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 CO₂ tolerance remains to be determined in strawberries during storage at low temperature. Total, free and esterified FAs in neutral (triacylglycerols) and polar (membrane lipids) were characterized and quantified, before their relationship with straight-chain esters was examined in CO₂-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 CO₂ (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 CO₂ 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-CO₂ fruit. By contrast, the application of a 2 d 20 kPaCO₂ 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 °C. 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 CO₂ 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, CO₂, esters, MDA, strawberries, energy supply.

1. Introduction

Short CO₂ 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 CO₂-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 CO₂ 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 CO₂ used and the length of exposure.
Indeed, when excess CO₂ 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 CO₂ 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, CO₂ and O₂ 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 CO₂ 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 CO₂, much work remains to better understand the metabolic alterations associated to CO₂ stress.
Therefore, we set out to analyze whether different CO₂ 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 CO₂ and low temperature. This study provides new insights into the beneficial effect of 2 d 20 kPa CO₂ treatment on the enhancement of PUFAs in the polar lipid fraction and on the emission of esters other than ethyl acetate, while stressful CO₂ 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 m³ treatment container at 0 °C (±0.5) and 95 % relative humidity (RH). Four different treatments were analyzed: 3 d 0.03 kPa CO₂ + 20 kPa O₂ in N₂ (T1); 2 d 20 kPa CO₂ + 20 kPa O₂ in N₂ (T2); 3 d 20 kPa CO₂ + 20 kPa O₂ in N₂ (T3); 3 d 40 kPa CO₂ + 20 kPa O₂ in N₂ (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 CO₂ concentration was
measured with a Check Mate 9900 O₂, O₂/CO₂ Headspace Analyzer (Dansensor España, S.L.U.). At the end of the 2- or 3-d sampling period, 15 strawberries were assessed for firmness, while another 45 were removed at random from each of the four treatments and divided into three batches of 15 berries. The 15 strawberries from each batch were used as biological replicates and each replicate was mixed, frozen in liquid nitrogen and stored at ~80 °C for further analysis.

2.2 Determination of firmness

Firmness was assessed by measuring the maximum shear force using a Kramer shear cell of a TA. HD Plus Stable Microsystems Analyzer (Stable Microsystems Ltd; Surrey, England), expressing the results in Newtons (N). For each assay, five intact strawberries of a similar size (50 g in total) were placed in the Kramer shear cell and measurements were made in triplicate.

2.3 Determination of phosphorylated nucleotides

ATP, ADP and AMP were quantified by HPLC performed as described previously (Blanch et al., 2015). The adenylate energy charge was calculated according to Pradet and Raymond (1983): ([ATP] + 0.5 [ADP])/([ATP] + [ADP] + [AMP]). Measurements were made in triplicate, expressing the results as mg kg⁻¹ fresh weight.

2.4 Ethanol and acetaldehyde content

Ethanol and acetaldehyde were quantified in the headspace of 5 mL of juice from three replicates of 15 strawberries without calyx in 10 mL vials. Gas Chromatography was performed as described previously (Blanch et al., 2015) and triplicate measurements were taken, expressing the results as g 100 L⁻¹ of juice.
2.5 Fatty acid analysis

Frozen berry samples (approximately 10 g) were homogenized in 18 mL of MeOH and 18 mL of CHCl₃, and the mixture was then vortexed for two min. Deionized water was added 5 mL, vortexed for 2 min and cooled over 24 hours. The homogenate was then centrifuged at 1684 g for 15 min at 4 °C to separate the methanolic, aqueous and chloroform phases. The total lipids were recovered in the chloroform phase and 15 mL of this phase was concentrated in an evaporator under nitrogen gas at 40 °C. A SPE Bond Elut NH₂ 500 mg column was used for FA fractionating, eluting: neutral lipids with 10 mL chloroform:isopropanol (2:1); free FAs with 10 mL diethyl ether containing 2 % acetic acid; and polar FAs, mainly phospholipids, with 10 mL methanol containing 2 % HCl. To each sample, 300 µg of glycercyl tritridecanoate (1,2,3-Tritridecanoylglycerol), 50 µg of nonadecanoic acid and 700 µg of 1,2-dipentadecanoyl-sn-glycero-3-phosphatidylcholine were added as internal standards (purchased from Sigma, Germany and Cymit Química, Spain).

After evaporating their respective eluents, the samples were methylated for 15 min at 50 °C with 2 mL (neutral and polar FAs) or 1 mL (free FAs) of 0.5 M sodium methoxide in anhydrous methanol. Acetyl chloride in anhydrous methanol (1:10 v/v) 2 mL (to neutral and polar FAs) or 1 mL (to free FAs) was added before mixing thoroughly and heating for 1h at 60 °C. Hexane 1.8 mL (neutral and polar FAs) and 0.8 mL (free FAs), distilled water (1 mL) and anhydrous sodium sulfate (0.2 g) were added, mixing and then centrifuging for 5 min at 277 g. An aliquot of the organic solvent (top layer) was collected in an amber vial for subsequent gas chromatography.

