibited by extremely low oxygen that induced a little anaerobic metabolism during the nine months of storage (Wills, McGlasson, Graham, & Joyce, 1998).

The high ACC oxidase enzyme activity found in fruit stored in CA resulted in high ethylene production rate (Fig. 3B). Fruit stored under DCA-CF showed lower ethylene production when compared to CA, but higher when compared to DCA-RQ1.5 and DCA-RQ2.0. When assessing CA, DCA-CF and DCA-RQ on the same apple cultivar Weber et al. (2015) also found lower ethylene production in fruit stored under DCA in comparison to CA, but they did not find a significant difference between the DCA-CF and DCA-RQ methods. Perhaps, the differences between their findings are due to the different RQ levels (2.0, 4.0 and 6.0) used by Weber et al. (2015) in contrast to our work (1.5 and 2.0). Once again, the low ethylene production may be related to the ethanol production in fruit stored under DCA. Previous research raised the hypothesis that this compound can significantly decrease ethylene production (Jin, Lv, Liu, Qi, & Bai, 2013; Thewes et al., 2015).

Similarly to ethylene production, the respiration rate was higher in fruit stored under CA, in comparison to the two DCA methods tested (Fig. 3C). This occurs because the rise in the respiration rate is an ethylene-triggered event. Prior studies have found an analogous response between ethylene and respiration rate (Thewes et al., 2015; Weber et al., 2015). Fruit stored under DCA-RQ 1.5 showed the lowest respiration rate, showing a lower metabolism and consequently a better storage condition. When the RQ level was increased to 2.0 the respiration rate, measured by CO_2 production, also increased, showing that the RQ 2.0 promoted more fermentation. The average levels of O_2 during storage were 0.41; 0.22 and 0.13 kPa for DCA-CF; DCA-RQ1.5 and DCA-RQ2.0, respectively (Fig. 1). Lower respiration rate in fruit stored under DCA-RQ 2.0, in comparison to CA, was found by Weber et al. (2015) when evaluating different RQ levels. However, at higher RQ (4.0 and 6.0) fermentation processes were promoted and

