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Emission of VOCs and quality evolution in response to repeated oxygen pull downs on 'Conference' pears during long-term cold storage

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Abstract

The effect of long-term storage of 'Conference' pears under ultra-low oxygen levels with or without multiple oxygen pull downs of different duration on fruit quality, ethylene emission, fermentative metabolites and volatile organic compounds (VOCs) was investigated. Pears were cold stored for seven months under three different atmospheres: initial low oxygen stress (ILOS), dynamic low oxygen pull downs monitored with a chlorophyll fluorescence sensor ($DLOS_1$) and extended dynamic or repeated low oxygen pull downs ($DLOS_2$). Overall, the application of repeated oxygen pull downs in the atmosphere composition ($DLOS_1$ and $DLOS_2$) did not affect the fruit firmness upon removal from cold storage.

Our results showed that fruit submitted to multiple oxygen pull downs ($DLOS_1$ and $DLOS_2$) ripened slower when further placed at 20 ºC, as indicated by changes in index of absorbance difference (I_{AD}), ethylene production capacity and the accumulation of ethanol within the fruit pulp. Moreover, the in-atmosphere detected concentrations of specific VOCs (butyl acetate, hexyl propanoate and α-farnesene) correlated well with ripening parameters (I_{AD}), thereby suggesting that specific VOCs could be used as fruit ripening state markers for real-time monitoring throughout the storage of 'Conference' pears.

Keywords: chlorophyll fluorescence, DCA, ethanol, esters, ripening marker.

1. Introduction

Cold storage (CS) of pear fruit is a common practice to satisfy the market demand of pears all year round. It is well known that the storage of pears at low temperatures reduces fruit metabolism and that high relative humidity avoids weight loss, helping to maintain optimal fruit quality (Mohapatra et al., 2013). However, cold storage can lead to the appearance of certain physiological disorders commonly known as chilling injuries, such as superficial scald in pears (Lurie and Watkins, 2012). In this sense, the combination of CS with controlled atmosphere (CA) (i.e. 2 kPa O_2 and 3 kPa CO_2) can partially control the appearance of these disorders and further extend the storability of the fresh product (Ke et al., 1994; Lau, 1990). However, most pear varieties are very sensitive to low oxygen partial pressure (P_{O2}) and high carbon dioxide partial pressure (P_{CO2}) and under such conditions may develop some other physiological disorders such as core or internal breakdown (Lum et al., 2016). The development of these internal physiological disorders is mainly caused by anoxia and the induction of fruit fermentative metabolism (Deuchande et al., 2016) together with the cell death due to energy shortage(Ho et al., 2013).

To avoid the induction of fermentative metabolism, recent trends in CA storage aim to dynamically adjust the O_2 levels inside the cold room in order to keep the oxygen level of the atmosphere to the minimum tolerated by the fruit, also called lower oxygen level (LOL), and keeping it as close as possible to the anaerobic compensation point (Prange et al., 2011). Storing fruit under dynamic controlled atmosphere (DCA) conditions prevents physiological disorders such as superficial scald, fruit off-flavours and even extends the produce storability (Deuchande et al., 2016). The key point of that technology is how to know the LOL of fruit at each time during the cold storage. Currently, there are three commercial variants of this technology each based on

74 monitoring a different biochemical parameter of the fruit which is assumed to change
75 when the shift from anaerobic to fermentative metabolism begins: chlorophyll
76 fluorescence, respiratory quotient and ethanol content, the latter being measured either in
77 the fruit pulp or in the cold room atmosphere (Rizzolo et al., 2015a; Van Schaik et al.,
78 2015; Veltman et al., 2003). While DCA storage has been widely applied in apples
79 (Mditshwa et al., 2018), scarce information is currently available on its use in pears
80 (Prange et al., 2013; Saquet, 2019).

81 It is well recognized that low temperature and restricted or enhanced levels of O_2
82 and CO_2, respectively, during fruit storage act as important stress factors (Larrigaudiere
83 et al., 2001). In response to biotic and abiotic stresses, fruit shift and alter their functional
84 metabolic pathways leading to the synthesis of specific stress-induced volatile
85 compounds (López et al., 2015; Spinelli et al., 2011). Induced volatile organic compounds
86 (IVOCs) include alkenes, alkanes, carboxylic acids, nitrogen-containing compounds and
87 alcohols, together with isoprene and terpenes (Holopainen and Gershenzon, 2010). In
88 pears, fatty acids appear to serve as ester precursors, catabolized through to main and
89 different pathways, β-oxidation and the lipoxygenase system (Jennings, 1967; Sanz et al.,
90 1997) and therefore, their concentration may be greatly affected by a reduction of the
91 oxygen levels within the atmosphere.

92 A better understanding of the synthesis and emission of these compounds in pear
93 fruit exposed to ultra-low oxygen levels during storage may assist on developing new
94 DCA monitoring technologies capable of accurately determine the fruit physiological
95 state prior and during the induced stress.

96 Accordingly, the objectives of this study were: 1) To evaluate the quality
97 parameters, ethylene production and fermentative metabolites of ‘Conference’ pears

during long-term storage under different imposed oxygen pull downs in different storage conditions; initial low oxygen stress (ILOS), dynamic low oxygen stresses ($DLOS_1$) and extended dynamic low oxygen stresses ($DLOS_2$). 2) To check the reliability of the chlorophyll fluorescence sensor to monitor a DCA cold room. 3) To determine if specific volatile compounds are emitted or enhanced in response to such oxygen pull downs and their suitability as ripening markers of 'Conference' pears during dynamic controlled atmosphere

2. Materials and methods

2.1 Plant material and storage conditions

'Conference' pears (*Pyrus communis* L.) were harvested in August 2016 at a commercial orchard near Lleida (NE of Spain). All fruit was picked up at the same day at optimum commercial maturity according to local growers' recommendations for long-term storage which are basically assessed in terms of firmness and sugars content (firmness ≈ 55-65 N and total soluble solids > 13 %). Thereafter, fruit was transported to IRTA research institute, cooled down within 2-3h after harvest and stored in three experimental containers named as ILOS, $DLOS_1$ and $DLOS_2$, each with a volume of 350 L (Fig. 1) and located inside a semi-commercial cold room (4x4x3 m) at 0 ºC and at 95 % of relative humidity (RH). Approximately 20 kg of fruit were stored in each experimental container and kept for up to seven months under the following atmosphere conditions:

- ILOS: 0.4 kPa O_2 and 1 kPa CO_2 for the first 14 d, thereafter storage at 2 kPa O_2 and 2 kPa CO_2 (Fig. 2A).

- Storage atmospheres $DLOS_1$ and $DLOS_2$: set point was kept at 0.5 kPa O_2 and 0.5 kPa CO_2 although the system did not always reproduce it exactly. Oxygen partial pressure

was lowered five times (<0.5 kPa) during the storage period simultaneously in containers $DLOS_1$ and $DLOS_2$ (Fig. 2B, 2C). In $DLOS_2$ container the low oxygen level was kept for a longer time than in $DLOS_1$ container. To compare the extension of oxygen, pull downs in $DLOS_1$ and $DLOS_2$ containers the index of oxygen depletion (I_{OD}) was used. This index was evaluated at pull down *i* as,

$$I_{OD_i} = \int_{t_{0_i}}^{t_{0_i}+\Delta t_{max}} \left(P_{O_2}ref - P_{O_2}\right)dt \quad (1)$$

Where $P_{O_2}ref$ (Pa) is the oxygen partial pressure set point, P_{O_2} (Pa) is the oxygen partial pressure during the *i* pull down period (*i*= 1,…, 5) starting at time t_{0_i} (d) and ending at time $t_{0_i+\Delta t_{max}}$ (d).In order to compare the importance of the pull downs all intervals have been evaluated over the same time interval $\Delta t_{max} = 10$.

