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Volatile Emission after Controlled Atmosphere Storage of Mondial Gala Apples (*Malus domestica*): Relationship to Some Involved Enzyme Activities

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Mondial Gala apples were harvested at commercial maturity and stored at 1 °C under either air or controlled atmosphere (CA) conditions (2 kPa O_2/2 kPa CO_2 and 1 kPa O_2/1 kPa CO_2), where they remained for 3 or 6 months. Data on emission of selected volatile esters, alcohol precursors, and activity of some aroma-related enzymes in both peel and pulp tissues were obtained during subsequent shelf life of fruit and submitted to multivariate analysis procedures. CA storage caused a decrease in the emission of volatile esters in comparison to storage in air. Results suggest that lessened ester production was the consequence of modifications in activities of alcohol *o*-acyltransferase (AAT) and lipoxygenase (LOX) activities. For short-term storage, inhibition of lipoxygenase activity in CA stored fruit possibly led to a shortage of lipid-derived substrates, resulting in decreased production of volatile esters in spite of substantial ester-forming capacity that allowed for some recovery of fruit capacity for ester emission during the shelf life. For long-term storage, strong inhibition of AAT activity in CA stored fruit in combination with low LOX activities resulted in unrecoverable diminution of biosynthesis of volatile esters.

KEYWORDS: Alcohol *o*-acyltransferase; alcohol precursors; aroma; controlled atmosphere; lipoxygenase; *Malus domestica*; volatile esters

INTRODUCTION

Aroma is an important factor affecting the final sensory quality of fruit produce and hence consumer satisfaction. The aroma profile of a fruit is complex and depends on the combination of all volatile compounds emitted, as well as on the concentration and odor threshold of each individual compound emitted. In apple (*Malus domestica* L. Borkh) fruit, over 300 volatile compounds have been detected in the aroma profile, including alcohols, aldehydes, carboxylic esters, ketones, and ethers (*1*). Among these, esters are associated with the "fruity" attributes of fruit flavor and are the most conspicuous contributors to the aroma profile of intact apples, in both quantitative and qualitative terms (*2*−*6*). Esters produced by a ripening apple fruit can be broadly separated into straight-chain and branched-chain types. Whereas straight-chain esters are thought to be derived from fatty acids via the lipoxygenase (LOX) and β-oxidation pathways, branched-chain esters are thought to arise from the metabolism of branched-chain amino acids (*7*−*9*). Alcohol *o*-acyltransferase (AAT) catalyzes the final linkage of an acyl moiety from a donor (acyl CoA) to the hydroxyl group of an acceptor alcohol contributed by these or other biosynthetic pathways and is thus directly responsible for the production of volatile esters by fruit tissues.

Two apple genes encoding for AAT have recently been isolated, characterized, and shown to be ethylene-regulated (*10*, *11*). Both genes are almost identical at the amino acid level and are most closely related to other AATs isolated from fruits such as melon (*Cucumis melo* L.) (*12*), cultivated strawberry (*Fragaria ananassa* Duch.) (*13*), wild strawberry (*Fragaria vesca* L.), or banana (*Musa sapientum* L.) (*14*). In all instances, broad substrate preferences have been reported for the corresponding enzyme, and further evidence has been provided that this substrate preference is not necessarily reflected in the representation of esters in the corresponding fruit volatile profile. The maturity stage at harvest is critical in this regard, as gene expression of the involved enzymes and thus production of the corresponding intermediates depends in part on the developmental stage of tissues. These findings suggest that the supply of precursors and thus availability of alcohol substrates may play a major role in determining the specific esters emitted by fruit. This is also supported by numerous feeding experiments in which the addition of intermediates enhanced the production of specific volatile compounds. For instance, treatment of apple fruit or tissue sections with the vapors of alcohols, aldehydes, or carboxylic acids significantly increased concentrations of the corresponding volatile esters (*1*, *15*−*18*). Similarly, incorporation of deuterium into both straight- and branched-chain ester volatiles has been demonstrated after the incubation of apple fruit tissue with deuterated flavor precursors (*7*−*8*, *19*).

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Table 1. Chemical Standards Used

compound	CAS no.	source
ethyl acetate	141-79-6	99.5% Fluka
ethanol	64-17-5	99.9% Panreac
tert-butyl propanoate	20487-40-5	99% Fluka
propyl acetate	109-60-4	98% Fluka
2-methylpropyl acetate	110-19-0	99% Avocado
1-propanol	71-23-8	99% Fluka
ethyl butanoate	105-54-4	98% Fluka
ethyl 2-methylbutanoate	7452-79-1	95% Fluka
butyl acetate	000123-86-4	98.5% Fluka
2-methyl-1-propanol	78-83-1	99.5% Fluka
2-methylbutyl acetate	123-92-2	98% Aldrich
1-butanol	71-36-3	99.5% Fluka
butyl propanoate	590-01-2	99% Aldrich
pentyl acetate	628-68-7	99% Fluka
2-methylbutyl propanoate	105-68-0	98% Aldrich
2-methyl-1-butanol	13-94-5	98% Fluka
D-limonene	5989-27-5	98% Aldrich
butyl butanoate	109-21-7	99% Fluka
butyl 2-methylbutanoate	15706-73-7	97% Aldrich
1-pentanol	71-41-0	99% Aldrich
hexyl acetate	142-92-7	99% Fluka
hexyl propanoate	1040036	99.64% Extrasynthese
1-hexanol	000111-27-3	99% Fluka
butyl hexanoate	626-82-4	98% Aldrich
hexyl butanoate	2639-63-6	98% Aldrich
hexyl 2-methylbutanoate	49-7729-970	95% Fluka
hexyl hexanoate	6378-65-0	97% Aldrich

Consumer acceptance of apple fruit has been reported to correlate with the production of some esters (*5*). This observation is of commercial importance because, despite the beneficial effects of controlled atmosphere (CA) storage on a number of fruit quality aspects, this storage technology is also known to result in partial inhibition of the capacity for ester biosynthesis in several apple cultivars (*5*, *20*−*25*). Therefore, CA storage may lead to decreased commercial value of produce through a lessening in production of some aroma volatiles. This lessening in volatile emission is likely to arise to some extent from partial suppression of oxidative processes required for substrate production. For instance, partial inhibition of LOX activity was found in CA stored Fuji apple (*26*) and Doyenne du Comice pear (*Pyrus communis* L.) (*27*), leading to abnormal development of fruit aroma after transfer from hypoxia to air. This is in agreement with reports showing that the recovery capability for volatile biosynthesis after CA storage was more pronounced for amino acid-derived than for fatty acid-derived volatiles (*24*). These previous works evidenced the relevance of precursor supply for aroma volatile production throughout the shelf life of fruit after storage.

