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Long-Term Storage of Pink Lady Apples Modifies Volatile-Involved Enzyme Activities: Consequences on Production of Volatile Esters

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Pink Lady apples were harvested at commercial maturity and stored at 1 °C and 92% relative humidity under either air or controlled atmosphere conditions (2 kPa O_2:2 kPa CO_2 and 1 kPa O_2:1 kPa CO_2) for 27 weeks. Data on the emission of volatile compounds and on the activity of some related enzymes in both skin and flesh tissues were obtained during subsequent shelf life at 20 °C. Major effects of storage atmosphere and poststorage period were observed on the emission of volatile esters and their precursors. Changes in the production of volatile esters were partly due to alterations in the activity of alcohol *o*-acyltransferase, but the specific esters emitted by fruit after storage also resulted largely from modifications in the supply of the corresponding substrates. Samples stored under air were characterized by higher availability of acetaldehyde, whereas those stored under CA showed enhanced emission of the alcohol precursors ethanol and 1-hexanol (2 kPa O_2) and 1-butanol (1 kPa O_2), with accordingly higher production of ethyl, hexyl, and butyl esters. Multivariate analysis revealed that a large part of the observed differences in precursor availability arose from modifications in the activity of the enzymes considered. Higher pyruvate decarboxylase activity in air-stored fruit possibly accounted for higher acetaldehyde levels in these samples, while storage under 1 kPa O_2 led to significantly decreased lipoxygenase activity and thus to lessened production of 1-hexanol and hexyl esters. Low acetaldehyde availability together with enhanced hydroperoxide lyase and alcohol dehydrogenase levels in these fruits are suggested to have led to higher emission of 1-butanol and butyl esters.

KEYWORDS: Alcohol dehydrogenase; alcohol *o*-acyltransferase; controlled atmosphere; hydroperoxide lyase; lipoxygenase; pyruvate decarboxylase; *Malus* × *domestica*; Pink Lady apple; volatile compounds

INTRODUCTION

Apple (*Malus* × *domestica* Borkh.) fruits of the Pink Lady cultivar are characterized by a brilliant pink skin color, a balanced sweet−tart flavor, and a crunchy texture (*1*−*3*). This variety originated in Australia in 1986 and has become widely accepted because of its appealing appearance, good sensory quality ratings, and potential for long-term storage (*1*).

Most volatile compounds contributing to apple aroma are esters, the formation of which is dependent on the availability of C_2−C_8 acids and alcohols (*4*). The precursors for the main volatile esters produced by apple fruit are derived from the metabolism of fatty acids (*5*) and specific amino acids (*6*). Fatty acid-derived substrates originate mainly from lipoxygenase (LOX) activity, β-oxidation, and α-oxidation (*5*), and previous investigations have shown that the supply of these substrates may be a major limiting factor for the production of aroma volatiles (*7*). The significant contribution of esters to the volatile fraction emitted by apple fruit confers the enzyme alcohol *o*-acyltransferase (AAT), which catalyzes the formation of ester bonds, a major role in the development of flavor (*4, 8*−*10*). However, although final ester composition in the volatile profile results from the balance between ester synthesis and hydrolysis, the availability of alcohols, aldehydes, and other minor compounds needed as substrates for ester formation is also a key factor. Thus, the enzyme activities involved in the synthesis of these precursor compounds, such as LOX, hydroperoxide lyase (HPL), pyruvate decarboxylase (PDC), and alcohol dehydrogenase (ADH), may also play important roles in the biosynthesis of flavor-contributing volatiles (*11*−*14*).

Storage of apples under controlled atmosphere (CA) can result in both the enhancement (*15*−*17*) and the suppression of particular flavor volatiles (*16*−*19*). Reduced volatile production after CA storage has been suggested to result from a limiting supply of immediate precursors rather than from degradation or inactivation of AAT and ADH (*13, 14, 20*), in agreement with reports that treatment of fruit or tissue sections with deuterated flavor precursors (*5, 6, 21*) or with the vapors of

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aldehydes, alcohols, and carboxylic acids (*4, 22*) significantly enhanced concentrations of the corresponding volatile esters.

The storage of Pink Lady apples under CA with low (LO) (2 kPa) or ultralow (ULO) (1 kPa) oxygen concentrations, combined with similar CO_2 levels, has been shown to extend the commercial life of fruit beyond 6 months and to preserve both instrumental (*23, 24*) and sensory quality (*25, 26*). However, in spite of the good storage potential of this apple cultivar, important modifications were found in the production of volatile compounds by CA-stored samples in comparison to fruit stored in air (*25*). These differences were not overcome during the poststorage period at 20 °C, showing a permanent residual effect of CA on the capacity of fruit for biosynthesis of volatile esters. For other apple cultivars, such as Fuji (*13*) and Mondial Gala (*14*), similar results have been found to arise to some extent from the inhibition of LOX activity in hypoxic conditions, leading to a shortage of lipid-derived substrates for AAT-catalyzed esterification. However, the response of the biochemical machinery of fruit to storage conditions may differ between cultivars with different storage potential. Thus, the purpose of this work was to examine the modifications in the capacity for volatile ester production after long-term CA storage of Pink Lady apples, with special emphasis focused on the alterations induced by storage conditions in some related enzyme activities.

