Involvement of fatty acids in the mechanism of tolerance to high carbon dioxide in strawberries stored at low temperature.

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Abstract

Fatty acids (FAs) have a great impact not only on changing membrane integrity and fluidity but also on modulating cellular energy. They can also affect aroma by modifying the production of specific FA-dependent esters. However, the role of FAs in high CO_2 tolerance remains to be determined in strawberries during storage at low temperature. Total, free and esterified FAs in neutral (triacylglyerols) and polar (membrane lipids) were characterized and quantified, before their relationship with straight-chain esters was examined in CO_2-treated strawberries exhibiting different metabolic energy. Our data show an imbalance in the saturated/unsaturated ratio of polar lipids and a decrease in the amount of free polyunsaturated fatty acids (PUFAs) linked to a prevalence of butanoates and hexanoates esterified to long alcohols in strawberries stored without added CO_2 (T1). Moreover, our results indicate a strong negative correlation ($P \leq 0.01$; $r \geq 0.7$) between butanoate esters and free PUFAs. A 3 d 40 kPa CO_2 treatment (T4) depleted the FAs from all the lipid fractions, increasing lipid peroxidation in association with severe low ATP levels, suggesting an active lipid breakdown which could play a causal role in the reported increased leakage of cellular water into intercellular air spaces that occurs in stressed-CO_2 fruit. By contrast, the application of a 2 d 20 kPaCO_2 treatment (T2) increased the amount of PUFAs from the neutral and polar fractions, with a preference for α-linolenic acid (18:3n3) which results in a rise in the 18:3/18:2 ratio, and which can confer membrane stability during storage at 0 ºC. Furthermore, our results also showed a strong positive correlation ($P \leq 0.01$; $r \geq 0.75$) between α-linolenic acid (18:3n3) of polar lipids and ethyl esters other than ethyl acetate. Thus, a 2 d 20 kPa CO_2 treatment during storage at low temperature could improve membrane fluidity and on account of the strong correlations with specific ester

volatiles, this could contribute to enriching aroma which, together with greater flesh firmness, represent an important challenge in postharvest.

Keywords: Fatty acids, CO_2, esters, MDA, strawberries, energy supply.

1. Introduction

Short CO_2 treatments are used commercially to reduce fruit decay incidence during low temperature storage in part due to the induction of defense compounds. Some of them are volatile compounds and polymers of (epi)catechin units with ability to inhibit microbial growth (Alejo-Armijo et al., 2018; Almenar et al., 2006; Blanch et al., 2012a; Prusky et al., 1991). In avocado, the presence of antifungal diene compounds has been reported in CO_2-treated fruit (Prusky et al., 1991) and the prevention of decay appears to be regulated by the presence of epicatechin, an inhibitor of lipoxygenase activity. Other important beneficial effects of high CO_2 treatments include an increase in flesh firmness (Larsen and Watkins, 1995) and the preservation of cellular structure and volume, linked to water status and accumulation of metabolites with an osmolyte function (Blanch et al., 2012b; Navarro et al., 2015). However, the accumulation of protective molecules varies according to the concentration of CO_2 used and the length of exposure. Indeed, when excess CO_2 is applied to strawberries it may produce a sharp decrease in bound water content, in association with intracellular liquid leakage (Blanch et al., 2015). We hypothesize that these alterations caused by excess CO_2 can be explained by changes in membrane permeability and by extension, through a modification of their lipid composition. In addition to the effect of lipids on the organization of water at the membrane (Disalvo et al., 2008; Lee et al., 2008), FAs play an important role in maintaining membrane fluidity. Membranes must be maintained in the fluid state to

function properly at low temperature, and those containing many PUFAs will be more fluid and less viscous. Modulating membrane fluidity by altering the relative proportions of different lipid classes in the lipid bilayer, and through the degree of unsaturation of the fatty acyl groups in polar lipids, represents a means of adaptation to different stresses (Li et al., 2016; Murakami et al., 2000). More specifically, controlling membrane fluidity has been considered an important strategy to adapt to temperature stress (Murata and Los, 1997).

In addition to being essential membrane structural components, FAs and their derivatives play another important roles for energy supply and acting as precursors for esters and molecules precursors involved in stress responses (Hamilton-Kemp et al., 1996; Song and Bangerth, 2003). Indeed, it has been reported that the presence of straight-chain esters is largely dependent on an adequate supply of FA-derived precursors (Schwab et al., 2008). Storage conditions like temperature, CO_2 and O_2 are critical factors in determining the predominant esters (Forney et al., 2000; Larsen and Watkins, 1995), which are quantitatively the most important group of volatile compounds responsible for strawberry aroma. In this context, the dynamic changes to FAs and their correlation with volatile esters acquires special relevance to improve flavor and aroma, an important postharvest challenge. This feature is particularly relevant to Mara des Bois strawberry, an aromatic cultivar of *Fragaria vesca* that relies on the accumulation of the esters responsible for its intense aroma. Accordingly, this cultivar represents an excellent system to analyze the effect of low temperature storage and high CO_2 concentrations on the impact of FAs, and on their relationship with volatile esters. Moreover, considering that strawberries can be subjected to intermittent periods of unfavorable conditions during the commercial application of CO_2, much work remains to better understand the metabolic alterations associated to CO_2 stress.

Therefore, we set out to analyze whether different CO_2 treatments induce qualitative and quantitative changes to the FAs in the neutral, free and polar lipid fractions of Mara des Bois strawberries, and their impact on the emission of esters, energy status and membrane oxidative damage. In addition, we try to distinguish the level and type of FAs and the FA-derived esters that might be considered as markers of tolerance to high CO_2 and low temperature. This study provides new insights into the beneficial effect of 2 d 20 kPa CO_2 treatment on the enhancement of PUFAs in the polar lipid fraction and on the emission of esters other than ethyl acetate, while stressful CO_2 is associated with inducible FAs degradation.

2. Materials and methods

2.1. Plant material and treatments

Strawberries (*Fragaria vesca* L. cv. Mara des Bois) were grown at an organic orchard in San Sebastián de los Reyes (Madrid, Spain) according to the guidelines of the Regulatory Committee on Organic Production. Ripe, fully red strawberries from the second harvest, were collected and transported to the Institute of Food Science Technology and Nutrition (ICTAN-CSIC) within two hours of harvest. The ripe fruit, free of defects, was placed in plastic boxes (0.5 kg of fruit per box, approximately 45 per box), and 15 plastic boxes of strawberries were placed in a 1 m^3 treatment container at 0 °C (±0.5) and 95 % relative humidity (RH). Four different treatments were analyzed: 3 d 0.03 kPa CO_2 + 20 kPa O_2 in N_2 (T1); 2 d 20 kPa CO_2 + 20 kPa O_2 in N_2 (T2); 3 d 20 kPa CO_2 + 20 kPa O_2 in N_2 (T3); 3 d 40 kPa CO_2 + 20 kPa O_2 in N_2 (T4), applied at a constant flow rate of 100 mL min^{-1}. The four treatments were compared with strawberries harvested at a commercial stage (CH). The CO_2 concentration was

119 measured with a Check Mate 9900 O_2, O_2/CO_2 Headspace Analyzer (Dansensor
120 España, S.L.U.). At the end of the 2- or 3 d sampling period, 15 strawberries were
121 assessed for firmness, while another 45 were removed at random from each of the four
122 treatments and divided into three batches of 15 berries. The 15 strawberries from each
123 batch were used as biological replicates and each replicate was mixed, frozen in liquid
124 nitrogen and stored at −80 °C for further analysis.
125
126 *2.2 Determination of firmness*
127 Firmness was assessed by measuring the maximum shear force using a Kramer shear
128 cell of a TA.HD Plus Stable Microsystems Analyzer (Stable Microsystems Ltd; Surrey.
129 England), expressing the results in Newtons (N). For each assay, five intact strawberries
130 of a similar size (50 g in total) were placed in the Kramer shear cell and measurements
131 were made in triplicate.
132
133 *2.3 Determination of phosphorylated nucleotides*
134 ATP, ADP and AMP were quantified by HPLC performed as described previously
135 (Blanch et al., 2015). The adenylate energy charge was calculated according to Pradet
136 and Raymond (1983): ([ATP] + 0.5 [ADP])/([ATP] + [ADP] + [AMP]). Measurements
137 were made in triplicate, expressing the results as mg kg^{-1} fresh weight.
138 *2.4 Ethanol and acetaldehyde content*
139 Ethanol and acetaldehyde were quantified in the headspace of 5 mL of juice from three
140 replicates of 15 strawberries without calyx in 10 mL vials. Gas Chromatography was
141 performed as described previously (Blanch et al., 2015) and triplicate measurements
142 were taken, expressing the results as g 100 L^{-1} of juice.
143

144 *2.5 Fatty acid analysis*

145 Frozen berry samples (approximately 10 g) were homogenized in 18 mL of MeOH and
146 18 mL of $CHCl_3$, and the mixture was then vortexed for two min. Deionized water was
147 added 5 mL, vortexed for 2 min and cooled over 24 hours. The homogenate was then
148 centrifuged at 1684 *g* for 15 min at 4 ºC to separate the methanolic, aqueous and
149 chloroform phases. The total lipids were recovered in the chloroform phase and 15 mL
150 of this phase was concentrated in an evaporator under nitrogen gas at 40 ºC. A SPE
151 Bond Elut NH2 500 mg column was used for FA fractionating, eluting: neutral lipids
152 with 10 mL chloroform:isopropanol (2:1); free FAs with 10 mL diethyl ether containing
153 2 % acetic acid; and polar FAs, mainly phospholipids, with 10 mL methanol containing
154 2 % HCl. To each sample, 300 µg of glyceryl tritridecanoate (1,2,3-
155 Tritridecanoylglycerol), 50 µg of nonadecanoic acid and 700 µg of 1,2-
156 dipentadecanoyl-sn-glycero-3-phosphatidylcholine were added as internal standards
157 (purchased from Sigma, Germany and Cymit Química, Spain).
158 After evaporating their respective eluents, the samples were methylated for 15 min at 50
159 ºC with 2 mL (neutral and polar FAs) or 1 mL (free FAs) of 0.5 M sodium methoxide in
160 anhydrous methanol. Acetyl chloride in anhydrous methanol (1:10 v/v) 2 mL (to neutral
161 and polar FAs) or 1 mL (to free FAs) was added before mixing thoroughly and heating
162 for 1h at 60 ºC. Hexane 1.8 mL (neutral and polar FAs) and 0.8 mL (free FAs), distilled
163 water (1 mL) and anhydrous sodium sulfate (0.2 g) were added, mixing and then
164 centrifuging for 5 min at 277 *g*. An aliquot of the organic solvent (top layer) was
165 collected in an amber vial for subsequent gas chromatography.
166 Fatty acid methyl esters (FAMEs) were analyzed by gas chromatography on an Agilent
167 gas chromatography apparatus (7820A model and EZChrom Elite software), using a
168 flame ionization detector (FID) and a capillary fused silica column with a cyanopropyl-

169 methylpolysiloxane stationary phase (GC-12 CP-SIL88, 100m x 250 μm x 0.2 μm:
170 Agilent Technologies, Waldbronn, Germany).
171 The optimal conditions determined were a column temperature of 100 ºC that increased
172 to 220 ºC at a rate of 4 ºC min^{-1}, which was held for 5 min, increased to 240 at 4 ºC min^{-1}
173 and held for 10 min. The total analysis time was 50 min. The carrier gas was helium,
174 at a constant flow of 1 mL min^{-1}, and the samples were injected (1 μL) in the split mode
175 1:20. The detector temperature was set at 270 ºC and the injector oven temperature at
176 260 ºC. The calibration of all FAMEs was carried out relative to the internal standards:
177 Glyceryl tritridecanoate, nonadecanoic acid and 1,2-dipentadecanoyl-sn-glycero-3-
178 phosphatidylcholine. Individual FAMEs were identified by comparing their retention
179 times with those of mixed FAME standards (FAME 37 SUPELCO + PUFA Nº 2
180 Animal Source Sigma + PUFA Nº 3 Menhaden oil Sigma). The compounds analyzed
181 were expressed as g kg^{-1} of fresh weight or as a percentage. The data represent the
182 means of three biological replicates with two different technical measurements for each.
183
184 *2.6. Analysis of volatile esters in strawberry juice*
185 Strawberry juice (5 mL) from three replicates of 15 strawberries without their calyx was
186 obtained immediately after harvest or following each treatment. The juice was
187 transferred to 22 mL glass vials with rubber stoppers and aluminium caps and frozen at
188 -80 °C. Volatile esters were sampled using solid-phase microextraction (SPME) and
189 analyzed using an Agilent 6890N Series gas chromatograph equipped to an Agilent
190 5973 Series mass selective detector (Agilent Technologies, Germany) and coupled with
191 a TurboMatrix 40 Trap Headspace sampler (Perkin Elmer, USA). After thawing at room
192 temperature, 2-octanone was added to each vial as an internal standard and the sample
193 was maintained at 80 °C for 25 min into the headspace. Chromatographic separation of

194 volatile compounds was achieved on an HP-5MS capillary column, with a detector
195 operated in electron impact ionization mode at 70 eV and using full-scan acquisition
196 mode in the 30 to 550 m/z range. The identification of peaks in the chromatograms was
197 performed by injecting commercial standards, comparing the spectra with different
198 Mass Spectral Libraries (Wiley Registry 7th Edition and NIST, 2005, USA), and by
199 calculating the linear retention indices (LRI) using retention time data from a series of
200 alkane standards (C6 to C20) run under the same chromatographic conditions. The
201 normalized peak area of each compound was then calculated as the ratio of its peak area
202 to the area of the internal standard.
203
204 *2.7. Determination of Malondialdehyde (MDA)*
205 MDA formation was assayed using a modified thiobarbituric acid (TBA) method
206 (Ederli *et al.,* 1997), with some modifications. Strawberry samples (c.a. 0.5 g) were
207 homogenized in 1.5 mL 1 % cold trichloroacetic acid (TCA) and after centrifugation,
208 the supernatant was collected and 150 μL was mixed with 600 μL of 0.5 % TBA in 20
209 % TCA. The mixture was heated for 30 min and then cooled quickly before determining
210 its absorbance at 532 nm and adjusting for non-specific absorbance at 600 nm. Three
211 independent extractions of each sample were performed and extracts were analyzed in
212 duplicate. The MDA content was estimated using a molar extinction coefficient of 155
213 mmol L^{-1} cm^{-1}, and expressed as the mg kg^{-1} of the fresh weight of the sample. The
214 data represent the means of three biological replicates with two different technical
215 measurements for each.
216
217
218

2.8. Statistical analysis

The results were presented as the mean ± standard deviation (SD) and the statistical analysis was carried out with SPSS Software version 24.0. One-way analysis of variance (ANOVA) was performed and a multiple comparison of the means were performed with the Tukey's test at a significance level of 0.05. A Pearson's test was used to find the correlation between the FAs in the free, polar and neutral lipid fractions with straight-chain esters.

