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Lipoxygenase Activity Is Involved in the Regeneration of Volatile Ester-Synthesizing Capacity after Ultra-Low Oxygen Storage of 'Fuji' Apple

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An extra period under cold air after ultra-low oxygen storage has been shown to increase the concentration of some volatile compounds emitted by stored 'Fuji' apples. The purpose of this work was to assess the role, if any, of lipoxygenase and hydroperoxide lyase activities in the regeneration of fruit capacity for volatile production after ultra-low oxygen storage. Fruits were stored at 1 °C and 92% relative humidity under ultra-low oxygen (1 kPa of O_2/1 kPa of CO_2); one lot was kept under hypoxia for 19 or 30 weeks, a second lot was maintained for 17 or 28 weeks under these conditions and then stored for 2 weeks in cold air, and a third lot remained for either 15 or 26 weeks under ultra-low oxygen followed by 4 weeks under cold air. Samples were placed subsequently at 20 °C, and analyses of volatile emission and enzyme activities were undertaken 1 and 7 days thereafter. Fruit stored during 4 weeks in cold air after ultra-low oxygen storage showed the highest capacity for volatile regeneration. Higher emission of volatiles by these samples was concomitant with higher levels of lipoxygenase activity. Results suggest that lipoxygenase activity, particularly in the flesh tissue, was strongly related to the regeneration of the emission of volatile compounds allowed by the extra period in cold air after ultra-low oxygen storage and, thus, appears to be a key control point for successful recovery of fruit ability for volatile compound production.

KEYWORDS: Apple; aroma regeneration; cold air storage; hydroperoxide lyase; lipoxygenase; ultra-low-oxygen storage; volatile emission

INTRODUCTION

'Fuji' apples (*Malus* × *domestica* Borkh.) display interesting quality attributes (crispy texture, sweet and aromatic flavor) and good potential for postharvest storage. Owing to these favorable quality and marketing characteristics, this cultivar is widely cultivated in the main producing areas of the world. Controlled atmosphere (CA) is recommended for the storage of a wide range of apple cultivars due to substantial lengthening of storage period and for retaining fruit quality, especially in terms of preservation of flesh firmness and epidermis color and of lowered oxidation of organic acids. During the past decades, the application of new CA techniques involving very low oxygen levels (around 0.8−1.2%) in the storage atmosphere has been increasingly adopted by the fruit industry. However, the negative impact of CA storage on the biosynthesis of some volatile compounds, both under standard and ultra-low oxygen (ULO) conditions, is also well documented for a number of apple cultivars (*1*−*6*). These changes in volatiles during and after storage are important from a commercial point of view, as they play a key role in the consumer perception of fruit flavor (*7*, *8*). Moreover, prolonged storage of apples in very low O_2 and/or high CO_2 atmospheres may cause detrimental effects on fruit quality as a consequence of the onset of fermentative processes, leading to ethanol and acetaldehyde accumulation, development of off-flavor, failure to ripen after removal to air, and development of low-O_2 and/or high-CO_2 injury (*9*).

Several techniques have been tested to improve the regeneration of aroma volatile compounds after CA storage of apples, including exposure to exogenous ethylene during or after storage (*2*, *10*), treatment with precursors of ester compounds (*10*−*12*), or exposure to hypoxia with atmospheres up to 100% CO_2 (*13*, *14*). Similarly, some regeneration of volatile production is achieved when fruit is placed in air after storage in atmospheres with low O_2 and/or high CO_2 (*3*, *10*, *15*−*17*). Among these strategies, the extra period under refrigerated air after ULO storage has the additional advantage of its easy application in commercial cold stores.

Volatile esters account for up to 80, 88, 90, and 98% of total volatiles in 'Golden Delicious' (*18*), 'Granny Smith' (*19*), 'Fuji' (*7*), and 'Starking Delicious' (*20*), respectively. Volatile esters are known to be the most significant contributors to apple aroma (*3*, *21*), together with some alcohols [2-methylpropanol, (*Z*)-hexen-3-ol, 1-hexanol, and 1-octanol] and aldehydes (1-hexanal) that are considered to be principal odorant compounds in aroma extracts of 'Golden Delicious', 'Fuji', and 'Braeburn' (*22*). Esters produced by a ripening apple fruit can be broadly separated into straight-chain and branched-chain types, the formation of which is partly dependent on the availability of the corresponding acid and alcohol precursors (*13*). In general, fatty acids are considered to be major precursors of

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straight-chain volatile esters in apples, the biosynthetic pathways including β-oxidation, hydroperoxy acid cleavage, and lipoxygenase (LOX) action to form the respective aldehydes, ketones, acids, alcohols, and esters (*21*, *23*−*25*). Branched-chain esters are thought to arise from the metabolism of amino acids (*24*, *26*). The capacity to regenerate the biosynthesis of volatile compounds, especially that of esters, which is of great importance in the preservation of the eating quality of fruit, is reduced after long-term storage (*27*), which influences negatively consumer acceptability of produce.

