Metabolic reconfiguration of strawberry physiology in response to postharvest practices

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Abstract

The strawberry fruit is perishable due to its high water content and soft texture, yet exhibits pleasant organoleptic and nutritional profile. Here we conducted a metabolomics-driven analysis followed by linear modelling to dissect the molecular processes in strawberry postharvest. Fruits from five cultivars were harvested and refrigerated during a ten-day period under three different atmospheres: ambient, CO_2-enriched and O_3-enriched. These analyses revealed that metabolites involved in, (i) organoleptic and nutritional properties; (ii) stress tolerance displayed duration and postharvest treatment-dependent levels. Ozone-enriched atmosphere appears to counteract postharvest negative effects, with fruits exhibiting lower levels of fermentative metabolites when compared to fruits kept in an ambient atmosphere. Furthermore, metabolic reconfiguration towards the synthesis of protective metabolites of those fruits can possibly confer enhanced tolerance to postharvest abiotic stresses. Finally, results from the linear modelling identified metabolites which could be used as biomarkers to assess strawberry quality during its postharvest shelf life.

Keywords: Strawberry; Postharvest; Quality; Metabolites

1 Introduction

The cultivated strawberry (*Fragaria* × *ananassa*) is the most consumed berry worldwide, with a global annual production exceeding 9 million tonnes in recent years (FAOSTAT, 2017; http://faostat.fao.org). This soft fruit with a delicate taste and aroma is a source of important health-promoting nutrients such as minerals, vitamins and polyphenols that appeal to consumers (Fait et al., 2008; Ulrich & Olbricht, 2016; Vallarino, de Abreu e Lima et al., 2018).

The ripening berry originates from the developing flower receptacle in which the dry achenes (the 'real' fruits) are embedded, and undergoes physiological changes that improve its palatability (Fait et al., 2008). While pectin depolymerisation and solubilisation are responsible for receptacle softening, changes in fruit metabolism contribute to the storage of sugars, organic acids and anthocyanins that confer the bright red colour to the mature strawberry and a multitude of other metabolites, including polyphenols, vitamins (i.e. vitamin C and folate) and minerals, which contribute to its organoleptic and nutritional characteristics (Fait et al., 2008; Giampieri et al., 2012; Schwieterman et al., 2014). In addition, the ripe fruit releases a complex mixture of hundreds of volatiles, where esters and furanones together with terpenoids, alcohols and aldehydes are the dominating aroma compounds (Ulrich & Olbricht, 2016). Fruit ripening, including the accumulation of highly valuable metabolites, is a complex process, influenced by genotype, environment and interaction between both of them (Krüger et al., 2012).

As a non-climacteric fruit, strawberry growth and ripening is driven by the interplay between auxin, abscisic acid (ABA), ethylene and gibberellins (Estrada-Johnson et al., 2017). In particular, harvested berries display increased ABA levels compared to *in planta* conditions, leading to a faster ripening process and consequently

fruit senescence (Chen et al., 2014). As a result, strawberry is highly perishable and has a short shelf-life that incurs important economic losses for the industry (Yan et al., 2019).

Upon harvest, due to their high respiration rate, strawberries become susceptible to water loss, mechanical damage and fungal deterioration that can lead to a short postharvest life (Yan et al., 2019). In addition, unlike climacteric fruits, strawberries must be harvested almost fully ripe, therefore heavily limiting shelf-life. Therefore, the extension of strawberry shelf-life embodies a major economic goal, and several commercial practices are currently adopted to arrest strawberry senescence. These practices include the deployment of a controlled atmosphere combined with optimal temperatures (Pedreschi & Lurie, 2015). Refrigerated storage is most commonly employed to slow down both fruit metabolism and pathogen development. In strawberry, this strategy seems to improve quality by decreasing the loss of soluble solids observed during postharvest (Ayala-Zavala, Wang, Wang, & González-Aguilar, 2004). However, exposure to low temperatures can also induce chilling injury, which negatively affects fruit quality (Bustamante et al., 2016). To prevent chilling injury, modified atmospheres are often combined with cold storage. Elevated CO_2 has been suggested to improve strawberry quality, by reducing respiration rate and total acidity loss during postharvest (Li et al., 2019). On the other hand, strawberry fruits stored in elevated O_3 showed a three-fold increase of ascorbic acid compared to fruits kept in a non-modified atmosphere (Pérez, Sanz, Ríos, Olías, & Olías, 1999). However, adverse effects on fruit colour, aroma and taste have also been observed due to exposure to modified atmospheres, and strawberry fruit metabolism under postharvest strategies is still elusive.

Fruit removal from the plant triggers a series of stress-related physiological changes, including water loss due to transpiration and nutrient deprivation (Aghdam, Jannatizadeh, Luo, & Paliyath, 2018). In addition, both cold and modified storage atmospheres act as abiotic stressors and activation of specific pathways take place to maintain fruit metabolic homeostasis (Pedreschi & Lurie, 2015). Under stress conditions, plants synthesise protective compounds, including secondary (e.g., polyphenols) and compatible (e.g., sorbitol, trehalose, proline, polyamines) metabolites. The accumulation of antioxidant compounds that scavenge reactive oxygen species (ROS) is also commonly observed (Pedreschi & Lurie, 2015). Stress responses were observed in the transcriptome and metabolome of strawberry fruits after a three-hour long CO_2 exposure followed by extended storage at 10 °C. Changes in carbohydrate, amino and quinic acid metabolism in particular were observed, followed by the accumulation of defence-related metabolites (Bang, Lim, Yi, Lee, & Lee, 2019).

High-throughput metabolite profiling is a powerful and widely used method, that enables the quantification of up to thousands of metabolites and improves our understanding of the regulation of metabolic networks in plant tissues or organs (Vallarino, de Abreu e Lima et al., 2018). In particular, gas (GC) and liquid chromatography (LC) followed by mass spectrometry (MS) have been widely adopted to unravel fruit metabolic reconfiguration during ripening and senescence (Fait et al., 2008; Osorio et al., 2019). As small polar molecules can be made volatile through a derivatisation process, GC–MS is effective for measuring compounds such as sugars, amino and organic acids, which comprise a major portion of plant central primary metabolism (Osorio et al., 2019). In addition, GC–MS can be coupled with headspace solid-phase microextraction (HS-SPME), allowing the detection and quantification of volatiles (Vallarino, Erban et al., 2018). Both volatile and primary metabolite profiling during postharvest have been described in different fruit-bearing species, with these two technologies helping to determine the levels of key taste and aroma

compounds that directly influence consumer acceptance (Brizzolara et al., 2017; Bustamante et al., 2016; Li et al., 2019; Obenland, Collin, Mackey, Sievert, & Arpaia, 2011; Pérez et al., 1999).