3. Results and discussion

3.1. Characterization of the FAs in the total lipids extracted from Mara des Bois strawberries

The composition and content of the individual FAs was assessed in the total lipids extracted from strawberries at commercial harvest (CH), and after storage at 0 ºC for 3 d with 0 kPa CO_2 (T1), 2 d 20 kPa CO_2 (T2), 3 d 20 kPa CO_2 (T3) and 3 d 40 kPa CO_2 (T4) (Table 1 and Table 2). When the content of total FAs was expressed on a percentage basis (Table 1), strawberries at CH had 11.2 % of saturated FAs (SFAs), predominantly palmitic acid (C16:0) (7.56 %), followed by stearic acid (C18:0) (2.15 %), arachidic acid (C20:0) (1.03 %). Mara des Bois strawberries also had very long FAs (VLFAs) like behenic acid (C20:0) (0.27 %), lignoceric acid (C24:0) (0.15 %) and lignoceric acid (C24:0) (0.33). VLFAs are involved in lipid molecules deposited at the primary plant surfaces that serve as a barrier to prevent excess water loss and pathogen attack (Beaudoin et al., 2009). Modified VLFAs are also present in suberin, an extracellular plant polyester that controls water and solute flux in plant tissues (Bakan and Marion, 2017). These VLFAs are formed by elongation of C16 and C18 fatty acids and they are thought to be physically associated in a complex referred to as the FA

elongase. The levels of monounsaturated FAs (MUFAs) in strawberries at CH reached 27.1 %, predominantly oleic acid (C18:1n9) (25.46 %), followed by *cis*-vaccenic acid (C18:1n7) (1.30 %) and paullinic acid (C20:1) (0.33 %). Paullinic acid is an abundant FA in the seeds of plants of the genus *Paullinia*. PUFAs constituted up to 61.7 % of the total FAs, included linoleic acid (C18:2n6) (38.36 %) and α-linolenic acid (C18:3n3) (23.38 %). Following postharvest treatment, the FAs in the total lipids did not change markedly with respect to the values of strawberries at CH.

When the content of FAs in the total lipids was expressed as g per kg fresh weight (FW) (Table 2), the predominant unsaturated FAs in Mara des Bois strawberries at CH, oleic (18:1), linoleic (18:2), and α-linolenic (18:3) acids reached values of 18.50, 27.89, and 16.99 g kg^{-1} fresh weight, respectively. Among the saturated FAs, palmitic (16:0) and stearic (18:0) acids reached values of 5.48 and 1.56 g kg^{-1} fresh weight, respectively. Following postharvest treatment, the total FAs in strawberries did not change significantly at T1 relative to CH. Interestingly, there was a significant increase in the amount of α-linolenic acid (18:3) in the strawberries at T2, reaching values of 20.97 g kg^{-1} fresh weight. The stearic acid (18:0) content also significantly increased, reaching values of 1.82 g kg^{-1} fresh weight, while there was only a mild increase in oleic (18:1) and linoleic acid (18:2) with respect to CH. By contrast, there was a significant decrease in α-linolenic (18:3), linoleic (18:2) and oleic (18:1) acids in strawberries at T4.

Table 1: Composition and content of FAs, expressed as percent of the FA from the total lipid, extracted from *Mara des Bois* strawberries at commercial harvest (CH), stored for 3 d in 0 kPa CO_2 (T1), 2 d in 20 kPa CO_2 (T2), 3 d in 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4).

Fatty acids		***CONTENT (%)***				
		CH	**T1**	**T2**	**T3**	**T4**
C16:0	Palmitic	7.56±0.3	7.65±0.1	7.67±0.4	7.64±0.6	7.91±0.6
C18:0	Stearic	2.15±0.0	2.19±0.0	2.16±0.0	2.29±0.1	2.34±0.1
C18:1n9	Oleic	25.46±0.1	25.19±0.1	24.09±0.4	24.16±0.3	23.68±0.1
C18:1n7	*cis*-Vaccenic	1.30±0.0	1.42±0.0	1.31±0.1	1.28±0.1	1.33±0.1
C18:2n6	Linoleic	38.36±0.4	38.03±0.2	37.97±0.7	38.28±0.6	38.29±0.3
C18:3n3	α-Linolenic	23.38±0.1	23.67±0.0	24.87±0.4	24.50±0.4	24.44±0.7
C20:0	Arachidic	1.03±0.0	1.02±0.0	1.09±0.1	1.11±0.1	1.11±0.0
C20:1	Paullinic	0.33±0.0	0.36±0.0	0.39±0.1	0.28±0.0	0.40±0.1
C22:0	Behenic	0.27±0.0	0.29±0.0	0.29±0.0	0.30±0.0	0.33±0.1
C24:0	Lignoceric	0.15±0.0	0.18±0.0	0.16±0.0	0.17±0.0	0.16±0.0

Each value represents the mean ± SD of the three biological replicates with two different technical measurements.

Note: m and n in "*Cm:n*" respectively represented carbon atom number and unsaturated bond number in fatty acid, for example, C18:1 representing there were 18 carbon atom numbers and 1 unsaturated bond number in oleic acid.

Table 2: Composition and content of FAs, expressed as g kg^{-1} fresh weight, in the total lipid fraction, extracted from *Mara des Bois* strawberries at commercial harvest (CH), stored for 3 d in 0 kPa CO_2 (T1), 2 d in 20 kPa CO_2 (T2), 3 d in 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4).

Fatty acids		***CONTENT(g kg^{-1} fresh weight)***				
		CH	**T1**	**T2**	**T3**	**T4**
C16:0	Palmitic	5.48±0.2ab	5.55±0.3ab	6.46±0.5b	5.60±1.0ab	4.20±0.4a
C18:0	Stearic	1.56±0.1ab	1.58±0.1bc	1.82±0.1c	1.66±0.1bc	1.24±0.0a
C18:1n9	Oleic	18.50±1.4b	18.28±1.2b	20.30±1.2b	17.65±2.2b	12.60±1.1a
C18:1n7	*cis*-Vaccenic	0.94±0.0ab	1.03±0.0b	1.09±0.1b	0.94±0.2ab	0.70±0.0a
C18:2n6	Linoleic	27.89±2.3b	27.58±1.5b	32.01±2.1b	27.91±2.8b	20.38±1.9a
C18:3n3	α-Linolenic	16.99±1.3b	17.17±1.0b	20.97±1.4c	17.86±1.8b	13.01±1.3a
C20:0	Arachidic	0.74±0.0ab	0.73±0.0ab	0.92±0.0c	0.80±0.0bc	0.59±0.0a
C20:1	Paullinic	0.23±0.0a	0.26±0.0a	0.32±0.1b	0.20±0.0a	0.21±0.0a
C22:0	Behenic	0.19±0.0a	0.21±0.0a	0.24±0.0a	0.21±0.0a	0.17±0.0a
C24:0	Lignoceric	0.11±0.0ab	0.13±0.0b	0.13±0.0b	0.12±0.0b	0.08±0.0a

Each value represents the mean ± SD of the three biological replicates with two different technical measurements. Different letters in rows indicate significant differences between the means determined by the Tukey's test ($P < 0.05$).
Note: m and n in "*Cm:n*" respectively represented carbon atom number and unsaturated bond number in fatty acid, for example, C18:1 representing there were 18 carbon atom numbers and 1 unsaturated bond number in oleic acid.

3.2. *Fatty acid composition and content in the different lipid fractions extracted from strawberries subjected to different CO_2 treatments*

Considering the overall changes in the unsaturated FAs following CO_2 treatments we wanted to identify the lipid fraction to which the aforementioned changes in FAs can be attributed to. As such, the FA composition and content (expressed as g per kg fresh weight) was determined in the neutral lipid fraction (triacylglycerols) (1A), free FAs (unesterified FAs) (1B) and polar (membrane lipids) (1C) of strawberries subjected to different CO_2 treatments (Figure 1A, B, C). The corresponding neutral (S1A) and free FAs (S1B) expressed on a percentage basis is indicated in Supplementary Fig. S1A, B.

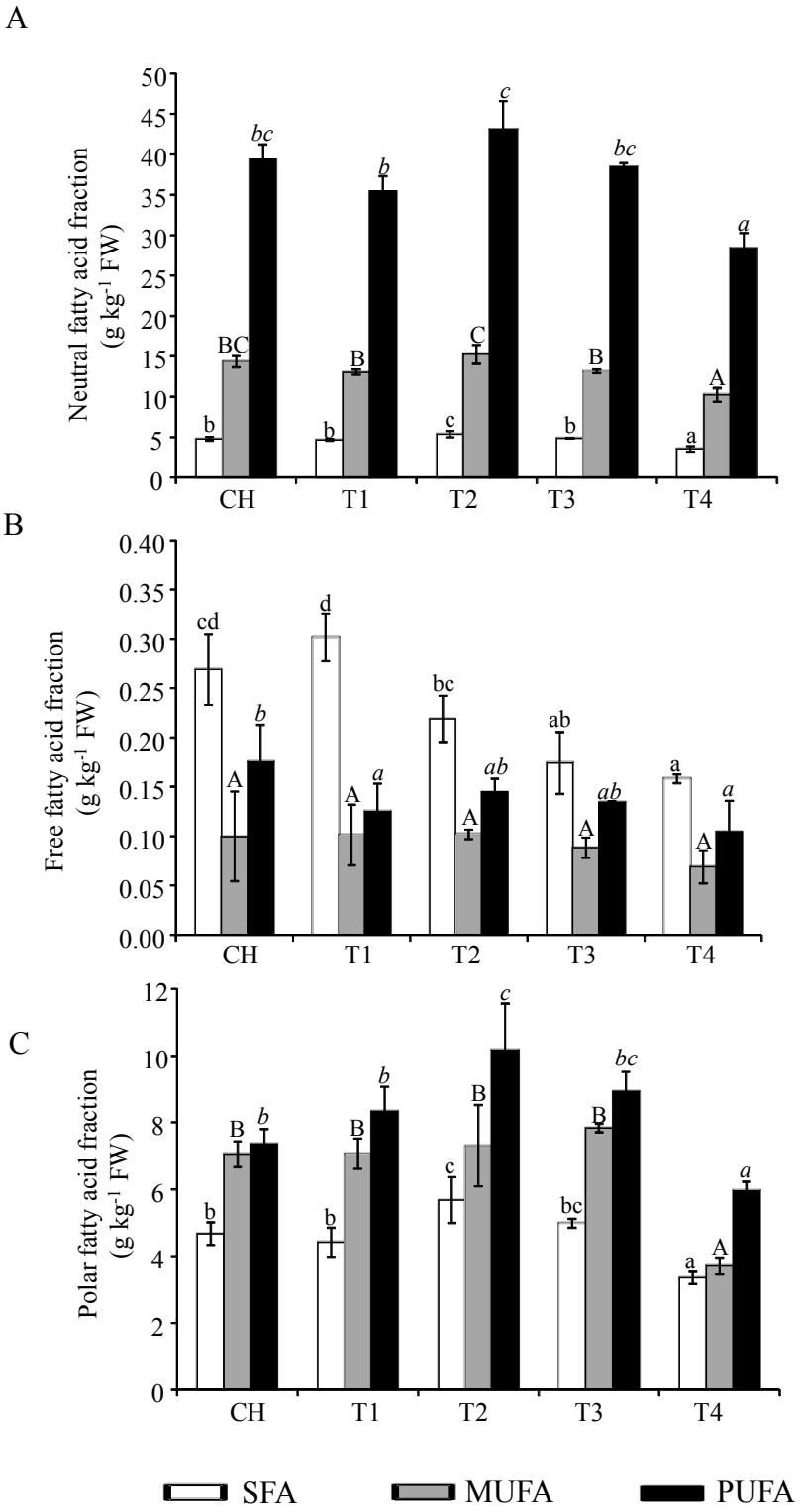
PUFAs are the dominant FAs esterified in the triacylglycerols, reaching values of 39.39 g kg^{-1} fresh weight, followed by MUFA with 14.33 g kg^{-1} fresh weight and SFA with only 4.48 g kg^{-1} fresh weight (Figure 1A). Following postharvest treatments, the PUFA content decreased in T1, mainly due to a lower α-linolenic acid content. By contrast, strawberries at T2 showed the highest SFA, MUFA and PUFA content, mainly α-linolenic acid that reached values of 17.2 g kg^{-1} fresh weight. Strawberries at T4 had the lowest levels of FAs in this fraction. When the content of FAs was expressed on a percentage basis (Supplementary Fig. S1A), the PUFA content decreased in T1. The decrease in PUFAs in T1 was concurrent with an increase in SFA. The percentage of FAs at T4 was virtually unchanged with respect to the strawberries at CH.

The levels of free FAs in Mara des Bois strawberries were 100 times lower than those of FAs in neutral lipid fraction (Figure 1B and Supplementary Figure S1B). SFAs are the dominant free FAs, reaching values of 0.26 g kg^{-1} fresh weight, followed by PUFA with 0.17 g kg^{-1} fresh weight and MUFA with 0.10 g kg^{-1} fresh weight. In CH fruit, the levels of PUFAs ranged between 0.07 g kg^{-1} fresh weight for α-linolenic acid and 0.16 g kg^{-1} fresh weight for oleic acid (Figure 1B). When free FAs were expressed on a percentage basis (supplementary SF1B), the largest proportion corresponded to SFAs, representing 49 % of the total FAs, of which 24 % was palmitic acid, 23 % stearic acid and 2 % arachidic acid. PUFAs account for a 32 % of the total free FAs, with 20 % linoleic acid, 12 % α-linolenic acid and 2 % paullinic acid. MUFAs account for 18 % of the total free FAs, while no cis-vaccenic acid was detected. Following postharvest treatment (Figure 1B), while the MUFA content (18:1) was maintained, the there was a significant decrease in the PUFA content of strawberries at T1, mainly in the α-linolenic acid values. Such a decrease in PUFAs was concurrent with a slightly increase in SFAs,

341 mainly in the stearic acid content. The largest amount of SFAs was quantified in fruit at

342 T1, greater than at CH and significantly more than in CO_2-treated fruit.

Figure 1



343

344 Figure 1: SFA, MUFA and PUFA content (g kg^{-1} fresh weight) in neutral lipids
345 (triacylglycerols) (A), free FAs (unesterified FAs) (B) and polar (membrane lipids) (C)
346 in strawberries at commercial harvest (CH), and after storage at 0 ºC for 3 d with 0 kPa
347 CO_2 (T1), 2 d 20 kPa CO_2 (T2), 3 d 20 kPa CO_2 (T3) and 3 d 40 kPa CO_2 (T4) The error
348 bars represent the standard deviation of the mean and each letter indicates the
349 significant differences between the means determined with a Tukey‘s test ($P < 0.05$).