Decreased production of volatiles after CA storage of apple fruit is to some extent the consequence of limited lipid-derived substrate availability arising from partial arrest of some related enzyme activities (*6*, *13*, *28*). LOX activity, which catalyzes the hydroperoxidation of polyunsaturated fatty acids, has been found to be essential for the recovery of the ability to synthesize volatile esters after CA storage of apple (*6*, *28*) and pear (*Pyrus communis* L.) (*29*). Cleavage of fatty acid hydroperoxides produced by LOX into aldehydes and oxo acids by hydroperoxide lyase (HPL) is likely to be another control point in the biosynthesis of volatile esters through the LOX system (*30*, *31*). The availability of lipid-derived substrates obtained through this pathway thus appears to be a major factor affecting volatile ester production after storage.

The present study investigated the influence of different periods in cold air subsequent to ULO storage on volatile production by 'Fuji' apples. We also aimed at evaluating a possible relationship to modifications in LOX and HPL activities in fruit tissues, and at finding out whether it might be possible to predict the potential for the regeneration of the main volatile compounds from these two enzyme activities.

MATERIALS AND METHODS

Plant Material and Storage Conditions. Apple (*Malus* × *domestica* Borkh. cv. 'Fuji Kiku 8') fruits were harvested in 2006 at commercial maturity, 182 days after full bloom (dafb), from 5-year-old trees grown on M-9 EMLA rootstock at an experimental orchard (Estació Experimental IRTA-Mollerussa) in the area of Lleida (northeastern Spain). Flesh firmness at harvest averaged 69.3 N, soluble solids content was 16.3 g 100 g of fresh weight $(FW)^{-1}$, and titratable acidity was 2.9 g of malic acid L^{-1}. Immediately after harvest, three lots of 100 kg of apples were selected for size, color uniformity, and absence of defects, and stored at 1 °C and 92% relative humidity (RH) in ULO atmosphere (1 kPa of O_2/1 kPa of CO_2) for up to either 19 or 30 weeks. One lot of ULO-stored fruit remained under these conditions for the whole 19 or 30 weeks. A second lot was kept for either 17 or 28 weeks under ULO conditions and then stored for 2 weeks in cold air (ULO + 2w). A third batch of fruit was kept for either 15 or 26 weeks in ULO followed by a further 4 weeks in cold air (ULO + 4w). After storage, fruits were stored at 20 °C to simulate commercial shelf life during 1 or 7 days. Volatile compound emission, enzyme activities, and content of acetaldehyde in fruit juice were measured at harvest and after removal from storage plus 1 and 7 days at 20 °C.

Chemicals. All of the standards for the volatile compounds studied in this work were of analytical grade and were purchased at the highest quality available. Ethyl acetate, *trans*-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, hexyl acetate, 1-hexanol, hexyl 2-methylbutanoate, and 2-ethyl-1-hexanol were obtained from Fluka (Buchs, Switzerland). Ethanol was purchased from Panreac Química, S.A. (Castellar del Vallès, Spain). 2-Methylpropyl acetate was obtained from Avocado Research Chemicals Ltd. (Madrid, Spain). The rest of the compounds (up to 49) were supplied by Sigma-Aldrich (Steinheim, Germany).

Analysis of Volatile Compounds. Eight kilograms of apples (2 kg/replicate × 4 replicates) per treatment (atmosphere × storage period × shelf life period) was selected for analysis of volatile compounds both at harvest and after removal from storage. Intact fruits were placed in an 8 L Pyrex container through which an air stream (900 mL min^{-1}) was passed for 4 h. The resulting 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 desorbed by agitation for 40 min with 0.5 mL of diethyl ether. The identification and quantification of volatile compounds was performed on a HP 5890 series II gas chromatograph (Hewlett-Packard Co., Barcelona, Spain) equipped with a flame ionization detector (GC-FID), using a polyethylene glycol capillary column with cross-linked free fatty acid as the stationary phase (FFAP; 50 m × 0.2 mm × 0.33 μm) into which a volume of 1 μL of the extract was injected in all of the analyses. The oven program was set at 70 °C (1 min), and the temperature was first raised by 3 °C min^{-1} to 142 °C and later by 5 °C min^{-1} to 225 °C and then kept constant for 10 min at this latter temperature. 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. A second capillary column (SGE, Milton Keynes, U.K.) 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. Compounds were identified by comparing their respective retention indices with those of standards and by enriching apple extract with authentic samples. Quantification was carried out by adding 25 μL of a 0.2% solution of butylbenzene (assay > 99.5%, Fluka) as an internal standard. A GC-MS system was used for compound confirmation, using the same capillary column as in the GC analyses. Analysis was carried out using an Agilent 6890N gas chromatograph interfaced to a 5973N mass selective detector. Mass spectrometric data were collected in full scan. Scan ranged from 30 to 500 amu. The scan rate was 3.1 scans s^{-1}. Mass spectra were obtained by electron impact ionization at 70 eV. Helium was used as the carrier gas (42 cm s^{-1}), following the same temperature gradient program as described previously. Spectrometric data were recorded (Hewlett-Packard 3398 GC Chemstation) and compared with those from the NIST HP59943C original library mass spectra. Results were expressed as micrograms per kilogram.