Although apple cultivars in the Gala group show excellent organoleptic properties including strong, pleasant aroma and flavor (*28*), they also display rapid softening rates resulting in poor fruit quality after extended periods of storage (*29*). The purpose of this work was to examine the suitability of Mondial Gala apples for long-term CA storage with regard to the emission of aroma volatile compounds during the subsequent shelf life of fruit. Some related enzyme activities were analyzed to identify possible key points controlling ester production after storage.

MATERIALS AND METHODS

Plant Material. Apple fruits (*Malus domestica* cv. Mondial Gala) were picked in 2003 from a commercial orchard near Lleida (northeast Spain) and selected for uniformity and the absence of defects. Harvest took place at the usual commercial maturity in the area, at approximately 120 days after full bloom (dafb). Firmness at harvest averaged 77.2 N, soluble solids content was 12.3 g/100 g FW^{-1}, and titratable acidity was 3.6 g malic acid/L^{-1}. Immediately after harvest, fruit were placed at 1 °C and about 92% RH under either air or two different controlled atmosphere conditions, namely, 2 kPa O_2/2 kPa CO_2 and 1 kPa O_2/1 kPa CO_2. The O_2 and CO_2 concentrations were monitored and automatically corrected using N_2 from a tank and by scrubbing off excess CO_2 with a charcoal system. Samples were removed from storage after 3 or 6 months and placed at 20 °C to simulate commercial shelf life. Analyses were carried out 1 and 7 days thereafter. Aroma-related enzyme activities were determined additionally upon removal from storage (day 0).

Table 2. Meaning of *X*-, *Y*-, and *Z*-Values for the Sample Generic Labels

	1	2	3
X[a]	air	2:2	1:1
Y[b]	3	6	
Z[c]	1	7	

[a] Storage atmosphere conditions as described in Materials and Methods (O_2/CO_2). [b] Storage period (months). [c] Shelf life period (days).

Table 3. Emission of Aroma Volatile Compounds (μg kg^{-1}) by Mondial Gala Apples 7 days after Harvest

compound	RI[a]	OTh[b] (μg L^{-1})	amount[c] (μg kg^{-1})	OU[d]	code[e]
ethyl acetate	898	13500 (a)	12.0		etac
ethanol	932	100000 (b)	10.7		etOH
tert-butyl propanoate	964	19 (c)	5.2		
propyl acetate	984	2000 (a)	69.3		
2-methylpropyl acetate	1020	65 (d)	40.6		
1-propanol	1036	9000 (b)	27.6		
ethyl butanoate	1043	1 (e)	4.5	4.5	etbut
ethyl 2-methylbutanoate	1059	0.006 (d)	5.4	900	et2mebut
butyl acetate	1082	66 (a)	2177.4	33	butac
2-methyl-1-propanol	1091	250 (f)	22.1		
2-methylbutyl acetate	1131	11 (d)	847.0	77	2mebutac
1-butanol	1144	500 (b)	259		buOH
butyl propanoate	1148	25 (b)	126.8	5.1	butpro
pentyl acetate	1183	43 (a)	116.5	2.7	petac
2-methylbutyl propanoate	1199	19 (c)	15.4		
2-methyl-1-butanol	1210	250 (e)	43.5		2mebuOH
D-limonene	1219	34 (e)	1.2		
butyl butanoate	1228	100 (g)	98.1		
butyl 2-methylbutanoate	1240	17 (g)	90.9	5.3	but2mebut
1-pentanol	1253	4000 (f)	5.3		peOH
hexyl acetate	1283	2 (f)	931.0	465.5	hexac
hexyl propanoate	1349	8 (h)	92.6	11.6	hexpro
1-hexanol	1358	500 (f)	26.4		heOH
butyl hexanoate	1423	700 (h)	320.0		
hexyl butanoate	1426	250 (d)	107.9		
hexyl 2-methylbutanoate	1436	6 (g)	90.6	15.1	hex2mebut
hexyl hexanoate	1621	–	140.3		
total aroma volatiles[f]		5687.1			

[a] Kovats retention index in a cross-linked FFAP column (*40*). [b] Odor thresholds as reported in (a) ref *41*, (b) ref *42*, (c) ref *43*, (d) ref *44*, (e) ref *45*, (f) ref *46*, (g) ref *47*, and (h) ref *48*. –, not found. [c] Values represent means of four replicates. [d] Odor units = amount/OTh. Only values >1 are indicated. [e] Codes used for multivariate analysis. [f] Total amount of all aroma volatile compounds detected during chromatographic analyses.

Chemical Standards and Reagents. The standards for the volatile compounds studied in this work were supplied by Sigma-Aldrich (Steinheim, Germany), Fluka (Chemie, Switzerland), Panreac Química, S.A. (Castellar del Vallès, Spain), Avocado Research Chemicals Ltd. (Madrid, Spain), and Extrasynthese (Genay, France). The CAS number, commercial source, and purity of each compound are shown in **Table 1**. All standards used were of analytical grade. Reagents used for analysis of enzyme activity were purchased from Sigma-Aldrich and Bio-Rad (Bio-Rad Laboratories Inc., Hercules, CA).