However, fruits also carry a vast amount of diverse secondary metabolites that are mainly responsible for its health benefits, in addition to contributing to their appearance, taste and aroma (Giampieri et al., 2012; Pott, Osorio, & Vallarino, 2019). In this sense, LC-MS approaches allow us to measure a broader range of compounds compared to GC–MS, and have been widely used to monitor postharvest impact on the main classes of secondary metabolites, including polyphenols and carotenoids (Osorio et al., 2019).

In the current study, we monitored fruit senescence in five commercial strawberry cultivars under commonly used postharvest storage treatments and compared the cultivars based on gathered time-resolved metabolomics data. Based on linear modelling analysis, we determined which of the three factors, i.e. cultivar (genotype), postharvest treatment, and its duration, affect the metabolic response of cultivars, and identified biomarkers that can be used to assess fruit quality. The different storage atmospheres were also discussed based on their impact on fruit metabolism reconfiguration.

2 Material and methods

2.1 Plant material and treatments

The plants were grown under commercial conditions in Moguer (Huelva, Spain). The following described treatments were performed with fruits harvested twice during the commercial strawberry season. For this, a randomised complete block design was employed with three panel blocks (13–19 plants/block) per strawberry cultivar (*F.* × *ananassa*, Duch. cv 'Amiga', 'Camarosa', 'Candonga', 'Fortuna' and 'Santa Clara'; Fig. 1, Fig. S1 and Table S1). Between 30 and 40 fully mature fruits were harvested from each block and transported in refrigerated conditions and kept at 4 °C under dark storage. In the day after harvest control fruits of each block per cultivar were frozen in liquid nitrogen (T_0 fruits), while the rest of the fruits were kept at 4 °C in dark conditions and 90% relative humidity and sorted into the different postharvest atmospheres: (i) non-modified atmosphere (henceforth referred as cold treatment; 4 °C), (ii) CO_2-enriched atmosphere (CO_2 atmosphere) and (iii) O_3-enriched atmosphere (O_3 atmosphere). For ozone atmosphere, fruits were kept in 0.35 ppm as described in Pérez et al. (1999). For CO_2 atmosphere, fruits were stored in an atmosphere of 10% CO_2 and 11% O_2 as reported in Almenar, Hernández-Muñoz, Lagarón, Catalá, and Gavara (2006). Each block of every cultivar (C_xB_x, Fig. S2) was handled and treated independently. For each treatment, between 6 and 10 fruits were held in a separate chamber for three (T_3), six (T_6) and ten (T_{10}) days. After treatment, fruits were immediately frozen in liquid nitrogen. Additionally, an independent harvest was performed late in the commercial strawberry season. Subsequently, a second round of identical treatments was performed as is described above. Finally, frozen fruits coming from both independent experiments were mixed and ground into powder using a TissueLyser II (Qiagen) and stored at −80 °C until analysis.

Fig. 1

Effect of cold treatment, CO_2 and O_3 atmospheres on the appearance of 'Camarosa' fruits.

2.2 Physiological parameters during postharvest: firmness and soluble solid content

Strawberry fruit firmness (g mm^{-1}) from control and treated fruits was measured at the equatorial zone using a penetrometer with a 3-mm-wide probe (FDP500; Effegi, Peschiera Borromeo, Italy). Soluble solid content (SSC, in °Brix) was evaluated with a refractometer (PR32; Atago Co., Ltd, Tokyo, Japan) by adding a few drops onto the lens. For each measure, a pool of ten fruits was used.

2.3 Metabolome analysis

Primary and secondary metabolite extraction and analysis by GC–TOF-MS and UPLC–Orbitrap-MS/MS was carried out as previously reported by Vallarino, de Abreu e Lima et al. (2018). Volatile analysis by HS-SPME–GC–MS was carried out as Vallarino, Erban et al. (2018). Metabolite levels were normalised to dry weight. Full documentation of metabolite profiling data acquisition and interpretation is provided in Tables S2–S4.

2.4 Statistical analysis

Metabolite levels were $\log_{10}$-transformed after setting null values to missing. Next, samples were median-centred and metabolites with more than 20% of missing values in the gathered samples were discarded. Remaining missing values were imputed using the Bayesian principal component analysis (PCA) method from the R package pcaMethods (Stacklies, Redestig, Scholz, Walther, & Selbig, 2007) using the first five principal components after standardisation, i.e. mean-centring and scaling to unit-variance per metabolite. Both hierarchical clustering and PCA were performed on the sample median values, each comprising three replicates, followed by standardisation.

For every standardised metabolite indexed by i, we then built three competing nested linear models with successively added two-way and three-way interaction effects. The base additive model has the form

$$met_i = G + E + T$$

the gene-by-environment two-way interaction model has the form

$$met_i = G + E + T + G \times E$$

while the gene-by-environment-by-time three-way interaction model the form

$$met_i = G + E + T + G \times E + G \times E \times T$$

where G expands as the effects from the 'Amiga' (A), 'Camarosa' (C), 'Candonga' (G), 'Fortuna' (F) and 'Santa Clara' (SC) cultivars, E as the effects from and O_3 and CO_2 and T denotes time (days of post-harvest). Apart from time, which was treated as continuous, all effects were encoded as binary dummy variables. All three competing models were devoid of intercept, and were fit and compared using the R package limma (Ritchie et al., 2015). From the three, the model with the overall smallest Bayesian information criterion value was selected for inference from all metabolites, using the function selectModel.

To qualitatively determine metabolic up- and down-regulation under the separate O_3, CO_2 and time effects, the latter contrasts were set prior to model fitting, and the resulting coefficients subjected to decideTests using the "global" method, following a Benjamini-Hochberg MHC P-value adjustment with $\alpha = 0.01$ (Benjamini & Hochberg, 1995).

To quantitatively address the impact of O_3 and CO_2 and their differences/similarities, alongside genotypic and time effects, we extended the model procedure to additionally extract the marginal F-values from the genotypic effects only (i.e. A, C, G, F and SC), resulting in our measure of genotypic variance.