Supplementary Fig S1

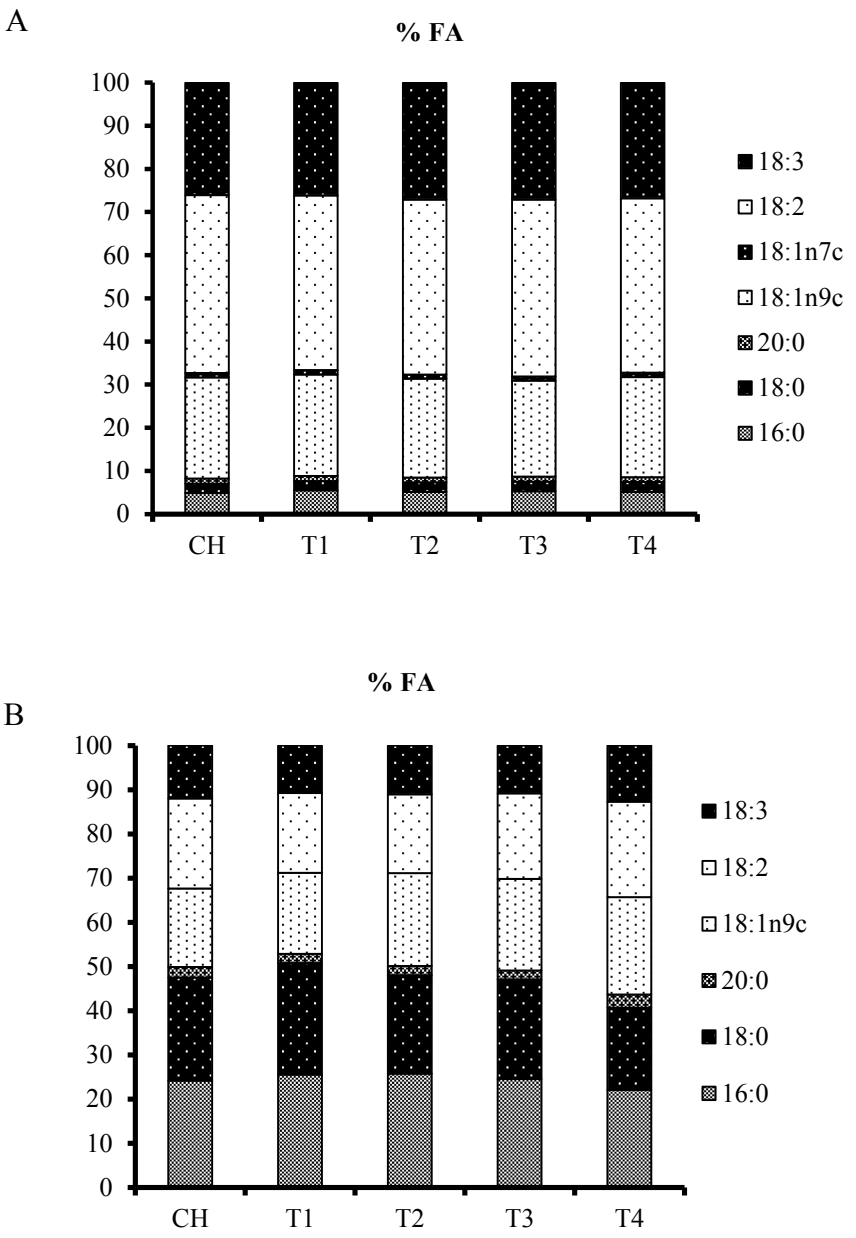


Fig S1: Composition and content (expressed on a percentage basis) of FAs esterified in triacylglycerols (A) and free FAs (B) in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the

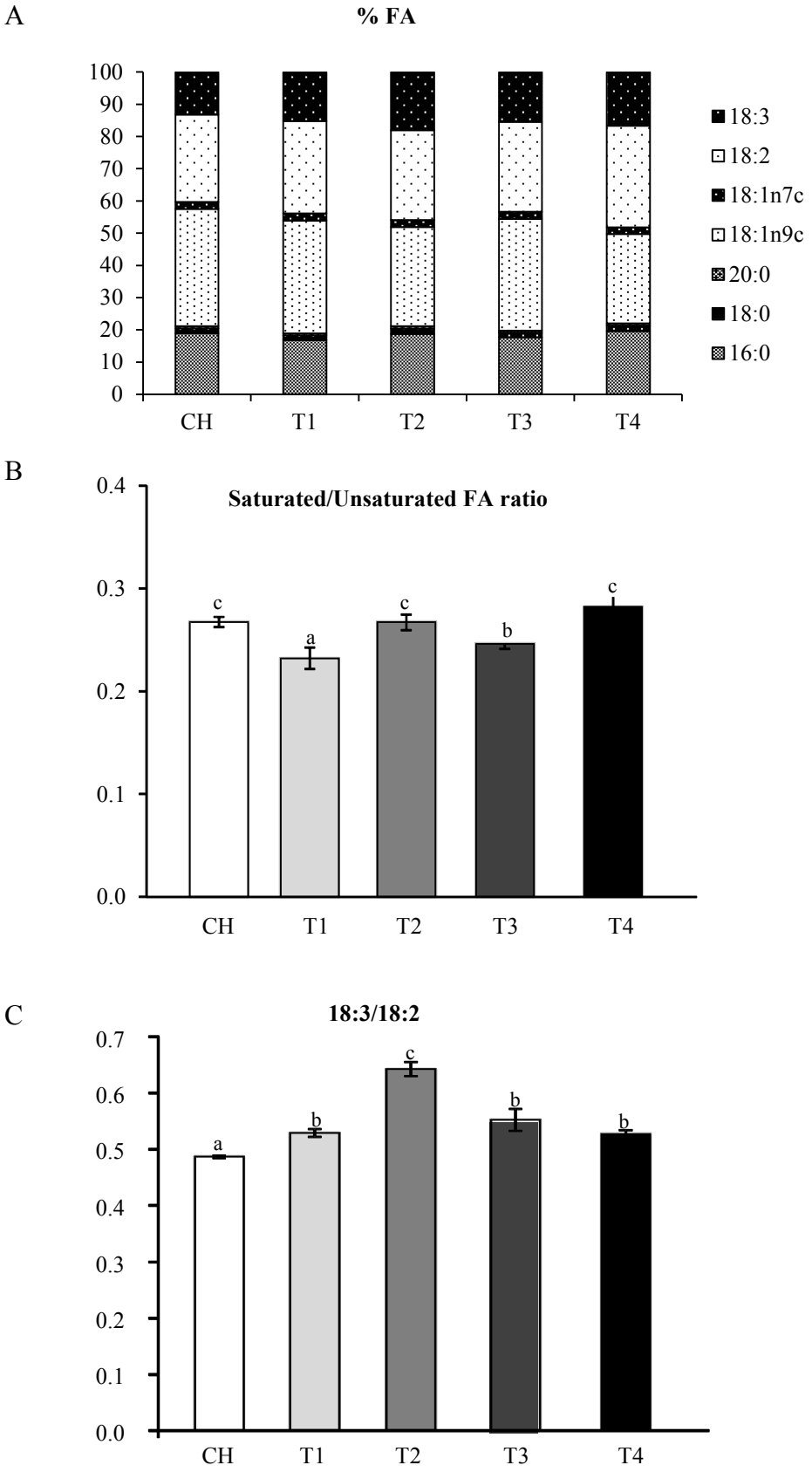
355 standard deviation of the mean and each letter indicates significant differences between
356 the means determined with a Tukey‘s test ($P < 0.05$).

357 The loss of unesterified linoleic and α-linolenic acids by the enzymes of the
358 lypoxygenase (LOX) pathway could be operating in strawberries at T1. By contrast, at
359 T2 only a slight decrease in the PUFAs and SFAs was quantified in the fruit. When the
360 dose or length of application of CO_2 treatment increased, the decrease in PUFAs and
361 SFAs became more pronounced. In strawberries at T4 there was a significant decrease
362 in the saturated and unsaturated free FA content, such that the proportion of SFAs,
363 MUFAs and PUFAs remained virtually unchanged when compared to strawberries at
364 CH (supplementary SF1B).

365 The FA composition and content determined in the polar lipid fraction (phospholipids
366 and glucolipids) of strawberries subjected to different CO_2-treatments is indicated in
367 Figure 1C and Figure 2. In this fraction, the proportion of MUFAs was more of less
368 similar to that of PUFAs with values of 7.05 g kg^{-1} fresh weight and 7.36 g kg^{-1} fresh
369 weight, respectively. The proportion of SFA was 21 % with values of 4.67 g kg^{-1} fresh
370 weight. With respect to MUFAs, there was up to 6.65 g kg^{-1} fresh weight of oleic acid,
371 the highest levels of the individual FAs, representing 36.4 % of total FAs in the polar
372 fraction in strawberries at CH. The *cis*-vaccenic acid detected in this fraction
373 represented 2.2 %. Following postharvest storage, the significant increase in the levels
374 of PUFAs in strawberries at T2 is noteworthy, mainly due to the increase in α-linolenic
375 acid that reached values of 4 g kg^{-1} fresh weight, 65 % higher than in strawberries at
376 CH. By contrast, a marked decrease in the PUFA and MUFA content was detected in
377 strawberries at T4, mainly in oleic acid that reached values as low as 3.7 g kg^{-1} FW. In
378 strawberries at T1, all SFAs had decreased, mainly palmitic acid (Figure 2A).
379 Consequently, the ratio of saturated:unsaturated FAs dropped significantly (Figure 2B).

Considering that a relatively stable saturated:unsaturated ratio can reflect the maintenance of the membrane, the decrease in this ratio observed in strawberries at T1 in the absence of CO_2 might denote altered membrane properties. However, such a decrease in saturated FAs in favor of unsaturated FAs in membrane lipids may also be a metabolic strategy of strawberries, classified as chilling tolerant, to overcome suboptimal low temperatures. As to function appropriately, membrane must be maintained in the fluid state, the decrease in the proportion of saturated FAs observed in strawberries at T1 will prevent the membrane from being too rigid at the temperature of 0 °C.

Figure 2


A
% FA
100
90
80
70
60
50
40
30
20
10
0
CH
T1
T2
T3
T4
18:3
18:2
18:1n7c
18:1n9c
20:0
18:0
16:0
B
Saturated/Unsaturated FA ratio
0.4
0.3
0.2
0.1
0.0
c
a
c
b
c
C
18:3/18:2
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
c
b
b

389

Figure 2: FA composition and content (expressed on a percentage basis) in the membrane lipids (A), the saturated:unsaturated ratio (B) and the α-linolenic acid (18:3)/linoleic acid (18:2) ratio (C) in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the standard deviation of the mean and each letter indicates the significant differences between the means determined with a Tukey's test ($P < 0.05$).

Such a decrease in the saturated:unsaturated ratio could be particularly significant for membrane-bound ATP-dependent enzymes. In previous work (Blanch et al., 2012b) reported modification in the monovalent ions K^+ and Na^+ leakage and in the K^+/Na^+ ratio in strawberries stored in air at 0 ºC without added CO_2. In the case of strawberries at T4, although the saturation:unsaturation rate was similar to that found at CH. In addition, the loss of unsaturated FAs could have important consequences on membrane functionality and integrity, and might explain the consequent water cellular leakage reported previously (Blanch et al., 2015a). In strawberries at T2, the enhanced SFA and PUFA level maintains the saturated:unsaturated FA ratio similar to that of fruit at CH (Figure 2B).

The above mentioned increase in the content of α-linolenic acid (18:3) in strawberries at T2 (Figure 1C), 65 % higher than in strawberries at CH, might explain the significant increase in 8:3/18:2 ratio (Figure 2C). The major increase in the ratio 18:3/18:2 in strawberries at T2 suggests an activation of estimated ω-3 desaturase. There is much interest in FA desaturation in response to several abiotic and biotic factors due to the activity of various FA desaturases (Dar et al., 2017; Domínguez et al., 2010; Wang et al., 2004). Lipid desaturases may regulate membrane fluidity by increasing the double bonds in the phospholipid tails. Treatments with 20 kPa CO_2 for 2 or 3 d (T2 and T3)

415 improves postharvest storage by increasing the double bonds in the phospholipid tails,
416 thereby ensuring that the membrane remains fluid at low temperature. Furthermore,
417 these treatments and especially 2 d exposure to 20 kPa CO_2 also improves fruit quality
418 by increasing (ω3) α-linolenic acid that is essential for human health as it cannot be
419 synthesized and have to be supplied through diet as an important ingredient of
420 docosahexaenoic acid and eicosapentaenoic acid, involved in the treatment of many
421 diseases including autoimmune diseases and inflammatory disorders.

422

423 *3.3. Esters from strawberries at commercial harvest and following CO_2 treatment*
424 *during storage at 0 ºC*

425 As esters play a major role in fruit flavor and aroma, and FAs can serve as ester
426 precursors, we analyzed the changes in unsaturated FAs in the light of both the type and
427 the level of volatile esters formed by strawberries after different high CO_2 treatments
428 (Table 3). An approach based on correlations has been shown to be useful to gain
429 insight into metabolic pathways and networks (Weckwerth et al., 2004). As such, we
430 subjected the straight-chain ester data to a Pearson correlation with oleic, linoleic and
431 linolenic acids from the polar, free and neutral lipid fractions.

432 There was a strong negative correlation (99.9 %) (Table 3) between unesterifed PUFAs
433 and butanoate and hexanoate derivatives like octyl butanoate, decyl butanoate, octyl
434 hexanoate and butyl butanoate, with the highest levels of these esters quantified in
435 strawberries at T1 (Figure 3). Other esters like hexyl acetate ($P<0.05$; $r = -0.54$), were
436 also negatively correlated with unesterified PUFAs. These esters were produced more
437 intensely in strawberries at T1 that showed an aforementioned significant decline in
438 PUFAs in the free fraction (see Figure 1C and Figure 3). The negative strong

439 correlations between the free PUFAs and volatiles butanoates and hexanoates esterified
440 to long alcohols suggests that free linoleic and α-linolenic would be implicated in the
441 production of volatile aldehydes derived from the activities of some enzymes in the
442 LOX pathway. The synthesis of aldehydes from free fatty acids by the action of
443 enzymes of the LOX pathway has been reported in disrupted tissues (Contreras and
444 Beaudry, 2013; Contreras et al., 2016). A broad range of aldehydes can be converted to
445 aroma volatile alcohols by alcohol dehydrogenase (ADH) activity (Prestage et al.,
446 1999). We previously reported (Blanch et a., 2015b) that *ADH* expression was 23-fold
447 greater in strawberries stored at 0 ºC in air without added CO_2 compared with fruit at
448 harvest and approximately 3-fold greater than that found in 3 d 20 kPa CO_2
449 strawberries. Enhancement of volatiles products derived from the peroxidation PUFAs
450 is typical in many stress responses (Loreto and Schnitzler, 2010) although the quantity
451 and composition of volatiles released can differs with the type of fruit and with the
452 intensity of stress. The high proportion of butanoate esters at T1 could confer specific
453 flavor and aroma associated with these particular aromatic compounds.