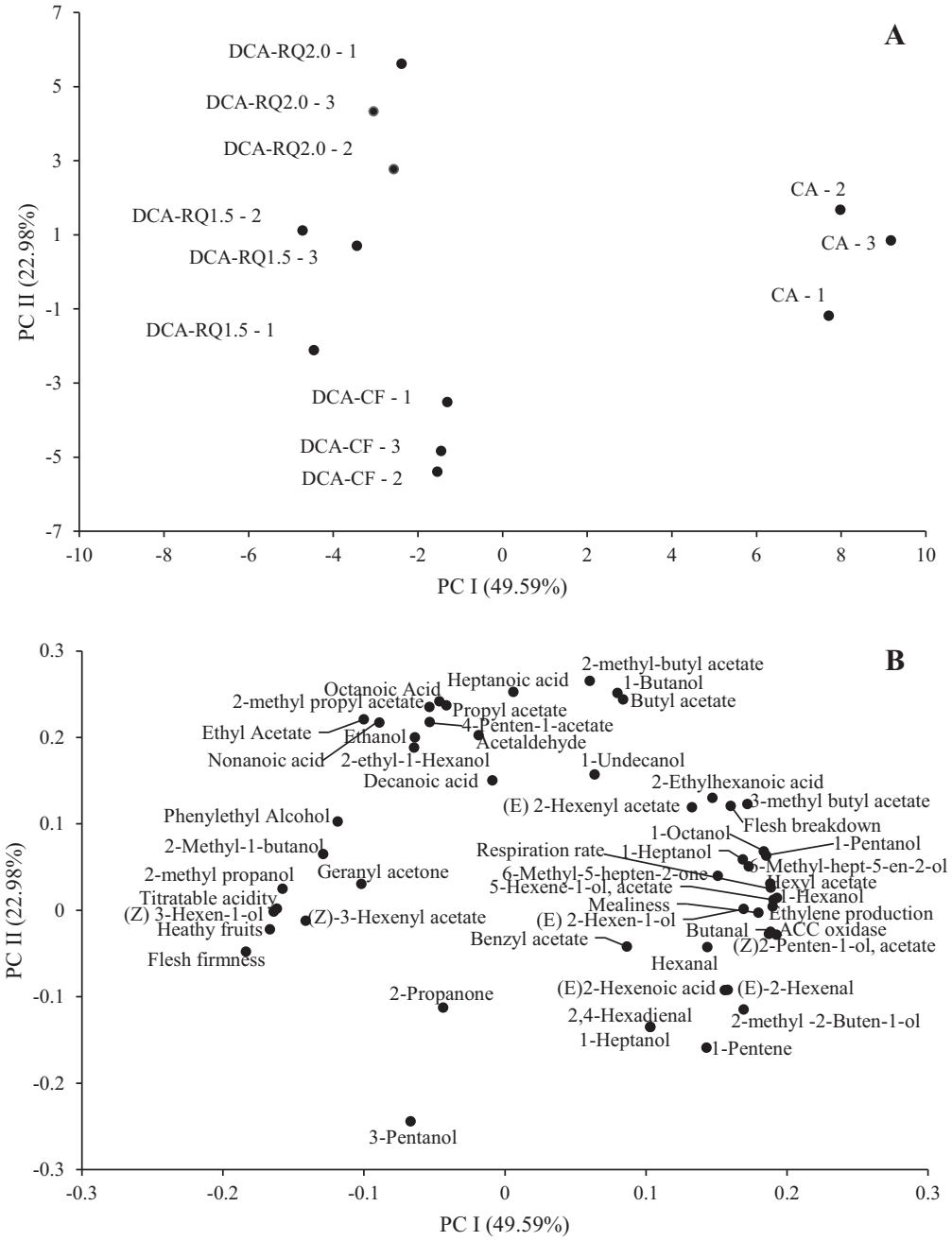

Fig. 2. Scores of treatments (A) and weights of the compounds (B) of 'Royal Gala' apple after 9 months of storage in static controlled atmosphere (CA) and dynamic controlled atmosphere (DCA) monitored by chlorophyll fluorescence (DCA-CF), respiratory quotient of 1.5 (DCA-RQ 1.5) and 2.0 (DCA-RQ 2.0), at temperature 1.0 ± 0.1 °C plus 7 days of shelf life at 20 °C. Numbers 1; 2 and 3 in A represent the 3 replicates of each treatment.

consequently enhanced the production of CO_2, which was no longer different from CA.

3.3. Quality analyses

Flesh firmness is a key indicator of fruit physical quality; therefore the development of a storage method that can reduce fruit softening is crucial. Fruit stored under DCA-RQ 1.5 was found to be the firmest, differing from the ones stored under CA and DCA-RQ 2.0 (Fig. 4A). Fruit stored in DCA-CF and DCA-RQ 2.0 showed higher flesh firmness compared to CA, but there was no difference between each other. In Granny Smith apple, Bessemans et al. (2016) also reported a higher firmness in fruits stored under DCA-RQ in comparison to regular CA, after 7 and 14 days of shelf life. The lower flesh firmness in fruit stored in CA is a result of the high ethylene production and respiration by these fruit. Nevertheless, the lower ethylene in DCA-RQ 2.0 as compared to DCA-CF did not correspond to higher firmness, but a slightly lower firmness. The ethylene triggers fruit ripening, increasing the cell wall enzyme activity (Payasi, Mishra, Chaves, & Singh, 2009; Wei et al., 2010). Previous studies have found higher flesh firmness in fruit stored under DCA-CF (Thewes et al., 2015; Weber et al., 2015). However, in our work there is a trend of DCA-RQ 1.5 to maintain higher flesh firmness in comparison to DCA-CF.

According to Harker, Kupferman, Marin, Gunson, and Triggs (2008), flesh firmness is one of the most important factors in consumer acceptance for apple; however, the soluble solids and titratable acidity also play a key role. In the present research the soluble

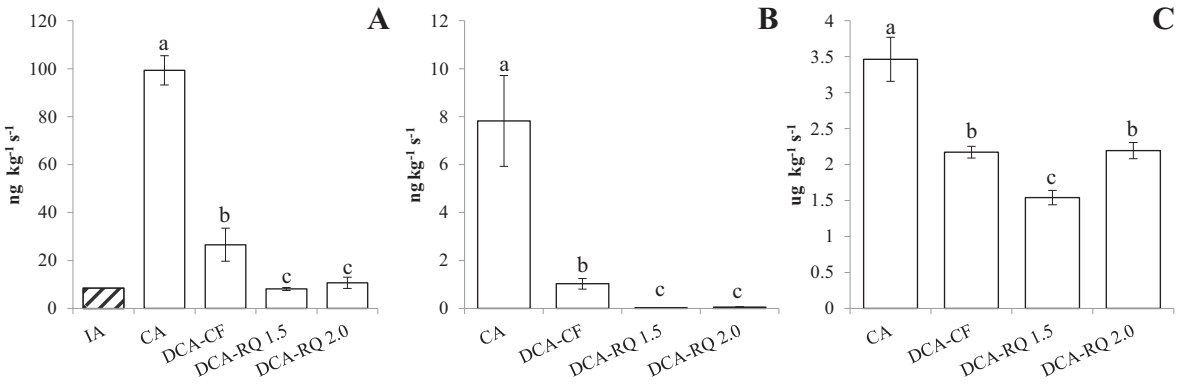

Fig. 3. ACC oxidase enzyme activity (A), ethylene production (B) and respiration rate (C) of 'Royal Gala' apple after 9 months of storage in static controlled atmosphere (CA) and dynamic controlled atmosphere (DCA) monitored by chlorophyll fluorescence (DCA-CF), respiratory quotient of 1.5 (DCA-RQ 1.5) and 2.0 (DCA-RQ 2.0) at temperature 1.0 ± 0.1 °C plus 7 days of shelf life at 20 °C (IA = initial analysis). Means followed by equal letters, after storage, do not differ by Tukey's test at 5% of error probability.

