UNIVERSITAT DE LLEIDA
Escola Tècnica Superior d'Enginyeria Agrària
Departament de Tecnologia d'Aliments

Avaluació de la qualitat aromàtica, estàndard, sensorial i sanitària de poma 'Pink Lady®' durant la maduració i la frigoconservació

Memòria presentada per:

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Per optar al grau de Doctora Enginyera Agrònoma

Directora: Dra. Mª Luisa López Fructuoso
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Lleida, Desembre de 2008

La present memòria que porta per títol 'Avaluació de la qualitat aromàtica estàndard, sensorial i sanitària de poma 'Pink Lady®' durant la maduració i la frigoconservació' constitueix la memòria que presenta **Carmen Villatoro González**, estudiant del programa de Sistemes Agrícoles, Alimentaris i Forestals de la Universitat de Lleida, per a optar al grau de Doctora. La part experimental del programa de doctorat s'ha realitzat en el centre UdL-IRTA sota la direcció de la **Dra. Mª Luisa López Fructuoso** (Departament de Tecnologia d'Aliments) i la **Dra. Isabel Lara i Ayala** (Departament de Química). Ambdues autoritzen la presentació de la citada memòria de tesi degut a que reuneix les condicions necessàries per a la seva defensa.

Carmen Villatoro González
Doctoranda

Directores de Tesi

Mª Luisa López Fructuoso Isabel Lara i Ayala

Lleida, Octubre 2008

Vigila, esperit, vigila,

no perdis mai el teu nord,
no et deixis dur a la tranquil·la
aigua mansa de cap port.

Gira, gira els ulls enlaire,
no miris les platges roïns,
dóna el front en el gran aire,
sempre, sempre mar endins.

Sempre amb les veles suspeses,
del cel al mar transparent,
sempre entorn aigües esteses
que es moguin eternament.

Fuig-ne de la terra innoble,
fuig dels horitzons mesquins:
sempre al mar, al gran mar noble;
sempre, sempre mar endins.

Fora terres, fora platja,
oblida't de ton regrés:
no s'acaba el teu viatge,
no s'acabarà mai més.

Joan Maragall

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Finalment després de quatre anys ha arribat el moment d'acabar una etapa. Durant aquests anys nombroses persones han estat al meu costat i de manera directa o indirecta m'han ajudat a arribar fins aquí.

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To Richard Newcomb, for giving me the chance to work with your team. To Edwige Souleyre for your technical support and your help during my stay in HortResearch.

A l'AGAUR i la Universitat de Lleida pel seu ajut econòmic.

A l'IRTA, pel seu suport en la realització d'aquesta tesi.

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A TOTS, MOLTES GRÀCIES.

La Tesi Doctoral s'ha desenvolupat dintre de la línia d'investigació 'Avaluació de la qualitat aromàtica, estàndard, sensorial i sanitària de poma 'Pink Lady®' durant la maduració i la frigoconservació' ha estat realitzat al laboratori de Tecnologia i Aromes de l'Àrea de Postcollita del Centre UdL-IRTA.

El treball experimental s'ha realitzat als laboratoris i càmares frigorífiques comercials de l'empresa Nufri, S.A.T i experimentals del àrea de Postcollita (Centre UdL-IRTA) rebent el finançament de l'AGAUR.

RESUM

L'objectiu principal d'aquesta Tesi va ser estudiar la qualitat estàndard, aromàtica, sensorial i la seguretat abiòtica de la poma 'Pink Lady®' durant tres campanyes (2003-2004, 2004-2005 i 2005-2006), tant durant la maduració en camp com després de la frigoconservació en diferents atmosferes de conservació, períodes d'emmagatzemament i de permanència a 20 ºC.

Les atmosferes controlades amb nivells de oxigen i diòxid de carboni entre 1-3% van permetre una molt bona retenció de la fermesa de la polpa, del contingut de sòlid solubles, de l'acidesa i del color de fons de l'epidermis durant la frigoconservació. Els fruits conservats sota la tecnologia de fred normal varen presentar una caiguda significativa de l'acidesa i la fermesa, sobretot en emmagatzematges llargs (25-28 setmanes) i després de 7 dies a 20 ºC.

Els ésters volàtils més importants tant durant la maduració en camp com durant la frigoconservació van ser l'acetat de butil, l'hexanoat de butil, l'acetat de 2-metilbutil, l'acetat d'hexil, el propanoat d'hexil, el butanoat d'hexil, l'hexanoat d'hexil i el 2-metilbutanoat d'hexil. La màxima producció de compostos volàtils aromàtics es va aconseguir després de 13-15 setmanes de frigoconservació independentment de les condicions d'atmosfera. No obstant, el fred normal va ser la tecnologia més recomanable per a obtenir major concentració total de compostos volàtils. La biosíntesi dels compostos volàtils aromàtics al llarg de la maduració en camp i durant la frigoconservació va estar condicionada fonamentalment per la disponibilitat dels precursors dels compostos volàtils, més que per l'activitat de l'alcohol o-aciltransferasa (AAT), l'enzim responsable de forma directa amb la producció d'ésters volàtils.

En relació amb l'acceptació sensorial, l'atmosfera controla amb baix oxigen (2%) i molt baix oxigen (1%) van ser les tecnologies de conservació que han proporcionat pomes amb més acceptació sensorial després de la frigoconservació. Els fruits més apreciats pels consumidors no sempre van ser els que mostraven una producció de compostos volàtils aromàtics més elevada. Per tant, es suggereix que la concentració d'alguns compostos volàtils aromàtics va ser més important que l'emissió total a l'hora de determinar l'acceptació general del fruit. Així, els compostos volàtils aromàtics que van permetre diferenciar les pomes 'Pink Lady®' més acceptades van ser el propanoat d'hexil, el hexanoat d'hexil, el 2-metilbutanoat de butil i el 2-metilbutanoat d'hexil. Cal destacar l'elevada influència de l'hexanoat d'etil i el 2-metilpropanoat de propil sobre l'acceptació sensorial per part del consumidor durant la 3ª campanya. Tots ells contribueixen de forma predominant en l'aroma de la 'Pink Lady®' aportant un aroma característic a 'poma' i 'afruitat' amb notes a 'poma verda'. La fermesa, l'acidesa i el contingut en sòlids solubles també van influir positivament en l'acceptación per part dels consumidors.

Respecte a la seguretat abiòtica dels fruits, els resultats indiquen que el contingut de difenilamina, folpet i imazalil es van retenir majorment a la pell. La concentració de difenilamina a la pell dels fruits conservats en fred normal va ser menor que a les mostres conservades en les atmosferes controlades. El contingut de folpet a la pell va disminuir de forma marcada després de 13-15 setmanes de conservació més 1 dia a 20 ºC en totes les atmosferes de conservació estudiades, amb una reducció del 80%. L'imazalil va ser més persistent que el folpet durant la conservació frigorífica. Després de 13 setmanes en atmosfera controlada amb baix contingut d'oxigen més 4 setmanes en fred normal es va reduir la concentració de difenilamina als fruits conservats amb les atmosferes controlades en baix oxigen (LO) i també de folpet en els els fruits procedents d'atmosfera controlada amb molt baix oxigen (ULO). Després de 27 setmanes sota atmosfera ULO més 4 setmanes en fred normal es va reduir la concentració d'imazalil a la pell als fruits. En tots els casos, Els nivells de residus en fruit fresc sencer procedents de tractaments postcollita van respectar els límits màxims fixats per la legislació.

RESUMEN

El objetivo principal de esta Tesis fue estudiar la calidad estándar, aromática, sensorial y seguridad abiòtica de la manzana 'Pink Lady®' durante tres campañas (2003-2004, 2004-2005 y 2005-2006), tanto durante la maduración en campo como después de la frigoconservació en diferentes atmósferas de conservación, periodos de almacenamiento y días de permanencia a 20 ºC.

Las atmósferas controladas con niveles de oxígeno y dióxido de carbono entre 1-3% permitieron una muy buena retención de la firmeza de la pulpa, del contenido en sólidos solubles, de la acidez y del color de fondo de la epidermis durante toda la frigoconservación. Los frutos conservados bajo la tecnologia de frío normal presentaron una caída significativa de la acidez y la firmeza sobretodo en largos periodos de almacenamiento y 7 días a 20 ºC.

Los ésteres volátiles más importantes tanto durante la maduración en campo como durante la frigoconservación fueron el acetato de butilo, el hexanoato de butilo, el acetato de 2-metilbutilo, el acetato de hexilo, el propanoato de hexilo, el butanoato de hexilo, el hexanoato de hexilo y el 2-metilbutanoato de hexilo. La máxima producción de compuestos volátiles aromáticos fue después de 13-15 semanas de frigoconservación independientemente de las condiciones de atmósfera. No obstante, el frío normal fue la tecnología más recomendable para obtener mayor concentración total de compuestos volátiles. La biosíntesis de compuestos volátiles aromáticos a lo largo de la maduración en campo y durante la frigoconservación estuvo condicionada fundamentalmente por la disponibilidad de los precursores de los compuestos volátiles, más que por la actividad del alcohol o-aciltransferasa (AAT), el enzima responsable de forma directa con la producción de ésteres volátiles.

En relación con la aceptación sensorial, la atmósfera controlada con bajo oxígeno (2%) y muy bajo oxígeno (1%) fueron las tecnologías de conservación que han proporcionado manzanas con más aceptación sensorial después de la frigoconservación. Los frutos más apreciados por los consumidores no siempre mostraron una producción de compuestos volátiles aromáticos más elevada. Por tanto, se sugiere que la concentración de algunos compuestos volátiles aromáticos fue más importante que la emisión total a la hora de determinar la aceptación general del fruto. De esta manera, los compuestos volátiles aromáticos que permitieron diferenciar las manzanas 'Pink Lady®' más aceptadas fueron el propanoato de hexilo, el hexanoato de hexilo, el 2-metilbutanoato de butilo y el 2-metilbutanoato de hexilo. Cabe destacar la elevada influencia del hexanoato de etilo y el 2-metilpropanoato de propilo sobre la aceptación sensorial por parte del consumidor la 3ª campaña. Todos ellos contribuyeron de forma predominante en el aroma de la 'Pink Lady®', aportando un aroma característico a 'manzana' y 'afrutado' con notas a manzana 'verde'. La firmeza, la acidez y el contenido en sólidos solubles también influyeron positivamente en la aceptación por parte de los consumidores.

Respecto a la seguridad abiótica de los frutos, los resultados indican que el contenido de difenilamina, folpet y imazalil se retuvo mayormente en la piel. La concentración de difenilamina en la piel de los frutos conservats en frío normal fue menor que las muestras conservadas en átmosfera controlada. El contenido de folpet en la piel disminuyó de forma marcada después de 13-15 semanas de conservación en todas las atmósferas de conservación estudiadas, con una reducción del 80%. El imazalil fue más persistente que el folpet durante la conservación frigorífica. Después de 13 semanas en atmósfera controlada con bajo contenido más 4 semanas en frío normal redujo la concentración de difenilamina en los frutos conservados en atmósfera controlada con bajo oxígeno (LO) y también de folpet en los frutos procedentes de atmósfera controlada con muy bajo contenido de oxígeno (ULO). Después de 27 semanas bajo atmósfera ULO más 4 semanas en frío normal se redujo la concentración de imazalil en la piel de los frutos. En todos los casos, los niveles de residuos en fruto fresco entero procedentes de tratamientos postcosecha respectaron los límites máximos fijados por la legislación.

SUMMARY

The main objective of this thesis was to study the changes in the standard quality parameters, volatile compounds emitted, consumer acceptance and abiotic safety of 'Pink Lady®' apples picked at three consecutive seasons (2003-2004, 2004-2005 and 2005-2006) during on-tree maturation and after storage under different conditions, including atmosphere composition, storage period and ripening time at 20 ºC

Controlled atmospheres with oxygen and carbon dioxide levels in the range 1-3% allowed very good retention of firmness, soluble solids content, titratable acidity and background colour during cold storage. Cold storage under air led to a drop in acidity levels after long-term storage (25-28 weeks) plus 7 days at 20 ºC.

The most important volatile esters in quantitative terms emitted both during on-tree maturation and after cold storage were butyl acetate, butyl hexanoate, 2-methylbutyl acetate, hexyl acetate, hexyl propanoate, hexyl butanoate, hexyl hexanoate and hexyl 2-methylbutanoate. In addition, the maximum total concentration of aroma volatile compounds was found after 13-15 weeks of cold storage irrespective of atmosphere composition; nevertheless, storage under cold air was the most advisable technology to obtain the maximum production of total aroma compounds. The biosynthesis of aroma volatile compounds both during on-tree maturation and after cold storage was conditioned by the availability of the necessary precursors rather than by the activity of alcohol o-acyltransferase (AAT), the direct enzyme responsible for the production of volatile esters.

In connection with consumer's acceptance, CA storage appeared as highly advisable in order to get the best sensory quality of 'Pink Lady®' apples after cold storage. The best accepted fruit did not always show the highest production of aroma volatile compounds, suggesting that the concentration of some specific volatile compounds is more important than total aroma volatile emission in determining overall fruit acceptability. Some specific aroma volatile compounds, namely hexyl propanoate, hexyl hexanoate, butyl 2-methylbutanoate, hexyl 2-methyllbutanoate, ethyl hexanoate and propyl 2-methylpropanoate (only during the last experimental season), accounted for the differentiation between well-accepted and only marginally accepted samples. These compounds contributed to the aroma profile of 'Pink Lady®' apples by conferring a characteristic aroma of 'apple' and 'fresh-green fruity'. Firmness, titratable acidity and soluble solids content were found to have a positive influence on acceptability.

Regarding abiotic safety of 'Pink Lady®' apples following treatments with different agrochemicals, results indicate that diphenylamine, folpet and imazalil were retained mainly in the skin. Diphenylamine concentration in the skin of air-stored fruit was smaller than that of CA-stored samples. Folpet concentration in the skin diminished after 13-15 weeks of cold storage plus 1 day at 20 ºC in all the atmosphere conditions studied, with a reduction of 80%. Imazalil was more persistent during storage than folpet. After 13 weeks in controlled atmosphere with low oxygen plus 4 weeks in normal air, diphenylamine and folpet contents in skin were reduced in low oxygen (LO) and ultra low oxygen (ULO) stored samples. Imazalil content in the skin of fruit was reduced after 27 weeks in ULO plus 4 weeks in normal air. In all cases, residue levels in the whole fresh fruit were lower than the maximum residue limits established by the Spanish legislation.

Recompte

Part dels resultats d'aquesta Tesi han estat inclosos a les següents publicacions o manuscrits:

Capítol 1.- Changes in biosynthesis of aroma volatile compounds during on-tree maturation of 'Pink Lady®' apples.
Publicat a Postharvest Biology and Technology 47 (2008), 286-295.

Capítol 3.- Volatile compounds quality parameters and consumer acceptance of 'Pink Lady®' apples stored in different conditions.
Publicat a Postharvest Biology and Technology 43 (2007), 55-66.

Capítol 4.- Effect of controlled atmospheres and shelf life period in the concentration of the volatile substances released by 'Pink Lady®' apples and on the consumer acceptance.
Enviat a European Food Research and Technology.

Capítulo 6.- Long-term storage of 'Pink lady®' apples modifies volatile-involved enzyme activities: consequences on production of volatile esters.
Publicat a Journal Agriculture and Food Chemistry 58, 9166-9174.

Capítulo 7.- Cold storage conditions affect the persistence of diphenylamine, folpet and imazalil residues in 'Pink Lady®' apples.
Acceptat a LWT- Food Science and Technology (en premsa).
doi:10.1016/j.lwt.2008.07.014.

Capítulo 8.- Influence of the combination of different atmospheres on Diphenylamine, Folpet and Imazalil content in cold-stored 'Pink Lady®' apples.
Acceptat a Postharvest Biology and Technology (en premsa).
doi:10.1016/j.postharvbio.2008.05.016.

ABREVIATURES

AAT: Alcohol o-aciltransferasa (EC 2.3.1.84)
AC: Atmosfera controlada
ADH: Alcohol deshidrogenasa (EC 1.1.1.1)
AGAUR: Agència de Gestió d'Ajuts Universitaris i de Recerca
APPLE: Associació Pink Lady® Europa
ANOVA: Anàlisi de Variança
BSA: Seroalbúmina bovina
CA: Controlled atmosphere
CBB: Blau Brillant de Coomassie G-250
CeRTA: Centre de Referència en Tecnología d'Aliments
CoA: Coenzim A
CTIFL: Centre Technique Interprofessionel des Fruits et Légumes
CTs: trienos conjugats
cv: cultivar
DAR: Departament d'Agricultura, Alimentació i Acció Rural
DPA: Difenilamina
ddpf: dies después de plena floració
DTNB: Àcid-2-nitro-ditiobenzoïc
DTT: Ditiotreitol
EDTA: Àcid etilendiaminotetraacètic
FAO: Organització de les Nacions Unides per l'Agricultura i Alimentació
FN: Fred normal
GC-MS: Cromatografia de gasos acoplada amb espectometria de mases
HPL: Hidroperòxid liasa (EC no assignat)
HR: Humitat relativa
IPLA: International Pink Lady® Alliance
IRTA: Institut de Recerca i Tecnología Agroalimentàries
LMR: Límits màxim de residus
LO: Low Oxygen (Baix oxigen)
LOX: Lipoxigenasa (EC 2.13.11.12)
LSD: Least Significant Difference
MES: Àcid morfolino-età-sulfònic
NADH: Nicotinadenin-dinucleòtid (forma reduïda)
NAD(P)H: Nicotinamina Adenina Dinucleòtid Fosfat (forma reduïda)
PCA: Principal Component Analysis
PDC: Piruvat descarboxilasa (EC 4.1.1.1)
PLSR: Partial Least Square Regression
PSA: Pressure Swing Adsorption
PVPP: Polivinilpolipirrolidona
SSC: Soluble Solid Concentration
Tª: Temperatura
TPP: Pirofosfat de tiamina
U.a: Unitats d'activitat enzimàtica
UdL: Universitat de Lleida
UE: Unió Europea
UIQPA: Unión Internacional de Química Pura y Aplicada.
ULO: Ultra Low Oxygen (Ultra baix oxigen)
UV/Vis : Ultravioleta-visible

ÍNDEX

Resultats

A los que han entendido la importancia de este esfuerzo,

y especialmente a los que la han compartido.

INTRODUCCIÓ GENERAL

1. TRETS BÀSICS DE LA VARIETAT 'PINK LADY®'

1.1. ORIGEN I CARACTERÍSTIQUES DEL FRUIT

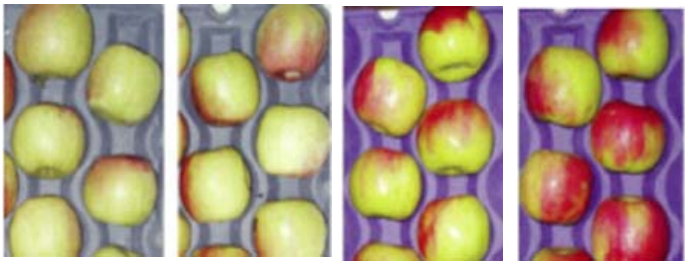
Cripp's Pink porta el nom del seu creador-productor, John Cripps, i fou seleccionada del programa original de producció de pomes (*Malus domestica,* Borkh) en la Horticultural Research Station of Stoneville de Austràlia Occidental, al 1979, i posada en circulació per a la seva avaluació comercial l'any 1986 (Cripps i col., 1993). Procedeix de l'encreuament entre 'Lady Williams' i 'Golden Delicious', amb el qual es buscava combinar la fermesa, potencial d'emmagatzematge i la baixa susceptibilitat a 'bitter pit' de 'Lady Williams' (Jobling i col., 2004), amb la bona qualitat organolèptica i baixa incidència de l'escaldat de 'Golden Delicious' (Cripps i col., 1993; Moggia i Pereira, 2003).

La poma 'Pink Lady®' és una varietat de maduració tardana d'un calibre mitjà a gran (70-75 mm de diàmetre) i de forma cònica allargada, amb àrees verdes a la pell i un sòlid color roig-rosat. L'epidermis, prima i llisa, es torna cerosa amb l'avanç de la maduresa. La polpa és blanca, densa, ferma, moderadament sucosa i dolça amb un balanç àcid (13% de contingut en sòlids solubles totals (SSC) i 7.3 g L^{-1} àcid màlic). La poma 'Pink Lady®' va ser descrita com a 'fresca' i 'cruixent' amb una fermesa de 83 N a la collita (Cripps i col., 1993).

Fotografia 1: **Poma 'Pink Lady®'** (Font: Iglesias, 2007).

La poma 'Pink Lady®' ha de tenir coloració a la pell rosa-roig entre un 40% a 70% de la superfície del fruit i un color de fons verd-groc sobre un 30% a 60% (Cripps i col.,

1993; Burmeister i col., 2001). A la fotografía 2 podem veure com evoluciona la coloració conforme es produceix la maduració.



***Fotografia 2*: Escala de color de la cara colorejada i ombrejada de pomes 'Pink Lady'™** (Font: Calvo i col., 2008).

1.2. LA PRODUCCIÓ I COMERCIALITZACIÓ

Espanya és el sisè país productor de pomes de la Unió Europea-25. La producció total de poma al 2007 en la UE-15 va ser de 6802 millers de tones (FAO, 2007; www.fao.org).

Dins de la producció de poma europea, la varietat més produïda, i amb diferència, és 'Golden Delicious' (1986 millers de tones). Altres varietats que destacarien o tindrien produccions més elevades, entre 300 i 800 milers de tones anuals, són el grup 'Gala' (814), 'Red Delicious' (562), 'Jonagold' (751), 'Elstar' (362) i la 'Granny Smith' (307) (Eurofel, 2007). La varietat 'Pink Lady®' no és una de les més produïdes, ja que a la campanya de 2007 se n'obtingueren 73.000 t el que representa l'1% de la producció total Europea. Ara bé, el que sí es pot observar és com la producció de 'Pink Lady®' ha anat augmentant al llarg d'aquests anys passant de 26.000 t l'any 2000 a 73.000 t l'any 2007 (Eurofel, 2007). Els principals mercats europeus per la 'Pink Lady®' són Alemanya (35%), França (28%) i Gran Bretanya (20%). (www.pinklady-europe.com; Crabos i Salon, 2007).

Aquest varietat ha estat pionera en establir la seva producció sobre un suport de marketing i la seva expansió és supervisada per la International Pink Lady® Alliance

(IPLA) amb l'objectiu de seguir els estàndards de qualitat. L'any 1995 es van realitzar les primeres plantacions en l'àmbit Europeu i al 1997 el grup creà l'Associació Pink Lady® Europa (APLE), la qual du a terme una política de producte que engloba tota la cadena productiva. El seu fruit pot ser comercialitzat a escala mundial sota la marca registrada 'Pink Lady®', complint estrictes requisits comercials, legals i de qualitat. La marca registrada 'Pink Lady®' és propietat de Apple and Pear Australia Ltd i els agricultors paguen la llicència i els costos de marketing associats amb la marca. La combinació de les característiques excepcionals de qualitat d'aquesta varietat i el marketing internacional fan que 'Pink Lady®' sigui una de les varietats més populars arreu del món. Per a mantenir la qualitat d'aquesta varietat és per tant, essencial assegurar la satisfacció del consumidor i la fidelitat de la marca 'Pink Lady®'.

És una varietat de poma protegida legalment sota 'drets d'hibridació' a Sudàfrica, Nova Zelanda, Sud Amèrica, Estats Units i la Unió Europea. L'Agricultural Western Australia posseeix una llicència exclusiva a cada un d'aquests territoris per la propagació i venta d'arbres a la indústria local.

2. QUALITAT DE LA POMA 'PINK LADY®'

2.1. PARÀMETRES DE QUALITAT ESTÀNDARD

És important resaltar que el consumidor aprecia l'estat de maduresa com el factor que més influeix en la qualitat de la fruita. En general, l'estat de maduresa de la poma al moment de la recol·lecció té una influència determinant en la seva aptitud per a la conservació frigorífica i la qualitat final del fruit. Al cas concret de 'Pink Lady®', la seva maduresa de recol·lecció es situa entre 200 i 220 dies després de plena floració (Cripps i col., 1993; Vayesse i Laudry, 2004).

Els índexs de maduresa habitualment utilitzats per determinar la data òptima de collita són, entre d'altres, el calibre, el color de fons i superficial, l'índex de midó, el contingut

de sòlids solubles, l'acidesa i la fermesa de la polpa. L'índex de midó és el paràmetre que més s'utilitza per determinar la data de collita de 'Pink Lady®' (Burmeister i col., 2001; Drake i col., 2002), encara que alguns autors afirmen que és un indicador variable en funció de la zona (James i col., 2005a). Els límits recomanats pel Centre Technique Interprofessionnel des Fruits et Légumes (Ctifl) són de 5 a 6 en una carta de color sobre 10. Per una òptima i llarga conservació frigorífica de la poma 'Pink Lady®'es recomana que l'índex de midó a la collita sigui de 3.5 amb una fermesa de la polpa més gran de 64 N (Jobling i Hannah, 2007). Altres autors van afirmar que en el cas de la poma 'Pink Lady®', el paràmetre fonamental per la recol·lecció és el color de fons, a més, ja que marca la diferència entre 'Pink Lady®' i 'Cripps Pink®' (Calvo i col. 2008), i és un índex recomanat per alguns autors en altres varietats de poma (Watkins i col., 1993; Iglesias i col., 2000). A la taula 1 es mostren els paràmetres òptims de recol·lecció de la 'Pink Lady®'.

***Taula 1*: Paràmetres òptims de recol·lecció de la 'Pink Lady®'** (Mathieu i col., 1998; Vayesse i Laudry, 2004).

Paràmetre de qualitat	Valor
Dies després de plena floració (ddpf)	210-220
Índex de midó	5-6 (escala 1-10 Eurofru)
Fermesa	70-80 N cm^{-2}
Sòlids solubles	> 13.5 ºBrix
Acidesa	6-7 g àcid màlic L^{-1}
Color de fons	De verd a groc F_3-F_4 (escala Ctifl de 1 a 7)
Intensitat de color	Rosa intens R_4-R_5 (escala Ctifl de 1 a 8)

Per a l'exportació de la poma 'Cripps Pink' sota la marca 'Pink Lady®', el fruit ha de tenir un mínim de fermesa de 6.8 kg (66.7 N) i una mitjana de 7.0 kg (68.8 N) i una coloració de fons de la pell verd pàlid (Hurndall i Fourie, 2003).

La climatologia determina el ritme de desenvolupament de la coloració vermella i, conseqüentment, la maduresa del fruit al moment de la collita comercial. El desig per aconseguir la totalitat de color roig a la poma 'Pink Lady®' que demanen els mercats, és

un factor limitant per la qualitat del fruit, perquè el fruit es converteix en sobremadur quan es retarda la data de collita comercial (Shafiq i Singh, 2005; De Castro i col., 2007a). Aquest fet fa que el potencial de conservació i altres atributs de qualitat com la fermesa o el color de fons es puguin veure afectats, a més a més de que es desenvoluparia greixositat i/o embruniment intern durant la conservació (Brown i col., 2005). D'altra banda, els fruits massa immadurs podrien no arribar a un 13% de SSC que requereix el mercat i són fruits més propensos a desenvolupar escaldat superficial (Burmeister i col., 2001).

2.2. INFLUÈNCIA DE LES CONDICIONS DE FRIGOCONSERVACIÓ

La frigoconservació de la poma 'Pink Lady®' amb atmosfera controlada, amb baixos nivells d'O_2 i de CO_2 d'1 a 3% s'utilitza amb la finalitat de millorar els atributs de qualitat del fruit com la fermesa, el color de fons, i reduir alguns desordres com l'escaldat superficial (Burmeister i col., 2001).

Els últims anys, s'estàn portant a terme moltes investigacions sobre els canvis en la qualitat de la poma 'Pink Lady®' com a resultat dels tractaments postcollita, incloent frigoconservació tant amb fred normal com amb atmosfera controlada o en combinació amb l'aplicació d'inhibidors d'etilè (aminoetoxivinilglicina -AVG- i 1-metilciclopropè -1-MCP-) (Drake i col., 2002; Crouch, 2003; Golding i col., 2005; Gualanduzzi i col., 2005). Malgrat aquests estudis, encara no ha sorgit un clar enteniment del comportament postcollita d'aquesta varietat.

En general, la poma 'Pink Lady®' pot frigoconservar-se en atmosfera controlada de 8 a 10 mesos depenent de les combinacions d'O_2 i CO_2 a la cambra (Cripps i col., 1993; Moggia i Pereira, 2003; Brackmann i col., 2005; Saftner i col., 2005). L'atmosfera controlada proporciona fruits amb major fermesa en relació al fred normal, independenment de la concentració de CO_2 (De Castro i col., 2007a), a més a més de mantenir millor acidesa i color (Meheriuk, 1993; Brackmann i Streif, 1994). L'efecte de

l'atmosfera controlada sobre la fermesa és degut a una reducció en la respiració o una menor degradació de les pectines de la paret cel·lular (Brackmann i col., 2005). Tot i així, la 'Pink Lady®' també manté una qualitat acceptable en condicions de fred normal després de 4-6 mesos de frigoconservació (Cripps i col., 1993; Drake i col., 2002; Moggia i Pereira, 2003; Clavo i col., 2008). Durant la frigoconservació, els principals problemes que presenta la 'Pink Lady®' *s*ón la pèrdua de fermesa i l'epidermis cerosa essencialment a les collites tardanes (Tronel i Mazollier, 2003).

Altres autors van estudiar les condicions òptimes de conservació de les pomes 'Pink Lady®' a diferents zones del món, fet que probablement reflecteix les diferències entre les zones de creixement i els estats de maduresa dels fruits al moment de la collita (Taula 2). Des del Centre Technique Interprofessionnel des Fruits et Légumes (Ctifl, 2004), situat a França, s'han divulgat les condicions de frigoconservació adequades per aquesta varietat de poma (Taula 3).

Taula 2: **Condicions òptimes de conservació de les pomes 'Pink Lady®' segons diversos autors.**

País	O_2 (%)	CO_2 (%)	T (ºC)	Període (mesos)	Referència bibliogràfica
Argentina	1.5-2	0.8-1	-	-	Candán i col. (2006)
Brasil	1.5	1-2	-0.5-0.5	9	Brackmann i col. (2005)
EE.UU	2	1	-	-	Meheriuk (1993)
EE.UU	1	1-3	-	-	Drake i col (2002)
Francia	1-2	0.5-1	2-3	6-8	Tronel i Mazollier (2003)
Itàlia	1.2	0.8	0-1	6	Testoni i col. (2002)
Itàlia	2-2.5	1.5-1.8	0-1	8	Sansavini i Asirelli (1998)
Itàlia	1.8	< 1.3	2.5	6	Zanella i col. (2003)
Sudàfrica	3	1	-	6	Crouch (2003)
Sudàfrica	≥ 1.5	1	0.5	8	Hurndall i Fourie (2003)
Xile	1-2	0.5-3	-	8-9	Moggia i Pereira (2003)

on: - = dada no disponible.

Taula 3: **Paràmetres òptims d'estocatge de les pomes 'Pink Lady®' en funció de la tecnologia de frigoconservació** (Ctifl, 2004; Vayesse i Laudry, 2004).

Tipus de conservació	T ºC	O_2 (%)	CO_2 (%)	Període (mesos)
FN	2 a 3 ºC	21%	0.03%	4-5
AC	2 a 3 ºC	2-3%	1.5-2%	6
ULO	2 a 3 ºC	1.5-1.8%	1%	6-7

FN: fred normal; AC: atmosfera controlada; ULO: ultra-low oxygen.

2.3. ALTERACIONS, FISIOPATIES I MALALTIES DE LA POMA 'PINK LADY®' EN POSTCOLLITA

Un dels principals problemes que presenta la varietat 'Pink Lady®' en el moment de la collita és la seva alta sensibilitat als cops, per això és molt important tenir cura durant les manipulacions posteriors (Vayesse, 2000; Moggia i Pereira, 2003). Aquesta varietat té la mateixa sensibilitat als cops que un dels seus parentals, 'Golden Delicious' (Candán i col., 2006) i en canvi no és susceptible als danys per fred com l'altre parental 'Lady Williams'. Maguire i col. (2000) van estudiar la permeabilitat al vapor d'aigua i la subsequent pèrdua de pes de la poma 'Pink Lady®' i van revelar una permeabilitat menor comparada amb altres varietats. Ambdós factors contribueixen a la reducció del desenvolupament d'arruges a la pell durant la frigoconservació de la poma 'Pink Lady®'. No és susceptible al cop de sol, al russeting, a ruptures de l'epidermis ('surface cracking'), ni al 'bitter pit' (Cripps i col., 1993; Campbell, 2005).

Durant la frigoconservació de les pomes es fa precisa l'aplicació de tractaments químics en postrecolecció degut a la gran incidència de podridures causades principalment per fongs dels gèneres *Penicillium expansum*, *Botrytis cinerea* i *Rhizopus stolonifer*. A més, poden aparèixer una sèrie de desordres fisiològics (embruniment intern, escaldat superficial, etc.). Tot això ocasiona les majors pèrdues durant el període de conservació frigorífica (De Castro i col., 2005).

L'escaldat superficial és un desordre fisiològic més comú en postcollita d'algunes varietats de pomes, que es manifesta com pardejaments de la pell sense tenir influència a la polpa. Aquest desordre és esporàdic a la natura, afectat per la campanya, la climatologia i la collita. Encara que el mecanisme del desenvolupament del escaldat no és conegut amb precisió, s'accepta generalment que sigui causat per productes d'oxidació de l'α-farnesè, un metabolit promogut per l'etilè. Ja que és un reacció d'oxigenació, en reduir la disponibilitat de l'O_2 (per l'aplicació d'atmosfera controlada) es redueix la taxa de reacció i el desenvolupament de l'escaldat (Watkins i col. 2000).

Fotografia 3: **'Pink Lady®' afectada per escaldat superficial sever després del període de 'shelf life'** (Font: East, 2006).

El símptoma apareix, generalment, després de períodes perllongats d'emmagatzematge iguals o superiors a 4 mesos (Ingle i D'Souza, 1989). Segons diversos autors, la varietat 'Pink Lady®' pateix certa susceptibilitat a l'escaldat superficial, principalment en collites primerenques i conservades en fred normal (Folchi i col., 2003; Hurndall, 2003; Gualanduzzi i col., 2005), fins a un 30% del fruit podria mostrar un lleuger escaldat al 50% de la seva superfície (Cripps i col., 1993), o atmosfera controlada (1.5% O_2 i 1% CO_2) després de 7 mesos més 7 dies de 'shelf life' arribant fins un 80% dels fruits afectats (Zanella i col., 2002).

Addicionalment, la imatge de marca de la poma 'Pink Lady®' està actualment en risc com a resultat de l'embruniment intern, degut a que aquest desordre està sent molt important a les regions d'Austràlia (Brown i col., 2003). Les causes del desenvolupament de l'embruniment intern de la 'Pink Lady®' ha estat un tema

important d'investigació arreu del món (Jobling i col. 2004; De Castro i col., 2005; James i col., 2005b). Investigacions recents han demostrat que el factor que més contribueix a la incidència a l'embruniment intern són les condicions climàtiques durant el creixement del fruit. No obstant, l'estat de maduresa és també important i el risc a l'embruniment podria reduir-se si el fruit es cull en el moment òptim (Jobling i Hannah, 2007).

L'embruniment intern és esporàdic a la natura i es veu afectat per una combinació de factors pre- i postcollita com les condicions climàtiques de la campanya, el contingut mineral (De Castro i col., 2007b; James i col., 2005ab), la varietat (Brown i col., 2003) o la regió (Jobling i col., 2004), sent així un desordre no predible i intermitent. Les investigacions realitzades per Jobling i col. (2004) van trobar que la maduresa del fruit a la collita és un factor crític que predisposa el fruit al desenvolupament del desordre durant la frigoconservació. Altres autors van trobar que el nivell de CO_2 a l'atmosfera de conservació també és un factor significant per al desenvolupament del desordre (De Castro i Mitcham, 2004). Zanella (2004) i James (2007) van mostrar que la temperatura de conservació és també un altre factor significant a tenir en compte.

L'embruniment intern es va definir com 'embruniment intern difús' ('diffuse browning'), 'embruniment intern radial' ('radial browning') i 'danys per CO_2' segons l'expressió visual del símptoma (fotografía 4). Cada desordre té un tipus específic de dany fisiològic que resulta en el desenvolupament de l'embruniment intern (Jobling i James, 2004). Tant l'embruniment intern difús com el radial es manifesten tant en condicions de fred normal com d'atmosfera controlada, mentre que el tercer tipus (danys per CO_2) només s'ha trobat en atmosfera controlada. Es recomanable que la concentració del CO_2 es mantingui per sota d'1% amb la finalitat de reduir la probabilitat del desenvolupament de danys interns produïts pel CO_2 (Jobling i Hannah, 2007).

El tipus d'embruniment difús és un dany per fred ('chilling injury'), que té lloc quan les pomes 'Pink Lady®' susceptibles són frigoconservades por sota de 3 ºC durant més de 4 mesos. Aquest desordre té lloc a les regions fredes que acumulen menys de 1100 dies a més de 10 ºC durant la campanya. La conservació del fruit a 3 ºC pot reduir la incidència a aquest tipus d'embruniment intern, però amb el risc d'una reducció de la qualitat i del potencial de conservació. La nutrició del fruit ha tingut també una influència en el desenvolupament de l'embruniment intern difús de la poma 'Pink Lady®', incrementant amb baixos quocients de Ca/K i Ca/Mg (James, 2007).

L'embruniment intern de tipus radial és principalment un desordre de senescència, que té lloc quan la poma 'Pink Lady®' es cull sobremadura i es conserva per sota d'1 ºC i amb alts nivells de CO_2 per un període superior a 4 mesos. Aquest desordre és molt comú en zones càlides que acumulen més de 1400 dies per sobre de 10 ºC per temporada (James, 2007). Aquest desordre afecta les cèl·lules adjacents al teixit vascular del fruit i es caracteritza per un dany de les parets de les cèl·lules. La conservació del fruit a 1 ºC pot reduir la incidència a aquest tipus d'embruniment intern. La maduresa a la collita i el nivell de CO_2 en l'atmosfera de conservació són factors que influeixen en el desenvolupament a l'embruniment intern radial (Brown i col., 2005; Jobling i col, 2004; De Castro i col., 2007ab; James, 2007) (fotografía 4).

***Fotografia 4*: Embruniment intern a la poma 'Pink Lady®'. Esquerra: embruniment intern difús, centre: embruniment intern radial, dreta: danys per CO_2.** Imatges adaptades per Jobling i James (2004).

Els estudis realitzats per Zanella i col. (2002) i East i col. (2005) mostren una incidència a l'embruniment intern del 90% en collites tardanes i frigoconservades 7 mesos en atmosfera controlada (1.5% O_2 i 1% CO_2) més 7 dies de 'shelf life', i del 70% als fruits

frigoconservats 7 mesos en fred normal, respectivament. S'ha establert que l'embruniment intern es desenvolupa durant la frigoconservació i la incidència augmenta amb el temps de conservació (Kupferman, 2003; East i col., 2005; James, 2007) i les collites tardanes (Drake i col., 2002; Brown i col., 2005; Gualanduzzi i col. 2005; Jobling i col. 2004; James i col. 2005b; James, 2007). East (2006) ha estudiat detalladament com va augmentar la incidència de l'embruniment intern amb el temps de frigoconservació, i als 2, 4 i 6 mesos la incidència va augmentar fins al 4%, 8% i 13%, respectivament. Quan la collita es retardava la incidència a l'embruniment intern augmentava (10% als 2 mesos, 28% als 4 mesos i 40% als 6 mesos).

2.4. ELS PRODUCTES FITOSANITARIS APLICATS EN POSTCOLLITA

Per tal de determinar la qualitat sanitària dels fruits es determinen les concentracions de l'antioxidant difenilamina (DPA) i dels fungicides imazalil i folpet, productes utilitzats habitualment per a la conservació de fruita en cambra.

2.4.1. MARC LEGAL

Tant a nivell europeu com estatal, s'ha establert una sèrie de disposicions per harmonitzar la legislació sobre límits de residus entre els països comunitaris per tal de garantir la lliure circulació de mercaderies. La legislació europea i estatal que estableix els límits màxim de residus (LMR) dels productes fitosanitaris emprats i els mètodes de mostratge és la següent (Taula 4).

Els LMRs permesos per les tres matèries actives estudiades en aquesta tesi, tant a nivell europeu com estatal, són 5 mg kg^{-1} de DPA, 2 mg kg^{-1} d'imazalil i 3 mg kg^{-1} de folpet. En la Norma Tècnica per a la producció integrada de fruita de llavor per a l'any 2007 (Departament d'agricultura, alimentació i acció rural-DAR, 2007) consten les matèries actives admeses per a tractaments en postcollita on, entre d'altres, hi ha presents la DPA, l'imazalil i el folpet. En la mateixa norma tècnica s'indica que el LMR dels

productes aplicats en postcollita serà com a màxim el 50% del LMR autoritzat per la legislació vigent.

Taula 4: **Legislació europea i estatal que fixa el límits màxims de residus**

Legislació Europea	*Legislació Estatal*
Directiva 08/149/CEE de la Comissió del 29 de gener de 2008, per la que es modifica el Reglament (CE) nº 396/2005 del Parlament Europeu i del Consell mitjançant l'establiment dels annexos II, III i IV que estipulen límits màxims de residus per als productes que figuren a l'annex I del Reglament L58, 1-398, Brusel·les, Bèlgica.	Reial Decret 280/1994, del 18 de febrer de 1994, pel qual s'estableixen els límits màxims de residus de plaguicides i el seu control en determinats productes d'origen vegetal (publicat al BOE, 09/03/94) i les seves modificacions.
Directiva 07/73/CEE de la Comissió del 13 de Decembre, per la que s'estableix els límits màxims d'imazalil al fruit . L329, 40-51, Brusel·les, Bèlgica.	Ordre PRE/1402/2008, de 20 de maig, pel qual es modifica l'annex II del Reial Decret 280/1994, de 18 de febrer, pel qual s'estableixen els límits màxims de residus de plaguicides i el seu control en determinats productes d'origen vegetal.
Directiva 2002/63/CEE de la Comissió de l'11 de juliol de 2002, per la que s'estableixen els mètodes comunitaris de mostreig pel control oficial de residus de plaguicides en els productes d'origen vegetal i animal L187, 30-43 Brusel·les.	Reial Decret 290/2003, del 7 de març de 2003, pel qual s'estableix els mètodes de mostratge per al control de residus de plaguicides als productes d'origen vegetal i animal.

2.4.2. ANTIOXIDANTS: DIFENILAMINA

La difenilamina o N-fenilanilina, també anomenada DPA (Figura 1), és utilitzada per al control preventiu de l'escaldat superficial en pomes (Smock, 1955; Huelin i Coggiola, 1970; Johnson i col., 1980; Ingle i D'Souza, 1989; Curry i Kupferman, 1993) degut a que inhibeix l'oxidació de l'α-farnesè als seus trienos conjugats (CTs) (Whitaker, 2000). Ja que la varietat 'Pink Lady®' es considera propensa a l'escaldat superficial (Crouch, 2003), en certs casos, la fruita s'ha de tractar amb DPA abans de la conservació.

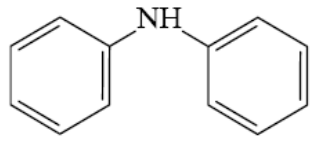


Figura 1: **Estructura molecular de la DPA**

Tot i estar registrada com a antioxidant també inhibeix desordres, tant interns com externs, causats pel CO_2 en pomes frigoconservades (Watkins i col., 1997; Fernández-Trujillo i col., 2001). A més, la DPA redueix el desenvolupament de l'escaldat tou ('soft scald') en poma 'Honeycrisp' (Watkins i col., 2004) i millora la retenció de la fermesa durant la frigoconservació (DeEll i col., 2005). Actualment, l'ús de la DPA s'ha mostrat útil per al control de l'embruniment intern en pomes 'Pink Lady®' (De Castro i col., 2007ab; De Castro i col., 2008). La DPA elimina l'embruniment intern induït pel CO_2 com a conseqüència d'una disminució més accentuada de la senescència relacionada amb l'embruniment (Jobling i Hannah, 2007).