Analysis of Acetaldehyde Concentration. At each sampling date, juice from 20 fruits per treatment (atmosphere × storage period × shelf life period) was individually obtained, and a 5 mL sample was introduced in a 10 mL test tube, which was closed with an elastic cap and frozen at −20 °C until analysis of the acetaldehyde content as described in ref *32*. 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 6890 gas chromatograph, equipped with a column containing Carbowax (5%) on Carbopack (60:80, 2 m × 2 m 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, 80 °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 microliters per liter.

Extraction and Assay of Volatile-Related Enzyme Activities. Lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities were determined 1 and 7 days after removal from storage. Samples of both skin and flesh 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 individual assay. LOX activity was determined as described elsewhere (*29*). Briefly, the sample was homogenized in 1 mL of extraction solution [0.1 M phosphate, pH 7.5, 2 mM DTT, 1 mM EDTA, 0.1% (v/v) Triton X-100, and 1% (w/v) polyvinyl polypyrrolidone (PVPP)]. After centrifugation, 100 μL of the supernatant was diluted in 0.1 M phosphate buffer (pH 8.0), and LOX activity was measured as the increase in absorbance at 234 nm over time, due to formation of hydroperoxides from linoleic acid as the substrate. For the analysis of HPL activity, tissue was homogenized in 1 mL of extraction solution [85 mM MES, pH 6.0, 5 mM DTT, 0.1% (v/v) Triton X-100, and 1% (w/v) PVPP]. After centrifugation, 100 μL of the supernatant was used for the determination of HPL activity according to the coupled assay described by Vick (*33*). The reduction of NADH, in the presence of a commercial ADH and the aldehyde substrates generated by the HPL-catalyzed reaction, was measured by following the increase in

Table 1. Compounds Identified in the Volatile Fraction Emitted by 'Fuji Kiku 8' Apples

	RI_1[a]	RI_2[b]	code		RI_1[a]	RI_2[b]	code
straight-chain esters				**branched-chain esters**			
methyl acetate	854	–	mA	*trans*-butyl propanoate	928	717	tbPr
ethyl acetate	882	609	eA	2-methylpropyl acetate	976	691	2mprA
propyl acetate	994	649	prA	ethyl 2-methylbutanoate	1015	845	e2mB
methyl butanoate	955	656	mB	2-methylpropyl propanoate	1046	865	2mprPr
ethyl butanoate	1002	803	eB	2-methylbutyl acetate	1096	876	2mbA
propyl propanoate	1008	809	prPr	butyl 2-methylpropanoate	1129	1009	b2mPr
butyl acetate	1040	813	bA	2-methylpropyl butanoate	1140	954	2mprB
butyl propanoate	1123	910	bPr	2-methylbutyl propanoate	1180	950	2mbPr
pentyl acetate	1161	914	pA	2-methylbutyl 2-methylpropanoate	1183	1016	2mb2mPr
butyl butanoate	1218	1000	bB	butyl 2-methylbutanoate	1235	1042	b2mB
ethyl hexanoate	1239	1002	eHx	2-methylbutyl 2-methylbutanoate	1300	1106	2mb2mB
pentyl propanoate	1247	969	pPr	2-methylpropyl hexanoate	1399	1153	2mprHx
hexyl acetate	1292	1015	hxA	hexyl 2-methylbutanoate	1488	1239	hx2mB
propyl hexanoate	1353	1099	prHx	heptyl 2-methylbutanoate	1505	1249	hp2mB
hexyl propanoate	1379	1109	hxPr	**alcohols**			
butyl hexanoate	1473	1196	bHx	ethanol	912	–	etOH
hexyl butanoate	1477	1197	hxB	1-propanol	992	–	1prOH
ethyl octanoate	1499	1201	eO	2-methyl-1-propanol	1054	996	2mprOH
octyl acetate	1549	1215	oA	2-pentanol	1087	–	2pOH
pentyl hexanoate	1590	1293	pHx	1-butanol	1119	626	1bOH
hexyl hexanoate	1687	1392	hxHx	2-methyl-1-butanol	1199	667	2mbOH
butyl octanoate	1690	1394	bO	1-pentanol	1262	688	pOH
hexyl octanoate	1840	1707	hxO	2-heptanol	1350	1198	2hpOH
terpenes and ketones				1-hexanol	1392	869	1hxOH
6-methyl-5-hepten-2-one	1395	795	mhone	2-ethyl-1-hexanol	1565	1031	2ehxOH
α-farnasene	1785	1647	farna				

[a] Kovats retention index (*38*) in cross-linked FFAP column. [b] Kovats retention index (*38*) in BPX5 column. –, eluted with the solvent.

absorbance at 340 nm over time. Total protein content in the enzyme extract was determined with the Bradford method (*34*), using BSA as a standard. In both cases, one activity unit (U) was defined as the variation in one unit of absorbance per minute, as measured in an Ati Unicam UV 2 UV/Vis spectrophotometer (Thermo Fisher Scientific, Alcobendas, Spain). Each determination was done in triplicate, and results were expressed as specific activity (U mg of protein^{-1}).