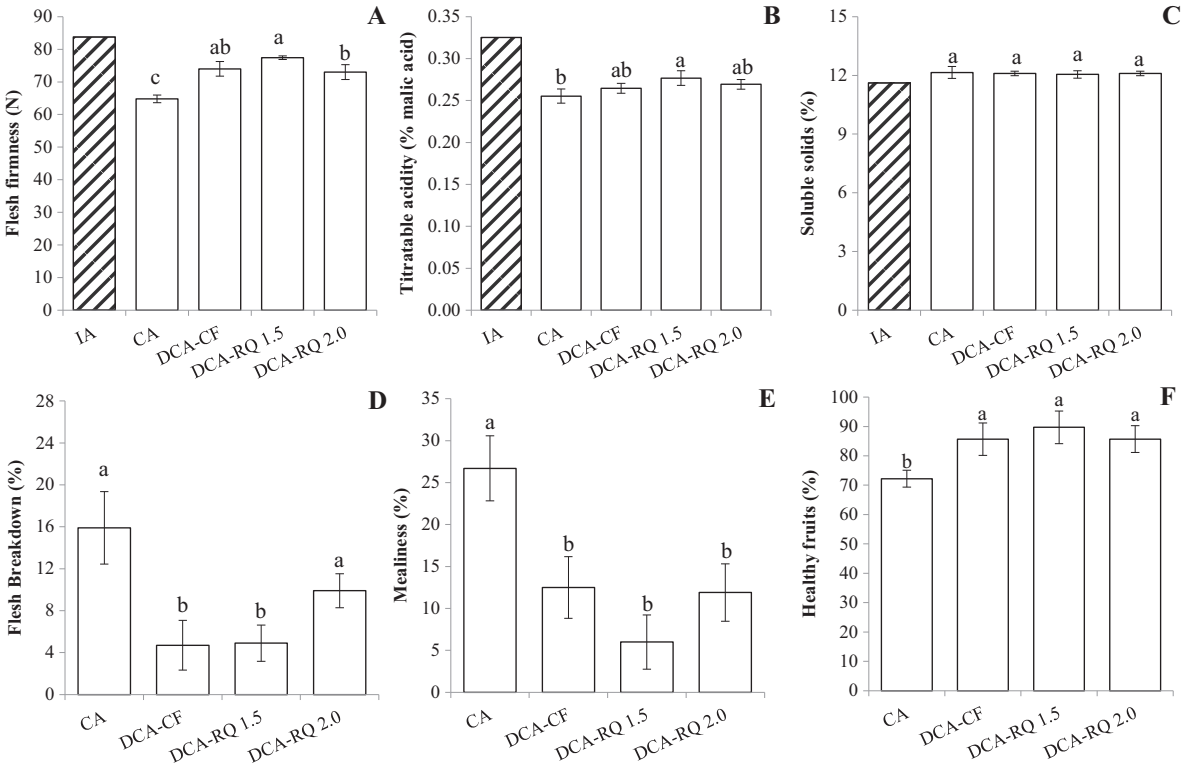

Fig. 4. Flesh firmness (A), titratable acidity (B), soluble solids (C), flesh breakdown (D), mealiness (E) and healthy fruit (F) of 'Royal Gala' apple after 9 months of storage in static controlled atmosphere (CA) and dynamic controlled atmosphere (DCA) monitored by chlorophyll fluorescence (DCA-CF), respiratory quotient of 1.5 (DCA-RQ 1.5) and 2.0 (DCA-RQ 2.0), at temperature 1.0 ± 0.1 °C plus 7 days of shelf life at 20 °C (IA = initial analysis). Means followed by equal letters, after storage, do not differ by Tukey's test at 5% of error probability.

solids did not differ among treatments (Fig. 4C), but fruit stored under DCA-RQ 1.5 showed the highest titratable acidity, differing from fruit stored under CA (Fig. 4B). Lumpkin et al. (2014) also reported higher flesh firmness and acidity of 'Scarlett Spur Red Delicious' apple stored in CA with 0.3 kPa O_2 compared to higher O_2 levels and this is related to the lower ethylene production. The oxygen lowering probably reduced the respiration rate and consequently the depletion of organic acids through the tricarboxylic acid cycle.