La persistència de la DPA en pomes tractades, i conseqüentment els nivells dels residus als fruits durant la frigoconservació i posterior maduració a 20 ºC, depén en gran mesura de la formulació, la dosi aplicada, la varietat (Johnson i col., 1997), el teixit (Flath i col., 1967), l'estat de maduresa i les condicions de frigoconservació (FAO, 1984a; Rudell i col., 2006; Mattheis i Rudell, 2008). El contingut de DPA generalment va decrèixer durant la frigoconservació (Hanekom i col., 1976; Johnson i col., 1997; Kim-Kang i col., 1998; Papadopoulou-Mourkidou, 1991; Whitaker, 2000) i posterior maduració a 20 ºC (Rudell, i col., 2006). S'ha observat que la DPA no és eliminada totalment, sinò que es converteix a conjugats glicosílics de diversos metabolits de DPA hidroxilada (OH-DPA) (Kim-Kang i col.,1998; Rudell i col., 2006; Mattheis i Rudell, 2008). El metabolit polar identificat en major quantitat en pomes emmagatzemades va ser un conjugat de glucosa de 4-hidroxidifenilamina (4-OH-DPA) i el seu contingut sembla veure's afectat per la frigoconservació en atmosfera controlada (Rudell i col.,

2006; Mattheis i Rudell, 2008). Cal considerar, segons un estudi de Rudell i col. (2005), que la posició dels grups funcionals i les característiques dels derivats de la DPA afecten a la seva habilitat per controlar l'escaldat suprficial.
Segons la FAO (1984a), la DPA es considera un producte tòxic amb una dosi màxima d'ingesta per persona i dia de 0.02 mg kg^{-1}.

2.4.3. FUNGICIDES: FOLPET I IMAZALIL

El folpet, N-(triclorometiltio)ftalmida (segons la UIQPA), és un fungicida de contacte que ha estat àmpliament utilitzat en raïm els últims 50 anys (Canal-Raffin i col., 2007). S'ha utilitzat els últims 50 anys a Europa i encara s'utilitza avui dia com a tractament preventiu i curatiu contra nombroses malalties d'origen fúngic, entre les que destaquen *Alternaria* sp., *Botryotinia fuckeliana*, *Gloeosporium* sp., *Penicillium expansium*, etc. (De Liñán, 2006; Canal-Raffin i col., 2007) (Figura 2).

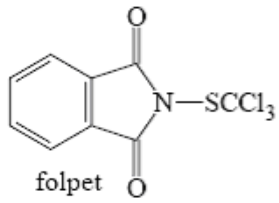


***Figura 2*: Estructura molecular del folpet.**

S'han trobat pocs estudis referents al folpet, i menys encara en frigoconservació. Es pot citar l'estudi de Palazón i col. (1984), on es mostrava una disminució del contingut del folpet en 'Golden Delicious' després de 6 mesos de frigoconservació en fred normal, encara que aquesta disminució no es va observar en els fruits d'atmosfera controlada. Cabras i col. (2000), van determinar la distribució del folpet en raïm després d'un tractament en camp. Van concloure que el folpet presentava una baixa penetració en el fruit i que gairebé tot es trobava en la superfície. També presentava una elevada resistència a ser rentat per acció de la pluja, de manera que la disminució de residus per

aquest factor va ser negligible. Altres investigacions han trobat productes de degradació del folpet al kiwi, suggerint que aquestos no s'eliminen totalment sinò que es converteixen en altres compostos (Akiyama i col., 1998). La degradació del folpet consisteix en una hidròlisi per donar clorur de tiocarbonil i 1,3-isoindolediona (phthalimide). El phthalimide s'hidrolitza a àcid phthalamic i àcid i phthalic.
El folpet està classificat com una substància nociva per inhalació i amb possibles riscos d'efectes irreversibles. A més, estudis realitzats in vitro en cèl·lules de mamífers han mostrat que el folpet té efectes mutagènics (Canal-Raffin i col., 2007). La dosi màxima d'ingesta per persona i dia és de 0.01 mg kg^{-1}.

L'imazalil, (*RS*)-1-(β-alliloxi-2,4-diclorofeniletil) imidazol (segons la UIQPA), és un fungicida sistèmic d'ampli espectre que actua per contacte i és actiu pel control de malalties produïdes per fongs endo i ectoparàsits (Figura 3).

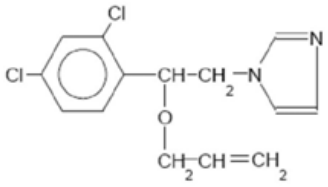


Figura 3: **Estructura molecular de l'imazalil.**

La seva aplicació en postcollita resulta efectiva en la protecció de fruits cítrics, pomes, melons, peres i plàtans pel control de podridures degudes a: *Alternaria* sp., *Botryosphaeria rhodina* (diplodiosis), *Diaporthe citri* (fomopsis), *Fusarium* sp., *Glomerella* sp., *Penicillium digitatum*, *Penicillium expansium* i *Verticillium* sp (FAO, 1977; De Liñán, 2006). Ha estat un fungicida estudiat i molt utilitzat en cítrics, trobant-se referències des de fa més de 30 anys, degut a la seva elevada activitat fúngica davant els fongs que amb major freqüència causen podridures en aquest tipus de fruits en postcollita (*Penicillium italicum* i *Penicillium digitatum*) (Lafuente i col., 1984; Cabras i col., 1999; Ghosoph i col., 2007). En pomes s'utilitza, bàsicament, per evitar la podridura blava causada per *Penicillium expansum* a més de possibles atacs de

Botryosphaeria rhodina, *Diaporthe perniciosa*, *Glomerella cingulata* i *Penicillium digitatum* entre altres (De Liñán, 2006).

La quantitat i el grau de dissipació dels residus d'imazalil en pomes frigoconservades està influenciada per la formulació utilitzada pel tractament, el tipus d'aplicació realitzada (immersió, dutxa, esprai), la varietat i les condicions d'atmosfera (Papadopoulou-Mourkidou, 1991; López i Riba, 1999). Diversos investigadors han detectat el seu metabolit majoritari (1-(2,4-diclorofenill)-1H-imidazol-1-etanol) en poma i cítrics (Woestenborghs i col., 1988; Matsumoto, 2001). Aquest producte de degradació del imazalil en poma va ser evident a partir de 4 mesos de frigoconservació representant el 10% del residu total (FAO, 1984b).
L'imazalil es considera un fungicida moderadament tòxic amb una dosi màxima d'ingesta per persona i dia de 0.01 mg kg^{-1} (FAO, 1984b).

2.5. QUALITAT AROMÀTICA

Atès que l'aroma de les pomes és un important atribut de qualitat, són moltes les investigacions que en els últims anys s'estan portant a terme sobre la composició dels compostos volàtils aromàtics a les pomes. Aquests compostos són responsables de l'olor, i contribueixen a l'aroma, la qualitat del fruit i la seva percepció final pel consumidor (Baldwin, 2002).

L'aroma del fruit resulta del conjunt de nombroses substàncies volàtils amb olor, específiques per a cada espècie i varietat (Berger i Drawert, 1984; Dixon i Hewett, 2000; Fellman i col., 2000), i està influït per diversos factors precollita (zona de cultiu, sòl, estat de maduresa del fruit...) i de manipulació del fruit com la data de collita (Fellman i col., 2003; Echeverría i col., 2004b) i les condicions de conservació (Brackmann i col., 1993; Fellman i col., 2000; Echeverría i col., 2004a).

Buttery (1993) va constatar que un compost volàtil no necessita produïr-se en alts nivells per causar un impacte en el sabor d'un fruit. Això s'explica pel fet de que la contribució de cada compost volàtil a l'aroma ve definida per les seves unitats d'olor, quocient entre la seva concentració al fruit i el seu llindar de percepció olfactiva (mínima concentració que es percebuda per l'olfacte) (Guadagni i col., 1966). S'assumeix que els compostos volàtils aromàtics amb logaritme decimal (log_{10}) de les unitats d'olor positiu són els que més contribueixen al sabor dels fruits, mentre que els que tenen valors negatius contribueixen només proporcionant el que es denomina 'notes de fons' (Baldwin, 2000). De totes maneres, només uns quants dels compostos volàtils emesos tenen un impacte decisiu en la qualitat sensorial de les pomes, sent aquestos designats com a 'compostos impacte' (Cunningham i col., 1985). Per tant, el perfil aromàtic final del fruit serà el resultat d'un equilibri metabòlic, i qualsevol canvi en aquest equilibri conduirà a canvis en l'aroma, i molt probablement a la seva acceptació sensorial.

2.5.1. COMPOSTOS VOLÀTILS DELS FRUITS

Nombrosos autors han realitzat estudis per caracteritzar la composició aromàtica de diferents varietats de pomes com 'Bisbee Delicious' (Mattheis i col., 1995), 'Golden Delicious' i 'Granny Smith' (López i col., 1998a), 'Starking Delicious' (López i col., 1998b), 'Gala' (Plotto i col., 2000; Lo Scalzo i col., 2003), 'Fuji' (Echeverría i col., 2004a), entre d'altres. S'han identificat més de 300 compostos volàtils en pomes, on els ésters representen un 80% del total (Dirink, 1989) i són els responsables de les 'notes afruitades' del perfil aromàtic del fruit (Mattheis i col., 1991). D'aquests, l'acetat de butil, l'acetat de 2-metilbutil, l'acetat d'hexil i el 2-metilbutanoat d'etil són els compostos que contribueixen en major mesura a l'aroma i sabor característics de moltes varietats de pomes (Mattheis i col., 1991; Young i col., 1996; Plotto i col., 1999; Fellman i col., 2000; López i col., 2000). L'acetat d'hexil i l'acetat de butil representen el 60% del total de compostos volàtils aromàtics a la poma 'Golden Delicious', un dels parentals de la poma 'Pink Lady®' (Brackmann i col., 1993). El 2-metilbutanoat d'etil

ha estat identificat com un compost impacte en varietats de poma del grup Delicious. Aquest compost té un llindar olfactiu molt baix ($6 \cdot 10^{-6}$ μg L^{-1}) (Takeoka i col., 1992) i correspon a una olor caracteritzada com a 'poma intensa' (Flath i col., 1967) i 'madura' (Paillard, 1990).

Si ens centrem en la varietat d'estudi d'aquest Tesi, els ésters més importants, tant qualitativament com quantitativament, identificats en poma 'Pink Lady®' eren l'acetat d'hexil, l'acetat de butil, l'acetat de 2-metilbutil, el butanoat d'hexil, el 2-metilbutanoat de butil, el 2-metilbutanoat d'hexil, l'hexanoat de butil i l'hexanoat d'hexil (Young i col., 2004; Saftner i col., 2005).

2.5.2. BIOSÍNTESI DELS COMPOSTOS VOLÀTILS AROMÀTICS

La biosíntesi d'ésters volàtils durant la maduració dels fruits climatèrics està ben establerta (Rowan i col., 1996; Sanz i col., 1997). No obstant, els factors que controlen la composició tant qualitativa com quantitativa del perfil dels ésters, que en molts casos determina el caràcter i la qualitat percebuda pels fruits, no està totalment determinada. Segons Sanz i col. (1997) i Fellman i col. (1993), la importància de cada compost volàtil dins el perfil aromàtic, depèn de l'activitat dels enzims implicats i de l'especificitat i disponibilitat de substrat. Els volàtils que en major proporció contribueixen a l'aroma del fruit són sintetitzats a partir dels lípids de membrana, aminoàcids i carbohidrats; essent els ésters, la fracció majoritària en la producció aromàtica total de la poma (Dixon i Hewett, 2000). Els ésters del fruit es produeixen per l'esterificació d'alcohols amb acetil CoA, derivats tant del metabolisme dels àcids grassos com el dels aminoàcids (Sanz et al., 1997), en una reacció catalitzada per l'enzim alcohol o-aciltransferasa (AAT) que realitza el pas d'alcohol i acil CoA a ésters volàtils (Fellman i col., 1993; Wyllie i Fellman, 2000). Segons Fellman i col. (2000), els àcids grassos representen els majors precursors per a la fracció volàtil durant la maduració. En primer lloc, les lipases alliberen àcids grassos a partir dels lípids de

membrana. Després hi ha dues possibles rutes metabòliques per l'obtenció d'ésters de cadena lineal:

▪ La ß-oxidació dels àcids grassos saturats o insaturats és el principal procés biosintètic per produir alcohols, àcids grassos de cadena curta i acetil CoA per a la síntesi d'ésters (Sanz i col., 1997). L'acil CoA és reduït per l'acil CoA reductasa a aldehids, els quals són reduïts a alcohols i aldehids mitjançant l'enzim alcohol dehidrogenasa i piruvat decarboxilasa (ADH; EC 1.1.1.1; PDC; EC 1.2.4.1), i posteriorment esterificats per l'acció de l'enzim alcohol aciltransferasa (AAT; EC 2.3.1.84) (Bartley i col., 1985; Ke i col., 1994). Aquest enzim és capaç de combinar diferents alcohols i acil-CoAs sintetitzant així un ampli rang d'ésters.

▪ L'enzim lipoxigenasa (LOX; EC 1.13.11.12) juga un paper clau en la determinació de la composició dels compostos volàtils en poma. Aquest enzim catalitza la hidroperoxidació dels àcids grassos poliinsaturats, essent l'àcid linoleic (18:2) i linolènic (18:3) els substrats principals en teixits vegetals (Fellman i col., 2000). Això condueix a l'obtenció de 9- i 13-hidroperòxids d'àcid gras, que són posteriorment metabolitzats a través de almenys 6 rutes (Porta i Rocha-Sosa, 2002). Aquests hidroperòxids són transformats a oxo-àcids o bé a aldehids per l'acció de les hidroperòxid liases (HPL). Els oxo-àcids, principalment l'àcid pirúvic, poden ser descarboxilats a aldehids en una reacció catalitzada per la piruvat descarboxilasa (PDC). Aquests aldehids passaran a alcohols per acció de l'enzim alcohol deshidrogenasa (ADH). L'última etapa de la ruta de biosíntesi consisteix en l'esterificació, catalitzada per l'enzim alcohol o-aciltransferasa (AAT), d'alcohols i acils-CoA, donant lloc a ésters volàtils (Figura 4).

Per la via d'oxidació dels àcids grassos s'obtenen àcids orgànics que seran els precursors de la formació d'ésters de cadena no ramificada. Els ésters i alcohols ramificats venen produïts per la via del metabolisme dels aminoàcids (Figura 5). El primer pas d'aquesta via és la transaminació d'aminoàcids a α-cetoàcids de cadena ramificada que passaran a ser aldehids ramificats o a acils-CoA ramificats. Els aldehids ramificats passen a alcohols ramificats per acció de l'enzim ADH. Els alcohols i l'acil

CoA són els substrats amb els quals l'AAT formarà ésters de cadena ramificada (Wyllie i Fellman, 2000).

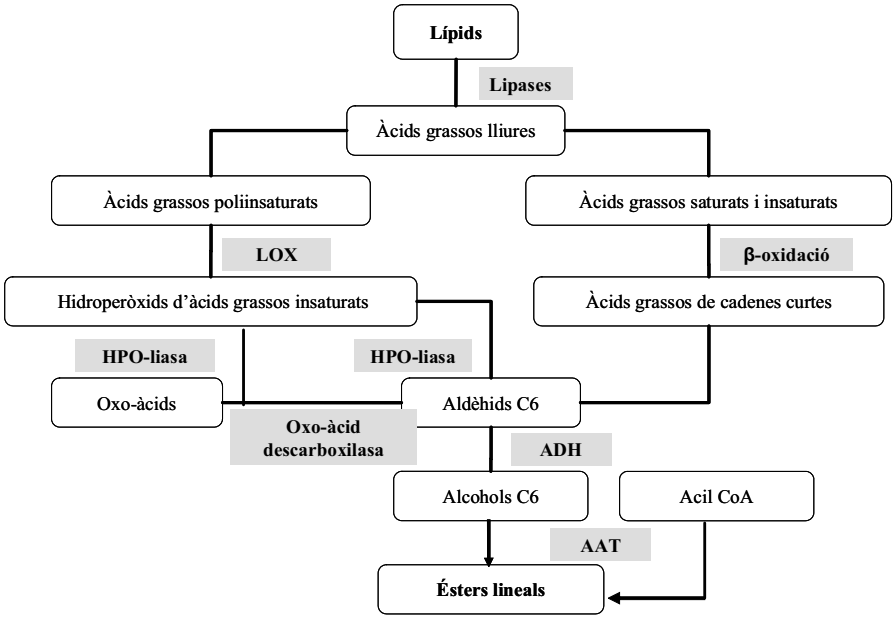


***Figura 4*: Síntesi d'ésters de cadena lineal en fruits** (Fellman, 2000).

Els factors que influeixen en la biosíntesi de compostos volàtils aromàtics han estat revisats per Yahia (1994), Fellman i col. (2000) i Dixon i Hewett (2000) i inclouen la varietat, els factors precollita, l'estat de maduresa, la temperatura, el període i les condicions de conservació. Saquet i col. (2003) van observar una disminució en la biosíntesi d'àcids grassos, amb conseqüències sobre la producció de compostos volàtils, en condicions d'atmosfera controlada i en pomes preclimatèrics (Song i Bangerth, 2003). Igualment, Lara i col., (2006, 2007) han demostrat que les condicions d'atmosfera controlada causen una disminució en la biosíntesi d'ésters volàtils a partir d'àcids grassos.

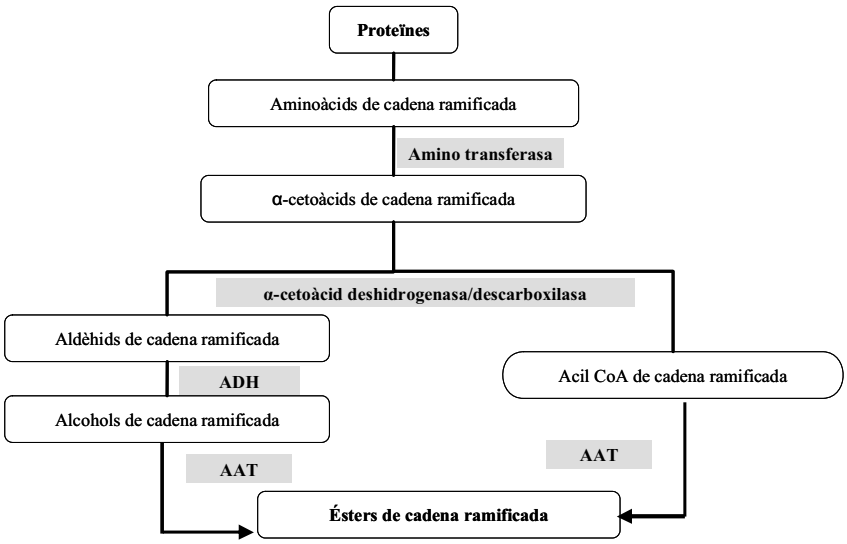


***Figura 5*: Síntesi d'ésters de cadena ramificada en fruits** (Wyllie i Fellman i col., 2000).

2.5.2.1. EVOLUCIÓ ENZIMÀTICA DURANT LA MADURACIÓ EN CAMP I LA CONSERVACIÓ FRIGORÍFICA

Segons diferents autors, l'activitat AAT augmenta amb l'inici de la maduració en diferents varietats de pomes (Fellman i col., 2000); en canvi, no s'han trobat variacions en l'activitat AAT durant la maduració en camp de la poma 'Fuji' (Echeverría i col., 2004e) ni 'Mondial Gala' (Lara i col., 2008). Per tant, la disponibilitat dels precursors també pot ser un factor limitant en la producció d'ésters volàtils als fruits immadurs (Song i Bangerth, 1994, 2003). L'AAT va mostrar una especificitat de substrat segons la varietat amb la conseqüent diferència en el perfil dels ésters volàtils (Pérez i col., 1993).

Durant la frigoconservació en atmosfera controlada l'activitat AAT es va reduir (Fellman i col., 2000; Defilippi i col., 2005), encara que l'activitat AAT es va reactivar

després de la sortida de cambra, particularment per aquells fruits amb baixa activitat enzimàtica abans de la conservació (Fellman i Mattheis, 1995). És per això que la manca d'aroma després de llargs emmagatzemaments en atmosfera controlada podria ser atribuida a una inhibició de l'activitat AAT causant una reducció en l'emissió d'ésters (Mattheis i col., 1991; Brackman i col., 1993; Fellman i col., 1993).
A més, l'activitat ADH va ser més elevada en atmosfera controlada comparada amb el fred normal (Golding i col., 2003) suggerint que l'augment en l'activitat d'aquest enzim podria estar relacionat amb l'inici de processos fermentatius en condicions d'hipòxia (Lara i col., 2007). Altres estudis mostren com les activitats PDC i ADH es veuen incrementades per l'exposició a alts nivells de CO_2, amb la corresponent acumulació d'etanol i acetaldehid, degut a l'alta sensibilitat de l'activitat d'aquests enzims als canvis del pH cel·lular (Ke i col., 1994; Saquet i Streif., 2008). En canvi, l'activitat LOX va ser parcialment inhibida en condicions d'hipòxia (Lara i col., 2006, 2007). Aquesta inhibició parcial de l'activitat LOX a la poma 'Fuji' (Lara i col., 2006) i 'Mondial Gala' (Lara i col., 2007) frigoconservades en atmosfera controlada va portar al desenvolupament d'un perfil aromàtic anormal després de la trasnferència del fruit a condicions d'aire. La disponibilitat limitada de precursors derivats d'àcids grassos podria ser un factor important de restricció de la producció d'ésters volàtils també als fruits immadurs (Song i Bangerth, 1994; 2003).

Altres estudis han revelat també que la supressió de la biosíntesi d'ésters volàtils produïda per l'atmosfera amb molt baix O_2 (ULO) és causada per una falta de precursors en lloc d'una degradació dels enzims responsables (Brackman i col., 1993; Fellman i Mattheis, 1995). Així, Song i Bangerth (2003) van demostrar que l'activitat AAT no va ser un factor limitant en la producció de volàtils. Per tant, sembla que la disponibilitat dels precursors és més limitant per a la síntesi de compostos volàtils (Wyllie i Fellman , 2000; Lara i col., 2006, 2007; Matich i Rowan, 2007). Williams i Knee (1977) van ser els primers en suggerir que la pèrdua d'aroma després de la conservació es va produir per un esgotament del subministre apropiat de substrat per la biosíntesi de compostos volàtils. Knee i Hatfield (1981) van demostrar que la proporció

d'alcohols exògena tant a la pell com als fruits sencers va incrementar l'emisió d'ésters volàtils. En experiments posteriors (De Pooter i col., 1983; Bangerth i col.,1998; Harb i col., 2000), la producció de compostos volàtils aromàtics va incrementar amb el subministre d'àcids orgànics, d'aldèhids i d'alcohols exògens als fruits. De Pooter i col. (1987) van observar una disminució dels compostos volàtils aromàtics en 'Golden Delicious' conservats amb alts nivells de CO_2. Així, es va concloure que la reducció en la síntesis d'ésters pels fruits frigoconservats es va produir per una reducció en el metabolisme de l'àcid carboxílic, i els alts nivells de CO_2 a l'atmosfera de conservació reduïen l'activitat ADH, i per tant, els fruits són incapaços de reduir els aldèhids a alcohols.

2.5.3. FACTORS QUE AFECTEN A LA PRODUCCIÓ DE COMPOSTOS VOLÀTILS

La formació dels compostos responsables de l'aroma de la fruita està relacionada amb la maduració del fruit i, per tant, està influenciada per diversos factors interns i externs (Kader, 2008). Els factors interns es refereixen a la regulació metabòlica de la maduració, els quals estan genèticament controlats a cada varietat (Paillard, 1981) i la data de collita (estat de maduresa). Els factors externs inclouen diversos factors precollita (per exemple, clima, sòl o fertilització) (Yamada i col., 1994; Mattheis i col., 1995; Fellman i col., 2000; Fellman i col., 2003) i els factors postcollita (per exemple, tractaments postcollita, període i tecnologia de frigoconservació, condicions de 'shelf life', etc...) (Plotto i col., 1995; Kader, 2008).

2.5.3.1. Diferències genètiques

Tot i que el perfil volàtil del fruit és funció de la varietat (Kakiuchi i col., 1986; Dixon i Hewett, 2000; Fellman i col., 2000), existeixen algunes similaritats. Per exemple, el butanoat d'etil, l'acetat de butil, el 2-metilbutanoat d'etil i l'acetat de 2-metilbutil s'han identificat a la fracció volàtil emesa per la pell de 9 varietats de poma (Guadagni i col.,

1971). Un altre estudi va revelar que 11 compostos volàtils contribueixen a l'aroma de més de 40 varietats diferents de pomes, mentre que 27 compostos volàtils només es van trobar al perfil de certs genotips (Cunningham i col., 1985).
Alguns autors van trobar una relació entre el color de la pell de diferents mutants de Delicious i el contingut d'èsters: els que tenien una major coloració tenien un menor aroma. Van concloure per tant que el tipus i quantitat de volàtils emesos per les pomes depenen de la varietat i dels clons de les mateixes (Fellman i col., 2000).

2.5.3.2. Fisiologia del fruit

En general, la producció de volàtils és més elevada a la pell que a la polpa (Guadagni i col., 1971; Fan i col., 1997; Rudell i col., 2002; Matich i Rowan, 2007; Lo Bianco i col., 2008), indicant que l'activitat enzimàtica relacionada i la disponibilitat dels principals precursors per a la síntesi de compostos volàtils, com ara els àcids grassos, és superior en aquest teixit (Rudell i col., 2002; Defilippi i col., 2005). La concentració d'aminoàcids i lípids a la pell del fruit podria representar un factor limitant per a la producció de volàtils. D'altra banda, el contingut de compostos volàtils aromàtics també es veu afectat pel tipus d'irrigació (Lo Bianco i col., 2008).

2.5.3.3. Efectes ambientals

La influència del clima sobre la composició de la fracció aromàtica es va estudiar en un treball realitzat per Rizzolo i Visai (1990) en 'Golden Delicious', on es va demostrar que els compostos volàtils emesos estaven influïts per l'altitud on es cultivaven les pomes, i que els fruits cultivats en muntanya posseïen una millor qualitat aromàtica tant en la collita com al final de la conservació. Un altre estudi va revelar que els fruits procedents de llocs més freds (segons latitud i/o altitud) produïen menys compostos volàtils, mentre que aquells procedents de llocs més càlids presentaven una emissió lleugerament major d'ésters després d'una frigoconservació en atmosfera controlada (Fellman i col., 1997).

Estudis realitzats a Estats Units mostren que una elevada aplicació de nitrogen augmenta la producció aromàtica en pomes 'McIntosh' (Somogyi i col., 1964). D'altra banda, el dèficit d'irrigació augmenta quantitativament el nombre de compostos volàtils en termes de concentració i qualitativament en termes d'unitat d'olor (Bussakorn i col., 2002). En un altre estudi realitzat a Dinamarca en pomes 'Jonagold' es va observar que reduint la càrrega de l'arbre augmentava la producció de compostos volàtils; arbres amb menor rati fruit/fulla produïen més acetat de butil i acetat d'hexil que arbres amb una càrrega més elevada (Hewett i col., 1999).

2.5.3.4. Estat de maduresa

La síntesi del compostos volàtils aromàtics depèn de l'estat de maduresa en el moment de la collita, ja que està associada amb la maduració (Dirink i Schamp, 1989), i el perfil aromàtic canvia al llarg del desenvolupament del fruit. L'estat de maduresa del fruit en la data de collita és un factor crític que afecta a la maduració i al desenvolupament dels compostos volàtils aromàtics al llarg del període de postcollita, particularment als fruits climatèrics on la maduració és regulada per l'etilè (Mattheis i Fellman, 1999; Echeverría i col., 2004c). L'etilè és el responsable de l'activació d'algunes de les activitats enzimàtiques implicades a la biosíntesi d'aromes, raó per la qual aquesta està relacionada amb la crisi climatèrica (Song i Bangerth, 1996; Fan i col., 1998; Defilippi i col., 2004; Mattheis i col., 2005). A mesura que la producció d'etilè i la taxa respiratòria augmenten, la quantitat d'aromes emesos també és major, observant-se que alguns compostos aromàtics es troben en nivells màxims just després del pic climatèric (Mattheis i col., 1991; Fellman i Mattheis, 1995; Song i Bangerth, 1996; Fellman i col., 2000). Una collita primerenca pot produir una marcada deficiència en el desenvolupament de l'aroma. Per tant, és preferible retardar la recol·lecció, encara que això pot provocar pèrdues de certs atributs de qualitat (fermesa i acidesa) durant la frigconservació (Song i Bangerth, 1994 i 1996; Bangerth i col., 1998; Mattheis i Fellman, 1999).

La família de compostos volàtils produïda de manera preferent canvia segons l'estat de creixement del fruit. En fruits preclimatèrics predominen, generalment, els aldehids com l'acetaldehid, l'hexanal i el E-2-hexanal (De Pooter i col 1987) . D'altra banda, a mesura que s'avança cap a l'estat climatèric i postclimatèric, la concentració d'ésters de tipus acetat s'incrementa progressivament (Fellman i col., 2000).

2.5.3.5. Condicions de frigoconservació

L'atmosfera controlada amb baix oxigen prolonga la conservació frigorífica dels fruits, a més de mantenir millor fermesa, acidesa, color i altres paràmetres de qualitat (Meheriuk, 1993; Brackmann i Streif, 1994). No obstant, s'ha demostrat en diferents estudis que l'atmosfera controlada redueix de forma marcada la producció de compostos volàtils aromàtics en diferents varietats de poma (Yahia i col., 1990; Bangerth i col., 1998; Fellman i col., 2000; Lo Scalzo i col., 2003; Mattheis i col., 2005; Graell i col., 2008). La magnitut de la resposta és depenent de varis factors que inclouen la maduresa a la collita (Yahia i col., 1990), les concentracions d'O_2 i CO_2 (Beaudry, 1999) i el període de frigoconservació (Streif i Bangerth, 1988). Patterson i col. (1974) van atribuir aquesta disminució a una pèrdua de substrat o d'enzims essencials per a la formació d'ésters. Segons Knee i Hatfield (1981) la producció d'ésters en atmòsferes amb baix oxigen és limitada per la disponibilitat d'alcohols necessaris per a la seva producció.

La severitat en la supressió de la producció de compostos volàtils aromàtics per part de l'atmosfera controlada és depenent de les condicions atmosfèriques i de la durada del període de frigoconservació. Com més baix és la concentració d'O_2 i/o més alta és la concentració d'CO_2 i més llarga és la conservació en atmosfera controlada, major és la reducció en l'emissió dels compostos volàtils (Yahia i col., 1990; Saquet i col., 2003). Aquesta resposta també es va observar per Patterson i col. (1974), Lidster i col. (1983) i Streif i Bangerth (1988). Però sembla ser que, si abans de finalitzar el període d'emmagatzemament s'augmentava el nivell d'O_2 o es transferia a condicions normals,

es provocava una millor regeneració de l'aroma de la poma (Streif i Bangerth, 1988; Fellman i col., 1993; Mattheis i col., 1995; Lau, 1998; Plotto i col., 1999; Mattheis i Fellman, 2000; Fellman i col., 2003; Young i col., 2004), que acabava de millorar amb la permanència posterior en condicions de 'shelf life' (López i col., 1998a).

La síntesi dels alcohols (via β-oxidació dels àcids grassos) i dels ésters (via esterificació d'alcohols i àcids carboxílics) són processos que depenen de l'O_2 (Brackmann i col., 1993; Fellman i col., 1993). Els mateixos autors confirmen els resultats obtinguts per Streif i Bangerth (1988), mostrant que l'emissió d'ésters de cadena lineal es va veure reduïda pel baix O_2 (1%) i l'alt nivell de CO_2 (3%). En canvi, l'emissió d'ésters de cadena ramificada es van veure afectats per l'alt nivell de CO_2, però no per l'O_2 (Hansen i col., 1992; Mattheis i col., 1995; Mattheis i Fellman, 2000). Si el nivell d'O_2 és molt reduit i el de CO_2 molt elevat fins a nivells d'estrés pot causar l'aparició de 'off-flavor' com a resultat de processos fermentatius en condicions anaeròbiques (Ke i col., 1991; Brackmann i col., 1993).

2.5.3.6. Diferències en la composició volàtil segons el mètode d'extracció

El contingut i composició aromàtica de la poma difereix estudi a estudi en funció del mètode de determinació. Un estudi sobre diverses varietats de poma posa en evidència les diferències entre els mètodes d'extracció. Segons Medina i col. (1996) els ésters representaven un 81-96% del total dels compostos volàtils aromàtics quan s'utilitzava el mètode d'extracció d'espai de cap i només entre el 11-33% quan s'utilitzava el mètode de destil·lació. En canvi, els alcohols representeaven un 48.3-75.5% quan s'utilitzava el mètode de destil.lació i al voltant d'un 10% en el d'extracció d'espai de cap. Kakiuchi i col. (1986) van realitzar un estudi sobre els compostos volàtils aromàtics emesos per cinc varietats de pomes ('Golden Delicious', 'Hatsuaki', 'Kogyoku', 'Mutsu' i 'Fuji') amb el mètode de destil·lació al buit i d'espai de cap dinàmic. El contingut total d'ésters va constituir el 78-96% dels compostos volàtils aromàtics de la poma quan s'utilitzaven tècniques d'espai de cap dinàmic i, en canvi, aquest contingut va disminuir d'un 11 a un

33% quan s'utilitzaven tècniques de destil·lació. Els alcohols són el segon grup de compostos en importància en l'aroma de les pomes. Però, es poden convertir en el primer grup quan el mètode d'extracció utilitzat és la destil·lació al buit, els quals representen el 53-76% del contingut total extret mitjançant la destil·lació al buit i només un 1-5% en el cas del mètode d'espai de cap dinàmic.

3. QUALITAT ORGANOLÈPTICA DEL FRUITS

La qualitat organolèptica està composta per molts atributs, tant intrínsecs com extrínsecs. Aquests atributs variaran depenent de les expectacions i la memòria del consumidor. Les característiques intrínseques del producte inclouen atributs com el color, la forma i el tamany. Addicionalment, atributs interns inclouen la textura, la dolçor, l'acidesa, l'aroma, la maduració a 20 ºC ('shelf life') i el valor nutricional (Hewett, 2006). De totes maneres, cada vegada més es persegueix obtenir uns bons atributs de sabor i aroma (els quals és una barreja complexa de sucres, àcids i compostos volàtils) que són bàsics per la qualitat organolèptica del producte (Baldwin, 2002).

3.1. ATRIBUTS ORGANOLÈPTICS

L'aroma constitueix un dels atributs més importants en la percepció de la qualitat sensorial per part del consumidor (Stow, 1995). L'avaluació sensorial és necessaria per entendre la qualitat del fruit i la percepció de l'aroma (Baldwin i col., 2007). La combinació de l'anàlisis instrumental amb un test sensorial proporciona millors perspectives sobre l'impacte dels compostos volàtils a l'aroma del fruit.

La qualitat sensorial del fruit està integrada per una sèrie d'atributs sensorials (dolçor, acidesa, aroma, fermesa i color) que es desenvolupen principalment durant la maduració del fruit. Aquests atributs sensorials es poden agrupar en tres categories principals: sabor, textura i aparença (Kays i Wang, 2000). En el cas del sabor, aquest

atribut és resultat d'una barreja complexa de sensacions de gust i olor del producte (Durán i Costell, 1999; Beaudry, 2000). La textura és un dels paràmetres que més influència té en la qualitat sensorial (Zerbini i col., 1999). Encara que molts consumidors diuen que el sabor és el component més important en la qualitat del fruit, algunes proves indiquen que els consumidors són més sensibles a les diferències de textura que de sabor (Shewfelt, 1999).

Els consumidors prefereixen la poma 'Pink Lady®' amb un mínim de 13% de sucres, més de 60% de rubor roig i que no sigui greixosa al tacte (Melvin-Carter i Little, 1997). La quantitat i la intensitat del rubor roig del fruit de 'Pink Lady®' és un atribut favorable comercialment i pot portar a grans retorns econòmics (Golding i col., 2005; Shafiq i Singh, 2005).

3.2. MÈTODES D'ANÀLISI SENSORIAL

L'avaluació sensorial de la fruita pretén, per una part, identificar i valorar les característiques organolèptiques d'un fruit, i, per l'altra, expressar la satisfacció percebuda pels consumidors després de la degustació. És, per tant, una eina molt interessant per a avaluar la qualitat del producte, aspecte bàsic per a optimitzar la producció, el maneig, l'emmagazetmament, i la comercialització de la fruita. Per tal d'identificar i valorar les característiques organolèptiques d'un fruit, i, expressar la satisfacció percebuda pels consumidors després de la degustació es realitzen avaluacions sensorials (Stebbins i col. 1991). L'avaluació sensorial en fruites és complicada degut a les nombroses fonts de variabilitat existents, entre elles l'abre i el fruit (Denver i col., 1995). Echeverría i col. (2004a) van observar en pomes 'Fuji' un elevat grau de variabilitat entre els jutges pel que fa a la preferència en general.

De tipus de proves usades en l'anàlisi sensorial n'hi ha moltes, però ens centrarem només en les que s'han utilitzat en aquesta tesi: les proves d'acceptació o hedòniques, que s'inclouen dins les proves afectives. Les proves hedòniques s'usen per avaluar

l'acceptació o refús d'un producte determinat. Tot i que la seva realització pugui semblar rutinària, el plantejament és molt complex i s'ha de fer de manera rigorosa per tal d'obtenir dades significatives (Sancho i col., 1999). En el moment en que es planteja una prova hedònica s'ha de tenir en compte una sèrie d'aspectes importants com precisar de forma inequívoca la naturalesa de la qüestió a resoldre i analitzar el comportament i tipus de consum del producte estudiat; utilitzar només grups ben definits de subjectes no entrenats; plantejar preguntes hedòniques senzilles o demanar comparacions fàcils i finalment tenir consciència de les limitacions pel que fa a la validesa dels resultats en funció de la situació artificial imposada als individus (Sancho i col. 1999). Aquestes proves presenten una gran variabilitat en els resultats i la seva interpretació, ja que es tracta d'apreciacions completament subjectives (Anzaldúa-Morales i Brennan, 1984).

Amb la finalitat de determinar quins atributs sensorials proporcionen una millor acceptació al fruit, diversos investigadors han realitzat avaluacions sensorials amb noves varietats de poma (Stebbins i col., 1991; Echeverría i col., 2004ad) o, també amb diferents clons de la varietat 'Fuji' (Cliff i col., 1998). Altres autors han avaluat l'efecte del període i de la tecnologia de frigoconservació sobre la qualitat sensorial del fruit al final de l'emmagatzemament i durant el període de 'shelf life' (Cliff i col., 1998; López i col., 2000; Saftner i col., 2002).

D'altra banda, existeixen nombrosos estudis que correlacionen els atributs sensorials i les mesures instrumentals dels paràmetres de qualitat de les pomes (Lavilla i col., 1999; Harker i col., 2003a; Harker i col., 2003b). A més, s'ha de tenir en compte que l'alta variació existent en una mostra (fruit a fruit) origina dificultats al intentar correlacionar els dos tipus de mesures (Bourne, 1979).

3.3. INFLUÈNCIA DE FACTORS AGRONÒMICS I TECNOLÒGICS

L'opció del consumidor de pomes a l'hora de comprar té en compte la relació entre el preu i la qualitat (Harker i col., 2003a). Els resultats d'aquests estudis reforcen la importància de les creences dels consumidors, les actituds, les percepcions i les preferències en la seva opció per la fruita. Atributs com la fermesa, el sabor i l'aroma requereix que els consumidor sigui habitual al producte per poder fer un judici de la qualitat del producte, i per tant aquests atributs no són fàcils de valorar experimentalment. Molts estudis han demostrat que la qualitat és més important pel consumidor que el preu, quan el preu és variable segons un rang comercial esperat. No obstant, el preu que el consumidor està preparat a pagar varia d'una persona a altra. Diversos investigadors han identificat que les preferències per la qualitat estan dividides en diferents grups de consumidors, per exemple, aquells que prefereixen les pomes cruixents i dolces i aquells que prefereixen les pomes sucoses i àcides (Harker i col., 2003a).

Segons els resultats obtinguts per Harker i col. (2008), la fermesa de la polpa va ser el factor dominant en l'acceptació del consumidor de pomes, però el contingut de sòlids solubles (SSC) i l'acidesa també van jugar un paper a la definició de la qualitat específica de la varietat. Els autors van observar que l'acceptabilitat dels consumidors incrementa amb valors alts de fermesa i si, a més a més, els valors de SSC són elevats, podria incrementar l'acceptabilitat.

Stainer i col. (2000) van destacar les diferències en els atributs sensorials de pomes cultivades en 3 zones diferents d'Itàlia: les pomes cultivades en zones de poca altitud (Bolònia) destaquen pels seus atributs de dolçor i sucositat, i les zones amb més altitud (valls de Laimburg) presenten millors puntuacions de color, fermesa, acidesa i crocanticitat.

L'acceptació sensorial per part dels consumidors ha estat correlacionada amb la producció d'alguns ésters (Echeverría i col., 2004a). Precisament, alguns ésters com el 2-metilbutanoat d'etil, l'hexanoat d'etil i l'acetat d'hexil tenen una contribució sensorial molt important degut als baixos llindars olfactius, que es sitúen a $6 \cdot 10^{-6}$ μg L^{-1}, $1 \cdot 10^{-3}$ μg L^{-1} i $2 \cdot 10^{-3}$ μg L^{-1}, respectivament (Takeoka i col., 1992).

Respecte a la tecnologia de frigoconservació, estudis anteriors mostren que els fruits frigoconservats en atmosfera controlada tenen una puntuació d'acceptació sensorial més elevada respecte als fruits conservats en fred normal, malgrat tenir menys compostos volàtils aromàtics (Cliff i col., 1998; Lau, 1998; Plotto i col., 1999; Echeverría i col., 2004a). Per això, es creu que la concentració d'alguns compostos volàtils aromàtics concrets és més important que l'emissió total d'aromes per determinar l'acceptació general del fruit. Una explicació podria estar relacionada amb la interacció de l'acidesa i la percepció de l'aroma (Cliff i col., 1998; Saftner i col., 2002). Pel contrari, l'anàlisi sensorial va revelar un perfil aromàtic similar en pomes 'Gravenstein' tant en fred normal com en atmosfera controlada. Això indica que les pomes de fred normal que tenien més altes concentracions de compostos volàtils respecte als fruits d'atmosfera controlada no necessàriament eren les més acceptades, ja que la concentració dels compostos volàtils que van contribuir a l'aroma va ser la mateixa (Aaby i col., 2002).

Altres estudis sensorials van demostrar que els fruits frigoconservats en atmosfera controlada tenien menys intensitat dels descriptors afruitats i florals després de 10 setmanes, mentre que l'acidesa i l'astringència fou major comparat amb els fruits frigoconservats en aire (Baldwin i col., 2007).

Un dels pocs estudis sensorials realitzats amb 'Pink Lady®' van ser els realitzats per Corrigan i col. (1997) on dóna a la poma 'Pink Lady®' juntament amb la 'Braeburn' i 'Fuji' els millors valors de textura, un excel·lent balanç de sucres-àcid i sabor, però s'obtenia una menor puntuació per la sucositat, tot i que és una poma ferma i crocant i molt valorada per la seva aparença (Cripps i col., 1993). D'altra banda, els consumidors

van preferir a la poma 'Pink Lady®', 'Fuji' i 'Braeburn' que a la 'Granny Smith' o la 'Red Doughert', sent la poma 'Pink Lady®' la més acceptada. L'anàlisi sensorial va indicar que la poma 'Pink Lady®' té un alt nivell d'acceptabilitat comparat amb altres varietats tardanes (Corrigan i col., 1997). Com a resultat, els consumidors van indicar que comprarien la poma 'Pink Lady®' més freqüentment que altres varietats de poma i estarien disposats a pagar un preu més alt.