Statistical Analyses. A multifactorial design was used to statistically analyze the results. The factors considered were storage period, storage atmosphere, and shelf-life period. All data were tested by analysis of variance (GLM-ANOVA procedure) using the SAS program package (*35*). Means were separated by the LSD test at $p \leq 0.05$. For multivariate analysis, samples were characterized according to the averages for the parameters assessed (volatile compounds, acetaldehyde content, and enzyme activities). To provide a general overview of the samples after cold storage, a principal component analysis (PCA) was developed. Two PCA models were performed for each of the two poststorage periods at 20 °C (1 and 7 days) considered. Fifty-four variables (volatile compounds emissions, acetaldehyde content, and LOX and HPL activities both in skin and flesh tissues) were considered in each case for sample characterization. Partial least-squares regression (PLSR) was also used as a predictive method to relate ester emission (Y) to LOX and HPL activities (X) as potentially explanatory variables. Unscrambler vers. 7.6a software (*36*) was used to develop these models. As a pretreatment, data were centered and weighted using the inverse of the standard deviation of each variable to avoid the influence of the different scales used for the variables (*37*). Full cross-validation was run as a validation procedure.

RESULTS AND DISCUSSION

Biosynthesis of Volatile Compounds after Ultra-Low Oxygen Storage. Forty-nine volatile compounds (37 esters, 10 alcohols, 1 terpene, and 1 ketone) were identified in the volatile fraction emitted by 'Fuji' apples at harvest and after cold storage (**Table 1**). Quantitatively, the most important volatile compounds after storage were 2-methylbutyl acetate, hexyl acetate, and hexyl 2-methylbutanoate, accounting together for up to 67% of the total volatile compound emission. No clear trend for the different compounds was found 1 day after removal from cold storage when the different treatments were compared. After 7 days, contrarily, considered as the mean time for fruit to arrive to consumers, an increase in some volatile compounds was observed for fruit kept under ULO + 4w treatment and, to a lesser extent, under ULO + 2w, as compared to ULO-stored samples. All detected volatile compounds were included in the subsequent analyses because, whereas only some compounds are considered to have an impact on 'Fuji' flavor, the rest contribute as background notes.

All 49 volatile compounds and acetaldehyde content in fruit juice, as well as LOX and HPL activities in both skin and flesh tissues, were used to characterize fruit at harvest and after storage (13 samples × 54 variables). A preliminary PCA model (data not shown) revealed that stored fruit separated clearly from freshly harvested samples, indicating modifications in the fruit capacity to synthesize volatile compounds after storage. To help interpretation of results, two further PCA models were developed for fruit kept for 1 and 7 days at 20 °C subsequent to cold storage (**Figure 1**), from which samples at harvest were excluded. The amounts of total variability explained by principal components 1 (PC1) and 2 (PC2) in these models were 72 and 75%, respectively.

After 1 day at 20 °C (**Figure 1A**), the storage period was the main factor for sample differentiation along PC1, whereas discrimination along the PC2 axis was given mainly by storage atmospheres. Fruits kept for 30 weeks under ULO + 4w were associated with higher HPL activity in the flesh tissue, which was highly correlated to some volatile compounds such as 2-methylbutyl acetate ($r = 0.93$), 2-methylpropyl acetate ($r = 0.92$), hexyl 2-methylbutanoate ($r = 0.86$), pentyl hexanoate ($r = 0.84$), 2-methylbutyl 2-methylbutanoate ($r = 0.83$), butyl 2-methylbutanoate ($r = 0.82$), and butyl hexanaote ($r = 0.81$). Some of these compounds have been described as conferring "fruity" notes in apple aroma (*22*, *39*, *40*).