2.2 Experimental setup

The three experimental containers were equipped with a volatile organic compounds (VOCs) extraction system which consisted in an air-recirculating pump that forced the air through two adsorption tubes in parallel (Fig. 1).

At the top of each container three small chambers with a capacity of 21.4 L were installed and filled with approximately 10kg of fruit each. The chambers were connected to each other and with the container's atmosphere sharing the same gas composition (Fig. 1). Connections were made through flexible pipes with a system of taps, in order to maintain the tightness of the container when removing the fruit for intermediate analysis, at 30, 60 and 158 d (except ILOS, which was provided with only one chamber and fruit was analysed only after 30 d).

2.3 Management of oxygen pull downs in DLOS containers

The Fruit Observer chlorophyll fluorescence (CF) sensor (Besseling, Netherlands) was installed inside the $DLOS_1$ container. The fluorescence monitoring system is assumed to detect a reaction of the chlorophyll when the LOL has been reached (Thompson et al., 2018). It was used following Besseling's protocol with some modifications (Fig. 2). Briefly, the system was activated and after the stabilization of the ambient conditions, which normally occurred after 24h, the first pull down was applied, establishing P_{O2} and P_{CO2} at 3 and 1 kPa, respectively, over a 48 h period, thereafter P_{O2} was reduced to 1.5 kPa and P_{CO2} to 0.8 kPa. After 17 d from harvest O_2 level was reduced to 0.5 kPa and after a week the first oxygen pull down was applied lowering the P_{O2} and P_{CO2} levels to 0.2 and 0.4 kPa, respectively. Thereafter, the P_{O2} was increased up to 0.5 kPa and P_{CO2} to 0.5 kPa when the fluorescence signal presented a peak or after 48 h if the sensor did not register any peak. After the stabilization of the ambient conditions, and in parallel with $DLOS_1$ container, the oxygen level was initially reduced to 3 kPa and CO_2 to 1 kPa in the $DLOS_2$ container and after 17 d from harvest O_2 and CO_2 levels were reduced to 0.5 kPa. A week later (t=24d) the first pull down was applied lowering the P_{O2} and P_{CO2} levels to 0.2 and 0.4 kPa, respectively and these levels were kept longer than in $DLOS_1$ (Fig. 2, Table 1) before re-establishing the set values (0.5 kPa O_2, 0.5 kPa CO_2). Pull downs lowering the oxygen level according to that pattern were applied in both DLOS containers at days 24, 54 and 78. After 152 d a prolonged oxygen pull down was applied in both DLOS containers and a final pull down to nearly 0 kPa of P_{O2} was done at 178 d of storage aiming to force the induction of the chlorophyll signal and the emission of specific VOCs (Fig. 2).

2.4 Fruit quality measurements

Fruit quality parameters, firmness (F), apparent maturity (I_{AD}), total soluble solids (TSS) and total titratable acidity (TTA) were measured as described elsewhere

(Torregrosa et al., 2019). Quality measurements were done immediately after harvest, at intermediate samplings points during the cold storage period, at the end of cold storage period (202 d) and after the cold storage period plus 5 d of shelf life (SL). For ILOS, intermediate sampling was analysed after 30 d. While intermediate samplings for fruit from DLOS chambers were done at days 30, 60 and 158 (6d after initiating the oxygen pull downs). At sampling point 20 fruit were analysed.

2.5 Ethylene production capacity

At harvest and at each sampling date (days 30, 60 and 202 days of cold storage) fruit ethylene production capacity was measured daily during 15 d (Fig. 2). Three 1.5 L flasks per container were used, each containing two fruit previously weighted. The flasks were continuously aerated with humidified air at a flow rate of 1.5 $L \cdot h^{-1}$ and kept at room temperature (20 ºC). The amount of ethylene produced by the fruit was measured by taking a 1 mL sample of gas from the headspace of each flask and injecting it into a gas chromatograph fitted with a FID detector (Agilent Technologies 6890, Wilmington, DE, USA) and an alumina column 80/100 (2m ×3mm) (Teknokroma, Barcelona, Spain) as described by Torregrosa et al. (2019).

2.6 Determination of fermentative metabolites

Ethanol (ET) and acetaldehyde (AA) pulp content were determined at the same sampling dates as other quality measurements (Fig. 2) following the methodology described by Deuchande et al. (2017). Briefly, frozen juices were incubated in a water bath at 65 °C for 1 h, thereafter, 1 mL of headspace gas sample was taken with a 1 mL glass syringe for chromatographic determination. Nitrogen was used as the gas carrier, and the operating conditions were as follows: oven temperature: 90 °C; injector temperature: 250 °C; detector temperature: 220 °C. The in liquid

concentrations were calculated using a standard curve generated by injecting standard solutions of known concentrations (acetaldehyde standards ranging between 0.5–15 μL L^{-1}; ethanol standards ranging between 2.5–250 μL L^{-1}).

2.7 VOCs extraction and quantification

VOCs extraction was done just before each oxygen pull down (t=24, 54, 78, 152 and 178 d) and 6 d after the oxygen pull down initiation (t= 30, 60, 84, 158 and 184 d) simultaneously in the three containers.

The extraction was conducted by inserting the two adsorption tubes filled with 350 mg Tenax TA porous polymer adsorbent (2, 6-diphenyl-p-phenylene oxide) and Carbograph 1TD outside of each container. During the extraction the pump was turned on and a 250 ml/min airflow was then forced through each container of pears and forced out through the absorption tubes to collect the volatiles contained therein (Fig. 1). The airflow was passed through the tubes for 60 min. The adsorption tubes were kept at 4 ºC until they were desorbed (Cano-Salazar et al., 2013).

Volatile compounds desorption was done using an automated UNITY Markes thermal desorption system (Markes International Ltd., Llantrisant, United Kingdom) at 275 ºC for 15 min. Identification and quantification were done with an Agilent 7890B gas chromatograph coupled to a 5977A mass spectrometer (MSD) (Agilent Technologies, Inc., Barcelona, Spain). Volatile compounds separation was performed with a capillary column with cross-linked free fatty acid as the stationary phase (FFAP; 50 m×0.2 mm×0.33 μm). Helium was used as the carrier gas, at a flow speed of 42 cm s^{-1}. Both the injector and detector were kept at 240 ºC. The analysis was conducted according to the following program: 40 ºC (1 min); 40-115 ºC (2.5 ºC min^{-1}); 115-225 ºC (8 ºC min^{-1}); 225 ºC (10 min). Mass spectra was obtained by electron impact ionization at 70 eV, using the

same flow of helium and following the same temperature gradient program as the ones used in the separation. Volatile compounds identification was carried out by comparing the spectrometric data recorded to those from the original NIST HP59943C library mass spectra and by matching their respective retention index with those of standards. All of the standards for the volatile compounds studied in this work were analytical grade or the highest quality available. Quantification was performed using individual calibration curves, with correlation coefficient higher than 0.95, for each identified compound.