454

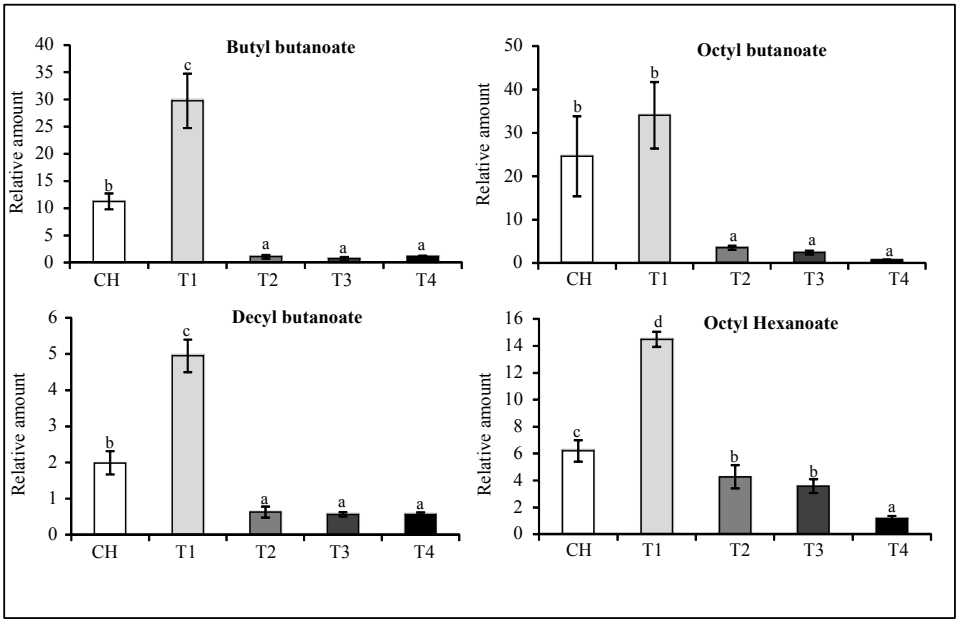
455 Table 3: Pearson correlation coefficients of oleic (18:1), linoleic (18:2) and α-linolenic (18:3) from free, polar and neutral fractions with FA-
456 with straight-chain esters in *Mara des Bois* strawberries at commercial harvest and after storage under different CO_2 treatments.

	Free Fatty Acid Fraction			***Polar Fatty Acid Fraction***			***Neutral Fatty Acid Fraction***		
	C18:1n9	C18:2n6	C18:3n3	C18:1n9	C18:2n6	C18:3n3	C18:1n9	C18:2n6	C18:3n3
Methyl acetate	0.64**	0.40	0.29	-0.76**	0.62*	0.61*	-0.46	-0.50	0.72**
Ethyl acetate	0.54*	0.42	0.38	-0.48	0.09	0.78**	-0.76**	-0.25	0.89**
Methyl butanoate	-0.50*	0.01	0.01	0.67**	-0.62*	-0.72**	0.37	0.61*	-0.59*
Ethyl butanoate	0.46	-0.06	-0.01	-0.58*	0.39	0.87**	-0.39	-0.49	0.57*
Ethyl (2E)-2-butenoate	0.40	0.07	0.04	-0.76**	0.71**	0.75**	-0.06	-0.55*	0.39
Methyl hexanoate	-0.42	-0.31	-0.23	0.27	-0.46	0.03	0.46	0.11	-0.42
Butyl butanoate	-0.45	-0.78**	-0.68**	0.48	-0.10	-0.42	0.57*	0.03	-0.70**
Ethyl hexanoate	0.38	0.19	0.14	-0.46	-0.01	0.88**	-0.34	-0.38	0.59*
Hexyl acetate	0.06	-0.54*	-0.53*	0.31	-0.28	0.19	-0.07	-0.21	0.02
Methyl octanoate	0.34	0.44	0.42	-0.49	-0.02	0.72**	-0.31	-0.19	0.56*
Hexyl butanoate	-0.45	-0.31	-0.25	0.61*	-0.44	-0.71**	0.61*	0.42	-0.82**
Ethyl octanoate	0.31	0.18	0.11	-0.25	-0.27	0.76**	-0.30	-0.28	0.53*
Octyl acetate	0.45	0.12	0.09	-0.14	-0.05	0.60*	-0.79**	-0.19	0.76**
Octyl butanoate	-0.34	-0.73**	-0.68**	0.59*	-0.26	-0.58*	0.69**	0.09	-0.83**
Ethyl decanoate	0.37	0.10	0.08	-0.27	-0.21	0.84**	-0.45	-0.30	0.60*
Decyl acetate	0.44	0.21	0.19	-0.05	-0.32	0.60*	-0.79**	-0.07	0.74**
Octyl Hexanoate	-0.35	-0.84**	-0.75**	0.56*	-0.27	-0.32	0.46	-0.02	-0.61*
Decyl butanoate	-0.31	-0.82**	-0.74**	0.47	-0.12	-0.40	0.61*	-0.11	-0.70**

457 The symbol * and ** shows significant differences according to the independent sample t-test ($P < 0.05$ and $P < 0.01$, respectively).

458

Figure 3


Butyl butanoate
Relative amount
40
35
30
25
20
15
10
5
0
b
c
a
a
a
CH
T1
T2
T3
T4
Octyl butanoate
Relative amount
50
40
30
20
10
0
b
b
a
a
a
CH
T1
T2
T3
T4
Decyl butanoate
Relative amount
6
5
4
3
2
1
0
b
c
a
a
a
CH
T1
T2
T3
T4
Octyl Hexanoate
Relative amount
16
14
12
10
8
6
4
2
0
c
d
b
b
a
CH
T1
T2
T3
T4

459

460 Figure 3: Relative amount of butyl butanoate, octyl butanoate, decyl butanoate and octyl
461 hexanoate in Mara des Bois strawberries at commercial harvest (CH), and after storage
462 for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3
463 d with 40 kPa CO_2 (T4). The error bars represent the standard deviation of the mean and
464 each letter indicates significant differences between the means determined with a
465 Tukey‘s test ($P < 0.05$).

466

467 Linoleic acid from the polar lipid fraction was strongly and positively correlated ($P <$
468 0.01 and r value > 0.7) with the presence of ethyl esters like ethyl hexanoate ($r = 0.88$),
469 ethyl octanoate ($r = 0.76$), ethyl decanoate ($r = 0.84$) and ethyl butanoate ($r = 0.87$:
470 Table 2). The levels of these ethyl esters increased significantly in strawberries after a 2
471 d 20 kPa CO_2 treatment (T2) (Figure 4). An aforementioned enhancement of α-linolenic
472 acid in the polar fraction was evident in strawberries at T2 (see Figure 1C and Figure 2).
473 Some of these esters have been identified as key constituents of fresh strawberry aroma
474 (Kim et al., 2013) and they have a typical strawberry-like odor (Larsen and Watkins,
475 1995; Schieberle and Hofmann, 1997;). Other esters that were closely correlated to the
476 α-linolenic acid in the polar fraction were methyl octanoate ($r = 0.72$) and ethyl (2E)-2-
477 butenoate ($r = 0.75$), both of which increased in strawberries after harvest. Ethyl (2E)-2-
478 butanoate is strongly correlated with linoleic (positive) and oleic acid (negative).
479 Indeed, 2-enoates are relatively rare constituents of aromas and they have generally only
480 been detected in tropical and subtropical fruits like *Annona muricata*.

481

482

483

Figure 4

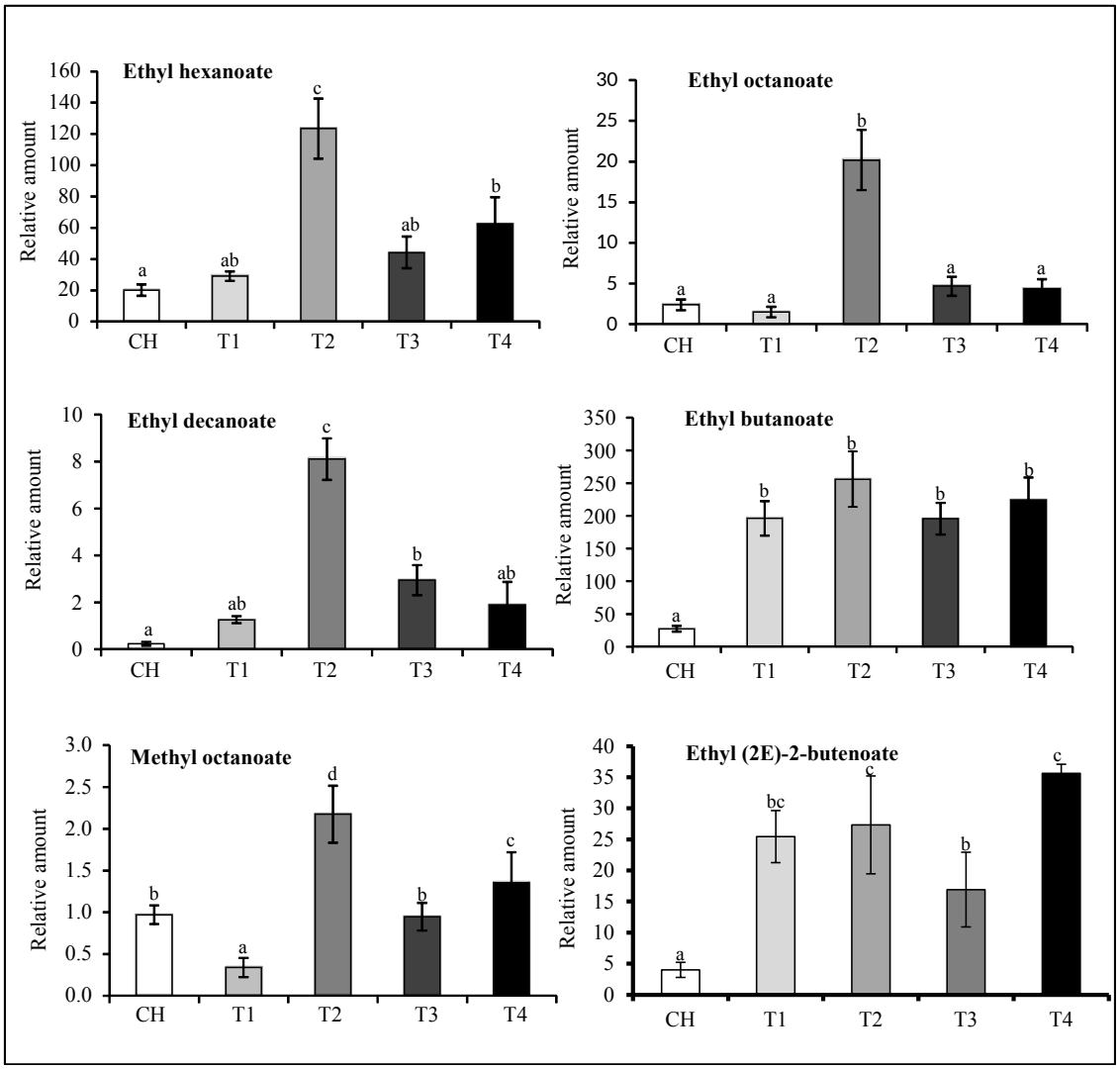
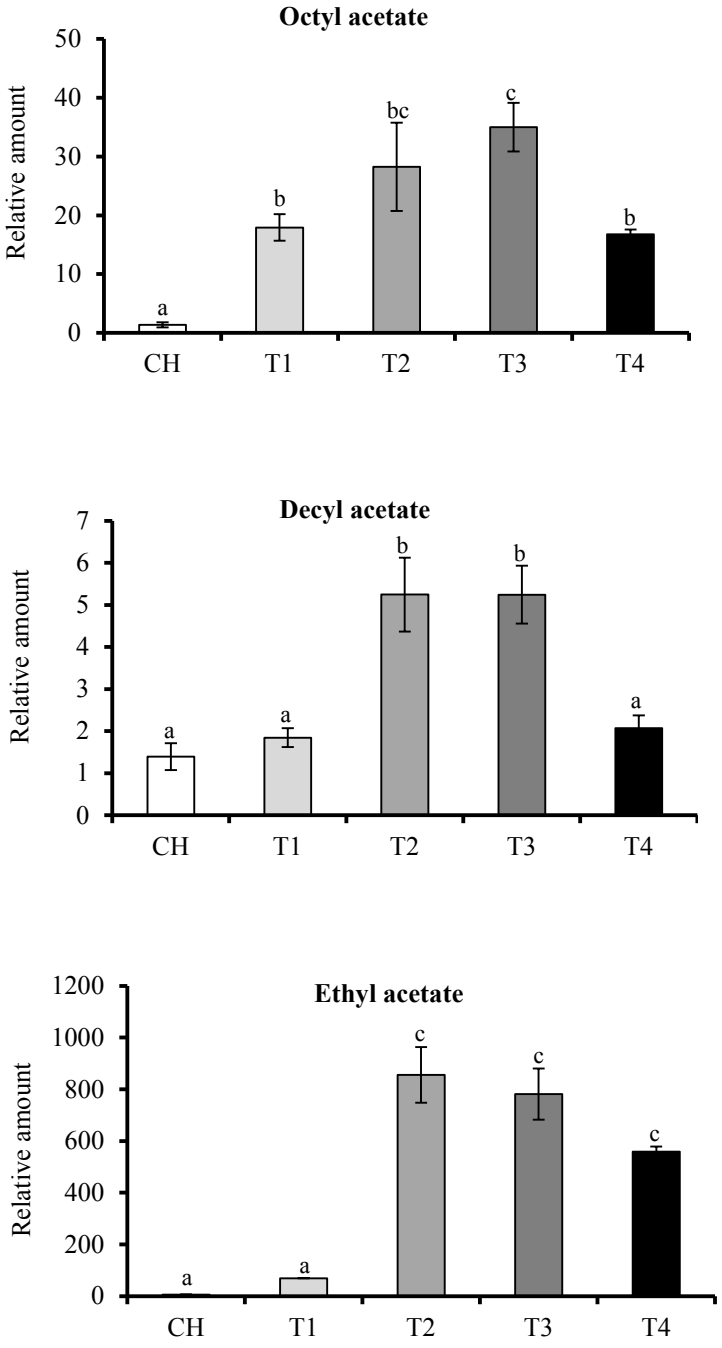


Figure 4: Relative amounts of ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl butanoate, methyl octanoate and ethyl (2E)-2-butenoate in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the standard deviation of the mean and each letter indicates significant differences between the means determined with a Tukey‘s test ($P < 0.05$).

493 With respect to the neutral fraction, there was a strong correlation ($P < 0.01$) of α-
494 linolenic acid (positive) and oleic acid (negative) with the volatile acetates, such as octyl
495 acetate (r=0.76), decyl acetate (r=0.74) and ethyl acetate (r=0.89). These esters
496 accumulated more strongly in CO_2 treated strawberries, with higher levels at T2 and T3
497 than at T4 (Figure 5). In strawberries at T4, an aforementioned decrease in α-linolenic
498 acid from the neutral fraction was reported (see Figure A and Supplementary Fig S1).
499 Considering the strong positive correlation between these esters and α-linolenic acid
500 from the neutral fraction, the levels of which were significantly higher in strawberries at
501 T2 relative to those at T4 and T1, the changes in acetate esters may be explained by the
502 ready availability of 18:3 substrate, which might be an important factor to improve the
503 production of these specific esters. In addition to substrate availability, changes in the
504 activity of alcohol acetyltransferases (AATs) contribute to ester biosynthesis (Defilippi,
505 et al., 2005; Pérez et al., 1996). AATs utilize acetyl-CoA to acetylate several alcohols
506 and in particular, octyl acetate is a genuine product of AATs (Aharoni et al., 2000). The
507 accumulation of octyl acetate has been reported in several cultivars of *Fragaria ves*ca
508 (Dong et al., 2013; Negri et al., 2015), and we found an increase in octyl acetate in
509 strawberries subjected to a 2 d 20 kPa CO_2 treatment. Some of these esters have a floral,
510 orange-rose odor and characteristic flavor, such as decyl acetate.