MATERIALS AND METHODS

Plant Material. Apple fruits (*Malus* × *domestica* Borkh., cv. Pink Lady) were hand-harvested at a commercial orchard near Lleida (NE Spain). The harvest took place in October 2005, at the usual commercial maturity in the area, corresponding to 214 days after full bloom. Fruits were selected according to the Association Pink Lady Europe (diameter, >70 mm; 50% diffuse pink or 30% intense pink; background color turning from green to yellow; starch index, 5−5.8 on a 1−10 scale; flesh firmness, >80 N; and absence of defects). Firmness at harvest averaged 82.5 N, the soluble solids content was 13.9 g 100 g FW^{-1}, and the titratable acidity was 5.9 g malic acid L^{-1}. Immediately after harvest, fruits were placed at 1 °C and about 92% relative humidity (RH) under either air or two different CA conditions, namely, 2 kPa O_2/2 kPa CO_2 (LO) and 1 kPa O_2/1 kPa CO_2 (ULO). The experimental chambers (20 m^3) available at the UdL-IRTA research center were used for storage of fruit. Atmospheres were established within 48 h of harvest. The O_2 and CO_2 concentrations were generated, monitored continuously, and corrected automatically using N_2 from a tank and by scrubbing off excess CO_2 with a charcoal system. A humidifier was used to maintain RH to constant levels. Samples were removed from storage after 27 weeks and transferred to a room at 20 °C to simulate commercial shelf life. Analyses as described below were performed 1 and 7 days thereafter, as well as 1 and 7 days after harvest. Volatile-related enzyme activities were determined additionally upon removal from storage (day 0).

Chemical Standards and Reagents. All of the standards for the volatile compounds studied in this work were of analytical grade and were purchased at the highest quality available from Sigma-Aldrich (Steinheim, Germany) unless indicated otherwise. Ethyl acetate, *t*-butyl propanoate, propyl acetate, 1-propanol, ethyl butanoate, ethyl 2-methylbutanoate, butyl acetate, 2-methyl-1-propanol, 1-butanol, pentyl acetate, 2-methyl-1-butanol, butyl butanoate, hexyl acetate, 1-hexanol, and 2-ethyl-1-hexanol were obtained from Fluka (Buchs, Switzerland). Ethanol and 2-methylpropyl acetate were supplied by Panreac Química, S.A. (Castellar del Vallès, Spain) and Avocado Research Chemicals Ltd. (Madrid, Spain), respectively. 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 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 deadsorbed by agitation for 40 min with 0.5 mL of diethyl ether. Identification and quantification of volatile compounds were achieved on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector and a polyethyleneglycol column with cross-linked free fatty acid as the stationary phase (FFAP; 50 m × 0.2 mm i.d. × 0.33 μm), where a volume of 1 μL from the extract was injected in all of the analyses. Helium was used as the carrier gas (42 cm s^{-1}), with a split ratio of 40:1. 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). A second capillary column (SGE, Milton Keynes, United Kingdom) with 5% phenyl polysilphenylene-siloxane as the stationary phase (BPX5; 30 m × 0.25 mm i.d. × 0.25 μm) was also used for compound identification under the same operating conditions as described above. Volatile compounds were identified by comparing retention indices with those of standards and by enriching apple extracts with authentic samples. The quantification was made using butylbenzene (assay >99.5%, Fluka) as the internal standard. A gas chromatography−mass spectrometry system (Hewlett-Packard 5890) was used for compound confirmation, in which the same capillary columns were 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 above. 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 (AA) Concentration. Juice from 20 fruits per treatment (atmosphere × shelf life period) was obtained individually. A 5 mL sample was introduced in a 10 mL test tube closed with a rubber cap and frozen at −20 °C until analysis of AA content as described in ref *27*. Frozen juice from each fruit was thawed 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. AA was identified and quantified by comparison with an external standard, and results were expressed as μL L^{-1}.

Extraction and Assay of Volatile-Related Enzyme Activities. LOX, HPL, PDC, ADH, and AAT activities were determined on days 0, 1, and 7 after removal from storage. Samples of both skin and flesh tissue were taken separately from four apples, frozen in liquid nitrogen, lyophilized, and powdered. 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 (*28*). HPL activity was extracted and assayed according to ref *29*. The total protein content in the enzyme extract was determined with the Bradford method (*30*), using bovine serum albumin as the 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. All data were tested by analysis of variance (GLM-ANOVA) according to standard SAS-STAT procedures (*31*), with storage atmosphere and shelf life period as the main factors. Means were separated by least significant differences (LSD) test at $p \leq 0.05$. Multivariate analysis procedures were also used to help the interpretation of results. Sample names were coded as $X \cdot Y$, where X and Y refer to storage atmosphere and days of shelf life, respectively. Volatile compounds analyzed were labeled as specified in **Table 1**. A general visualization of all of the information contained in the data set was provided by means of principal component analysis (PCA). Partial least-squares 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 7.6 software (CAMO ASA, Norway) was used for developing these

Table 1. Emission of Volatile Compounds (μg kg^{-1}) by Pink Lady Apples 1 and 7 Days after Harvest