Physiological disorders are one of the most important problems faced when storing apples, so their reduction is important to prevent product loss and offer high quality products to the market. Higher flesh breakdown incidence was observed in fruit stored under CA and DCA-RQ 2.0 in comparison to fruit stored under DCA-CF and DCA-RQ 1.5 (Fig. 4D). Previous researchers have found a high flesh breakdown incidence in fruit stored under CA when compared to DCA (Thewes et al., 2015; Weber et al., 2015). The higher flesh breakdown in fruit stored under DCA-RQ 2.0 in comparison to DCA-RQ 1.5 and DCA-CF is due to high fermentation that leads to excessive acetaldehyde and ethanol accumulation and might result in flesh breakdown (Franck et al., 2007). Another explanation is that fermentative metabolism resulted in insufficient energy production to maintain cell integrity, triggering the incidence of flesh breakdown (Ho, Verboven, Verlinden, Schenk, & Nicolaï, 2013; Pedreschi et al., 2009).

Mealiness in an attribute signalling texture quality degradation and its incidence is closely related to fruit ripening. Fruit stored

under CA showed the highest mealiness incidence after 9 months of storage plus 7 days of shelf life at 20 °C in comparison to fruit stored under DCA, independently of the DCA method, although there is a tendency of lower disorder in DCA-RQ 1.5 (Fig. 4E). This shows that fruit stored under CA remained in an advanced ripening stage. Perhaps, the high mealiness incidence of fruit stored in CA is a result of higher ethylene production by these fruit, once the high ethylene sets off the cell wall enzymes that degrade the middle lamella of cell (Goulao & Oliveira, 2008; Payasi et al., 2009; Wei et al., 2010), resulting in a mealy pulp aspect. Analysing the mealiness and flesh breakdown of fruit stored under DCA-RQ 2.0, is proof that the higher flesh breakdown in these fruits is due to the higher anaerobic metabolism. Therefore we can raise the hypothesis that flesh breakdown incidence in this storage condition occurs due to excess ethanol, as shown in Table 1 and Fig. 2B.

Fruit stored in DCA, regardless of the method used, showed a higher amount of healthy fruit in comparison to CA (Fig. 4F). The higher percentage of healthy fruit is a result of lower mealiness and flesh breakdown in fruit stored in DCA-CF and DCA-RQ 1.5. Another study also reported a higher percentage of healthy fruit in DCA-RQ and DCA-CF in comparison to CA (Weber et al., 2015). Thewes et al. (2015) found a higher healthy fruit amount in 'Royal Gala' apples that were stored in DCA-CF in comparison to CA, but for 'Galaxy' apple, there was no significant difference between DCA-CF and CA, showing that fruit response to DCA-CF is cultivar dependent. Research carried out with 'Topaz', 'Otava' and 'Ariane' apple cultivars, also showed that the effect of DCA in quality maintenance is cultivar dependent (Gasser & Von Arx, 2015).

3.4. Volatile compounds analysis

The apple volatile profile was evaluated at harvest and after 9 months of storage plus 7 days of shelf life (Table 1). In the analysis carried out at harvest, 18 esters, 14 alcohols, 3 aldehydes, 4 ketones and 6 acids were identified and quantified by GC/MS. After storage plus shelf life, a lower number of esters (13) and ketones (3) and a higher number of alcohols (16 alcohols) were detected. This result is in accordance with the literature, which shows that the storage of apple under CA significantly decreases the ester production (Bangerth et al., 2012; Brackmann, Streif, & Bangerth, 1993; Fellmann et al., 2003; Song & Bangerth, 1996). However, there are very few reports in the literature that take into account the increase of alcohols after storage, as described in our work. The higher number of alcohols found after storage is likely related to the low oxygen partial pressure maintained during storage in this new DCA storage method, resulting in anaerobic metabolism, which could provide some alcohol production.

Esters are the main apple odour compounds and for 'Gala' apples, such as 'Royal Gala', the esters of major impact are butyl acetate, hexyl acetate and 2-methylbutyl acetate (Aprea et al., 2012; Mehinagic, Royer, Symoneaux, Jourjon, & Prost, 2006). These three esters are dramatically reduced by storage, independently of the conditions used (Table 1). Butyl acetate was strongly reduced under all storage conditions as compared to harvest. After storage, fruit of DCA-CF and DCA-RQ 1.5 showed the lowest butyl acetate, but when fruit were stored under DCA-RQ 2.0 no difference was observed when compared to fruit stored under CA. This result shows that oxygen reduction in DCA, with RQ 2.0 level, did not reduce the butyl acetate concentration. The lower butyl acetate production by apple stored under DCA-CF and DCA-RQ1.5 is probably a result of lower precursor concentration (1-butanol). Another previous study has also reported that the main concern for high volatile compound production is not the enzyme activity, but the precursor concentration (Echeverría, Graell, López, & Lara, 2004). Both et al. (2014) and Raffo et al. (2009) while working with 'Royal Gala' and 'Pinova' apple, respectively, also found a significant butyl acetate concentration reduction in ULO (0.8, 0.7 and 0.5 kPa O_2) and DCA-CF compared to CA, corroborating the result obtained in our work. However, in our work when the oxygen partial pressure was decreased to 0.13 kPa (RQ 2.0), there was an increase in the butyl acetate concentration due to the precursor concentration increment. Additionally, this is the first literature report of butyl acetate increase by the use of extremely low oxygen partial pressure.