2.8 Statistical and data analysis

Means were compared by analysis of variance (ANOVA). When the analysis was statistically significant, the Student t-test (LSD) and the Tukey's Honestly Significant Difference (HSD) at $P \leq 0.05$ were performed for separation of means using JMP® 13.1.0 SAS Institute Inc. (SAS Institute, 2013).

A Principal Component Analysis (PCA) was conducted in order to establish a preliminary relationship between VOC's emitted from the three experimental conditions ($DLOS_1$, $DLOS_2$ and ILOS), after the application of each pull down.

3. Results and discussion

3.1 Impact of initial vs dynamic oxygen pull downs on fruit quality and ripening capacity upon removal from cold storage

Maturity at harvest determines the suitability of fruit for long-term storage (Kader, 1999), and in the case of pear fruit is commonly measured in terms of firmness or the I_{AD} index (Costa et al., 2016; Zerbini, 2002). In our study, the average firmness of pears at harvest was 61.3 N, in agreement with local recommendations as well as those published by other authors for long-term storage of 'Conference' pears (55-65 N; Rizzolo et al.

2015, Torregrosa et al. 2019). Firmness evolution throughout the storage period followed a similar trend in the three containers (Table 2). Even though, firmness was significantly higher in fruit from ILOS just after cold storage, differences of 6 N are not relevant from an organoleptic point of view (Harker et al., 2002).During the shelf life period (SL) at 20 ºC after long term storage, fruit firmness decreased from 65 N to approximately 17.5 N in 5 d and regardless of the storage conditions (Table 2), thereby in agreement to the pattern of firmness loss reported in other studies (Torregrosa et al., 2019). The I_{AD} index at harvest was 2.10±0.07, hence within the range considered to be optimal in 'Conference' pears (Torregrosa et al., 2019) and 'Barlett' pears (Wang et al., 2015) for long term storage. In our experiments, ILOS stored fruit had significantly lower I_{AD} values at day 5 of SL than DLOS stored fruit (Table 2).

The TSS/TTA ratio did not show significant differences during the storage period except after 5 d at SL, when fruit stored under more extreme conditions ($DLOS_2$) showed significantly lower values (8.5±0.4), suggesting that fruit were less ripe (Table 2).

Ethylene triggers the initiation of ripening in climacteric fruit with the associate physical and physiological changes in pears. 'Conference' pear, require a chilling period to start ripening (Villalobos-Acuña and Mitcham, 2008). In agreement, our results showed that pears harvested at the optimal commercial maturity and maintained at 20 ºC without a cold period did not produce ethylene until day 15 (Fig. 3A).

After 30 d under cold storage, fruit from all storage conditions started to produce ethylene at day 4 of SL, confirming the short-chilling requirement for ripening of this pear variety. ILOS stored fruit showed the climacteric peak one day earlier than DLOS stored fruit (Fig. 3B). After the second sampling, at day 60 of cold storage, fruit started to produce ethylene just after 1 d in SL and fruit from $DLOS_1$ container reached the

climacteric peak approximately two days earlier than fruit from $DLOS_2$ (Fig. 3C) thereby confirming that the lower the oxygen levels increase the inhibition of the fruit ripening capacity. After 202 d in cold storage fruit from all storage conditions showed a postclimacteric behaviour (Fig. 3D), characterized by a decrease in ethylene production just after the cold storage period which is typical for long-term stored 'Conference' pears (Torregrosa et al., 2019).

Our results showed an increase in ET content and AA concentrations after 5 d of SL following long-term (202 d) cold storage (Fig. 2 and 4) thereby highlighting that fruit was undergoing normal ripening (Pesis, 2005). Significant lower ET and AA levels were found in $DLOS_1$ and $DLOS_2$ containers, reflecting a slower ripening pattern of the fruit following storage under more restrictive storage conditions (Fig. 4). Our results are in accordance with the ones reported by Chervin et al. (1999), who found lower ethanol levels in Packham's Triumph pears stored for 2 m at 3 kPa O_2 and <0.2 kPa CO_2 plus 18 d in SL (12.5 µmol g^{-1}) than under normal air (20 µmol g^{-1}). It has been reported that 'Conference' pears stored under different conditions produced ET levels in the range of 0-50 µL L^{-1} during the cold storage period and AA levels in the range of 1-3 µL L^{-1} (Saquet and Streif, 2006), which is in accordance with our results during the whole storage period; 0.2-42 µL L^{-1} and 0.5-3 µL L^{-1}, respectively (data not shown). In all our experiments no physiological disorders such as internal browning were detected, what is consistent with the measured concentrations of ET and AA.

3.2 Chlorophyll fluorescence and evolution of fruit ethanol content

Continuous evolution of O_2 and CO_2, ethanol content in fruit at intermediate samplings in the three storage containers as well as the CF signal evolution in $DLOS_1$ container during the cold storage period are shown in Fig. 2. Oxygen levels in both DLOS containers were pulled down at days 24, 54, 78, 152 and 178 with extended ultra-low

conditions applied at $DLOS_2$. That fact is reflected in the values of the I_{OD} index of $DLOS_1$ and $DLOS_2$ (Table 1). In the ILOS chamber an initial pull down was applied and thereafter was maintained at 2 kPa O_2 and 2 kPa CO_2.

Fruit Observer signal exhibited a peak after the first oxygen pull down (PD_1) at t=24 d. At day 35 the CF sensor showed an unexpected peak which could not be explained by O_2 or CO_2 variations nor by temperature or RH shifts within the storage container. However, it could be due to light interaction as previously reported by Zerbini and Grassi (2010). At day 54, when the second pull down was applied (PD_2) no CF peak was observed. The third pull down (PD_3) was applied at day 78 and the CF signal peaked 4 d afterwards. After 152 d the oxygen level was lowered (PD_4) but again the CF did not show any peak, for this reason, levels of O_2 were maintained at 0.4 kPa for 22 d with the aim to see a CF reaction by the sensor. Since the sensor did not show any peak, at t =178 d the P_{O2} and P_{CO2} levels were increased to 1 kPa and 1 kPa, respectively, for 2 d and subsequently dropped again to 0.2 kPa of O_2 and 0.4 kPa of CO_2 (PD_5). Then, the CF signal peaked afterwards.

Based on the data depicted in Fig. 2, the CF signal peaked three times out of the five oxygen pull downs, hence highlighting that pears harvested at the commercial maturity from the Lleida region are either very resistant to atmospheres with low oxygen level, as pointed out in recent studies (Torregrosa et al., 2019) or that the CF signal, as given by the sensor used herein, do not precisely monitor the fruit response to ultra-low O_2 levels. However, these results should be confirmed with other pear varieties and pears from different regions. Despite the dissimilarities between species, Prange et al. (2003), also reported that chlorophyll fluorescence sensor did not show any peak until oxygen levels were near 0.1 kPa in stored cabbage, they attributed this fact to an hysteresis effect.