compound	RI$_1$[a]	RI$_2$[b]	OTh[c] (μg kg^{-1})	1 day[d]	OU[e]	7 days[d]	OU[e]	code[f]
methyl acetate	773	–	8300	29.0 a		16.5 b		
ethyl acetate	803	609	13500	25.5 a		22.1 a		ea
α-pinene	810	937	–	3.5 a		2.4 b		
ethanol	838	–	10000 (a)	23.7 a		15.1 b		etOH
t-butylpropanoate	867	717	19	7.7 a		2.7 b		
propyl acetate	889	649	2000	11.4 b		35.7 a		
methyl butanoate	902	656	76 (b)	8.4 b		16.6 a		
2-methylpropyl acetate	923	691	65	12.0a		10.9 a		
3-methyl-2-butanol	928	636	–	1.5 a		1.5 a		
1-propanol	940	–	9000	6.1 b		18.2 a		
ethyl butanoate	946	803	1	2.7 a	2.7	4.2 a	4.2	eb
propyl propanoate	954	809	57 (a)	17.4 a		5.1 b		
ethyl 2-methylbutanoate	963	845	0.006	4.9 a	810.1	4.8 a	803.6	e2mb
butyl acetate	986	813	66	96.1 b	1.5	358.5 a	5.4	ba
2-methylpropyl propanoate	991	865	–	6.8 a		3.6 b		
2-methyl-1-propanol	996	614	250	2.0 a		1.3 b		
2-methylbutyl acetate	1023	876	11	281.6 a	25.6	379.4 a	34.5	2mba
1-butanol	1034	626	500	13.0 b		26.3 a		bOH
butyl propanoate	1052	910	25	35.3 b	1.4	58.5 a	2.3	bpr
butyl 2-methylpropanoate	1057	1009	80 (b)	5.1 a		4.1 a		
2-methylpropyl butanoate	1070	954	–	3.6 b		10.6 a		
pentyl acetate	1087	914	43	16.0 b		36.8 a		
heptanal	1093	909	–	1.9 a		1.5 a		
2-methylbutyl propanoate	1103	950	19	6.2 b		20.6 a		
2-methylbutyl 2-methylpropanoate	1106	1016	–	1.2 a		1.6 a		
2-methyl-1-butanol	1113	667	250	8.7 a		8.8 a		2mbOH
D-limonene	1118	1035	34	Tr[g]		Tr[g]		
butyl butanoate	1130	1000	100	21.2 b		43.6 a		
pentyl propanoate	1135	969	–	2.2 a		1.7 a		
butyl 2-methylbutanote	1143	1042	17	31.4 b	2.1	108.6 a	6.4	b2mb
ethyl hexanoate	1145	1002	1	2.8 b	2.8	3.9 a	3.9	eh
1-pentanol	1157	688	4000	1.5 a		1.1 a		
2-methylbutyl butanoate	1178	1058	–	1.1 a		0.7 a		
hexyl acetate	1186	1015	2	269.8 b	134.9	811.5 a	405.8	ha
2-methylbutyl 2-methylbutanoate	1203	1106	–	7.1 b		30.1 a		
propyl hexanoate	1231	1099	–	3.7 a		2.7 a		
hexyl propanoate	1251	1109	8	84.5 b	10.6	113.3 a	14.2	hpr
1-hexanol	1262	869	500	2.4 a		1.4 b		hOH
2-methylpropyl hexanoate	1264	1153	–	13.8 b		18.6 a		
heptyl acetate	1292	1115	–	1.2 a		0.7 b		
butyl hexanoate	1325	1196	700	80.4 a		93.5 a		bh
hexyl butanoate	1328	1197	250	115.7 a		81.9 b		hb
hexyl 2-methylbutanoate	1339	1239	6	163.6 b	27.3	258.6 a	43.1	h2mb
ethyl octanoate	1347	1201	–	1.5 b		7.3 a		
heptyl 2-methylpropanoate	1352	1249	–	1.9 a		1.3 b		
octyl acetate	1388	1215	12 (c)	4.1 a		1.6 b		
2-ethyl-1-hexanol	1398	1031	–	13.1 a		5.8 b		
pentyl hexanoate	1424	1293	–	9.6 a		8.5 a		
hexyl hexanoate	1524	1392	6400 (d)	95.8 a		80.3 a		hh
butyl octanoate	1528	1394	–	10.5 a		10.3 a		
3-methylbutyl octanoate	1550	1453	–	4.6 a		2.7 a		

[a] Kovats retention indices (*44*) in a FFAP column. [b] Kovats retention indices (*44*) in a BPX5 column; –, eluted with the solvent. [c] Odor thresholds as reviewed in ref *25*, except for (a) ref *45*, (b) *46*, (c) *47*, and (d) *48*; –, not found. [d] Values represent means of four replicates. Means within the same row showing different letters are significantly different at $P \leq 0.05$ (LSD test). [e] Odor units = amount/OTh (*33*). Only values >1 are indicated. [f] Codes used for multivariate analysis. [g] Traces ($\leq$0.5 μg kg^{-1}).

models. Data were centered and weighed by the inverse of the standard deviation of each variable to avoid dependence on measured units (*32*), and full-cross validation was run as a validation procedure.

RESULTS AND DISCUSSION

Modifications in Production of Volatile Compounds after Cold Storage of Pink Lady Apples. A total of 51 volatile compounds (39 esters, nine alcohols, two terpenes, and one aldehyde) were identified in the volatile fraction emitted by Pink Lady apples at harvest (**Table 1**). Some of these compounds were selected to examine fruit capacity for volatile biosynthesis after long-term storage. Ten of them were chosen on the basis of having odor units >1 and thus being likely to have an impact on fruit flavor (*33*). All of them were esters, namely, ethyl butanoate, ethyl hexanoate, ethyl 2-methylbutanoate, butyl acetate, butyl propanoate, butyl 2-methylbutanoate, 2-methylbutyl acetate, hexyl acetate, hexyl propanoate, and hexyl 2-methylbutanoate (**Table 1**). Butyl hexanoate, hexyl butanoate, and hexyl hexanoate were also selected on account of their quantitative importance in the volatile fraction ($\geq$50 μg kg^{-1}), together with ethyl acetate as an indicator of possible fermentative processes in CA-stored fruit and AA and some alcohols (ethanol, 1-butanol, 1-hexanol, and 2-methyl-1-butanol) as the precursors to these compounds. The 18 volatile esters and alcohols chosen for sample characterization after storage accounted together for almost 90% of total volatiles produced by fruit 7 days after harvest (**Table 1**), and six of them (hexyl 2-methylbutanoate, hexyl hexanoate, hexyl propanoate, butyl