Figure 5



511

512 Figure 5: Relative amounts of octyl acetate, decyl acetate ethyl acetate and in Mara des
513 Bois strawberries at commercial harvest (CH), and after t storage for 3 d with 0 kPa
514 CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa
515 CO_2 (T4). The error bars represent the standard deviation of the mean and each letter

516 indicates significant differences between the means determined with a Tukey‘s test ($P < 0.05$).

517

518

519 Ethyl acetate is synthesized by esterification with ethanol, which is induced by low-
520 oxygen atmospheres and high levels of CO_2 (Ke et al., 1994; Larsen and Watkins, 1995;
521 Ueda and Bai, 1993). Our results suggest that ethanol produced in response to a 2 or 3 d
522 20 kPa CO_2 treatment is not sufficient to displace other alcohols in the esterification
523 reactions. These acetate esters are also negatively correlated with oleic acid from neutral
524 fraction.

525 Our data indicate that methyl acetate was correlated positively and negatively with
526 different FAs from the different fraction (Table 3). Unlike the other volatile acetates,
527 this ester accumulated most strongly in strawberries at T4 (Supplementary Fig.S2). A
528 remarkable increase in this ester may be a sign of senescence-associated membrane
529 deterioration and cell wall disorganization.

530

Supplementary Fig S2

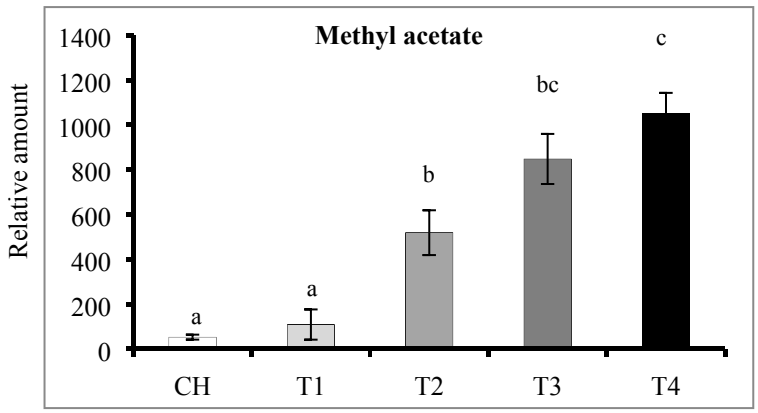
531

532

533

534

535



536 Fig S2: Relative amount of methyl acetate in Mara des Bois strawberries at commercial
537 harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2),

538 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the
539 standard deviation of the mean and each letter indicates significant differences between
540 the means determined with a Tukey‘s test ($P < 0.05$).

541

542 There are several esters that reached their highest levels in strawberries at CH (Figure
543 6). Both methyl butanoate and hexyl butanoate were negatively correlated ($P < 0.01$ and
544 r value > 0.70) with α-linolenic acid from the polar fraction (Table 3), while methyl
545 hexanoate was not correlated with any unsaturated FAs from the free, polar or neutral
546 fractions. Methyl hexanoate is one of the three major esters in Mara des Bois
547 strawberries at CH and it was maintained in strawberries at T2 before dropping
548 significantly in those at T3 and T4 when treatment with high CO_2 was prolonged or
549 increased. This ester is the second most abundant volatile ester in other strawberry
550 cultivars (Song et al., 1998). By maintaining the initial levels of this ester, or even
551 slightly increasing them in strawberries subjected to a 2 d 20 kPa CO_2 treatment, it is
552 possible that methyl hexanoate might serve as a biomarker to define the adequate
553 concentration external CO_2 to be applied. It has been reported that the amounts of
554 several methyl esters changes along with the storage temperature in apple (Forney et al.,
555 2000).

Figure 6

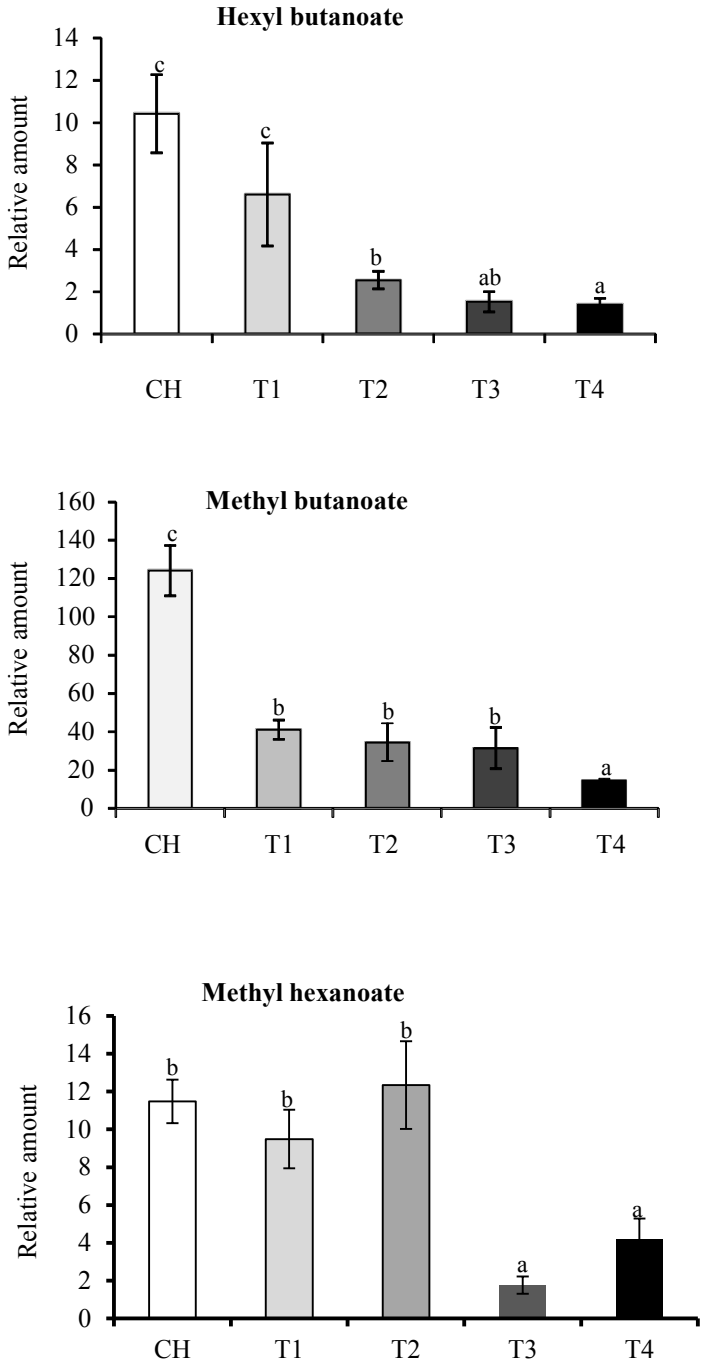


Figure 6: Relative amounts of hexyl butanoate, methyl butanoate and methyl hexanoate in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the standard deviation of the mean and each

letter indicates significant differences between the means determined with a Tukey‘s test ($P < 0.05$).

3.4. Lipid peroxidation

The presence of malondialdehyde (MDA), a secondary end product of the oxidation of PUFAs, is a useful marker of lipid peroxidation. The only significant increase in MDA is in strawberries at T4 (Figure 7), indicative of enhanced lipid peroxidation. Lipid peroxidation is one of the best studied consequences of the action of enhanced reactive oxygen species (ROS) on membranes. Stressful environmental conditions increase the production of ROS and the deleterious effects of lipid peroxidation in the damage associated with low temperature storage is well known (Marangoni et al., 1996).

Figure 7

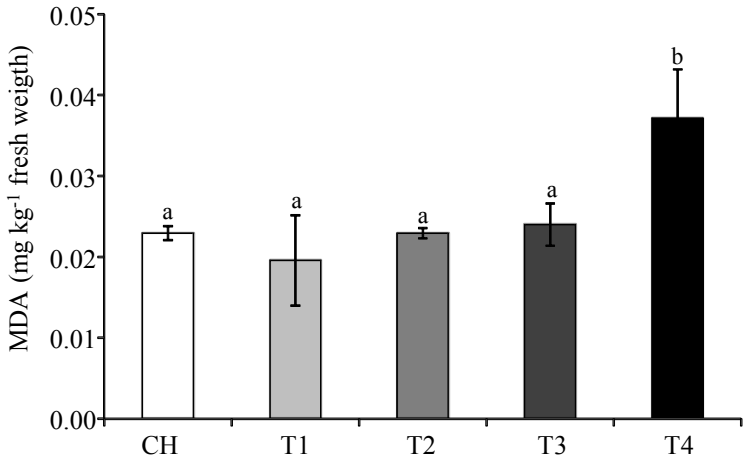


Figure 7: MDA content (mg kg^{-1} FW) in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the standard deviation of the mean and each letter indicates significant differences between the means determined with a Tukey‘s test ($P < 0.05$).

577 Our results indicate low levels of lipid peroxidation in strawberries after 3 d at 0 ºC,
578 whereas the lipid peroxidation associated with the stress of high CO_2 concentrations
579 may be responsible for the degradation of unsaturated fatty acids in the polar fraction
580 (see Figure 1C). The deleterious effects of lipid peroxidation can lead to a disruption of
581 membrane structure and function. The increase in the SFAs in the polar fraction of
582 strawberries at T1, not exposed to CO_2, could have consequences in terms of reduced
583 lipid peroxidation.

584 *3.5. Energy and metabolic status of strawberries at commercial harvest and following*
585 *different high CO_2 treatments during storage at 0 ºC*

586 We compared the firmness, energy and fermentative status of Mara des Bois
587 strawberries at CH and after storage at 0 ºC with or without CO_2 treatments T1, T2, T3
588 and T4 (Table 4). The ethanol:acetaldehyde ratio was markedly altered by low
589 temperature storage at atmospheric CO_2 concentrations and the ratio shifted from 1:0.6
590 at CH to 1:10 in strawberries at T1. Treatment with high concentrations of CO_2 strongly
591 influenced the levels of ethanol and the ethanol:acetaldehyde ratio decreased following
592 all three treatments (T2, T3 and T4). We previously indicated that ethanol did not rise in
593 strawberries stored at 0 ºC, although an increase in the abundance of transcripts for
594 pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) was detected. By
595 contrast, CO_2-treatment prevented the increase in *PDC* expression and the *ADH*
596 expression was less pronounced (Blanch et al., 2015b). Our results indicate that
597 strawberries activated fermentative metabolism under the three high CO_2 treatments
598 analyzed, confirmed by the accumulation of ethanol (Table 4) and ethanol production
599 was not influenced by oxygen availability.

600

601 Table 4: Firmness (N, expressed as relative accumulation), ATP and ADP (mg kg^{-1} FW), ADP/ATP
602 ratio, energy charge, ethanol:acetaldehyde ratio ratio in *Mara des Bois* strawberries at commercial
603 harvest (CH), stored for 3 d in 0 kPa CO_2 (T1), 2 d in 20 kPa CO_2 (T2), 3 d in 20 kPa CO_2 (T3) and 3 d
604 in 40 kPa CO_2 (T4).

	Firmness	*Energy status*				*Fermentation metabolism*
		ATP	ADP	ADP/ATP	Energy charge	Ethanol:acetaldehyde
CH	1	22.44±2.2c	1.02±0.1b	0.05b	0.82±0.0e	1:0.60
T1	1.14	22.65±2.2c	0.69±0.0a	0.03a	0.78±0.0d	1:10
T2	1.97	8.94±0.3ab	0.91±0.5ab	0.10c	0.62±0.0b	1:0.13
T3	2.12	11.84±1.7b	0.88±0.0ab	0.07d	0.68±0.0c	1:0.07
T4	1.84	7.06±0.7a	1.09±0.0b	0.15e	0.56±0.0a	1:0.14

605 Each value represents the mean ± SD of the three biological replicates with two different technical measurements.
606 Firmness results were expressed as the relative fold-change with respect to firmness value of strawberries at
607 commercial harvest. Different letters in columns indicate significant differences between the means determined
608 by the Tukey‘s test ($P < 0.05$).
609

610

611

612

As fermentation produces ATP inefficiently, the ATP values were markedly lower in CO_2-treated fruit than in strawberries without added CO_2, although the magnitude of energy production depends on the duration and concentration of CO_2 to which they were exposed. Among CO_2-treated strawberries, those at T4 had the lowest ATP and energy charge, which leads us to assume that the rate of anaerobic respiration seems to be insufficient to meet the energy requirements of FA biosynthesis. The involvement of adenylate nucleotides (Song and Bangerth, 2003) and oxygen in FA synthesis is well established. As the atmospheric O_2 concentration is maintained constant in all treatments, the sharp decrease in ATP in fruit at T4 may be responsible for the imbalance between lipid biosynthetic and degradative pathways. By contrast, ATP levels in fruit at T3 and T2 can be enough for fatty acid and lipid biosynthesis. Given the increase in FAs in strawberries especially at T2 (Figure 1 A, B, C and Table 1), we suggest that one of the biosynthetic routes favored by storage for 2 d in 20 kPa CO_2 is the synthesis of FAs.

The low levels of ATP in strawberries at T4, the lipid breakdown and the loss of PUFAs in membrane lipids can lead to altered membrane properties and may render fruit vulnerable to intracellular water leakage, as previously seen in LT-SEM studies (Blanch et al., 2015a). However, such a structural disassembly is not reflected in the texture of strawberries at T4. Textural data (Table 4) showed the efficacy of all CO_2 treatments in maintaining flesh firmness during storage at 0 ºC, as CO_2-treated strawberries were firmer than at CH regardless of the CO_2 concentrations used and in accordance with previous reports (Larsen and Watkins, 1995). Our data indicate that while exposure to low temperature without added CO_2 (T1) drives a mild increase in firmness, the application of high CO_2 concentrations significantly increases the flesh firmness. Since similar increase in firmness was obtained in strawberries at T2, T3 and T4, a treatment

638 as short as 2 d in 20 kPa CO_2 (T2) appears to be sufficient to significantly enhance flesh
639 firmness.

640 **Conclusions**

641 Results of this lipidomics study, carried out on Mara des Bois strawberries, have proved
642 evidence for the important role of FAs in mediating tolerance to CO_2 during storage at
643 low temperature.