313 Ethanol in fruit pulp is a common indicator of fermentative damage induced by
314 low P_{O2} levels (Deuchande et al., 2016). At the first sampling, no ethanol accumulation
315 in the fruit pulp was observed for any storage condition even though the CF signal peaked
316 at day 26 in parallel to the restriction in O_2 levels. Enhanced ethanol production has been
317 previously linked to alterations in the chlorophyll fluorescence signal during apple
318 storage (Fan et al., 2005). After the second oxygen pull down ethanol remained low in
319 both DLOS storage conditions, which was in accordance with the absence of the CF peak
320 given by the sensor. However, at the third sampling, after the fourth pull down, higher
321 ethanol accumulation was found in fruit from $DLOS_1$ but with no significant differences
322 between DLOS containers. Ethanol levels in fruit from $DLOS_1$ were above 20 $\mu L\ L^{-1}$
323 which was recently defined as the critical level for the induction of internal disorders in
324 'Rocha' pears by Deuchande et al. (2016). However, the ethanol levels registered in this
325 study for $DLOS_1$-stored fruit, or any of the other conditions tested, were not accompanied
326 by fruit internal damage even at the end of the cold storage period and shelf-life (data not
327 shown). After the last sampling, significant differences in ET content were found among
328 the three containers. ILOS stored fruit had the higher ethanol content followed by $DLOS_1$
329 and $DLOS_2$ (37, 30 and 21 ppm, respectively). This result may seem contradictory since
330 reduced oxygen level in the storage atmosphere enhance fermentative pathways leading
331 to AA and ET accumulation within the fruit (Imahori et al., 2013). Fruit, including pears,
332 accumulate ET not only in response to anoxia or high CO_2 but also during normal ripening
333 (Nanos et al., 1992; Pesis, 2005) via enhanced pyruvate decarboxylase and alcohol
334 dehydrogenase enzyme activity. Therefore, it is likely that the higher ET content observed
335 in ILOS stored fruit was associated to an enhanced fruit maturity stage at the end of
336 storage.

Fruit from different maturities and grown under different agroclimatic conditions are known to show a different susceptibility to both external and internal physiological disorders (Kadam et al., 1995), hence likely explaining the absence of physiological disorders observed in our experiments. Deuchande et al. (2016), also reported the absence of internal browning disorders in 'Rocha' pears stored under dynamic controlled atmosphere at 0.5 kPa of CO_2 with the aid of HarvestWatch chlorophyll monitoring sensor. In our study, no clear correlation was found between ET content and the chlorophyll signal, as given by the Besseling sensor, during the cold storage of 'Conference' pears. Similar results were obtained by Prange et al. (2003) who stored 'Summerland McIntosh' apples for 9 months under three different CA treatments and did not find a clear relationship between ethanol pulp content and changes in chlorophyll fluorescence. As shown in the Fig. 2, our results strongly suggest that chlorophyll fluorescence, albeit representing alterations at the chloroplastic level, do not always depict changes from aerobic to anaerobic respiration (ethanol accumulation) in 'Conference' pears stored under ultra-low oxygen levels. Further research is needed to find more suitable markers of the low oxygen level (LOL) tolerated by the fruit under O_2-depleted atmospheres.

3.3 Can emitted volatiles within the storage atmosphere be used as a marker of the LOL or the fruit ripening behaviour?

In the context of developing novel markers to depict the LOL tolerated by the fruit or the ripening behaviour under ultra-low O_2 atmospheres, changes in the emission of VOCs during storage were investigated. It is well documented that low oxygen levels during the cold storage period affect the fruit metabolism, inhibiting the synthesis of some volatile esters and affecting their emission during the subsequent shelf life period (Chervin et al., 2000; Hendges et al., 2018; Rizzolo et al., 1991). The emission rates of

not only straight esters but overall VOCs inside the cold storage atmosphere (Fig. 5) were up to one thousand times lower than the ones emitted by the same pear variety during ripening at 20ºC in normal air atmospheres (Torregrosa et al., 2019). This result is not surprising but clearly showed that very sensitive equipment is needed when looking at the volatiles within cold-storage rooms (Harren and Cristescu, 2013).

Despite their lower concentration, 22 active odour compounds were identified inside the storage rooms (Fig. 5), including 12 esters, 3 aldehydes, 3 terpenes, 3 alcohols and 1 acid. Esters are the main contributors to the ripe pear aroma (El Hadi et al., 2013; Zlatić et al., 2016) and aldehydes generate a green and an herbaceous aroma which are typical for unripe fruit (El Hadi et al., 2013; Hendges et al., 2018). Quantitatively speaking, the main compounds detected were the two esters, butyl propanoate and 2-methylpropyl butanoate and the two alcohols benzyl alcohol and benzoic acid.

A PCA model was developed to obtain a global view of the pear volatiles emission distribution after each of the five pull downs for each storage condition. In this PCA, the volatiles emissions were used to characterize the different cold storage scenarios (three storage atmospheres) and the different samplings at 30, 60, 84, 158 and 184 d, numbered from PD_1 to PD_5, respectively. The biplot of the two principal components captured 48.7 % of the total variability (Fig. 6). This relatively low explained variance could be due to an overlap in the information relating to the volatile compounds included in the PCA, yet it was sufficient for our qualitative purposes. The corresponding biplot showed that the main factor accounting for sample differentiation was the sampling dates; this finding is consistent with the higher concentration of hexanal, α-farnesene and hexyl propanoate of the pears stored for 184 d and, in particular, for those kept under the less restricted O_2 atmosphere (ILOS). Hexanal was the main volatile compound accounting for sampling date differentiation. The higher hexanal concentration found in pears at PD_5 could be due

387 to high stress experienced by the fruit due to low O_2 concentrations since it is well known
388 that the emission of the C_6 aldehydes, alcohols and esters derived from fatty acids through
389 the action of lipoxygenases (Holopainen, 2004) may be increased during some biotic and
390 abiotic stresses (Laothawornkitkul et al., 2008). However, the emission of the ester (Z)-
391 2-hexen-1-yl acetate, other important C_6 ester, was higher after the two first oxygen pull
392 downs or sampling dates (Fig. 6), especially in pears from ILOS and $DLOS_1$ but was low
393 at PD_5. This higher concentration of (Z)-2-hexen-1-yl acetate after the first two oxygen
394 pull downs could be due to its independence from the availability of linoleic acid, which
395 is more available as a substrate for LOX earlier in the storage period.

396 From the PCA biplot, it can also be observed that after the first oxygen pull down
397 (PD_1), the $DLOS_2$ atmosphere appears located in the lower part of the PCA-biplot,
398 meaning that for this sampling date, the PC2 was important to differentiate pears stored
399 under the three storage conditions. The pears from $DLOS_2$ showed higher concentrations
400 of three esters (butyl propanoate, methyl butanoate and 2-methylpropyl acetate) (Fig. 5
401 and 6) all known to be characteristic of the pear aroma (El Hadi et al., 2013). The higher
402 amounts of 2-methylpropyl acetate, methyl butanoate and butyl propanoate esters emitted
403 by pears from $DLOS_2$ can likely be attributed to the higher oxygen level (3 kPa) observed
404 in this container during the first 17 d of storage since the two main biosynthetic pathways
405 of esters from fatty acids (β-oxidation and the lipoxygenase (LOX) pathway), are oxygen
406 dependent.