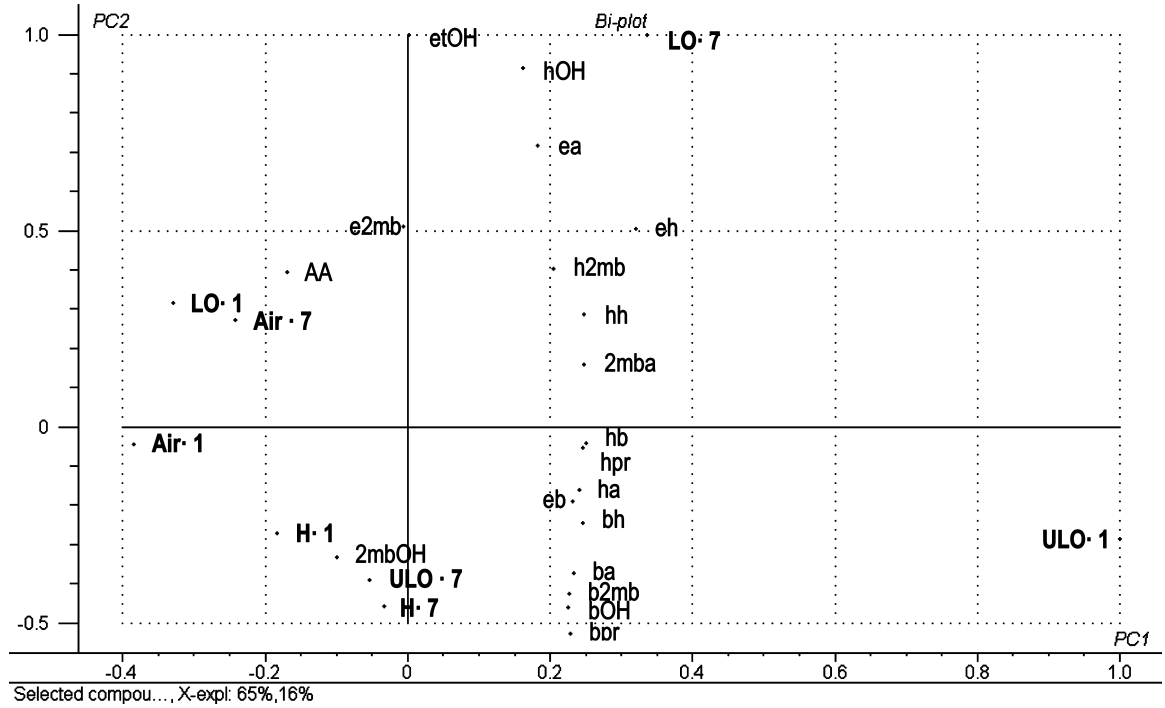

Figure 1. Biplot (scores and loadings) of PC1 vs PC2 corresponding to a PCA model for volatile compounds emitted by Pink Lady apple fruit at harvest (H) and after 27 weeks of storage under different conditions. For sample labels, the numerical suffix refers to the period at 20 °C (days) following harvest or storage. Volatile compounds are coded as indicated in **Table 1**.

2-methylbutanoate, butyl propanoate, and 2-methylbutyl acetate) have been shown to positively influence the sensory acceptability of Pink Lady apples (*25*).

Selected volatile compounds were used to characterize samples both at harvest and after storage (eight samples × 19 variables) by means of a PCA model. The two first principal components (PC1) accounted together for 81% of total variability among samples. The biplot for this model (**Figure 1**) suggests interactions between storage atmosphere and shelf life period in sample differentiation. Fruit kept 1 day at 20 °C after cold storage separated according to storage atmosphere along PC1, which explained 65% of total variance. Samples stored under air or 2 kPa O_2 grouped together on the left side of the plot, clearly away from ULO-stored apples, and were characterized mainly by higher levels of AA and 2-methylbutanol. Air- and LO-stored fruit differentiated along the second principal component (PC2), primarily as a function of ethanol and 1-hexanol levels, which were the variables showing most weight for differentiation along the second PC, and were higher for fruit stored under LO.

As to fruit kept at 20 °C for a whole week after cold storage, samples stored under 2 kPa O_2 were characterized by higher emission of most volatiles selected for this work and were separated from air- and ULO-stored apples along PC1 (**Figure 1**). Separation among storage conditions was not as broad as for fruit kept at 20 °C for only 1 day, indicating partial equalization of the capacity for volatile biosynthesis along the poststorage period. The two CA conditions considered herein differentiated along PC2, showing differences in the emission of volatile compounds in response to storage atmosphere. Generally speaking, ULO-stored samples were characterized by higher emission of butyl esters, in accordance with higher levels of 1-butanol, their alcohol precursor, whereas LO-stored fruit showed higher production of some ethyl (ethyl acetate, ethyl 2-methylbutanoate, and ethyl hexanoate) and hexyl (hexyl 2-methylbutanoate and hexyl hexanoate) esters, concomitantly with higher availability of ethanol and 1-hexanol. These results