Fatty acid methyl esters (FAMEs) were analyzed by gas chromatography on an Agilent gas chromatography apparatus (7820A model and EZChrom Elite software), using a flame ionization detector (FID) and a capillary fused silica column with a cyanopropyl-
methylpolysiloxane stationary phase (GC-12 CP-SIL88, 100 m x 250 μm x 0.2 μm: Agilent Technologies, Waldbronn, Germany).

The optimal conditions determined were a column temperature of 100 °C that increased to 220 °C at a rate of 4 °C min⁻¹, which was held for 5 min, increased to 240 at 4 °C min⁻¹ and held for 10 min. The total analysis time was 50 min. The carrier gas was helium, at a constant flow of 1 mL min⁻¹, and the samples were injected (1 μL) in the split mode 1:20. The detector temperature was set at 270 °C and the injector oven temperature at 260 °C. The calibration of all FAMEs was carried out relative to the internal standards: Glyceryl tritridecanoate, nonadecanoic acid and 1,2-dipentadecanoyl-sn-glycero-3-phosphatidylcholine. Individual FAMEs were identified by comparing their retention times with those of mixed FAME standards (FAME 37 SUPELCO + PUFA Nº 2 Animal Source Sigma + PUFA Nº 3 Menhaden oil Sigma). The compounds analyzed were expressed as g kg⁻¹ of fresh weight or as a percentage. The data represent the means of three biological replicates with two different technical measurements for each.

2.6. Analysis of volatile esters in strawberry juice

Strawberry juice (5 mL) from three replicates of 15 strawberries without their calyx was obtained immediately after harvest or following each treatment. The juice was transferred to 22 mL glass vials with rubber stoppers and aluminium caps and frozen at -80 °C. Volatile esters were sampled using solid-phase microextraction (SPME) and analyzed using an Agilent 6890N Series gas chromatograph equipped to an Agilent 5973 Series mass selective detector (Agilent Technologies, Germany) and coupled with a TurboMatrix 40 Trap Headspace sampler (Perkin Elmer, USA). After thawing at room temperature, 2-octanone was added to each vial as an internal standard and the sample was maintained at 80 °C for 25 min into the headspace. Chromatographic separation of
volatile compounds was achieved on an HP-5MS capillary column, with a detector
operated in electron impact ionization mode at 70 eV and using full-scan acquisition
mode in the 30 to 550 m/z range. The identification of peaks in the chromatograms was
performed by injecting commercial standards, comparing the spectra with different
Mass Spectral Libraries (Wiley Registry 7th Edition and NIST, 2005, USA), and by
calculating the linear retention indices (LRI) using retention time data from a series of
alkane standards (C6 to C20) run under the same chromatographic conditions. The
normalized peak area of each compound was then calculated as the ratio of its peak area
to the area of the internal standard.

2.7. Determination of Malondialdehyde (MDA)
MDA formation was assayed using a modified thiobarbituric acid (TBA) method
(Ederli et al., 1997), with some modifications. Strawberry samples (c.a. 0.5 g) were
homogenized in 1.5 mL 1 % cold trichloroacetic acid (TCA) and after centrifugation,
the supernatant was collected and 150 μL was mixed with 600 μL of 0.5 % TBA in 20
% TCA. The mixture was heated for 30 min and then cooled quickly before determining
its absorbance at 532 nm and adjusting for non-specific absorbance at 600 nm. Three
independent extractions of each sample were performed and extracts were analyzed in
duplicate. The MDA content was estimated using a molar extinction coefficient of 155
mmol L⁻¹ cm⁻¹, and expressed as the mg kg⁻¹ of the fresh weight of the sample. The
data represent the means of three biological replicates with two different technical
measurements for each.
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 °C for 3 d with 0 kPa CO₂ (T1), 2 d 20 kPa CO₂ (T2), 3 d 20 kPa CO₂ (T3) and 3 d 40 kPa CO₂ (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 CO₂ (T1), 2 d in 20 kPa CO₂ (T2), 3 d in 20 kPa CO₂ (T3) and 3 d with 40 kPa CO₂ (T4).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>CONTENT (%)</th>
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<tbody>
<tr>
<td></td>
<td>CH</td>
</tr>
<tr>
<td>C16:0</td>
<td>Palmitic</td>
</tr>
<tr>
<td>C18:0</td>
<td>Stearic</td>
</tr>
<tr>
<td>C18:1n9</td>
<td>Oleic</td>
</tr>
<tr>
<td>C18:1n7</td>
<td>cis-Vaccenic</td>
</tr>
<tr>
<td>C18:2n6</td>
<td>Linoleic</td>
</tr>
<tr>
<td>C18:3n3</td>
<td>α-Linolenic</td>
</tr>
<tr>
<td>C20:0</td>
<td>Arachidic</td>
</tr>
<tr>
<td>C20:1</td>
<td>Paullinic</td>
</tr>
<tr>
<td>C22:0</td>
<td>Behenic</td>
</tr>
<tr>
<td>C24:0</td>
<td>Lignoceric</td>
</tr>
</tbody>
</table>