644 Strawberries at T1, not exposed to added CO_2, had the lowest PUFA and MUFA values
645 in the neutral fraction, indicative of active FA breakdown. Another important change
646 caused by low temperature is an alteration to the polar lipid composition, with an
647 increase in the amount of SFAs and consequently a decrease in the saturated:unsaturated
648 ratio. Strawberries at T1 showed the highest levels in volatile butanoates and hexanoates
649 esterified to long alcohols (C_8-C_{10}), which are strongly and negatively correlated with
650 unesterified unsaturated FAs.

651 As a result of the active breakdown of FAs from triacylglycerols and the strong
652 production of ethanol, the highest levels of ethyl acetate accumulate in strawberries at
653 T4. The ethanol produced in strawberries at T4 was sufficient to displace other alcohols
654 in the esterification reactions. Moreover, the enhanced lipid peroxidation in response to
655 high CO_2 stress, as evident following T4 treatment, may be responsible for the
656 degradation of unsaturated FAs in the polar fraction, disrupting membrane integrity and
657 permeability.

658 Interestingly, the increase in unsaturated and saturated FAs of triacylglycerols and
659 membrane lipids indicates an active FA synthesis involving desaturases in strawberries
660 subjected to a 2 d 20 kPa CO_2 treatment. This represents the first report wherein the
661 application of 2 d 20 kPa CO_2 + 20 kPa O_2 induces adjustment of fruit metabolism that

662 results in linoleic acid and (ω3) α-linolenic acid accumulation that confer membrane
663 stability combined with health benefits and enhanced volatile ester production. This
664 application has also a positive effect on flesh firmness. Whether such beneficial effects
665 on quality parameters by this short high CO_2 treatment are maintained during shelf-life
666 period requires further analyses.

667

668 **Funding**

669 This work was financed by Spanish Project AGL2017-85291-R
670 (MINECO/AEI/FEDER, UE).

671

672 **Figure Legends**

673 Figure 1: SFA, MUFA and PUFA content (g kg^{-1} fresh weight) in neutral lipids
674 (triacylglycerols) (A), free FAs (unesterified FAs) (B) and polar (membrane lipids) (C)
675 in strawberries at commercial harvest (CH), and after storage at 0 ºC for 3 d with 0 kPa
676 CO_2 (T1), 2 d 20 kPa CO_2 (T2), 3 d 20 kPa CO_2 (T3) and 3 d 40 kPa CO_2 (T4) The error
677 bars represent the standard deviation of the mean and each letter indicates the
678 significant differences between the means determined with a Tukey‘s test ($P < 0.05$).

679 Figure 2: FA composition and content (expressed on a percentage basis) in the
680 membrane lipids (A), the saturated:unsaturated ratio (B) and the linolenic/linoleic ratio
681 (C) in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d
682 with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3 d with
683 40 kPa CO_2 (T4). The error bars represent the standard deviation of the mean and each
684 letter indicates the significant differences between the means determined with a Tukey‘s
685 test ($P < 0.05$).

Figure 3: Relative amounts of butyl butanoate, octyl butanoate, decyl butanoate and octyl hexanoate in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the standard deviation of the mean and each letter indicates significant differences between the means determined with a Tukey‘s test ($P < 0.05$).

Figure 4: Relative amounts of ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl butanoate, methyl octanoate and ethyl (2E)-2-butenoate in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the standard deviation of the mean and each letter indicates significant differences between the means determined with a Tukey‘s test ($P < 0.05$).

Figure 5: Relative amounts of octyl acetate, decyl acetate ethyl acetate and in Mara des Bois strawberries at commercial harvest (CH), and after t storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the standard deviation of the mean and each letter indicates significant differences between the means determined with a Tukey‘s test ($P < 0.05$).

Figure 6: Relative amounts of hexyl butanoate, methyl butanoate and methyl hexanoate in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2), 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the standard deviation of the mean and each letter indicates significant differences between the means determined with a Tukey‘s test ($P < 0.05$).

710 Figure 7: MDA content (mg kg^{-1} FW) in Mara des Bois strawberries at commercial
711 harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2),
712 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the
713 standard deviation of the mean and each letter indicates significant differences between
714 the means determined with a Tukey‘s test ($P < 0.05$).

715 **Supplementary data**

716 Fig S1: Composition and content (expressed on a percentage basis) of FAs esterified in
717 triacylglycerols (A) and free FAs (B) in Mara des Bois strawberries at commercial
718 harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2),
719 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the
720 standard deviation of the mean and each letter indicates significant differences between
721 the means determined with a Tukey‘s test ($P < 0.05$).

722 Fig S2: Relative amount of methyl acetate in Mara des Bois strawberries at commercial
723 harvest (CH), and after storage for 3 d with 0 kPa CO_2 (T1), 2 d with 20 kPa CO_2 (T2),
724 3 d with 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4). The error bars represent the
725 standard deviation of the mean and each letter indicates significant differences between
726 the means determined with a Tukey‘s test ($P < 0.05$).

727

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729

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731

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846

Figure 2

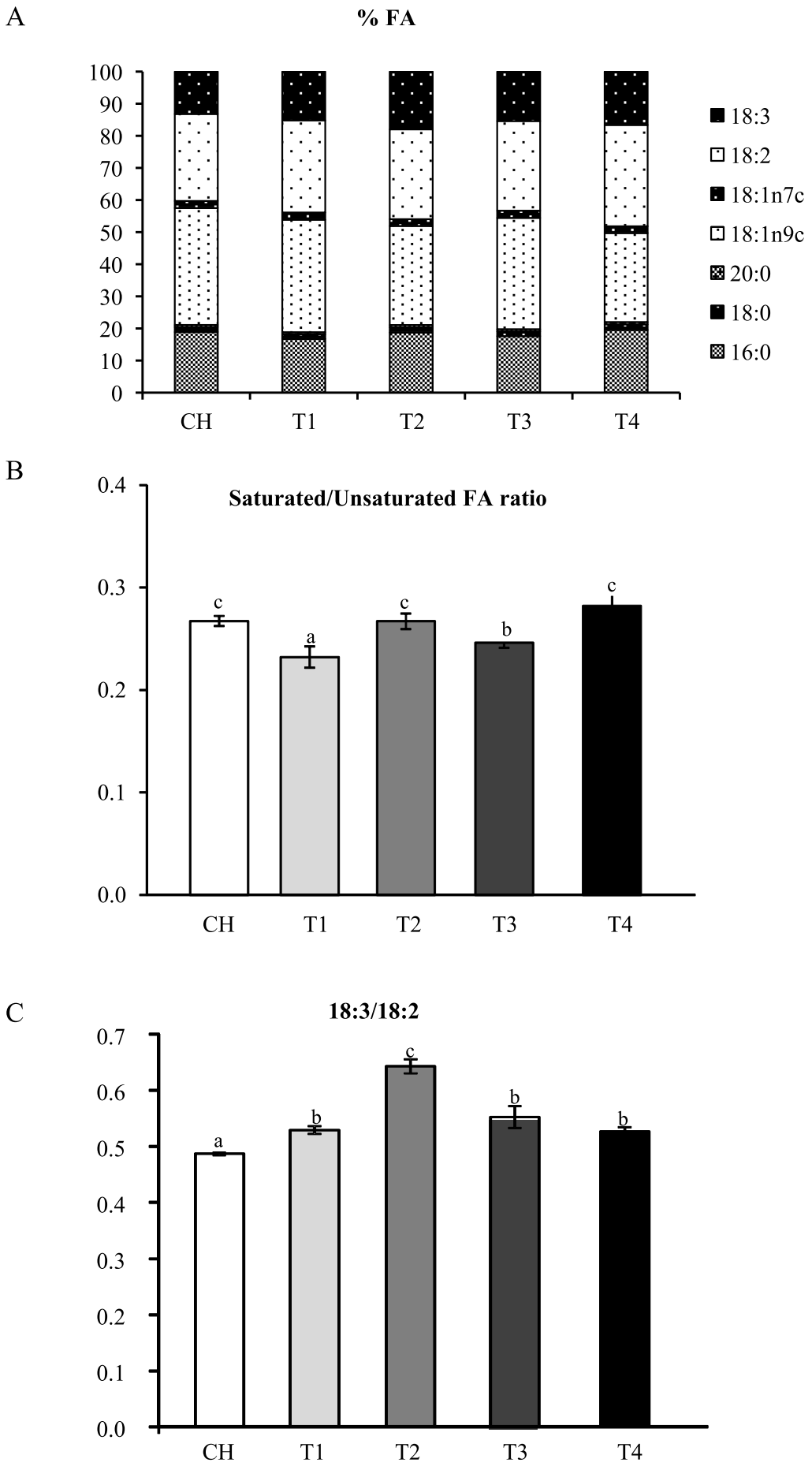

A
% FA
100
90
80
70
60
50
40
30
20
10
0
CH
T1
T2
T3
T4
18:3
18:2
18:1n7c
18:1n9c
20:0
18:0
16:0
B
Saturated/Unsaturated FA ratio
0.4
0.3
0.2
0.1
0.0
c
a
c
b
c
CH
T1
T2
T3
T4
C
18:3/18:2
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
c
b
b
CH
T1
T2
T3
T4

Figure 3

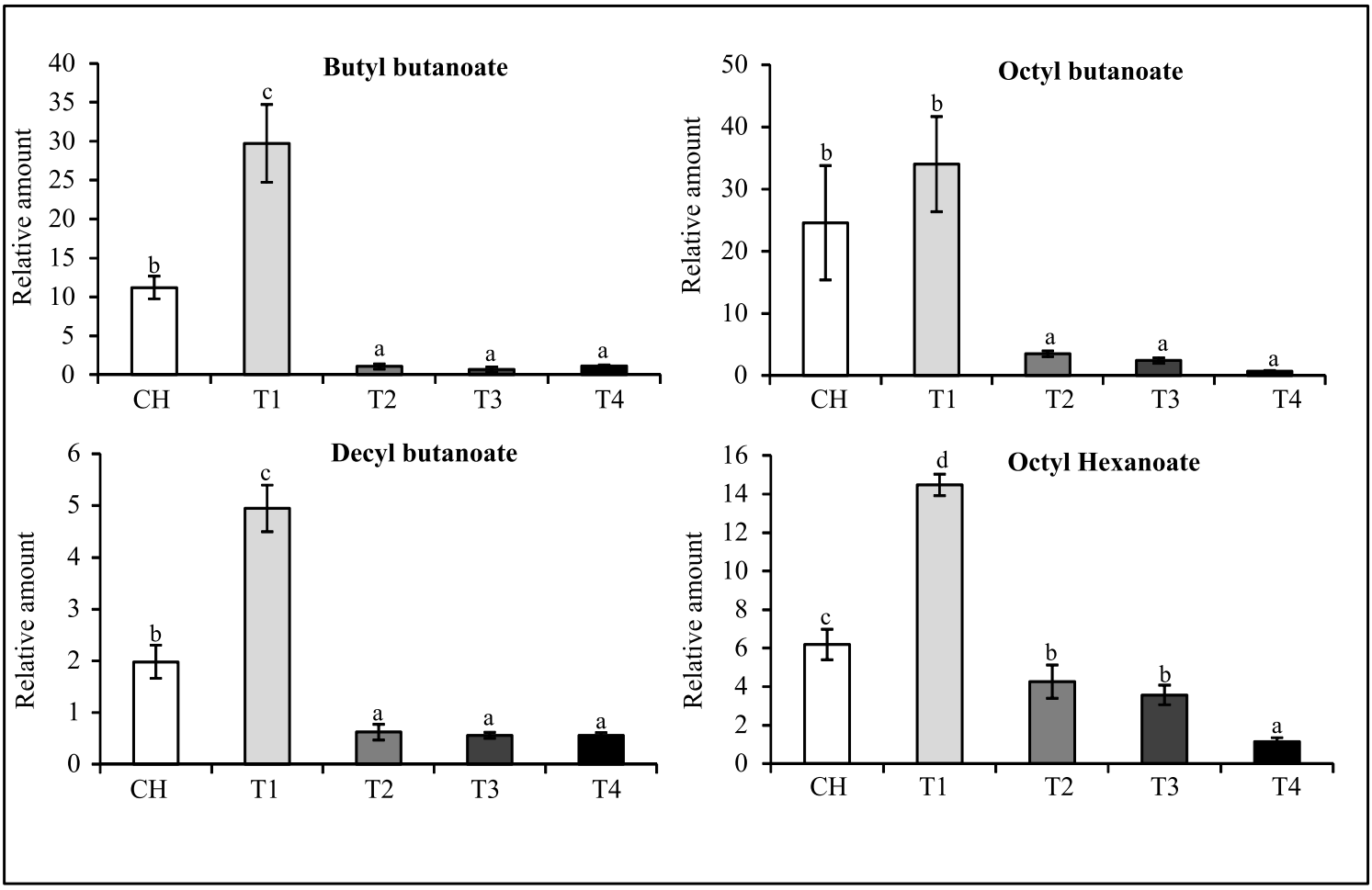

Butyl butanoate
Relative amount
40
35
30
25
20
15
10
5
0
b
c
a
a
a
CH
T1
T2
T3
T4
Octyl butanoate
Relative amount
50
40
30
20
10
0
b
b
a
a
a
CH
T1
T2
T3
T4
Decyl butanoate
Relative amount
6
5
4
3
2
1
0
b
c
a
a
a
CH
T1
T2
T3
T4
Octyl Hexanoate
Relative amount
16
14
12
10
8
6
4
2
0
c
d
b
b
a
CH
T1
T2
T3
T4