Table 2. AAT Specific Activity (U mg Protein^{-1}) in Skin and Flesh Tissues of Pink Lady Apple Fruit after Cold Storage for 27 Weeks[a]

shelf life period[b]	0	1	7
	Skin		
H[c]		0.35 Ab	0.29 Ab
Air	0.47 Ab	0.35 Bb	0.46 Aa
LO	0.59 Aa	0.43 Bb	0.53 Aa
ULO	0.53 Aab	0.53 Aa	0.44 Aa
	Flesh		
H[c]		0.21 Aab	0.26 Aa
Air	0.21 Ab	0.19 Ab	0.19 Ab
LO	0.25 Aa	0.25 Aa	0.25 Aa
ULO	0.26 Aa	0.24 Aa	0.22 Aab

[a] Values represent means of three replicates. Means within the same row followed by different capital letters are significantly different at $P \leq 0.05$ (LSD test). Means within the same column for a given tissue followed by different small letters are significantly different at $P \leq 0.05$ (LSD test). [b] Days at 20 °C following harvest or cold storage. [c] At harvest.

are interesting, since some of these compounds have been found to have a positive influence on the acceptability of Pink Lady apples (*25*), and indeed, acceptability scores of CA-stored fruit were higher than those of samples stored in air (results not shown).

To confirm the apparent relationship between differential emission of the chosen volatile esters both at harvest and across storage conditions and the availability of the selected precursors, a PLSR model was developed in which AA and alcohols (*X* variables) were related to esters emitted (*Y* variables). The corresponding biplot (**Figure 2**) shows that 76% of variability in ester emission could be attributed to precursor availability. Nonstored and air-stored fruit separated from CA-stored samples along PC1, which alone explained 66% of sample differentiation. CA conditions considered separated mainly along PC2. The variables showing most weight for sample separation along PC1 were 1-butanol and AA (regression coefficients = 0.67 and −0.50, respectively). Air-stored fruit were characterized by

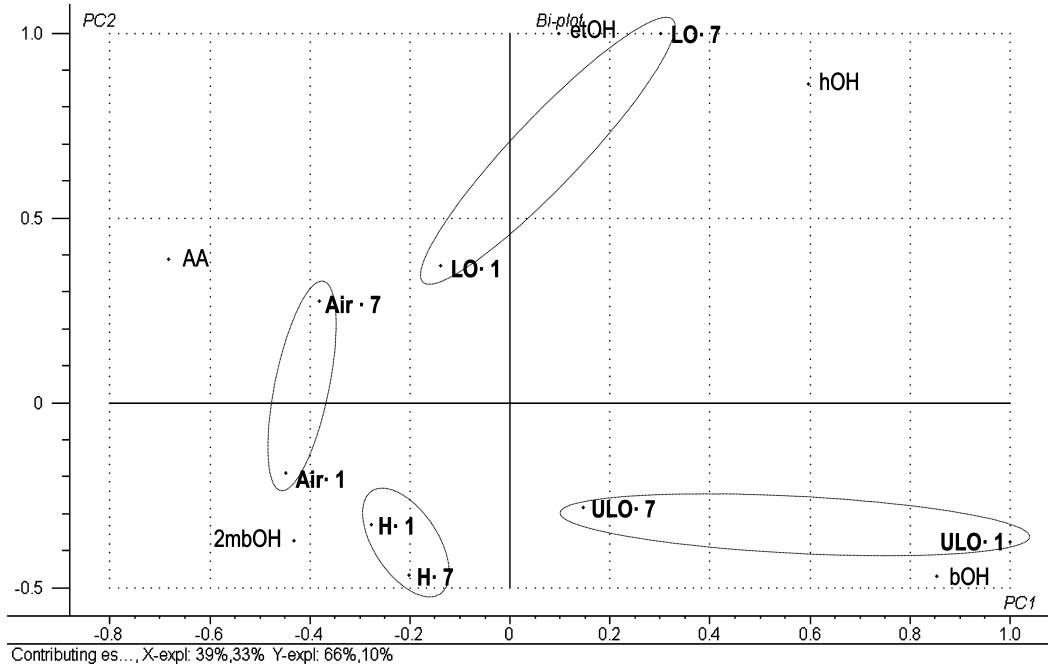

Figure 2. Biplot (scores and loadings) of PC1 vs PC2 corresponding to a PLSR model of volatile compounds emitted (*Y* variables) vs precursors available (*X* variables) in Pink Lady apple fruit at harvest (H) and after 27 weeks of storage under different conditions. For sample labels, the numerical suffix refers to the period at 20 °C (days) following harvest or storage. Volatile compounds are coded as indicated in **Table 1**.

higher levels of AA and 2-methylbutanol, in accordance with previous reports on other apple cultivars with long-term storage potential such as Fuji (*13*). Contrarily, preferential accumulation of AA in CA- as compared to air-stored fruit has been observed for cultivars not as well-suited for extended storage such as Mondial Gala (*14*). Higher emission of ethanol by LO- in comparison to air- or ULO-stored fruit, particularly after 7 days at 20 °C (**Figure 2**), is also in agreement with previous reports on Fuji. In contrast, samples stored under CA were characterized by higher availability of 1-hexanol (LO) and 1-butanol (ULO), which disagrees with observations on Fuji, where these two alcohols characterized fruit stored in air (*13*). These differences may be related to the different composition of the volatile fraction emitted by Fuji and Pink Lady apples: whereas hexyl and butyl esters were very prominent both quantitatively and qualitatively in Pink Lady fruit (**Table 1**), some ethyl and acetate esters were found to be the major contributors to the volatile profile of Fuji apples at harvest (*13*).