Finally, to estimate the significance of the joint effects of CO_2 and O_3 per metabolite, and assuming the normality of the data following the $\log_{10}$-transformation, we devised a permutation scheme whereby 100,000

samples from the standard normal distribution $X\ N(0,1)$ were used in place of the standardised metabolites and modelled as aforementioned. The resulting coefficients were then analysed as a bivariate distribution using the R package ellipse, from which we estimated the 90%, 95% and 99% confidence intervals, used to delineate the significance of the observed pairs of coefficients.

The analysis can be reproduced using the R code available under https://github.com/monogenea/strawberryPostharvest.

3 Results

3.1 Physiological parameters during postharvest: firmness and soluble solid content

After three, six and ten days of cold, CO_2 and O_3 atmospheres treatments fruits were frozen to be further analysed for metabolite profiling. Complementarily, fruit firmness and soluble solid content (SSC) were measured in the five cultivars (Figs. S3 and S4). Despite significant differences in firmness and SSC between T0 fruits of the different cultivars ('Amiga' and 'Santa Clara' fruits were firmer, while 'Fortuna' and 'Santa Clara' had the lowest SSC in comparison with the other cultivars), they displayed a similar behaviour along postharvest (Fig. 1 for 'Camarosa' cultivar; Fig. S1 for the remaining cultivars). Fruit softening symptoms were more evident in cold treatment than in the two modified atmospheres after 6 days (Fig. 1, Fig. S1), even if no visible differences in firmness were observed (Fig. S3). After 10 days of postharvest, fruits from the three treatments were equally damaged (Fig. 1 for 'Camarosa' cultivar; Fig. S1), concomitant with the decrease of firmness (Fig. S3).

The decrease of SSC observed during postharvest was associated to storage time, with the exception of 'Candonga'. CO_2 and O_3 atmospheres had a positive impact on SSC in 'Amiga', as it was partially recovered between 6 and 10 days, reaching 8.1° and 7.6° values, respectively, when compared with the 5.9° value in cold-treated fruits and the 8.6° value in T0 fruits (Fig. S4).

3.2 Variation in the metabolite levels of the five commercial strawberry cultivars during postharvest

Metabolite profiling of the T0 and postharvest treated fruits was performed using (i) gas chromatography–time-of-flight-mass spectrometry (GC–TOF-MS) to identify 49 primary metabolites, (ii) UPLC–Orbitrap-MS/MS which allowed the identification of 132 secondary metabolites and (iii) automated headspace solid-phase micro-extraction (HS-SPME) sampling coupled to GC–MS for the detection of 70 volatiles. For further analysis, the mean value of the three biological replicates per sample for each metabolite was used (Tables S2–S4).

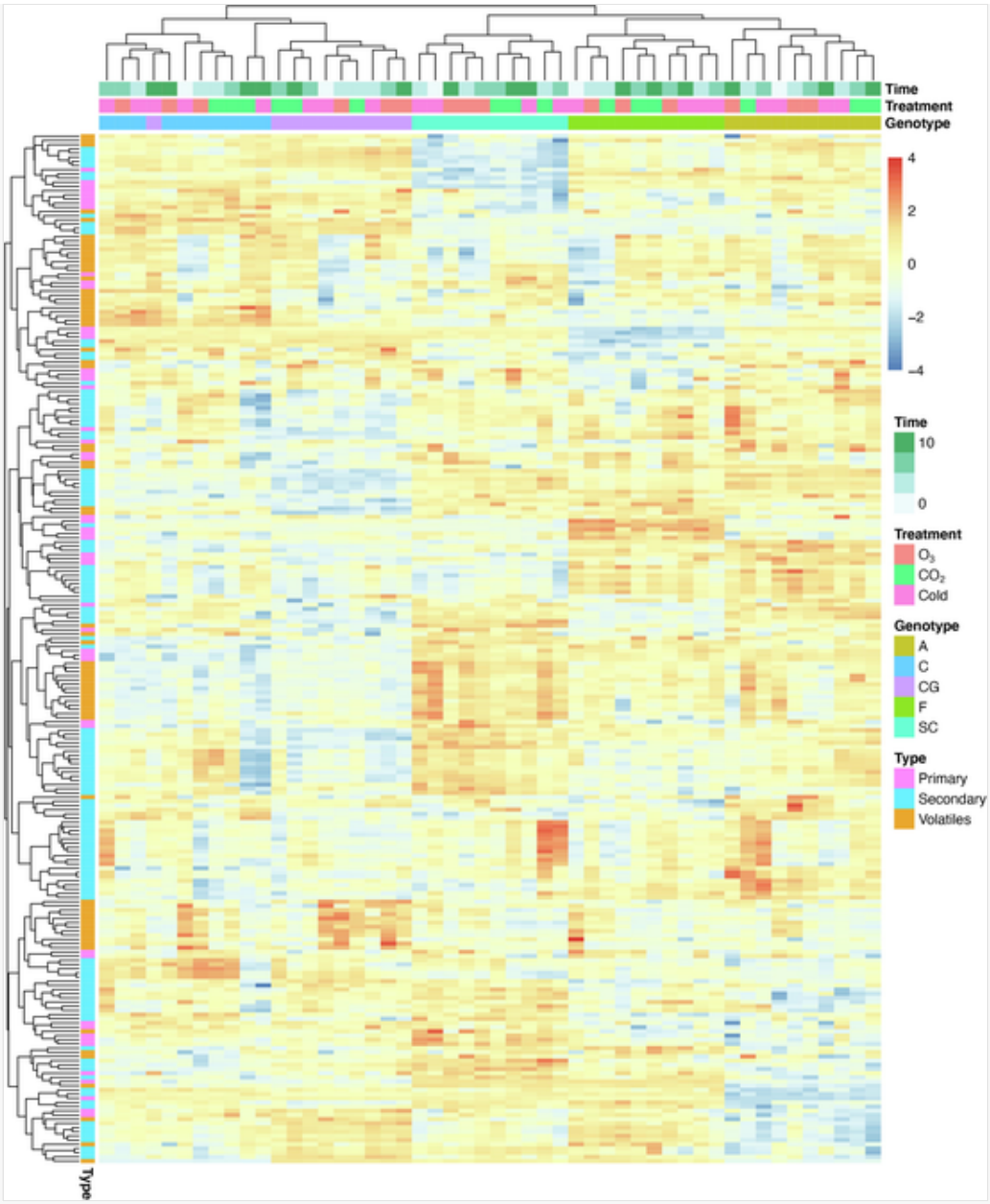
Detected primary metabolites included 19 amino acids, 18 sugars and sugars derivatives, 11 organic acids and one polyamine (putrescine). Secondary metabolites comprised mainly of polyphenols, with the exception of five terpenoids. Phenolic compounds were grouped into flavonoids (59), soluble tannins (including precursors and derivatives, 47) and phenolic acids (including hydroxycinnamic and benzoic acid derivatives, 21).