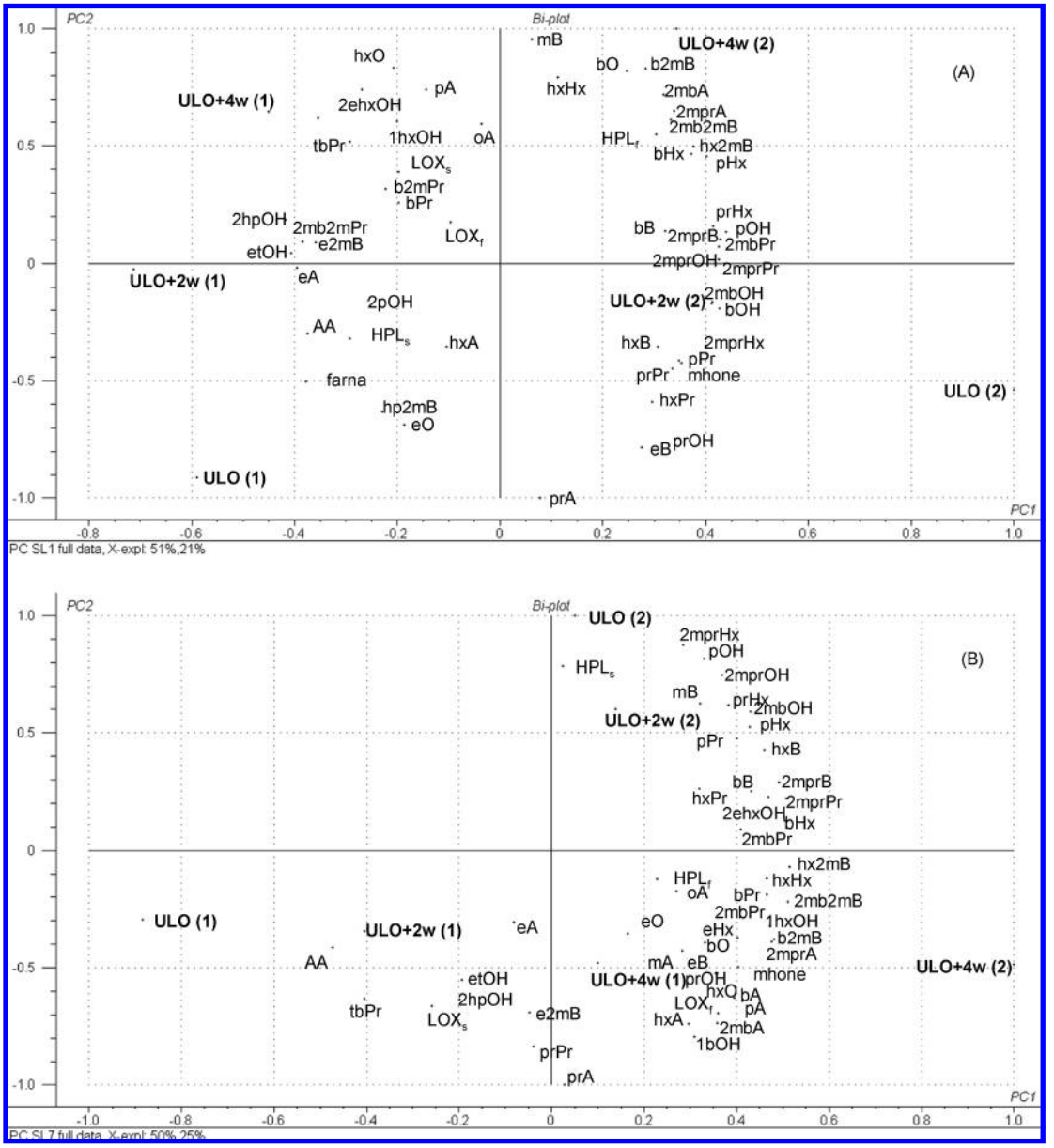

Figure 1. Biplot (scores and loadings) corresponding to a PCA model for 'Fuji Kiku 8' apples after storage plus 1 (**A**) and 7 (**B**) days at 20 °C. Volatiles are coded as indicated in **Table 1** (AA, acetaldehyde). Suffix "s" or "f" in enzyme labels refers to skin or flesh tissue, respectively. Sample labels are defined under Materials and Methods. Numbers within parentheses refer to cold storage period (1, 19 weeks; 2, 30 weeks).

Particularly, 2-methylbutyl acetate is one of the volatile compounds mostly contributing to 'Fuji' flavor (*4*). Pentyl hexanoate, adversely affected by anoxic conditions as reported elsewhere (*41*), displayed a larger increase upon removal to air (**Figure 1A**).

In contrast, HPL activity in the fruit skin was associated with samples stored for 19 weeks in ULO and ULO + 2w, and related mainly to ethyl acetate (r = 0.88) and ethanol (r = 0.84). Previous works (*42*) reported an increase in ethyl ester production, especially ethyl acetate, in whole apple fruit after hypoxic storage. It should also be noted that ethyl 2-methylbutanoate, one of the most important compounds characterizing the aroma of the variety due to its low odor threshold (0.006 μg L^{-1} according to ref *43*), correlated well with ethanol (r = 0.90) and ethyl acetate (r = 0.86). Previous studies have shown a steady decline for this compound during fruit maturation (*44*, *45*); this would be in accordance with results reported herein, showing lower emission of ethyl 2-methylbutanoate for fruit at a more advanced maturity stage after storage (ULO + 4w stored for 30 weeks). CA conditions, while leading to increased biosynthesis of ethanol, may actually limit the formation of ethyl esters as long as these storage conditions are maintained. More importantly, the procedure by which the fruits are exposed to air following CA storage may have a great effect on the final flavor profile of fruit after CA storage (*41*).