The fruits stored under DCA, regardless of the method, showed lower concentration of hexyl acetate, but the concentration remained above the odour threshold (Table 1). This may be a result of lower 1-hexanol concentration in fruit stored under these conditions. Perhaps, there is a problem in hexanal conversion into 1-hexanol, by the enzyme alcohol dehydrogenase (ADH) (Schaffer et al., 2007), once there is no reduction in hexanal concentration by storage under DCA. The ADH gene family is composed of 10 genes, where 9 genes are induced by ethylene and 1 is decreased in response to ethylene (ADH1) (Defilippi, Kader, & Dandekar, 2005; Schaffer et al., 2007). In our work, the ethylene production was significantly lower in fruit stored under DCA (Fig. 3B), which may result in lower ADH gene expression culminating in a lower hexanal conversion into 1-hexanol and consequently decreasing hexyl acetate production. Corroborating our findings, another study also verified lower 1-hexanol and hexyl acetate concentration when comparing DCA-CF to CA (1.5 kPa O_2 + 1.3 kPa CO_2) (Raffo et al., 2009).

2-Methylbutyl acetate was the most abundant ester detected in 'Royal Gala' apple after 9 months of storage plus 7 days of shelf life (Table 1). According to Young, Gilbert, Murray, and Ball (1996) 2-methylbutyl acetate had the greatest effect on eight of nine sensory attributes for 'Royal Gala' apple after storage. Fruit stored under CA, DCA-RQ1.5 and DCA-RQ2.0 showed higher amounts of 2-methylbutyl acetate in comparison to fruit stored under DCA-CF (Table 1) and these changes could have an effect of the sensory quality of fruit stored under DCA-CF. The low 2-methylbutyl acetate observed in fruit stored under DCA-CF may be a result of lower alcohol acyl transferases (AAT), once 2-methyl-1-butanol was not influenced by the storage conditions. Literature reports that activity of AAT is triggered by ethylene (Defilippi et al., 2005), but in the present work the treatments with the lowest levels of ethylene production (Fig. 3B) did not show lower 2-methylbutyl acetate concentration. The low ethylene production by fruits stored under DCA-RQ 1.5 and DCA-RQ 2.0 was probably enough to start the AAT activity, leading to high 2-methylbutyl acetate production. The oxygen lowering to 0.7 kPa also led to an increase in 2-methylbutyl acetate, but when the oxygen was below 0.7, this ester concentration decreased (Both et al., 2014). In this work DCA-RQ allowed oxygen reduction to 0.22 and 0.13 kPa (means in RQ 1.5 and 2.0, respectively), along with maintenance of a high level of 2-methylbutyl acetate.

Ethyl acetate is a volatile compound closely related to fermentative metabolism and to the development of off-flavour during storage (Wright et al., 2015), but in all storage conditions studied in the present work the ethyl acetate concentration was far below the odour threshold (Table 1). Fruit stored under CA and DCA-CF showed the lowest ethyl acetate production, with no significant difference between each other (Table 1). Previous studies also found no difference between CA and DCA-CF for ethyl acetate production (Raffo et al., 2009; Thewes et al., 2015). On the other hand, fruit stored under DCA-RQ 1.5 and 2.0 showed a sharp increase in ethyl acetate concentration, especially in DCA-RQ 2.0. The higher ethyl acetate production by these fruit is a result of higher ethanol concentration, once the ethyl acetate is formed by the linkage of an ethanol to an acyl moiety by AAT (Defilippi et al., 2005). The ethyl acetate and ethanol production by fruit stored under DCA-RQ 2.0

Table 1
Volatile compounds determination (μg L^{-1}) of 'Royal Gala' apple at harvest and after 9 months of storage in static controlled atmosphere (CA) and dynamic controlled atmosphere (DCA) monitored by chlorophyll fluorescence (DCA-CF), respiratory quotient of 1.5 (DCA-RQ 1.5) and 2.0 (DCA-RQ 2.0), at temperature 1.0 ± 0.1 °C, plus 7 days of shelf life, at 20 °C.