En d'altres estudis realitzats amb pomes 'Fuji' es va trobar que la concentració en sòlids solubles, l'acidesa, la fermesa de la polpa i el color de fons de la banda ombrejada tenen una influència positiva en l'acceptació sensorial del consumidor (Echeverría i col., 2004d). Segons Drake i col. (2002), la relació entre el contingut de sòlids solubles i l'acidesa del fruit és important, ja que valors elevats d'aquest quocient impliquen una millor preferència del consumidor. Altres estudis van suggerir que l'acceptabilitat de les pomes 'Cripp's Pink' podria predir-se mitjançant determinacions de fermesa i acidesa. En canvi, els sòlids solubles no serien bons predictors de la qualitat organolèptica d'aquesta varietat (Calvo i col., 2008).

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OBJECTIUS

Objectius

L'objectiu general d'aquesta tesi va ser determinar les condicions d'atmosfera controlada (composició i període) i període de maduració a 20 ºC òptims per a la conservació de la poma 'Pink Lady®', amb la finalitat de preservar-ne la qualitat estàndard, aromàtica, sensorial i sanitària. Es pretenia també avaluar com evolucionen diversos paràmetres relacionats amb la qualitat aromàtica i estàndard durant la maduració del fruit. Per tal d'assolir aquests objectius generals es van fixar els següents objectius específics:

1. Determinar el perfil aromàtic de la poma 'Pink Lady®' durant el període de maduració en camp, en el moment de la collita comercial i després de l'emmagatzemament frigorífic.
2. Estudiar l'activitat d'alguns enzims relacionats amb la biosíntesi dels compostos volàtils aromàtics emesos per la poma 'Pink Lady®' amb la finalitat d'avaluar la seva influència sobre la qualitat aromàtica durant els períodes esmentats al punt 1.
3. Determinar l'evolució de la qualitat estàndard després de la conservació frigorífica i la seva influència sobre l'acceptació sensorial amb la finalitat d'aconseguir una avaluació global de la qualitat de la poma 'Pink Lady®'.
4. Avaluar l'eficàcia de la tecnologia de frigoconservació a la persistencia dels residus en poma 'Pink Lady®' procedents de tractaments postcollita.

Globalment, els resultats ens permetran obtenir un millor coneixement científic de la incidència de les diferents tecnologies d'atmosfera controlada sobre la qualitat global de la poma 'Pink Lady®'. Això hauria de permetra optimitzar les condicions de règim de gasos i període de conservació per a la seva aplicació pel sector frigorista, i permetre mantenir al màxim la qualitat estàndard, aromàtica, sensorial i sanitària dels fruits d'aquesta varietat d'implantació creixent.

DISSENY EXPERIMENTAL
I
MATERIAL VEGETAL

1. Disseny experimental

L'estudi s'ha realitzat durant tres campanyes frutícoles consecutives (1ª campanya: 2003-2004; 2ª campanya: 2004-2005 i 3ª campanya: 2005-2006). Per a la realització dels objectius anteriorment esmentats es va seguir el següent plà de treball (Figura 1).

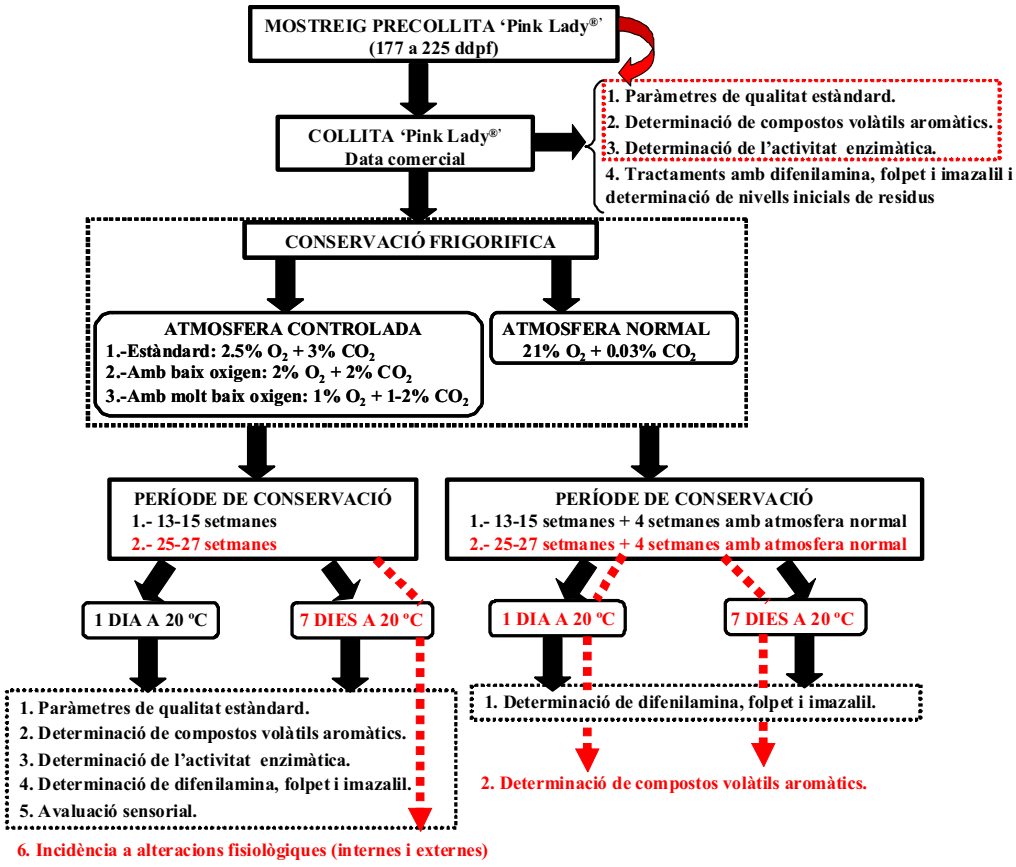


Figura 1. **Esquema del disseny experimental realitzat durant les tres campanyes. (La part marcada en vermell són els anàlisis addicionals determinats en el període assenyalat).**

2. Material vegetal

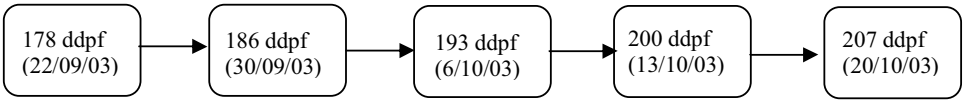
Els fruits utilitzats per al present estudi es van obtenir en una parcel·la comercial situada a la partida d'Albi del terme municipal de Lleida. Les dades de mostreig i de collita van estar compreses entre octubre de 2003 i octubre de 2005.

Es van utilitzar pomes de la varietat 'Pink Lady®' (*Malus* x *domestica* Borkh.). Les característiques de la plantació eren les següents:

- Any de plantació: 1998.
- Patró o porta-empelt: M-9 EMLA.
- Sistema de formació: solaxe.
- Marc de plantació: 4 x 1.4 m
- Reg: goteig.

3. Campanya fructícola 2003-2004

► Dades de mostreig en camp:



► Data de recol·lecció comercial: 28/10/2003

► Atmosferes de conservació: cambres comercials (750 m^3) i 180 t amb tres tipus d'atmosfera diferents:

- Fred normal.
- Atmosfera controlada amb baix oxigen: 2% O_2 + 2% CO_2.
- Atmosfera controlada amb molt baix oxigen: 1% O_2 + 1% CO_2.

► Període de conservació: 14 i 25 setmanes

► Període de post-emmagatzemament: 1 i 7 dies a 20 ºC.

►Determinacions analítiques:

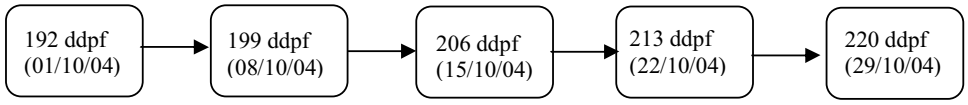
Les anàlisis es van dur a terme durant el mostreig en camp, a la collita i a cada sortida de cambra. Els paràmetres analitzats van ser els següents (Taula 1):

Taula 1. **Determinacions analítiques durant els mostrejos precollita, la collita i la frigoconservació de la 1ª campanya.**

Mostreig precollita	Collita	Frigoconservació
1.- Paràmetres físico-químics de qualitat: fermesa, acidesa, sòlids solubles, midó i color.	1.-Paràmetres físico-químics de qualitat: fermesa, acidesa, sòlids solubles, midó i color.	1.-Paràmetres físico-químics de qualitat: fermesa, acidesa, sòlids solubles i color.
2.- Producció de compostos volàtils aromàtics.	2.-Producció d'etilè.	2.-Producció d'etilè.
	3.- Producció de compostos volàtils aromàtics.	3.- Producció de compostos volàtils aromàtics.
	4.- Activitat AAT, ADH, HPL, PDC i LOX.	4.- Activitat AAT, ADH, HPL, PDC i LOX.
		5.- Anàlisi sensorial: 100 jutges consumidors habituals de pomes.
		6.- Incidència d'alteracions fisiològiques (externes e internes).

4. Campanya fructícola 2004-2005

►Dades de mostreig en camp:



►Data de recol·lecció comercial: 04/11/2004.

►Atmosferes de conservació: cambres comercials (750 m^3) i 180 t amb tres tipus d'atmosfera diferents:

- Fred normal.

- Atmosfera controlada estàndard: 2.5% O_2 + 3% CO_2.
- Atmosfera controlada amb molt baix oxigen: 1% O_2 + 2% CO_2.

► Període de conservació: 15 i 28 setmanes

► Període de post-emmagatzemament: 1 i 7 dies a 20 ºC.

► Període de permanència post-emmagatzemament: 10, 17, 24 i 50 dies a 20 ºC (només després de 28 setmanes)

► Determinacions analítiques:

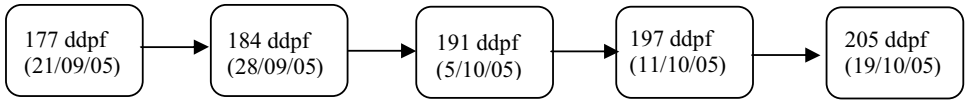
Les anàlisis es van dur a terme durant el mostreig en camp, a la collita i a cada sortida de cambra. Els paràmetres analitzats van ser els següents (Taula 2):

***Taula 2*. Determinacions analítiques durant els mostrejos precollita, la collita i la frigoconservació de la 2ª campanya.**

Mostreig precollita	Collita	Frigoconservació
1.- Paràmetres físico-químics de qualitat: fermesa, acidesa, sòlids solubles, midó i color.	1.-Paràmetres físico-químics de qualitat: fermesa, acidesa, sòlids solubles, midó i color.	1.-Paràmetres físico-químics de qualitat: fermesa, acidesa, sòlids solubles i color.
2.- Producció de compostos volàtils aromàtics.	2.-Producció d'etilè.	2.-Producció d'etilè.
3.- Activitat enzimàtica: AAT, ADH, HPL, PDC i LOX..	3.- Producció de compostos volàtils aromàtics.	3.- Producció de compostos volàtils aromàtics.
	4.- Activitat enzimàtica: AAT, ADH, HPL, PDC i LOX.	4.- Activitat enzimàtica: AAT, ADH, HPL, PDC i LOX.
	5.- Determinació de difenilamina, folpet e imazalil.	5.- Determinació de difenilamina, folpet e imazalil.
		6.- Anàlisis sensorial: 61 jutges consumidors habituals de pomes.
		7.- Incidència a alteracions fisiològiques (externes e internes)

5. Campanya fructícola 2005-2006

►Dades de mostreig en camp:



►Data de recol·lecció comercial: 27/10/2005.

►Atmosferes de conservació: cambres experimentals (22 m^3) i 4 t amb tres tipus d'atmosfera diferents:

- Fred normal
- Atmosfera controlada amb baix oxigen: 2% O_2 + 2% CO_2.
- Atmosfera controlada amb molt baix oxigen: 1% O_2 + 1% CO_2.

►Període de conservació: 13 i 27 setmanes i 13+4 i 27+4 setmanes.

►Període de post-emmagatzemament: 1 i 7 dies a 20 ºC.

►Determinacions analítiques:

Les anàlisis es van dur a terme durant el mostreig en camp, a la collita i a cada sortida de cambra. Els paràmetres analitzats van ser els següents (Taula 3):

***Taula 3*. Determinacions analítiques durant el mostreig precollita, la collita i la frigoconservació de la 3ª campanya.**

Mostreig precollita	Collita	Frigoconservació
1.- Paràmetres físico-químics de qualitat: fermesa, acidesa, sòlids solubles, midó i color.	1.-Paràmetres físico-químics de qualitat: fermesa, acidesa, sòlids solubles, midó i color.	1.-Paràmetres físico-químics de qualitat: fermesa, acidesa, sòlids solubles, midó i color.
2.- Producció de compostos volàtils aromàtics.	2.-Producció d'etilè.	2.-Producció d'etilè.
	3.- Producció de compostos volàtils aromàtics.	3.- Producció de compostos volàtils aromàtics.
	4.- Activitat AAT, ADH, HPL, PDC i LOX.	4.- Activitat AAT, ADH, HPL, PDC i LOX.
	5.- Determinació de difenilamina, folpet e imazalil.	5.- Determinació de difenilamina, folpet e imazalil.
		6.- Anàlisi sensorial: 40 jutges consumidors habituals de pomes.
		7.- Incidència d'alteracions fisiològiques (externes e internes)

RESULTATS

CAPÍTOL 1

Changes in biosyntesis of aroma volatile compounds during on-tree maturation of 'Pink Lady®' apples.

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SUMMARY

The production of aroma volatile compounds and standard quality parameters, in addition to lipoxygenase (LOX), hydroperoxide lyase (HPL), pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH) and alcohol o-acyltransferase (AAT) activities, were assessed during maturation of 'Pink Lady®' apples. Low production of aroma volatiles was observed in early-harvested fruit, which gradually increased as ripeness approached. Hexyl acetate, hexyl 2-methylbutanoate, hexyl hexanoate, hexyl butanoate, 2-methylbutyl acetate and butyl acetate were prominent within the blend of volatiles produced by fruit throughout maturation. Multivariate analysis showed these compounds had the highest influence on differentiation of maturity stages, indicating aroma volatile emission is an important factor for definition of fruit ripeness, which suggests production of these esters might be useful as an index of maturity. No large variations in AAT activity were found throughout experimental period despite increasing ester emission, suggesting the enhancement of ester production by 'Pink Lady' apples at ripening arises mainly from greater availability of substrates. Increased LOX activity was observed at later stages of fruit development, and the possible role of this enzyme activity on enhanced capacity for aroma volatile biosynthesis in more mature fruit is discussed.

Keywords: Aroma; Alcohol dehydrogenase; Alcohol *o*-acyltransferase; Hydroperoxide lyase; Lipoxygenase; Pyruvate decarboxylase; *Malus* × *domestica*; Maturation; 'Pink Lady®' apple; Quality; Volatile compounds

1. Introduction

'Pink Lady', originated from a cross between 'Lady Williams' and 'Golden Delicious' (Cripps et al., 1993), is a new, late maturing apple (*Malus* × *domestica* Borkh.) cultivar increasingly cultivated in many apple-producing areas of the world owing to its excellent flavour and sensory attributes. Commercial interest is thus focused on developing suitable criteria for harvest maturity as well as appropriate storage procedures in order to assure quality of final produce. Production of aroma volatile compounds is an important factor determining final sensory quality of fruit produce and hence consumer satisfaction, and is directly influenced by fruit maturity (Mattheis et al., 1991; Echeverría et al., 2004). Whereas premature harvesting may result in pronounced lack of flavour development, late-harvested fruit undergo rapid firmness loss during storage (Mattheis et al., 1995). Deficient aroma volatile production in immature fruit, suggested to arise from low rates of precursor synthesis, is gradually overcome as fruit approach the optimal harvest date (Song and Bangerth, 1994), with maximum emission taking place at the climacteric peak (Fellman et al., 2000; Dixon and Hewett, 2000).

The total number, identity and concentration of volatile compounds emitted by ripening apple fruit are cultivar-specific (Dixon and Hewett, 2000), although esters, associated with "fruity" attributes of fruit flavour, can account for up to 98% of total volatiles emitted by intact ripe fruit (López et al., 1998). The contribution of each compound to the specific aroma profile of each cultivar depends on the activity and substrate specificity of the relevant enzymes in the biosynthetic pathway, the substrate availability, the odour threshold above which the compound can be detected by smell, and the presence of other compounds (Rizzolo et al., 2006). For the 'Pink Lady®' cultivar, ethyl butanoate, ethyl 2-methylbutanoate, 2-methylbutyl acetate, hexyl acetate, hexyl propanoate, hexyl hexanoate and hexyl 2-methylbutanoate have been identified as the primary contributors to fruit aroma at commercial harvest (López et al., 2007).

Volatile esters are generated by esterification of alcohols and acyl-CoAs, catalysed by alcohol o-acyltransferase (AAT; EC 2.3.1.84). Substrates for this esterification are thought to derive primarily from both fatty acids and amino acids (Sanz et al., 1997). Although AAT activity has been shown to follow a clear pattern concomitant with ethylene regulation in 'Greensleeves' apples (Defilippi et al., 2005), results of that work also indicated there are ethylene-independent regulatory processes involved in aroma production. Furthermore, while AAT activity has been reported to increase transiently with the onset of ripening in some apple cultivars (Fellman et al., 2000), no large variations were found in AAT activity during on-tree maturation of 'Fuji' apples (Echeverría et al., 2004). Therefore, in addition to enzyme activity, ester biosynthesis may be also limited in part by the supply of the required substrates, suggesting that some critical steps for ester emission may be located upstream in the biosynthetic pathway.

It has been suggested that low ability for biosynthesis of precursor fatty acids may be a major factor limiting production of volatile esters in immature apple fruit (Song and Bangerth, 1994, 2003). Accordingly, transgenic modification of fatty acid biosynthesis in tomato leaves led to significant changes in emitted volatiles (Wang et al., 2001). The relevance of fatty acid metabolism for aroma production is further illustrated by observations on CA-induced inhibition of lipoxygenase (LOX; EC 1.13.11.12) activity in 'Fuji' (Lara et al., 2006) and 'Mondial Gala' (Lara et al., in press) apples, leading to abnormal fruit aroma after transfer from hypoxia to air.

In this work, production of aroma volatile compounds and activity of some related enzymes were assessed throughout fruit maturation of 'Pink Lady' apples, with the general purpose of studying the progress of the ability to produce aroma volatiles, and of identifying which enzymes in the biosynthetic pathway are important for ripening-related increase of the capacity for ester biosynthesis in this apple cultivar.

2. Materials and Methods

2.1. Plant material

Apple fruit (*Malus* × *domestica* Borkh. cv. 'Pink Lady®'), selected for uniformity of size and absence of defects, were picked weekly from six-year old trees grown on M-9 EMLA rootstocks at a commercial orchard near Lleida (NE Spain). The sampling period was from 16th September to 4th November 2004, corresponding to 177 and 226 days after full bloom (dafb), respectively. At each sampling date, 8 kg of apples (2 kg/replicate × 4 replicates) were taken for analysis of aroma compounds. In addition 25 fruit were taken for analysis of enzyme activity and standard quality parameters.

2.2. Analysis of standard q uality parameters

Twenty fruit per sampling date were used individually for the analysis of flesh firmness, soluble solids content (SSC), titratable acidity (TA), skin colour and starch index. Flesh firmness was measured on two opposite sides of each fruit with a penetrometer (Effegi, Milan, Italy) equipped with an 11-mm diameter plunger tip; results were expressed in N. SSC and TA were assessed in juice pressed from the whole fruit. SSC was determined using a hand refractometer (Atago, Tokyo, Japan), and results were expressed as g · 100 g^{-1}. TA was analysed by titration of 10 ml of juice with 0.1N NaOH to pH 8.1 with 1% (v/v) phenolphthaleine as an indicator, and data are given as g malic acid · l^{-1}. Hue values were calculated from a* and b* parameters measured with a CR-200 chromameter (Minolta Co., Osaka, Japan) on both the exposed and the shaded sides of each fruit, using standard CIE illuminant and 8 mm viewing aperture diameter. Starch hydrolysis was rated visually using a 1–10 EUROFRU scale (1, full starch; 10, no starch) (Planton, 1995), after dipping of cross-sectional fruit halves in 0.6% (w/v) I_2-1.5% (w/v) KI solution for 30 s.

2.3. Analysis of aroma volatile compounds

The extraction of volatile aroma 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 ionisation detector and a polyethyleneglycol column with cross-linked free fatty acid as the stationary phase (FFAP; 50m × 0.2mm i.d. × 0.33µm), where a volume of 1 µl from the extract was injected in all the analyses. Helium was used as the carrier gas (0.8 ml min^{-1}), with a split ratio of 40:1. The injector and detector were held at 220 and 240 ºC, respectively. The analysis was conducted according to the following programme: 70 ºC (1 min); 70–142 ºC (3 ºC min^{-1}); 142–225 ºC (5 ºC min^{-1}); 225 ºC (10 min), as described elsewhere (Echeverría et al., 2002). Volatile compounds were identified by comparing retention indexes with those of standards and by enriching apple extract with authentic samples. The quantification was made using butylbenzene (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 ionisation at 70 eV. Helium was used as the carrier gas (0.8 ml min^{-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}.

2.4. Analysis of acetaldehyde concentration

At each sampling date, juice was obtained individually from twenty fruit and frozen at -20 ºC until analysis of acetaldehyde content as described by Ke et al. (1994). Frozen juice from each fruit was thawed, and a 5-ml sample was introduced in a 10-ml test tube, which was closed with a rubber cap and incubated at 65 ºC 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, 2m×2mm i.d.) as the stationary phase, and a flame ionisation detector. Nitrogen was used as the carrier gas (45 ml min^{-1}), and operating conditions were as follows: oven temperature 110 ºC, injector temperature 180 ºC, detector temperature 220 ºC. Acetaldehyde was identified and quantified by comparison with an external standard, and results were expressed as $\mu l\ l^{-1}$.

2.5. Extraction and assay of aroma-related enzyme activities

Lipoxygenase (LOX), hydroperoxide lyase (HPL), pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH) and alcohol o-acyltransferase (AAT) activities were determined at each sampling date. Samples of both skin and flesh tissue were taken separately from four apples, frozen in liquid nitrogen, lyophilised, powdered, and kept at -80 ºC until processing. Weight loss after lyophilisation was consistently around 80% (skin) and 87% (flesh). One hundred milligrams of lyophilised 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 (Lara et al., 2003). HPL activity was extracted and assayed according to Vick (1991). Total protein content in the enzyme extract was determined with the Bradford method (1976), 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 ($U \cdot mg\ protein^{-1}$).

2.6. Statistical and multivariate analyses

A factorial design with sampling date and replication as factors was used to statistically analyse results. All data were tested by analysis of variance (GLM-ANOVA), using the SAS program package (SAS Institute, Inc., 1987). Means were separated by L.S.D. test at $p \leq 0.05$. To provide a general visualisation of all the information contained in the data set obtained, principal component analysis (PCA) was used. Partial least-square regression (PLSR) was 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 vers. 6.11a software (CAMO ASA, 1997) was used for developing these models. Samples were coded H1 to H6, corresponding to fruit picked between 192 and 226 dafb, respectively. Variables were labelled as specified in Tables 1 and 2. As a pre-treatment, data were centred and weighed by the inverse of the standard deviation of each variable in order to avoid dependence on measured units (Martens and Naes, 1989). Leverage correction was run as a validation procedure.

3. Results and Discussion

3.1. Fruit quality during tree maturation of 'Pink Lady' apples

Fruit picked at early dates showed higher TA, firmness and hue values, and lower SSC and SI as compared with fruit picked at commercial maturity (Table 1). SSC, TA and SI at commercial harvest, which took place at 226 dafb, were indicative of an appropriate stage of maturity according to Centre Technique Interprofessionnel des Fruits et Légumes (CTIFL) recommendations (Mathieu et al., 1998), although firmness values were higher than those considered therein (68.6-78.4 N). Colorimetric data indicated that background colour was changing from green to yellow, which is similarly considered to be a good maturity index for deciding commercial harvest of 'Pink Lady' apples.

Fruit aroma is also an important factor affecting final sensory quality of produce. Total production of aroma volatiles remained low and steady until approximately 200 dafb, but increased sharply afterwards (Table 2), possibly signalling the onset of the ripening process. In order to define maturity stages during fruit development on the tree, a PCA model was developed in which samples were characterised by standard quality parameters and aroma volatile compounds emitted. Principal components 1 (PC1) and 2 (PC2) accounted for 52% and 14% respectively of total variability. The corresponding scores plot (Fig. 1A) shows that at least four different maturity stages could be defined by the variables studied: H1-H2, H3-H4, H5 and H6.

***Table 1*. Standard quality and maturity parameters of 'Pink Lady®' apples at different sampling dates (dafb: days after full bloom).**

Parameters	Code[b]		H1	H2	H3	H4	H5	H6
		177 dafb	192 dafb	199 dafb	206 dafb	213 dafb	220 dafb	226 dafb
Weight (g)	we	130.2 e	144.8 d	167.7 c	170.0 c	175.0 bc	181.7 ab	187.4 a
Size (mm)	Cal	65.6 d	69.6 c	72.3 b	72.0 b	73.9 ab	75.2 a	75.3 a
Firmness (N)	firm	10.9 a	10.7 ab	10.5 ab	10.9 a	10.1 bc	9.7 c	8.9 d
SSC(g $100g^{-1}$)	SSC	11.5 e	12.2 d	12.9 c	13.6 b	13.7 b	15.0 a	14.7 a
TA (g L^{-1})	TA	8.7 a	7.8 bc	7.5 cd	8.9 a	7.3 cd	8.4 ab	6.9 d
Starch Index	SI	1.0 c	1.6 c	1.6 c	3.5 b	4.2 b	6.4 a	6.8 a
Hue (SS)[c]	Hue SS	114.8 a	113.7 a	114.5 a	110.0 a	103.0 b	94.4 bc	97.1 c
Hue (ES)[d]	Hue ES	106.1 a	82.9 c	94.0 b	50.9 d	36.4 e	34.1 e	29.7 e

[a]Values represent means of 20 replicates. Means followee by different letters for a given parameter are significantly different al $P \leq 0.05$ (LSD test).
[b] Variable codes used for multivariate analyses. [c] SS: shaded side.[d] ES: exposed side.

The variables having most influence on sample differentiation were four hexyl esters (hexyl acetate, hexyl butanoate, hexyl 2-methylbutanoate and hexyl hexanoate), two acetate esters (butyl and 2-methylbutyl acetates) and two ethyl esters (ethyl butanoate and ethyl hexanoate), all of which were higher for more advanced maturity stages (Fig. 1B). Ethanol availability and some standard quality parameters (firmness, SI, SSC and hue) were also observed to have some weight on sample differentiation along PC1. This PCA model thus demonstrated aroma volatile emission to be an important factor for definition of fruit ripeness, and suggests it might be useful as an index of maturity reflecting the current physiological stage of development (Mattheis et al., 1991).

3.2. Modifications in production of aroma volatile compounds during tree maturation of 'Pink Lady' apples

Up to 28 volatile aroma compounds (21 esters, six alcohols and one terpene) were identified and quantified in the volatile fraction emitted during tree maturation of 'Pink Lady' apples. Emission of most compounds increased along the process (Table 2). Early-harvested fruit showed low capacity for aroma production which was gradually overcome as ripeness approached, the highest emission of esters corresponding to fruit picked at commercial maturity (226 dafb). Fully ripe 'Pink Lady' apples produced high amounts of hexyl esters (Table 2), which have been reported to confer a characteristic "apple" odour (Plotto, 1999, 2000). Hexyl esters have been shown to be important in the aroma volatile fraction emitted by other bicolour apple cultivars such as 'McIntosh' and 'Cortland', in which hexyl acetate has been reported to be the main ester in quantitative terms (Yahia et al., 1990), while this ester was observed to be the third predominant compound in the aroma profile of 'Fuji' apples at commercial harvest, after 2-methylbutyl and butyl acetates (Echeverría et al., 2004).

***Table 2*. Aroma volatile production (µg · kg^{-1}) by 'Pink Lady®' apples at different sampling dates (dafb: days after full bloom).**

Volatile compound[a]	Code[b]	H1 192 dafb	H2 199 dafb	H3 206 dafb	H4 213 dafb	H5 220 dafb	H6 226 dafb
Methyl acetate	ma	ND	ND	ND	ND	0.75 a	1.35 a
Ethyl acetate	ea	1.62 c	0.93 c	6.33 a	1.18 c	3.37 b	3.76 b
Ethanol	etOH	10.80 a	8.63 ab	13.68 a	12.22 a	2.59 bc	0.76 c
Propyl acetate	pra	ND	ND	2.83 b	ND	3.63 b	8.66 a
2-Methylpropyl acetate	2mpra	ND	ND	ND	ND	4.31 b	25.70 a
1-Propanol	prOH	ND	ND	ND	ND	ND	3.49 a
Ethyl butanoate	eb	1.94 b	1.46 b	2.35 b	1.20 b	2.51 b	5.46 a
Ethyl 2-methylbutanoate	e2mb	ND	ND	21.85 a	ND	1.79 c	11.95 b
Butyl acetate	bs	3.43 b	0.97 b	ND	1.62 b	4.39 b	196.75 a
2-Methyl-1-propanol	2mprOH	ND	ND	ND	ND	2.79 b	9.35 a
2-Methylbutyl acetate	2mba	7.43 c	3.59 c	ND	3.31 c	122.70 b	255.15 a
1-Butanol	buOH	ND	ND	ND	ND	13.51 b	30.33 a
Butyl propanoate	bp	ND	ND	ND	ND	10.02 b	62.91 a
Pentyl acetate	pa	2.02 d	6.45 a	5.32 ab	4.39 bc	3.55 bcd	2.49 cd
2-Methylbutyl propanoate	2mbp	ND	ND	ND	ND	2.39 b	8.92 a
2-Methyl-1-butanol	2mbuOH	ND	ND	ND	ND	6.32 a	0.84 b
D-limonene	lim	ND	ND	ND	ND	4.78 a	8.09 a
Butyl butanoate	bb	ND	ND	ND	ND	27.20 b	76.66 a
Ethyl hexanoate	eh	1.44 b	1.62 b	ND	ND	24.78 b	76.93 a
Hexyl acetate	ha	13.84 c	7.99 c	ND	ND	194.70 b	549.31 a
Hexyl propanoate	hp	ND	ND	ND	ND	7.12 a	2.41 a
Hexyl 2-methylpropanoate	h2mp	ND	ND	ND	ND	15.73 b	148.74 a
1-Hexanol	heOH	ND	ND	ND	ND	7.09 a	3.89 a
2-Methylpropyl hexanoate	2mprh	ND	ND	ND	ND	ND	17.80 a
Butyl hexanoate	bh	10.28 c	ND	ND	ND	57,15 b	96.44 a
Hexyl butanoate	hb	5.06 c	5.55 c	3.87 c	3.52 c	46.44 b	142.74 a
Hexyl 2-methylbutanoate	h2mb	8.72 c	ND	ND	ND	127.12 b	382.45 a
Hexyl hexanoate	hh	7.45 c	3.02 c	ND	ND	71.48 b	187.67 a
Total aroma volatiles[c]		**74.03**	**40.21**	**56.23**	**27.44**	**768.21**	**2321.0**

[a] Values represent means of four replicates (ND: non-detectable). Means followed by different letters for a given compound are significantly different at $p \leq 0.05$ (LSD test).[b] Variable code used for multivariate analyses. [c] Total amount of all volatile compounds detected during chromatographic analyses.

Hexyl acetate, hexyl 2-methylbutanoate, hexyl hexanoate and hexyl butanoate, in addition to 2-methylbutyl and butyl acetates, were prominent quantitatively among the rest of compounds emitted by 'Pink Lady' apples along maturation, accounting for 52-74 % of total emission of aroma volatiles, depending on harvest date (Table 2). These compounds, together with ethyl butanoate and ethyl hexanoate, were also found to have the highest weight for differentiation among maturity stages (Fig. 1B). This is interesting, as the odour thresholds for ethyl butanoate, ethyl hexanoate, hexyl acetate, hexyl 2-methylbutanoate, 2-methylbutyl acetate and butyl acetate are reportedly of 1, 1,

2, 6, 11 and 66 $\mu g \cdot l^{-1}$, respectively (Takeoka et al., 1990, 1992, 1996; Buttery, 1993; Rychlik et al., 1998), indicating they were likely to have an impact on overall flavour of fruit at commercial maturity on the basis of odour units present (Buttery, 1993). Some of these compounds have also been reported to contribute to the overall flavour of ripe 'Golden Delicious' fruit, one of the parentals of the 'Pink Lady' cultivar (López et al., 2000; Kakiuchi et al., 1986); however, the prominence of hexyl esters in the volatile fraction emitted by 'Pink Lady' fruit is a difference with respect to its parent. 'Golden Delicious' apples also produce significant amounts of butyl butanoate throughout fruit maturation (Song and Bangerth, 1996), which was detected in 'Pink Lady' fruit in moderate concentrations and only at later maturity stages (Table 2).

Ethyl 2-methylbutanoate showed an irregular pattern throughout the experimental period (Table 2), with a transient increase three weeks before commercial harvest. Release of this compound increased again at later stages of development (H5-H6), in contrast with previous observations for 'Fuji' apples, where a steady decrease was observed during fruit maturation (Echeverría et al., 2004). In spite of low production observed during sampling period (Table 2), the extremely low odour threshold for this compound (0.006 $\mu g \cdot l^{-1}$) (Takeoka et al., 1992) indicates it might have likewise contributed to the characteristic aroma profile of 'Pink Lady' apples.

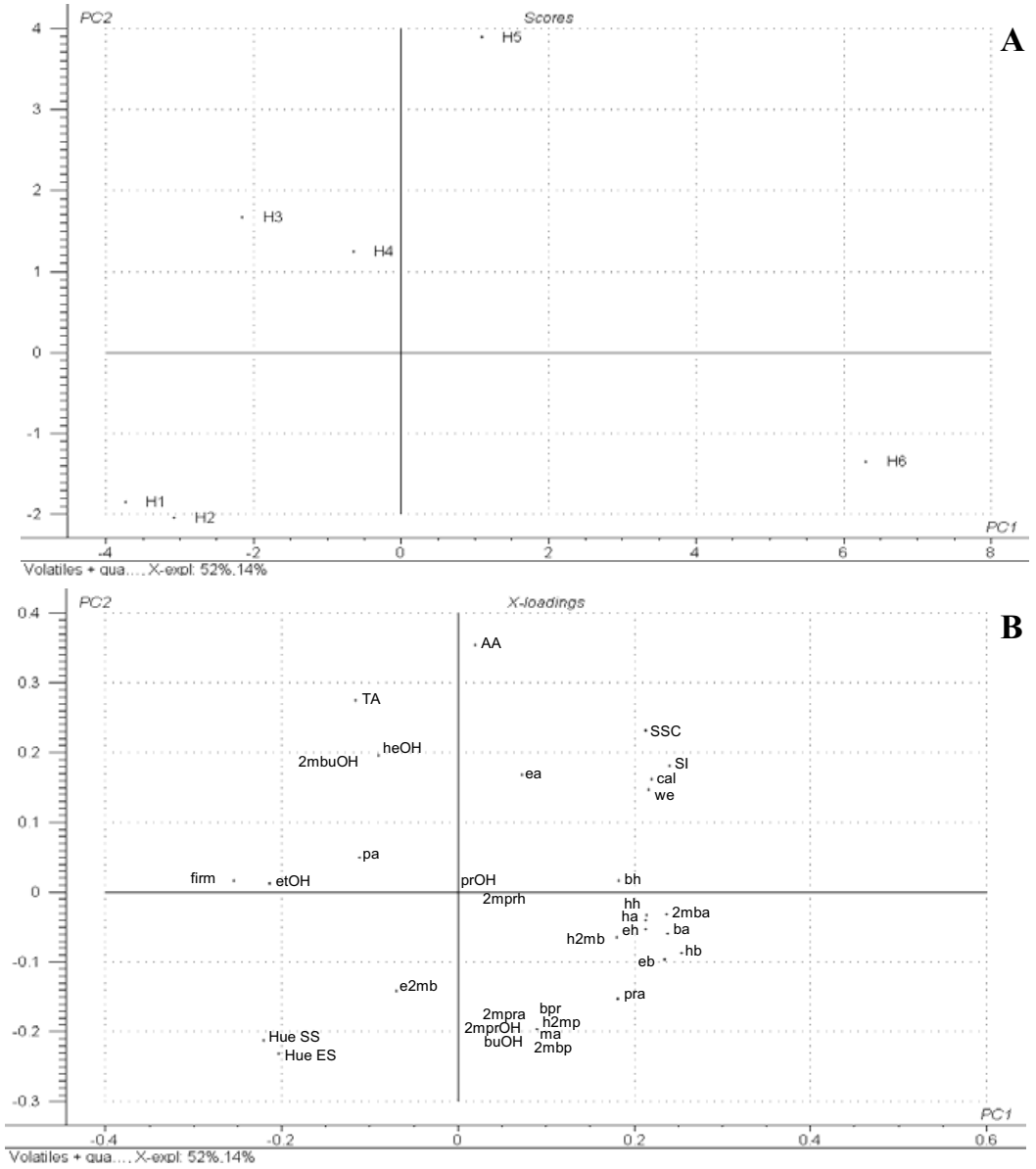

Figure 1. **Scores (A) and loadings (B) plot of PC1 vs. PC2 corresponding to a PCA model for emission of aroma volatile compounds and standard quality of 'Pink Lady' apples at different sampling dates. Samples are coded H1 to H6 according to harvest date (H1, earlier; H6, later). Variables are labelled as indicated in Tables 1 and 2 (AA, acetaldehyde).**

3.3. Modifications in aroma-related enzyme activities during tree maturation of 'Pink Lady' apples

Some hexyl, butyl and 2-methylbutyl esters were present already in the aroma volatile fraction of very early samples, although the corresponding alcohol precursors (1-hexanol, 1-butanol and 2-methylbutanol, respectively) were detectable only at the most mature stages (H5 and H6) (Table 2). This observation indicates ester-synthesizing capacity was present at early stages, and indeed AAT activity was detectable throughout the experimental period both in skin and flesh tissues (Fig. 2). Because no large variations in this enzyme activity were found during fruit maturation in spite of generally increasing ester emission, it is suggested the enhancement of ester emission arose mainly from greater availability of substrates. This might explain why precursor alcohols were not detectable in early maturity stages, as low or moderate levels produced would be used for ester biosynthesis by AAT.

Ethanol production decreased during the experimental period, in accordance with previous reports on 'Bisbee Delicious' apples (Mattheis et al., 1991), but in contrast with observations for 'Fuji' (Echeverría et al., 2004). The evolution of ethanol emission was not paralleled by that of ethyl esters, which increased during fruit maturation. However, acetaldehyde content was higher in more advanced maturity stages (Fig. 3), with a maximum at 220 dafb. Acetaldehyde can be obtained either from pyruvic acid through the action of PDC, from fatty acids via the LOX/HPL pathway, or by enzymatic oxidation of ethanol, the reverse reaction of alcoholic fermentation catalysed by ADH. Therefore, the drop in ethanol production throughout maturation might be indicative that it was being diverted to acetaldehyde production. Plant tissues have been demonstrated to use carbon from acetaldehyde to produce acetate (Kreuzwieser et al., 1999), which is subsequently available for the synthesis of acetyl-CoA. This would be in agreement with the observation that emission of most acetate esters increased significantly during the experimental period.

Although developmental changes in AAT activity levels have been associated with ripening in a number of fruits such as melon (*Cucumis melo* L.) (Shalit et al., 2001), strawberry (*Fragaria* × *ananassa* Duch.) (Aharoni et al., 2000), banana (*Musa* L. spp., AAA group) (Jayanty et al., 2002) and apple (Defilipi et al., 2005), results reported here suggest that higher ester production throughout maturation arose mainly from increased availability of substrate for enzyme action. Broad substrate preferences have been reported for apple AAT (Defilippi et al., 2005; Souleyre et al., 2005), in accordance with findings for other fruit species, and evidence has been provided that substrate preference is not necessarily reflected in the representation of esters in the corresponding volatile profile of fruit, suggesting that specific esters emitted are dependent upon precursors supplied. For instance, treatment of apple fruit or tissue sections with the vapors of alcohols, aldehydes or carboxylic acids significantly increases concentrations of the corresponding volatile esters (Berger et al., 1984; Bartley et al., 1985; Kollmannsberger and Berger, 1992; Harb et al., 1994).

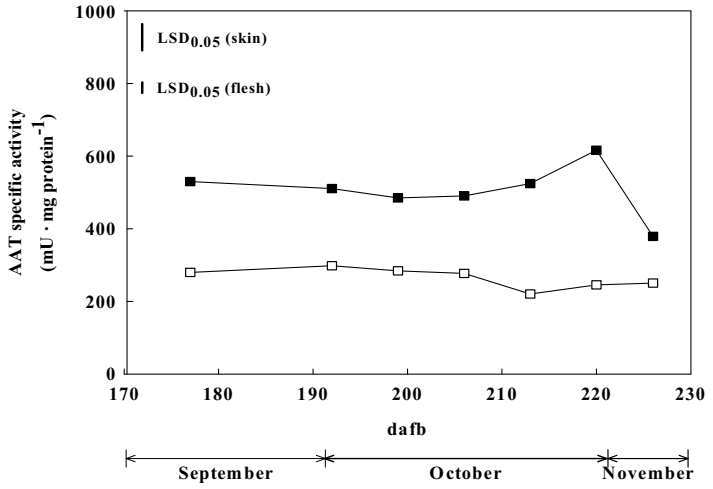

Figure 2. **Alcohol o-acyltransferase specific activity in skin (■) and flesh (□) of 'Pink Lady' apples at different sampling dates. Values represent means of three replicates. Vertical bars indicate $LSD_{0.05}$.**

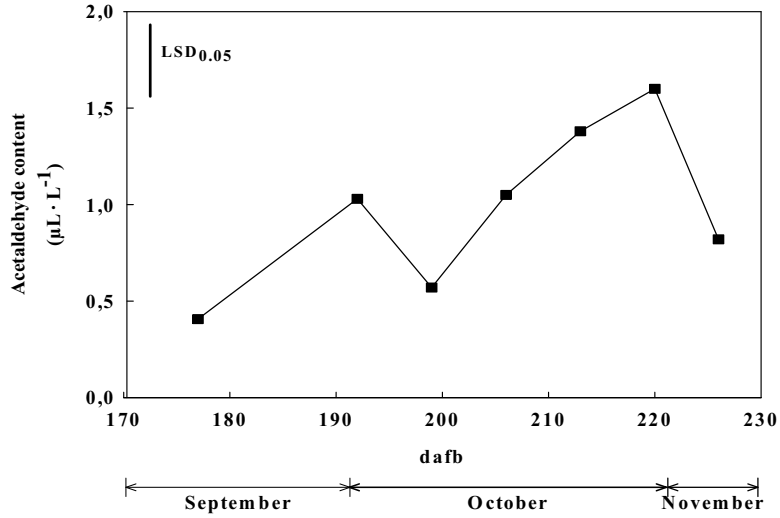

***Figure 3*. Acetaldehyde concentration in 'Pink Lady' apples at different sampling dates. Values represent means of 20 replicates. Vertical bar indicates $LSD_{0.05}$.**

The importance of an adequate substrate supply for ester biosynthesis was illustrated when a PLSR model was developed with alcohols and acetaldehyde as X xariables and esters as Y variables. The corresponding biplot (Fig. 4A) shows that 93% of total variability in production of volatile esters could be explained by availability of precursors. PC1 and PC2 accounted for 50 and 43%, respectively, of total variability. Two groups of samples separated along PC2, corresponding to immature (H1-H3) and mature (H4-H6) fruit. Acetaldehyde content (r = 0.48), along with availability of 1-hexanol and 2-methylbutanol (r = 0.42 in both instances), were the main factors accounting for this differentiation. Within the group of mature fruit, commercially ripe (H6) samples separated clearly from H4-H5 ones along PC1. H4-H5 samples were characterised by higher levels of 1-hexanol, 2-methylbutanol and acetaldehyde, whereas H6 fruit had higher productions of 1-butanol and 2-methylpropanol.