Acetaldehyde content was correlated positively to α-farnesene (r = 0.91) and 2-heptanol (r = 0.81), but correlated inversely to most of the volatile compounds associated with ULO + 4w samples. Thus, the extra period of 4 weeks under air caused a decline in acetaldehyde content. Acetaldehyde has been shown to be used by plant tissues to produce acetates (*46*), and indeed an apparent relationship has been reported between acetaldehyde concentration and the emission of acetate esters by apples after CA storage (*6*, *28*, *31*). Accordingly, and despite the overall enhanced emission of volatile esters by ULO + 4w fruit after storage for 30 weeks, most of the detected acetate esters were not associated with these samples (**Figure 1A**).

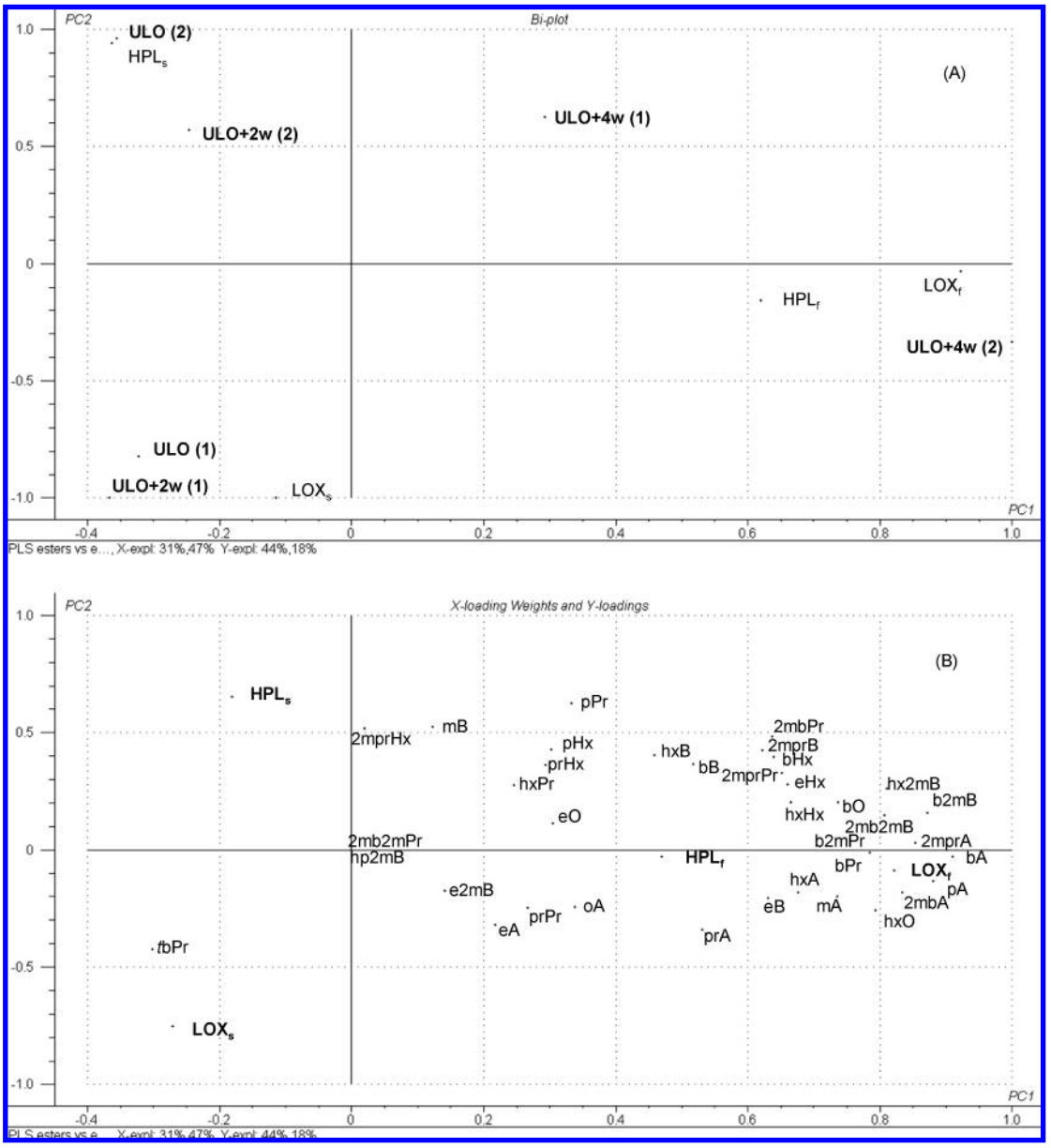

Figure 2. Biplot (**A**) and loading weights plot (**B**) of PC1 versus PC2 for a PLSR model of LOX and HPL activities (*X* variables) versus ester emission (*Y* variable) for 'Fuji Kiku 8' apples after cold storage plus 7 days at 20 °C. Volatile esters are coded as indicated in **Table 1** (AA, acetaldehyde). Suffix "s" or "f" in enzyme labels refers to skin or flesh tissue, respectively. Sample labels are defined under Materials and Methods. Numbers within parentheses refer to cold storage period (1, 19 weeks; 2, 30 weeks).