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 CO₂ (T1), 2 d in 20 kPa CO₂ (T2), 3 d in 20 kPa CO₂ (T3) and 3 d with 40 kPa CO₂ (T4).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>CONTENT (g kg⁻¹ fresh weight)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CH</td>
</tr>
<tr>
<td>C16:0 Palmitic</td>
<td>5.48±0.2ab</td>
</tr>
<tr>
<td>C18:0 Stearic</td>
<td>1.56±0.1ab</td>
</tr>
<tr>
<td>C18:1n9 Oleic</td>
<td>18.50±1.4b</td>
</tr>
<tr>
<td>C18:1n7 cis-Vaccenic</td>
<td>0.94±0.0ab</td>
</tr>
<tr>
<td>C18:2n6 Linoleic</td>
<td>27.89±2.3b</td>
</tr>
<tr>
<td>C18:3n3 α-Linolenic</td>
<td>16.99±1.3b</td>
</tr>
<tr>
<td>C20:0 Arachidic</td>
<td>0.74±0.0ab</td>
</tr>
<tr>
<td>C20:1 Palminic</td>
<td>0.23±0.0a</td>
</tr>
<tr>
<td>C22:0 Behenic</td>
<td>0.19±0.0a</td>
</tr>
<tr>
<td>C24:0 Lignoceric</td>
<td>0.11±0.0ab</td>
</tr>
</tbody>
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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 CO₂ treatments

Considering the overall changes in the unsaturated FAs following CO₂ 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 CO₂ 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 \(\alpha\)-linolenic acid content. By contrast, strawberries at T2 showed the highest SFA, MUFA and PUFA content, mainly \(\alpha\)-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 \(\alpha\)-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 % \(\alpha\)-linolenic acid and 2 % paullinic acid. MUFA 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 \(\alpha\)-linolenic acid values. Such a decrease in PUFAs was concurrent with a slightly increase in SFAs,
mainly in the stearic acid content. The largest amount of SFAs was quantified in fruit at T1, greater than at CH and significantly more than in CO₂-treated fruit.

Figure 1

A

Neutral fatty acid fraction (g kg⁻¹ FW)

B

Free fatty acid fraction (g kg⁻¹ FW)

C

Polar fatty acid fraction (g kg⁻¹ FW)

SFA - MUFA - PUFA
Figure 1: SFA, MUFA and PUFA content (g kg\(^{-1}\) fresh weight) in neutral lipids (triacylglycerols) (A), free FAs (unesterified FAs) (B) and polar (membrane lipids) (C) in strawberries at commercial harvest (CH), and after storage at 0 °C 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) 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).
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 CO₂ (T1), 2 d with 20 kPa CO₂ (T2), 3 d with 20 kPa CO₂ (T3) and 3 d with 40 kPa CO₂ (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).

The loss of unesterified linoleic and α-linolenic acids by the enzymes of the
lypoxigenase (LOX) pathway could be operating in strawberries at T1. By contrast, at
T2 only a slight decrease in the PUFAs and SFAs was quantified in the fruit. When the
dose or length of application of CO2 treatment increased, the decrease in PUFAs and
SFAs became more pronounced. In strawberries at T4 there was a significant decrease
in the saturated and unsaturated free FA content, such that the proportion of SFAs,
MUFAs and PUFAs remained virtually unchanged when compared to strawberries at
CH (supplementary SF1B).

The FA composition and content determined in the polar lipid fraction (phospholipids
and glucolipids) of strawberries subjected to different CO2-treatments is indicated in
Figure 1C and Figure 2. In this fraction, the proportion of MUFAs was more of less
similar to that of PUFAs with values of 7.05 g kg\(^{-1}\) fresh weight and 7.36 g kg\(^{-1}\) fresh
weight, respectively. The proportion of SFA was 21 % with values of 4.67 g kg\(^{-1}\) fresh
weight. With respect to MUFAs, there was up to 6.65 g kg\(^{-1}\) fresh weight of oleic acid,
the highest levels of the individual FAs, representing 36.4 % of total FAs in the polar
fraction in strawberries at CH. The cis-vaccenic acid detected in this fraction
represented 2.2 %. Following postharvest storage, the significant increase in the levels
of PUFAs in strawberries at T2 is noteworthy, mainly due to the increase in α-linolenic
acid that reached values of 4 g kg\(^{-1}\) fresh weight, 65 % higher than in strawberries at
CH. By contrast, a marked decrease in the PUFA and MUFA content was detected in
strawberries at T4, mainly in oleic acid that reached values as low as 3.7 g kg\(^{-1}\) FW. In
strawberries at T1, all SFAs had decreased, mainly palmitic acid (Figure 2A).
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 CO₂ 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