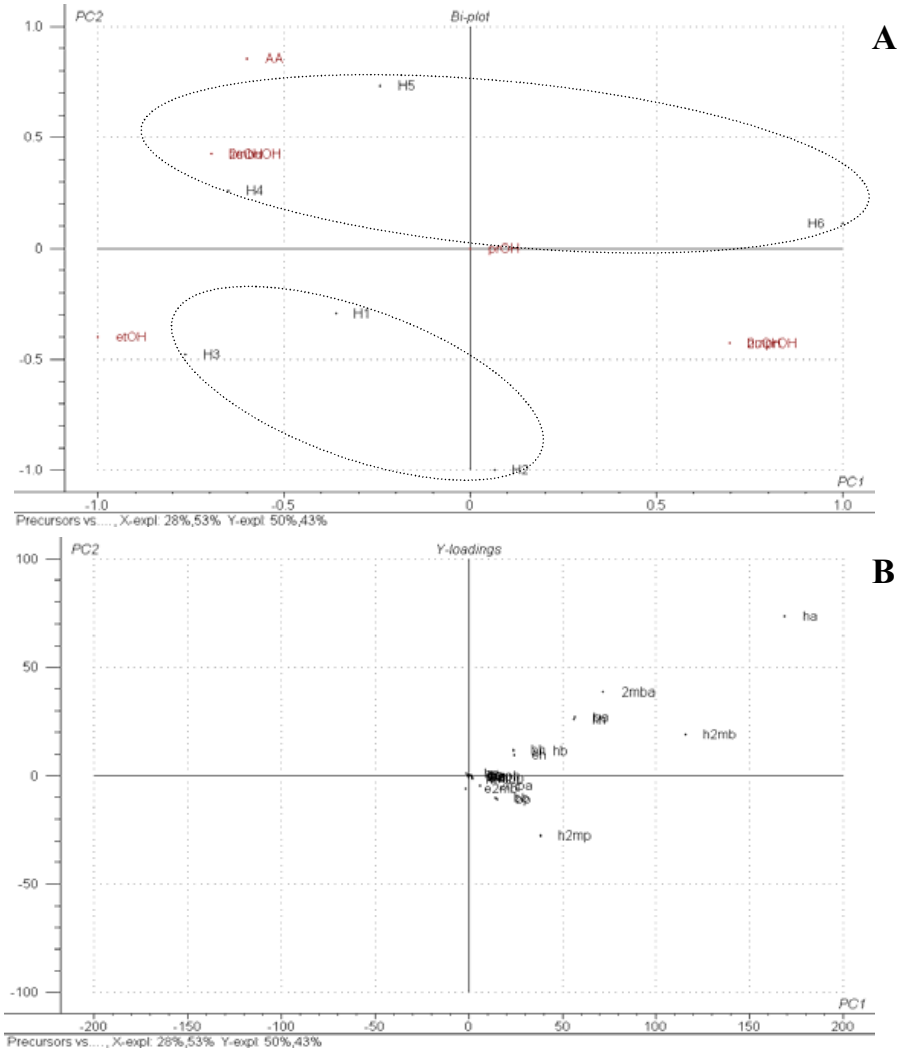

***Figure 4*. Biplot (scores and X-loadings) (A) and Y-loadings plot (B) corresponding to a PLSR model of volatile ester emission (Y variables) vs. precursor availability (X variables) in 'Pink Lady®' fruit at different sampling dates. Samples are coded H1 to H6 according to harvest date (H1, earlier; H6, later). Variables are labelled as indicated in Table 2 (AA, acetaldehyde).**

Because hexyl and acetate esters had been found to be prominent quantitatively among the rest of volatile compounds emitted by 'Pink Lady®' fruit (Table 2), these results suggest some important precursors (1-hexanol, 2-methylbutanol and acetaldehyde) were synthesised prior to the onset of ripening-related emission of aroma compounds, rendering them available for volatile ester biosynthesis at later maturity stages. Indeed, the Y-loadings plot (Fig. 4B) shows that all four hexyl esters identified as having most influence on sample differentiation, along with butyl and 2-methylbutyl acetates (Fig. 1), were associated to H6 fruit.

Fatty acids are major precursors of aroma volatiles in most fruit species (Sanz et al., 1997; Dixon and Hewett, 2000), the involved pathways including ß-oxidation and the LOX system and leading to the formation of aldehydes, acids, alcohols and esters. It is considered that, during fruit maturation, enzymes and substrates of the LOX pathway have different subcellular locations. Therefore, no LOX-related volatile emission would be possible, thus rendering ß-oxidation as the main metabolic pathway for aroma production (Sanz et al., 1997). However, lipid biosynthesis and membrane fluidity increase as apples ripen (Bartley, 1985), allowing the LOX pathway to become active and to function as an alternative to ß-oxidation. This is in accordance with results reported here, showing an important albeit transient upsurge in LOX activity both in skin and flesh tissues at later stages of fruit development (Fig. 5A). Because this upsurge coincided chronologically, or was immediately followed by, the rise in the production of most volatile esters (Table 2), it is suggested it might have accounted for increased capacity of fruit for aroma volatile biosynthesis. These results are interesting in the light of prominence of hexyl esters in the aroma volatile fraction emitted by mature 'Pink Lady' apples (Table 2), as hexyl esters have been reported to associate with lipid-degrading enzymes (Olías et al., 1993). Additionally, LOX activity has been found to be essential for recovery of the ability to synthesize volatile esters after controlled atmosphere storage of apple (Lara et al., 2006) and pear (*Pyrus communis* L.) (Lara et al., 2003). Low oxidation rates for fatty acids might account for a shortage of precursors to the biosynthetic pathway and thus to ester production (Brackmann et

al., 1993; Fellman et al., 1993). Cleavage of fatty acid hydroperoxides into aldehydes by hydroperoxide lyase (HPL) is likely to be another control point in the biosynthesis of aroma compounds through the LOX system. HPL activity increased both in skin and flesh tissues until approximately one month before commercial harvest (Fig. 5B).

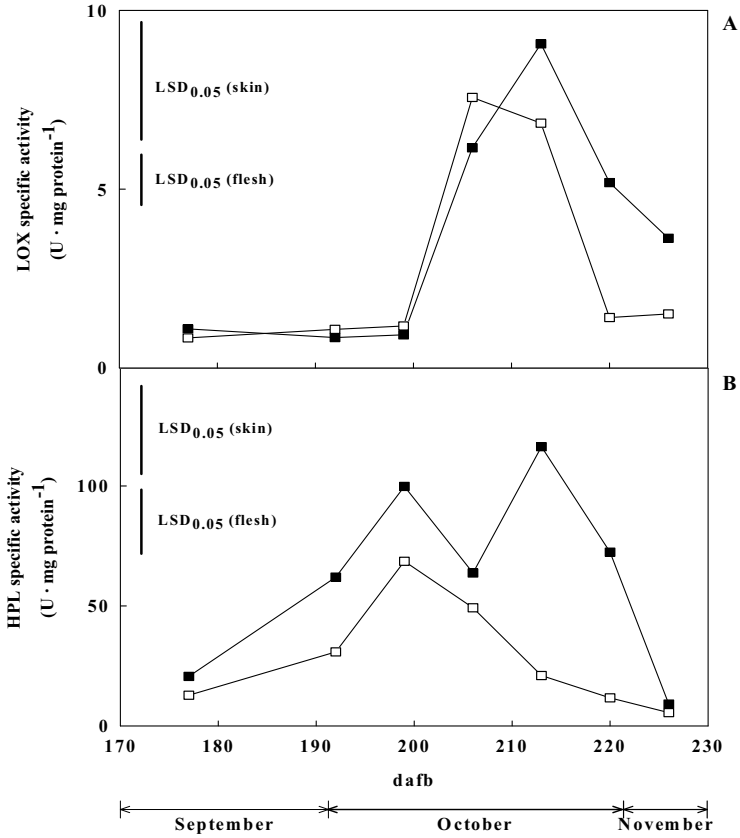

Figure 5. **Lipoxygenase (top) and hydroperoxide lyase (bottom) specific activities in skin (■) and flesh (□) of 'Pink Lady' apples at different sampling dates. Values represent means of three replicates. Vertical bars indicate $LSD_{0.05}$.**

These increases preceded those of LOX activity by approximately a week, which might suggest that LOX activity was activated as a mechanism to restore the hydroperoxide pool consumed by HPL. A transient increase in acetaldehyde content (Fig. 3) was found

at early maturity stages, which however did not coincide temporally with that of HPL activity. Instead, enhanced PDC activity (Fig. 6A) was observed in the skin tissue of fruit at the same time, which might be signalling the onset of the metabolic modifications leading to the respiratory climacteric. A second augment in acetaldehyde content was noticed approximately one week before commercial harvest, immediately after upsurges in LOX, HPL (Fig. 5) and ADH activities (Fig. 6B).

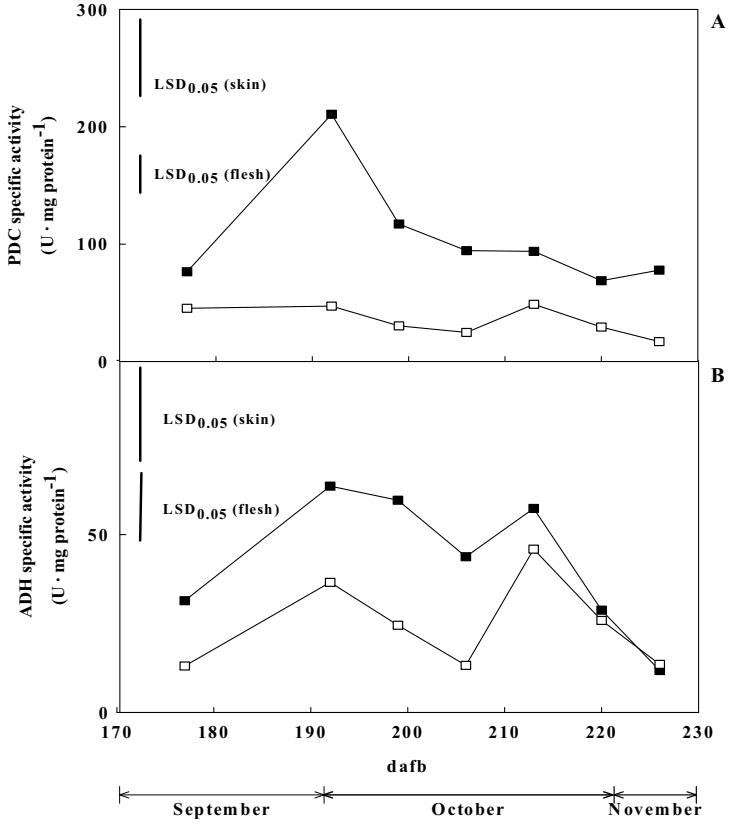

Figure 6. **Pyruvate decarboxylase (top) and alcohol dehydrogenase (bottom) specific activities in skin (■) and flesh (□) of 'Pink Lady' apples at different sampling dates. Values represent means of three replicates. Vertical bars indicate $LSD_{0.05}$.**

As no significant increase in PDC activity was found concomitantly (Fig. 6A), results might be indicative that acetaldehyde production in advanced maturity stages was related to HPL rather than to PDC. A partial least squares regression (PLSR) model was developed for acetaldehyde content (Y variable) and HPL activity in both skin and flesh (X variables). The corresponding predicted vs. measured plot (Fig. 7) shows that the correlation coefficient for acetaldehyde content according to this model was 0.82, suggesting concentrations of this precursor could be indeed predicted from levels of HPL activity.

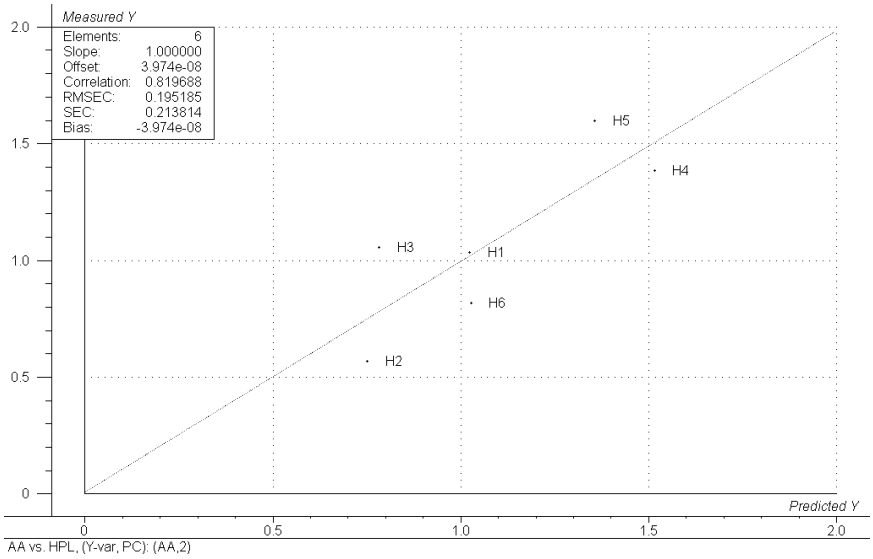

Figure 7. **Predicted vs. measured plot corresponding to a PLSR model of acetaldehyde content (Y variable) vs. hydroperoxide lyase activity (X variable) in 'Pink Lady®' fruit at different sampling dates. Samples are coded H1 to H6 according to harvest date (H1, earlier; H6, later).**

In conclusion, results indicate that modifications in AAT activity alone could not explain observed changes in the production of volatile esters by 'Pink Lady®' apple fruit. Although a moderate increase in AAT activity was observed in later maturity stages, data suggest variations in this enzyme activity are not the main factor leading to increased emission of volatile esters throughout maturation. It is suggested that

precursors were synthesised prior to the onset of ripening-related emission of aroma compounds, rendering them available for volatile ester biosynthesis at later maturity stages.

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CAPÍTOL 2

AAT involucrades en la biosíntesi d'ésters volàtils en 'Royal Gala'.

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Resum

L'aroma de la poma està format per combinacions de diferents compostos volàtils, majoritàriament ésters, el quals incrementen notablement durant la maduració del fruit. Molts ésters volàtils són sintetitzats a partir de productes de la ruta de lipoxigenació, de degradació d'aminoàcids i d'acils CoA. El pas final de la biosíntesi dels ésters és catalitzat per l'alcohol o-aciltransferasa (AAT), que utilitza donants i receptors de grups acil com a substrats. Es va utilitzat una tècnica genòmica amb la finalitat d'identificar isogens d'AAT que juguen un paper clau en la producció d'ésters volàtils durant la maduració de poma 'Royal Gala'. Es van identificat dotze ADNc de seqüència completa potencialment codificants per a AATs a partir d'una base de seqüències EST (Expressed Sequence Tags), que mostren canvis a la seva taxa d'etilè en pell i en polpa. Aquests ADNc van ser seqüenciats i sotmesos a RT-PCR (real time-polymerase chain reaction) en presència d'oligonucleòtids específics per a *MpAAT1*, aïllat de poma 'Royal Gala', amb l'objectiu d'estudiar l'expressió de cada gen després d'un tractament amb etilè i durant la maduració en camp del fruit. La majoria del gens estudiats van mostrar un patró de regulació depenent d'etilè. Els gens putatius *MpAT2*, *MpAT5*, *MpAT9* i *MpAT11* van mostrar un patró d'expressió genètica similar amb increments a partir d'estadis de maduració mitjana seguits d'una disminució en fruit madur. Altres gens van mostrar patrons d'expressió diferents. Aquestes dades suggereixen que més d'un gen *AAT* està involucrat en la biosíntesi d'ésters volàtils en la poma 'Royal Gala'.

1. Introducció

La maduració dels fruits està caracteritzada per un gran nombre de processos bioquímics que resulten en canvis de color, textura, flavor i aroma. Entre els factors que determinen la qualitat del fruit i l'acceptació final del consumidor es troba la producció de compostos volàtils aromàtics (Baldwin, 2002). Les propietats de l'aroma d'un fruit depenen de la combinació dels compostos volàtils, de la seva concentració i del llindar de percepció olfactiva per a cada compost volàtil. Els compostos volàtils majoritaris de les pomes són els ésters que augmenten la seva concentració durant la maduració i arriben al màxim quan es troben al pic climatèric (Dixon i Hewett, 2000; Fellman i col., 2000).

Un altre aspecte important en la biosíntesi d'aromes és la disponibilitat de precursors, incloent-hi àcids grassos i aminoàcids, la qual és altament regulada durant el desenvolupament del fruit (Song i Bangerth, 2003). El pas final en la biosíntesi d'ésters està catalitzada per l'alcohol o-aciltransferasa (AAT). Aquest enzim transfereix un grup acil d'un donador acil-CoA a grups hidroxil, amino o tiol per a la formació de l'éster corresponent. S'ha observat activitat AAT a teixits vegetals com ara flors o fruits (Dudareva i col., 1998; Aharoni i col., 2000; Yahyaoui i col., 2002; Beekwilder i col., 2004) i s'ha estudiat en algunes varietats de poma incloent-hi 'Fuji' (Echeverría i col., 2004), Royal Gala', 'Golden Delicious', 'Granny Smith' i 'Pacific Rose' (Holland i col., 2005), 'Mondial Gala' (Lara i col., 2007) i 'Pink Lady' (Villatoro i col., 2008).

Tot i la presència d'activitat AAT en diferents varietats de poma, encara no es coneix si un sol producte AAT és responsable de tota la producció d'ésters, o si hi estan involucrats diferents tipus d'AATs. Segons Holland i col. (2005), existeix més d'un tipus d'AAT implicat en la formació d'ésters a la poma. Les diferents activitats AAT observades en els diferents teixits i varietats de poma contribuirien a la variació observada en l'acumulació de compostos volàtils.

Les plantes contenen un gran nombre d'acil transferases, 88 de les quals es troben a l'*Arapidopsis* i més de 40 a l'arròs. Només unes quantes acil transferases trobades a l'*Arapidopsis* han estat caracteritzades per a funcions bioquímiques (St-Pierre i De Luca, 2000). Fins ara, només uns quants gens directament influenciats en la biosíntesi de compostos volàtils han estat estudiats als fruits. Recentment, el gen *MpAAT1* fou clonat amb èxit a la varietat de poma (cv. 'Royal Gala'). Aquest gen fou expressat en fulles, flors i fruits i va produir una proteïna que conté característiques d'altres aciltransferases en plantes. Té l'habilitat d'utilitzar un ampli rang de substrats des d'alcohols de cadena lineal (C_3-C_{10}) a alcohols ramificats i àcids de CoA per produir ésters trobats a la poma (Souleyre i col., 2005). Es va trobar que aquestes *MpAAT1* canvien significativament, incrementant la seva expressió amb l'addició d'etilè com també es va trobar prèviament per Defilippi i col. (2005a).

La preferència del gen *MpAAT1* pels substrats d'alcohol és depenent de la concentració de substrat, la qual determina el perfil aromàtic del fruit. És possible que altres *AATs* presents al genoma de la poma puguin contribuir a la biosíntesi d'ésters (Souleyre i col., 2005). Un altre gen *AAT*, *MdAAT2* fou clonat a la 'Golden Delicious' i a diferència d'altres varietats de poma, es va expressar exclusivament al fruit i es va trobar localitzat a la pell. L'acumulació de *MdAAT2* es va veure incrementada durant el desenvolupament del fruit (Li i col., 2006).

També es va aïllar i caracteritzat altres gens que codifiquen per a l'enzim AAT en altres fruits com ara plàtan (*Ban-AAT*) (Beekwilder i col., 2004), meló (*CM-AAT1 i CM-AAT2*) (Yahyaoui i col., 2002) i maduixa (*SAAT* i *VAAT*) (Aharoni i col., 2000). D'aquests estudis es van trobar diferents *AATs* que poden contribuir a la biosíntesi d'ésters volàtils i que tenen l'habilitat d'utilitzar un ampli rang de substrats, suggerint que les diferències observades en la composició de volàtils depenen també de la disponibilitat de substrats fonalmentalment els alcohols generats per ADH (Defilippi i col., 2005a; Souleyre i col., 2005; Lara i col., 2006). Segons Wyllie i Fellman (2000), la

disponibilitat del substrat i/o l'especificitat de substrat dels enzims podrien influir en la quantitat i el tipus d'ésters volàtils produïts.

L'últim pas de la biosíntesi d'ésters està també regulada per l'etilè. L'etilè està associat amb molts processos fisiològics i bioquímics de les plantes i juga un paper especialment important en els processos de maduració dels fruits climatèrics (Fellman i col., 2000; Defilippi i col., 2004). Estudis previs van mostrar una reducció en els nivells d'ésters volàtils en pomes tractades amb inhibidors de la biosíntesi o de l'acció de l'etilè (Fan i col., 1998; Lurie i col., 2002) i en pomes transgèniques que bloquegen la biosíntesi d'etilè, l'AAT es va veure regulada per l'etilè (Dandekar i col., 2004; Defilippi i col., 2004; Defilippi i col., 2005ab).

L'expressió dels gens que codifiquen per a AATs identificats tant a 'Royal Gala' (*MpAAT1*) com a 'Golden Delicious' (*MdAAT2*) van ser també depenent d'etilè (Souleyre i col., 2005; Li i col., 2006). Aquests resultats van suggerir que les reduccions en els nivells d'ésters volàtils observades en condicions de supressió de producció d'etilè podrien ser causades per una reducció en l'activitat o l'expressió d'*AAT* (Defilippi i col., 2005a).

L'objectiu d'aquest estudi fou aïllar i caracteritzar gens *AAT* putatius de la varietat 'Royal Gala' a partir de bases de seqüències EST (Expressed Sequence Tags) en relació amb la producció d'ésters volàtils aromàtics.

2. Material i mètodes

2.1. Material vegetal

Es van fer 2 estudis:

2.1.1. Els fruits de la varietat 'Royal Gala' utilitzats pertanyien a una línia transgènica (AO3) que produïa nivells d'etilè no detectables, generada per introducció d'un gen d'ACC oxidasa en orientació antisentit, i que per tant resultava

en un fenotipus en el qual no es donaven els canvis de maduració depenents d'etilè. La maduració dels fruits transgènics AO3 va ser induïda mitjançant un corrent continu de 120 mg m^{-3} d'etilè exogen. Dues mostres de 3 fruits per repetició es van seleccionar a 0 h, 3 h, 18 h, 96 h (4 dies) i 192 h (8 dies) tant a la pell com a la polpa. Per aquest experiment, les pomes AO3 que no es van tractar amb etilè es van analitzar 192 h després de l'inici de l'experiment. Tan els fruits transgènics AO3 com els fruits control es van emmagatzemar a 22 ºC amb la finalitat d'estimular el climatèri respiratori.

2.1.2. Es va estudiar l'expressió dels mateixos gens *AAT* putatius en diferents estadis fisiològics de fruits de poma 'Royal Gala', corresponents a 0, 14, 25, 35, 60, 87, 132 i 146 dies després de plena floració (ddpf).

2.2. Extracció d'ARN total de la poma 'Royal Gala'

L'ARN total es va extreure utilitzant el kit RNAeasy (Qiagen) seguint les recomanacions del fabricant. La preparació d'ARN resultant es va quantificar mesurant l'absorbància a 260 nm (A_{260}). Es va comprovar l'eliminació de proteïnes mesurant a 280 nm (A_{280}). La concentració d'ARN es va estimar considerant que una unitat A_{260} correspon a 40 µg d'ARN per mL i es va comprovar la seva integritat mitjançant electroforesi sobre 1% (p/v) d'agarosa en TAE (1x).

Per a l'extracció d'ARN total a partir del fruit, es van homogeneïtzar 300 mg de teixit de pell i polpa de poma 'Royal Gala' amb 3 mL de tampó d'extracció (473 g GI (4.0 M), 16.5 g CH_3COONa (0.2 M), 9.3 g d'EDTA (25 mM), 25 g de PVPP i aigua milliQ fins a 1000 mL, pH 5.0), d'acord amb Mackenzie i col. (1997). Es va centrifugar l'homogenat a 13000 rpm durant 3 minuts. Es van transferir 500 µL de sobrenedant a un tub de centrífuga al qual es van afegir 100 µL de 20 % (p/v) SDS. La mostra es va incubar durant 10 minuts a 70 ºC amb agitació intermitent i posterior refredament durant 5 minuts en gel. Un cop fred, es va centrifugar 10 min a 13000 rpm. 300 µL del

sobrenedant es van afegir a 300 µL de tampó alt en sal (90 g NaI (6.0 M), 2 g Na_2SO_3 i aigua milliQ fins a 100 mL), 150 µL d'etanol absolut i 25 µL de llet de silica (10 g de partícules de diòxid de silicona 1-5 µM i 10 mL de tampó (Gly (100 mM), NaCl (100 mM), HCl (100 mM) i aigua milliQ fins a 100 mL, es va incubar el tub a temperatura ambient durant 10 min amb agitació intermitent i es va tornar a centrifugar durant 3 minuts a 6000 rpm descartant el sobrenedant. Es va resuspendre el pellet de silica amb 300 µL de tampó de rentat (Tris, EDTA (100 mM stock), NaCl, etanol absolut i aigua milliQ fins a 1000 mL (pH 7.5)), i es va centrifugar durant 1 minut a 4600 rpm. Es va tornar a resuspendre la silica amb 150 µL de solució tampó (Tris, EDTA (100 mM stock) i aigua milliQ fins arribar a 1 L (pH 7.5)). Finalment les mostres es van incubar a 70 ºC durant 4 minuts i es van centrifugar a 13000 rpm durant 5 minuts. 100 µL del sobrenedant final es van congelar a -70 ºC abans de ser amplificats per RT-PCR.

Per tal d'eliminar l'ADN durant el procés de purificació previ a l'amplificació de l'ARN total es va realitzar una digestió amb DNasa. Es van afegir a 1 µg d'ARN total, 1µL de solució tampó (200 mM Tris-HCl, pH 8.4, 20 mM $MgCl_2$ i 500 mM KCl), 1 U de DNasa (Invitrogen) i d'aigua lliure de RNases tractada amb DEPC fins a un volum de 10 µL. Es van incubar els tubs durant 15 minuts a temperatura ambient i posteriorment es va inactivar la DNasa afegint 1 µL de d'EDTA 25 mM. Es van escalfar les mostres durant 10 minuts a 65 ºC i finalment es van incubar aproximadament 2 h a -80 ºC fins a la seva utilització per a retrotranscripció prèvia a l'amplificació per PCR.

2.3. Síntesi d'ADN complementari (ADNc)

Després de la digestió amb DNasa es va fer la retrotranscripció de l'ARN extret. Es va realitzar un control negatiu en absència de retrotanscriptasa per assegurar que no hi havia contaminació genòmica. Com a control positiu per obtenir l'ADNc, es van afegir a 0.05 µg d'ARN total 1 µL d'oligo$(dT)_{20}$ (50 µM) (Invitrogen), 1 µL de dNTP (10 mM) (Invitrogen) i 14 µL d'aigua lliure de RNases tractada amb DEPC. Seguidament es va escalfar a 65 ºC durant 5 minuts i es van incubar les mostres amb gel durant

mínim 1 minut. Posteriorment s'hi van afegir 4 µL de solució tampó (200 mM Tris-HCl, pH 8.4, i 500 mM KCl), 1 µL DTT (0.1 M) i 200 U de retrotranscriptasa SuperScript III (Invitrogen). Es va incubar la mostra a 50 ºC durant 60 minuts i es va aturar la reacció escalfant a 70 ºC durant 15 minuts. Finalment les mostres es van incubar a -80 ºC fins a la seva amplificació per RT-PCR.

2.4. Test PCR

Aquest test es va realitzar per comprovar si els oligonucleòtids específics per a *MpAAT1* amplificaven correctament els ADNc a estudiar. Es va preparar una solució mare amb 165 µL de solució tampó (200 mM Tris-HCl, pH 8.4, i 500 mM KCl), 49.5 µL de $MgCl_2$ (50 mM), 33 µL de dNTP (10 mM), 66 U de Taq ADN polimerasa (Invitrogen) i 927.3 µL d' d'aigua lliure de RNases tractada amb DEPC. Seguidament es van afegir 36 µL de la solució mare a cada tub, 2 µL de cada oligonucleòtid (reverse i forward) (10 µM), 10 µL d'ADNc i d'aigua lliure de RNases tractada amb DEPC fins a un volum total de 50 µL (Invitrogen). Després es va procedir a incubar els tubs en un termociclador a 94 ºC durant 2 minuts amb la finalitat d'activar la polimerasa. Es va realitzar un pre-escalfament a 94 ºC durant 2 minuts seguits per 40 cicles d'amplificació de PCR amb les condicions següents: desnaturalització (94 ºC, 20 segons), hibridació (72 ºC, 30 segons) i elongació (72 ºC, 30 segons), seguits d'una elongació final a 72 ºC durant 10 minuts. Un cop finalitzada la incubació, es va comprovar l'amplificació i es van visualitzar els fragments amplificats per electroforesi sobre agarosa al 1 % (p/v) en TAE (1x). Es carregaven al gel 12 µL de cada producte.

2.5. Anàlisi de l'expressió genètica per RT-PCR.

Es va utilitzar 5 µL d'ADNc (15 ng μL^{-1}) com a motlle en 20 µL de reacció que contenia 15 µL d'una solució formada per 279 µL d' d'aigua lliure de RNases tractada amb DEPC, 60 µL de tampó (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 60 µL dNTP (2mM) (Invitrogen), 18 µL de $MgCl_2$ (1.5 mM), 12 µL de cada oligonucleòtid (10 µM),

6 µL de SYBR® Green I (Molecular Probes) al 0.1 % (p/v) i 3 µL de Taq ADN polimerasa (Invitrogen).

Es va mesurar per quadruplicat la densitat òptica entre 500 nm i 660 nm de cada ADNc per a cada mostra amb un detector de fluorescència ABI Prism 7900HT (Applied Biosystems). Les condicions d'amplificació incloïen un escalfament inicial a 95 °C durant 2 minuts, seguit de 40 cicles de 94 °C durant 15 segons, 55 °C durant 20 segons i 72 °C durant 30 segons. Finalment, a cada reacció de RT-PCR s'afegia una anàlisi de corba de dissociació per a cada producte amplificat. Això va involucrar la desnaturalització a 95 ºC durant 15 s, un refredament a 55 ºC durant 20 s i seguidament un escalfament gradual a 0.01 ºC s^{-1} fins a 95 ºC. Finalment es va comprovar que els productes obtinguts per RT-PCR sol amplificaven un producte.

Es van seleccionar dos gens de referència (actina i GAPDH de *Malus*) a cada reacció de RT-PCR per tal de normalitzar l'expressió dels gens. El factors de normalització es calculaven prenent la mitjana geomètrica dels dos gens de referència que mostraven menys variabilitat utilitzant el programa informàtic geNorm v3.4 (Vandesompele i col., 2002). Els resultats es van expressar com a nivells d'expressió relativa segons el mètode de Pfafft (2001), i amb la correcció de diferents eficiències d'amplificació (Ramakers i col., 2003).

3. Resultats i discussió

3.1.- Canvis a l'expressió dels gens que codificquen per a l'enzim AAT a la pell de la poma 'Royal Gala' després d'un tractament amb etilè

Es va seleccionar un total de 12 gens potencialment codificants per a AAT en base a la seva homologia a *MpAAT1*, que són probables punts de control etileno-depenent de la producció d'esters volàtils a les pomes (Taula 1). La identificació de les seqüències clonades com a AATs potencials es va basar en l'homologia de seqüència amb *MpAAT1*, pel producte gènic de la qual es va demostrar la capacitat per a produir ésters

volàtils a partir de diversos substrats (Souleyre i col., 2005). Tots els gens *AAT* caracteritzats fins el moment semblen ser claus en les rutes de biosíntesi dels diversos ésters volàtils aromàtics que proporcionen a la fruita el gust i olor característics.

***Taula 1*.- Característiques dels gens seleccionats com a potencialment codificants per a AAT en poma 'Royal Gala'**

Gen	*Seqüència del oligonucleotid (5'-3')*	*Longitud de la seqüència EST*	*Tm[a] producte*
MpAT2	5′-TAAGGTAAAATATGCCAATGA-3′	127 bp	70.4 ºC
MpAT3	5′-GCCAAAAACTCCCGTGAAAG-3′	140 bp	74.7 ºC
MpAT4	5′-ACGAAGACGAAATGAAAGTG-3′	143 bp	71.5 ºC
MpAT5	5′-GCTAAGTAGGGTGGTAATGG-3′	138 bp	73.7 ºC
MpAT8	5′-CCTGATAATGGAACAAATGG-3′	230 bp	71.4 ºC
MpAT9	5′-TTCATTTCTTGCTGTTGGTGC-3′	159 bp	68.6 ºC
MpAT10	5′-ACCTGCTCTCGTATGCTTC-3′	220 bp	73.4 ºC
MpAT11	5′-TATGTGGGAACAGATTTGGG-3′	178 bp	74.1 ºC
MpAT12	5′-GGGTGTTCTGTTTGTTGAG-3′	283 bp	79.0 ºC
MpAT13	5′-TGTGGTGGTGTTAGTCTTAG-3′	263 bp	75.9 ºC
MpAT14	5′-AACCTACCTGATTCCAAAAC-3′	85 bp	75.1 ºC
MpAT15	5′-AAGCCCAACAAGAAGATAGG-3′	232 bp	77.3 ºC

[a]Tm: temperatura de fusió.

De les 12 seqüències seleccionades (Taula 1), 6 gens potencialment codificants per a AAT es van detectar correctament a la pell de poma 'Royal Gala' (Taula 2).

***Taula 2*.- Característiques dels gens expressats a la pell de poma 'Royal Gala' en resposta a l'etilè**

Gen	*Seqüència del oligonucleotid (5'-3')*	*Longitud de la seqüència EST*	*Tm[a] producte*
MpAT3	5′-GCCAAAAACTCCCGTGAAAG-3′	140 bp	74.7 ºC
MpAT4	5′-ACGAAGACGAAATGAAAGTG-3′	143 bp	71.5 ºC
MpAT8	5′-CCTGATAATGGAACAAATGG-3′	230 bp	71.4 ºC
MpAT11	5′-TATGTGGGAACAGATTTGGG-3′	178 bp	74.1 ºC
MpAT12	5′-GGGTGTTCTGTTTGTTGAG-3′	283 bp	79.0 ºC
MpAT14	5′-AACCTACCTGATTCCAAAAC-3′	85 bp	75.1 ºC

[a]Tm: temperatura de fusió.

L'expressió dels gens *MpAT3*, *MpAT4*, *MpAT8*, *MpAT12* i *MpAT14*, va restar inhibida als fruits no tractats amb etilè (0 h i 192 h control), mostrant que l'expressió del gen

corresponent va tenir un patró de regulació depenent de l'etilè. Només el gen *MpAT11* va mostrar una expressió elevada als fruits no tractats amb etilè (0 h i 192 h control) (Fig. 1). Defilippi i col. (2005b) van observar que els fruits de línies transgèniques amb supressio de la producció d'etilè mostraven una reducció molt important en l'emissió de tots els grups de volàtils, especialment d'ésters i alcohols. Els nivells d'expressió *AAT* foren majors en fruits no transformats respecte a les línies transgèniques, concloent que *AAT* van ser regulats per l'etilè.

Altres estudis van mostrar un augment significatiu de la producció de volàtils totals en poma 'Royal Gala' després de 192 h d'exposició a etilè respecte als fruits control (Schaffer i col., 2007). El 80% dels volàtils va arribar als seus màxims de concentració entre 96 i 192 h d'exposició a etilè. L'expressió relativa dels gens *MpAAT1* i *MpAT6*, identificats prèviament per Souleyre i col. (2005) a la pell de la varietat 'Royal Gala', va mostrar un augment progressiu a mida que s'allargava el temps del fruit a 22 ºC, mentre que l'expressió de *MpAAT1* restava inhibida als fruits sense tractament amb etilè (0 h i 192 h control).

Es va demostrar que línies transgèniques amb supressió dels enzims encarregats de la biosíntesi d'etilè, l'ACC sintasa i ACC oxidasa proporcionen una evidència addicional que l'etilè regula la producció d'ésters volàtils. La producció d'ésters totals va ser inhibida un 65-70% en els fruits transgènics. Aquestes investigacions confirmen que la producció d'ésters volàtils als fruits és un procés depenent de l'etilè (Dandekar i col., 2004).

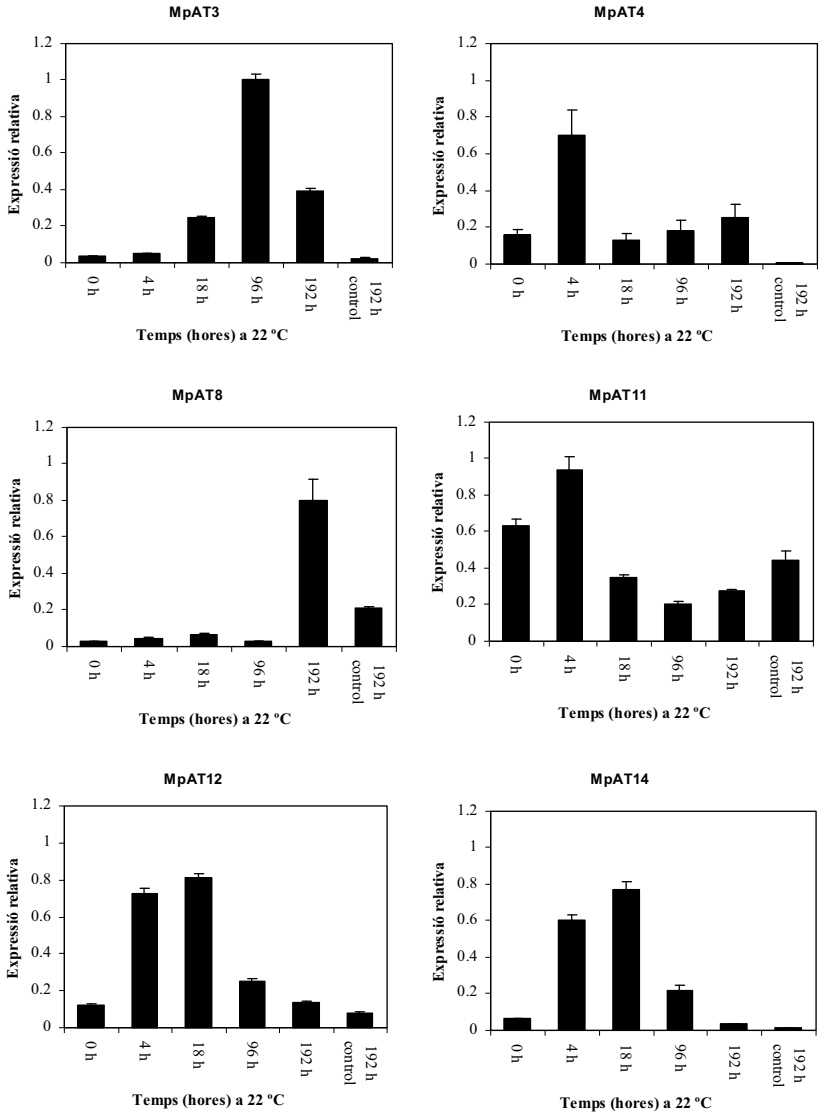

***Figura 1.*- Perfil d'expressió relativa dels gens *MpAT3*, *MpAT4*, *MpAT8*, *MpAT11*, *MpAT12* i *MpAT14* a la pell de poma 'Royal Gala' tractada amb 100 ppm d'etilè després de 0, 4, 18, 96 i 192 h a 22 ºC (192 h control = 192 h sense tractament). Les barres verticals indiquen l'error estàndard. Els valors representen mitjanes de 4 mesures.**

3.2.- Canvis a l'expressió dels gens que codificquen per a l'enzim AAT a la polpa de la poma 'Royal Gala' després d'un tractament amb etilè

Dels 12 gens potencialment codificants per l'AAT en base al seu homòleg *MpAAT1* (Taula 1), 3 d'ells es van detectar correctament a la polpa de poma 'Royal Gala' (Taula 3).

***Taula 3.*- Característiques dels gens expressats a la polpa de poma 'Royal Gala' en resposta a l'etilè**

Gen	*Seqüència del oligonucleotid (5'-3')*	*Longitud de la seqüència EST*	*Tm[a] producte*
MpAT3	5'-GCCAAAAACTCCCGTGAAAG-3´	140 bp	74.7 ºC
MpAT11	5´-TATGTGGGAACAGATTTGGG-3´	178 bp	74.1 ºC
MpAT15	5´-AAGCCCAACAAGAAGATAGG-3´	232 bp	77.3 ºC

[a]Tm: temperatura de fusió.

L'expressió del gen *MpAT15* va ser severament inhibida als fruits després de 0, 4, 18, 96 i 192 h a 22 ºC posteriors als tractaments. Només les mostres a 192 h no tractades durant tot el període posterior al tractament amb etilè van mostrar una expressió elevada resultat que suggereix que l'expressió d'aquest gen és inhibida per l'etilè. El gen *MpAT3* va mostrar una expressió aproximadament constant. El gen *MpAT11* només va mostrar una expressió més important als fruits sense tractament (0 h i 192 h control) o a les 4 h de tractament amb etilè (Fig. 2). Aquest fet fa pensar que podria ser un gen inhibit per l'etilè tal i com han demostrat altres autors (Yahyaoui i col., 2002; Souleyre i col., 2005; Defilippi i col., 2005a).

L'expressió relativa dels gens *MpAT1* i *MpAT6* identificats prèviament per Souleyre i col. (2005) a la polpa de poma 'Royal Gala', va mostrar un augment progressiu a mida que avançava el temps a 22 ºC, mostrant un màxim a les 192 h sense tractament amb etilè (control). Altres autors van observar una reducció important a l'expressió dels gens *AAT* en polpa (Defilippi i col., 2005a) durant la maduració a 22 ºC. L'aplicació exògena d'etilè suposa una acumulació massiva del transcrit corresponent a aquests gens, juntament amb un augment en l'activitat AAT, amb nivells significativament més alts que a la pell.

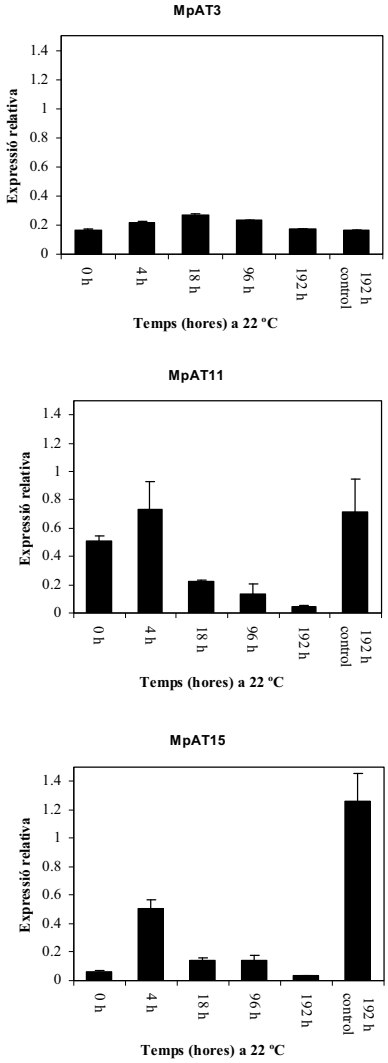

***Figura 2*.- Perfil d'expressió relativa dels gens *MpAT3*, *MpAT11 i MpAT15* a la polpa de poma 'Royal Gala' tractada amb 100 ppm d'etilè després de 0, 4, 18, 92, 192 h a 22 ºC (192 h control = 192 h sense tractament). Les barres verticals indiquen l'error estàndard. Els valors representen mitjanes de 4 mesures.**

3.3.- Canvis a l'expressió dels gens que codificquen per a l'enzim AAT a la 'Royal Gala' al llarg de la maduració en camp

Es van seleccionar un total de 12 gens potencialment codificants per l'AAT en base al seu homòleg *MpAAT1*, (Taula 1). D'aquests 12 gens, 9 d'ells es van detectar correctament durant la maduració en camp en poma 'Royal Gala' (Taula 4).