407 After the second oxygen pull down, only three acetates (2-methylpropyl, butyl and
408 hexyl acetates) and the aldehyde hexanal showed significant differences between storage
409 conditions (Fig. 5). ILOS stored pears produced higher concentrations of 2-methylpropyl
410 and hexyl acetates, while pears from $DLOS_1$ exhibited greater emissions of butyl acetate
411 and hexanal. After the third oxygen pull down, differences along storage conditions were

observed again along the PC2, since the VOCs emitted by ILOS stored fruit clearly differed from the ones emitted by fruit stored under DLOS. DLOS fruit were mainly characterized by the emission of 2-ethylhexanal and 2-ethylhexanol (Fig. 6), even though significant differences between fruit from ILOS and DLOS were only detected in butyl acetate and hexyl 2-methylbutanoate, which showed higher concentration in DLOS stored pears, and in 2-methylpropyl acetate and α-farnesene, with higher emission in ILOS stored pears. The lower oxygen levels in $DLOS_1$ and $DLOS_2$, significantly inhibited α-farnesene emission which is consistent with the results reported by Chervin et al. (2000) and Larrigaudière et al. (2019). α-farnesene tends to accumulate as fruit ripens after harvest and hence the higher content of this compound observed in ILOS stored fruit suggest that the fruit was in a more advanced maturity stage. Even though terpenes are considered as important IVOCs (Holopainen and Gershenzon, 2010), the results from this study suggest that α-farnesene was not emitted in response to the imposed oxygen pull down conditions. At t=152 d an enlarged pull down was applied in both DLOS containers but no clear separation between storage containers were observed (Fig. 6), coinciding with the fact that no CF peak was detected upon the application of this oxygen pull down. However, some volatile compounds showed statistical differences in their concentration (Fig. 5). For instance, hexyl acetate and 2-ethylhexanol were detected only in the headspace from DLOS containers. After the application of the fifth oxygen pull down at 178 d, Fig. 4 shows a clear separation between ILOS stored fruit and DLOS stored ($DLOS_1$ and $DLOS_2$) along the PC1. Fruit from ILOS container exhibited significantly higher amounts of some VOCs, such as ethyl and butyl acetate, which are typical ripening-related esters (Saquet, 2017; Torregrosa et al., 2019), hexyl propanoate, 2-methyl-1-butanol as well as α-farnesene. The high emitted amounts of ethyl and butyl acetate together with the lower I_{AD} values (Table 2) and the ethylene production pattern

437 (Figure 5) exhibited by ILOS stored fruit confirmed that the lower the oxygen levels
438 during storage the higher the inhibition of the fruit ripening capacity.

439 Although oxygen level was forced to lower five times in $DLOS_1$ and $DLOS_2$
440 stored fruit, none of the volatile compounds detected showed a repeated maximum or
441 minimum in parallel or after the fluorescence peaks. After the third oxygen pull down
442 (t=84), when ethanol accumulation in fruit pulp was higher, the emission of butyl
443 hexanoate and 2-ethylhexanol were also higher in DLOS stored fruit. Methyl butanoate
444 and benzyl alcohol were emitted at higher levels in fruit from the most restrictive
445 container ($DLOS_2$) at this specific sampling (Fig. 5). These results suggest that not only
446 the amount of ethanol within the fruit pulp but also the concentration of some emitted
447 volatiles into the storage atmosphere may be employed as markers of fruit ripening during
448 the storage of 'Conference' pears.

449 Although further research is needed, our results showed that IVOCs, such as ethyl
450 acetate, butyl acetate and hexyl propanoate, among others, could be used to monitor the
451 fruit ripening stage during storage.

452 **4. Conclusions**

453 The application of periodic oxygen pull downs, as generally done during dynamic
454 controlled atmosphere storage, slow down the ripening capacity of 'Conference' pears,
455 during cold as well as after subsequent shelf-life storage, as indicated by the changes in
456 the I_{AD} values and the ethylene production pattern or even the synthesis of some typical
457 pear ripening related volatiles (ethyl and butyl acetate). Our data clearly show that the
458 lower and longer the oxygen depletion period established, the higher the inhibition of the
459 fruit ripening capacity.

460 Despite its usage within packinghouses in ‘Conference’ pears, CF signal, as given
461 by the sensor and the conditions used herein, did not peak after all the oxygen pull downs,
462 and was poorly correlated with the ethanol flesh content. Neither, CF peaks were always
463 in accordance with the induction of specific VOCs emission as highlighted by our
464 multivariate data analysis.

465 The levels of VOCs emissions inside the storage atmosphere did not follow a clear
466 pattern after the oxygen pull downs. However, our data suggest that changes in the
467 emission of butyl acetate, hexyl propanoate and α-farnesene along the cold storage period
468 had a good correlation with ripening parameters (I_{AD}) so they could be used as ripening
469 markers of ‘Conference’ pears.

470 To further develop these markers as predictors of fruit ripening evolution during
471 long-term cold storage, additional research is needed to define seasonal, cultivar, and
472 maturity effects on these ripening markers.

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Table 1 Duration of each oxygen pull down (Δ_{t_i}) in (d) and oxygen depletion index (I_{OD_i}) in (Pa·d), during the five oxygen pulled downs applied at containers dynamic low oxygen stresses ($DLOS_1$) and extended dynamic low oxygen stresses ($DLOS_2$). The I_{OD_i} were calculated using eq. (1) with P_{O2ref}=0.6 kPa and $\Delta_{t_{max}} = 10d$.

	Δt_i (d)		I_{ODi} (Pa·d)	
O_2 pull down (d)	$DLOS_1$	$DLOS_2$	$DLOS_1$	$DLOS_2$
PD_1 (24)	3.0	3.0	48.4	74.8
PD_2 (54)	3.0	9.0	1281.3	3480.2
PD_3 (78)	5.0	9.5	1040.5	2384.7
PD_4 (152)	10.5	10.5	1227.2	1277.6
PD_5 (178)	3.5	6.5	1397.3	2596.6
		$\sum_{i=1}^{5} I_{OD_i}$	4994.7	9813.9

Table 2 Physicochemical parameters evolution, (Firmness (F), I_{AD} index and TSS/TTA ratio), in 'Conference pears under three different storage atmospheres: initial low oxygen stress (ILOS), dynamic low oxygen stresses monitored with a CF sensor ($DLOS_1$) and extended dynamic low oxygen stresses ($DLOS_2$); oxygen pull downs were applied at t=24, 54, 78, 152 and 178 d in $DLOS_1$ and $DLOS_2$. Mean ± standard deviation (n=20 for F and I_{AD}) (n=4 for TSS/TTA). Different letters indicate significant differences $P \leq 0.05$ (LSD test) between storage atmospheres for each parameter. No letter indicates the absence of significant differences. *= not measured.

	F (N)			**I_{AD} (-)**			**TSS/TTA**		
Time (d)	**ILOS**	**$DLOS_1$**	**$DLOS_2$**	**ILOS**	**$DLOS_1$**	**$DLOS_2$**	**ILOS**	**$DLOS_1$**	**$DLOS_2$**
OHD t=0	61.3±6.2	61.3±6.2	61.3±6.2	2.1±0.07	2.1±0.07	2.1±0.07	6.1±0.8	6.1±0.8	6.1±0.8
30	67.7±7.1	71.2±7.6	70.3±4.8	[b]2.02±0.08	[a]2.22±0.10	[a]2.16±0.10	5.2±0.8	5.2±1.0	6.0±0.6
60	*	73.2±11.1	71.5±6.7	*	2.07±0.05	2.11±0.07	*	5.8±0.8	5.4±0.5
158	*	64.0±7.0	67.8±5.4	*	1.95±0.13	1.93±0.15	*	6.9±0.5	6.8±1.0
202	[a]70.1±6.0	[b]63.5±5.3	[b]63.7±6.7	[b]1.69±0.20	[a]2.00±0.13	[a]1.93±0.19	7.5±0.7	7.0±0.9	7.1±1.0
202+5	17.9±3.4	17.7±1.9	16.6±2.6	[b]1.30±0.27	[a]1.57±0.20	[a]1.60±0.21	[a]9.6±0.8	[a]9.5±0.6	[b]8.5±0.4

Figure 1 Schematic representation of the experimental setup used for the volatile organic compound's extraction from the experimental container atmosphere. The three small chambers were located at the top of each container. The chambers were connected to each other and with the container's atmosphere in order to standardize the headspace air.