B

Saturated/Unsaturated FA ratio

C

18:3/18:2

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 CO₂ (T1), 2 d with 20 kPa CO₂ (T2), 3 d with 20 kPa CO₂ (T3) and 3 d with 40 kPa CO₂ (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 °C without added CO₂. 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 CO₂ for 2 or 3 d (T2 and T3)
improves postharvest storage by increasing the double bonds in the phospholipid tails, thereby ensuring that the membrane remains fluid at low temperature. Furthermore, these treatments and especially 2 d exposure to 20 kPa CO$_2$ also improves fruit quality by increasing (ω3) ω-linolenic acid that is essential for human health as it cannot be synthesized and have to be supplied through diet as an important ingredient of docosahexaenoic acid and eicosapentaenoic acid, involved in the treatment of many diseases including autoimmune diseases and inflammatory disorders.

3.3. Esters from strawberries at commercial harvest and following CO$_2$ treatment during storage at 0 °C

As esters play a major role in fruit flavor and aroma, and FAs can serve as ester precursors, we analyzed the changes in unsaturated FAs in the light of both the type and the level of volatile esters formed by strawberries after different high CO$_2$ treatments (Table 3). An approach based on correlations has been shown to be useful to gain insight into metabolic pathways and networks (Weckwerth et al., 2004). As such, we subjected the straight-chain ester data to a Pearson correlation with oleic, linoleic and linolenic acids from the polar, free and neutral lipid fractions.

There was a strong negative correlation (99.9 %) (Table 3) between unesterified PUFAs and butanoate and hexanoate derivatives like octyl butanoate, decyl butanoate, octyl hexanoate and butyl butanoate, with the highest levels of these esters quantified in strawberries at T1 (Figure 3). Other esters like hexyl acetate (P<0.05; r = -0.54), were also negatively correlated with unesterified PUFAs. These esters were produced more intensely in strawberries at T1 that showed an aforementioned significant decline in PUFAs in the free fraction (see Figure 1C and Figure 3). The negative strong
correlations between the free PUFAs and volatiles butanoates and hexanoates esterified
to long alcohols suggests that free linoleic and α-linolenic would be implicated in the
production of volatile aldehydes derived from the activities of some enzymes in the
LOX pathway. The synthesis of aldehydes from free fatty acids by the action of
enzymes of the LOX pathway has been reported in disrupted tissues (Contreras and
Beaudry, 2013; Contreras et al., 2016). A broad range of aldehydes can be converted to
aroma volatile alcohols by alcohol dehydrogenase (ADH) activity (Prestage et al.,
1999). We previously reported (Blanch et al., 2015b) that ADH expression was 23-fold
greater in strawberries stored at 0 °C in air without added CO₂ compared with fruit at
harvest and approximately 3-fold greater than that found in 3 d 20 kPa CO₂
strawberries. Enhancement of volatiles products derived from the peroxidation PUFAs
is typical in many stress responses (Loreto and Schnitzler, 2010) although the quantity
and composition of volatiles released can differs with the type of fruit and with the
intensity of stress. The high proportion of butanoate esters at T1 could confer specific
flavor and aroma associated with these particular aromatic compounds.
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- with straight-chain esters in *Musa* *Dar Bois* strawberries at commercial harvest and after storage under different CO$_2$ treatments.

<table>
<thead>
<tr>
<th></th>
<th><strong>Free Fatty Acid Fraction</strong></th>
<th><strong>Polar Fatty Acid Fraction</strong></th>
<th><strong>Neutral Fatty Acid Fraction</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C18:1e9</td>
<td>C18:2n6</td>
<td>C18:3n3</td>
</tr>
<tr>
<td>Methyl acetate</td>
<td>0.64**</td>
<td>0.40</td>
<td>0.29</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.54*</td>
<td>0.42</td>
<td>0.38</td>
</tr>
<tr>
<td>Methyl butanoate</td>
<td>-0.50*</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Ethyl butanoate</td>
<td>0.46</td>
<td>-0.06</td>
<td>-0.01</td>
</tr>
<tr>
<td>Ethyl (2E)-2-butenoate</td>
<td>0.40</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>Methyl hexanoate</td>
<td>-0.42</td>
<td>-0.31</td>
<td>-0.23</td>
</tr>
<tr>
<td>Butyl butanoate</td>
<td>-0.45</td>
<td>-0.78**</td>
<td>-0.68**</td>
</tr>
<tr>
<td>Ethyl hexanoate</td>
<td>0.38</td>
<td>0.19</td>
<td>0.14</td>
</tr>
<tr>
<td>Hexyl acetate</td>
<td>0.06</td>
<td>-0.54*</td>
<td>-0.53*</td>
</tr>
<tr>
<td>Methyl octanoate</td>
<td>0.34</td>
<td>0.44</td>
<td>0.42</td>
</tr>
<tr>
<td>Hexyl butanoate</td>
<td>-0.45</td>
<td>-0.31</td>
<td>-0.25</td>
</tr>
<tr>
<td>Ethyl octanoate</td>
<td>0.31</td>
<td>0.18</td>
<td>0.11</td>
</tr>
<tr>
<td>Octyl acetate</td>
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<td>0.12</td>
<td>0.09</td>
</tr>
<tr>
<td>Octyl butanoate</td>
<td>-0.34</td>
<td>-0.73**</td>
<td>-0.68**</td>
</tr>
<tr>
<td>Ethyl decanoate</td>
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<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>Decyl acetate</td>
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<td>0.21</td>
<td>0.19</td>
</tr>
<tr>
<td>Octyl Hexanoate</td>
<td>-0.35</td>
<td>-0.84**</td>
<td>-0.79**</td>
</tr>
<tr>
<td>Decyl butanoate</td>
<td>-0.31</td>
<td>-0.82**</td>
<td>-0.74**</td>
</tr>
</tbody>
</table>