***Taula 4*.- Característiques dels gens expressats en poma 'Royal Gala' durant la maduració en camp.**

Gen	*Seqüència del oligonucleotid (5'-3')*	*Longitud de la seqüència EST*	*Tm[a] Producte*
MpAT2	5´-TAAGGTAAAATATGCCAATG-3´	127 bp	70.4 ºC
MpAT3	5'-GCCAAAAACTCCCGTGAAAG-3´	140 bp	74.7 ºC
MpAT5	5´-GCTAAGTAGGGTGGTAATGG-3´	138 bp	73.7 ºC
MpAT8	5´-CCTGATAATGGAACAAATGG-3´	230 bp	71.4 ºC
MpAT9	5´-TTCATTTCTTGCTGTTGGTGCT-3´	159 bp	68.6 ºC
MpAT1	5´-TATGTGGGAACAGATTTGGG-3´	178 bp	74.1 ºC
MpAT1	5´-GGGTGTTCTGTTTGTTGAG-3´	283 bp	79.0 ºC
MpAT1	5´-AACCTACCTGATTCCAAAAC-3´	85 bp	75.1 ºC
MpAT1	5´-AAGCCCAACAAGAAGATAGG-3´	232 bp	77.3 ºC

[a]Tm: temperatura de fusió.

Per a aquests 9 gens es van observar 3 tipus diferents de patrons d'expressió al llarg del període experimental (Fig. 3). Així, els gens *MpAT2*, *MpAT5*, *MpAT9* i *MpAT11*van mostrar n nivell d'expressió màxim en estadis mitjans de maduració (entre 60 i 87 ddpf segons el gen), seguit d'una disminució en estadis més avançats. L'expressió de *MpAT3* i *MpAT15* va augmentar en estadis finals de maduració, sent quasi bé nul·la fins a 60 ddpf. Els gens *MpAT8*, *MpAT12* i *MpAT14* es van expressar preferentment a estadis de maduració primerencs, disminuint de forma progressiva a mida que el fruit madurava. El gen *MpAT14*, fins i tot, només va mostrar expressió detectable a 0 ddpf, sent nul·la per a la resta d'estadis de maduració en camp (Fig. 3).

Tot i que es va observar una expressió elevada en estadis de maduració considerats avançats (132 i 146 ddpf) per als gens *MpAT3* i *MpAT15*, els resultats indiquen que en tot moment al llarg del període experimental hi va haver expressió d'algun gen

codificant per a AAT. Aquest fet podria explicar els nivells aproximadament constants d'activitat AAT trobats durant la maduració d'altres varietats de poma, com ara 'Pink Lady®' (Villatoro i col., 2008), 'Mondial Gala' (Lara i col., 2008) i 'Fuji' (Echeverría i col., 2004), tot i que en altres casos sí que s'ha observat un increment d'activitat a l'inici de la maduració (Fellman i col., 2000). Com que, no obstant, la producció d'ésters volàtils sí que s'incrementa en estadis avançats de desenvolupament (Fellman i col., 2000; Echeverría i col., 2004; Lara i col., 2008; Villatoro i col., 2008), aquestes dades suggereixen que l'activitat AAT és necessària però no suficient per a la biosínteisi d'aquest compostos.

La biosíntesi d'ésters podria estar limitada pel subministre i disponibilitat dels substrats. Un altre factor a tenir en compte per explicar els canvis en la producció d'ésters al llarg de la maduració és l'especificitat de substrat de les diferents isoformes d'AAT que es sintetitzen durant el procès.

Estudis realitzats per Li i col. (2006) van mostrar que l'acumulació d'ARNm del gen *MdAAT2*, va augmentar durant el desenvolupament del fruit, encara que disminuïa durant la posterior maduració postcollita de poma 'Golden Delicious'. Segons Holland i col. (2005) no es va observar activitat AAT durant estadis de maduració de la poma 'Fuji' i 'Granny Smith' poc avançats ni a la pell ni a la polpa, i els nivells d'AAT van incrementar amb la maduració i el desenvolupament del fruit. Altres gens (*MpAAT1*, *MpAT6* i *MpAT7*) identificats prèviament per Souleyre i col. (2005) durant la maduració en camp de poma 'Royal Gala' foren fortament expressats a partir de 132 ddpf i per tant podrien contribuir fortament a l'aroma del fruit.

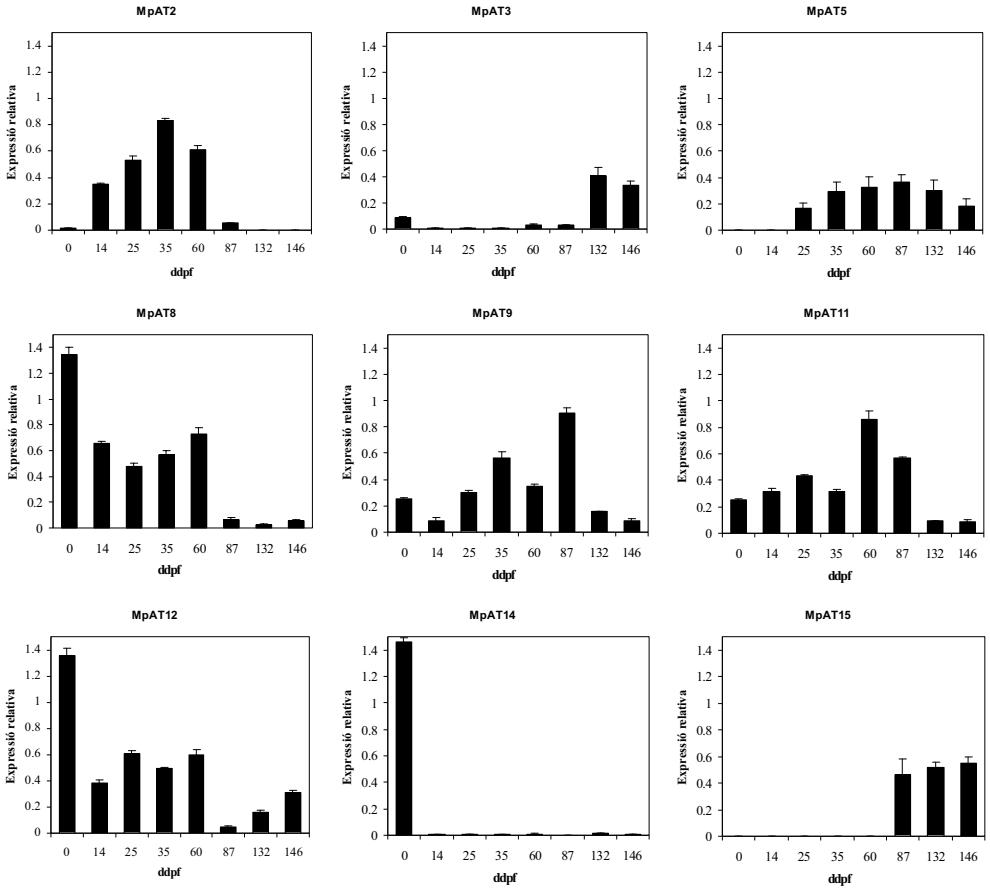

Figura 3.- **Perfil d'expressió d'AAT determinades durant la maduració en camp de poma 'Royal Gala'. Els controls no retrotranscrits, indicatius de posible contaminació per ADN genòmic, no van mostrar amplificació (dades no mostrades). Per a les mostres de 0 ddpf, els borrons es van eliminar dels pètals i pistils. Per a 14, 25, 35 i 60 ddpf es va utilitzar el fruit sense pecíol. Per a 87, 132 i 146 ddpf, es van analitzar només els teixits del còrtex. *MpAT4*, *MpAT10* i *MpAT13* no van ser detectats durant el desenvolupament del fruit. Les barres verticals indiquen l'error estàndard. Els valors representen mitjanes de 4 mesures.**

4. Conclusions

La majoria del gens putatius identificats a la pell de poma 'Royal Gala' (*MpAT3, MpAT4, MpAT8 i MpAT12 i MpAT14*) van mostrar un patró de regulació depenent d'etilè i per tant van estar involucrats en el procés de maduració dels fruits i la síntesis d'ésters volàtils aromàtics. En canvi a la polpa, el gens putatius *MpAT3, MpAT11 i MpAT15* es van veure inhibits per l'etilè.

Els gens putatius identificats durant la maduració en camp (*MpAT2, MpAT5, MpAT9 i MpAT11*) van mostrar un patró d'expressió genètica similar amb increments a partir d'estadis de maduració mitjana seguits d'una disminució en fruit madur.

Aquests resultats indiquen que hi ha diverses isoformes d'AAT, probablement amb diferents característiques. Existeixen molts factors que influencien la biosíntesi d'ésters. La disponibilitat de substrats (alcohols i àcids, per exemple), la de precursors inicials com els àcids grassos o els aminoàcids, el nombre d'isoformes d'AAT presents, la seva regulació i les diferents característiques cinètiques d'aquests enzims sota diferents concentracions de substrat són els més importants. Les diferències significatives en els nivells de volàtils observades entre la pell i la polpa, els precursors i els enzims relacionats amb la biosíntesi d'aromes indiquen que el mecanisme de regulació podria diferir entre teixits.

5. Abreviatures

CH_3-COONa: acetat de sodi; ddpf: dies després de plena floració; DEPC: dietilpirocarbonat; DNasa: desoxiribonucleasa; EDTA: àcid etilen-diaminotetracétic; GAPDH: Gliceraldehid-3-fosfat dehidrogenasa; GI: isotiocianat de guanidi; Gly: glicina; HCl: àcid clorhídric; KCl: clorur de potassi; $MgCl_2$: clorur de magnesi; NaCl: clorur de sodi; NaI: iodur sòdic; Na_2SO_3: sulfit sòdic; PVPP: polivinilpolipirrolidona; SDS: dodecilsulfat de sodi; Taq: Thermus aquaticus; Tris: (hidroximetil)aminometà;

oligo dT; dNTP; Deoxyribonucleotide triphosphate; DTT: 1,4-ditiotreitol; RT-PCR: real time polimerase chain reaction; TAE; Tris-acetat-EDTA.

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CAPÍTOL 3

Volatile compounds quality parameters and consumer acceptance of 'Pink Lady®' apples stored in different conditions.

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SUMMARY

Standard quality parameters, consumer acceptance, and volatile compound emission of 'Pink Lady®' apples (*Malus* × *domestica* Borkh.) were measured at harvest and after 14 and 25 weeks of cold storage in three different atmospheres. After storage, fruit were left to ripen for 1 and 7 days at 20 ºC before instrumental and sensory measurements were performed. Data were subjected to Principal Component Analysis (PCA) and Partial Least Square Regression (PLSR). PLSR results indicated that the parameters positively influencing acceptability were soluble solid content, titratable acidity, background colour, and emission of hexyl 2-methylbutanoate, hexyl hexanoate, hexyl propanoate, butyl 2-methylbutanoate, 2-methylbutyl acetate and butyl propanoate. Results of sensory analyses revealed the treatments considered in this work could be split into two levels of acceptability.

Keywords: Acceptability, Controlled atmosphere, Quality parameters, Multivariate analysis, Shelf life, Storage period, Volatile compounds.

Introduction

'Pink Lady®' apples originated from a cross between 'Lady Williams' and 'Golden Delicious' made by J.E.L. Cripps. The aim of the cross was to combine sweetness and scald-free surface of 'Golden Delicious' with firmness and long-storage potential of 'Lady Williams' (Cripps et al., 1993). Since 1990, this variety has been extensively cultivated in the main apple-producing areas of the world, on account of its excellent sensory attributes: it is firm, has fine dense flesh, is crisp and juicy, provides excellent flavour, and has a high sugar-acid balance. 'Pink Lady®' has been rated higher for acceptability than 'Granny Smith' and 'Red Doughert' by a consumer panel in New Zealand (Corrigan et al., 1997).

In the area of Lleida (NE Spain), controlled-atmosphere (CA) storage of apples under low (2 kPa) or ultralow (1 kPa) oxygen concentrations, combined with similar CO_2 levels, is becoming increasingly used in detriment of 'traditional' CA (3 kPa O_2 + 3 kPa CO_2), in an attempt to extend the storage period beyond 7 months without a decrease in apple quality and sensory characteristics (López et al., 1998a). Most studies involving 'Pink Lady®' have been concerned with storage disorders (Jobling et al., 2004), but a few have focused on its aroma volatile composition and changes taking place therein during cold storage. Boamfa et al. (2004) showed that even when exposed to a brief (24 h) anaerobic treatment, 'Pink Lady'® fruit suffered low oxygen injuries, with a concomitant loss of aroma and flavour. Saftner et al. (2005) reported that 'Pink Lady®' is a promising new apple cultivar, retaining high quality after cold storage in regular atmosphere.

Apple flavour results from a complex combination of taste and odour. Although taste and texture are important for apple quality, the presence of trace amounts of volatiles, which are responsible for the characteristic odour of an apple, gives fruit much of their perceived quality (Dimick and Hoskin, 1982). The relative contribution of volatile compounds to overall aroma is expressed by the odour unit, which can be determined

by calculating the concentration ratio of a particular food component to its odour threshold (Takeoka et al., 1992; Echeverría et al., 2004a). This representation of the volatile pattern is only an approximation, but it serves a practical purpose when selecting the most important volatile contributors to aroma (Guadagni et al., 1966). Gas chromatography and mass spectrometry have made it possible to identify more than 300 volatile compounds present in apples (Dimick and Hoskin, 1982), but only a few of these, about 20-40 compounds, have been shown to be responsible for fruit aroma (Cunningham et al., 1986). When the logarithm of odour unit value is > 0, these compounds are likely to contribute to flavour. However, compounds with negative odour units may still contribute to overall food flavour as background notes (Buttery, 1993.)

Sensory analysis is used to measure the components of taste, texture and aroma, and to predict eating quality with instrumental measurements. Consumers prefer additive-free fruit showing a faultless appearance, having a high nutritional value and exhibiting texture and flavour typical for each particular cultivar. Floury fruit are undesirable, as are fruit harvested when preclimacteric, before ripening-related ethylene production has begun, although these fruit are more likely to have a longer shelf life than those harvested at a later developmental stage (Jobling, 2002).

The influence of maturity stage, storage conditions and storage period on consumer acceptance has been the subject of several other works (Plotto et al., 1999; Saftner et al., 2002). However, studies relating consumer preference to instrumental measurements for 'Pink Lady®' apple have been scarce so far. The objective of this study was therefore to assess possible relationships between standard quality parameters, aroma volatile compounds and consumer acceptance of 'Pink Lady®'apples.

2. Materials and methods

2.1. Plant material and storage conditions

Apples (*Malus* × *domestica* Borkh. cv. 'Pink Lady®') were harvested at commercial date (215 days after full bloom) from 5 year-old trees grown on M-9 EMLA rootstock at a commercial orchard in Lleida (NE Spain). Immediately after harvest, 4 lots (100 kg each) of apples were selected in accordance with the Association Pink Lady Europe (calibre >70 mm; 50% of diffuse pink or 30% intense pink; background colour: revolving-between green and yellow; starch index 5-5.8; firmness > 80 N, and absence of defects). Three of these lots were stored at 1 ºC and 92-93 % relative humidity in cold-storage chambers. Three different storage atmospheres were tested: normal atmosphere (AIR): 21 kPa O_2+0.03 kPa CO_2 ; low oxygen (LO): 2 kPa O_2+2 kPa CO_2 and ultra-low oxygen (ULO): 1 kPa O_2+1 kPa. Fruit samples were taken from each storage chamber after 14 and 25 weeks, and analysed after being kept at 20 ºC for 1 or 7 days (shelf life period).

2.2. Maturity and standard quality parameter analyses

Twenty fruit per treatment were analysed individually for flesh firmness, soluble solid content (SSC), titratable acidity (TA) and skin colour, both at harvest and after removal from cold storage (3 atmospheres × 2 storage periods × 2 shelf life periods). Starch index and ethylene production were also analysed at harvest.

Flesh firmness was measured on two opposite sides of each fruit with a penetrometer (Effegi, Milan, Italy) equipped with an 11-mm diameter plunger tip; results were expressed in N. SSC and TA were measured in juice pressed from the whole fruit. SSC was determined with a hand refractometer (Atago, Tokyo, Japan) and results were expressed as a % of sucrose in an equivalent solution. TA was determined by titrating 10 ml of juice with 0.1 N NaOH to pH 8.1 using 1% (v/v) phenolphthaleine; results

were expressed as g malic acid per litre. Fruit colour was determined with a portable tristimulus colorimeter (Chroma Meter CR-200, Minolta Corp, Osaka, Japan) using CIE illuminant D_{65} and an 8 mm diameter measuring aperture. Skin colour was measured on each fruit at two equatorial locations 180º apart and that corresponds to the side exposed to sunlight (ES) and the shaded side (SS). Hue angle on the exposed side and Hue angle on the shaded side were respectively used as measurements of surface and background fruit colour. Starch index was determined in twenty apples by dipping cross-sectional fruit halves in an iodine solution (15 g KI + 6 g I_2 per litre) for 30 s; starch hydrolysis was rated using a 1-10 Eurofru scale.

2.3. Analysis of volatile compounds

Eight kg of apples (4 replicates × 2 kg/replicate) per treatment (atmosphere × storage period × shelf life period) were taken for volatile compound analysis both at harvest and after removal from storage. Intact fruit were placed in a 10 l Pyrex container and exposed to an air stream (900 ml/min) for 4h: the effluent was then passed through an ORBO-32 adsorption tube filled with 100 mg of activated charcoal (20/40 mesh). Volatile compounds were de-adsorbed by agitation with 0.5 ml of diethyl ether for 40 min. Identification and quantification of volatile compounds was performed in a gas chromatograph H-P 5890 series II (Hewlet-Packard Co., Barcelona, Spain) equipped with a flame ionisation detector (GC-FID), using a cross-linked FFAP capillary column (50 m × 0.2 mm × 0.33 μm). The oven program was set at 70 ºC (1 min) and the temperature was initially raised by 3 ºC/ min to 142 ºC and then by 5 ºC/min to 225 ºC. It was then kept constant for 10 min at this final temperature. Helium was used as the carrier gas at a flow rate of 0.8 ml/min (42 cm/s), with a split ratio of 40:1, in the presence of air (400 ml/min) and H_2 (32 ml/min). The injector and detector were held at 220 ºC and 240 ºC, respectively. Compounds were identified by comparing their respective Kovats retention indices with those of standards, and by enriching apple extract with authentic samples. Quantification was carried out using butylbenzene (assay>99.5%, Fluka) as an internal standard. Spectra were recorded with a Hewlett-

Packard 3398GC Chemstation. The identity of volatile compounds detected was confirmed by comparing their GC retention indices and their mass spectra with those of an external standard injected into a Hewlett-Packard 5890 gas chromatograph (GC-MS) under the same conditions as described above, and by comparing spectra with those in a registered database (NIST HP59943C original mass spectral library). GC-MS was equipped with the same capillary column as in GC-FID analyses. Mass spectra were obtained by electron impact ionization at 70 eV. Helium was used as the carrier gas. Results were expressed as μg/Kg.

To measure ethylene production, 8 apples were divided into 2 replicates (about 1 kg per replication) and placed in 5-l jars continuously aerated with humidified air at a rate of ~ 2 l/h at 20 ºC. Ethylene production was measured by taking gas samples from the effluent air with a 1-ml syringe, followed by injection into a Hewlett-Packard 5890 GC/FID equipped with an alumina column 80/100 (2 m × 3 mm) (Teknokroma, Barcelona, Spain). Gas analyses were conducted isothermally at 100 ºC. N_2 was used as the carrier gas, with air and H_2, at a flow of 45, 400, and 45 ml/min, in that order. The injector and detector were held at 120 ºC and 180 ºC, respectively.

2.4. Sensory measurements

For sensory evaluation, fruit samples were kept in a room at 20 ºC for 1 or 7 days after removal from storage atmosphere. Twenty-five apples per treatment (atmosphere × storage period × shelf life period) were used for sensory analysis. Each fruit was divided into 4 pieces, which were then given to 4 panellists for taste evaluation. Three pieces (one per treatment) were placed on white plates and immediately presented to a consumer panel comprised of 100 judges. All participating judges were every-day apple consumers, selected among UdL-IRTA Research Institute staff and UdL students. The panel consisted of 58 women and 42 men, aged between 18 and 58 years. Each piece was identified by a random three-digit code. The order in which the three parts were presented to each judge was randomised. Mineral water was used as a palate cleanser

between samples. The judges were asked to rate overall fruit acceptability according to a hedonic test (9, like very much; …; 1, dislike very much). The samples could be retested as often as desired. All evaluations were conducted in individual booths under white illumination and at room temperature.

2.5. Statistical analysis

A multi-factorial design was used to statistically analyse results. Factors considered were storage period, storage atmosphere, shelf life period, and replication. All data were tested by analysis of variance (GLM-ANOVA procedure) with the SAS program package (SAS Institute, Cary, NC, USA, 1988). Means were separated by the LSD test at $P \leq 0.05$. For multivariate analysis, samples were characterized by their average measurement (instrumental analyses) or by their average score among all judges (sensory analyses). Before calculating the average score for all judges, scores non exhibiting a normal distribution were eliminated. A Principal Component Analysis (PCA) was performed, involving 14 samples (2 at harvest and 12 after storage) and 17 variables (11 aroma volatile compounds, 5 standard quality parameters and consumer acceptability), using full cross-validation as a validation method. Partial least-square regression (PLSR) was used to quantify the correlation between instrumental parameters and consumer acceptability. Variables and samples analysed were labelled as specified in Table 1. Unscrambler vers. 6.11a software was used (CAMO ASA, 1997) to develop these models. Aroma compounds and standard quality parameters were used as X variables and correlated to acceptability as the Y variable by PLS1 regression. As a pre-treatment, data were centred and weighted by the inverse of the standard deviation of each variable in order to avoid dependence on measured units (Martens and Naes, 1989).

***Table 1*. Variable and sample codes used for PCA and PLS analyses.**

Variable	Code	Sample	Code
Ethyl butanoate	eb	Harvest	HARV
Ethyl hexanoate	eh	1 day at 20 ºC	SL1
Butyl acetate	ba	7 days at 20 ºC	SL7
Butyl propanoate	bp	14 weeks of storage	S14
Hexyl acetate	ha	25 weeks of storage	S25
Hexyl propanoate	hp	21 kPa O_2 + 0.03 kPa CO_2	AIR
Hexyl hexanoate	hh	2 kPa O_2 + 2 kPa CO_2	LO
Ethyl 2-methylbutanoate	e2mb	1 kPa O_2 + 1 kPa CO_2	ULO
Butyl 2-methylbutanoate	b2mb		
2-methylbutyl acetate	2ma		
Hexyl 2-methylbutanoate	h2mb		
Flesh firmness	Firmness		
Titratable acidity	TA		
Soluble solid content	SSC		
Hue angle (shaded side)	SS		
Hue angle (exposed side)	ES		
Consumer acceptance	Acceptability		

3. Results and discussion

3.1. Volatile emission at harvest

Thirty volatile compounds were detected at harvest, namely 21 esters (eight acetates, four propanoates, six butanoates and three hexanoates), seven alcohols, one terpenoid and one aldehyde (Table 2).

Esters represented more than 98% of total volatile compounds detected. The main compound emitted during ripening at 20 ºC (17% and 25% after one and seven days, respectively) was hexyl acetate, which also predominates in 'Golden Delicious' apples (López et al., 1998b). The next most important esters in quantitative terms were hexyl hexanoate, hexyl 2-methylbutanoate, hexyl butanoate, 2-methylbutyl acetate, butyl acetate and hexyl propanoate. Together, these seven compounds contributed 81.5% and 83% of total volatile compounds after one and seven days at 20 ºC, respectively. Ester compounds were hence largely predominant in the aroma profile of 'Pink Lady®' apples, and conferred a characteristic "apple" odour due to the presence of hexyl hexanoate, hexyl butanoate, butyl acetate and hexyl propanoate. There was also a touch

of banana odour owing to 2-methylbutyl acetate, and a fruity flavour associated to hexyl acetate and hexyl 2-methylbutanoate. In the present study, we also determined the log odour units of volatiles detectable in 'Pink Lady'®apples (Table 2). Ethyl butanoate, ethyl 2-methylbutanoate, 2-methylbutyl acetate, hexyl acetate, hexyl propanoate and hexyl 2-methylbutanoate were found to have log odour units >0 and were therefore likely to contribute to the flavour of 'Pink Lady®'apples.

***Table 2*. Volatile compounds emitted (µg/kg), odour threshold (OTH), log_{10} odour units[a] (in brackets) and odour description for 'Pink Lady®' apples at harvest.**

Compounds	Kovats Index	OTH[b] (µg/kg)	Amount (µg/kg) 1 day at 20°C	Amount (µg/kg) 7 days at 20°C	Odour descriptor[b]
Methyl acetate LSD= 5.4	834	8300(h)	2.6 B(-3.5)	13.5 A(-2.8)	
Ethyl acetate LSD= 3.5	898	13500(c)	2.8 A(-3.6)	4.8 A(-3.4)	Ethereal-fruity(i)
Tert-butyl propanoate LSD= 1.9	964	19(h)	0.9 A(-1.3)	2.7 A((-0.8)	
Propyl acetate LSD= 6.9	984	2000(c)	1.5 B(-3.1)	10.2 A(-2.3)	Pear-raspberry (o)
2-methylpropyl acetate LSD= 2.7	1020	65(b)	3.0 A(-1.3)	4.3 A(-1.1)	Fruity (o)
1-propanol LSD= 1.2	1036	9000(a)	> 0.5	2.8 (-3.5)	Sweet (i)
Ethyl butanoate LSD= 3.7	1043	1(d)	1.6 A(0.2)	4.2 A(0.6)	Fruity (k), apple-like(n)
Ethyl 2-methylbutanoate LSD= 4.0	1059	0.006(b)	2.8 A(2.7)	5.1 A(2.9)	Ripe apple (a)
Butyl acetate LSD= 57.3	1082	66(c)	21.2 B(-0.5)	121.0 A(0.3)	Red apple aroma (j)
2-methyl-1-propanol LSD= 1.6	1091	250(f)	0.9 A(-2.4)	1.6 A(-2.2)	Chemical (p)
Hexanal LSD= 0.1	1101	10.5(d)	not detected	> 0.5	Green (j),(k),(n)
2-methybutyl acetate LSD= 51.8	1131	11(b)	50.3 B(0.7)	111.6 A(1.0)	Banana (i)
1-butanol LSD= 5.3	1144	500(a)	1.4 B(-2.5)	7.8 A(-1.8)	Sweet aroma(k), (o)
Butyl propanoate LSD= 12.5	1148	25(a)	9.4 B(-0.4)	26.0 A(0.02)	Faintly sweet odour (o)
4-methyl-2-pentanol LSD= 0.25	1163		> 0.5	0.5	
Pentyl acetate LSD= 7.6	1183	43(c)	3.3 B(-1.1)	18.7 A(-0.4)	Apple, fruity (k)
2-methylbutyl propanoate LSD= 5.9	1199	19(h)	6.8 A(-0.4)	11.5 A(-0.2)	
2-methyl-1-butanol LSD= 2.5	1210	250(d)	2.0 A(-2.1)	3.8 A(-1.8)	Highly diluted-pleasant (i)
D-limonene LSD= 0.2	1219	34(d)	> 0.5	> 0.5	Citrus-like (m)
Butyl butanoate LSD= 7.5	1228	100(e)	6.1 B(-1.2)	22.6 A(-0.6)	Rotten apple (l)
Butyl 2-methylbutanoate LSD= 28.2	1240	17(e)	9.8 B(-0.2)	62.5 A(0.6)	Fruity, apple (l)
Ethyl hexanoate	1243	1(b)	1.7 (0.2)	> 0.5	Fruity (m)
1-pentanol	1253	4000(f)	> 0.5	> 0.5	
Hexyl acetate LSD= 104.3	1283	2(f)	68.8 B(0.3)	392.3 A(2.3)	Fruity (i)
Hexyl propanoate LSD= 22.6	1349	8(g)	26.0 B(0.5)	87.4 A(1.0)	Apple (l)
1-hexanol LSD= 2.9	1358	500(f)	1.7 B(-2.5)	5.2 A(-2.0)	Grassy (i)
Butyl hexanoate LSD= 7.2	1423	700(e)	16.0 B(-1.6)	56.1 A(-1.1)	Green apple (l)
Hexyl butanoate LSD= 20.8	1426	250(b)	35.9 B(-0.8)	127.2 A(-0.3)	Apple (l)
Hexyl 2-methylbutanoate LSD= 85.7	1436	6(e)	59.7 B(1.0)	273.4 A(1.6)	Fresh-green fruity(i)
Hexyl hexanoate LSD= 30.7	1621		70.0 B	170.2 A	Apple (l)
Total ester compounds			400.2	1525,8	
Total alcohol compounds			6.5	21.7	
Total volatile compounds			407.3	1547.5	

[a] Log_{10} of odour unit value = log_{10} [amount / OTH].

[b] Odour threshold and odour descriptor reported by: (a): Flath et al., 1967, (b): Takeoka et al., 1992, (c): Takeoka et al., 1996, (d): Rychlik et al., 1998, (e): Takeoka et al., 1990, (f): Buttery R.G., 1993, (g): Van Gemert and Nettenbreijer, 1977, (h): Schnabel et al., 1988; (i): Dimick and Hoskin, 1982. (j): Young et al., 1996. (k): Rizzolo et al. 1989. (l): Plotto (1998). (m): Buettner and Schieberle, 2001. (n): Wang et al. 2005. (o): Burdock, 2002. (p): Rizzolo et al., 1997.

Means within the same row followed by the same capital letters are not significantly different at $p \leq 0.05$ (LSD test).

After 7 days of ripening at 20 ºC, there was an increase in total emission of ester compounds (Table 2). Similar trends were observed for ethylene production as ripening progressed (0.43 µl/kg.h and 95.22 µl/kg.h, after 1 and 7 days at 20 ºC, respectively). These results suggest that ester production in 'Pink Lady®' apples is an ethylene-dependent process, in accordance with observations for other varieties (Defilippi et al., 2005). Production of most butyl esters (butyl acetate, 2-methylbutyl acetate, butyl propanoate, butyl butanoate, butyl 2-methylbutanoate and butyl hexanoate), hexyl esters (hexyl acetate, hexyl propanoate, hexyl butanoate, hexyl 2-methylbutanoate and hexyl hexanoate), ethyl acetate, propyl acetate and pentyl acetate was significantly higher for longer shelf life periods. This increase in the emission of hexyl and butyl esters as well as of propyl acetate was facilitated by the availability of the necessary alcohol precursors, as the productions of 1-hexanol, 1-butanol and 1-propanol paralleled those of the corresponding esters (Table 2), in agreement with previous reports for 'Gala' (Fellman et al., 2000), 'Greensleeves' (Defilippi et al., 2005) and 'Fuji' (Lara et al., 2006) apples.

3.2. Influence of different storage periods and atmospheres on emission of aroma volatile compounds

A total of 31 volatile compounds were detected after cold storage, including 23 esters (eight acetates, five propanoates, six butanoates and four hexanoates), six alcohols, one terpenoid and one aldehyde (Tables 3 and 4). All factors considered in this work (storage conditions and shelf life periods) influenced the emissions of these compounds.

An increase in the number of straight-chain esters detectable was observed after one day at 20 ºC, regardless of storage period, in comparison with fruit at harvest (Tables 2 and 3). Increases in the emission of methyl acetate, butyl acetate, butyl butanoate, pentyl acetate, hexyl acetate and hexyl butanoate were also found for fruit kept under LO conditions.

***Table 3*. Straight-chain ester emitted and hexanal (μg/kg) and log_{10} odour units (in brackets) by 'Pink Lady®' apples after cold storage.**

Compounds	Days at 20 °C	Storage (weeks)	AIR ($21kPaO_2+0.03\ kPaCO_2$)	LO ($2kPaO_2+2kPaCO_2$)	ULO ($1kPaO_2+1kPaCO_2$)
methyl acetate	1	14	5.4 aA	4.7 aA	2.7 aB
LSD= 3.3	1	25	2.6 bA	3.7 abA	6.9 aA
OTH = 8300 μg/kg	7	14	7.8aA	8.1aA	7.7aA
	7	25	2.3aB	2.9aB	3.7aB
ethyl acetate	1	14	4.4 aA	2.2 bA	3.5 abA
LSD= 1.3	1	25	3.8 aA	1.7 bA	1.7 bB
OTH = 13500 μg/kg	7	14	4.0aA	3.1abA	1.9bA
	7	25	2.2aB	1.0abB	0.7aA
ethyl butanoate	1	14	5.1 aA (0.7)	1.1 bA (0.04)	2.1 bA (0.3)
LSD= 1.5	1	25	4.0 aA (0.6)	1.6 bA (0.2)	0.6 bA (-0.2)
OTH = 1 μg/kg	7	14	7.4 aA (0.8)	1.6 bA (0.2)	0.6 bA (-0.2)
	7	25	4.6 aB (0.7)	0.7 bA (-0.1)	> 0.5
ethyl hexanoate	1	14	2.5 aB (0.4)	2.8 aB (0.4)	1.7 aB (0.2)
LSD= 5.4	1	25	38.4 aA (1.6)	27.6 bA (1.4)	22.3 bA (1.3)
OTH = 1 μg/kg	7	14	1.5 aB (0.2)	1.1 aB (0.04)	1.1 aB (0.04)
	7	25	33.6 aA (1.5)	33.9 aA (1.5)	30.6 aA (1.5)
propyl acetate	1	14	3.7 aA	0.9 bA	1.9 bA
LSD= 1.3	1	25	2.4 aA	1.0 bA	1.1 abA
OTH = 2000 μg/kg	7	14	6.5 aA	2.5 bA	0.9 cA
	7	25	2.9 aB	0.8 bB	0.5 bA
butyl acetate	1	14	219.1 aA (0.5)	52.9 bA (-0.1)	47.8 bA (-0.1)
LSD= 34.1	1	25	121.5 aB (0.3)	32.9 bA (-0.3)	8.8 bB (-0.9)
OTH = 66 μg/kg	7	14	142.7aA (0.3)	40.4 bA (-0.2)	15.9 bA (-0.6)
	7	25	54.5 aB (-0.1)	10.4 bA (-0.8)	1.7 bA (-1.6)
butyl propanoate	1	14	25.6 aA (0.01)	3.0 bA (-0.9)	2.5 bA (-0.1)
LSD= 6.5	1	25	12.4 aB (-0.3)	2.3 bA (-0.1)	0.5 bA (-1.7)
OTH = 25 μg/kg	7	14	33.8 aA (0.1)	7.7 bA (-0.5)	2.3 bA (-1.0)
	7	25	11.3 aB (-0.3)	1.7 bA (-1.2)	0.5 bA (-1.0)
butyl butanoate	1	14	67.3 aA	12.7 bA	7.3 bA
LSD= 12.1	1	25	42.8 aB	10.0 bA	2.7 bA
OTH = 100 μg/kg	7	14	48.7 aA	14.0 bA	4.5 bA
	7	25	20.8 aB	4.0 bA	2.4 bA
butyl hexanoate	1	14	87.8 aA	14.4 bA	17.0 bA
LSD= 19.5	1	25	45.5 aB	16.0 bA	8.7 bA
OTH = 700 μg/kg	7	14	79.4 aA	37.5 bA	16.2 cA
	7	25	27.4 aB	14.3 aB	10.8 aA
pentyl acetate	1	14	17.4 aA	6.9 bA	7.1 bA
LSD= 2.6	1	25	9.7 aB	4.7 bA	2.2 bB
OTH = 43 μg/kg	7	14	16.3 aA	9.8 bA	4.9 cA
	7	25	6.7 aB	2.8 bB	1.2 bB
hexyl acetate	1	14	457.6 aA (2.3)	288.9 bA (2.1)	322.3 bA (2.2)
LSD= 76.7	1	25	278.1 aB (2.1)	255.3 aA (2.1)	113.4 bB (1.7)
OTH = 2 μg/kg	7	14	363.8 aA (2.2)	208.3 bA (2.0)	113.8 cA (1.7)
	7	25	138.4 aB (1.8)	67.3 abB (1.5)	32.6 bB (1.2)
hexyl propanoate	1	14	52.0 aA (0.8)	15.0 bA (0.3)	22.6 bA (0.4)
LSD= 16.0	1	25	37.2 aA (0.7)	22.9 abA (0.4)	12.0 b A (0.2)
OTH = 8 μg/kg	7	14	83.8 aA (1.0)	43.3 bA (0.7)	19.4 cA (0.4)
	7	25	35.6 aB (0.6)	22.3 abB (0.4)	8.7 bA (0.04)
hexyl butanoate	1	14	211.3 aA	57.2 bA	64.1 bA
LSD= 43.5	1	25	130.8 aB	62.0 bA	28.0 bA
OTH = 43.5 μg/kg	7	14	213.0 aA	109.8 bA	59.3 cA
	7	25	77.7 aB	37.2 abB	27.0 bA

Table 3 (*Continued*)

Compounds	Days at 20 ºC	Storage (weeks)	AIR ($21kPaO_2+0.03\ kPaCO_2$)	LO ($2kPaO_2+2kPaCO_2$)	ULO ($1kPaO_2+1kPaCO_2$)
hexyl hexanoate	1	14	209.4 aA	44.9 bA	60.2 bA
LSD= 51.6	1	25	108.8 aB	48.7 bA	312 bA
OTH not determined	7	14	205.6 aA	138.8 bA	103.5 bA
	7	25	66.5 aB	53.0 aB	44.9 aB
hexanal	1	14	> 0.5	0.5 aB	> 0.5
LSD= 0.8	1	25	2.4 aA	1.5 bA	1.8 abA
OTH = 10.5 µg/kg	7	14	0.6 aA	> 0.5	> 0.5
	7	25	0.8 aA	1.2 aA	1.3 aA

Means within the same storage atmosphere and day at 20 ºC followed by the same capital letters and means within the same storage period and day at 20 ºC followed by the same small letters are not significantly different at $p \leq 0.05$ (LSD test). Log_{10} of odour unit value = log_{10} [amount / OTH]; OTH: Odour threshold reported by literature (Table 1).

Most straight-chain esters (93%) were especially prevalent after 14 weeks of storage in AIR atmosphere (Table 3). Tressl et al., (1970) explained similar results as the consequence of CA-induced changes in the biosynthetic pathways of these compounds. Straight-chain organic acid precursors are produced either by β-oxidation of fatty acids and/or through the lipoxygenase pathway, both of which require O_2 and therefore are presumably slowed down during LO and ULO storage.

Extending storage to 25 weeks led to an increase in the emission of ethyl hexanoate regardless of storage atmosphere (Table 3). This increase probably induced changes in the sensory quality of fruit, as this compound was deemed likely to contribute to 'Pink Lady®' apple flavour on the basis of its showing positive values for log odour units.

Longer storage period also led to the highest emissions of ethyl butanoate, ethyl hexanoate, propyl acetate, butyl acetate, butyl propanoate and hexyl hexanoate in AIR-stored fruit, while no differences were observed for hexyl acetate in comparison with LO-stored apples (Table 3), which was the main in quantitative terms volatile compound present both at harvest and after cold storage.

In contrast to observations for fruit at harvest, ripening at 20 ºC after cold storage did not result in an increase in the emission of all straight-chain ester. For instance, ethyl butanoate, propyl acetate, butyl propanoate and hexyl propanoate increased in AIR-stored fruit after 7 days at 20 ºC, whereas emission of hexyl acetate was highest one day after removal from storage, regardless of conditions (Table 3).

***Table 4*. Branched-chain ester emission, alcohol and D-limonene (μg/kg) and log_{10} odour units (in brackets) by 'Pink Lady®' apples after cold storage.**

Compounds	Days at 20 ºC	Storage (weeks)	AIR (21kPaO_2+0.03 kPaCO_2)	LO (2kPaO_2+2kPaCO_2)	ULO (1kPaO_2+1kPaCO_2)
ethyl 2-methylbutanoate	1	14	2.5 aA (2.6)	1.6 aA (2.4)	3.6 aA (2.8)
LSD= 2.4	1	25	1.2 aA (2.3)	0.5 aA (1.9)	> 0.5
OTH = 0.006 μg/kg	7	14	3.6aA (2.8)	2.5aA (2.6)	2.5aA (2.6)
	7	25	1.6aB (2.4)	0.7aA (2.1)	> 0.5
2-methylpropyl acetate	1	14	13.2 aA	3.2 bA	5.3 bA
LSD= 2.6	1	25	7.3 aB	2.6 bA	2.4 bB
OTH = 65 μg/kg	7	14	5.7 aA	5.3 aA	2.9 bA
	7	25	2.1 aB	1.7 aB	0.8 aA
2-methylpropyl hexanoate	1	14	1.5 aB	not detected	> 0.5
LSD= 1.1	1	25	4.4 aA	3.4 aA	3.6 aA
OTH not determined	7	14	1.3 aB	1.1 aB	0.7 aB
	7	25	6.0 aA	6.9 aA	6.1 aA
butyl 2-methylbutanoate	1	14	22.0 aA (0.1)	8.9 bA (-0.3)	8.0 bA (-0.1)
LSD= 7.8	1	25	16.4 aA (-0.01)	7.4 bA (-0.3)	4.6 bA (-0.5)
OTH = 9.8 μg/kg	7	14	39.3 aA (0.4)	18.5 bA (0.03)	6.5 cA (-0.4)
	7	25	15.1 aB (-0.05)	8.9 abB (-0.3)	6.0 bA (-0.4)
2-methylbutyl acetate	1	14	145.9 aA (1.1)	57.7 cA (0.7)	92.3 bA (0.9)
LSD= 26.4	1	25	66.0 aB (0.8)	38.2 bA (0.5)	37.9 bB (0.5)
OTH = 11 μg/kg	7	14	93.1 aA (0.9)	100.8 aA (1.0)	62.6 bA (0.7)
	7	25	33.5 aB (0.5)	30.8 aB (0.4)	14.7 aB (0.1)
tert-butyl propanoate	1	14	2.6 aA	4.5 aA	4,3 aA
LSD= 2.6	1	25	not detected	0.9 abB	3,3 aA
OTH = 19 μg/kg	7	14	3.9aA	4.0aA	5.9aA
	7	25	1.0aB	0.8aB	1.7aB
2-methylbutyl propanoate	1	14	10.2 aA	2.1 bB	2.5 bB
LSD= 3.5	1	25	9.3 aA	7.0 aA	7.6 aA
OTH = 19 μg/kg	7	14	9.6 aA	10.8 aA	5.9 bA
	7	25	6.3 aA	7.2 aB	4.8 aA
hexyl 2-methylbutanoate	1	14	104.3 aA (1.2)	48.8 bA (0.9)	65.1 bA (1.0)
LSD= 42,5	1	25	51.7 aB (0.9)	46.5 aA (0.9)	24.7 aA (0.6)
OTH = 6 μg/kg	7	14	180.8 aA (1.5)	139.7 aA (1.4)	85.0 bA (1.1)
	7	25	53.3 aB (0.9)	46.0 aB (0.9)	28.5 aB (0.7)
2-methylbutyl 2-methylpropanoate	1	14	0.7 aB	0.6 aA	> 0.5
LSD= 0.7	1	25	1.5 aA	0.6 bA	0.8 abA
OTH not determined	7	14	0.8 aA	0.8 aA	0.5 aA
	7	25	1.0 aA	1.1 aA	1.0 aA
D-limonene	1	14	0.7 aA	> 0.5	> 0.5
LSD= 0.3	1	25	> 0.5	not detected	not detected
OTH = 34 μg/kg	7	14	> 0.5	> 0.5	> 0.5
	7	25	> 0.5	> 0.5	> 0.5
1-propanol	1	14	1.0 aA	1.2 aB	> 0.5
LSD= 1.2	1	25	> 0.5	2.9 aA	not detected
OTH = 9000 μg/kg	7	14	2.7aA	1.7abA	0.7bA
	7	25	2.8aA	0.9bA	not detected
1-butanol	1	14	9.1 aA	3.7 bA	2.8 bA
LSD= 3.3	1	25	11.4 aA	4.9 bA	2.2 bA
OTH = 500μg/kg	7	14	15.1 aA	5.7 bA	2.1 cA
	7	25	17.3 aA	3.0 bA	1.8 bA
1-hexanol	1	14	8.5 a B	6.2 abB	5.5 bA
LSD= 2.5	1	25	14.4 aA	10.0 bA	5.8 cA
OTH = 500 μg/kg	7	14	13.9 aB	8.7 bA	4.7 cA
	7	25	16.5 aA	10.1 bA	7.2 cA

***Table 4* (Continued)**

Compounds	Days at 20 ºC	Storage (weeks)	AIR ($21kPaO_2+0.03\ kPaCO_2$)	LO ($2kPaO_2+2kPaCO_2$)	ULO ($1kPaO_2+1kPaCO_2$)
2-methyl-1-propanol	1	14	3.6 aA	1.0 bA	1.2 bA
LSD= 1,0	1	25	2.9 aA	0.9 bA	0.9 bA
OTH = 250 µg/kg	7	14	3.0aA	3.3aA	1.5bA
	7	25	2.4aA	1.2bB	0.9cA
2-methyl-1-butanol	1	14	4.3 aA	3.2 aA	3.6 aA
LSD= 2.2	1	25	4.1 aA	3.6 aA	2.7 aA
OTH = 250 µg/kg	7	14	6.2 bA	11.0 aA	6.2 bA
	7	25	5.1 aA	4.8 aB	5.1 aA
4-methyl-2-pentanol	1	14	0.5 aB	0.4 aB	> 0.5
LSD= 0.4	1	25	1.9 aA	1.3 bA	1.2 bA
OTH not determined	7	14	> 0.5	0.5 aB	> 0.5
	7	25	0.9aA	1.3 aA	1.1 aA

Means within the same storage atmosphere and day at 20 ºC followed by the same capital letters and means within the same storage period and day at 20 ºC followed by the same small letters are not significantly different at $p \leq 0.05$ (LSD test). Log_{10} of odour unit value = log_{10} [amount / OTH]; OTH: Odour threshold reported by literature (Table 1).