Compounds	LRI	OT (μg kg^{-1})[1]	At harvest	CA	DCA-CF[2]	DCA-RQ 1.5[2]	DCA-RQ 2.0[2]
Esters							
Ethyl acetate	899	13,500[a]	1.8[*] ± 0.2	7.8 ± 0.8c[**]	9.7 ± 0.7c	510.2 ± 137.6b	1,400.8 ± 31.4a
Propyl acetate	970	2000[a]	4.8 ± 0.1	1.2 ± 0.3b	0.3 ± 0.1c	1.1 ± 0.2b	4.8 ± 0.1a
2-Methylpropyl acetate	1019	65[a]	7.65 ± 0.4	8.8 ± 1.1b	5.4 ± 0.7c	11.8 ± 0.6a	11.4 ± 0.1a
Butyl acetate	1084	66[a]	2,053.5 ± 36.7	45.0 ± 8.8a	6.2 ± 2.2b	14.6 ± 3.6b	52.2 ± 5.5a
2-Methylbutyl acetate	1131	11[a]	327.1 ± 11.2	146.4 ± 22.2ab	71.9 ± 7.4c	113.9 ± 4.5b	158.8 ± 10.6a
3-Methylbutyl acetate	1181	2[d]	109.7 ± 3.3	12.1 ± 2.7a	1.7 ± 0.4c	2.7 ± 0.2bc	5.6 ± 0.7b
4-Penten-1-yl acetate	1181	nf	7.02 ± 0.2	0.8 ± 0.04b	0.4 ± 0.03c	1.2 ± 0.1a	1.0 ± 0.06a
(*Z*) 2-penten-1-ol acetate	1255	nf	7.87 ± 0.4	24.2 ± 4.4a	6.9 ± 2.2b	2.3 ± 0.02b	0.9 ± 0.02b
Hexyl acetate	1277	2[a]	499.1 ± 23.4	92.2 ± 15.4a	26.9 ± 4.4b	24.4 ± 1.1b	23.9 ± 3.5b
(Z) 3-hexenyl acetate	1322	8[c]	8.21 ± 0.4	1.9 ± 0.3b	2.8 ± 0.6b	4.3 ± 0.4a	2.7 ± 0.4b
5-Hexen-1-ol acetate	1334	nf	10.5 ± 0.3	5.8 ± 0.9a	1.6 ± 0.4b	1.1 ± 0.1b	1.2 ± 0.2b
(*E*) 2-hexenyl acetate	1342	7[c]	4.3 ± 0.1	8.5 ± 1.1a	5.4 ± 0.9b	6.9 ± 0.6ab	6.5 ± 0.6ab
Benzyl acetate	1745	364[c]	1.5 ± 0.03	1.1 ± 0.3a	1.0 ± 0.1a	0.9 ± 0.2a	0.8 ± 0.1a
Alcohols							
Ethanol	943	100,000[d]	0.3 ± 0.03	1.9 ± 0.2c	3.6 ± 0.8c	17.1 ± 3.5b	67.5 ± 6.9a
2-Methyl-1-propanol	1107	250[a]	–	1.3 ± 0.7b	2.3 ± 0.5ab	3.1 ± 0.3a	2.3 ± 0.3ab
3-Pentanol	1110	nf	–	0.4 ± 0.03c	1.4 ± 0.04a	0.8 ± 0.2b	0.3 ± 0.01c
1-Butanol	1155	500[a]	51.4 ± 3.1	16.6 ± 3.0a	5.8 ± 1.9b	8.5 ± 1.9b	18.3 ± 2.6a
2-Methyl-1-butanol	1215	250[a]	20.1 ± 1.4	45.2 ± 1.1a	57.1 ± 3.9a	63.9 ± 21.3a	64.4 ± 4.9a
1-Pentanol	1257	4000[a]	3.7 ± 0.2	3.1 ± 0.5a	1.18 ± 0.2b	1.2 ± 0.1b	1.4 ± 0.1b
2-Methyl-2-buten-1-ol	1332	nf	–	8.3 ± 0.8a	5.1 ± 1.4b	1.1 ± 0.1c	0.4 ± 0.02c
1-Hexanol	1362	500[a]	118.0 ± 9.7	81.6 ± 5.7a	35.5 ± 1.3b	29.8 ± 4.3b	32.4 ± 5.0b