Figure 4

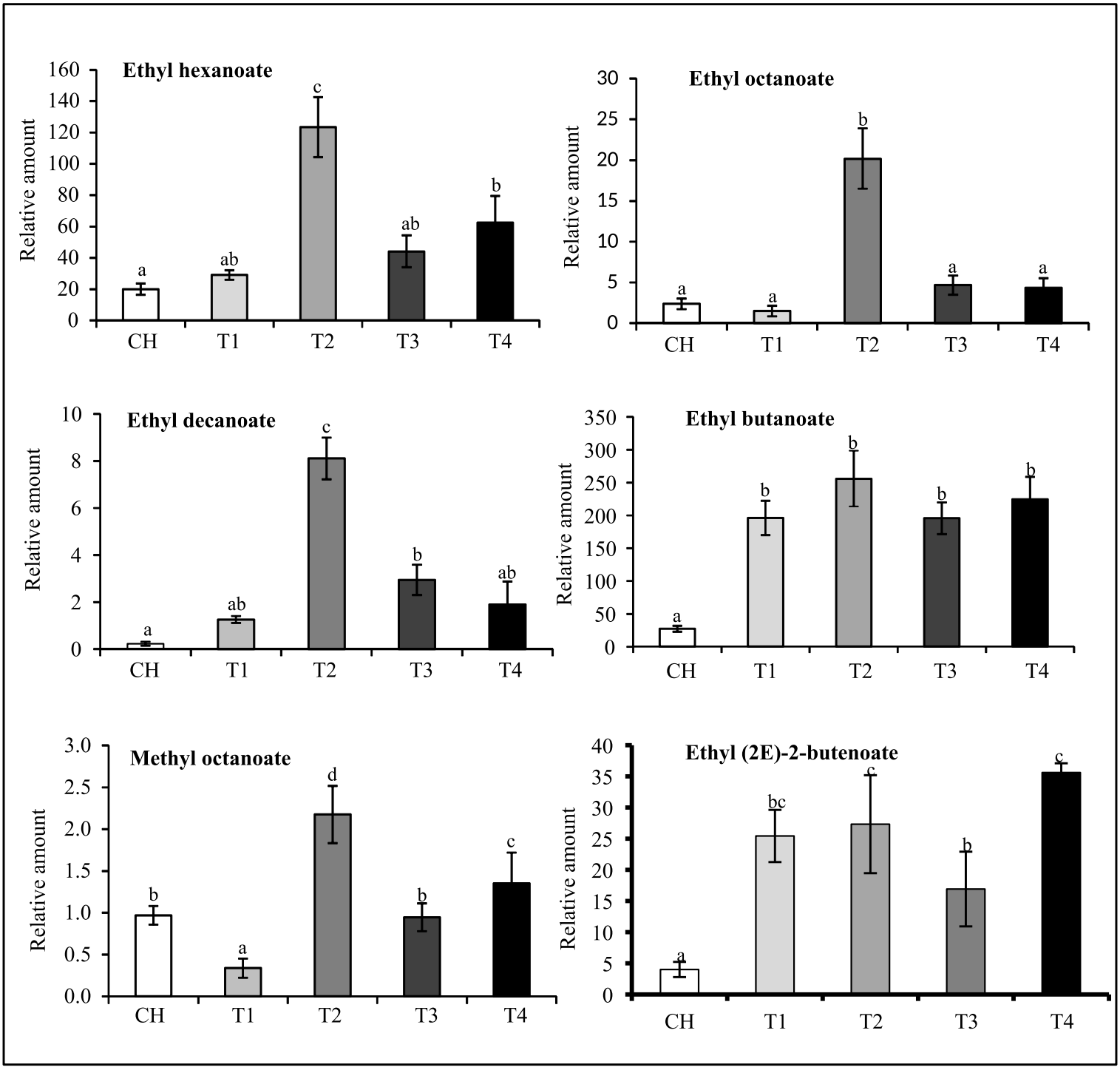

Ethyl hexanoate
Relative amount
160
140
120
100
80
60
40
20
0
a
ab
c
ab
b
CH
T1
T2
T3
T4
Ethyl octanoate
Relative amount
30
25
20
15
10
5
0
a
a
b
a
a
CH
T1
T2
T3
T4
Ethyl decanoate
Relative amount
10
8
6
4
2
0
a
ab
c
b
ab
CH
T1
T2
T3
T4
Ethyl butanoate
Relative amount
350
300
250
200
150
100
50
0
a
b
b
b
b
CH
T1
T2
T3
T4
Methyl octanoate
Relative amount
3.0
2.5
2.0
1.5
1.0
0.5
0.0
b
a
d
b
c
CH
T1
T2
T3
T4
Ethyl (2E)-2-butenoate
Relative amount
40
35
30
25
20
15
10
5
0
a
bc
c
b
c
CH
T1
T2
T3
T4

Figure 5

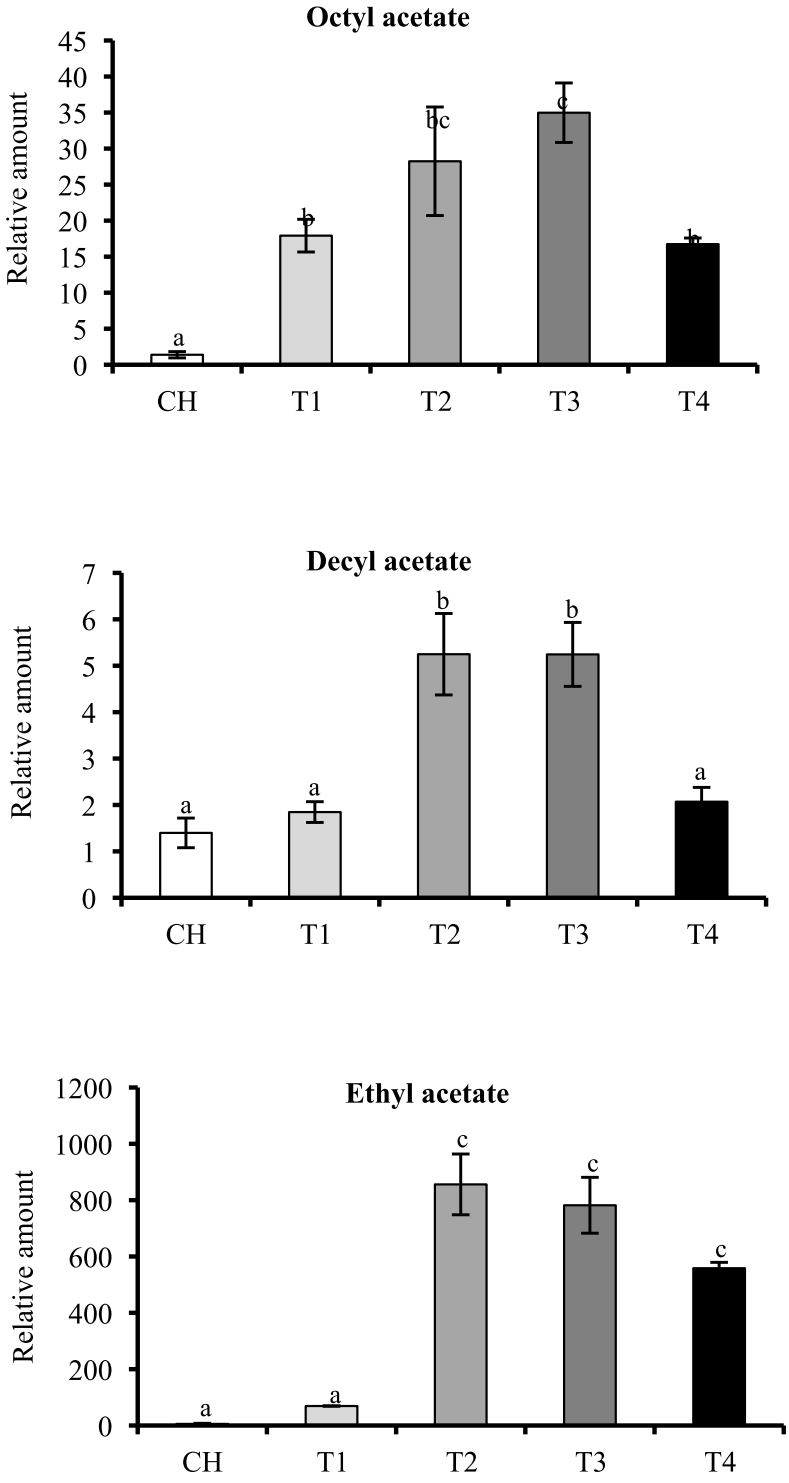
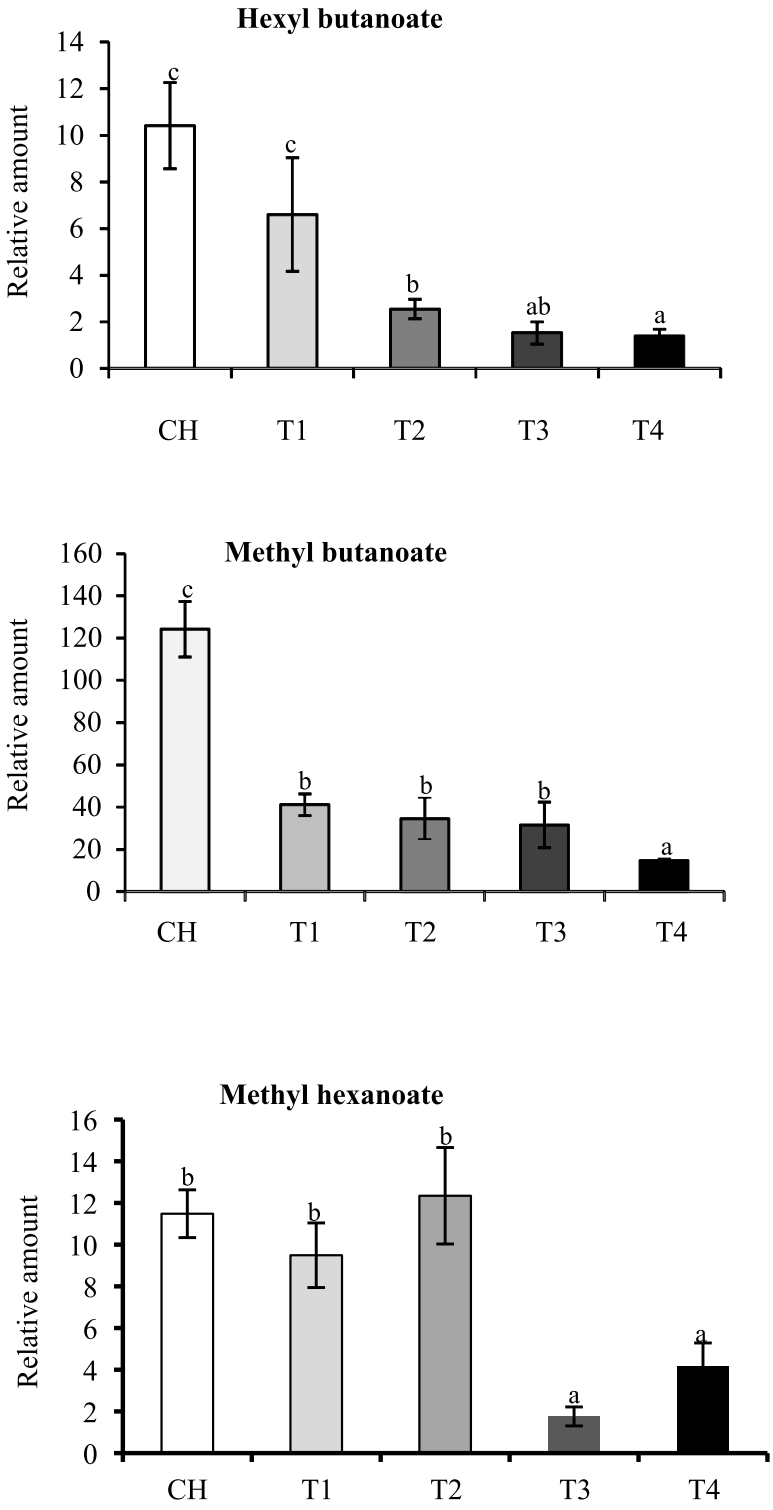
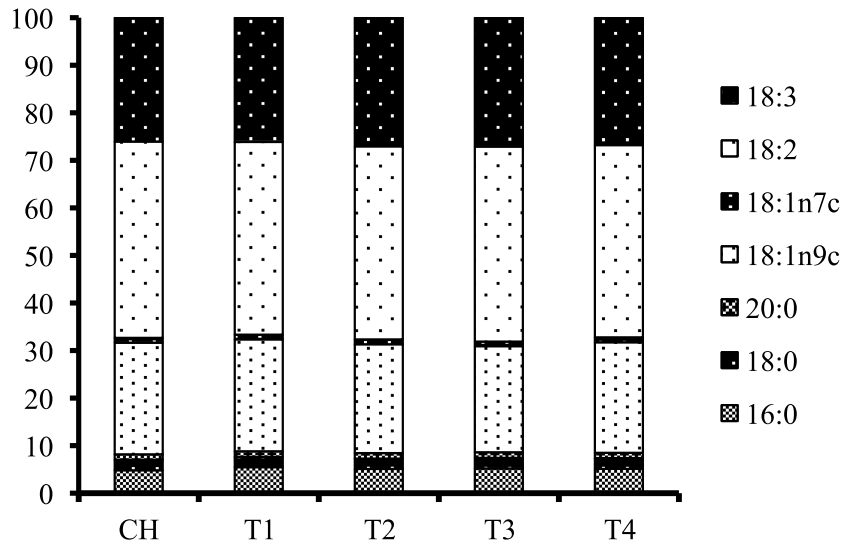

Octyl acetate
Relative amount
45
40
35
30
25
20
15
10
5
0
a
b
bc
c
b
CH
T1
T2
T3
T4
Decyl acetate
Relative amount
7
6
5
4
3
2
1
0
a
a
b
b
a
CH
T1
T2
T3
T4
Ethyl acetate
Relative amount
1200
1000
800
600
400
200
0
a
a
c
c
c
CH
T1
T2
T3
T4

Figure 6

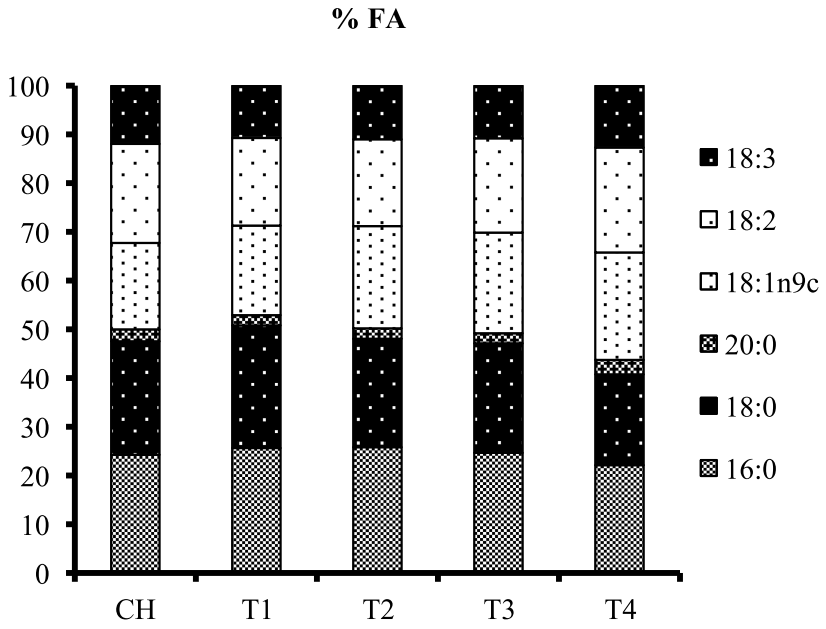

Hexyl butanoate
Relative amount
14
12
10
8
6
4
2
0
c
c
b
ab
a
CH
T1
T2
T3
T4
Methyl butanoate
Relative amount
160
140
120
100
80
60
40
20
0
c
b
b
b
a
CH
T1
T2
T3
T4
Methyl hexanoate
Relative amount
16
14
12
10
8
6
4
2
0
b
b
b
a
a
CH
T1
T2
T3
T4