The symbol * and ** shows significant differences according to the independent sample t-test (P < 0.05 and P < 0.01, respectively).
Figure 3
Figure 3: Relative amount 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 CO₂ (T1), 2 d with 20 kPa CO₂ (T2), 3 d with 20 kPa CO₂ (T3) and 3 d with 40 kPa CO₂ (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).

Linoleic acid from the polar lipid fraction was strongly and positively correlated (P < 0.01 and r value > 0.7) with the presence of ethyl esters like ethyl hexanoate (r = 0.88), ethyl octanoate (r = 0.76), ethyl decanoate (r = 0.84) and ethyl butanoate (r = 0.87; Table 2). The levels of these ethyl esters increased significantly in strawberries after a 2 d 20 kPa CO₂ treatment (T2) (Figure 4). An aforementioned enhancement of α-linolenic acid in the polar fraction was evident in strawberries at T2 (see Figure 1C and Figure 2). Some of these esters have been identified as key constituents of fresh strawberry aroma (Kim et al., 2013) and they have a typical strawberry-like odor (Larsen and Watkins, 1995; Schieberle and Hofmann, 1997;). Other esters that were closely correlated to the α-linolenic acid in the polar fraction were methyl octanoate (r = 0.72) and ethyl (2E)-2-butenoate (r = 0.75), both of which increased in strawberries after harvest. Ethyl (2E)-2-butenoate is strongly correlated with linoleic (positive) and oleic acid (negative). Indeed, 2-enoates are relatively rare constituents of aromas and they have generally only been detected in tropical and subtropical fruits like Annona muricata.
Figure 4: Relative amounts of ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl butanoate, methyl octanoate and ethyl (2E)-2-butenol in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d with 0 kPa CO₂ (T1), 2 d with 20 kPa CO₂ (T2), 3 d with 20 kPa CO₂ (T3) and 3 d with 40 kPa CO₂ (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).
With respect to the neutral fraction, there was a strong correlation (P < 0.01) of \( \alpha \)-linolenic acid (positive) and oleic acid (negative) with the volatile acetates, such as octyl acetate (r=0.76), decyl acetate (r=0.74) and ethyl acetate (r=0.89). These esters accumulated more strongly in CO\(_2\) treated strawberries, with higher levels at T2 and T3 than at T4 (Figure 5). In strawberries at T4, an aforementioned decrease in \( \alpha \)-linolenic acid from the neutral fraction was reported (see Figure A and Supplementary Fig S1). Considering the strong positive correlation between these esters and \( \alpha \)-linolenic acid from the neutral fraction, the levels of which were significantly higher in strawberries at T2 relative to those at T4 and T1, the changes in acetate esters may be explained by the ready availability of 18:3 substrate, which might be an important factor to improve the production of these specific esters. In addition to substrate availability, changes in the activity of alcohol acetyltransferases (AATs) contribute to ester biosynthesis (Defilippi, et al., 2005; Pérez et al., 1996). AATs utilize acetyl-CoA to acetylate several alcohols and in particular, octyl acetate is a genuine product of AATs (Aharoni et al., 2000). The accumulation of octyl acetate has been reported in several cultivars of *Fragaria vesca* (Dong et al., 2013; Negri et al., 2015), and we found an increase in octyl acetate in strawberries subjected to a 2 d 20 kPa CO\(_2\) treatment. Some of these esters have a floral, orange-rose odor and characteristic flavor, such as decyl acetate.
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 CO₂ (T1), 2 d with 20 kPa CO₂ (T2), 3 d with 20 kPa CO₂ (T3) and 3 d with 40 kPa CO₂ (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).

Ethyl acetate is synthesized by esterification with ethanol, which is induced by low-oxygen atmospheres and high levels of CO₂ (Ke et al., 1994; Larsen and Watkins, 1995; Ueda and Bai, 1993). Our results suggest that ethanol produced in response to a 2 or 3 d 20 kPa CO₂ treatment is not sufficient to displace other alcohols in the esterification reactions. These acetate esters are also negatively correlated with oleic acid from neutral fraction.