Hexanal was first detected 7 days after harvest, its production being higher after extended (25 weeks) cold storage. As regards the terpenoid D-limonene, it was detectable only in trace amounts, both at harvest and after cold-storage (Tables 2 and 3).

Two branched-chain esters not found at harvest (2-methylpropyl hexanoate and 2-methylbutyl 2-methylpropanoate) were detected for the first time one day after removal from cold storage during 14 weeks (Tables 2 and 4). In general, extending storage from 14 to 25 weeks reduced emissions of branched-chain esters, with the exception of 2-methylpropyl hexanoate and 2-methylbutyl 2-methylpropanoate (Table 4).

The highest production of ethyl 2-methylbutanoate was found for AIR- and ULO-stored fruit after 14 weeks of storage regardless of shelf life period. LO storage conditions had significantly favourable effects on the emission of *tert*-butyl propanoate (after one day at 20 ºC) as well as of 2-methylbutyl propanoate and hexyl 2-methylbutanoate (after 7 days at 20 ºC), in agreement with previous results showing that production of branched acetate esters was not suppressed by low O_2 (Fellman et al., 2000; Echeverría et al., 2004a).

Alcohol emission was higher in AIR- than in CA-stored fruit (Table 4), whereas the effect of storage period was far more variable. The influence of shelf life period was

also variable: for example, whereas a longer shelf life period (7 days) resulted in an increase in 1-propanol, 1-butanol and 1-hexanol after AIR storage, the same effect was not observable for branched-chain alcohols.

3.3. Influence of different storage periods and atmospheres on standard quality parameters

Fruit firmness and background colour at harvest (Table 5) were indicative of an appropriate stage of maturity for long-term cold storage, according to CTIFL recommendations (Mathieu et al., 1998). In addition, fruit also had low starch indices (5.80, on the 1-10 Eurofru-scale) and ethylene production (0.43 µl/kg· h).

Table 5. **Standard quality parameters of 'Pink Lady®' apples at harvest[a] and after storage in different atmospheres plus 1 and 7 days at 20 ºC.**

Standard quality parameters	At harvest[a]	Days at 20 ºC	Storage (weeks)	AIR ($21kPaO_2+0.03\ kPaCO_2$)	LO ($2kPaO_2+2kPaCO_2$)	ULO ($1kPaO_2+1kPaCO_2$)
Flesh firmness (N)	91.7	1	14	83.8bAB	90.8aA	96.2aA
			25	88.4aA	88.2aA	93.4aA
		7	14	80.0bBC	88.4aA	85.7abB
			25	74.8bC	75.0bB	87.7aAB
Titratable acidity (g malic acid/l)	6.5	1	14	5.1abAB	4.9bB	5.5aA
			25	5.3aA	4.1bC	5.3aAB
		7	14	4.7bB	5.4aA	5.1abAB
			25	3.6cC	4.3bC	5.0aB
Soluble solid content (%)	14.0	1	14	14.6bB	14.9aB	15.2aAB
			25	15.2aA	15.4aA	15.5aA
		7	14	15.0bAB	15.6aA	14.9bB
			25	14.1cC	14.9bB	15.4aA
Hue angle (º) (shaded side)	95.7	1	14	92.1aA	96.2aA	96.0aA
			25	88.0bA	97.6aA	96.4aA
		7	14	90.1bA	96.2aA	86.4bB
			25	90.1aA	94.5aA	93.2aA
Hue angle (º) (exposed side)	32.3	1	14	40.0aA	40.1aA	34.4bA
			25	25.9bC	31.0aB	26.2bB
		7	14	33.8aB	37.6aA	34.8aA
			25	33.0aB	29.5aB	29.2aB

[a] Means within the same day at 20 ºC followed by the same small letters are not significantly different at $p<0.05$ (LSD test). Means within the same storage period and the same day at 20 ºC, followed by the same small letters are not significantly different at $p\leq0.05$ (LSD test). Means within the same day at 20 ºC and the same atmosphere, followed by the same capital letters are not significantly different at $p\leq0.05$ (LSD test).

For short-term (14 weeks) storage, ULO-stored apples retained higher firmness, TA, SSC and pink surface colour values than those stored in AIR one day after removal from storage. LO-stored fruit also displayed significantly higher firmness and SSC than fruit stored in AIR. When shelf life period was extended to 7 days, LO-stored fruit showed the best preservation of standard quality, consistent with high values for flesh firmness, TA, SSC and with greener colour on their shaded sides in comparison with AIR-stored fruit.

After a longer (25 weeks) storage period, AIR- and ULO-stored fruit showed the highest degrees of pink surface colouring and TA values after 1 day at 20 ºC. CA-stored fruit retained a greener background colour than those stored in AIR. No storage atmosphere-related differences were found in surface or background colour after 7 days at 20 ºC, whereas ULO-stored fruit had the highest values for firmness, TA and SSC.

It should be noted, however, that the lowest levels of flesh firmness, found for AIR-stored apples (74.80 N), were still very satisfactory, which is indicative of the characteristically good firmness retention potential of this apple cultivar, even after long storage under regular air. In contrast, TA was badly preserved (3.6 g/l) after 25 weeks of storage in AIR, which probably would result in low consumer acceptance. Therefore, storage under ULO would appear as necessary in order to maintain satisfactory fruit quality, in accordance with Drake et al., (2002) who reported that ULO atmosphere led to better preservation of the quality of 'Pink Lady®' apples during extended storage.

3.4. Relationship between consumer acceptability and standard quality parameters and aroma volatile compounds

Results obtained from sensory analyses indicate that, one week after removal from long-term (25 weeks) storage, CA-stored fruit scored higher for acceptability than AIR-stored samples (Table 6). In order to find out the instrumental quality parameter(s)

mainly influencing acceptability, PCA and PLSR models were developed, for which 11 volatile compounds, 5 standard quality parameters as well as consumer acceptability were selected (Table 1). The volatile compounds included in these models (ethyl butanoate, ethyl hexanoate, hexyl acetate, hexyl propanoate, ethyl 2-methylbutanoate, 2-methylbutyl acetate and hexyl 2-methylbutanoate) were chosen on the basis of their having log odour unit >0 after cold storage and thus being likely to contribute to the overall flavour of 'Pink Lady®' apples. Butyl acetate, butyl propanoate and 2-methylbutanoate were also selected as they had log odour unit >0 for AIR-stored fruit, and so was hexyl hexanoate on account of its quantitative importance in the volatile fraction (Tables 3 and 4). All eleven chosen compounds, in addition to the five standard quality parameters (SSC, TA, firmness, and hue on both the exposed and shaded sides) and consumer acceptability were used fruit characterisation both at harvest and after storage.

***Table 6*. Mean sensory scores for 'Pink Lady®' apples stored in different atmospheres for 14, and 25 weeks plus 1 and 7 days at 20 ºC[a].**

	Storage (weeks)	Days at 20 ºC	AIR ($21kPaO_2$+$0.03\ kPaCO_2$)	LO ($2kPaO_2$+$2kPaCO_2$)	ULO ($1kPaO_2$+$1kPaCO_2$)
SCORES	14	1	7.1 aAB	6.6 bB	6.7 abA
		7	7.2 aA	7.1 aA	7.0 aA
LSD= 0.4	25	1	6.7 aBC	6.9 aAB	7.1 aA
		7	6.6 bC	7.3 aA	7.0 aA

[a] Means within the same storage period and days at 20ºC followed by the same small letters are not significantly different at $p \leq 0.05$ (LSD's test). Means within the same storage atmosphere and shelf-life period followed by the same capital letters are not significantly different at $P \leq 0.05$ (LSD's test).

A full-data PCA model was developed to provide a global overview of the different samples and variables. Principal components 1 (PC1) and 2 (PC2) accounted respectively for 45% and 15% of total variance. The biplot of PC1 vs. PC2 for this PCA model (Figure 1) shows four well-differentiated groups: group 'A', including non-stored fruit after 7 days at 20 ºC (HARVSL7) as well as fruit stored in AIR for 14 weeks; group 'B', comprising non-stored fruit after 1 day at 20 ºC (HARVSL1) together with fruit stored under CA for 14 weeks; group 'C', consisting of samples

stored in AIR for 25 weeks; and finally group 'D', containing fruit stored under CA for 25 weeks. No significant differentiation was found between stored and non-stored samples grouped together in groups 'A' or 'B', in contrast with previous results obtained for other apple cultivars such as 'Fuji', where clear differences were observed between fruit at harvest and after cold storage (Echeverría et al., 2004b).

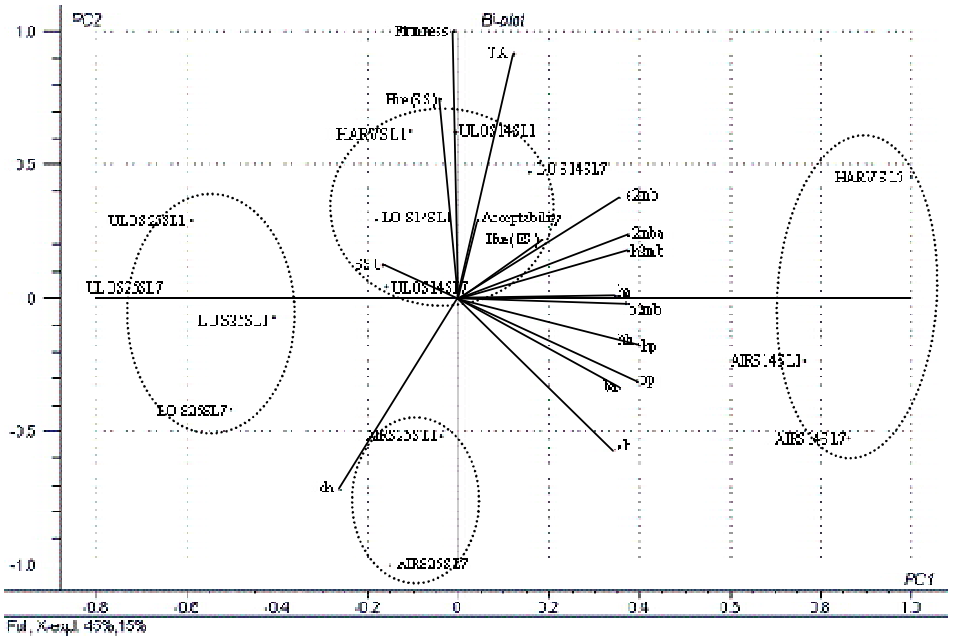

***Figure 1*. Biplot (scores and loadings) of PC1 vs. PC2 corresponding to a full-data PCA model for 'Pink Lady®' apples at harvest and after cold storage. Samples and variables are coded as indicated in Table 1.**

With the exception of ethyl hexanoate, the highest concentrations of the aroma volatile compounds included in the model were found for short-term AIR-stored fruit and for non-stored fruit 7 days after harvest (group 'A'). Conversely, samples contained in group 'D' showed the lowest emissions of these same compounds, regardless of shelf life period applied.

We were interested in determining the instrumental parameters having the greatest influence on consumer acceptability. A PLS1 regression model was therefore built up in an attempt to correlate acceptability to the standard quality parameters and the chosen aroma volatile compounds (Figure 2). The validation step indicated that two PLS factors were relevant in the model. The percentage of explained variance was 59 %. This value was more than twice that of variance in acceptability explained by SSC, which was the instrumental measurement that correlated best to acceptability (r^2 = 0.26). Although some instrumental variables, such as hexyl 2-methylbutanoate, explained around 18% of the Y-variance, all variables taken together explained 59% of total variance, indicating the existence of a non-negligible correlation amongst them; stated in other words, the instrumental variables contained repeated information. Biological variability associated to fruit is also a factor affecting the identification of statistical significant differences (Harker et al., 2005).

The PLS model developed allowed the identification of those variables mainly affecting consumer acceptance. The parameters having most influence on acceptability were soluble solid content (SSC), hexyl 2-methylbutanoate (h2mb), hexyl hexanoate (hh), hexyl propanoate (hp), butyl 2-methylbutanoate (b2mb) and titratable acidity (TA) (Figure 2A). These results were in agreement with previous reports (Alavoine et al., 1990) suggesting that sugar content may be the best determinant of consumer acceptance. The importance of some aroma volatile compounds for consumer acceptability has also been reported in earlier works on 'Fuji' apples (Echeverría et al., 2004b). Firmness correlated negatively to acceptability; this observation might have been due to the apparently small effect of storage conditions considered in this work on firmness of 'Pink Lady®' apples (Table 5).

The plot of predicted versus measured acceptability (Figure 2B) revealed two groups of samples associated with higher (labelled '**a**' in the Figure) and lower (labelled '**b**') levels of acceptability. The correlation coefficient between measured and predicted acceptability was 0.86 and the RMSECV was 0.21 measuring units. Scores for all

treatments considered in this study were higher than 6.5 in the hedonic test, indicating good acceptability levels for all of them.

However, the plot shows good separation between samples scoring above and below 7 points in the hedonic test, threshold value chosen as indicative of differences between well-accepted fruit and fruit only marginally acceptable. The group of best valued samples (**a**) included fruit stored for 14 weeks in either AIR (irrespective of shelf life period) or under CA plus 7 days at 20 ºC, as well as apples stored under CA for 25 weeks. The group of less accepted samples (**b**) contained fruit stored in AIR for 25 weeks (regardless of shelf life period) in addition to samples kept under CA during 14 weeks plus 1 day at 20 ºC. Lower acceptability scores for apples in group '**b**' could have arisen from lower firmness values for these fruit, as the difference between both groups was higher than 4.9 N (Table 4), and it has been reported that the human senses can detect differences in texture between two apples when the difference in firmness is equal or higher than this value (Harker et al., 2002).

AIR-stored fruit showed significant firmness and TA losses (Table 5), in accordance with previous reports for 'Fuji' apples (Echeverría et al., 2004b). CA-stored fruit displayed significantly lower emissions of most aroma volatile compounds selected in this work (Tables 3 and 4). In spite of these losses, and according to the present results, CA storage appears as highly advisable in order to get the best consumer acceptance of 'Pink Lady®' apples after long storage periods.

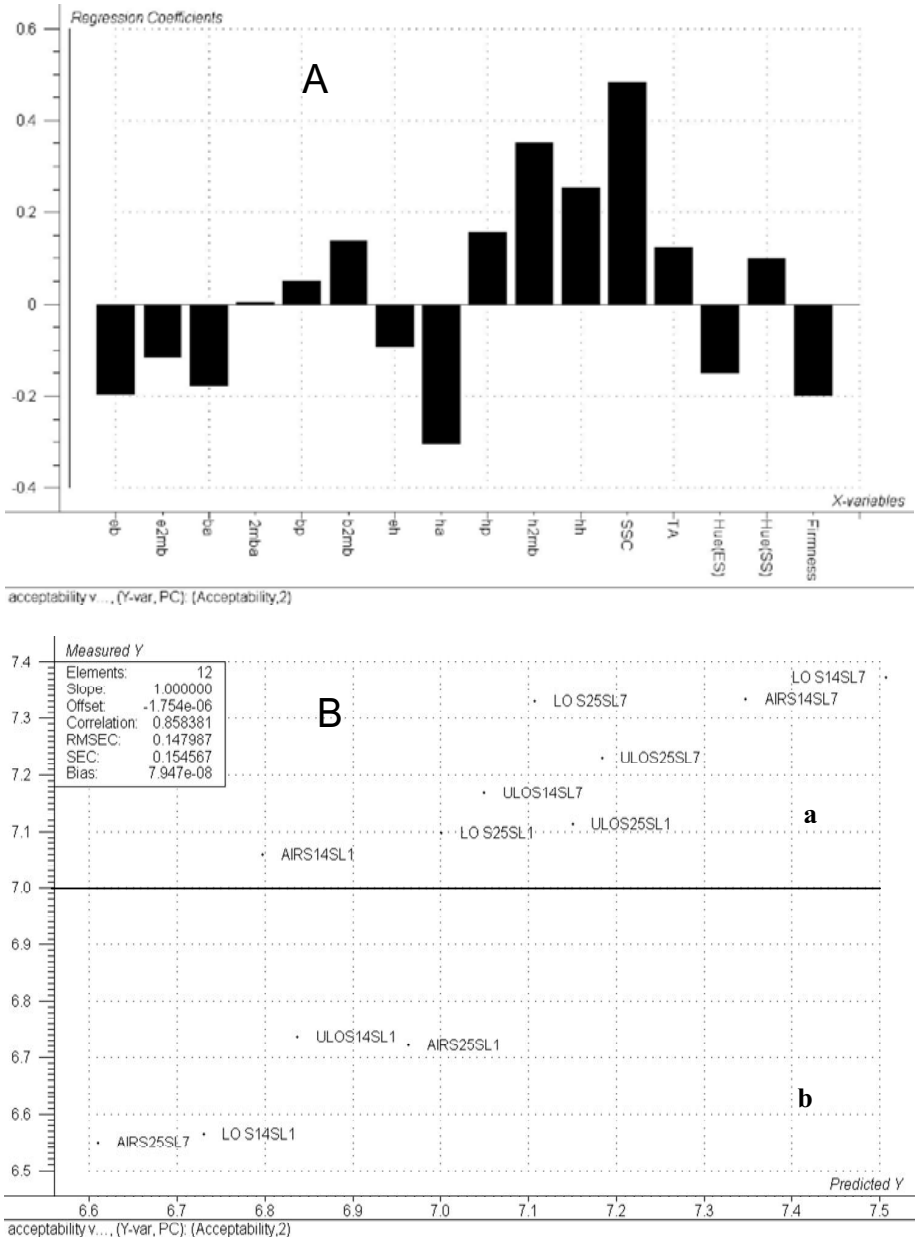

Figure 2. **(A) Regression coefficient plot of PC1 vs. PC2 corresponding to a PLS model for acceptability. Variables and samples are labelled as defined in Table 1. (B) Predicted vs. measured acceptability of 'Pink Lady®' apples. Samples and variables are coded as indicated in Table 1.**

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CAPÍTOL 4

Effect of controlled atmospheres and shelf life period on concentration of volatile substances released by 'Pink Lady®' apples and on the consumer acceptance.

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SUMMARY

'Pink Lady®' (Malus × domestica Borkh.) apples were harvested at commercial maturity and stored at 1 ºC under either air or controlled atmosphere (CA) conditions (2.5 kPa O_2 : 3 kPa CO_2 and 1 kPa O_2 : 2 kPa CO_2) for 15 or 28 weeks. Standard quality parameters, consumer acceptance and volatile compound emission were evaluated after cold storage plus shelf life period at 20 ºC. A period of 17 days of shelf life after long-term storage in controlled atmospheres allowed the characteristic esters associated with this variety to regenerate. Sixty-five % of consumers preferred apples with high emissions of aroma compounds, despite the fact that these apples displayed low standard quality values. These samples correspond to fruit stored in air for 15 weeks, regardless of the number of days at 20 ºC, samples stored in air atmosphere for 28 weeks plus 1 day at 20 ºC, and in controlled atmosphere (2.5 kPa O_2 : 3 kPa CO_2) for 15 weeks plus 7 days at 20 ºC. It is believed that concentrations of certain specific aroma volatile compounds are more important than total aroma emission for determining the general acceptability of 'Pink Lady®' apples.

Keywords Acceptability · Controlled atmosphere · Internal preference mapping · Quality parameters · Shelf life · Volatile compounds

1. Introduction

Nowadays, only a small percentage of apple fruits are marketed fresh, the majority is put into cold storage to keep fruit available to the market for an extended period [Knee, 1993]. Controlled atmosphere (CA) storage is recommended for the commercial storage of apples due to numerous advantages in maintaining firmness, color, acidity and many other qualities attributes as compared to fruit in air atmosphere [Smock, 1979; Kader, 1986; Dixon and Hewett, 2000]. The optimum set point for O_2 (0.7-3 kPa) and CO_2 (0-3kPa) vary with cultivar and CA-effects are largely dependent on the storage and shelf life periods [Plotto et al., 1999; Harb et al., 2000; Aaby et al., 2002; Lo Scalzo et al., 2003; Echeverría et al., 2004a; Mattheis et al., 2005; López et al., 2007].

Fruit aroma is a complex mixture of volatile compounds that contribute to the overall sensory quality of fruit and is especies and cultivar specific [Sanz et al., 1997]. It is generally accepted that only volatile compounds present in concentrations above their odour thresholds tend to contribute to overall apple aroma in different cultivars [Rizzolo et al., 1989; Plotto et al., 2000; Mehinagic et al., 2006]. Changes in the volatile compounds that contribute to fruit aroma during cold storage play an important role in the consumer perception of fruit taste [Mattheis et al., 2005; Harb et al., 2008]. The production of straight-chain esters tends to decrease after long-term CA storage; (Brackmann et al., 1993; Fellman et al., 2003; López et al., 1998; Echeverría et al., 2004b). Young et al. (1996) reported that hexyl and 2-methyl acetates were identified by a tasting panel as having the greatest impact on the attractiveness of ripe 'Royal Gala' apples.

'Pink Lady®' is potentially important as a late-maturing, long-storing cultivar, which is ready for harvest after the traditional late-season cultivars [Corrigan et al., 1997]. Storage life in air at 0-1 ºC is 4 months and in CA , it is 8 - 9 months, although it has been stored longer [Cripps et al., 1993]. Despite the decline in total volatile production

in 'Pink Lady®' apples after 4 months of storage in air at 0 ºC, the fruit tends to maintain a good apple aroma even, after 12-months storage [Saftner et al., 2005]. CA-storage under low (2 kPa) or ultra-low (1 kPa) oxygen concentrations combined with similar CO_2 levels, has been shown to preserve both standard [Drake et al., 2002] and sensory quality of fruit [López et al., 2007] better than air apple after six months.

The importance of sensory evaluation in apple production and processing is obvious if consistent high-quality product is required [Dimick and Hoskin, 1983]. Sensory evaluation methods offer a way of collecting information about the sensory attributes of food samples as perceived by the human senses. However, the consumer population of a given product is often heterogeneous in its likes and dislikes. Consequently, a variety of techniques have been developed to assist scientists in understanding the variables (descriptive sensory attributes and instrumental measurements) that influence consumer preferences [Schlich, 1995; McEwan, 1996; Murray and Delahunty, 2000]. Ones of these techniques include internal and external preference mapping [Arditti, 1997]. Internal preference mapping implies the analysis of only preference data, and provides a summary of the main preference directions and the associated consumer segments [Greenhoff and MacFie, 1994]. Although, these techniques have been implemented in several research studies on apples [Dalliant-Sprinnler et al., 1996; Jaeger et al., 1998], little research has up till now been performed using preference mapping techniques to understand consumer perception and acceptance of the flavour profiles of cold-stored apples.

The objectives of this study were: to determine volatile compound emission, standard quality parameters and consumer acceptance in 'Pink Lady®' apples kept in cold storage under air and two CA conditions, to assess the relationships between sensory and instrumental quality of cold stored fruits by multivariate analysis, and to examine the efficacy of post-storage fruit exposure to air at 20 ºC in order to stimulate volatile production after long-term storage.

2. Materials and methods

2.1. Plant material and storage conditions

Apple (Malus domestica Borkh. cv. 'Pink Lady®') fruits were hand-harvested at commercial date (4th November, 226 days after full bloom) from 6 year-old trees grown on M-9 EMLA rootstock at a commercial orchard in Lleida (NE Spain). Immediately after harvest, four lots (100 kg each) of apples were selected in accordance with norms established by the Association Pink Lady Europe (diameter >70 mm; 50% of diffuse pink or 30% intense pink; background colour: turning from green to yellow; starch index 5-5.8 in a 1-10 scale; flesh firmness > 80 N; and absence of defects).

Three of these lots were stored at 1 ºC and 92-93% relative humidity in three commercial cold-storage chambers: AIR (21 kPa O_2 : 0.03 kPa CO_2) and controlled atmospheres SCA (2.5 kPa O_2 : 3 kPa CO_2) and ULO (1 kPa O_2 : 2 kPa CO_2). The capacity and volume of the commercial cold-storage chamber were 180 t and 750 m3, respectively. The storage chamber atmospheres were established within 72 h of harvest, O_2 and CO_2 concentrations were monitored and automatically corrected using N_2 supplied from a tank, excess CO_2 being scrubbed off using a charcoal system.

Samples were removed from storage after 15 or 28 weeks and transferred at 20 ºC to simulate commercial shelf life. Analyses were carried out 1 and 7 days thereafter.

2.2. Analysis of volatile compounds

Eight kilograms of apples (2 kg × replicate × 4 replicates) per treatment (atmosphere × storage period × shelf life period) were selected to analyze volatile compounds at both harvest and after removal from storage. Volatile compounds were also measured in fruits after 28 weeks of cold storage plus 10, 17, 24 and 50 days at 20 ºC. 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 de-adsorbed by agitation for 40 min with 0.5 ml of diethyl ether. The identification and quantification of volatile compounds was performed on a Hewlett Packard 5890 series II gas chromatograph equipped with a flame ionisation detector (GC-FID) and a polyethylene glycol column with cross-linked free fatty acid as the stationary phase (FFAP; 50m × 0.2mm i.d. × 0.33μm) into which a volume of 1 μL of the extract was injected in all the analyses. Helium was used as the carrier gas, at a flow rate of 0.8 ml min^{-1} (42 cm s^{-1}), with a split ratio of 40:1, in the presence of air (400 ml min^{-1}) and H_2 (32 ml min^{-1}). The injector and detector were held at 220 and 240 ºC, respectively. The analysis was conducted according to the following program: 70 ºC (1 min); 70–142 ºC (3 ºC min^{-1}); 142–225 ºC (5 ºC min^{-1}); 225 ºC (10 min), as described elsewhere [Echeverría et al., 2004b]. Volatile compounds were identified by comparing retention indexes 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 butyl benzene (assay > 99.5%, Fluka) as an internal standard. A GC–MS system (Hewlett Packard 5890) was used for compound confirmation, using the same capillary column as in the GC-FID analyses. Mass spectra were obtained by electron impact ionisation 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 μg kg^{-1}.

To measure ethylene production, eight apples were divided into 2 replicates (about 1 kg per replication) and placed in 5-L jars continuously aerated with humidified air at a rate of ~ 2 L h-1 at 20 ºC. Ethylene production was measured by taking gas samples from the effluent air with a 1-ml syringe, followed by injection into a Hewlett-Packard 5890 series II (GC-FID) equipped with an alumina column 80/100 (2m × 3mm) (Teknokroma, Barcelona, Spain). Gas analyses were conducted isothermally at 100 ºC.

N_2 was used as the carrier gas, with air and H_2, at a flow of 45, 400, and 45 ml min^{-1}, in that order. The injector and detector were held at 120 ºC and 180 ºC, respectively.

2.3. Maturity and standard quality parameter analyses

Twenty fruits per treatment were individually assessed for analyses of flesh firmness, soluble solids content (SSC), titratable acidity (TA), and skin colour, both at harvest and after removal from cold storage (atmosphere × storage period × shelf life period). Flesh firmness was measured on opposite sides of each fruit with a penetrometer (Effegi, Milan, Italy) equipped with an 11-mm diameter plunger tip; results were expressed in N. SSC and TA were assessed in juice pressed from the whole fruit. SSC was determined using a hand refractometer (Atago, Tokyo, Japan), and results were expressed as % sucrose in an equivalent solution. TA was determined by titration of 10 ml of juice with 0.1N NaOH to pH 8.1 with 1% (v/v) phenolphthaleine as an indicator, and results were given as g malic acid L^{-1}. Fruit epidermis color was determined with a portable tristimulus colorimeter (Croma Meter CR-200, Minolta Co., Osaka, Japan) using CIE illuminant D_{65} and an 8 mm measuring aperture diameter. Skin color was measured at two points on the equator of each fruit that were 180 º apart: one on the side exposed to sunlight (ES) and the other on the shaded side (SS). Hue angle was measured on both the side exposed to the sun and on the shaded side and the resulting values were respectively used as measurements of superficial and background color. Starch index was determined in twenty apples by dipping of cross-sectional fruit halves in an iodine solution (15 g KI + 6 g I_2 per litre) for 30 s; starch hydrolysis was rated using a 1–10 Eurofru scale (1, full starch; 10, no starch) (Planton, 1995).

2.4. Sensory measurements

For consumer evaluation, the fruit samples removed from each atmosphere and relating to each storage period were kept in a room at 20 ºC for 1 and 7 days. Twenty apples per treatment (atmosphere × storage period × shelf life period) were used for sensory

analysis. Prior to sensory evaluation, half of each fruit was instrumentally analyzed in relation to its standard quality parameters. Three pieces (one per atmosphere) were placed on white plates and immediately presented to a tasting panel of 61 consumers who conducted a sensory evaluation of fruit for both storage and shelf life periods. All 61 participants were the same for all treatments assessed. Consumers were volunteers from the staff working at the UdL-IRTA research institute and students from the University of Lleida. All the test participants were habitual (daily) apple consumers. Each piece was identified with a random three-digit code. The order of presentation of the three fruit parts presented on the white plate was randomized for each consumer. Mineral water was used as a palate cleanser between samples. All evaluations were conducted in individual booths under white illumination and at room temperature. Each consumer assessed all three samples and was asked to indicate his/her degree of liking/disliking using a 9-point hedonic scale (1-dislike extremely to 9-like extremely). The samples could be retested as often as desired.

2.5. Experimental design and statistical analyses

A multi-factorial design was used to statistically analyse results. The factors considered were storage period, storage atmosphere, shelf life period, and replication. All data were tested by analysis of variance (GLM-ANOVA procedure) with the SAS program package (SAS, 1988). Means were separated by the LSD test at $p \leq 0.05$. Agglomerative hierarchical clustering (AHC) was applied to the acceptability data in order to identify particular clusters of consumers who preferred one particular treatment. This analysis was made using the Euclidian distance and with Ward's method as aggregation criteria. The coordinates of the cluster centroids were used to calculate a principal component analysis (PCA) in order to characterize the preferences of each cluster for particular storage conditions. Internal preference mapping was carried out to project quality parameters and aroma volatile emissions on the map of consumer acceptance in order to get additional information on the preferences of consumers in each cluster. The variables analysed were labelled as specified in Table 1.

XLSTAT version 5.1, Addinsoft, New York, USA was used to develop these models (Crisosto et al., 2007).

3. Results and discussion

3.1. Modifications in emission of aroma volatile compounds at harvest and after cold storage of 'Pink Lady®' apples

A total of 39 aroma volatile compounds were quantified in freshly harvested fruit: 30 esters (8 acetates, 7 propanoates, 8 butananoates, 6 hexanoates and one octanoate), 7 alcohols, 1 terpene and 1 ketone (Table 1). The main volatile compound emitted during shelf life at 20 ºC (20.4% and 25.4% after 1 and 7 days, respectively) was hexyl acetate, which was subsequently predominant in the aroma profile of 'Pink Lady®' apples (López et al., 2007; Lo Bianco et al, 2008), and conferred a fruity odour (Table 1). The next most important esters, in quantitative terms, were: hexyl 2-methylbutanoate, 2-methylbutyl acetate, hexyl hexanoate, butyl acetate, hexyl butanoate, hexyl propanoate, butyl hexanoate and butyl 2-methylbutanoate. Together, these 9 ester compounds contributed 83% and 86% of total volatile fraction after 1 and 7 days at 20 ºC. Hexyl esters were therefore predominant in the aroma profile of 'Pink Lady®' apples (55%), and conferred a characteristic fresh-apple odour due to the presence of hexyl acetate, hexyl propanoate, hexyl butanoate, hexyl hexanoate and hexyl 2-methylbutanoate (Table 1). Hexyl esters have also been shown to be important in the aroma volatile fraction emitted by other bicolour apple cultivars such as 'McIntosh' and 'Cortland', in which hexyl acetate has been reported as the main ester in quantitative terms (Yahia et al., 1990). This ester was observed to be the third most predominant compound in the aroma profile of 'Fuji' apples at commercial harvest, after 2-methylbutyl and butyl acetates (Echeverría et al, 2004c).

The contribution of a particular volatile compound to overall flavour is expressed by the odour unit (Dimick and Hoskin, 1983; Plotto et al., 1999; Aaby et al., 2002; Lo Scalzo

et al., 2003; Echeverría et al., 2004a; López et al., 2007; Lo Bianco et al., 2008), which is the ratio of the concentration to its corresponding detection threshold. When the volatile concentrations were converted into odour units using the odour thresholds cited in the literature (Table 1), the aroma of 'Pink Lady®' apples was predominantly characterized by ethyl 2-methylbutanoate, 2-methylpropyl propanoate, 2-methylbutyl acetate, hexyl acetate, and hexyl 2-methylbutanoate. In line with our works, these ester compounds were considered the ones that most contribute to 'Pink Lady®' aroma in both the peel and flesh of non-stored fruit (Lo Bianco et al., 2008).

The total emission of ester compounds in 'Pink Lady®' apples showed an increase after 7 days of shelf life period at 20 ºC. The exact influence of shelf life period on apple aroma remains unclear: some authors have observed an increase of ester production with shelf life at 20 ºC up to 28 days in 'Delicious' and 'Golden Delicious' apples [Kondo et al., 2005], whereas aroma production from freshly harvested 'Gala' apple had a narrow peak after 7 days at 20 ºC and a decrease of total headspace volatile compounds up to 25 days of shelf life [Lo Scalzo et al., 2003]. Production of straight propyl esters (propyl acetate, propyl propanoate and propyl hexanoate), most butyl esters (butyl acetate, butyl propanoate, butyl butanoate, butyl hexanoate, 2-methylbutyl acetate and 2-methylbutyl propanoate), all pentyl and all hexyl esters was significantly higher for fruits ripened for 7 days at 20 ºC than for those obtained after one day at 20 ºC (Table 1). Emissions of propyl (acetate, propanoate and hexanoate), butyl (acetate, propanoate, butanoate, hexanoate and 2-methylbutanoate) and hexyl esters (acetate, propanoate, butanoate and hexanoate) were related to higher concentrations of their alcohol precursors, as emissions of 1-propanol, 1-butanol and 1-hexanol paralleled those of their corresponding esters (Table 1). This contribution of the alcohol precursors has been reported in previous reports on 'Pink Lady®' apples [López et al., 2007; Villatoro et al., 2008ab].

The same volatile compounds present at harvest were identified and quantified in the volatile fraction emitted by 'Pink Lady®' apples during cold storage (Tables 1 and 2).

After 15 weeks of storage plus 1 day at 20 ºC, total emission of volatile compounds were 4.7 times higher for air than for freshly harvested fruit and were 2.7 times higher for SCA and 1.9 times higher for ULO with respect to fruits at harvest. The nine esters identified as the quantitatively most important volatile compounds emitted by fruit at harvest contributed at least 78% (AIR, 28 weeks, 7 days at 20 ºC) and at most 89% (ULO, 15 weeks, 1 day at 20 ºC) of the total volatile fraction after cold storage. Hexyl esters tended to predominate (54%) in the aroma profile 'Pink Lady®' apples after cold storage (Table 2).

After 15 weeks in AIR, the concentration of 77% straight esters increased with respect to CA conditions. Hexyl acetate was the main volatile compound emitted by cold stored fruit, with the highest concentrations being registered after 15 weeks plus 1 day at 20 ºC under AIR and SCA conditions (Table 2). Storing fruit in SCA (2.5 kPa O_2 : 3 kPa CO_2) maintained its capacity to synthesize this ester beyong 15 weeks. Fruit storage in the ULO atmosphere produced a significant decrease in the amount of hexyl acetate. Straight-chain organic acid precursors are formed by the oxidation of acids and/or via lipoxygenase activity, both of which require oxygen and are presumably slowed down by ULO storage conditions [Brackmann et al., 1993].

Fruit from controlled atmospheres synthesized significantly smaller amounts of branched-chain esters than that stored in AIR for 15 weeks (Table 2). However, apples stored in controlled atmospheres synthesized the highest amounts of tert-butyl propanoate after 15 weeks in 2.5 kPa O_2 : 3 kPa CO_2 and 28 weeks in 1 kPa O_2 : 2 kPa CO_2 atmospheres followed by one day at 20 ºC (Table 2). The favourable effect of CA on the emission of this ester has also been reported for 'Pink Lady®' apples stored in a controlled atmosphere with low oxygen (2 kPa O_2 : 2 kPa CO_2) [López et al., 2007].

Table 1. **Volatile compounds emitted (μg kg^{-1}), retention index, codes using for PCA analyses, odour thresholds, odour units[b] (in brackets) and odour description for 'Pink Lady®' apples at harvest plus 1 and 7 days at 20 ºC**

Nº	Compound	RI[a]	Code[c]	OTH[d] (μg kg^{-1})	Amount (μg kg^{-1}) 1 day at 20 ºC	Amount (μg kg^{-1}) 7 day at 20 ºC	Odour descriptor[d]
1	Methyl butanoate	984	mb	76 (c)	2.2 A	0.5 B	
2	Ethyl acetate	834	ea	13500	15.3 A	13.5 A	Ethereal-fruity
3	Ethyl butanoate	1043	eb	1	2.6 B (2.6)	9.2 A (9.2)	Fruity, apple-like
4	Ethyl hexanoate	1243	eh	1	3.7 (3.7)	traces[e]	Fruity
5	Ethyl 2-methylbutanoate	1059	e2mb	0.006	9.9 A (1650)	10.9 A (1817)	Ripe apple
	Total ethyl esters				**31.5 A (1656.3)**	**33.6 A (1826.2)**	
6	Propyl acetate	964	pra	2000	7.3 B	51.2 A	Pear-raspberry
7	Propyl propanoate	1051	pp	57 (c)	5.7 B	30.1 A	Sweet, lift, fruity (g)
8	Propyl hexanoate	1316	prh		7.1 B	43.4 A	Sweet, fruity (g)
9	2-Methylpropyl acetate	1020	2mpa	65	19.2 A	19.0 A	Fruity
10	2-Methylpropyl propanoate	1091	2mpp	0.086 (g)	6.8 A (79.0)	6.6 A (76.7)	
11	2-Methylpropyl butanoate	1165	2mpb	8700 (g)	3.3 A	3.3 A	
12	2-Methylpropyl hexanoate	1359	2mph		1.7 A	2.3 A	
	Total propyl esters				**51.1 B (79.40)**	**156.0 A (76.7)**	
13	Butyl acetate	1082	ba	66	154.3 B (2.3)	563.8 A (8.5)	Red apple aroma
14	Butyl propanoate	1148	bp	25	53.7 B (2.1)	121.3 A (4.9)	Faintly sweet odour
15	Butyl butanoate	1228	bb	100	57.0 B	112.6 A (1.1)	Rotten apple
16	Butyl hexanoate	1423	bh	700	111.5 B	269.5 A	Green apple
17	Butyl octanoate	1623	bo		16.3 A	18.8 A	
18	*Tert*-butyl propanoate	932	tbp	19	1.7 A	7.9 B	
19	2-Methybutyl acetate	1131	2mba	11	210.8 B (19.2)	493.2 A (44.8)	Banana, ripe apple
20	2-Methylbutyl propanoate	1199	2mbp	19	7.5 A	8.5 A	
21	Butyl 2-methylpropanoate	1157	b2mp	80 (c)	5.2 A	6.6 A	Apple(g)
22	Butyl 2-methylbutanoate	1240	b2mb	17	61.6 B (3.6)	225.5 A (13.3)	Fruity, apple
	Total butyl esters				**679.6 B (25.2)**	**1827.7 A (72.6)**	
23	Pentyl acetate	1183	pa	43	22.5 B	63.7 A (1.5)	Apple, fruity
24	Pentyl hexanoate	1520	ph		11.8 B	27.1 A	Rosal, fresh sweet (e)
	Total pentyl esters				**34.3 B**	**90.8 A**	
25	Hexyl acetate	1283	ha	2	395.6 B (197.8)	1277.9 A (639.0)	Fruity
26	Hexyl propanoate	1349	ph	8	107.9 B (13.5)	227.9 A (28.5)	Apple
27	Hexyl butanoate	1426	hb	250	115.6 B	214.5 A	Apple
28	Hexyl hexanoate	1621	hh	6400 (g)	165.3 B	234.8 A	Apple
29	Hexyl 2-methylbutanoate	1436	h2mb	6	288.1 B (48.0)	822.4 A (137.1)	Fresh-green fruity
	Total hexyl esters				**1072.5 B (259.3)**	**2777.5 A (806.1)**	
30	Octyl acetate	1484	oa	12 (a)	1.0 A	1.4 A	Fruity (f)
31	Ethanol	898	etOH	100000 (b)	17.5 A	10.5 A	Slight (g)
32	1-Propanol	1036	prOH	9000	2.4 B	15.8 A	Sweet
33	1-Butanol	1141	buOH	500	20.3 B	54.5 A	Sweet aroma
34	1-Pentanol	1253	pOH	4000 (d)	2.9 A	3.1 A	
35	1-Hexanol	1358	hOH	500	14.7 B	26.7 A	Grassy
36	2-Ethyl-1-hexanol	1494	2eOH		1.7 A	0.7 B	
37	2-Methyl-1-butanol	1210	2mbOH	250	0.6 A	traces[e]	Highly diluted-pleasant
	Total alcohols				**61.1 B**	**112.7 A**	
38	D-limonene	1219	limon	34	6.8 B	11.5 A	Citrus-like
39	6-Methyl-5-hepten-2-ona	1391	6m5h2o	50	2.7 B	12.8 A	Citrus, strawberry-like (e)
	TOTAL [f]				**1941.8 B (2020.2)**	**5023.0 A (2781.6)**	

[a]RI, linear retention index based on a series of *n*-hydrocarbons. Means within the same row followed by the different capital letters are not significantly different at $p \leq 0.05$ (LSD test). [b] Odour units = [amount / OTH]. Only values > 1 are indicated. [c] Codes used for multivariate analysis.[d] Odour thresholds and odour descriptors as reported in López et al. (2007), excepting (a): Guadagni et al.. (1966) (b): Flath et al. (1967) (c): Takeoka et al. (1990) (d): Buttery (1993) (e): Mehinagic et al. (2006) (f): Moya-León et al. (2007) (g): Burdock (2002). [e] traces (≤ 0.5 μg kg^{-1}). [f] total amount of all volatile compounds detected during the chromatographic analyses.

Extending the cold storage from 15 to 28 weeks reduced emissions of total volatile compounds (Table 2), total butyl esters (Fig. 1C) and total hexyl esters (Fig. 2E) from fruits. However, when volatile concentrations were converted into odour units, the decrease was not significant for total hexyl esters (Fig. 2F) and total butyl esters, except for AIR-stored fruits after one day at 20 ºC (Fig. 1D).