(*Z*) 3-hexen-1-ol	1389	70[b]	0.2 ± 0.02	0.4 ± 0.1b	0.9 ± 0.3ab	1.4 ± 0.2a	1.0 ± 0.2a
(*E*) 2-hexen-1-ol	1414	400[d]	1.3 ± 0.3	5.1 ± 1.2a	2.5 ± 0.2b	2.3 ± 0.4b	2.6 ± 0.7b
1-Heptanol	1462	3[d]	4.1 ± 0.3	2.8 ± 0.9a	0.8 ± 0.1b	1.1 ± 0.4b	0.9 ± 0.2b
6-Methyl-5-hept-2-en-1-ol	1438	2000[d]	0.6 ± 0.05	0.8 ± 0.2a	0.4 ± 0.02b	0.3 ± 0.01b	0.4 ± 0.01b
2-Ethyl-1-hexanol	1495	270,000[d]	1.91 ± 0.2	0.98 ± 0.05a	0.76 ± 0.1a	1.39 ± 0.6a	1.36 ± 0.1a
1-Octanol	1565	130[d]	1.7 ± 0.1	1.1 ± 0.2a	0.4 ± 0.05b	0.4 ± 0.1b	0.5 ± 0.03b
Phenylethyl alcohol	1854	750[d]	0.2 ± 0.01	0.3 ± 0.07b	0.4 ± 0.06ab	0.4 ± 0.1ab	0.5 ± 0.1a
1-Undecanol	1973	nf	0.3 ± 0.05	0.4 ± 0.1a	0.2 ± 0.04a	0.3 ± 0.1a	0.3 ± 0.1a
Aldehydes							
Acetaldehyde	713	120[d]	–	2.2 ± 0.7b	1.6 ± 0.5b	1.6 ± 0.3b	4.5 ± 0.3a
Butanal	867	37[d]	–	0.8 ± 0.1a	0.3 ± 0.06b	0.2 ± 0.04b	0.2 ± 0.01b
Hexanal	1095	5[a]	110.6 ± 8.1	177.9 ± 40.2a	138.1 ± 20.3a	113.2 ± 23.2a	133.1 ± 10.5a
(*E*) 2-hexenal	1229	17[b]	288.0 ± 14.6	195.3 ± 35.9a	160.1 ± 22.0ab	114.7 ± 18.4b	129.8 ± 5.2b
(*E,E*) 2,4-hexadienal	1428	60[d]	3.2 ± 0.3	2.6 ± 0.6a	2.2 ± 0.5a	2.2 ± 0.3a	1.6 ± 0.2a
Ketones							
2-Propanone	827	500,000[d]	0.5 ± 0.06	1.0 ± 0.3b	1.2 ± 0.3b	2.4 ± 0.1a	0.1 ± 0.02c
6-Methyl-5-hepten-2-one	1348	50[b]	0.7 ± 0.1	1.0 ± 0.2a	0.6 ± 0.1b	0.7 ± 0.04ab	0.7 ± 0.1ab
Geranyl acetone	1868	9[d]	0.6 ± 0.2	0.5 ± 0.1a	0.6 ± 0.1a	0.8 ± 0.2a	0.7 ± 0.2a
Acids							
Hexanoic acid	1782	3000[d]	1.5 ± 0.2	1.9 ± 0.1a	1.7 ± 0.2a	1.8 ± 0.2a	1.9 ± 0.1a
2-Ethylhexanoic acid	1952	nf	0.98 ± 0.1	1.36 ± 0.16a	1.01 ± 0.08b	1.03 ± 0.08b	1.15 ± 0.07ab
Heptanoic acid	1958	3000[d]	0.4 ± 0.03	0.6 ± 0.1a	0.5 ± 0.04a	0.6 ± 0.1a	0.7 ± 0.1a
(*E*)-2-Hexenoic acid	1977	nf	0.4 ± 0.1	0.4 ± 0.1a	0.3 ± 0.04ab	0.3 ± 0.02b	0.2 ± 0.01b
Octanoic acid	1977	3000[d]	1.2 ± 0.1	1.9 ± 0.3a	1.5 ± 0.2a	2.2 ± 0.3a	2.2 ± 0.2a
Nonanoic acid	<2000	3000[d]	2.2 ± 0.2	3.8 ± 1.0a	2.9 ± 0.5a	4.8 ± 0.9a	4.6 ± 0.6a
Decanoic acid	<2000	10,000[d]	–	0.7 ± 0.3a	0.5 ± 0.3a	0.9 ± 0.3a	0.7 ± 0.2a