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

**Supplementary Fig S2**

![Graph showing methyl acetate levels](image)

**Fig S2:** Relative amount of methyl acetate in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d with 0 kPa CO₂ (T1), 2 d with 20 kPa CO₂ (T2),
3 d with 20 kPa CO₂ (T3) and 3 d with 40 kPa CO₂ (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).

There are several esters that reached their highest levels in strawberries at CH (Figure 6). Both methyl butanoate and hexyl butanoate were negatively correlated (P < 0.01 and r value > 0.70) with α-linolenic acid from the polar fraction (Table 3), while methyl hexanoate was not correlated with any unsaturated FAs from the free, polar or neutral fractions. Methyl hexanoate is one of the three major esters in Mara des Bois strawberries at CH and it was maintained in strawberries at T2 before dropping significantly in those at T3 and T4 when treatment with high CO₂ was prolonged or increased. This ester is the second most abundant volatile ester in other strawberry cultivars (Song et al., 1998). By maintaining the initial levels of this ester, or even slightly increasing them in strawberries subjected to a 2 d 20 kPa CO₂ treatment, it is possible that methyl hexanoate might serve as a biomarker to define the adequate concentration external CO₂ to be applied. It has been reported that the amounts of several methyl esters changes along with the storage temperature in apple (Forney et al., 2000).
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 CO₂ (T1), 2 d with 20 kPa CO₂ (T2), 3 d with 20 kPa CO₂ (T3) and 3 d with 40 kPa CO₂ (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).
Our results indicate low levels of lipid peroxidation in strawberries after 3 d at 0 °C, whereas the lipid peroxidation associated with the stress of high CO₂ concentrations may be responsible for the degradation of unsaturated fatty acids in the polar fraction (see Figure 1C). The deleterious effects of lipid peroxidation can lead to a disruption of membrane structure and function. The increase in the SFAs in the polar fraction of strawberries at T1, not exposed to CO₂, could have consequences in terms of reduced lipid peroxidation.

3.5. Energy and metabolic status of strawberries at commercial harvest and following different high CO₂ treatments during storage at 0 °C

We compared the firmness, energy and fermentative status of Mara des Bois strawberries at CH and after storage at 0 °C with or without CO₂ treatments T1, T2, T3 and T4 (Table 4). The ethanol:acetaldehyde ratio was markedly altered by low temperature storage at atmospheric CO₂ concentrations and the ratio shifted from 1:0.6 at CH to 1:10 in strawberries at T1. Treatment with high concentrations of CO₂ strongly influenced the levels of ethanol and the ethanol:acetaldehyde ratio decreased following all three treatments (T2, T3 and T4). We previously indicated that ethanol did not rise in strawberries stored at 0 °C, although an increase in the abundance of transcripts for pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) was detected. By contrast, CO₂-treatment prevented the increase in PDC expression and the ADH expression was less pronounced (Blanch et al., 2015b). Our results indicate that strawberries activated fermentative metabolism under the three high CO₂ treatments analyzed, confirmed by the accumulation of ethanol (Table 4) and ethanol production was not influenced by oxygen availability.
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 CO₂ (T1), 2 d in 20 kPa CO₂ (T2), 3 d in 20 kPa CO₂ (T3) and 3 d in 40 kPa CO₂ (T4).

<table>
<thead>
<tr>
<th>Firmness</th>
<th>Energy status</th>
<th>Fermentation metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>ADP</td>
</tr>
<tr>
<td>CH</td>
<td>1</td>
<td>22.4±2.2c</td>
</tr>
<tr>
<td>T1</td>
<td>1.14</td>
<td>22.65±2.2c</td>
</tr>
<tr>
<td>T2</td>
<td>1.97</td>
<td>8.94±0.3ab</td>
</tr>
<tr>
<td>T3</td>
<td>2.12</td>
<td>11.84±1.7b</td>
</tr>
<tr>
<td>T4</td>
<td>1.84</td>
<td>7.06±0.7a</td>
</tr>
</tbody>
</table>

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).
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 °C, 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
as short as 2 d in 20 kPa CO$_2$ (T2) appears to be sufficient to significantly enhance flesh
firmness.

Conclusions

Results of this lipidomics study, carried out on Mara des Bois strawberries, have proved
evidence for the important role of FAs in mediating tolerance to CO$_2$ during storage at
low temperature.

Strawberries at T1, not exposed to added CO$_2$, had the lowest PUFA and MUFA values
in the neutral fraction, indicative of active FA breakdown. Another important change
caused by low temperature is an alteration to the polar lipid composition, with an
increase in the amount of SFA$s$ and consequently a decrease in the saturated:unsaturated
ratio. Strawberries at T1 showed the highest levels in volatile butanoates and hexanoates
esterified to long alcohols (C$_8$-C$_{10}$), which are strongly and negatively correlated with
unesterified unsaturated FAs.