After 7 days at 20 °C (**Figure 1B**), samples stored in ULO + 4w got the highest production of most of the identified volatile compounds regardless of storage duration. Some of these compounds were those being reportedly the major contributors to 'Fuji' flavor, including ethyl hexanoate, ethyl butanoate, butyl acetate, butyl propanonate, hexyl acetate, ethyl 2-methylbutanoate, 2-methylpropyl acetate, 2-methylbutyl acetate, and 1-hexanol (*4*, *7*, *22*), suggesting that the ULO + 4w treatment helped overcome in part the characteristic ULO-induced loss in aroma quality (*1*, *4*, *5*). LOX activity in the flesh was well correlated to the flavor-contributing volatile esters in 'Fuji' apples, namely, 2-methylbutyl acetate (r = 0.95), hexyl acetate (r = 0.94), pentyl acetate (r = 0.92), butyl acetate (r = 0.91), butyl 2-methylbutanoate (r = 0.91), 2-methylpropyl acetate (r = 0.86), as well as to 1-hexanol (r = 0.86), and 1-butanol (r = 0.81), suggesting this enzyme activity may be related to the biosynthesis of these volatile compounds. During fruit ripening, cell membranes become more permeable to different substrates (*47*), increasing the chance of reaction between enzymes and substrates in the LOX pathway. In addition, storage under hypoxic conditions will lead to the partial arrest of oxygen-dependent processes and reactions, including LOX-catalyzed hydroperoxidation of fatty acids, and thus to the partial inhibition of the capacity for volatile emission (*6*, *28*, *29*). Accordingly, samples kept in air after ULO storage (ULO + 2w and ULO + 4w) were characterized by higher LOX activity in the flesh tissue, suggesting that the LOX pathway may be important for the regeneration of volatile biosynthesis after storage of 'Fuji' apples, although previous work suggested that LOX activity may be higher in the skin tissue (*41*).

HPL is also an important enzyme for the biosynthesis of volatile esters through the LOX pathway, as it processes fatty acid hydroperoxides produced by LOX and contributes to the supply of the necessary aldehyde substrates for subsequent alcohol dehydrogenase (ADH)-catalyzed production of the corresponding alcohols (*30*). In accordance with this view,

HPL activity in the flesh tissue was also associated with higher emission of most of the detected compounds after 7 days at 20 °C (**Figure 1B**). The emission of all detected esters increased dramatically after removal of fruit from hypoxia. This observation might be indicative of a sudden increase in the pool of substrates for the esterification reaction upon re-exposure to air (*41*).

Higher LOX activity was associated with higher emission of 1-hexanol. No apparent relationship was observed between the availability of 1-hexanol and the production of hexanoate esters, whereas strong correlations were found between the levels of 1-hexanol and those of some hexyl esters such as hexyl 2-methylbutanoate ($r = 0.93$), hexyl hexanoate ($r = 0.81$), and, to a lesser extent, hexyl acetate ($r = 0.67$). These correlations might have arisen from the substrate preferences of the alcohol acyltransferase (AAT) catalyzing the esterification reaction. It has been reported that a recently isolated apple AAT gene (*MpAAT1*) produces preferentially hexyl esters from middle-length acyl-CoAs at both low and high concentrations of alcohol substrate (*48*).

PLSR Model for the Prediction of Ester Emission Based on Enzyme Activities. Because 7 days is a usual period for stored fruit to reach the consumers, we chose to focus on samples kept for 7 days at 20 °C after cold storage. For a more in-depth study of the influence of LOX and HPL activities on the regeneration of the emission of volatile compounds after ULO storage, the PLSR method was used. This model considered HPL and LOX activities in both skin and flesh tissues as the potentially explanatory *X* variables and volatile ester emissions as the *Y* variables (**Figure 2**). The model developed indicated that the enzyme activities considered explained 62% of the total variability in ester production. The biplot (scores and loadings) for this model (**Figure 2A**) showed that fruit kept under ULO + 4w separated clearly from the rest of the samples along PC1. Samples stored under ULO + 4w were characterized by higher LOX and HPL activities in the flesh tissue regardless of storage period. These samples were also associated with increased concentrations of most volatile esters detected during the analyses, with LOX activity as the variable having the most weight on sample differentiation along PC1 (**Figure 2B**). In contrast, samples stored under ULO and ULO + 2w were characterized by higher LOX and HPL activity levels in the skin for short- and long-term storage, respectively. Because these fruits produced lower levels of volatile compounds, it is suggested that LOX and HPL activities in the skin had no influence on the development of aroma-synthesizing capacity during the poststorage period of 'Fuji' apples.