Esters that were detected only at harvest: butyl propanoate (3.96); butyl butyrate (3.56); 2-methylbutyl butyrate (1.38); 3-methyl-2-butenyl acetate (1.52) and heptyl acetate (0.96).
Alcohol that was present only at harvest: 1-octen-3-ol (0.79).
LRI: Linear retention index.
[*] Concentrations were calculated relative to an internal standard (3-octanol).
[**] Means followed by equal letters, after storage, in the same row do not differ by Tukey's test at 5% of error probability.
[1] Odour threshold. References: [a]López et al. (2007); [b]Mehinagic et al. (2006); [c]Pino & Quijano (2012); [d]Leffingwell & Leffingwell (1991). nf = not found.
[2] The carbon dioxide partial pressure was 1.2 kPa for all DCA treatments.

was probably too high, resulting in high flesh breakdown in these fruit (Fig. 4D). Another research also found higher ethyl acetate and ethanol production by 'Scarlett Spur Red Delicious' apple stored under 0.30 kPa O_2, compared to higher partial pressure (Lumpkin et al., 2014).

A significant reduction of the main esters production was also found in apple stored under DCA-CF in comparison to CA (1.5 kPa O_2 + 1.3 kPa CO_2) in 'Pinova' apple, where the concentration of volatile compounds remained higher in DCA-CF as compared to CA plus 1-methylcyclopropene (Raffo et al., 2009).

After 9 months of storage plus 7 days of shelf life 16 alcohols were identified and quantified by GC/MS (Table 1). Among the 16 alcohols, ethanol was the only one related to fermentation (Lumpkin et al., 2014). The concentration of ethanol was lower in fruit stored under CA and DCA-CF; an intermediary concentration was noticed in fruit stored under DCA-RQ1.5 and the highest was found in fruit stored under DCA-RQ 2.0, but its concentration was lower than the odour threshold.

Concerning the straight-chain alcohols produced by β-oxidation, generally there is a higher concentration in fruit stored under CA

compared to the ones stored under DCA, independently of the method (Table 1). This is a result of higher oxygen partial pressure during storage, allowing the β-oxidation of fatty acids, resulting in alcohols accumulation in these fruits. The compounds generated by β-oxidation are important straight-chain ester precursors (Brackmann et al., 1993; Song & Bangerth, 2003) and extremely low oxygen partial pressure could inhibit the synthesis of important substrates for ester production (Both et al., 2014). However, in our work that appears not to be true, especially at DCA-RQ 2.0, where the oxygen partial pressure is very low (0.13 kPa) and high volatile compounds synthesis was observed (Table 1). This is probably due to the variation of the oxygen according to the fruit metabolism during storage and the anaerobic metabolism induction, which could provide alcohols to produce volatile compounds.

Acetaldehyde is a toxic metabolite for fruit; therefore the higher its concentration, the greater is the risk of physiological disorders. Thus, the higher flesh breakdown in fruit stored under DCA-RQ 2.0 (Fig. 4D) may be related to higher acetaldehyde concentration (Table 1). The higher acetaldehyde in DCA-RQ 2.0 was substrate for higher ethanol production by ADH in this treatment. Another important aldehyde is hexanal, which confers a green aroma to the apple (Mehinagic et al., 2006), but there was no difference among treatments (Table 1). Nevertheless, fruit stored under DCA-RQ 1.5 and 2.0 showed lower (*E*)-2-hexenal concentration compared to fruit stored under CA. This result is not in accordance to the findings reported by Both et al. (2014), since they found a lower concentration in CA in comparison to extremely low oxygen partial pressures storage of 0.7 kPa. (*E*)-2-Hexenal confers a green leafy sensorial attribute to apple flavour (Amaro, Beaulieu, Grimm, Stein, & Almeida, 2012; Mehinagic et al., 2006).

Among the three ketones detected, geranyl acetone was not significantly affected by the treatments tested (Table 1). In 'Royal Gala' apple stored under extremely low oxygen partial pressures, this ketone was not affected by oxygen (Both et al., 2014). 6-Methyl-5-hepten-2-one is another important ketone originating from carotenoids oxidation. Higher 6-methyl-5-hepten-2-one was quantified in fruit stored under CA, differing only from those stored under DCA-CF (Table 1).

In relation to the acids, 7 compounds were detected and quantified (Table 1). In general terms there is little difference among the treatments concerning the acids concentration. 2-Ethylhexanoic acid showed lower concentration in fruit stored under DCA-CF and DCA-RQ 1.5 in comparison to fruit stored in CA. (*E*)-2-Hexenoic acid was also significantly affected by the storage conditions, with lower concentration in fruit stored under DCA RQ 1.5 and RQ 2.0 in comparison to CA.

4. Conclusions

The storage of 'Royal Gala' apple under DCA maintains higher flesh firmness, number of healthy fruit, and lower physiological disorders after 9 months storage plus 7 days of shelf life, especially if stored in DCA-RQ 1.5, due to the low ethylene production and respiration rate. DCA-RQ 2.0 promoted a higher anaerobic metabolism, increasing the ethanol and ethyl acetate production of the fruit.

Controlled atmosphere storage provides fruit with a higher concentration of volatile compounds. However, fruit stored under DCA-RQ also show high key volatile compounds, such as butyl acetate (RQ 2.0) and 2-methylbutyl acetate (RQ 1.5 and 2.0), with an increase in ethanol and ethyl acetate concentration, but far below the odour threshold. The storage of 'Royal Gala' apples in DCA-CF decreased the fruit ester production, especially 2-methylbutyl acetate, which is the most important for 'Royal Gala' apple flavour.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.08.009.

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