After 28 weeks of cold storage, there was an increase in total ethyl esters for AIR-fruits that were ripened for 7 days at 20 ºC (Fig. 1A). In general, extending storage time from 15 to 28 weeks increased the number of odour units for total ethyl esters (Fig. 1B). It is also important to note that the emission of ethyl 2-methylbutanoate led to an increase equivalent to extending storage to 28 weeks, regardless of the storage atmosphere (Table 2). As the odour unit of ethyl 2-methylbutanoate was very high (6117, 1967 and 2383 in fruit from AIR, SCA and ULO after 28 weeks plus 7 days at 20 ºC, respectively) this compound is likely to contribute to the characteristic ripe note of apple [Flath et al., 1967]. This branched-chain ester is reportedly one the main contributors to the aroma of 'Gravenstein' [Aaby et al., 2002], 'Fuji' [Echeverría et al., 2004ab; Mehinagic et al., 2006], 'Delicious' [López et al., 1998; Mehinagic et al., 2006], and 'Braeburn' apples [Mehinagic et al., 2006].

Apples stored in controlled atmospheres (SCA and ULO) registered lower levels of ethyl (Fig. 1A), total butyl (Fig. 1C) and total hexyl esters (Fig. 2E) than those stored in AIR. However, the odour unit of total ethyl esters when fruits were ripened for one day at 20 ºC was not significantly different with respect to other storage atmospheres (Fig. 1B). A similar result was obtained for the odour units of total hexyl esters for apples stored for 15 weeks (Fig. 2F). The observed decrease in the level of ethyl acetate in CA conditions is desirable given its solvent-like aroma [Verstrepen et al., 2003].

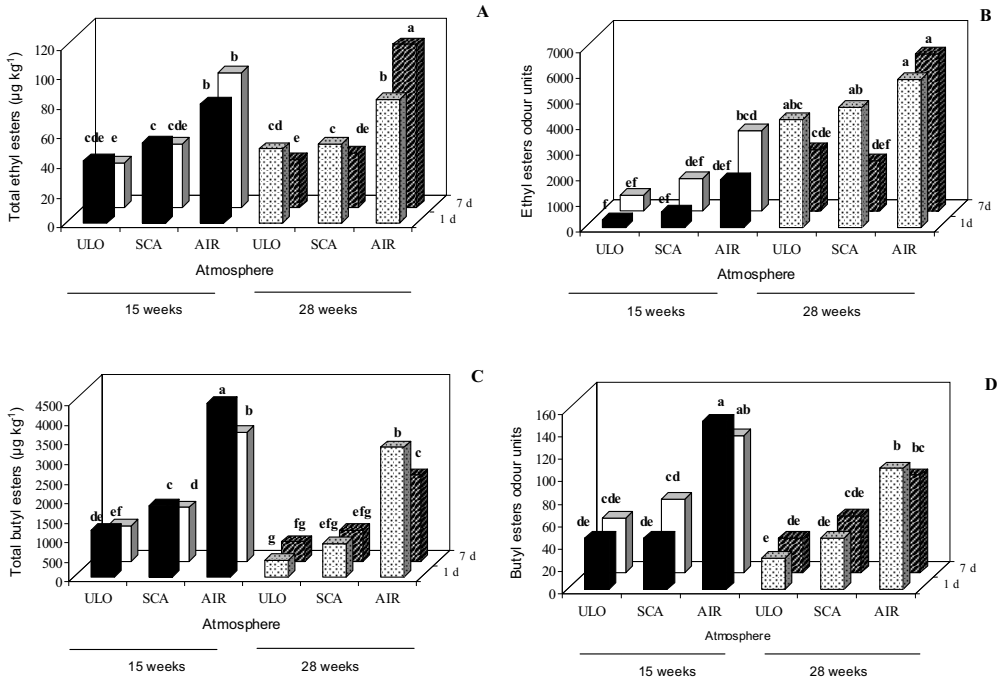

Figure 1. **Total ethyl (A) and butyl (C) ester concentrations (µg kg^{-1}) and odour units (B, D) after 15 and 28 weeks of cold storage in air (AIR: 2.5 kPa O_2 : 3 kPa CO_2) and controlled atmosphere (ULO: 1 kPa O_2 : 2 kPa CO_2 and SCA: 2.5 kPa O_2: 3 kPa CO_2) plus 1 and 7 days at 20 ºC. Means with different letter indicate significant difference between atmospheres conditions, cold storage weeks and days at 20 ºC at $p \leq 0.05$, least significant differences (LSD) test.**

In contrast to observations for fruit at harvest, 7 days of shelf life period after cold storage produced increased emissions of hexyl 2-methylbutanoate and propyl acetate for all treatments except fruit stored for 28 weeks under 2.5 kPa O_2 : 3 kPa CO_2 atmosphere (Table 2). The odour threshold of hexyl 2-methylbutanoate is very high (6 µh kg^{-1}), and it seems likely that this compound contributes to the characteristic fresh-green fruity note of apples (Table 1).

Table 2. **Volatile compounds (μg kg^{-1})[a] after storage from air and controlled atmosphere (kPa O_2: kPa CO_2) for 15 and 28 weeks plus 1 and 7 days at 20 ºC**

	Atmosphere	AIR	AIR	AIR	AIR	2.5:3	2.5:3	2.5:3	2.5:3	1:2	1:2	1:2	1:2
	Storage (weeks)	15	15	28	28	15	15	28	28	15	15	28	28
Nº	Days (20 ºC)	1	7	1	7	1	7	1	7	1	7	1	7
1	Methyl butanoate	14.1 a	4.9 d	12.2 b	7.4 c	4.2 de	2.5 fgh	3.8 def	2.0 ghij	1.5 ghij	0.7 j	3 efg	1.3 hij
2	Ethyl acetate	25.6 bcd	30.5 b	21.3 def	38.6 a	28.4 bc	21.0 def	21.1 def	20.5 def	27.0 bcd	18.8 ef	23.2 cde	15.2 f
3	Ethyl butanoate	23.8 c	29.4 b	27.6 bc	34.5 a	14.9 d	7.5 e	4.4 ef	4.4 ef	7.9 e	3.2 f	1.7 f	2.7 f
4	Ethyl hexanoate	20.0 a	12.3 b	ND	ND	7.5 c	6.4 c	ND	ND	5.6 cd	3.8 d	ND	ND
5	Ethyl 2-methylbutanoate	11.0 e	18.5 c	34.5 a	36.7 a	3.4 fg	7.6 ef	28.1 b	11.8 de	1.7 g	3.7 fg	25.2 b	14.3 cd
6	Propyl acetate	54.7 bc	86.7 a	44.8 cd	59.4 b	12.5 ef	35.0 d	3.9 f	12.8 ef	5.6 f	16.9 e	ND	8.6 ef
7	Propyl propanoate	24.1 c	76.5 a	14.5 d	33.4 b	3.3 e	15.1 d	tr	6.5 e	tr	6.5 e	ND	1.3 e
8	Propyl hexanoate	90.5 a	95.2 a	44.0 b	33.2 c	8.2 e	26.1 c	0.7 e	9.9 de	4.2 e	18.9 d	30.5 c	9.1 e
9	2-Methylpropyl acetate	60.3 a	34.7 b	35.0 b	21.3 c	18.3 cd	20.7 c	12.8 de	12.8 de	22.1 c	18.0 cd	8.2 e	8.6 e
10	2-Methylpropyl propanoate	10.8 cde	19.0 b	31.0 a	31.2 a	7.7 de	12.6 cd	10.4 cde	15.6 bc	6.7 e	8.0 de	7.4 de	9.2 de
11	2-Methylpropyl butanoate	11.9 a	8.4 bc	10.1 a	5.2 def	5.2 def	3.1 fg	4.6 ef	1.2 g	2.9 fg	3.0 fg	6.5 cde	7.3 cd
12	2-Methylpropyl hexanoate	6.8 a	5.5 b	ND	ND	ND	2.7 c	ND	ND	2.5 c	2.0 c	ND	ND
13	Butyl acetate	1653.0 a	1132.0 c	1482.5 b	846.7 d	845.6 d	422.0 ef	274.3 fg	124.6 gh	524.6 e	180.7 gh	81.1 h	47.9 h
14	Butyl propanoate	179.0 c	298.0 a	134.1 d	213.0 b	53.7 efg	59.0 ef	34.5 fghi	63.3 e	17.0 i	30.1 ghi	18.8 hi	42.5 efgh
15	Butyl butanoate	500.0 a	300.0 c	364.8 b	188.5 d	177.8 d	70.3 ef	44.4 fg	23.5 g	98.0 e	39.6 fg	16.8 g	13.7 g
16	Butyl hexanoate	744.9 a	432.3 b	372.6 c	180.8 d	190.0 d	145.7 de	46.9 f	39.7 f	121.0 e	122.8 e	30.8 f	48.2 f
17	Butyl octanoate	42.4 a	41.3 a	42.5 a	ND	14.5 b	13.3 b	ND	ND	12.7 b	12.2 b	ND	ND
18	Tert-butyl propanoate	7.2 de	12.3 cde	8.7 cde	17.5 bc	27.5 a	9.4 cde	13.3 bcd	8.6 cde	4.6 e	16.0 bcd	22.5 ab	9.0 cde
19	2-Methylbutyl acetate	1095.8 a	818.7 b	702.3 b	570.4 c	434.6 de	547.1 cd	399.6 ef	477.0 cde	394.2 ef	442.4 de	249.7 fg	282.7 fg
20	2-Methylbutyl propanoate	20.3 a	18.4 a	7.9 bc	6.7 bcd	4.1 de	10.0 b	4.2 de	6.1 cd	1.9 e	7.6 bc	2.0 e	6.4 cd
21	Butyl 2-methylpropanoate	10.8 b	9.2 bc	19.3 a	ND	6.0 cd	3.9 e	5.0 de	ND	3.8 e	2.6 ef	0.8 f	ND
22	Butyl 2-methylbutanoate	187.3 b	247.2 a	187.2 b	182.4 b	64.4 d	97.0 c	34.6 ef	45.4 def	23.7 f	57.8 de	17.9 f	38.9 def
23	Pentyl acetate	87.2 a	84.4 a	66.8 b	59.7 b	45.3 c	45.8 c	23.4 ef	25.5 e	37.0 cd	33.6 d	14.0 f	24.2 ef
24	Pentyl hexanoate	33.4 b	42.9 a	5.1e	5.4 e	11.4 d	20.4 c	ND	ND	7.5 de	19.0 c	ND	4.7 e
25	Hexyl acetate	2217.2 a	2063.5 a	1993.1 a	1328.2 bc	2113.7 a	987.2 cde	1158.3 cd	501.0 fg	1532.6 b	715.5 ef	794.1 def	335.3 g
26	Hexyl propanoate	238.6 b	501.8 a	148.6 de	198.9 bc	142.3 de	174.6 cd	76.7 fg	72.5 fg	59.5 g	110.6 ef	45.9 g	53.3 g
27	Hexyl butanoate	579.7 a	614.7 a	445.0 b	461.0 b	365.0 c	239.5 de	181.3 ef	119.6 f	290.2 d	159.8 f	112.6 f	124.1 f
28	Hexyl hexanoate	504.1 a	409.8 b	177.2 de	144.4 e	211.0 cd	258.4 c	89.0 f	61.2 fg	190.7 de	211.0 cd	34.1 g	95.3 f
29	Hexyl 2-methylbutanoate	499.5 c	1174.4 a	324.5 de	604.0 c	265.7 ef	797.9 b	174.3 ef	292.2 e	155.4 ef	488.5 cd	108.6 f	301.3 e
30	Octyl acetate	4.8 a	3.1 bc	ND	ND	2.1 cd	1.2 d	ND	ND	3.6 ab	1.3 d	ND	ND
31	Ethanol	10.3 cd	10.9 cd	9.0 cd	15.1 bc	21.6 a	8.1 d	13.4 cd	15.4 abc	10.9 cd	12.5 cd	20.1 ab	10.1 cd
32	1-Propanol	11.3 de	35.6 b	15.3 cd	41.4 a	38.9 ab	18.8 c	39.1 ab	8.5 ef	12.7 de	8.4 ef	8.1 ef	5.5 f
33	1-Butanol	92.7c	168.6 b	156.8 b	223.3 a	72.9 c	78.5 c	24.9 d	31.7 d	40.4 d	38.5 d	25.3 d	21.1 d
34	1-Pentanol	1.2 ab	2.5 a	tr	2.4 a	1.3 ab	1.5 ab	tr	tr	0.9 b	0.8 b	tr	1.3 ab
35	1-Hexanol	43.3 d	79.3 b	63.2 c	133.9 a	66.4 bc	45.0 d	42.5 de	22.0 f	43.0 de	20.8 f	34.8 def	29.1 ef
36	2-Ethyl-1-hexanol	3.1 abc	3.5 ab	ND	ND	4.5 a	3.5 ab	ND	ND	1.2 bc	0.8 c	ND	ND
37	2-Methyl-1-butanol	21.9 ef	35.9 cd	27.6 de	69.4 a	20.7 ef	19.4 ef	41.4 c	51.3 b	16.0 f	26.4 e	43.8 bc	37.0 c
38	D-limonene	1.3 ab	tr	ND	ND	1.6 ab	0.9 b	2.1 a	ND	0.9 b	0.6 b	tr	ND
39	6-methyl-5-hepten-2-one	7.4 def	10.6 abc	9.9 bcd	8.6 bcde	7.4 def	9.6 bcde	10.8 abc	8.4 cde	7.1 ef	12.6 a	5.6 f	11.2 ab
	Total volatile compounds	**9151.3 a**	**8972.5 a**	**7039.0 b**	**5802.6 c**	**5323.6 d**	**4248.3 e**	**2823.8 g**	**2095.0 h**	**3698.8 f**	**2876.0 g**	**1793.1 i**	**1620.4 i**

[a] Means within each row followed by different letters indicate significant differences between treatments and days at 20 ºC at $P \leq 0.05$, least significant difference (LSD) test
Volatile compounds not detected are indicated as ND and amounts of ≤ 0.5 μg kg^{-1} are indicates as trace (tr).

When fruit was ripened for 10 days at 20 ºC after 28 weeks under SCA (2.5 kPa O_2 : 3 kPa CO_2), total emissions of volatile compounds were 3.3 times higher than for fruit ripened for 7 days at 20 ºC and 4 times higher after 17 days of shelf life (Table 3). After 10 days of ripening at 20 ºC, total emissions of volatile compounds increased by a factor of 1.6 compared to ULO apples ripened for 7 days, while total emissions increased 2.5 times in fruits subjected to ULO+17 days at 20 ºC. The residual effect of controlled atmospheres on the production of volatile compounds depends on the cultivar, storage atmosphere combinations, and several others factors. Lo Scalzo el al. (2003) reported that 'Gala' apples subjected to long ULO (1.2 kPa O_2 : 1 kPa CO_2) treatment showed a subsequent decrease in ester levels after 17 days of shelf life at 20 ºC.

After 28 weeks, the large increase in total volatile compounds was mainly due to the increased amounts of propyl esters, butyl esters and hexyl esters under SCA (2.5 kPa O_2 : 3 kPa CO_2) plus 17 days at 20 ºC of shelf life period (Table 3). SCA-stored fruits after 17 days produced the greatest amounts of ethyl 2-methylbutanoate and 2-methylbutyl acetate, two of the four esters that most contribute of aroma in this cultivar.

After 15 and 28 weeks of storage plus 7 days at 20 ºC, total alcohol emissions (Fig. 2G) and odour units (Fig. 2H) for fruit stored in air were higher than for fruit stored in controlled atmospheres (SCA and ULO). The highest alcohol emissions were obtained after 28 weeks in AIR-stored apples subjected to 50 days at 20 ºC, especially for ethanol, 1-propanol, 1-butanol, 1-pentanol, 2-ethyl-1-hexanol and 2-methyl-1-butanol (Table 3). These findings confirmed previous results showing that alcohol production increases toward senescence in 'Golden Delicious' apples [Kondo et al., 2005].

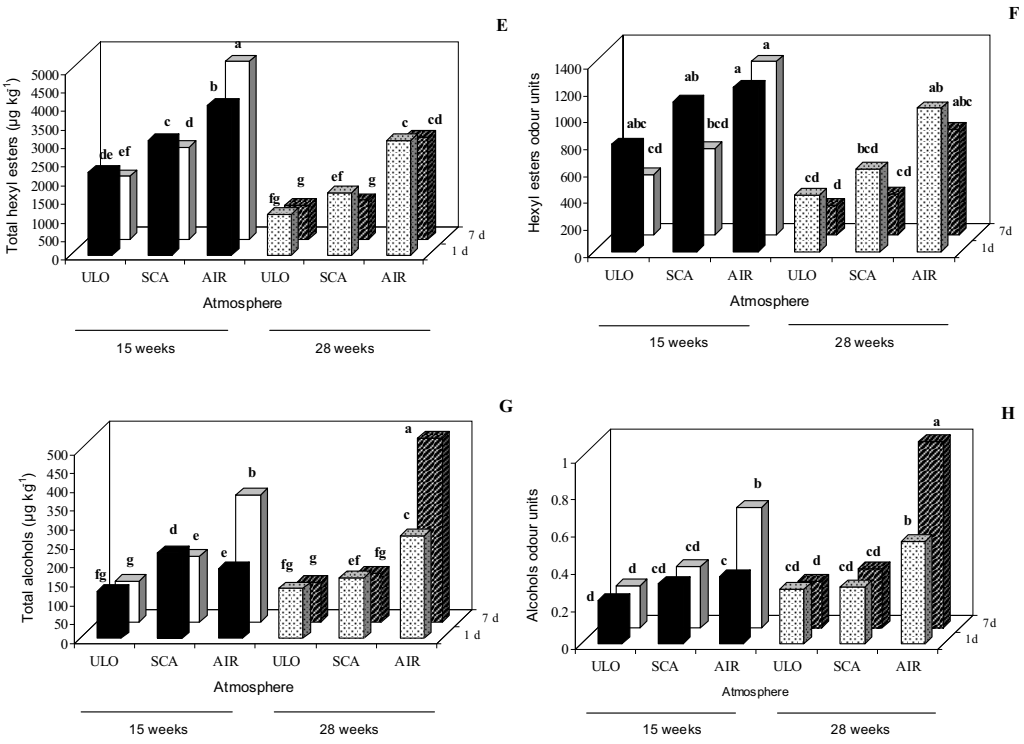

Figure 2. Total hexyl esters (E) and alcohol concentrations (G) (µg kg^{-1}) and odour units (F, H) after 15 and 28 weeks of cold storage from air (AIR: 2.5 kPa O_2 : 3 kPa CO_2) and controlled atmosphere (ULO: 1 kPa O_2 : 2 kPa CO_2 and SCA: 2.5 kPa O_2 : 3 kPa CO_2) after plus 1 and 7 days at 20 ºC Means with different letter indicate significant difference between atmospheres conditions, cold storage weeks and days at 20 ºC at $p \leq 0.05$, least significant differences (LSD) test.

3.2. Standard quality parameters at harvest and after cold storage

According to Centre Technique Interprofessionel des Fruits et Légumes (CTIFL) recommendation, fruit flesh firmness and background colour at harvest (Table 4) are indicative of an appropriate stage of maturity for long-term cold storage (Mathieu et al., 1998). Such fruit also tends to exhibit low starch indices (6.00, on the 1-10 Eurofru-scale) and ethylene production (0.55 µL kg^{-1} h^{-1}).

***Table 3*. Volatile compounds (µg kg^{-1}) after storage from air and controlled atmosphere (kPa O_2: kPa CO_2) after 28 weeks of storage plus 10, 17, 24 and 50 days at 20 ºC**

	Atmosphere	AIR	AIR	AIR	AIR	2.5:3	2.5:3	2.5:3	2.5:3	1:2	1:2	1:2	1:2
Nº	Days (20 ºC)	10	17	24	50	10	17	24	50	10	17	24	50
1	Methyl butanoate	3.4 d	11.6 c	12.3 bc	27.5 a	6.6 cd	18.8 b	7.4 cd	11.8 c	2.3 d	7.0 cd	8.6 cd	11.3 c
2	Ethyl acetate	89.5 de	198.1 b	50.5 e	334.9 a	158.8 bc	333.7 a	163.5 bc	71.0 e	76.0 e	155.1 bc	84.6 de	138.3 cd
3	Ethyl butanoate	34.7 cd	135.6 b	33.2 cd	180.6 a	6.6 d	29.8 cd	62.0 c	175.0 a	14.1 d	37.9 cd	37.1 cd	21.7 d
4	Ethyl hexanoate	6.8 e	31.9 bc	3.7 e	41.6 ab	13.6 de	23.1 cd	14.6 de	ND	11.3 e	13.7 de	24.5 cd	48.7 a
5	Ethyl 2-methylbutanoate	358.1 c	485.6 bc	385.0 c	303.8 c	607.9 ab	757.6 a	345.3 c	299.6 c	346.9 c	347.1 c	441.1 bc	449.4 bc
	Total ethyl esters	**489.1 d**	**851.2 b**	**472.4 d**	**860.9 b**	**786.9 bc**	**1144.2 a**	**585.4 cd**	**545.6 cd**	**448.3 d**	**553.8 cd**	**587.3 cd**	**658.1 bcd**
6	Propyl acetate	52.4 cd	82.5 a	26.4 ef	74.9 ab	49.6 cd	92.6 a	61.6 bc	27.3 ef	15.8 f	39.5 de	51.8 cd	62.2 bc
7	Propyl propanoate	29.2 cd	52.1 b	15.1 ef	28.2 cde	32.8 cd	71.5 a	54.2 b	11.8 f	8.6 f	27.3 de	41.1 bc	49.1 b
8	Propyl hexanoate	18.8 e	74.0 ab	11.7 e	33.6 de	71.0 ab	75.0 ab	84.5 a	18.6 e	31.8 de	62.8 abc	58.9 bc	44.7 cd
9	2-Methylpropyl acetate	16.9 bcd	23.2 bc	8.2 d	23.7 bc	47.9 a	59.4 a	21.6 bc	12.7 cd	18.9 bcd	27.5 b	22.5 bc	16.1 bcd
10	2-Methylpropyl propanoate	14.5 de	25.0 cd	11.9 e	70.3 a	32.2 c	46.1 b	21.6 cde	19.8 de	11.2 e	18.8 de	20.5 cde	20.8 cde
11	2-Methylpropyl butanoate	4.8 e	16.0 ab	7.3 cde	6.7 de	13.1 bc	21.1 a	8.1 cde	6.1 de	8.6 cde	12.1 bcd	11.7 bcd	6.1 de
12	2-Methylpropyl hexanoate	ND	1.8 e	ND	ND	13.9 a	11.6 b	5.0 d	ND	8.1 c	4.5 de	ND	ND
	Total propyl esters	**136.6 de**	**274.6 b**	**80.6 e**	**237.4 bc**	**260.5 bc**	**377.3 a**	**256.6 bc**	**96.3 e**	**103.0 e**	**192.5 cd**	**206.5 bcd**	**199.0 bcd**
13	Butyl acetate	493.2 ab	583.6 a	67.3 g	246.3 de	343.2 cd	446.0 bc	243.6 de	42.5 g	75.9 g	194.6 ef	200.3 ef	133.3 fg
14	Butyl propanoate	75.0 ab	94.7 a	ND	ND	86.0 ab	ND	71.5 ab	ND	20.0 c	5.8 c	56.6 b	9.5 c
15	Butyl butanoate	92.2 bc	157.5 a	23.3 fg	49.6 def	101.0 b	133.7 a	69.7 cd	13.3 g	40.6 defg	64.8 cde	51.9 def	35.8 efg
16	Butyl hexanoate	71.9 cd	179.2 b	19.6 d	50.6 d	303.7 a	301.3 a	142.7 bc	26.5 d	144.0 bc	137.2 bc	91.9 cd	46.4 d
17	Butyl octanoate	4.0 cde	10.1 b	1.4 e	2.6 e	21.5 a	19.6 a	9.7 bc	1.8 e	9.3 bcd	10.8 b	7.1 bcde	3.3 de
18	Tert-butyl propanoate	14.8 gh	63.7 c	23.7 fgh	172.6 a	18.6 gh	46.8 cde	31.6 defg	38.4 def	8.0 h	28.3 efg	48.9 cd	121.4 b
19	2-Methylbutyl acetate	416.9 bcd	656.5 b	92.3 e	268.8 de	1096.2 a	1245.9 a	624.8 bc	92.7 e	382.6 cd	549.7 bc	557.6 bc	392.5 bcd
20	2-Methylbutyl propanoate	7.3 fg	15.1 cdef	6.1 g	10.4 efg	33.3 b	41.1 a	20.3 c	7.7 fg	16.4 cde	18.3 cde	18.7 cd	12.0 defg
21	Butyl 2-methylpropanoate	3.2 d	7.3 abc	ND	8.6 ab	7.0 abc	10.3 a	4.6 cd	4.2 cd	3.1 d	ND	5.9 bcd	4.8 cd
22	Butyl 2-methylbutanoate	101.3 cd	165.0 a	29.3 e	69.4 cde	152.5 ab	188.5 a	109.5 bc	32.5 e	57.2 de	107.3 bc	108.5 bc	62.0 cde
	Total butyl esters	**1279.8 c**	**1932.7 b**	**263.0 e**	**878.9 cd**	**2163.0 ab**	**2433.2 a**	**1328.0 c**	**259.6 e**	**757.1 de**	**1116.8 cd**	**1147.4 cd**	**821.0 cd**
23	Pentyl acetate	41.2 de	70.3 c	15.8 e	22.0 e	99.3 b	154.8 a	73.5 bc	16.6 e	33.8 e	75.2 bc	62.7 cd	31.1 e
24	Pentyl hexanoate	5.3 c	16.2 bc	5.8 c	4.0 c	56.3 a	68.5 a	29.2 b	3.7 c	27.9 b	28.8 b	16.3 bc	5.5 c
	Total pentyl esters	**46.5 fg**	**86.5 de**	**21.6 h**	**26.0 h**	**155.6 b**	**223.3 a**	**102.7 cd**	**20.3 h**	**61.7 f**	**104.0 c**	**79.0 e**	**36.6 gh**
25	Hexyl acetate	620.9 bc	1370.9 a	189.0 d	391.2 cd	1265.6 a	1557.7 a	837.6 b	84.7 d	425.9 cd	875.4 b	684.4 bc	338.9 cd
26	Hexyl propanoate	68.8 de	193.6 bc	23.3 e	42.9 e	265.6 ab	329.3 a	194.4 bc	22.9 e	99.4 de	136.3 cd	121.7 cd	64.1 de
27	Hexyl butanoate	118.5 bcde	284.1 a	30.5 e	56.8 cde	317.8 a	337.9 a	176.1 b	26.5 e	128.8 bcd	150.4 bc	108.3 bcde	51.4 de
28	Hexyl hexanoate	50.8 cd	140.0 bc	13.9 d	15.4 d	337.9 a	311.3 a	146.9 bc	13.4 d	155.2 b	148.0 b	85.8 bcd	26.5 d
29	Hexyl 2-methylbutanoate	204.0 def	577.5 c	65.8 f	120.9 ef	889.0 ab	1029.9 a	654.5 bc	82.1 f	348.5 cdef	470.3 cd	407.0 cde	265.8 def
30	Octyl acetate	ND	1.6 ab	0.9 c	1.5 abc	ND	1.0 bc	1.7 a	tr	1.3 abc	1.3 abc	1.4 abc	1.3 abc
	Total hexyl esters	**1063.0 efg**	**2566.1 bc**	**322.5 g**	**627.2 fg**	**3075.9 ab**	**3566.1 a**	**2009.5 cd**	**229.6 g**	**1157.8 defg**	**1780.4 cde**	**1407.2 def**	**746.7 fg**
31	Ethanol	46.6 b	82.8 b	57.2 b	693.2 a	74.1 b	140.5 b	64.0 b	92.0 b	31.0 b	60.1 b	39.3 b	85.0 b
32	1-Propanol	49.8 ef	110.8 b	19.6 gh	255.4 a	38.0 fg	84.2 cd	70.2 de	35.0 fg	8.4 h	31.0 fgh	48.0 ef	102.0 bc
33	1-Butanol	218.5 cd	428.4 a	76.7 gh	447.0 a	131.9 fg	339.3 b	207.3 cde	82.6 gh	31.3 h	134.4 efg	175.3 def	272.9 bc
34	1-Pentanol	4.4 cd	12.6 b	4.4 cd	16.4 a	5.9 cd	11.4 b	6.6 c	4.4 cd	3.6 d	5.8 cd	6.1 cd	6.1 cd
35	1-Hexanol	71.8 d	225.6 a	46.1 de	158.0 b	68.4 d	105.8 c	69.3 d	25.2 e	22.0 e	44.1 de	46.8 de	51.1 de
36	2-Ethyl-1-hexanol	3.2 cd	7.7 ab	2.3 d	11.0 a	7.9 ab	5.4 bcd	4.6 bcd	2.9 d	7.8 ab	7.0 abc	4.0 bcd	6.2 bcd
37	2-Methyl-1-butanol	63.0 ef	118.9 abc	45.0 f	135.9 a	114.9 abc	132.9 ab	88.0 cde	65.3 ef	46.8 f	61.4 ef	72.7 def	101.6 bcd
	Total alcohols	**457.3 def**	**986.8 b**	**251.3 fg**	**1716.9 a**	**441.1 def**	**819.5 bc**	**510.0 de**	**307.4 efg**	**150.9 g**	**343.8 efg**	**392.2 defg**	**624.9 cd**
38	D-limonene	ND	2.3 b	ND	ND	0.8 cd	3.5 a	1.2 cd	0.6 d	0.8 cd	1.8 bc	1.9 bc	1.1 cd
39	6-methyl-5-hepten-2-one	2.9 def	5.5 cde	0.7 f	2.3 def	15.4 a	15.3 a	5.8 bcd	1.0 ef	10.3 b	8.8 bc	5.1 cdef	2.3 def
	Total volatile compounds	**3475.2 cd**	**6705.7 b**	**1412.1 e**	**4349.6 cd**	**6899.2 ab**	**8582.4 a**	**4799.2 c**	**1460.3 e**	**2689.9 de**	**4101.9 cd**	**3826.6 cd**	**3089.7 cde**

[a]Means within each row followed by different letters indicate significant differences between treatments and days at 20 ºC at $P \leq 0.05$, least significant difference (LSD) test. Volatile compounds not detected are indicated as ND.

***Table 4*. Standard quality parameters of 'Pink Lady®' apples at harvest and after storage in air and controlled atmospheres (kPa O_2 : kPaCO_2) plus 1 and 7 days at 20 ºC[a]**

Quality parameters	Harvest	Days (20 ºC)	Storage period (weeks)	AIR (21:0.03)	SCA (2.5:3)	ULO (1:2)
Flesh firmness (N)	86.9	1	15	62.9 d	75.6 b	79.4 b
			28	62.3 d	69.4 c	84.2 a
		7	15	64.7 d	75.0 b	84.2 a
			28	62.0 d	65.2 cd	79.1 b
Titratable acidity (g malic acid L^{-1})	6.9	1	15	4.7 bc	5.1 ab	5.5 a
			28	4.4 c	5.0 ab	5.0 ab
		7	15	4.5 bc	5.2 a	5.1 ab
			28	3.6 d	4.5 bc	4.7 bc
Soluble solid content (%)	14.6	1	15	14.1 d	15.5 b	15.7 b
			28	14.6 c	14.7 c	16.0 ab
		7	15	14.4 cd	15.5 b	14.7 c
			28	14.1 d	16.3 a	15.7 b
Hue (SS)[b]	97.2	1	15	86.3 de	85.0 de	93.9 bc
			28	79.4 f	96.6 ab	89.9 cd
		7	15	83.9 ef	96.5 ab	100.4 a
			28	88.2 cd	83.1 ef	94.1 bc
Hue (ES)[c]	29.7	1	15	31.6 b	35.1 b	35.9 b
			28	36.5 a	38.2 a	32.8 b
		7	15	34.8 b	38.2 a	46.5 a
			28	39.7 a	37.5 a	37.4 a

[a] Means followed by different small letters for each quality parameter are significantly different at $p \leq 0.05$ (LSD test). [b]SS: shaded side. [c]ES: exposed side.

For 15 weeks of storage plus 1 day at 20 ºC, ULO- and SCA-stored apples retained higher degrees of firmness and soluble solid content (SSC) than AIR-stored apples. After 7 days at 20 ºC, ULO-stored apples showed the best preservation of standard quality, which was consistent with the observed high values for flesh firmness (84.2 N), titratable acidity (TA) and the greener colour on their shaded sides compared to AIR-stored apples. After 28 weeks, ULO-stored apples showed the highest degrees of flesh firmness, TA and SSC, regardless of the lenght of shelf life at 20 ºC (Table 4).

The lowest level of flesh firmness was found in AIR-stored apples after 28 weeks plus 7 days at 20 ºC (62.0 N); this is indicative of a good firmness retention potential in this apple cultivar, even after long-term storage under AIR. In contrast, TA was not well preserved (3.6 g l^{-1}). However, this result did not affect consumer acceptance because there were no significant differences between AIR- and CA-stored apples after 28 weeks plus 7 days at 20 ºC (Table 5). ULO-stored apples seem to be the ones that best maintain the standard quality of 'Pink Lady®' apples, confirming the findings of Drake et al. [2002]. Even so, the acceptability of these apples was not always scored as the best received by consumers.

***Table 5*. Mean sensory scores for 'Pink Lady®' apples stored in air and controlled atmospheres (ULO: 1 kPa O_2 : 2 kPa CO_2 and SCA: 2.5 kPa O_2 : 3 kPa CO_2) plus 1 and 7 days at 20 ºC**

Storage period	Days at 20 ºC	AIR	SCA	ULO
15	1	7.1 a	6.2 bc	6.6 ab
	7	7.2 a	6.9 ab	6.3 abc
28	1	6.8 ab	5.6 c	6.3 abc
	7	6.1 bc	6.7 ab	6.5 abc

Means within the same small letters are not significantly different at $p \leq 0.05$ (LSD test)

3.3 Relationship between consumer acceptability, standard quality parameters and aroma volatile compounds

Consumer acceptance of 'Pink Lady®' apples was analysed by means of Internal Preference Mapping. Arrows showing the preference direction for each consumer were mainly concentrated in the area of positive scores for both dimensions (1 and 2). When consumers were segmentied using Agglomerative Hierarchical Clustering (AHC), four different consumer clusters were identified by Ward's method. These clusters were characterised by average values of consumer acceptance. When then performed principal component analysis (PCA), which revealed that PC1 and PC2 accounted for 74.9% of total variance (Fig. 3a). Cluster 1, which included the greatest number of

consumers (n=21), preferred apples stored in ULO atmosphere for 15 weeks. Consumers in cluster 2 (n=16) preferred apples stored in AIR + 1 day at 20 ºC, while those in cluster 3 (n=8) preferred apples stored in AIR during 28 weeks + 7 days at 20 ºC (Fig. 3b). For cluster 4 (n=16), the highest scores were found for samples stored in SCA atmosphere for 15 weeks + 7 days at 20 ºC.

We carried out internal preference mapping to obtain additional information on the characteristics of the samples preferred by each consumers cluster. This involved 39 volatile compounds and 5 standard quality parameters (SSC, TA, firmness, and hue on both the exposed and shaded sides), which were projected onto the map of consumer acceptance. The results confirmed that individuals in cluster 1 preferred ULO samples due to higher acidity and firmness, as shown in Table 4 and Figure 4. The acceptance of AIR samples was related to their greater aroma volatile emissions. Results from sensory analyses gave a maximum score for AIR-stored apples after 15 weeks + 7 days at 20 ºC, although did not significantly differ from those for fruit kept in SCA and ULO atmosphere, or for that stored under AIR for 15 weeks plus 1 day at 20 ºC (Table 5). In spite of not being significantly different from those for cold storage in SCA and ULO atmosphere, this maximum score was related to the highest concentrations of the aroma volatile compounds.

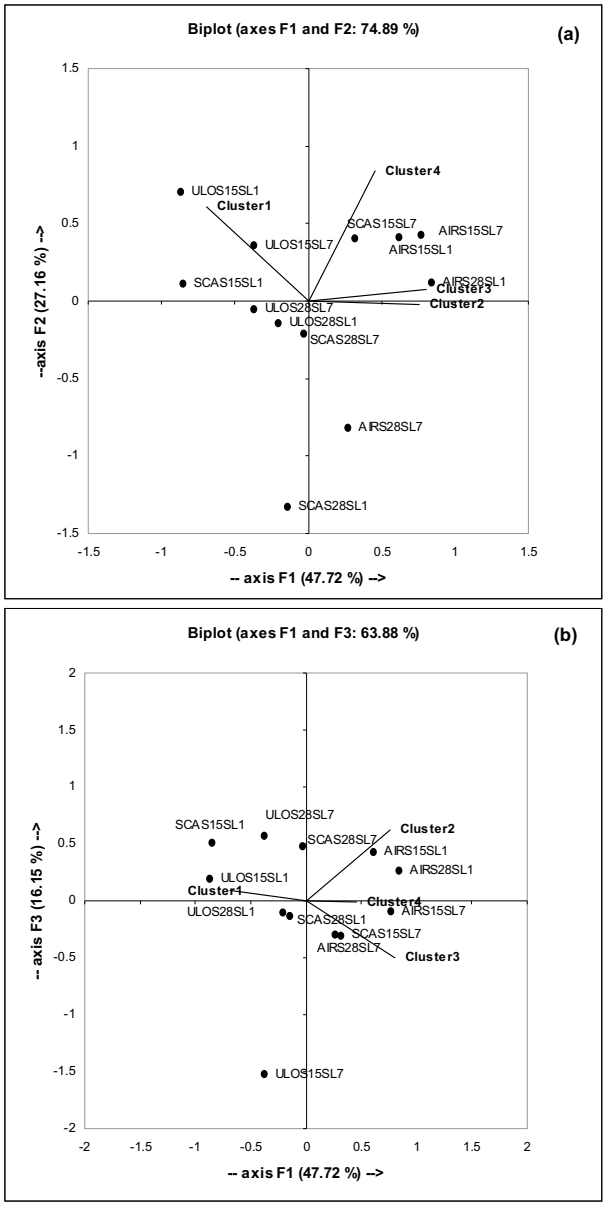

Figure 3. Biplot model for cold-stored fruit including consumer clusters (a) PC1 vs PC2 (b) PC1 vs PC3 (S15 and S28: 15 and 28 weeks of storage; SL1 and SL7: 1 and 7 days at 20 ºC). AIR: 21 kPa O_2 : 0.03 kPa CO_2, ULO: 1 kPa O_2 : 2 kPa CO_2, SCA: 2.5 kPa O_2 : 3 kPa CO_2).

As seen in Figure 4, the esters most closely related to the acceptance of AIR samples were propyl acetate, propyl hexanoate, butyl acetate, butyl butanoate, butyl hexanoate, butyl 2-methylbutanoate, hexyl propanoate, hexyl butanoate, 2-methylpropyl acetate, 2-methylbutyl acetate, 2-methylbutyl propanoate and hexyl 2-methylbutanoate. Coinciding with a previous report of 'Pink Lady®' apples, hexyl 2-methylbutanoate, hexyl propanoate, and butyl 2-methylbutanoate were found to have most influence on consumer acceptance [López et al., 2007]. Ethyl 2-methylbutanote had a positive influence on the acceptance of AIR-stored apples after 28 weeks plus 7 days at 20 ºC (Fig. 4), while that of SCA-stored apples after 15 weeks plus 7 days at 20 ºC were related to greater hexyl acetate emissions.

Several authors studied that although AIR-stored apples showed the highest emissions of volatile compounds, they were not always the fruit most appreciated by the panellist (Aaby et al., 2002; Echeverría et al., 2004d). For that reason, it is believed that the concentration of certain aroma volatile compounds is more important than total aroma volatile emission in determining the general acceptability of fruit. Accordingly, the contribution of each compound to the specific aroma profile of 'Pink Lady®' apples depends both on the odour threshold above which the compound can be detected by smell and the presence of other compounds. It is possible that differences in sensorial acceptance could also be due to changes in other attributes, such as flesh firmness, soluble solids content and titratable acidity and the different atmospheric conditions applied in other studies by Echeverría et al. (2004d).

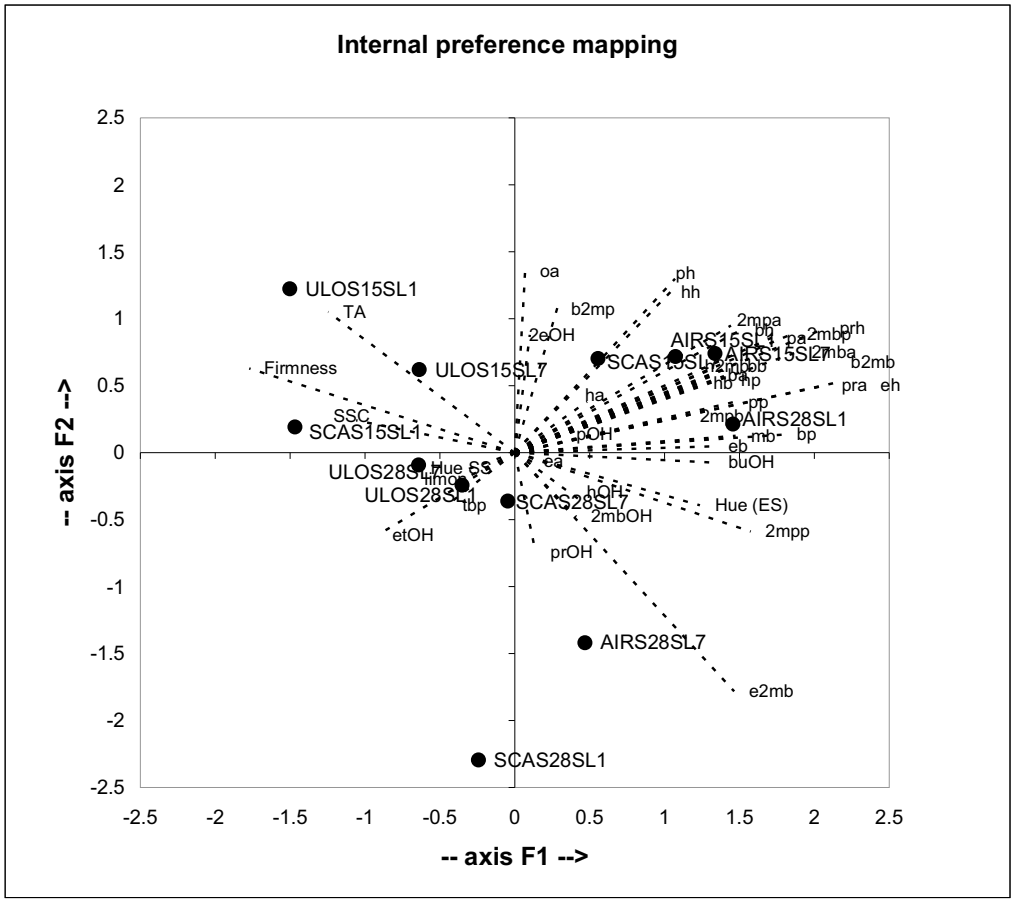

Figure 4. Internal preference mapping of perception of apple acceptability and instrumental variables (S15 and S28: 15 and 28 weeks of storage; SL1 and SL7: 1 and 7 days at 20 ºC; Hue: hue angle (exposed side), a* + b*: shaded side, TA: titratable acidity; SSC: soluble solid content). AIR: 21 kPa O_2 : 0.03 kPa CO_2, ULO: 1 kPa O_2 : 2 kPa CO_2 , SCA: 2.5 kPa O_2 : 3 kPa CO_2).

In conclusion, 65.5% of the consumers involved in this study preferred 'Pink Lady®' apples displaying high emissions of aroma compounds. Another group of consumers showed a preference for fruits with high firmness and acidity values. Even though AIR-stored apples had the lowest firmness and acidity values, they obtained the best levels

of acceptance after 15 weeks of storage, which was related to their high levels of aroma volatile production. It is important to underline the need to take into account several factors that seem to have a significant influenece on consumer acceptance: the presence of a good balance amongst the volatile compounds that make an important contribution to the aroma profile. For this variety, a period of 17 days of shelf life after a long period of storage in a controlled atmosphere allowed the regeneration of the mostcharacteristic esters.

Acknoledgements

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CAPÍTOL 5

Regeneration of aroma volatile compounds in 'Pink Lady®' apples after long-term storage following low and ultra low atmosphere.