Volatiles from the main classes impacting strawberry aroma were identified: 38 esters, 13 aldehydes, six ketones, five furans, four alcohols, and four terpenes.

To visualise similarities between samples during postharvest based on the metabolic profiles, principal component analysis (PCA) was performed with the primary, secondary metabolite and volatile datasets, separately (Fig. S5). Samples clustered according to the genotype, indicating that there is a strong genotypic effect driving the differences in metabolic profiles. We found that the first principal component (PC1) from the primary metabolites accounted for 23.2% of total variation therein and separated 'Fortuna' from the remaining cultivars, while PC2 (19.5% of variation) clearly isolated 'Santa Clara' from the rest (Fig. S5A), indicating that the metabolic profiles can separate the genotypes which exhibited lowest SCC. Furthermore, the genotypic effect also dominated the first two PCs for secondary metabolites (26.9% and 20.4% of the variation for PC1 and PC2, respectively; Fig. S5B). However, the PCA of volatile content showed an interesting tendency regarding postharvest duration. While PC1 marginally separated genotypes, indeed a time gradient was observed along PC2 (25.6.0% of total variation), where initial time points exhibited the highest scores (Fig. S5C). Together, these results suggest that strawberry aroma composition is highly dynamic under the tested conditions, being more impacted by postharvest duration than primary or secondary metabolites.

Next, hierarchical clustering analysis (HCA) using the results of primary, secondary metabolites and volatile profiles was used to further investigate the relationship between metabolites gathered from the different postharvest treatments (Fig. 2). As expected, metabolites were primarily grouped by genotype. In addition, the higher similarity existing between 'Candonga' and 'Camarosa' samples, was emphasised as they grouped together in a main cluster, while the other three remaining genotypes were in the second main cluster. Metabolite clustering showed that primary, secondary metabolites and volatiles were grouped together in several main clusters, pointing out the interconnections in fruit metabolism. Even if no obvious common metabolic pattern was observed due to storage treatments and their duration, a high degree of metabolic disparity was found between samples, depending on both genotype and postharvest conditions (Fig. 2).

Fig. 2



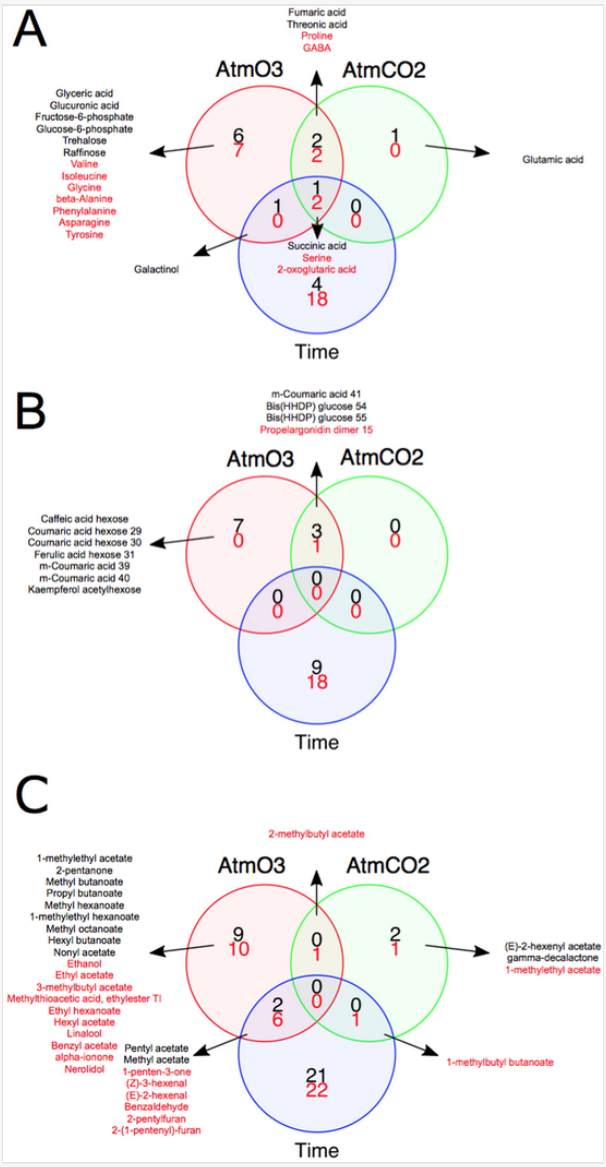
Heatmap visualisation of the primary, secondary metabolites and volatiles identified in the T0 fruits and postharvest samples. Each value represents the standardised median of the three biological replicates, with red and blue colours denoting relatively high and low intensities. Samples and metabolites were clustered using the Euclidean distance and complete clustering method. Genotypes (cultivars, A for 'Amiga', C for 'Camarosa', CG for 'Candonga', F for 'Fortuna' and SC for 'Santa Clara'), postharvest treatments (cold for cold storage, CO_2 for CO_2–enriched atmosphere and O_3 for O_3–enriched atmosphere) and postharvest duration (0 for T0 fruits, 3, 6 and 10 days) are indicated by different colour scales as explained in the legend.

3.3 Metabolic shifts during strawberry postharvest in cold storage under different atmospheres outlined time effects

To better visualise the main effects of cold storage under different atmospheric conditions on strawberry fruit metabolism, we represented the median-normalised metabolite levels on a heatmap (Figs. S6–S8). Next, we fitted three competing nested linear models: a base additive model, the additive model with a gene-by-environment interaction, and the additive model with a gene-by-environment-by-time interaction (cf.

Experimental Procedures). Based on the Bayes information criterion, the base additive model was the best fitting for at least 73.2% of secondary, 69.4% of primary and 72.8% of volatile metabolites (Tables S5). Finally, we determined significant changes of metabolite levels in response to either atmospheres or postharvest time using the optimal model (Fig. 3, Tables S6–S9).

Fig. 3



Venn diagrams representing significant (adjusted $p < 0.01$) increase and decrease of primary (A), secondary (B) metabolites and volatiles (C) in response to CO_2-enriched atmosphere ($AtmCO_2$), O_3-enriched atmosphere ($AtmO_3$) and/or time. Metabolites in red are significantly decreased, while metabolites in black are significantly increased.