To assess whether it was possible to predict the emission of volatile esters from the activity levels for each of the enzyme activities considered, we chose to focus on the main volatile compounds mostly contributing to 'Fuji' flavor, namely, ethyl butanoate, ethyl hexanoate, hexyl acetate, 2-methylbutyl acetate, and ethyl 2-methylbutanoate (*11*, *35*). A separate PLSR model was run for each impact compound, with enzyme activities (*X* variables) versus the production of each volatile ester. Two PLS factors were used for each PLSR model. The validation parameters for all five PLSR models are presented in **Table 2**. Prediction was excellent for all of the volatiles included in these models and particularly for hexyl acetate and 2-methylbutyl acetate, for which 96 and 98% of total variation, respectively, were explained by LOX and HPL activities. Regression coefficients (**Table 3**) showed strong relationships to LOX activity in the flesh of all compounds characterizing 'Fuji' flavor.

These results highlight the importance of this biosynthetic pathway for the regeneration of the production of aroma volatile compounds after ULO storage. Indeed, LOX activity increased in the flesh tissue after storage under ULO + 4w as compared to ULO and ULO + 2w treatments (**Figure 3**) for both storage periods. Enhanced activity levels were also found in ULO + 2w samples in comparison to ULO-stored fruit after 19, but not after 30, weeks of storage, suggesting that an additional period of 2 weeks in cold air was not effective for the recovery of

Table 2. Validation Parameters for PLSR Models of the Major Impact Volatile Compounds Contributing to 'Fuji Kiku 8' Flavor (*Y* Variables) versus LOX and HPL Activities (*X* Variables)

Y variable	correlation coefficient[a]	explained variance[b] (%)
ethyl butanoate	0.84	70
ethyl hexanoate	0.88	79
hexyl acetate	0.98	96
2-methylbutyl acetate	0.99	98
ethyl 2-methylbutanoate	0.96	93

[a] Correlation between predicted and measured values. [b] Amount of *Y* variance explained by the first two PLS factors.

Table 3. Regression Coefficients between LOX and HPL Activities (*X* Variables) and Impact Volatile Compounds (*Y* Variables) at $p < 0.05$

	Y variables			
	LOX		HPL	
aroma compound	flesh	skin	flesh	skin
ethyl butanoate	0.727	−0.023	−0.041	−0.284
ethyl hexanoate	0.777	−0.355	0.067	0.067
hexyl acetate	0.912	0.005	−0.185	−0.232
2-methylbutyl acetate	0.848	−0.055	0.149	−0.250
ethyl 2-methylbutanoate	0.669	0.236	−0.752	−0.105

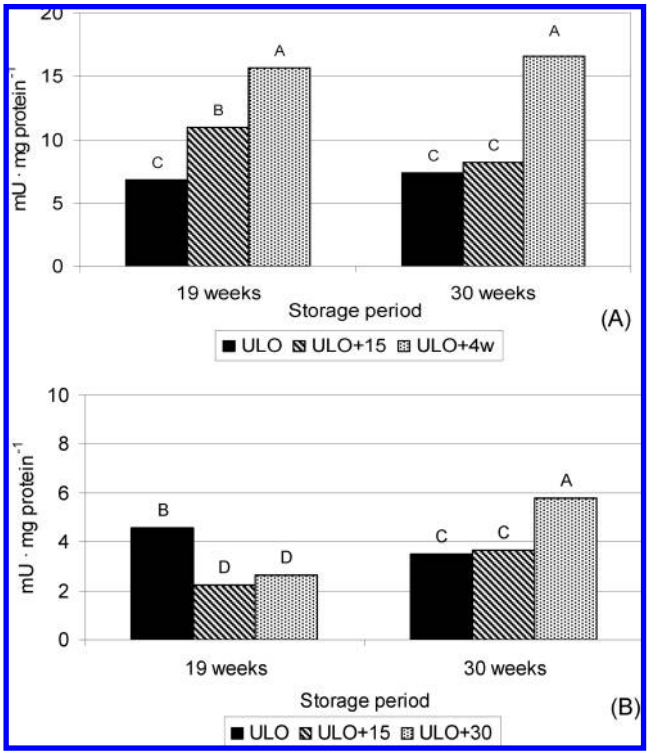

Figure 3. LOX (**A**) and HPL (**B**) activities in the flesh tissue of 'Fuji Kiku 8' apples after cold storage plus 7 days at 20 °C. Values represent means of three replicates. Means showing different letters for a given enzyme activity are significantly different at $p \leq 0.05$ (LSD test).

LOX activity, and thus for aroma regeneration, after long-term storage of 'Fuji' apples. Similarly, only ULO + 4w samples showed enhanced levels of HPL activity after long-term storage (**Figure 3**) in comparison to ULO-stored fruit, suggesting that subsequent cleavage of fatty acid hydroperoxides might also be a key control point for volatile ester production after extended periods. The concluding remark that the LOX pathway is central for the regeneration of flavor after ULO storage of 'Fuji' fruit is also supported by the observation that four of the five hexyl esters detected, among which are some major contributors to 'Fuji' flavor, as well as 1-hexanol, their alcohol precursor, were closely associated with LOX and HPL activities in the flesh tissue (**Figure 1B**).

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