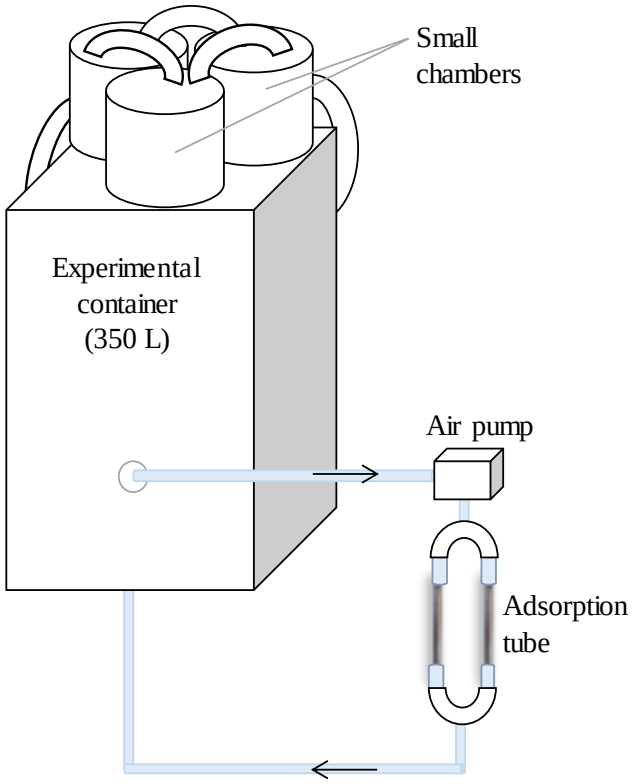
Figure 2 Oxygen and CO_2 partial pressure (left axis) and ethanol content (ET) in fruit pulp (right axis) in the three containers (storage atmospheres) used for cold storage. A) initial low oxygen stress (ILOS), B) dynamic low oxygen stresses ($DLOS_1$) monitored with chlorophyll fluorecence signal (right ofset axis) and C) enlarged dynamic low oxygen stresses ($DLOS_2$). Discontinuos vertical lines indicate the time of application of oxygen pull downs (t=24, 54, 78, 152 and 178 d) in DLOS. (☆) Indicates the time at which fruit samples were removed from the chambers/containers (t=0, 30, 60, 158 and 202), (●) indicates ethanol content in fruit pulp. Error bars indicate standard deviation for n=3, mean values with the same letter are not significantly different according to analysis of variance (ANOVA) and LSD test at $P \leq 0.05$. No letter indicates the absence of significant differences.

Figure 3 Ethylene production rate of 'Conference' pears during shelf life at 20ºC immediately after harvest (A) and after 30 (B), 60 (C) and 202 d (D) of cold storage under different atmospheres: initial low oxygen stress (ILOS,●), dynamic low oxygen stresses ($DLOS_1$,o), enlarged dynamic low oxygen stresses ($DLOS_2$,▼). Error bars represent the mean ± standard error (n=3).

Figure 4 A) Ethanol content (ET) and B) acetaldehyde (AA) content in 'Conference' pears after 202 d of storage under different storage atmospheres (initial low oxygen stress (ILOS), dynamic low oxygen stresses ($DLOS_1$) and enlarged dynamic low oxygen stresses ($DLOS_2$)) plus 5 d of shelf life. Error bars represent the mean ± standard deviation (n=3). Bars with different letters are significantly different based on an HSD test at $P \leq 0.05$.

Figure 5 Heat map of volatile organic compounds (VOCs) grouped by esters, aldehydes, terpenes, alcohols and acids. Each row represents one sampling date during storage and each column represents the different storage atmospheres (initial low oxygen stress (ILOS), dynamic low oxygen stresses ($DLOS_1$) and extended dynamic low oxygen stresses ($DLOS_2$)). Numbers in brackets under each VOC name represent the maximum emission rate in ng kg^{-1} h^{-1}. Variables of significance: * $P \leq 0.05$, ** $P \leq 0.01$ and the absence of asterisks means no significant differences, $P > 0.05$.

Figure 6 Biplot of the first principal component (PC1) and the second principal component (PC2) from a full data principal component analyses (PCA) model considering volatile organic compounds (n=22) after low oxygen pull down. The five sampling days were identified as PD_1 (t=30 d), PD_2 (t=60 d), PD_3 (t=84 d), PD_4 (t=158 d) and PD_5 (t=184 d), from three different atmosphere conditions: initial low oxygen stress (ILOS,●), dynamic low oxygen stresses ($DLOS_1$,o), enlarged dynamic low oxygen stresses ($DLOS_2$,▼).



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728 **Figure 1**

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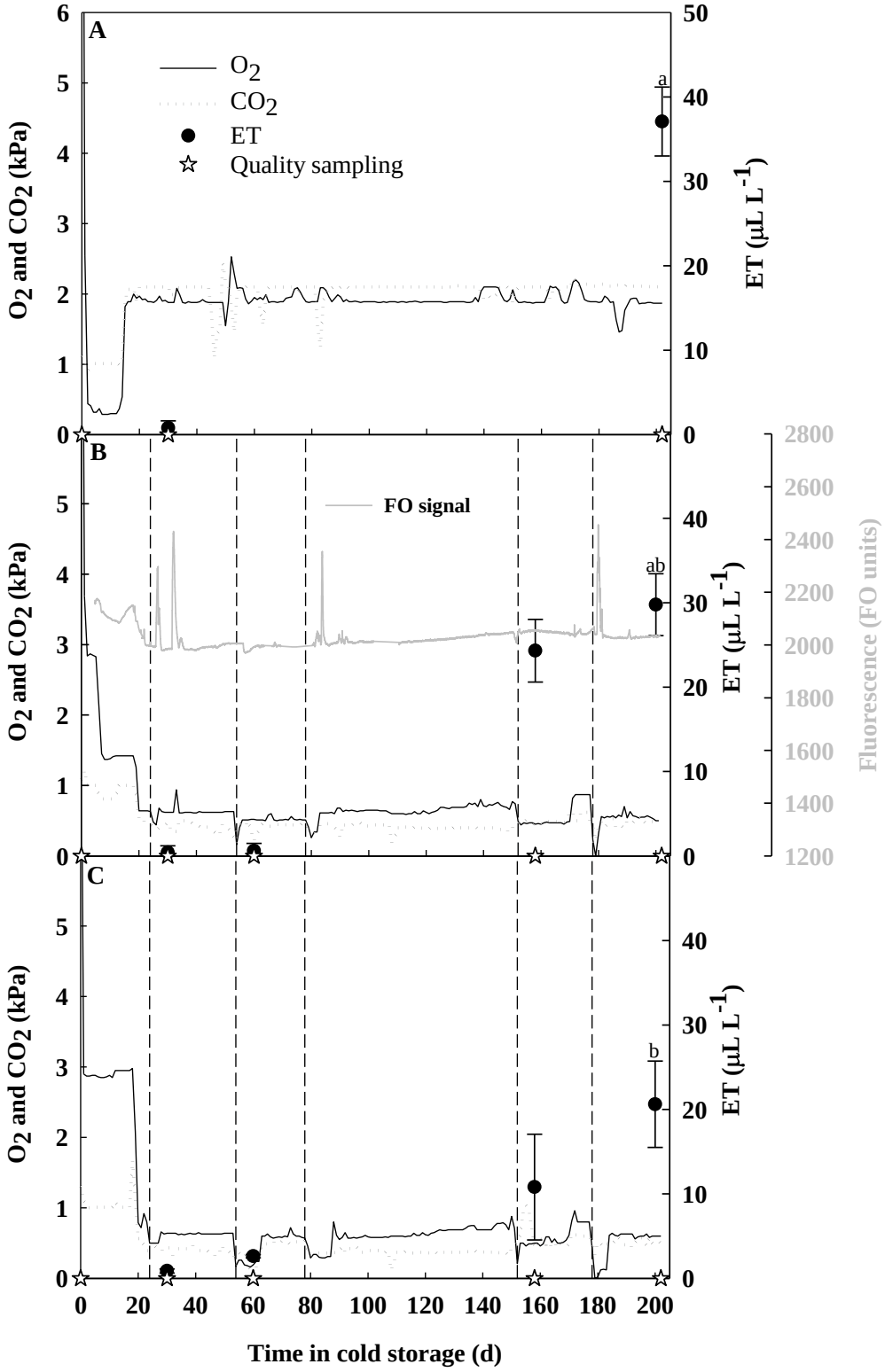