Supplementary Fig S1

A



B



Supplementary Fig S2

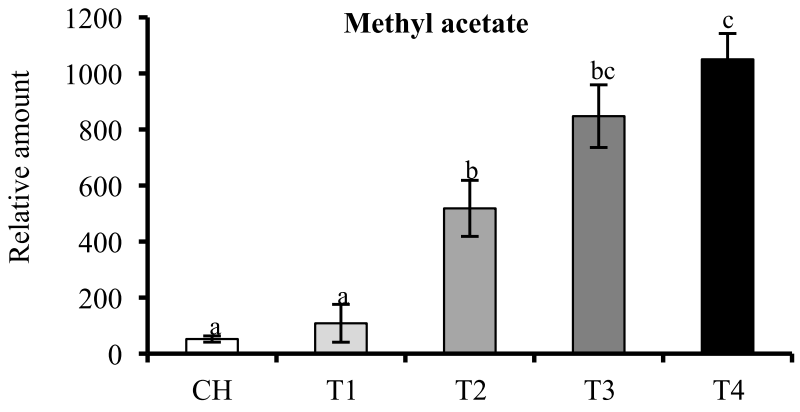


Figure 1

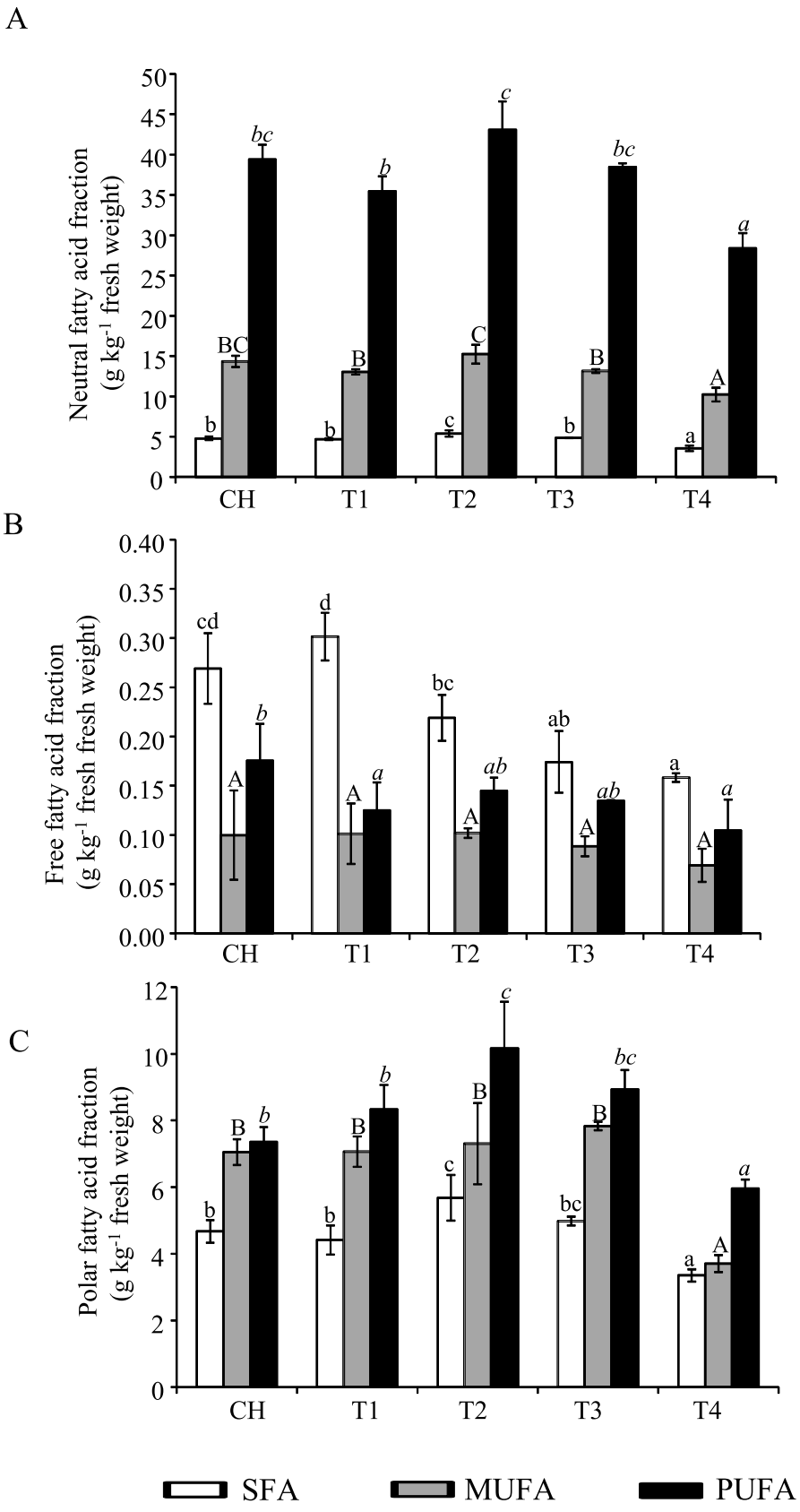

A
Neutral fatty acid fraction (g kg-1 fresh weight)
50
45
40
35
30
25
20
15
10
5
0
b
BC
bc
b
B
b
c
C
c
b
B
bc
a
A
a
CH
T1
T2
T3
T4
B
Free fatty acid fraction (g kg-1 fresh fresh weight)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
cd
A
b
d
A
a
bc
A
ab
ab
A
ab
a
A
a
CH
T1
T2
T3
T4
C
Polar fatty acid fraction (g kg-1 fresh weight)
12
10
8
6
4
2
0
b
B
b
b
B
b
c
B
c
bc
B
bc
a
A
a
CH
T1
T2
T3
T4
SFA
MUFA
PUFA

Figure 7

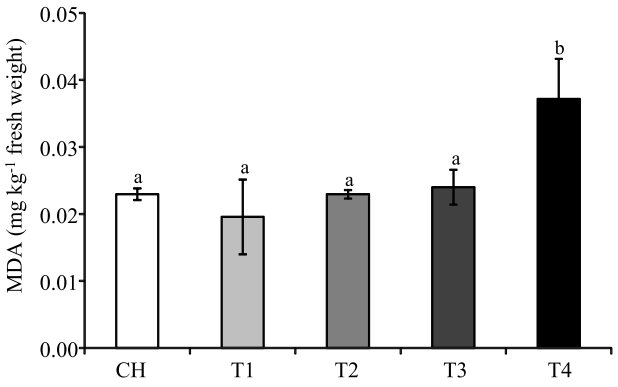

0.05
0.04
0.03
0.02
0.01
0.00
MDA (mg kg-1 fresh weight)
a
a
a
a
b
CH
T1
T2
T3
T4

Table 3: Pearson correlation coefficients of oleic, linoleic and α-linolenic from free, polar and neutral fractions with FA-esters straight chain volatiles in *Mara des Bois* strawberries at harvest and after storage under different CO_2 treatment.

	Free Fatty Acid Fraction			***Polar Fatty Acid Fraction***			***Neutral Fatty Acid Fraction***		
	C18:1n9	C18:2n6	C18:3n3	C18:1n9	C18:2n6	C18:3n3	C18:1n9	C18:2n6	C18:3n3
Methyl acetate	0.64**	0.40	0.29	-0.76**	0.62*	0.61*	-0.46	-0.50	0.72**
Ethyl acetate	0.54*	0.42	0.38	-0.48	0.09	0.78**	-0.76**	-0.25	0.89**
Methyl butanoate	-0.50*	0.01	0.01	0.67**	-0.62*	-0.72**	0.37	0.61*	-0.59*
Ethyl butanoate	0.46	-0.06	-0.01	-0.58*	0.39	0.87**	-0.39	-0.49	0.57*
Ethyl (2E)-2-butenoate	0.40	0.07	0.04	-0.76**	0.71**	0.75**	-0.06	-0.55*	0.39
Methyl hexanoate	-0.42	-0.31	-0.23	0.27	-0.46	0.03	0.46	0.11	-0.42
Butyl butanoate	-0.45	-0.78**	-0.68**	0.48	-0.10	-0.42	0.57*	0.03	-0.70**
Ethyl hexanoate	0.38	0.19	0.14	-0.46	-0.01	0.88**	-0.34	-0.38	0.59*
Hexyl acetate	0.06	-0.54*	-0.53*	0.31	-0.28	0.19	-0.07	-0.21	0.02
Methyl octanoate	0.34	0.44	0.42	-0.49	-0.02	0.72**	-0.31	-0.19	0.56*
Hexyl butanoate	-0.45	-0.31	-0.25	0.61*	-0.44	-0.71**	0.61*	0.42	-0.82**
Ethyl octanoate	0.31	0.18	0.11	-0.25	-0.27	0.76**	-0.30	-0.28	0.53*
Octyl acetate	0.45	0.12	0.09	-0.14	-0.05	0.60*	-0.79**	-0.19	0.76**
Octyl butanoate	-0.34	-0.73**	-0.68**	0.59*	-0.26	-0.58*	0.69**	0.09	-0.83**
Ethyl decanoate	0.37	0.10	0.08	-0.27	-0.21	0.84**	-0.45	-0.30	0.60*
Decyl acetate	0.44	0.21	0.19	-0.05	-0.32	0.60*	-0.79**	-0.07	0.74**
Octyl Hexanoate	-0.35	-0.84**	-0.75**	0.56*	-0.27	-0.32	0.46	-0.02	-0.61*
Decyl butanoate	-0.31	-0.82**	-0.74**	0.47	-0.12	-0.40	0.61*	-0.11	-0.70**

The symbol * and ** shows significant differences according to the independent sample t-test ($P < 0.05$ and $P < 0.01$, respectively).

Table 1: Composition and content of FAs, expressed as percent, from the total lipid extracted from *Mara des Bois* strawberries at commercial harvest (CH), stored for 3 d in 0 kPa CO_2 (T1), 2 d in 20 kPa CO_2 (T2), 3 d in 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4).

Fatty acids		***CONTENT (%)***				
		CH	**T1**	**T2**	**T3**	**T4**
C16:0	Palmitic	7.56±0.3	7.65±0.1	7.67±0.4	7.64±0.6	7.91±0.6
C18:0	Stearic	2.15±0.0	2.19±0.0	2.16±0.0	2.29±0.1	2.34±0.1
C18:1n9	Oleic	25.46±0.1	25.19±0.1	24.09±0.4	24.16±0.3	23.68±0.1
C18:1n7	*cis*-Vaccenic	1.30±0.0	1.42±0.0	1.31±0.1	1.28±0.1	1.33±0.1
C18:2n6	Linoleic	38.36±0.4	38.03±0.2	37.97±0.7	38.28±0.6	38.29±0.3
C18:3n3	α-Linolenic	23.38±0.1	23.67±0.0	24.87±0.4	24.50±0.4	24.44±0.7
C20:0	Arachidic	1.03±0.0	1.02±0.0	1.09±0.1	1.11±0.1	1.11±0.0
C20:1	Paullinic	0.33±0.0	0.36±0.0	0.39±0.1	0.28±0.0	0.40±0.1
C22:0	Behenic	0.27±0.0	0.29±0.0	0.29±0.0	0.30±0.0	0.33±0.1
C24:0	Lignoceric	0.15±0.0	0.18±0.0	0.16±0.0	0.17±0.0	0.16±0.0

Each value represents the mean ± SD of the three biological replicates with two different technical measurements.

Note: m and n in "*Cm:n*" respectively represented carbon atom number and unsaturated bond number in fatty acid, for example, C18:1 representing there were 18 carbon atom numbers and 1 unsaturated bond number in oleic acid.

Table 2: Composition and content of FAs, expressed as mg g^{-1} FW, in the total lipid fraction, extracted from *Mara des Bois* strawberries at commercial harvest (CH), stored for 3 d in 0 kPa CO_2 (T1), 2 d in 20 kPa CO_2 (T2), 3 d in 20 kPa CO_2 (T3) and 3 d with 40 kPa CO_2 (T4).

Fatty acids		***CONTENT(g kg^{-1} fresh weight)***				
		CH	**T1**	**T2**	**T3**	**T4**
C16:0	Palmitic	5.48±0.2ab	5.55±0.3ab	6.46±0.5b	5.60±1.0ab	4.20±0.4a
C18:0	Stearic	1.56±0.1ab	1.58±0.1bc	1.82±0.1c	1.66±0.1bc	1.24±0.0a
C18:1n9	Oleic	18.50±1.4b	18.28±1.2b	20.30±1.2b	17.65±2.2b	12.60±1.1a
C18:1n7	*cis*-Vaccenic	0.94±0.0ab	1.03±0.0b	1.09±0.1b	0.94±0.2ab	0.70±0.0a
C18:2n6	Linoleic	27.89±2.3b	27.58±1.5b	32.01±2.1b	27.91±2.8b	20.38±1.9a
C18:3n3	α-Linolenic	16.99±1.3b	17.17±1.0b	20.97±1.4c	17.86±1.8b	13.01±1.3a
C20:0	Arachidic	0.74±0.0ab	0.73±0.0ab	0.92±0.0c	0.80±0.0bc	0.59±0.0a
C20:1	Paullinic	0.23±0.0a	0.26±0.0a	0.32±0.1b	0.20±0.0a	0.21±0.0a
C22:0	Behenic	0.19±0.0a	0.21±0.0a	0.24±0.0a	0.21±0.0a	0.17±0.0a
C24:0	Lignoceric	0.11±0.0ab	0.13±0.0b	0.13±0.0b	0.12±0.0b	0.08±0.0a

Each value represents the mean ± SD of the three biological replicates with two different technical measurements. Different letters in rows indicate significant differences between the means determined by the Tukey's test ($P < 0.05$).

Note: m and n in "*Cm:n*" respectively represented carbon atom number and unsaturated bond number in fatty acid, for example, C18:1 representing there were 18 carbon atom numbers and 1 unsaturated bond number in oleic acid.

Table 4: Firmness (N, expressed as relative accumulation), ATP and ADP (mg kg^{-1} FW), ADP/ATP ratio, energy charge, ethanol:acetaldehyde ratio ratio in *Mara des Bois* strawberries at commercial harvest (CH), stored for 3 d in 0 kPa CO_2 (T1), 2 d in 20 kPa CO_2 (T2), 3 d in 20 kPa CO_2 (T3) and 3 d in 40 kPa CO_2 (T4).

	Firmness	*Energy status*				*Fermentation metabolism*
		ATP	ADP	ADP/ATP	Energy charge	Ethanol:acetaldehyde
CH	1	22.44±2.2c	1.02±0.1b	0.05b	0.82±0.0e	1:0.60
T1	1.14	22.65±2.2c	0.69±0.0a	0.03a	0.78±0.0d	1:10
T2	1.97	8.94±0.3ab	0.91±0.5ab	0.10c	0.62±0.0b	1:0.13
T3	2.12	11.84±1.7b	0.88±0.0ab	0.07d	0.68±0.0c	1:0.07
T4	1.84	7.06±0.7a	1.09±0.0b	0.15e	0.56±0.0a	1:0.14

Each value represents the mean ± SD of the three biological replicates with two different technical measurements. Firmness results were expressed as the relative fold-change with respect to firmness value of strawberries at commercial harvest. Different letters in columns indicate significant differences between the means determined by the Tukey‘s test ($P < 0.05$).