As a consequence of fruit metabolism and respiration during cold storage, the levels of 20 out of 49 primary metabolites, including major compounds affecting taste, decreased over time. With respect to carbohydrates, the contents of fructose and sucrose, the main sugars in ripe strawberry fruits, were reduced at later time points together with those of several minor sugars and their derivatives (e.g., trehalose, raffinose, maltose,

maltotriose, rhamnose, myo-inositol and 1-*O*-methyl-α-glucopyranoside). The levels of the two most abundant organic acids, citric and malic acids, were also significantly reduced over time, together with glyceric and 2-oxoglutaric acids and eight amino acids (serine, methionine, aspartic, pyroglutamic and glutamic acids). Finally, the levels of dehydroascorbic and threonic acids, two metabolites originating from ascorbic acid catabolism, were also significantly reduced. Taken together, these data suggest a gradual loss in fruit organoleptic and nutritional traits as a consequence of extended duration of postharvest. By contrast, six primary metabolites were significantly increased during storage, namely glycine, β-alanine, γ-aminobutyric acid (GABA), succinic acid and the sugar alcohols erythritol and galactinol (Fig. 3A, Table S9). Time coefficients extracted from the linear models were most negative for sucrose, trehalose, glyceric acid, malic acid, 1-*O*-methyl-α-glucopyranoside, glutamic acid and maltose, and highest for GABA, β-alanine, glycine and erythritol, which is indicative of their overall decrease and increase over time, respectively (Table S6).

Compared to primary metabolites and volatile compounds, postharvest duration influenced secondary metabolite levels to a smaller degree, possibly as a consequence of cold and dark storage (Fig. 3B, Table S7). Indeed, time coefficients for secondary metabolites showed both smaller positive and negative values, when compared to the values obtained for the other two classes of metabolites. However, several polyphenols belonging to the soluble tannin class, i.e. an isomer of galloyl hexose, two unknown ellagitannins, an isomer of castalagin and of galloyl-hexahydroxydiphenyl(HHDP)-glucose exhibited the most prominence decrease across postharvest time. By contrast, an isomer of sesquiterpenoid hexose was by far the most increased compound as reflected by its time coefficient (Table S7). Additionally, a derivative of cinnamic acid, a galloyl hexose, a derivative of (*epi*)afzelechin and two procyanidin oligomers showed relatively high time coefficients, suggesting that the tested postharvest treatments favoured their accumulation (Table S7).

The larger variance of time coefficients from volatile compounds is in agreement with PCA results, both indicating time had the largest impact on volatile compounds. This was confirmed by the greater variation in time coefficients observed for volatile compounds than for primary and especially secondary metabolites (Fig. S5C, Table S8). Cold storage duration in the three tested atmospheric conditions negatively impacted ten aldehydes, four ketones, two furans and 13 esters. Interestingly, most esters showing a decrease over time were methyl esters, including methyl butanoate and hexanoate, two of the key identified strawberry aroma compounds and with severely negative time coefficients, together with methyl octanoate and 1-methylethyl hexanoate (Fig. 3C, Table S8). By contrast, the content of 20 esters, including seven ethyl esters, such as ethyl butanoate and hexanoate, and hexyl acetate, three main strawberry volatiles, increased over time. Furthermore, mesifurane, one of the two most important contributors to strawberry aroma, increased significantly across the duration of the postharvest period. Finally, the accumulation of the two alcohols eugenol and ethanol was also significant over time. In particular, ethanol, together with the ester ethyl acetate, showed the strongest intensification along the postharvest period (Fig. 3C, Table S8). Both volatiles are considered off-aroma compounds originating from fermentative metabolism during postharvest storage and negatively impacting consumer acceptance (Almenar et al., 2006).

3.4 Metabolic changes in response to CO_2 and O_3-enriched atmospheres

To analyse the extent to which the use of carbon dioxide and ozone impacts on the metabolic content of strawberry fruits, CO_2-and O_3-treated samples were compared to cold treatment, and the metabolites significantly increased or decreased are included in the Venn diagrams (Fig. 3, Table S9). In addition, the set of CO_2 and O_3 coefficients were jointly analysed and contrasted to a permutation-based set in pursuit of discriminative features across the three major compound groups (Fig. S9). Interestingly, seven primary metabolites, four secondary metabolites and one volatile showed common responses in the two tested modified atmospheres (Fig. 3). While the content of GABA, proline, succinic and 2-oxoglutaric acids, an isomer of the flavonoid propelargonidin dimer 2 and the volatile 2-methylbutyl acetate decreased in CO_2 and O_3-treated samples when compared to cold-stored fruits, those of fumaric, succinic, threonic and *m*-coumaric acids, together with two ellagitannins (bis-HHDP-glucose) increased (Fig. 3).

In addition, the CO_2 atmosphere seemed to have a limited effect on the fruit metabolome in comparison with O_3 atmosphere, as only glutamic acid and the two volatiles, 1-methylbutyl butanoate and 1-methylethyl acetate, were decreased when compared to cold storage, while (*E*)-2-hexenyl acetate and the key aroma volatile γ-decalactone were increased (Fig. 3). In addition, CO_2 coefficients were generally smaller than the ones for O_3, especially in the case of primary metabolites and volatiles (Fig. S9, Tables S6–S8).

The O_3-enriched atmosphere had a stronger impact on strawberry fruit metabolome during postharvest, affecting the content of 14 primary metabolites, seven secondary metabolites and 25 volatiles (Fig. 3). Interestingly, metabolic redirection towards the synthesis of stress-related compounds could be observed as five sugars and sugar derivatives were increased (raffinose, galactinol, trehalose, glucose- and fructose-6-phosphate) in addition to seven polyphenols (the flavonol kaempferol acetylhexose, and six phenolic acid derivatives). By contrast the levels of seven amino acids were decreased, including the aromatic amino acids tyrosine and phenylalanine, which are the precursors for the synthesis of polyphenols (Fig. 3A-B).

Ozone impacts on the volatile profiles were also evident. It induced a decrease in total of 16 volatiles (three aldehydes, two furans, three terpenoids, six esters, ethanol and 1-penten-3-one), six of them were negatively influenced by storage time. Some of these volatiles have been described to be key components of strawberry scent, such as linalool, nerolidol, (*Z*)-3-hexenal and (*E*)-2-hexenal (Schwieterman et al., 2014; Ulrich & Olbricht, 2016). The off-aroma compounds, ethanol and ethyl acetate, were decreased in ozone-treated fruits compared to cold-stored samples, as were the key aroma volatiles ethyl hexanoate and hexyl acetate. By contrast, the O_3 atmosphere induced a relative increase in 11 volatiles if compared to cold storage values, ten esters and the ketone 2-pentanone. Interestingly, seven volatiles exhibited a decrease across the postharvest period. In addition, while CO_2-enriched atmosphere caused a decrease in 1-methylethyl acetate content, ozone atmosphere did the opposite (Fig. 3C).