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737 **Figure 2**

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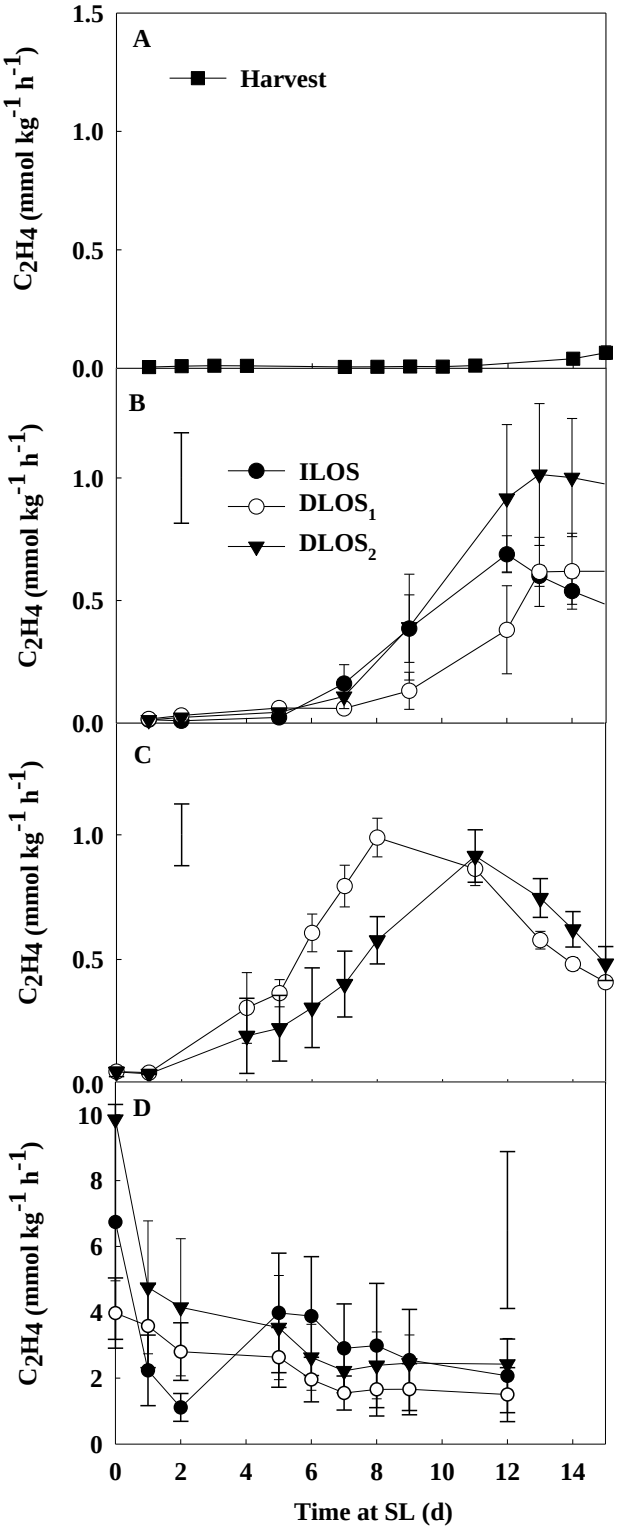

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740 **Figure 3**

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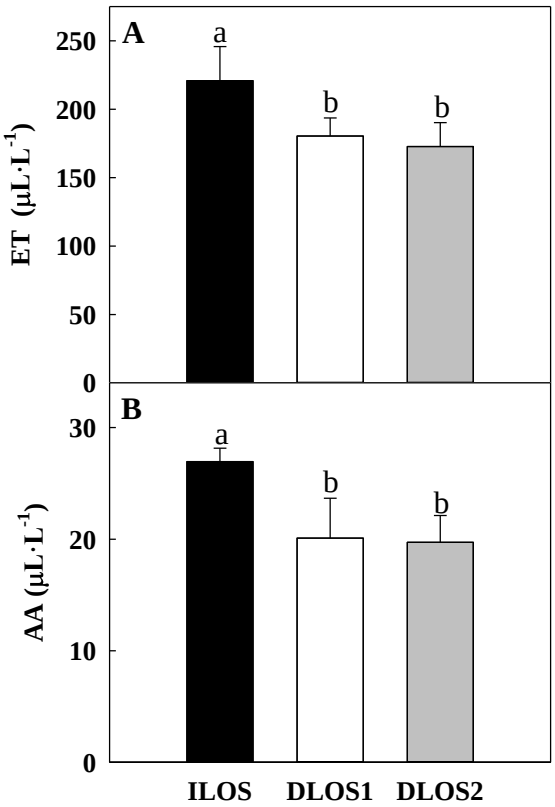