Modifications in Volatile-Related Enzyme Activities after Cold Storage of Pink Lady Apples. The good correspondence found between the differential emission of the chosen volatile esters and the availability of the selected precursors suggested rapid utilization of substrates upon removal from cold storage. The direct enzyme responsible for the production of volatile esters by fruit tissues is AAT, which catalyzes the final linkage of an acyl moiety to an alcohol. Therefore, the observed changes in production of volatile esters after storage could have arisen from modifications in AAT activity. Little differences (skin) or no differences at all (flesh) in AAT activity were observed during the poststorage period at 20 °C (**Table 2**), indicating that differential production of volatile esters resulted from biochemical modifications taking place during storage rather than from recovery of ester-synthesizing capacity upon transfer to air. Indeed, AAT activity upon removal from cold storage (day 0) was higher in CA- than in air-stored fruit (**Table 2**), which is agreement with previous results on Mondial Gala (*14*). Increased AAT activity in CA-stored fruit could have accounted at least partially for differences in ester emission after storage (**Figure 1**). The question arises whether this observation may be reflecting the potential of Pink Lady fruit for adequately regenerating the volatile biosynthesizing capacity after long-term storage: enhanced AAT activity in Mondial Gala fruit, a cultivar not well-suited for extended storage periods, was found after 3 months, whereas storage for 6 months led to sharply reduced enzyme activity both in skin and in flesh tissues (*14*) with concomitant unrecoverable diminution of biosynthesis of volatile esters.

However, differences observed in precursor availability (**Figure 2**) show that the actual ester composition of the volatile fraction emitted by fruit could also be controlled by other factors such as the availability of the necessary substrates or the substrate selectivity of the AAT isoforms present in the tissues (*10*). The products of all AAT genes isolated to date from fruit, including apple (*34*), melon (*Cucumis melo* L.) (*35*), cultivated strawberry (*Fragaria* × *ananassa* Duch.) (*36*), wild strawberry (*Fragaria vesca* L.), or banana (*Musa sapientum* L.) (*37*), show reportedly broad substrate preferences. For apple, it has also been reported that the binding of alcohol substrates is rate-limiting in comparison with that of acyl CoA substrates (*34*) and that the ultimate preference of the enzyme for alcohol precursors is dependent on substrate concentration, which thus determines the final volatile profile. Therefore, other enzymes situated upstream of AAT in the metabolic pathways leading to biosynthesis of volatile esters may be controlling production by providing or limiting the supply of the necessary aldehyde and alcohol precursors.

To assess the relationships between the activity of some related enzyme activities (*X* variables) and the availability of substrates for the esterification reaction (*Y* variables), a PLSR model was developed. This model revealed that the activity of the enzymes considered in this work accounted for up to 70% of the differences in precursor availability (**Figure 3**). Air- and LO-stored samples were characterized by higher levels of LOX and PDC activity and separated along PC1 from ULO-stored fruit, which were associated to greater HPL, ADH, and AAT activities. The variables showing most weight for differentiation