3.5 Biomarkers for strawberry cold storage under different atmospheres

The analysis of the metabolic profiles of strawberry at different stages of postharvest storage is instrumental to uncover mechanisms underlying senescence and to identify potential biomarkers to be used by the industry to more tightly control the fruit physiological state and the quality of fruit. An important biomarker characteristic to consider is its cost effectiveness for a wide spectrum of cultivars. The linear model proposed in this study allowed us to identify metabolites affected by storage time in cold conditions and the separate modified

atmospheres (CO_2 and O_3 atmospheres) under refrigeration. The genotypic effects from all five different cultivars were also estimated using *F*-values extracted from the model (Figs. S9 and S10). Regardless of atmosphere, the amino acid GABA, a key primary metabolite involved in carbon and nitrogen metabolism, an isomer of sesquiterpenoid hexose and ethyl acetate were the most increased primary, secondary metabolites and volatiles, respectively, throughout strawberry cold storage (Tables S6–S8). Enhanced GABA levels under postharvest storage were observed in the five cultivars, even if they were higher in 'Fortuna', and lower in 'Candonga' and 'Camarosa'. Ethanol and ethyl acetate accumulation were also a common response among genotypes, being accentuated in 'Santa Clara' and 'Amiga' (Table S8). GABA and the two aforementioned fermentative volatiles showed moderate *F*-values (Fig. S10A).

However, the profiles of other compounds with lower genotypic variance and increased levels over postharvest could be more useful to comprehensively assess the dynamics of strawberry senescence under cold storage. The metabolites include the amino acids β-alanine and glycine, an isomer of cinnamic acid hexose and a series of ethyl esters (ethyl dodecanoate, decanoate, octanoate and butanoate), together with 3-methylbutyl acetate (Fig. S10, Tables S6–S8).

The metabolites that best distinguished both modified CO_2 and O_3 atmospheres from cold storage under atmospheric conditions were proline and succinic acid for primary metabolites, two isomers of bis-HHDP-glucoses and *m*-coumaric acid for secondary metabolites and the volatile 2-methylbutyl acetate (Fig. 3, Fig. S9). The water-stress related amino acid proline was significantly decreased in both CO_2 and O_3 atmospheres, especially after three and six days of storage (Fig. S6). In addition, changes observed in proline levels were stable among genotypes as suggested by a relatively low *F*-value, suggesting that fruits kept under modified CO_2 and O_3 atmospheres could undergo higher water stress than fruits maintained in ambient conditions. In this light, proline content could be used as biomarker of fruit water homeostasis. Furthermore, the relatively high levels of *m*-coumaric acid levels and their reproducibility among the cultivars can also be used to evaluate improved quality in fruits stored under CO_2 and O_3 atmospheres.

The effect of ozone on the strawberry metabolome during postharvest could be mainly defined by the increase of glucose- and fructose-6-phosphate and a shift of volatile profiles (Fig. S9). Both hexose phosphates showed an increase after ten days of O_3 atmosphere, with a more pronounced increase in 'Fortuna' cultivar, even if the trend is conserved among genotypes, as suggested by relative low *F*-values (Figs. S6 and S10).

Regarding volatiles, ethanol concentration was remarkably decreased in ozone-treated samples, with the exception of 'Fortuna' cultivar. Even if this trend tended to diminish over time, this volatile could be used as a marker for fruit quality under modified atmosphere (Fig. S8). Additionally, 3-methylbutyl acetate content was also strikingly and extendedly diminished (with the exception of 'Fortuna' cultivar in which the decrease was not maintained after ten days of O_3 atmosphere) in the fruit kept in the O_3-modified atmosphere (Fig. S8). By contrast, esters hexyl butanoate and methyl acetate, butanoate and hexanoate are significantly increased in ozone-treated samples. Based on their low *F*-values, methyl acetate and hexyl acetate would be more robust indicators of ozone effect in different strawberry cultivars (Fig. S10, Table S8). However, the effect of O_3 on volatiles tends to diminish over time, as the increase of the aforementioned esters was more apparent after three days of treatment than latter in the experiment (Fig. S8).

4 Discussion

Strawberries are particularly affected by postharvest storage, mainly due to their high respiration rate and predisposition to water loss and pathogen development (Yan et al., 2019). Several studies established how strawberry fruit quality parameters, such as SSC, firmness, pH or volatiles, were affected by many common practices such as refrigerated storage and modified atmospheres during postharvest (Almenar et al., 2006; Pérez et al., 1999). However, the impact of cold storage alone or combined with modified atmospheres (the two main strategies followed by the industry during postharvest) on strawberry fruit metabolism is still poorly understood. In this study, a metabolomic approach allowed us to describe the metabolic changes occurring in five commercial strawberry cultivars over the course of a ten-day postharvest period under refrigerated conditions alone or in combination with CO_2- or O_3-enriched atmospheres. For each individual metabolite, a linear model was then built based on the genotype, time, CO_2 and O_3 effects, to highlight the influences of these different factors on strawberry metabolism. Although, as pointed out by PCA and HCA analysis, our metabolomic data highlights the strong genotypic effect on strawberry composition, as also observed by Diamanti et al. (2012), a metabolic reconfiguration of the fruit could also be detected in response to the different postharvest treatments. Independently of the storage atmosphere, the most important physiological changes observed in our study could be summarised as (i) a decrease of main sugars and acids, impacting directly fruit taste, (ii) the alteration of volatile emission, with strong time and ozone-treatment effects and (iii) the accumulation of protective and compatible metabolites.

4.1 Biomarkers of strawberry quality are negatively influenced by postharvest duration

Postharvest duration has a strong impact on metabolic composition, as the levels of 25 primary metabolites, 27 secondary metabolites and 52 volatiles were significantly different from those quantified in T0 fruits. Indeed, primary metabolites, such as sugars, lipids, organic and amino acids have been described to act as respiratory substrates during fruit senescence (Pedreschi & Lurie, 2015). In this light, the observed significant decrease of sugars and organic acids in the course of the treatment is unsurprising. However, remodelling of primary metabolism during postharvest negatively affects fruit taste, as this characteristic is mostly dependent on the ratio between sugars and acids (Bang et al., 2019; Bustamante et al., 2016). Moreover, two of the most abundant sugars present in ripe strawberries (i.e. sucrose, being the most decreased primary metabolite along postharvest, and fructose) and the two main organic acids (citric and malic acids) were negatively impacted along time, concordant with the significant decrease of SSC, suggesting a meaningful loss of fruit palatability.