As a result of the active breakdown of FAs from triacylglycerols and the strong
production of ethanol, the highest levels of ethyl acetate accumulate in strawberries at
T4. The ethanol produced in strawberries at T4 was sufficient to displace other alcohols
in the esterification reactions. Moreover, the enhanced lipid peroxidation in response to
high CO$_2$ stress, as evident following T4 treatment, may be responsible for the
degradation of unsaturated FAs in the polar fraction, disrupting membrane integrity and
permeability.

Interestingly, the increase in unsaturated and saturated FAs of triacylglycerols and
membrane lipids indicates an active FA synthesis involving desaturases in strawberries
subjected to a 2 d 20 kPa CO$_2$ treatment. This represents the first report wherein the
application of 2 d 20 kPa CO$_2$ + 20 kPa O$_2$ induces adjustment of fruit metabolism that
results in linoleic acid and (ω3) α-linolenic acid accumulation that confer membrane
stability combined with health benefits and enhanced volatile ester production. This
application has also a positive effect on flesh firmness. Whether such beneficial effects
on quality parameters by this short high CO₂ treatment are maintained during shelf-life
period requires further analyses.

Funding
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(MINECO/AEI/FEDER, UE).

Figure Legends
Figure 1: SFA, MUFA and PUFA content (g kg⁻¹ fresh weight) in neutral lipids
(triacylglycerols) (A), free FAs (unesterified FAs) (B) and polar (membrane lipids) (C)
in strawberries at commercial harvest (CH), and after storage at 0 °C for 3 d with 0 kPa
CO₂ (T1), 2 d 20 kPa CO₂ (T2), 3 d 20 kPa CO₂ (T3) and 3 d 40 kPa CO₂ (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).

Figure 2: FA composition and content (expressed on a percentage basis) in the
membrane lipids (A), the saturated:unsaturated ratio (B) and the linolenic/linoleic ratio
(C) in Mara des Bois strawberries at commercial harvest (CH), and after storage for 3 d
with 0 kPa CO₂ (T1), 2 d with 20 kPa CO₂ (T2), 3 d with 20 kPa CO₂ (T3) and 3 d with
40 kPa CO₂ (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).
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 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).
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).

**Supplementary data**

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

Fig S2: Relative amount of methyl acetate 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).
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stress: effects on xanthophyll cycle, scavenger enzymes and abscisic acid content in

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1996. Metabolism of natural volatile compounds by strawberry fruit. J. Agric. Food
Chem. 44, 2802–2805.


Figure 2

**A**  
\[
\text{% FA}
\]

**B**  
\[
\text{Saturated/Unsaturated FA ratio}
\]

**C**  
\[
\text{18:3/18:2}
\]
Figure 3

- Butyl butanoate
- Octyl butanoate
- Decyl butanoate
- Octyl Hexanoate

Comparative analysis of relative amounts of esters in different samples (CH, T1, T2, T3, T4) with statistical significance indicated by letters (a, b, c, d, e).
Figure 4

Ethyl hexanoate

Ethyl octanoate

Ethyl decanoate

Ethyl butanoate

Methyl octanoate

Ethyl (2E)-2-butenoate
Figure 5

Octyl acetate

Decyl acetate

Ethyl acetate
Figure 6

Hexyl butanoate

Methyl butanoate

Methyl hexanoate
Supplementary Fig S1

A

% FA

- 18:3
- 18:2
- 18:1n7c
- 18:1n9c
- 20:0
- 18:0
- 16:0

CH T1 T2 T3 T4

B

% FA

- 18:3
- 18:2
- 18:1n9c
- 20:0
- 18:0
- 16:0

CH T1 T2 T3 T4
Supplementary Fig S2

![Bar chart showing relative amounts of Methyl acetate for different groups (CH, T1, T2, T3, T4). The chart indicates significant differences among the groups with letters a, b, c, and h.](image-url)
Figure 1

A

Neutral fatty acid fraction
(g kg\(^{-1}\) fresh weight)

B

Free fatty acid fraction
(g kg\(^{-1}\) fresh weight)

C

Polar fatty acid fraction
(g kg\(^{-1}\) fresh weight)

Legend:
- SFA
- MUFA
- PUFA
Figure 7

![Graph showing MDA (mg kg⁻¹ fresh weight) for different treatments: CH, T1, T2, T3, T4. Each group is represented by a bar with error bars, indicating variability. The bars are labeled with lowercase letters, where 'a' and 'b' denote significant differences.](image-url)
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 CO₂ treatment.