Analysis of Volatile Compounds. The extraction of volatile

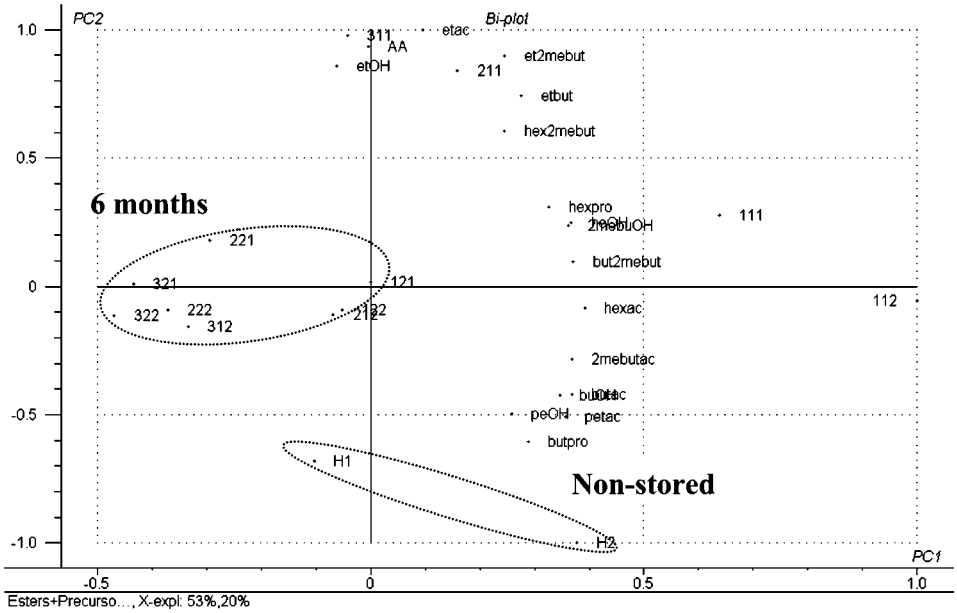

Figure 1. Biplot of PC1 vs PC2 corresponding to a PCA model for aroma volatile compounds emitted by Mondial Gala fruit. H1 and H2 stand for non-stored fruit, analyzed 1 and 7 days after harvest, correspondingly. Samples and volatile compounds are labeled as indicated in **Tables 2** and **3**, respectively (AA, acetaldehyde).

compounds was performed from a sample (2 kg × 4 replicates) of intact fruit according to the method of dynamic headspace. Each fruit sample was placed in a 8-l Pyrex glass container, and an air stream (900 mL min^{-1}) was passed through for 4 h; the effluent was then passed through an ORBO-32 adsorption tube filled with 100 mg of activated charcoal (20/40 mesh), from which volatile compounds were de-adsorbed by agitation for 40 min with 0.5 mL of diethyl ether. Identification and quantitation of volatile compounds were achieved on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector and a cross-linked free fatty acid phase (FFAP; 50 m × 0.2 mm i.d. × 0.33 μm) as the capillary column, where a volume of 1 μL from the extract was injected in all the analyses. Helium was used as the carrier gas (42 cm s^{-1}), with a split ratio of 1:40. The injector and detector were held at 220 and 240 °C, respectively. The analysis was conducted according to the following program: 70 °C (1 min); 70−142 °C (3 °C min^{-1}); 142−225 °C (5 °C min^{-1}); and 225 °C (10 min), as described elsewhere (*30*). Volatile compounds were identified by comparing retention indexes with those of standards and by enriching the apple extract with authentic samples. The quantification was made using butyl benzene (assay >99.5%, Fluka) as the internal standard. A GC−-MS system (Hewlett-Packard 5890) was used for compound confirmation, in which the same capillary column was used as in the GC analyses. Mass spectra were obtained by electron impact ionization at 70 eV. Helium was used as the carrier gas (42 cm s^{-1}), according to the same temperature gradient program as described previously. Spectrometric data were recorded (Hewlett-Packard 3398GC Chemstation) and compared with those from the NIST HP59943C original library mass spectra. Results were expressed as μg kg^{-1}.

Analysis of Acetaldehyde Concentration. Juice from 20 fruits per treatment (atmosphere × storage period × shelf life period) was individually obtained and frozen at −20 °C until analysis of the acetaldehyde content as described in ref *31*. Frozen juice from each fruit was thawed, and a 5 mL sample was introduced in a 10 mL test tube, which was closed with an elastic cap and incubated at 65 °C for 1 h. A 1 mL headspace gas sample was taken with a syringe and injected into a Hewlett-Packard 5890 gas chromatograph, equipped with a column containing Carbowax (5%) on Carbopack (60:80, 2 m × 2 mm i.d.) as the stationary phase, and a flame ionization detector. Nitrogen was used as the carrier gas (24 cm s^{-1}), and operating conditions were as follows: oven temperature 110 °C, injector temperature 180 °C, and detector temperature 220 °C. Acetaldehyde was identified and quantified by comparison with an external standard, and results were expressed as μL L^{-1}.

Extraction and Assay of Aroma-Related Enzyme Activities. LOX, hydroperoxide lyase (HPL), pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), and AAT activities were determined on days 0, 1, and 7 after removal from storage. Samples of both peel and pulp tissue were taken separately from four apples, frozen in liquid nitrogen, lyophilized, powdered, and kept at −80 °C until processing. One hundred milligrams of lyophilized powdered tissue was used for each determination. Extraction and assay of LOX, PDC, ADH, and AAT activities on crude enzyme extracts were performed as described elsewhere (*27*). HPL activity was extracted and assayed according to ref *32*. Total protein content in the enzyme extract was determined with the Bradford method (*33*), with modifications (BioRad Protein Assay kit) according to the manufacturer's instructions, using BSA as a standard. In all cases, one activity unit (U) was defined as the variation in one unit of absorbance per minute. Each determination was done in triplicate, and results were expressed as specific activity (U mg $protein^{-1}$).