In addition, as fruits undergo abiotic stress during postharvest due to low temperature storage or low O_2 levels combined with high CO_2 concentration, an adjustment of cellular metabolism from respiration to energy-inefficient fermentation can take place (Li, Limwachiranon, Li, Du, & Luo, 2016; Pedreschi & Lurie, 2015). Indeed, the final product of glycolysis, pyruvate, may be alternatively used as a substrate for anaerobic respiration and ATP production, *via* the fermentation of ethanol. Consequently, off-aroma compounds such as ethanol, acetaldehyde and ethyl acetate accumulate and critically lead to a decline in fruit quality (Almenar et al., 2006). In strawberry, the increase of fermentative volatiles has been described as a response to CO_2 exposure and appears to be cultivar-specific (Almenar et al., 2006; Li et al., 2020). However, in the present study, ethanol and ethyl acetate displayed a strong increase along time in the five tested cultivars, with no

significant differences between cold storage under atmospheric conditions and under high levels of CO_2, suggesting that fermentation takes place in senescent strawberries even at ambient oxygen concentrations. The relatively low percentage of CO_2 used in our study (10%) was chosen based on previous studies in strawberry, in order to prevent a high fermentative metabolism and consequently an excessive accumulation of off-aroma compounds (Almenar et al., 2006). According to our model, ethanol and ethyl acetate could be selected as potential biomarkers for loss in strawberry quality during cold storage. Interestingly, previous studies have also described these two volatiles as viable markers of freeze injury (Obenland et al., 2011; Tietel, Lewinsohn, Fallik, & Porat, 2012). Moreover, the ozone atmosphere seems to have a positive influence by significantly preventing off-aroma volatile formation at least until three days of treatment. Similar results were obtained by Pérez et al. (1999) in 'Camarosa' cultivar kept at 2 °C for three days with the same ozone atmosphere.

4.2 Time and ozone effects produce extensive changes on strawberry volatilome during postharvest

In agreement with previous studies in which it is well established that postharvest practices have a detrimental impact on the aroma (Ke, Zhou, & Kader, 1994; Miszczak, Forney, & Prange, 1995; Pérez et al., 1999), we also observed that several specific volatiles were changed in response to time effect, independently of the atmosphere. In particular, the decrease of six methyl esters and the increase of six ethyl esters was especially noticeable. Furthermore, the ratio between methyl and ethyl esters is cultivar-dependent, even if the environment also strongly influences it (Hakala, Lapveteläinen, & Kallio, 2002). Interestingly, Miszczak et al. (1995) observed also an increase in the ratio of ethyl to methyl esters during the postharvest period of 'Kent' strawberry cultivar stored at 15 °C in ambient atmosphere. Ke et al. (1994) correlated ethyl esters increasing with the induction of fermentative metabolism in strawberry fruits cv. 'Chandler' that were kept in low O_2 and/or high CO_2 concentrations. Moreover, increase of ethyl esters during postharvest cold storage also seems to be a common trend in other fruits and correlated with ethanol accumulation (Brizzolara et al., 2017). In addition, a detrimental postharvest effect was observed on strawberry aroma, as the levels of 1-methylbutyl butanoate and 1-penten-3-one, both sweetness enhancers were decreased and the levels of butyl acetate, pentyl acetate and ethyl octanoate, which show negative correlation with acceptance and sweetness perception, were increased (Schwieterman et al., 2014; Ulrich & Olbricht, 2016). Due to their lower *F*-value, our model suggests that ethyl octanoate and butyl acetate may be used as biomarkers to assess strawberry postharvest quality. Furthermore, the key compound mesifurane, described as one of the main contributors to strawberry aroma, was increased over time. This volatile imparts burnt or even fusty notes to the aroma, for which high concentration in the fruit is associated with a negative acceptance coefficient (Ulrich & Olbricht, 2016). As storage in the ozone atmosphere produced significant changes in 28 volatiles, including a significant decrease in fermentative compounds (ethanol and ethyl acetate) when compared to cold storage, we were highly interested in figuring out if the use of this modified atmosphere was able to alleviate the negative impact of postharvest on strawberry aroma. In particular, ozone-treated fruits presented significantly higher levels of hexyl and methyl butanoates, two esters associated with sweetness and flavour intensity, respectively (Schwieterman et al., 2014). This result is particularly interesting for methyl butanoate, one of the most abundant volatiles present in ripe strawberry (Schwieterman et al., 2014) In addition, storage in the ozone atmosphere significantly increased 1-methylethyl acetate, an ester conferring intense fruity and sweet notes,

while CO_2-atmosphere storage had a negative impact on it. Decrease of acetate esters following CO_2 storage has previously been described, with the authors suggesting that the accumulation of ethanol favoured ethyl acetate synthesis, and thus induced a decrease of other acetate esters, as they compete for acetyl CoA as a common substrate (Ke et al., 1994). In this sense, hexyl and methyl butanoates and 1-methylethyl acetate could be used as biomarkers to evaluate the positive effect of ozone-modified atmosphere. However, O_3 application may also impair the emission of several other volatiles, some of which are known to be essential in strawberry aroma perception. In particular, ozone-treated fruits showed a significant decrease in three terpenoids (linalool, nerolidol and α-ionone) which has been positively associated with acceptance and flavour intensity by strawberry sensory tests (Schwieterman et al., 2014; Ulrich & Olbricht, 2016). In addition, O_3-enriched atmosphere negatively impacts sweetness perception by diminishing 1-penten-3-one and hexyl acetate levels, both described as sweetness enhancers, and by increasing 2-pentanone levels, which negatively correlates with sweetness (Schwieterman et al., 2014).