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744 **Figure 4**

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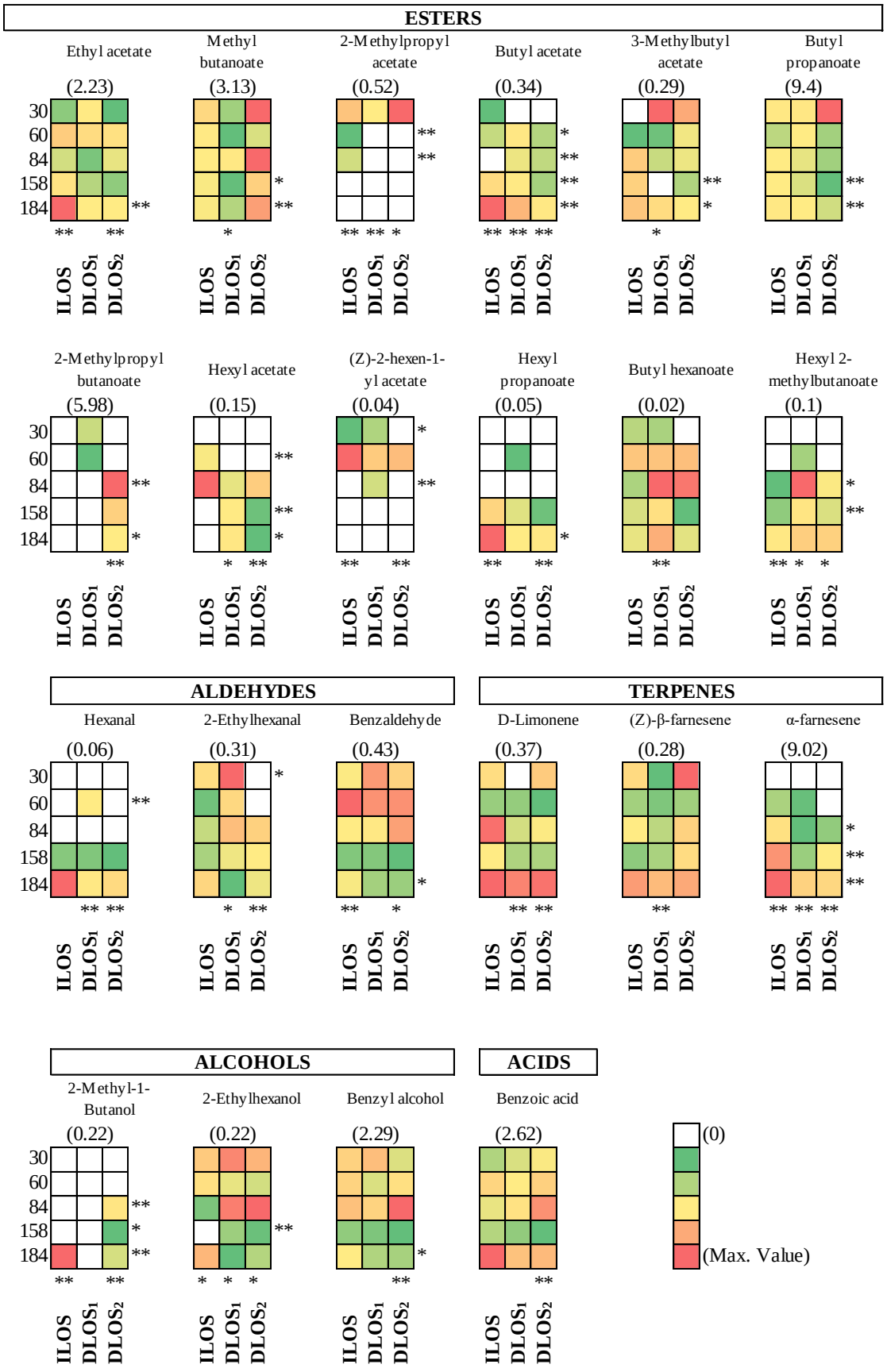
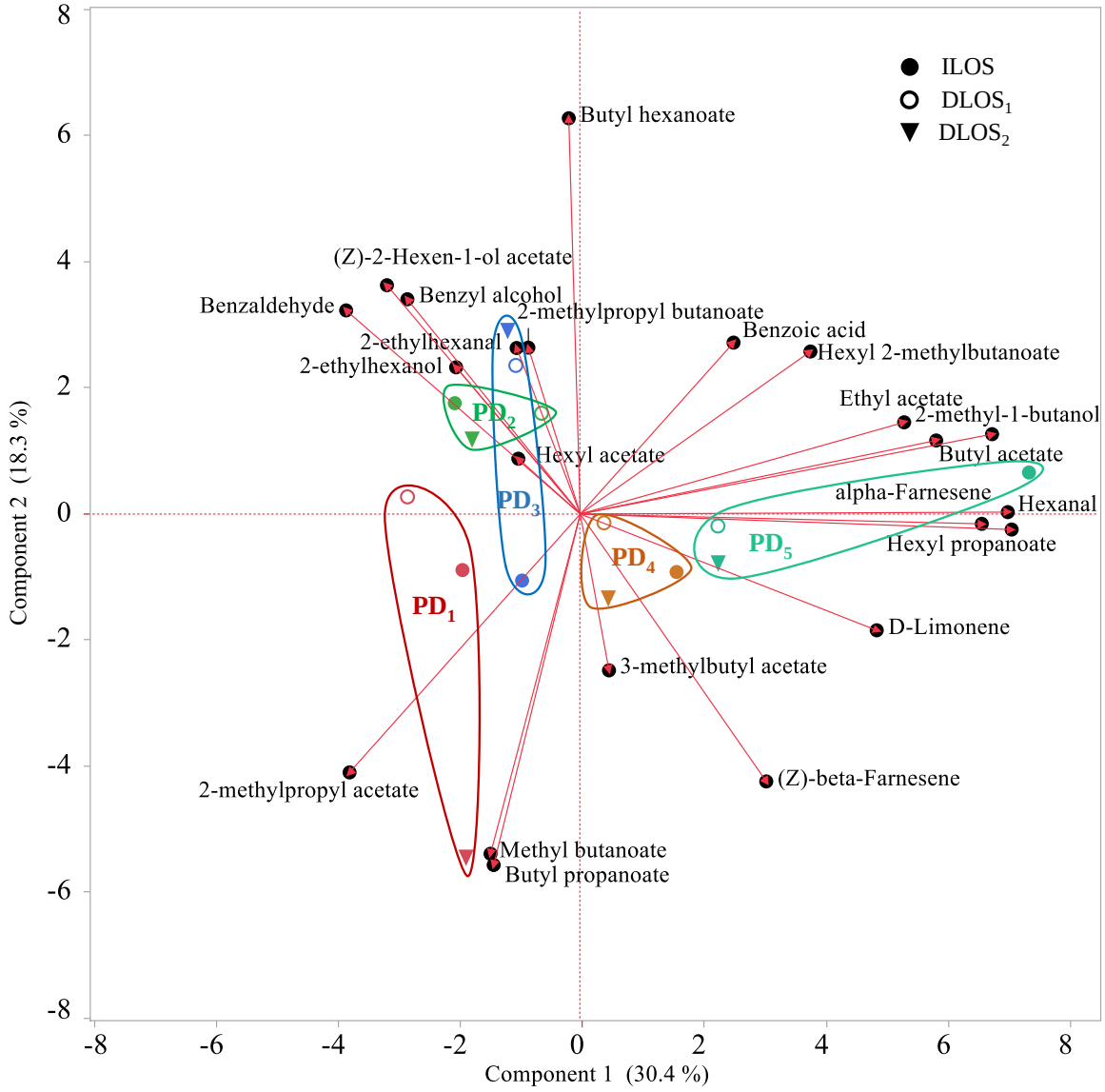

ESTERS
Ethyl acetate
(2.23)
Methyl butanoate
(3.13)
2-Methylpropyl acetate
(0.52)
Butyl acetate
(0.34)
3-Methylbutyl acetate
(0.29)
Butyl propanoate
(9.4)
2-Methylpropyl butanoate
(5.98)
Hexyl acetate
(0.15)
(Z)-2-hexen-1-yl acetate
(0.04)
Hexyl propanoate
(0.05)
Butyl hexanoate
(0.02)
Hexyl 2-methylbutanoate
(0.1)
ALDEHYDES
Hexanal
(0.06)
2-Ethylhexanal
(0.31)
Benzaldehyde
(0.43)
TERPENES
D-Limonene
(0.37)
(Z)-β-farnesene
(0.28)
α-farnesene
(9.02)
ALCOHOLS
2-Methyl-1-Butanol
(0.22)
2-Ethylhexanol
(0.22)
Benzyl alcohol
(2.29)
ACIDS
Benzoic acid
(2.62)
30
60
84
158
184
ILOS
DLOS1
DLOS2
(0)
(Max. Value)

Figure 5



758

759 **Figure 6**

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