Statistical Analyses. A multi-factorial design with storage atmosphere, storage period, shelf life period, and replication as factors was used to statistically analyze results. All data were tested by analysis of variance (GLM-ANOVA), according to standard SAS-STAT procedures (*34*). Means were separated by a L.S.D. test at $p \leq 0.05$. In addition, multivariate analysis procedures were used to help the interpretation of the information contained in the data set while avoiding the collinearity effects found in conventional multiple linear approaches (*35*). To provide a general visualization of all the information obtained, principal component analysis (PCA) was used. Sample names were coded as *XYZ*, where *X*, *Y*, and *Z* refer to storage atmosphere, storage period, and days of shelf life, respectively, and take values of 1, 2, or 3 as indicated in **Table 2**. Volatile compounds analyzed were labeled as specified in **Table 3**. Partial least-square regression (PLSR) was also used as a predictive method to relate a matrix of several dependent variables (*Y*) to a set of explanatory variables (*X*) in a single estimation procedure. Unscrambler version 6.11a software (*36*) was used for developing these models. As a pretreatment, data were centered and weighed by the inverse of the standard deviation of each variable to avoid dependence on measured units (*35*). Full cross-validation was run as a validation procedure.

RESULTS AND DISCUSSION

A total of 27 aroma compounds (19 esters, seven alcohols, and one terpene) were identified in the volatile fraction emitted

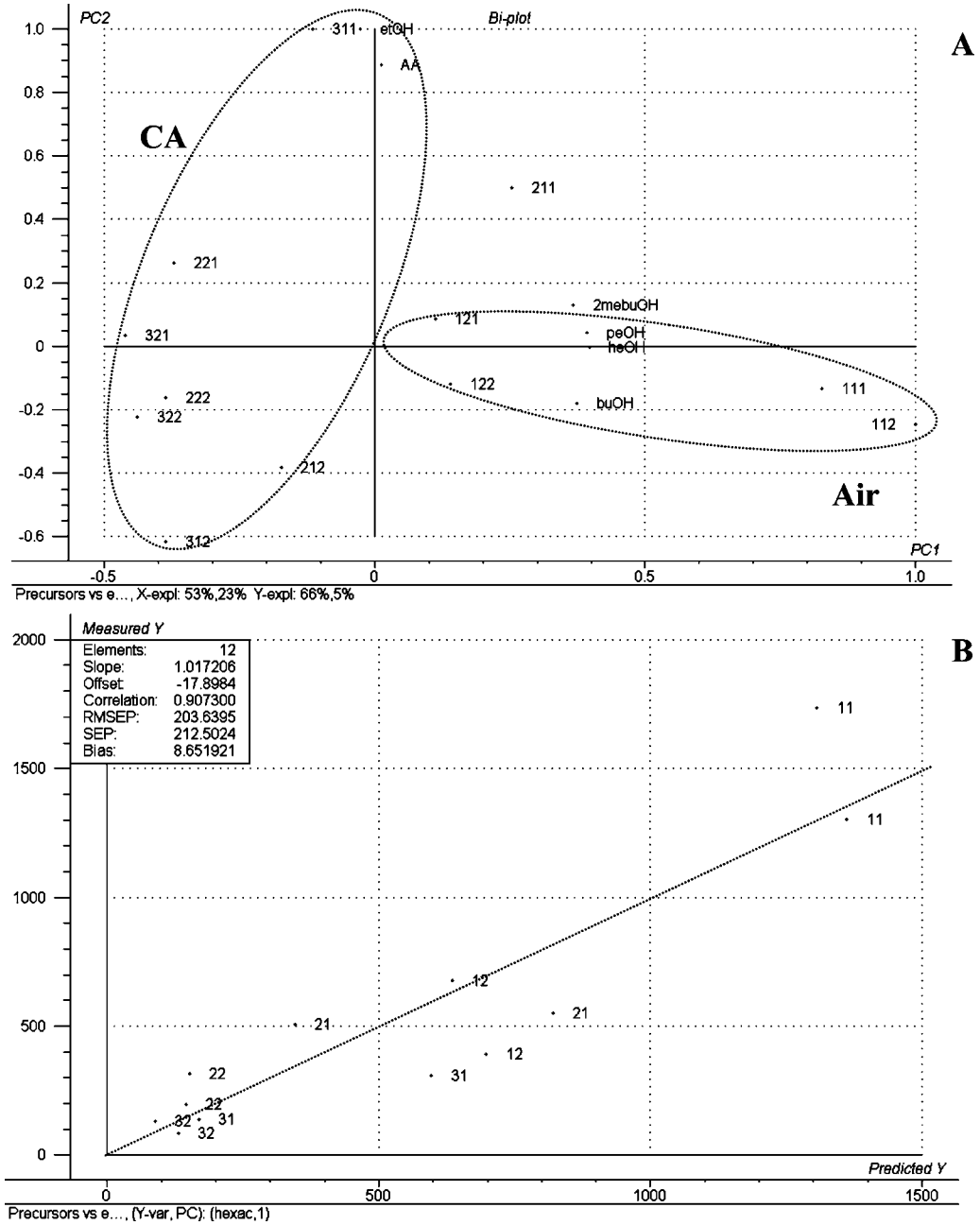

Figure 2. (**A**) Biplot (scores and loadings) of PC1 vs PC2 corresponding to a PLSR model of volatile esters emitted (*Y*) vs precursors available (*X*) in Mondial Gala fruit after storage. (**B**) Predicted vs measured hexyl acetate emitted by Mondial Gala fruit after storage, using full-cross validation. Samples and volatile compounds are labeled as indicated in **Tables 2** and **3**, respectively (AA, acetaldehyde). In panel **B**, for clarity, only the two first digits are shown.

by Mondial Gala apples at harvest (**Table 3**). Ten of them were deemed as likely to have an impact on overall flavor of non-stored fruit based on their showing odor units >1 (*37*) and therefore were chosen to examine changes in the biosynthesis of aroma volatiles after storage. All 10 chosen compounds were esters, namely, four acetates, two propanoates, one butanoate, and three 2-methylbutanoates (**Table 3**). In addition, six precursors to these compounds (ethanol, 1-butanol, 2-methylbutanol, 1-pentanol, 1-hexanol, and acetaldehyde), as well as ethyl acetate as an indicator of the possible onset of fermentative processes in CA stored fruit, were also included in the analyses. Selected compounds accounted together for over 85% of total volatiles produced by Mondial Gala apples 1 week after harvest (**Table 3**).