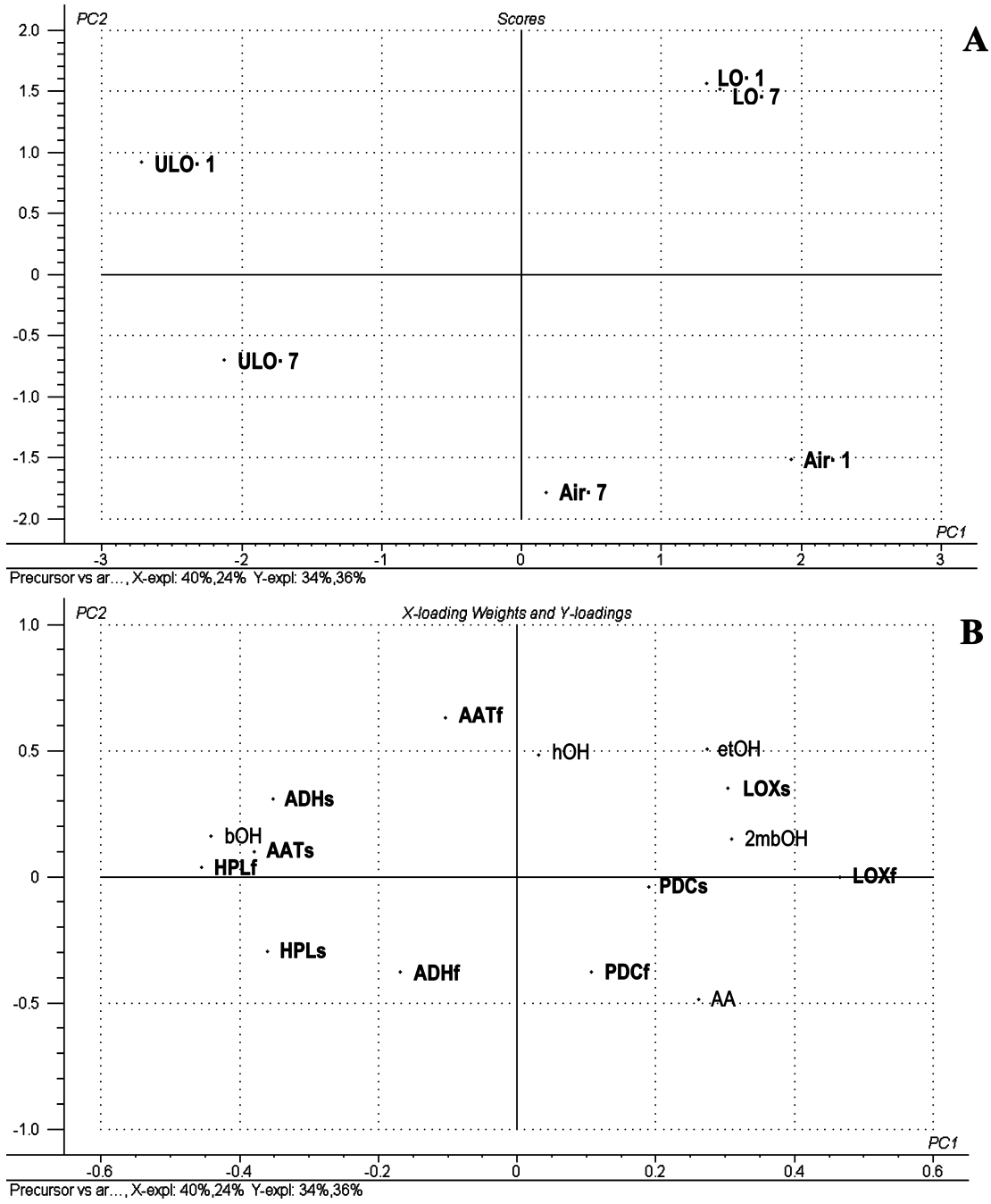

Figure 3. Scores (**A**) and loadings (**B**) plots of PC1 vs PC2 corresponding to a PLSR model of precursor availability (*Y* variables) vs volatile-related enzyme activities (*X* variables) in Pink Lady apple fruit after 27 weeks of storage under different conditions. Precursors are coded as indicated in **Table 1**. For sample labels, the numerical suffix refers to the period at 20 °C (days) following harvest or storage. For enzyme labels, the suffix "s" or "f" refers to the activity in the skin or the flesh, respectively.

along PC1 were LOX and HPL in the flesh tissue, with regression coefficients of 0.47 and −0.46, respectively. 1-Butanol was the precursor apparently most affected by these differences. Partial inhibition of LOX activity upon removal from storage under hypoxic conditions is consistent with the O_2 requirement for this enzyme activity and agrees with previous reports on other apple cultivars (*13, 14*), in which it has been shown to account for a shortage of fatty acid-derived precursors and thus for decreased biosynthesis of volatile esters after CA storage. No differences were detected in LOX activity in the flesh immediately after transfer to air regardless of storage atmosphere (**Table 3**). However, activity increased significantly 1 day thereafter both in air- and in LO-stored apples, whereas a steady diminution throughout ripening at 20 °C was noticed for ULO-stored samples, indicating that preservation under ultralow O_2 concentrations caused some unrecoverable alteration in the properties of the enzyme. The fact that both air-and LO-stored fruit had similar LOX activity levels suggests that a severe decrease in O_2 concentrations is required to result in significant inhibition of enzyme activity in Pink Lady fruit, which is a difference respecting observations for other apple cultivars (*13, 14*).

Air- and LO-stored fruit separated along the second PC, which accounted alone for 36% of total variability and hence was also important for sample differentiation. The main variable for sample separation along PC2 was AAT activity in the flesh tissue (regression coefficient = 0.63), although PDC and ADH activities in this same tissue also showed high regression coefficients (−0.38 in both cases). Air-stored fruits were

Table 3. LOX Specific Activity (U mg Protein^{-1}) in Skin and Flesh Tissues of Pink Lady Apple Fruit after Cold Storage for 27 Weeks[a]

shelf life period[b]	0	1	7
	Skin		
H[c]		13.79 Bc	25.88 Abc
Air	25.92 Ab	22.96 Ab	21.67 Ac
LO	49.93 Aa	52.76 Aa	41.68 Ba
ULO	28.82 Ab	18.85 Bbc	32.47 Ab
	Flesh		
H[c]		22.83 Bb	50.05 Aa
Air	28.70 Ba	42.17 Aa	35.62 ABb
LO	25.83 Ba	38.40 Aa	35.54 ABb
ULO	28.31 Aa	22.40 ABb	15.76 Bc

[a] Values represent means of three replicates. Means within the same row followed by different capital letters are significantly different at $P \leq 0.05$ (LSD test). Means within the same column for a given tissue followed by different small letters are significantly different at $P \leq 0.05$ (LSD test). [b] Days at 20 °C following harvest or cold storage. [c] At harvest.

Table 4. PDC Specific Activity (U mg Protein^{-1}) in Skin and Flesh Tissues of Pink Lady Apple Fruit after Cold Storage for 27 Weeks[a]

shelf life period[b]	0	1	7
	Skin		
H[c]		41.49 Aa	18.11 Ba
air	11.94 Ab	11.10 Ab	18.07 Aa
LO	11.44 Ab	11.48 Ab	14.26 Aab
ULO	53.63 Aa	11.71 Bb	8.20 Bb
	Flesh		
H[c]		26.04 Aa	10.70 Ba
air	16.74 Ba	24.94 Aa	10.00 Ba
LO	8.69 Ab	8.38 Ab	7.69 Aa
ULO	10.90 Aab	9.16 Ab	10.50 Aa

[a] Values represent means of three replicates. Means within the same row followed by different capital letters are significantly different at $P \leq 0.05$ (LSD test). Means within the same column for a given tissue followed by different small letters are significantly different at $P \leq 0.05$ (LSD test). [b] Days at 20 °C following harvest or cold storage. [c] At harvest.

characterized by higher PDC levels, which were associated to increased contents of AA (**Figure 3B**), possibly reflecting the main metabolic origin of this important precursor. Indeed, CA-stored fruit showed decreased levels of PDC activity in the flesh tissue upon removal from storage (**Table 4**), which did not recover throughout the shelf life considered herein, in contrast to samples stored in air. These data suggest that CA storage led to partial inhibition of either gene expression or activity of the gene product. The compounds most affected by these differences were ethanol, AA, and 1-hexanol (regression coefficients of 0.51, −0.49, and 0.48, correspondingly). 1-Hexanol characterized LO-stored samples, in agreement with higher emission of some hexyl esters by these fruit (**Figure 1**), and was associated to higher LOX activities, particularly in the skin tissue, which is in accordance with reports that production of hexyl esters is related to lipid-degrading enzymes (*38*).