<table>
<thead>
<tr>
<th></th>
<th>Free Fatty Acid Fraction</th>
<th>Polar Fatty Acid Fraction</th>
<th>Neutral Fatty Acid Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C18:1e9</td>
<td>C18:2n6</td>
<td>C18:3n3</td>
</tr>
<tr>
<td>Methyl acetate</td>
<td>0.64**</td>
<td>0.40</td>
<td>0.29</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.54*</td>
<td>0.42</td>
<td>0.38</td>
</tr>
<tr>
<td>Methyl butanoate</td>
<td>-0.50*</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Ethyl butanoate</td>
<td>0.46</td>
<td>-0.06</td>
<td>-0.01</td>
</tr>
<tr>
<td>Ethyl (2E)-2-butenoate</td>
<td>0.40</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>Methyl hexanoate</td>
<td>-0.42</td>
<td>-0.31</td>
<td>-0.23</td>
</tr>
<tr>
<td>Butyl butanoate</td>
<td>-0.45</td>
<td>-0.78**</td>
<td>-0.68**</td>
</tr>
<tr>
<td>Ethyl hexanoate</td>
<td>0.38</td>
<td>0.19</td>
<td>0.14</td>
</tr>
<tr>
<td>Hexyl acetate</td>
<td>0.06</td>
<td>-0.54*</td>
<td>-0.53*</td>
</tr>
<tr>
<td>Methyl octanoate</td>
<td>0.34</td>
<td>0.44</td>
<td>0.42</td>
</tr>
<tr>
<td>Hexyl butanoate</td>
<td>-0.45</td>
<td>-0.31</td>
<td>-0.25</td>
</tr>
<tr>
<td>Ethyl octanoate</td>
<td>0.31</td>
<td>0.18</td>
<td>0.11</td>
</tr>
<tr>
<td>Octyl acetate</td>
<td>0.45</td>
<td>0.12</td>
<td>0.09</td>
</tr>
<tr>
<td>Octyl butanoate</td>
<td>-0.34</td>
<td>-0.73**</td>
<td>-0.68**</td>
</tr>
<tr>
<td>Ethyl decanoate</td>
<td>0.37</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>Decyl acetate</td>
<td>0.44</td>
<td>0.21</td>
<td>0.19</td>
</tr>
<tr>
<td>Octyl Hexanoate</td>
<td>-0.35</td>
<td>-0.85**</td>
<td>-0.79**</td>
</tr>
<tr>
<td>Decyl butanoate</td>
<td>-0.31</td>
<td>-0.82**</td>
<td>-0.74**</td>
</tr>
</tbody>
</table>

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 CO₂ (T1), 2 d in 20 kPa CO₂ (T2), 3 d in 20 kPa CO₂ (T3) and 3 d with 40 kPa CO₂ (T4).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>CONTENT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH</td>
</tr>
<tr>
<td>C16:0</td>
<td>Palmitic</td>
</tr>
<tr>
<td>C18:0</td>
<td>Stearic</td>
</tr>
<tr>
<td>C18:1n9</td>
<td>Oleic</td>
</tr>
<tr>
<td>C18:1n7</td>
<td><em>cis</em>-Vaccenic</td>
</tr>
<tr>
<td>C18:2n6</td>
<td>Linoleic</td>
</tr>
<tr>
<td>C18:3n3</td>
<td>α-Linolenic</td>
</tr>
<tr>
<td>C20:0</td>
<td>Arachidic</td>
</tr>
<tr>
<td>C20:1</td>
<td>Paullinic</td>
</tr>
<tr>
<td>C22:0</td>
<td>Behenic</td>
</tr>
<tr>
<td>C24:0</td>
<td>Lignoceric</td>
</tr>
</tbody>
</table>

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 CO₂ (T1), 2 d in 20 kPa CO₂ (T2), 3 d in 20 kPa CO₂ (T3) and 3 d with 40 kPa CO₂ (T4).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>CONTENT (g kg⁻¹ fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH</td>
</tr>
<tr>
<td>C16:0</td>
<td>Palmitic</td>
</tr>
<tr>
<td>C18:0</td>
<td>Stearic</td>
</tr>
<tr>
<td>C18:1n9</td>
<td>Oleic</td>
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<tr>
<td>C18:1n7</td>
<td>cis-Vaccenic</td>
</tr>
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<td>C18:2n6</td>
<td>Linoleic</td>
</tr>
<tr>
<td>C18:3n3</td>
<td>α-Linolenic</td>
</tr>
<tr>
<td>C20:0</td>
<td>Arachidic</td>
</tr>
<tr>
<td>C20:1</td>
<td>Paullinic</td>
</tr>
<tr>
<td>C22:0</td>
<td>Behenic</td>
</tr>
<tr>
<td>C24:0</td>
<td>Lignoceric</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Firmness</th>
<th>Energy status</th>
<th>Fermentation metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>ADP</td>
</tr>
<tr>
<td>CH</td>
<td>1</td>
<td>22.4±2.2c</td>
</tr>
<tr>
<td>T1</td>
<td>1.14</td>
<td>22.65±2.2c</td>
</tr>
<tr>
<td>T2</td>
<td>1.97</td>
<td>8.94±0.3ab</td>
</tr>
<tr>
<td>T3</td>
<td>2.12</td>
<td>11.8±1.7b</td>
</tr>
<tr>
<td>T4</td>
<td>1.84</td>
<td>7.0±0.7a</td>
</tr>
</tbody>
</table>

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).