Selected esters and precursors were used to characterize samples both at harvest and after storage (14 samples × 17 variables) through the development of a PCA model, which allows a global visualization of results and of possible relationships among variables. Samples were coded as defined in **Table 1**. Principal components 1 (PC1) and 2 (PC2) accounted for 73% of total variability. The score plot of PC1 versus PC2 for this model (**Figure 1**) shows that non-stored samples (labeled H1 and H2 and corresponding to fruit analyzed 1 and 7 days after harvest, respectively) separated clearly from cold stored fruit along PC2, indicating differences in volatile emission after storage. Non-stored samples were characterized by higher production of butyl propanoate, butyl acetate, and pentyl acetate, in concomitance with higher levels of their alcohol precursors 1-butanol and 1-pentanol. These samples also had the lowest emission of all three selected ethyl esters, which were the variables showing the highest weight on differentiation along PC2.

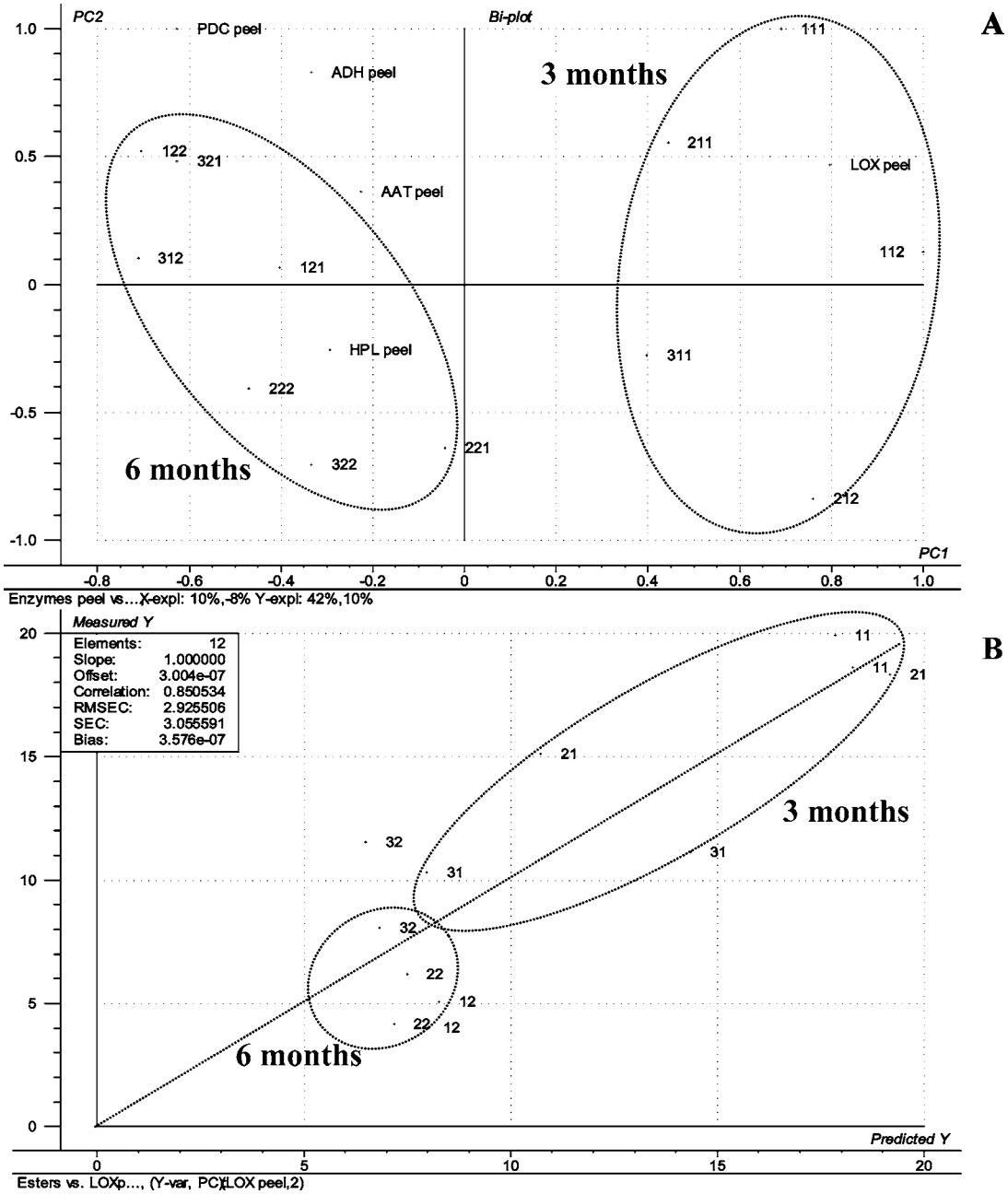

Figure 3. (**A**) Biplot (scores and loadings) of PC1 vs PC2 corresponding to a PLSR model of volatile esters emitted (*Y*) vs aroma-related enzyme activities (*X*) in the peel of Mondial Gala fruit after storage. (**B**) Predicted vs measured LOX activity in the peel of Mondial Gala fruit after storage. Samples are labeled as indicated in **Table 2**. In panel **B**, for clarity, only the two first digits are shown.