4.3 Postharvest strawberry fruits present metabolic stress signature, more pronounced in O_3-enriched atmosphere combined with cold storage

Under stress conditions, plants can redirect metabolism towards the synthesis of metabolites that play a role in osmoprotection, membrane protection, macromolecular structure stabilisation and requesting of reactive oxygen species (ROS) (Pedreschi & Lurie, 2015; Vazquez-Hernandez, Navarro, Sanchez-Ballesta, Merodio, & Escribano, 2018). These compounds include primary metabolites, such as sugars and amino acids, and were especially outlined in our model. GABA was significantly enhanced during cold storage, this increase being significantly moderated in both modified atmospheres, even if previous studies in strawberry fruits linked GABA accumulation with CO_2 atmosphere (Li et al., 2018). GABA is metabolised to NADH and succinic acid in the GABA shunt; succinic acid can subsequently enter the TCA cycle or act as an electron donor to the mitochondrial electron transport chain (Li et al., 2018). Interestingly, succinic acid increase was observed in the fruits from the three treatments, probably because of GABA shunt activity converting it to a putative strawberry postharvest biomarker. Indeed, GABA shunt activity during postharvest cold storage has been described as fundamental for maintaining fruit quality attributes, by providing intracellular ATP and carbon skeletons and thus delaying senescence (Li et al., 2018; Wang et al., 2019).

Proline levels displayed a significant decrease in both modified atmospheres when compared to cold storage. This amino acid is known to protect against dehydration and also to be an effective free-radical scavenger (Wang et al., 2017). It has been previously described that proline increase in grape stored under refrigerated conditions supplemented with CO_2 was related to enhanced tolerance to water deficit (Vazquez-Hernandez et al., 2018). In this sense, our result suggests that proline metabolism was somehow impaired in CO_2- and O_3-treated strawberries, which in turn may indicate a reduced tolerance of the fruits to postharvest-associated stresses (Wang et al., 2017).

Metabolic reconfiguration of strawberry physiology as a response to postharvest practices was more apparent in ozone-treated fruits. Interestingly, two sugars and two sugar phosphates (i.e. trehalose, raffinose, fructose-6-phosphate and glucose-6-phosphate) and the sugar alcohol galactinol, were significantly up-regulated under O_3 storage when compared to cold storage under non-modified atmosphere. Sugars play a role in plant cells

osmotic adjustment in response to temperature stress (Bustamante et al., 2016; Navarro et al., 2015). Due to its properties, the disaccharide trehalose is particularly efficient in protecting plant proteins against cold and dehydration stresses, and its increase has been reported in grape cold postharvest storage (Navarro et al., 2015). Galactinol has been described to be involved in cold response and, together with sucrose, it takes part in raffinose synthesis, which has been described as an essential cold-inducible biosynthetic route (Bustamante et al., 2016). Apart from their osmoprotective functions, galactinol and raffinose, together with the two detected phosphorylated hexoses (fructose- and glucose-6-phosphate) can act as antioxidant molecules, having the ability to scavenge hydroxyl radicals and thereby decrease the levels of ROS (Rohloff et al., 2012). In addition to changes in sugar metabolism, accumulation of several polyphenol compounds was particularly striking in ozone-treated fruits and would suggest a positive role for O_3 atmosphere by improving strawberry fruit nutritional characteristics, in spite of previous studies outlining a negative effect of ozone on total polyphenols and anthocyanins (Pérez et al., 1999). It is also worth noting that refrigerated and dark storage conditions in our experiment may have a detrimental effect on polyphenol accumulation (Ayala-Zavala et al., 2004; Sun et al., 2017). Furthermore, a decreased activity of phenylalanine ammonia lyase, the key enzyme of the phenylpropanoid pathway, has been described in response to CO_2 atmosphere in strawberry (Li et al., 2019). However, both modified atmospheres induced an increase of two ellagitannin isomers (bis(HHDP)glucose), while 16 soluble tannins, including one of the bis(HHDP) glucose isomers, were significantly decreased during the postharvest period. Increase of ellagitannins has previously been described during blackberry cold storage and was explained as a result of stress and/or pathogen defence (Segantini et al., 2017). Interestingly, soluble tannins and their derivatives are known to be powerful antioxidant molecules (Giampieri et al., 2012), as such their increase under modified atmosphere may be highly valuable. In this sense, bis(HHDP) glucose isomers could be considered as possible biomarkers for strawberry quality in CO_2 and O_3 storage.

5 Conclusions

The analysis of the metabolic reconfiguration of strawberry physiology in response to postharvest practices allows us to gain a better understanding of the different factors influencing fruit quality traits. In this sense, the potential biomarkers ethanol, ethyl, butyl and 1-methylethyl acetates, ethyl octanoate, hexyl and methyl butanoates, GABA, succinic acid and bis(HHDP)glucose that could help anticipate strawberry organoleptic deterioration were determined by using a linear modelling strategy. Moreover, the current challenge for modern breeding programs is to deliver commercial cultivars with improved flavour, but that maintain extended postharvest and yield. In this vein, our results open also new opportunities for identifying new genotypes with increased fruit shelf-life and fruit quality stability. In addition, the use of an ozone-modified atmosphere may have a positive impact on fruit aroma by reducing fermentative compounds and increasing one of the most abundant esters (methyl butanoate) as well as on fruit tolerance to the abiotic stresses generated by postharvest cold storage, by redirecting metabolism towards the accumulation of protective metabolites.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126747.

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Highlights

- Strawberry fruit is highly affected and perishable during postharvest.
- Metabolic reconfiguration of fruits in response to different postharvest treatments.
- Ozone atmosphere has a positive impact on fruit tolerance to cold stress and aroma.
- CO_2 atmosphere has a limited effect on the metabolome in contrast to O_3 atmosphere.
- Identification of 10 compounds as biomarkers to assess fruit quality postharvest.

Appendix A Supplementary data

The following are the Supplementary data to this article:

Multimedia Component 1

Supplementary data 1

Multimedia Component 2

Supplementary data 2

Multimedia Component 3

Supplementary data 3

Multimedia Component 4

Supplementary data 4

Multimedia Component 5

Supplementary data 5

Multimedia Component 6

Supplementary data 6

Multimedia Component 7

Supplementary data 7

Multimedia Component 8

Supplementary data 8

Queries and Answers

Query: Your article is registered as a regular item and is being processed for inclusion in a regular issue of the journal. If this is NOT correct and your article belongs to a Special Issue/Collection please contact d.krish@elsevier.com immediately prior to returning your corrections.

Answer: Yes

Query: The author names have been tagged as given names and surnames (surnames are highlighted in teal color). Please confirm if they have been identified correctly.

Answer: I have included thre modifications:

1. de Abreu e Lima

2. afilation for Francisco de Abreu e Lima should be "b, 1".

3. It has to be corrected vallarino´s mail. The correct mail is vallarino@uma.es