ULO-stored samples were characterized by higher ADH activity, particularly in the skin, which is consistent with previous findings that low oxygen exposure induces the expression of a number of genes, including those in the ethanolic fermentation pathway (*39*). However, ADH activity levels in both skin and flesh immediately after removal from storage (day 0) were significantly lower for CA- than for air-stored fruit (**Table 5**), suggesting that CA-induced transcripts would have been translated only after transfer to 20 °C. Furthermore, and with the exception of ethyl butanoate, fruits stored under ULO were not characterized by higher emission of either ethanol or ethyl esters (**Figure 1**), indicating that other factors in addition

Table 5. ADH Specific Activity (U mg Protein^{-1}) in Skin and Flesh Tissues of Pink Lady Apple Fruit after Cold Storage for 27 Weeks[a]

shelf life period[b]	0	1	7
	Skin		
H[c]		42.61 Ab	15.59 Bab
air	52.96 Aa	15.35 Bc	25.03 Ba
LO	20.51 Ab	17.72 Ac	23.56 Aa
ULO	17.42 Bb	66.15 Aa	10.80 Bb
	Flesh		
H[c]		9.48 Aa	7.01 Abc
air	13.25 Aa	5.79 Bb	14.45 Aa
LO	6.16 Ab	5.95 Ab	5.19 Ac
ULO	4.30 Bb	9.12 Aa	9.58 Ab

[a] Values represent means of three replicates. Means within the same row followed by different capital letters are significantly different at $P \leq 0.05$ (LSD test). Means within the same column for a given tissue followed by different small letters are significantly different at $P \leq 0.05$ (LSD test). [b] Days at 20 °C following harvest or cold storage. [c] At harvest.

Table 6. HPL Specific Activity (U mg Protein^{-1}) in Skin and Flesh Tissues of Pink Lady Apple Fruit after Cold Storage for 27 Weeks[a]

shelf life period[b]	0	1	7
	Skin		
H[c]		34.58 Aa	12.76 Bc
air	14.73 Ba	11.14 Bb	30.16 Ab
LO	18.74 Aa	12.53 ABb	10.77 Bc
ULO	18.44 Ca	28.40 Ba	52.15 Aa
	Flesh		
H[c]		8.94 Bc	23.97 Ab
air	11.44 Ab	12.76 Abc	11.51 Ac
LO	29.11 Aa	16.14 Bb	15.71 Bbc
ULO	20.25 Cb	32.64 Ba	40.15 Aa

[a] Values represent means of three replicates. Means within the same row followed by different capital letters are significantly different at $P \leq 0.05$ (LSD test). Means within the same column for a given tissue followed by different small letters are significantly different at $P \leq 0.05$ (LSD test). [b] Days at 20 °C following harvest or cold storage. [c] At harvest.

to ADH activity are involved in the production of these volatile esters, possibly including substrate supply and/or differential expression of ADH isogenes (*40*). Actually, it has been reported that ADH is not the limiting factor for ethanol production in pear (*Pyrus communis* L.) fruit stored under hypoxia (*41*).

ULO-stored fruit were also associated with higher production of 1-butanol (**Figure 3B**), concomitantly with higher HPL activity. HPL catalyzes cleavage of fatty acid hydroperoxides, resulting from the catalytic activity of LOX to aldehydes and oxoacids and is a membrane-bound enzyme present in small amounts in plant tissues (*12*). It has been reported that butanal and hexanal are derived from the LOX pathway and/or β-oxidation (*42*), and partially purified extracts of apple fruit ADH have been shown to have a higher affinity for AA than for larger straight-chain aldehydes (*43*). These facts are interesting in the light of results reported herein: Increased AA contents in air-stored fruit might have out-competed butanal and hexanal for ADH-catalyzed reduction. For ULO-stored samples, in contrast, lower AA concentrations in combination with enhanced HPL (**Table 6**) and ADH activities, arising at least partially from diminution in intracellular pH under hypoxic conditions, would have led to increased reduction of butanal, thus resulting in higher availability of 1-butanol and thus to higher emission of butyl esters by fruit.

In conclusion, CA storage of Pink Lady apples led to modifications in biosynthesis of volatile compounds during the subsequent shelf life under air. These alterations arose from

changes both in the ester-forming capacity of the tissues and in the supply of the necessary substrates, as a consequence of modifications in the activities of other related enzymes located upstream in the pathway. LOX and HPL were found to be key enzymes in the regulation of the actual composition of the volatile fraction emitted by fruit.

ABBREVIATIONS USED

AA, acetaldehyde; ADH, alcohol dehydrogenase; AAT, alcohol *o*-acyltransferase; CA, controlled atmosphere; 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; LO, low oxygen atmosphere; ULO, ultralow oxygen atmosphere.

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