Cold stored samples separated along PC1, the main factor for group differentiation being storage period rather than storage atmosphere (**Figure 1**). For each period, however, samples separated clearly along the PC1 axis as a function of storage conditions. Fruit stored for 3 months showed higher emission of all 10 selected esters as well as of their alcohol precursors, suggesting better preservation of the characteristic flavor of this apple cultivar as compared to long-term stored samples. For short-term stored fruit, CA conditions resulted in reduced emission of selected ethyl esters, probably facilitated by higher levels of ethanol and acetaldehyde in CA stored samples. However, these samples also had decreased production of the rest of the selected esters, indicating that the biosynthetic pathways involved in the development of fruit aroma were differentially affected by CA conditions and that this storage technology can result in both enhancement and suppression of specific flavor volatiles.

The shelf life period after cold storage also had an influence on volatile production, as shown by groupings along PC2, which accounted for 20% of the total variability (**Figure 1**). Samples kept at 20 °C during 1 day after cold storage were located above PC1 and were characterized by higher production of ethyl acetate, ethyl butanoate, ethyl 2-methylbutanoate, ethanol, and acetaldehyde. Contrarily, the production of butyl esters and 1-butanol, their alcohol precursor, was higher after 7 days of shelf life. The plot for this PCA model also shows that fruit kept at 20 °C for 7 days was situated closer to non-stored samples than those kept for only 1 day, suggesting partial recovery along the shelf life of the capacity for production of those volatiles characterizing non-stored fruit. This partial recovery might have arisen from the metabolization of ethanol and acetaldehyde accumulated during storage under hypoxic conditions, as suggested by a higher acetate ester emission in fruit 7 days after removal from storage (**Figure 1**).

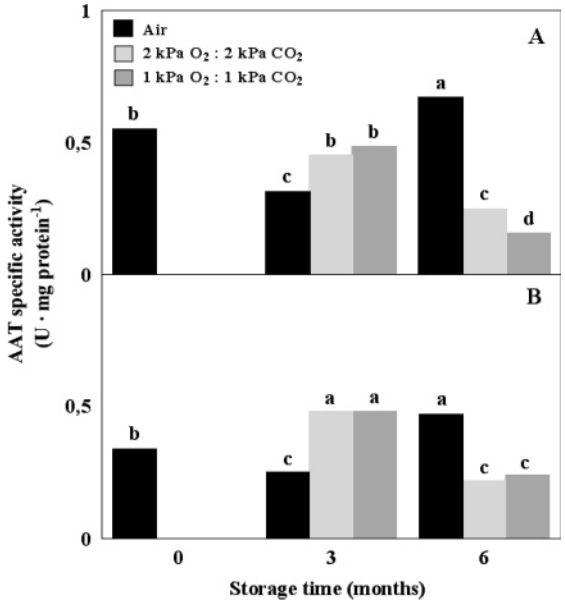

Figure 4. AAT activity in peel (**A**) and pulp (**B**) of Mondial Gala fruit upon removal from storage (day 0). Values represent means of three replicates. Means showing different letters (**a**–**c**) for a given tissue are significantly different at $P \leq 0.05$ (LSD test).

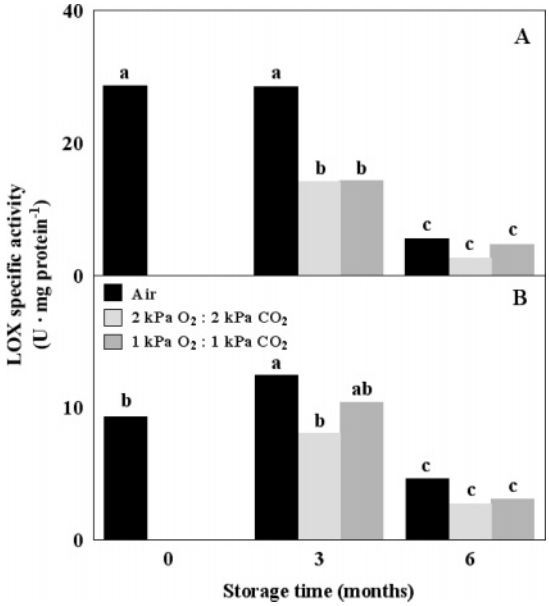

Figure 5. LOX activity in peel (**A**) and pulp (**B**) of Mondial Gala fruit upon removal from storage (day 0). Values represent means of three replicates. Means showing different letters (**a**–**c**) for a given tissue are significantly different at $P \leq 0.05$ (LSD test).

Role of Precursor Availability in Ester Biosynthesis during Shelf Life of Fruit. With the aim of assessing possible correlations between selected volatile esters (*Y* variables) and alcohols and acetaldehyde (*X* variables), a PLSR model was developed. This procedure allows the rapid assessment of relationships between a matrix of dependent variables (*Y*) to a set of potentially explanatory variables (*X*). According to this model, up to 71% of variability in ester emissions was explained by precursor availability (**Figure 2A**). The storage atmosphere was the main factor for sample differentiation, as air and CA stored fruit separated clearly along PC1, which accounted alone for 66% of the total variability. The shelf life period was the main factor for sample differentiation along PC2, but the low percentage of variability explained by this PC (5%) suggests a limited influence of this factor for overall sample differentiation.

Variables with the most weight for differentiation along PC1 were 1-hexanol, 1-pentanol, 2-methyl-butanol, and 1-butanol, which characterized air stored samples (**Figure 2A**), meaning that these fruit displayed higher emissions of these precursors than CA stored samples and suggesting that the availability of the precursors was an important factor explaining the production of volatile esters after storage. In addition, both air and CA stored samples split into two groups as a function of storage period. The plots of predicted versus measured ester emissions showed the highest correlation coefficients for hexyl acetate ($r = 0.91$), 2-methylbutyl acetate ($r = 0.89$), pentyl acetate ($r = 0.87$), and butyl acetate ($r = 0.81$). These plots confirmed that the production of volatile esters indeed could be predicted from levels of precursors present, which is in accordance with earlier works reporting that the supply of specific intermediates enhanced the production of the corresponding volatile compounds (*1*, *7*−*8*, *15*−19). In all cases, the levels of precursors were higher for air than for CA stored samples, as they were for short-term rather than for long-term storage. The plot for hexyl acetate (**Figure 2B**) is given as an example, similar results having been found for 2-methylbutyl acetate, pentyl acetate, butyl acetate, butyl 2-methylbutanoate, and, with lower correlation coefficients, for hexyl 2-methylbutanoate, hexyl propanoate, ethyl butanoate, and butyl propanoate.

Role of Enzyme Activity in Ester Biosynthesis during Shelf Life of Fruit. Separate PLSR models for peel and pulp tissues were developed to assess the relationships, if any, between some enzyme activities related to aroma biosynthesis (*X* variables) and precursor availability (*Y* variables). Values of residual variance (result not shown) indicated that the model for the peel tissue was not valid, suggesting that levels of alcohol precursors were not related to enzyme activity in this tissue. However, when relating the same activities (*X* variables) to esters emitted (*Y* variables), the corresponding PLSR model revealed that the activity of the enzymes considered in this work accounted for up to 52% of the variability in the emission of selected esters (**Figure 3A**). The biplot for this model also shows that the main factor for differentiation was the storage period rather than the storage atmosphere, as short- and long-stored samples separated along the first principal component, which explained alone 42% of total variance. LOX activity was the variable with most weight for this differentiation (regression coefficient = 0.83), and it was associated to fruit stored for 3 months, suggesting that this enzyme activity declined after extended storage periods. Ethyl butanoate, ethyl 2-methylbutanoate, and 2-methylbutyl acetate were the volatile esters apparently most dependent on LOX activity in the peel tissue (regression coefficients = 0.72, 0.72, and 0.76, respectively). The plot of predicted versus measured LOX activity (**Figure 3B**) shows that this enzyme activity indeed could be predicted from levels of volatile esters emitted and confirmed that samples grouped preferentially according to storage period. Within the group corresponding to samples stored for 3 months, some differentiation was also found according to the storage atmosphere. Air stored fruit displayed the highest levels of LOX activity, consistent with the fact that LOX is an O_2 requiring enzyme and in agreement with the idea that CA-induced lessening in volatile emissions arises to some extent from partial suppression of the oxidative processes required for substrate production.

The apparent lack of a clear relationship between enzyme activities in the peel tissue and availability of alcohol precursors might have arisen from rapid utilization of substrates during the subsequent shelf life. This suggestion is supported by data

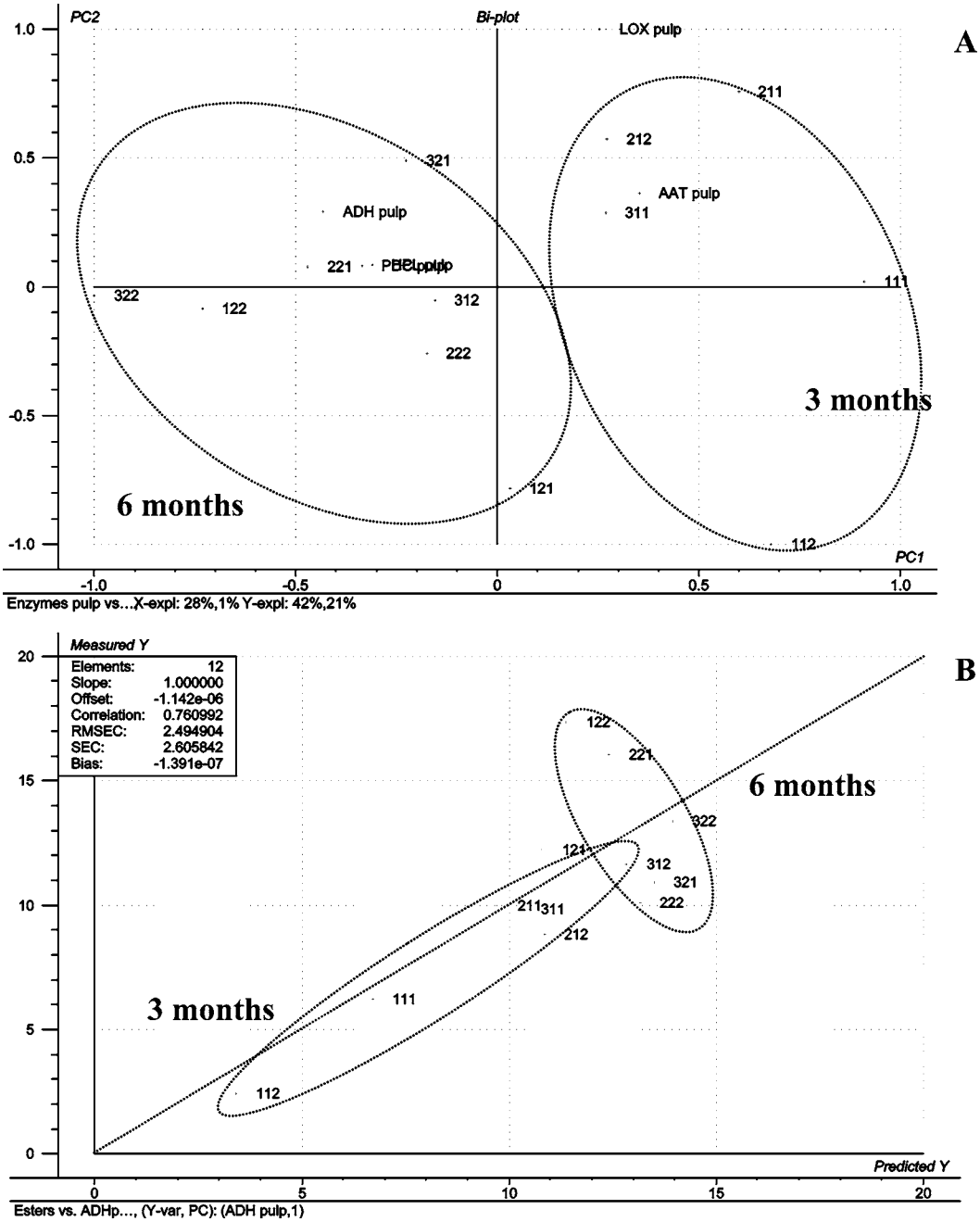

Figure 6. (**A**) Biplot (scores and loadings) of PC1 vs PC2 corresponding to a PLSR model of volatile esters emitted (*Y*) vs aroma-related enzyme activities (*X*) in the pulp of Mondial Gala fruit after storage. (**B**) Predicted vs measured ADH activity in the pulp of Mondial Gala fruit after storage. Samples are labeled as indicated in **Table 2**.

on AAT activity upon removal from storage (**Figure 4**). Results indicate that AAT activity was present during storage, allowing for immediate substrate processing during the shelf life of fruit. After storage for 3 months, the activity was actually higher in CA than in air stored fruit, and therefore, any reduction in ester emission after storage should be attributed to precursor shortage. In contrast, AAT activity was strongly inhibited in CA stored samples after long-term storage, which might have accounted in part for lowered ester emission in these fruit (**Figure 1**). It is thus apparent that the recovery of the capability for the production of volatile esters after CA storage of Mondial Gala fruit was strongly influenced by the storage period.

LOX activity, which was the enzyme activity in the peel tissue with the most influence on the emission of volatile esters during shelf life after storage (**Figure 3A**), was also found to be differentially affected by storage period (**Figure 5**). Whereas CA storage clearly inhibited LOX activity in fruit stored for 3 months, no significant differences between storage atmospheres were found for samples stored for 6 months. Activity levels immediately after removal from storage were significantly decreased in long-term stored samples in comparison with those after a shorter storage period, even when compared with CA stored fruit. In both cases, results suggest that the recovery of activity was not possible during the subsequent shelf life of fruit. Consequently, lowered levels of fatty acid-derived precursors might have accounted for decreased ester production, in combination with a reduced capacity for ester biosynthesis in long-term stored fruit.

With the aim of assessing possible tissue-related regulatory differences, a separate PLSR model was developed for the pulp in which enzyme activities in this tissue (*X* variables) were related to the availability of alcohols and acetaldehyde (*Y*

variables). As already found for the peel, residual variance values (results not shown) indicated that the model was not valid, again suggesting rapid processing of substrates upon removal from storage. Therefore, the enzyme activities considered (*X* variables) were related to the emission of selected esters (*Y* variables). This model revealed that the enzyme activities considered explained 63% of the total variability in ester production. The corresponding biplot (**Figure 6A**) shows that the main factor for sample differentiation was the storage period, as fruit stored for 3 and 6 months separated along PC1, which alone accounted for 42% of the total variance. The variable with the most weight for separation of samples was ADH activity, followed by HPL and PDC activities (regression coefficients = −0.66, −0.49, and −0.43, respectively), all of which were higher in fruit stored for 6 months. In contrast, apples stored for 3 months were characterized by higher LOX and AAT activities in the pulp tissue, possibly in relation to higher emissions of all selected esters in these fruit. The esters apparently most influenced by enzyme activities in the pulp were the same as for the peel, namely, ethyl butanoate, ethyl 2-methylbutanoate, and 2-methylbutyl acetate (regression coefficients = 0.61, 0.59, and 0.58, in that order). In addition, two hexyl esters (hexyl acetate and hexyl propanoate, with coefficients of 0.51 and 0.54, respectively) were also found to relate closely to modifications in enzyme activities in this tissue. This result is interesting and may be important for understanding the final aroma quality of samples, as hexyl acetate has been reported to associate with lipid-degrading enzymes (*38*), and it was the second most important volatile compound emitted by Mondial Gala apples in terms of odor units present (**Table 3**). The plot of predicted versus measured ADH activity (**Figure 6B**) shows that the enzyme activity increased with the storage period. Because higher ADH activity in the long-term stored apples was concomitant with a significantly lower volatile production, it is suggested that this enzyme activity may be related to the onset of fermentative processes after extended storage under hypoxia rather than to the aroma of the fruit. Low oxygen exposure is known to induce the expression of a number of genes, including those for PDC and ADH in the ethanolic fermentation pathway (*39*). In accordance, ADH activity was also higher in CA than in air stored samples after storage for 3 months. In contrast, no clear differentiation between air and CA stored fruit was found for samples stored for a longer period. Another explanation for the apparent lack of relationship between high ADH activity and the production of volatile esters after long-term storage, however, arises from a strongly inhibited AAT activity in those samples (**Figure 4**), which would prevent esterification of the alcohol precursors.

In conclusion, CA storage of Mondial Gala apples led to a decrease in the emission of volatile esters contributing to the aroma profile of non-stored fruit. LOX and AAT appeared to be the key enzymes involved in lessened ester production during the subsequent shelf life. The storage period was a key factor affecting these two enzyme activities and thus the fruit capacity for the biosynthesis of volatile esters. Results suggest that extended storage, especially under CA, led to unrecoverable loss of the ester-synthesizing ability during the subsequent shelf life of fruit.

ABBREVIATIONS USED

AA, acetaldehyde; ADH, alcohol dehydrogenase; AAT, alcohol *o*-acyltransferase; HPL, hydroperoxide lyase; LOX, lipoxygenase; PCA, principal component analysis; PC1, first principal component; PC2, second principal component; PDC, pyruvate decarboxylase; PLSR, partial least-squares regression.

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