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SUMMARY

'Pink Lady®' (*Malus* × *domestica* Borkh.) apples were harvested at commercial maturity and stored at 1 ºC 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) for 13 or 27 weeks. The standard quality parameters, sensory attributes and volatile compound emissions of fruits were evaluated after cold storage plus 1 and 7 days at 20 ºC. Multivariate analysis showed that the parameters positively influencing acceptability were ethyl hexanoate, 2-methylpropyl propanoate and soluble solid content. Results of consumer acceptance revealed that the highest scores were for fruit subjected to LO and ULO conditions for short- and long-term storage plus 7 days to 20 ºC, while the lowest scores were for fruit subjected to AIR conditions. The extra period of 31 weeks in an AIR atmosphere after ULO storage resulted in an increase in the concentration of the compounds that most contribute to the flavour of 'Pink Lady®' apples.

Keywords: acceptability, aroma compounds, regeneration, standard quality parameters.

1. Introduction

Numerous investigations have been carried about the composition of the volatile compounds of apples, since the aroma is an important factor affecting the final sensory quality of fruit produce and hence consumer satisfaction.

Apples are often held for several months at low temperature either in air or in controlled-atmosphere (CA) storage. During CA storage, the production of volatiles decreases and the capacity for their production after storage decline (Willaert et al., 1983; Brackmann et al., 1993; Yahia et al., 1990; Mattheis et al., 1991; Hansen et al., 1992; Mattheis et al., 1995; Plotto et al., 2000; Fellman et al., 2000; Aaby et al., 2002; Fellman et al., 2003), and there can be some increase in volatiles when they are subsequently placed into a regular atmosphere (Hansen et al., 1992; Brackmann et al., 1993, Plotto et al., 2000; Fellman et al., 2003; Altisent, 2008). Reduced emission of aroma volatiles has been reported as the factor most likely responsible for diminished flavour (Smith, 1984), and indeed higher consumer acceptance has been reported to correlate with production of some esters in 'Pink Lady®' apples (López et al., 2007). The severity of these detrimental effects of CA storage on emission of flavour compounds depends on storage atmosphere conditions and time. Lower O_2 and higher CO_2 concentrations and longer storage periods result in greater flavour suppression in apples (Streif and Bangerth, 1988; Brackmann et al., 1993; Fellman et al., 2000).

'Pink Lady' apples maintained good quality characteristics during at least 8 months storage in air and started to decay after removal from storage at 8 months (Saftner et al., 2005). The best controlled-atmosphere parameters in 'Pink Lady®' apples were 2-3% O_2 and 1.5-2% CO_2 for 6 months (Vayesse and Laudry, 2000). However, because CA storage strongly inhibits aromatic volatile production, the flavour quality, at least, of 'Pink Lady®' apples would probably have been compromised (Saftner et al., 2005).

It is very well-known the studies about the standard quality of 'Pink Lady®' apples in relation to maturity at harvest and controlled atmosphere during storage (De Castro et

al., 2007a), as well as the studies about how the CO_2 induces flesh browning (De Castro et al., 2007b, De Castro et al., 2008). Additionally, other studies compared the sensory quality of 'Pink Lady®' with that of four standard late-harvest apple cultivars (Corrigan et al., 1997). According to López et al. (2007), the parameters having most influence on acceptability of 'Pink Lady®' apples were soluble solid content, hexyl 2-methylbutanoate, hexyl hexanoate, hexyl propanoate, butyl 2-methylbutanoate and titratable acidity.

Because of the lack of recent studies relating aroma compounds, quality parameters and sensory evaluation and its importance for characterizing the 'Pink Lady®' apples after different storage conditions, we focus this work on evaluating aroma compounds and sensory evaluation of apples stored under different conditions, and of finding out the instrumental measurements having most influence thereupon and to verify whether an additional period under cold air conditions after controlled atmosphere storage could regenerate some of the aroma volatile compounds in 'Pink Lady®' apples.

2. Materials and methods

2.1. Plant material and storage conditions

Apple (*Malus domestica* Borkh. cv. 'Pink Lady®') fruits were hand-harvested at commercial date (27th October 2005, corresponding to 214 days after full bloom) from 7 year-old trees grown on M-9 EMLA rootstock at a commercial orchard in Lleida (NE Spain). Immediately after harvest, four lots (100 kg each) of apples were selected in accordance with the Association Pink Lady Europe (diameter >70 mm; 50% of diffuse pink or 30% intense pink; background colour: turning from green to yellow; starch index 5-5.8 in a 1-10 scale; flesh firmness > 80 N; and absence of defects).

Three of these lots were stored at 1 ºC and 92-93% relative humidity in three different conditions: AIR (21 kPa O_2 + 0.03 kPa CO_2) and controlled atmospheres (2 kPa O_2 + 2

kPa CO_2 and 1 kPa O_2 + 1 kPa CO_2). Samples were removed from storage after 13 or 27 weeks and transferred at 20 ºC to shelf life period when the analyses were carried out after 1 and 7 days. A fourth batch of fruit was kept 27 weeks in CA storage followed by a further 4 weeks in AIR (LO+4w and ULO+4w).

2.2. Analysis of volatile compounds

Eight kilograms of apples (2 kg × replicate × 4 replicates) per treatment (atmosphere × storage period × shelf life period) were 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 de-adsorbed 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 series II gas chromatograph equipped with a flame ionisation detector (GC-FID) and a polyethylene glycol column with cross-linked free fatty acid as the stationary phase (FFAP; 50m × 0.2mm i.d. × 0.33µm) into which a volume of 1 µL of the extract was injected in all the analyses. The oven program was set at 70 ºC (1 min), and the temperature was first raised by 3 ºC min^{-1} to 142 ºC and later by 5 ºC min^{-1} to 225 ºC. It was then kept constant for 10 min at this later 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 ºC. Compounds were identified by comparing their respective retention indexes 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 (Hewlett Packard 5890) was used for compound confirmation, using the same capillary column was used as in the GC-FID analyses. Mass spectra were obtained by electron impact ionisation 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 μg kg^{-1}.

2.3. Standard quality parameter analyses

Twenty fruits per treatment were individually assessed for the analyses of flesh firmness, soluble solids content (SSC), titratable acidity (TA), skin colour, both at harvest and after removal from cold storage (atmosphere × storage period × shelf life period). Flesh firmness was measured on opposite sides of each fruit with a penetrometer (Effegi, Milan, Italy) equipped with an 11-mm diameter plunger tip; results were expressed in N. SSC and TA were assessed in juice pressed from the whole fruit. SSC was determined using a hand refractometer (Atago, Tokyo, Japan), and results were expressed as % sucrose in an equivalent solution. TA was analysed by titration of 10 ml of juice with 0.1 N NaOH to pH 8.1 with 1% (v/v) phenolphthaleine as an indicator, and data are given as g malic acid L^{-1}. Fruit epidermis color was determined with a portable tristimulus colorimeter (Croma Meter CR-200, Minolta Co., Osaka, Japan) using CIE illuminant D_{65} and an 8 mm measuring aperture diameter. Skin color was measured at two points on the equator of each fruit that were 180 ºapart: one on the side exposed to sunlight (ES) and the other on the shaded side (SS). Hue angle was measured on both the side exposed to the sun and on the shaded side and the resulting values were respectively used as measurements of superficial and background color. Starch index was rated determined in twenty apples by dipping of cross-sectional fruit halves in an iodine solution (15 g KI + 6 g I_2 per litre) for 30 s; starch hydrolysis was rated using a 1–10 Eurofru scale (1, full starch; 10, no starch) (Planton, 1995).

2.4. Sensory measurements

For consumer evaluation, the fruit samples removed from each atmosphere and during each storage period were kept in a room at 20 ºC for 1 and 7 days. Twenty apples per treatment (atmosphere × storage period × shelf life period) were used for sensory

analysis. Prior to sensory evaluation, half of each fruit was instrumentally analyzed in relation to its standard quality parameters. Three pieces (one per atmosphere) were placed on white plates and immediately presented to a taste panel of 40 consumers who conducted a sensory evaluation of fruit for both storage and shelf life periods. All 40 participants were the same for all treatments assessed. Consumers were volunteers from the staff working at the UdL-IRTA research institute and students from the University of Lleida. All the test participants were habitual (daily) apple consumers. Each piece was identified with a random three-digit code. The order of presentation of the three fruit parts presented on the white plate was randomized for each consumer. Mineral water was used as a palate cleanser between samples. All evaluations were conducted in individual booths under white illumination and at room temperature. Each consumer assessed all three samples and was asked to indicate his/her degree of liking/disliking using a 9-point hedonic scale (1-dislike extremely to 9-like extremely). The samples could be retested as often as required.

2.5. Statistical analysis

A multifactorial design was used to statistically analyse results. The factors considered were storage period, storage atmosphere, shelf life period, and replication. All data were tested by analysis of variance (GLM-ANOVA procedure) with the SAS program package (SAS, 1988). Means were separated by the LSD test at $p \leq 0.05$. For multivariate analysis, samples were characterized according to average measurements (instrumental analyses) or by taking average scores for all the consumers (sensory analyses). A principal component analysis (PCA) model was performed to provide an easy visualization of the complete data set in a reduced dimension plot. The PCA model included the 12 samples stored for 13 and 27 weeks and 21 variables: 15 volatile compounds (selected by their quantitative importance in the volatile fraction and to have the odour units > 1), firmness, acidity, soluble solids content, hue on both the exposed and shaded side and consumer acceptability. Samples were labelled as specified in Plan material and storage conditions section. The variables analyzed were

labelled as specified in Table 1. Partial least-squares regression (PLSR) was used as a predictive method to relate consumer acceptability (Y) to a set of explanatory variables (X) that contains the volatile compounds, instrumental quality measurements, and sensory attributes within a single estimation procedure. Unscrambler, version 6.11a software (CAMO, 1997) was used to develop these models. As a pretreatment, data were centred and weighted by the inverse of the standard deviation of each variable in order to avoid dependence on measured units (Martens and Naes, 1989). Full cross-validation was run as a validation procedure.

3. Results and discussion

3.1. Volatile compounds emission at harvest and after cold storage

The volatile compounds identified and quantified at harvest are shown in Table 1. A total of 51 compounds were detected, of which 39 were esters (10 acetates, 10 propanoates, 10 butanoates, 6 hexanoates, and 3 octanoates) 9 alcohols, 2 terpenes and 1 aldehyde). Eleven of these compounds were chosen on the basis of having odor units > 1, and thus being likely to have an impact on fruit flavor (Buttery, 1993). All of them were esters, namely ethyl butanoate, ethyl hexanoate, ethyl 2-methylbutanoate, 2-methylpropyl propanoate, 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 its quantitative importance in the volatile fraction ($\geq$ 50 $\mu g\ kg^{-1}$), together with ethyl acetate as an indicator of possible fermentative processes in CA-stored fruit. They accounted for 88% of the total volatile fraction after 7 days at 20 ºC (Table 1) and six of them (hexyl 2-methylbutanoate, hexyl hexanoate, hexyl propanoate, butyl 2-methylbutanoate, butyl propanoate and 2-methylbutyl acetate) have been shown to influence positively the sensory acceptability of 'Pink Lady®' apples (López et al., 2007).

Table 1. **Volatile compounds emitted (μg kg^{-1}), odour threshold (OTH), odour units[a] (in brackets) and odour description for 'Pink Lady®' apples at harvest**

Compound	OTh [b] (μg L^{-1})	1 day at 20 ºC [c]	7 day at 20 ºC [c]	Odour descriptor [b]	Code [d]
Methyl acetate	8300	29.0 a	16.5 b		
Methyl butanoate	76 (b)	2.3 a	2.3 a		
Ethyl acetate	13500	25.5 a	22.1 a	Ethereal-fruity	ea
Ethyl butanoate	1	2.7 a (2.7)	4.2 a (4.2)	Fruity, apple-like	eb
Ethyl hexanoate	1	2.8 a (2.8)	3.9 a (3.9)	Fruity	eh
Ethyl octanoate	-	2.2 b	1.4 a		
Ethyl 2-methylbutanoate	0.006	4.9 a (816.7)	4.8 a (800.0)	Ripe apple	e2mb
Propyl acetate	2000	11.4 b	35.7 a	Pear-raspberry	
Propyl propanoate	57 (b)	5.0 a	17.4 b	Sweet, lift, fruity (c)	
Propyl hexanoate	-	3.7 a	2.7 a	Sweet, fruity (c)	
2-methylpropyl acetate	65	12.0 a	10.9 a	Fruity	
2-methylpropyl propanoate	0.086 (c)	3.5 a (41.9)	3.1 a (36.0)		2mprpr
2-methylpropyl butanoate	-	2.1 b	1.3 a		
2-methylpropyl hexanoate	-	13.8 b	18.6 a		
Butyl acetate	66	96.1 b (1.5)	358.5 a (5.4)	Red apple aroma	ba
Butyl propanoate	25	35.3 b (1.4)	58.5 a (2.3)	Faintly sweet odour	bpr
Butyl butanoate	100	21.2 b	43.6 a	Rotten apple	
Butyl hexanoate	700	80.4 a	93.5 a	Green apple	bh
Butyl octanoate	-	10.5 a	10.3 a		
Tert-butylpropanoate	19	7.7 a	2.7 b		
2-methylbutyl acetate	11	281.6 a (25.6)	379.4 a (34.5)	Banana	2mba
2-methylbutyl propanoate	19	7.6 b	6.4 a		
2-methylbutyl butanoate	-	1.1 a	0.7 a	Ethereal (f)	
3-methylbutyl octanoate	-	4.6 a	2.7 a		
Butyl 2-methylpropanoate	80 (b)	3.2 a	5.1 a	Apple (c)	
Butyl 2-methylbutanote	17	35.9 b (2.1)	108.6 a (6.4)	Fruity, apple	b2mb
2-methylbutyl 2-methylpropanoate	-	3.9 a	1.9 b		
2-methylbutyl 2-methylbutanoate	-	12.5 a	16.1 a		
Pentyl acetate	43	16.0 b	36.8 a	Apple, fruity	
Pentyl propanoate	-	2.2 a	1.7 a		
Pentyl hexanoate	-	9.6 a	8.5 a	Rosal, fresh, sweet (e)	
Hexyl acetate	2	269.8 b (134.9)	811.5 a (405.8)	Fruity	ha
Hexyl propanoate	8	84.5 a (10.6)	113.3 a (14.2)	Apple	hpr
Hexyl butanoate	250	115.7 a	81.9 a	Apple	hb
Hexyl hexanoate	6400 (c)	95.8 a	80.3 a	Apple	hh
Hexyl 2-methylbutanoate	6	163.6 b (27.3)	258.6 a (43.1)	Fresh-green fruity	h2mb
Heptyl acetate	-	1.2 a	0.7 a		
Heptyl 2-methylpropanoate	-	1.9 a	1.3 a		
Octyl acetate	12 (d)	4.1 a	1.6 b	Fruity (f)	
Total esters		**1393.1 b**	**2626.0 a**		
Ethanol	10000 (a)	23.7 a	15.1 b	Slight (c)	
1-propanol	9000	6.1 b	18.2 a	Sweet	
1-butanol	500	13.0 b	26.3 a	Sweet aroma	
1-pentanol	4000	1.5 a	1.1 a	Fatty-green grassy (g)	
1-hexanol	500	2.4 a	1.4 b	Grassy	
2-ethyl-1-hexanol	-	13.1 a	5.8 b		
2-methyl-1-propanol	250	2.0 a	1.3 a	Chemical	
2-methyl-1-butanol	250	8.7 a	8.8 a	Highly diluted-pleasant	
3-methyl-2-butanol	-	1.5 a	1.5 a		
Total alcohols		**72.0 a**	**79.5 b**		
Alpha-pinene		3.5 a	2.4 b	Pine tree (h)	
D-limonene	34	Tr [e]	Tr [e]	Citrus-like	
Heptanal	-	1.9 a	1.5 a	Citrus, strawberry-like (e)	
Total aroma volatile compounds [f]		**1465.1 b**	**2705.5 a**		

[a] Odor units = amount/OTh. Only values >1 are indicated. [b] Odour thresholds and descriptors as rewieved by López et al. (2007), excepting (a) Flath et al. (1967), (b) Takeoka et al. (1990), (c) Burdock (2002), (d) Fazzalari (1978), (e) Mehinagic et al. (2006), (f) Moya-León et al. (2007), (g) Dimick and Hoskin (1982), (h) Buettner and Schieberle (2001). -, not found. [c] Values represent means of four replicates. Means within the same row showing different letters are significantly different at $P \leq 0.05$ (LSD test). [d] Codes used for multivariate analysis. [e] Traces (≤ 0.5 μg kg^{-1}). [f] Total amount of all aroma volatile compounds detected during chromatographic analyses.

The main volatile compound emitted during shelf life at 20 ºC (18% and 30% after 1 and 7 days, respectively) was hexyl acetate, which was hence largely predominant in the aroma profile of 'Pink Lady®' apples (López et al., 2007; Lo Bianco et al, 2008), and conferred a fruity odour (Table 1).

Odour threshold of 11 compounds (listed in table 1) found in the literature were used to calculate the corresponding odour units in order to express the relative contribution of each volatile to the formation of the final aroma. Therefore, aroma of 'Pink Lady®' apples was characterized predominantly by ethyl 2-methylbutanoate, 2-methylpropyl propanoate, hexyl acetate, 2-methylbutyl acetate and hexyl 2-methylbutanoate. In previous works, these ester compounds were considered to be the compounds that most contribute to 'Pink Lady®' flavour (López et al., 2007) and of both peel and flesh tissue at harvest (Lo Bianco et al., 2008). These compounds also reportedly contribute to fresh green and fruity odours (Flath et al., 1967; Dimick and Hoskin, 1982; Plotto, 1998)

The same volatile compounds presents at harvest date, were identified and quantified in the volatile fraction emitted during cold storage of 'Pink Lady®' apples (Tables 1 and 2). The total straight-chain ester compounds were divided in 8 acetates, 4 propanoates, 4 butanoates, 5 hexanoates, and 2 octanoates, while the total branched-chain esters comprised 2 acetates, 6 propanoates, 6 butanoates, 1 hexanoate, and 1 octanoate. A further 10 alcohols were detected after the different storage periods (Table 2).

An increase in total emissions of volatile compounds was observed for LO (3953.1 µg kg^{-1}) with respect to samples stored in ULO (2923.4 µg kg^{-1}) after 13 and 27 weeks of storage plus 7 day at 20 ºC. However, there were not differences in total emissions of volatile compounds between LO and ULO after an additional period of 4 weeks regardless of shelf life period. This would seem to suggest that the capacity of fruit to synthesize volatile compounds was modified by these treatments. The emission of eight esters identified as the most important volatile compounds in quantitative terms in fruit at harvest namely butyl acetate, butyl hexanoate, 2-methylbutyl acetate, hexyl acetate, hexyl propanoate, hexyl butanoate, hexyl hexanoate and hexyl 2-methylbutanoate contributed as minimum of 67.1% (ULO, 27 weeks, 7 days at 20 ºC) and as maximum

of 88.3% (LO, 13 weeks, 1 day at 20 ºC) of total volatile emission after cold storage. Hexyl acetate was the main volatile compound emitted by cold stored fruit; their highest concentrations were after 13 weeks plus 1 day at 20 ºC irrespective of atmosphere conditions (Table 2). Both LO- and ULO-storage resulted in decreased of hexyl acetate concentration throughout cold storage period, therefore, contributing probably to the detrimental influence of CA storage on aroma development of apples.

Butyl acetate, hexyl acetate and 2-methylbutyl acetate have been identified to be primarily responsible for apple aroma in several cultivars including 'Golden Delicious' after cold storage, one of its 'Pink Lady®' parents (Brackmann et al., 1993; López et al., 1998, 1999, 2000;), agreed with the volatile compounds emission predominant in 'Pink Lady®' together with hexyl butanoate and hexyl 2-methylbutanoate reported by Young et al. (2004) and Saftner et al. (2005). Together, hexyl esters were hence largely predominant (49%) in the 'Pink Lady®' apples aroma profile after cold storage (Table 2).

Fruit from LO atmospheres synthesized significantly high amounts of volatile esters when compared to ULO for long-term storage (27 weeks) plus 7 days at 20 ºC (Table 2).

Ripening at 20 ºC favoured the synthesis of the majority of volatile compounds under LO throughout cold storage period as confirms the findings of previous reports (Young et al., 2004), but the ability of fruit to produce volatile compounds during shelf life after cold storage declined as storage time increased beyond 27 weeks. Our results showed an inhibiting effect on the synthesis of hexyl esters after lengthening the shelf life period to 7 days at 20 ºC in the case of long-term storage for ULO-stored fruit (Table 2). Nevertheless, the branched-chain esters, 2-methylbutyl acetate, butyl 2-methylbutanoate and hexyl 2-methylbutanoate showed an increase after shelf life period. These results confirm that production of esters with branched-chains was not suppressed by low O_2. The suppression of volatiles with straight C-chains under ULO could be related to the influence of low O_2 concentrations on lipid metabolism and/or synthesis (Brackmann et al., 1993).

***Table 2*. Aroma volatile compounds (µg kg^{-1}) of 'Pink Lady®' apples after storage from controlled atmosphere storage (kPa CO_2: kPa O_2) after 13 and 27 weeks of storage following 4 weeks in air.**

	Atmosphere	LO	LO	LO	LO	LO+AIR	LO+AIR	ULO	ULO	ULO	ULO	ULO+AIR	ULO+AIR
	Storage (weeks)	13	13	27	27	27+4	27+4	13	13	27	27	27+4	27+4
N°	Days (20 °C)	1	7	1	7	1	7	1	7	1	7	1	7
1	Methyl acetate	7.4 f	17.6 de	20.4 cde	59.3 a	30.2 c	22.4 cde	13.5 ef	22.9 cde	20.0 de	46.1 b	23.5 cd	21.2 cde
2	Methyl butanoate	2.8 bc	2.6 bc	2.8 bc	2.7 bc	5.6 b	2.6 bc	3.5 bc	1.7 c	5.6 b	9.5 a	5.3 bc	4.6 bc
3	Ethyl acetate	32.3 cd	37.0 c	19.9 ef	71.8 a	18.7 ef	26.5 de	36.7 c	37.9 c	24.3 def	49.1 b	17.5 ef	25.0 def
4	Ethyl butanoate	5.9 b	5.0 b	1.0 e	5.7 b	0.8 e	2.0 de	13.0 a	3.1 cd	2.6 cde	4.4 bc	2.3 de	6.1 b
5	Ethyl hexanoate	7.4 ab	nd	2.8 c	10.4 a	3.4 cd	7.0 b	6.8 b	nd	1.5 c	3.0 c	2.0 c	7.6 ab
6	Ethyl octanoate	nd	2.4 bc	0.5 d	3.6 a	0.9 d	3.0 ab	2.1 bc	2.3 bc	0.7 d	1.6 cd	1.4 cd	3.2 ab
7	Ethyl 2-methylbutanoate	16.4 ab	21.1 ab	32.7 ab	18.3 ab	8.2 b	12.1 b	14.6 b	18.4 ab	28.0 ab	16.7 ab	8.0 b	49.5 a
8	Propyl acetate	18.7 b	33.9 a	2.3 c	20.1 b	3.2 c	10.5 c	19.1 b	19.3 b	4.6 cd	8.1 cd	3.8 c	10.9 c
9	Propyl propanoate	10.7 ab	12.5 a	0.6 e	9.6 b	1.6 de	7.1 c	3.9 d	6.5 c	1.0 e	4.0 d	2.4 de	6.7 c
10	Propyl hexanoate	16.3 bc	16.2 bc	nd	26.0 a	3.7 d	23.7 ab	3.9 d	11.1 cd	nd	12.9 c	nd	21.1 ab
11	2-methylpropyl acetate	14.8 de	27.8 ab	7.4 f	30.5 a	20.4 cd	21.0 bc	18.1 cd	23.0 bc	9.2 ef	17.0 c	19.0 cd	19.1 cd
12	2-methylpropyl propanoate	7.3 a	4.1 bc	1.3 de	3.0 cd	1.1 de	2.9 cd	5.3 ab	3.5 bc	0.4 e	4.6 bc	1.2 de	2.8 cd
13	2-methylpropyl butanoate	4.5 a	2.7 abcd	1.1 d	4.0 ab	2.8 abcd	3.4 abc	3.6 ab	2.4 abc	1.4 cd	3.8 ab	2.1 bcd	3.3 abc
14	2-methylpropyl hexanoate	57.7 b	34.5 ef	21.8 g	38.1 de	50.1 bc	24.7 fg	67.7 a	28.8 efg	20.1 g	21.4 g	46.6 cd	28.3 fg
15	Butyl acetate	653.4 a	334.8 c	60.2 e	189.1 d	44.3 e	71.6 e	585.7 b	206.7 d	63.1 e	45.2 e	36.9 e	79.3 e
16	Butyl propanoate	22.7 bc	40.8 a	6.1 ef	35.2 a	4.5 f	16.1 cd	19.3 c	27.8 b	5.0 f	11.5 de	3.0 f	17.8 cd
17	Butyl butanoate	66.1 a	37.7 c	5.6 f	45.5 b	11.4 ef	24.4 d	63.7 a	26.2 d	6.8 ef	12.7 e	8.0 ef	26.0 d
18	Butyl hexanoate	68.1 de	148.1 ab	27.3 ef	167.4 a	39.6 ef	121.1 abc	59.7 def	77.9 cde	12.9 f	51.8 ef	45.6 ef	106.5 bcd
19	Butyl octanoate	19.2 b	13.1 bc	2.8 d	11.3 bcd	5.4 cd	12.2 bcd	31.7 a	21.1 b	3.3 d	5.4 cd	6.2 cd	8.5 cd
20	Tert-butylpropanoate	5.2 cd	8.6 bc	4.5 cd	10.5 ab	7.3 bcd	3.5 d	7.0 bcd	10.2 ab	3.5 d	14.0 a	5.4 cd	7.8 bc
21	2-methylbutyl acetate	414.2 de	562.0 abc	110.4 f	659.4 a	382.7 e	433.8 de	572.5 ab	516.1 bcd	173.7 f	319.9 e	390.2 de	438.8 cde
22	2-methylbutyl propanoate	8.3 bcd	9.9 b	1.6 e	16.9 a	6.8 bcd	15.3 a	3.6 de	9.3 b	2.2 e	9.1 bc	4.4 cde	17.2a
23	2-methylbutyl butanoate	nd	8.6 cd	2.2 e	22.2 a	12.0 bc	19.2 a	1.0 e	8.3 cd	4.4 de	13.3 b	8.7 cd	19.9 a
24	3-methylbutyl octanoate	nd	4.0 de	nd	16.1 a	11.2 bc	14.3 ab	3.6 de	6.2 de	1.7 e	7.0 cd	14.9 ab	18.2 a
25	Butyl 2-methylpropanoate	8.4 bc	25.5 a	nd	22.6 a	11.1 bc	13.0 b	nd	nd	nd	1.4 d	7.0 c	13.0 b
26	Butyl 2-methylbutanoate	26.6 c	83.7 a	5.2 d	60.6 b	2.4 d	9.4 d	30.9 c	52.7 b	8.3 d	23.0 c	nd	4.4 d
27	2-methylbutyl 2-methylpropanoate	2.6 cd	1.5 d	1.2 d	6.3 abc	7.9 ab	5.4 abcd	1.9 d	1.2 d	3.3 cd	7.5 ab	3.8 bcd	8.8 a
28	2-methylbutyl 2-methylbutanoate	nd	20.3 c	3.2 f	35.2 a	12.1 de	30.3 ab	12.8 de	19.3 cd	9.1 ef	22.7 bc	10.1 ef	27.1 bc
29	Pentyl acetate	47.1 ab	45.6 b	9.8 h	43.0 bc	26.3 ef	28.1 de	54.3 a	35.9 cd	13.5 gh	20.5 efg	18.4 fg	25.1 ef
30	Pentyl propanoate	nd	9.0 cde	0.9 g	9.4 cd	16.0 b	34.9 a	4.5 defg	7.0 cdef	2.1 f	3.2 efg	10.9 bc	36.7 a
31	Pently hexanoate	14.3 c	14.9 c	2.3 f	28.4 a	10.8 cde	23.0 b	6.5 ef	12.1 cd	2.2 f	9.1 de	8.8 de	19.4 b
32	Hexyl acetate	2402.7 a	1193.8 b	465.4 de	853.6 c	589.9 d	345.3 ef	2357.0 a	848.9 c	442.4 de	247.8 f	542.9 d	363.7 ef
33	Hexyl propanoate	117.9 b	181.9 a	17.6 g	149.0 b	66.5 def	87.7 cd	52.4 ef	116.6 bc	16.4 g	41.4 fg	53.9 def	82.7 cde
34	Hexyl butanoate	164.9 ab	148.1 b	29.6 f	173.9 a	90.2 cd	92.6 cd	143.8 b	109.3 c	23.7 f	59.0 e	76.7 de	88.0 cd
35	Hexyl hexanoate	162.6 bc	184.5 b	37.2 e	309.8 a	187.9 b	196.2 b	122.7 cd	152.5 bc	27.3 e	84.3 de	171.9 bc	183.1 b
36	Hexyl 2-methylbutanoate	134.9 d	514.2 a	46.1 e	473.2 a	241.6 c	226.8 c	120.9 de	352.8 b	42.0 e	115.2 de	226.2 c	218.4 c
37	Heptyl acetate	nd	15.5 a	3.9 d	13.6 a	8.3 bcd	11.6 abc	16.9 a	13.9 a	4.7 d	7.8 cd	8.2 bcd	14.4 a
38	Heptyl 2-methylpropanoate	nd	2.8 a	0.4 cd	2.5 a	0.5 cd	1.6 b	1.8 ab	1.7 b	0.2 d	0.9 bcd	1.3 bc	1.8 ab
39	Octyl acetate	2.6 bc	2.2 c	1.4 c	5.4 a	2.0 c	3.1 bc	4.1 ab	2.9 bc	1.7 c	2.0 c	2.3 bc	5.0 a
	Total esters	**4544.2 a**	**3828.6 a**	**959.6 e**	**3664.2 ab**	**1949.1 cd**	**2009.3 cd**	**4490.2 a**	**2817.3 bc**	**993.1 e**	**1337.7 de**	**1800.8 de**	**2041.2 cd**
40	Ethanol	18.0 bc	21.5 bc	128.0 a	125.2 a	11.5 c	11.9 c	20.9 bc	22.9 bc	13.7 c	33.2 b	13.7 c	14.3 c
41	1-propanol	6.4 ab	5.2 bc	1.7 efg	7.3 a	1.4 fg	3.4 cde	3.0 def	3.7 cd	0.3 g	0.5 g	0.3 g	0.7 g
42	1-butanol	31.4 c	54.1 a	4.4 e	17.4 d	3.3 e	8.0 e	45.1 b	35.3 c	6.6 e	5.6 e	4.0 e	8.8 e
43	1-pentanol	nd	4.0 cd	1.5 e	9.1 a	7.0 b	4.8 c	4.3 cd	4.8 c	2.5 de	4.5 c	5.9 bc	6.1 bc
44	2-pentanol	nd	2.0 de	1.1 e	3.8 cd	6.0 ab	4.7 bc	4.6 bc	1.9 de	1.5 e	2.3 de	7.4 a	6.0 ab
45	1-hexanol	42.9 a	1.6 ef	8.3 cd	24.7 b	6.0 de	13.3 c	1.2 f	1.7 ef	1.7 ef	6.2 de	5.2 def	12.0 c
46	2-ethyl-1-hexanol	6.9 c	4.4 c	4.4 c	15.3 ab	16.0 a	10.0 bc	7.7 c	4.5 c	7.7 c	14.1 ab	7.4 c	8.5 c
47	2-methyl-1-propanol	3.3 de	5.7 bcd	3.3 de	10.6 a	7.4 bc	8.5 ab	3.8 de	4.9 cde	2.6 e	6.2 bcd	7.9 ab	7.4 bc
48	2-methyl-1-butanol	13.3 b	21.2 a	7.7 c	2.1 e	3.5 de	0.8 e	23.8 a	21.3 a	6.5 cd	1.6 e	3.7 de	1.0 e
49	3-methyl-2-butanol	nd	nd	nd	3.1 b	13.2 a	nd	2.3 b	nd	nd	nd	nd	nd
	Total alcohol	**122.3 bc**	**119.8 bc**	**160.5 b**	**218.5 a**	**75.2 cde**	**65.4 de**	**116.6 bc**	**100.9 cd**	**43.1 e**	**74.2 cde**	**55.6 de**	**64.7 de**
50	Alpha-pinene	nd	3.3 c	3.6 c	7.2 ab	7.7 ab	4.8 bc	2.2 c	3.5 c	5.0 bc	7.6 ab	9.9 a	7.0 ab
51	D-limonene	nd	nd	3.6 c	26.3 a	19.9 ab	20.5 ab	nd	nd	7.7 c	17.9 b	21.3 ab	19.1 ab
52	Heptanal	nd	1.5 a	nd	nd	nd	nd	1.9 a	1.6 a	nd	nd	nd	nd
	Total aroma volatile compounds	**4666.6 a**	**3953.1 a**	**1127.4 cd**	**3916.3 a**	**2051.9 bc**	**2099.9 bc**	**4610.8 a**	**2923.4 a**	**1048.9 d**	**1437.5 cd**	**1887.5 cd**	**2132.0 bc**

[a] Means within each row followed by different letters indicate significant differences between treatments and days at 20 ºC at $P \leq 0.05$, least significant difference (LSD) test. Volatile compounds not detected are indicated as ND and amounts of ≤ 0.5 µg kg^{-1} are indicates as trace (tr).

The inhibiting effect of shelf life could be explained by insufficient substrate availability: substrate is necessary for aroma recovery after cold storage. The pool of available precursors may have been consumed earlier in the shelf life period.

The extra period under AIR conditions helped to enhance the aroma profile of the volatile esters hexyl acetate, hexyl hexanoate and hexyl 2-methylbutanoate after 27 weeks plus 1 day at 20 ºC and ethyl 2-methylbutanoate, butyl hexanoate, hexyl propanoate and hexyl butanoate after 27 weeks plus 7 days at 20 ºC for ULO-stored apples, showing regeneration. However, in general volatile esters decreased under an extra period of 4 weeks in LO (2 kPa O_2 + 2 kPa CO_2). The odour threshold of hexyl acetate (2 μg L^{-1}), hexyl 2-methylbutanoate (6 μg L^{-1}) and hexyl propanoate (8 μg L^{-1}) are likely to contribute to the characteristic fruity, fresh-green and apple odours (Dimick and Hoskin, 1982; Plotto, 1998). It is also important to note that ethyl 2-methylbutanoate is one of the main contributing to the flavour of Delicious apples characterized as having an apple or apple-like aroma (Flath et al., 1967) coupled with an 'impression of ripeness' (Paillard, 1990). This increase in volatile compounds in an AIR atmosphere after CA storage is also notable in 'Jonagold' (Hansen et al., 1992), 'Delicious' (Fellman et al., 2003), 'Royal Gala' (Young et al., 2004) and 'Fuji' (Altisent et al., 2008) where resulted in an increase in the concentration of the compounds that most contribute to the flavour of this variety after ULO storage.

3.2. Standard quality parameters at harvest and after cold storage

Table 3 shows values for standard quality parameters in apples at harvest and after cold storage plus periods of 1 or 7 days at 20 ºC (simulating their commercial life and final quality on reaching potential consumers). Fruit flesh firmness (77.4 N) and background colour at harvest were indicative of an appropriate stage of maturity for long-term cold storage, according to Centre Technique Interprofessionel des Fruits et Légumes (CTIFL) recommendations (Mathieu et al., 1998).

In general, fruit kept under ULO and LO conditions had higher firmness and titratable acidity (TA) than AIR fruits throughout cold storage period, which was indicative of a less advanced stage of ripening. However, no significant were observed for soluble solid content (SSC); fruit subjected to the CA atmosphere also showed higher values of background and superficial colour than those stored in AIR atmosphere after 13 weeks plus 7 days at 20 ºC (indicating more green and red on the epidermis). Extending the shelf life period produced a reduction in firmness and TA values and an increase in SSC values in fruits subjected to AIR. There was also a decline in superficial red colour (Hue ES) values in all the atmospheres studied and an increase in background colour (Hue SS) in fruit subjected to the CA conditions (Table 3).

***Table 3*. Standard quality parameters of 'Pink Lady®' apples at harvest and after storage in different atmospheres plus 1 and 7 days at 20 ºC**

Standard quality parameters	At harvest	Days at 20 ºC	Storage period	AIR	LO	ULO
Flesh firmness (N)	77.4	1	13	63.7 c	75.5 b	79.4 a
			27	60.8 cd	77.4 ab	79.4 a
		7	13	58.8 d	76.4 ab	77.4 ab
			27	54.9 e	79.4 a	78.4 ab
Titratable acidity (g malic acid/L)	5.9	1	13	5.1 bcd	6.0 a	5.4 b
			27	3.8 f	4.8 de	5.3 bc
		7	13	4.5 e	4.5 e	5.0 cd
			27	3.6 f	4.6 e	4.8 de
Soluble solid content (%)	13.9	1	13	14.7 bc	15.3 a	14.9 ab
			27	14.3 c	14.7 bc	14.6 bc
		7	13	14.7 bc	14.6 bc	14.7 bc
			27	14.9 ab	15.0 ab	14.9 ab
Hue angle (º) (shaded side)	156.6	1	13	84.8 bc	77.2 c	79.1 c
			27	91.3 bc	84.6 bc	98.3 b
		7	13	87.5 bc	124.3 a	124.5 a
			27	74.8 c	92.2 bc	86.5 bc
Hue angle (º) (exposed side)	14.8	1	13	34.3 bc	27.5 d	27.8 d
			27	36.6 ab	39.3 a	39.5 a
		7	13	13.0 f	17.8 e	21.8 e
			27	31.8 cd	40.0 a	33.2 bc

Means followed by the same letter for each standard quality parameters are not significantly different at $P \leq 0.05$ (LSD's test).

The lowest level of flesh firmness were found in AIR-stored apples after 27 weeks + 7 days at 20 ºC (54.9 N), which is indicative of a good firmness retention potential of this apple cultivar, even after long storage under AIR. In contrast, TA was badly preserved (3.6 g L^{-1}), which result in low acceptance. Indeed, CA-stored apples showed higher values of consumer acceptance than those stored in an AIR atmosphere after 27 weeks + 7 days at 20 ºC (Table 4). Therefore, storage under CA would appear as necessary in order to maintain satisfactory 'Pink Lady®' quality, in accordance with Drake et al. (2002).

3.3 Relationship between consumer acceptability, standard quality parameters and aroma volatile compounds

The PCA (Figure 1) revealed that 69% of the volatile compounds were located on the right-hand side of the graph which indicate a strong correlation with samples stored under LO conditions after 7 days at 20 ºC. The biplot of PC1 versus PC2 for this full-data model revealed that air-stored samples were clearly separated from those stored under ULO (1 kPa) O_2 along PC1, which accounted for 54% of the total variability (Figure 1). Moreover, samples stored under AIR showed the highest volatile emissions compared to CA-stored samples. Samples stored under LO were characterized by higher emission of ethyl hexanoate, hexyl acetate, hexyl 2-methylbutanoate, 2-methylbutyl acetate and ethyl acetate after 13 and 27 weeks plus 7 days at 20º C (Figure 1). These results are interesting, since some of these compounds stood out for their contribution to the aroma profile of 'Pink Lady®' apples (López et al., 2007).

Results obtained from sensory analyses indicated that the maximum score was for LO- and ULO-stored apples after 13 and 27 weeks + 7 days at 20 ºC (Table 4), agreed with previous studies in 'Gala' (Cliff et al., 1998; Saftner et al., 2002) or 'Fuji' apples (Echeverría et al., 2003). In addition, as found in Figure 1, samples stored under LO and ULO after 13 weeks were characterized by higher acceptability. LO-stored apples after 13 weeks + 1 day at 20 ºC showed a strong correlation with firmness, titratable

acidity, SSC and 2-methlypropyl propanoate. Lower acceptability scores for AIR-stored apples in the case of long-term storage (27 weeks) could have arisen from lower firmness values for these fruit, as the difference between both groups was higher than 19N (Table 4), and it has been reported that the human senses can detect differences in texture between two apples when the difference in firmness is equal or higher than 6N (Harker et al., 2002).

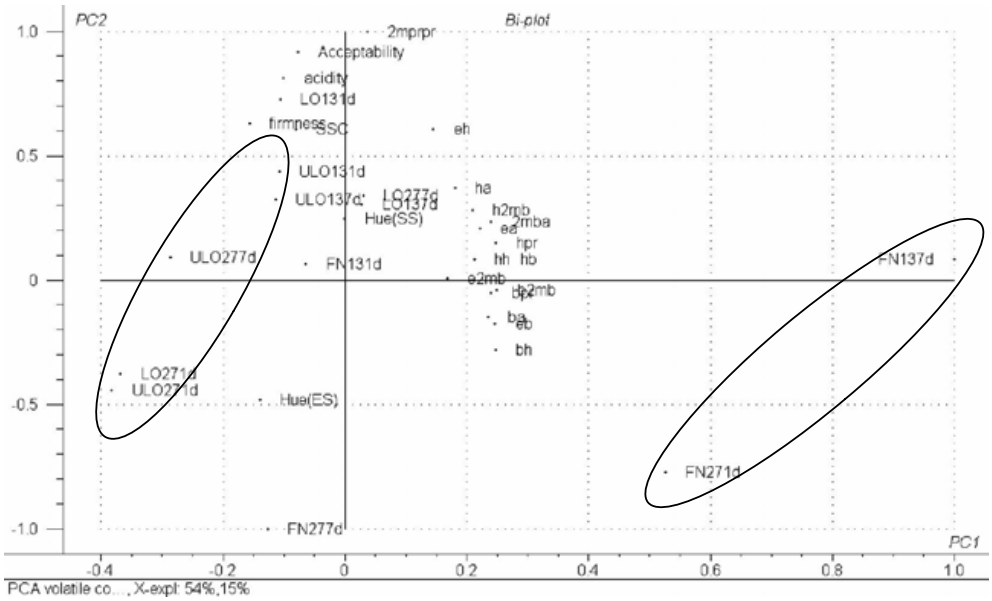

Figure 1. **Biplot (scores and loadings) of PC1 vs. PC2 corresponding to a full-data PCA model for 'Pink Lady®' apples after cold storage. Aroma volatile compounds are coded as indicated in Table 1.**

Table 4. **Global acceptability of 'Pink Lady®' apples after 13 and 27 weeks under different conditions after 1 and 7 days at 20 ºC**

Storage period	Days at 20 ºC	AIR	LO	ULO
13	1	6.8 ab	6.6 ab	6.9 ab
	7	6.2 bc	7.1 ab	7.2 a
27	1	5.4 cd	6.6 ab	6.2 bc
	7	5.0 d	7.1 ab	7.2 a

Means followed by the same capital letters are not significantly different at $P \leq 0.05$ (LSD's test).

Several authors studied that although AIR-stored apples showed the highest emission of volatile compounds; however, not always were the most appreciated fruit among the panellist (Aaby et al., 2002; Echeverría et al., 2004). For that reason, it is believed that the concentrations of certain specific volatile compounds are more important than total aroma volatile emissions in determining overall fruit acceptability. Accordingly, it is important to stand out that we should taking into account possible interactions and synergisms among volatile compounds that seem to be affected by human perception of the fruit flavour and consequently the consumer's acceptability. Additionally, it is possible that the differences in acceptability can also be owed to changes in other attributes like flesh firmness, the soluble solids content and titratable acidity as observed in previous works by Echeverría et al. (2004).

A PLSR model was used in an attempt to correlate acceptability (Y variable) to the standard quality parameters and the chosen aroma volatile compounds (X variables). This procedure allowed a rapid assessment of relationships between the dependent variable (Y) and a set of potentially explanatory variables (X). Higher acceptability scores were associated with fruit exhibiting higher emissions of ethyl hexanoate and 2-methylpropyl propanoate. The most important volatile compounds that showed the lowest weight on acceptability were butyl acetate and butyl hexanoate. The instrumental quality measurements that positively influenced acceptability were firmness, acidity, SSC and hue (shaded side) (Figure 2). This result confirmed the findings reported by Echeverria et al. (2004) suggesting that soluble solids content, titratable acidity, flesh firmness, and background colour of the shaded side had a positive influence on ‘Fuji’ acceptability.

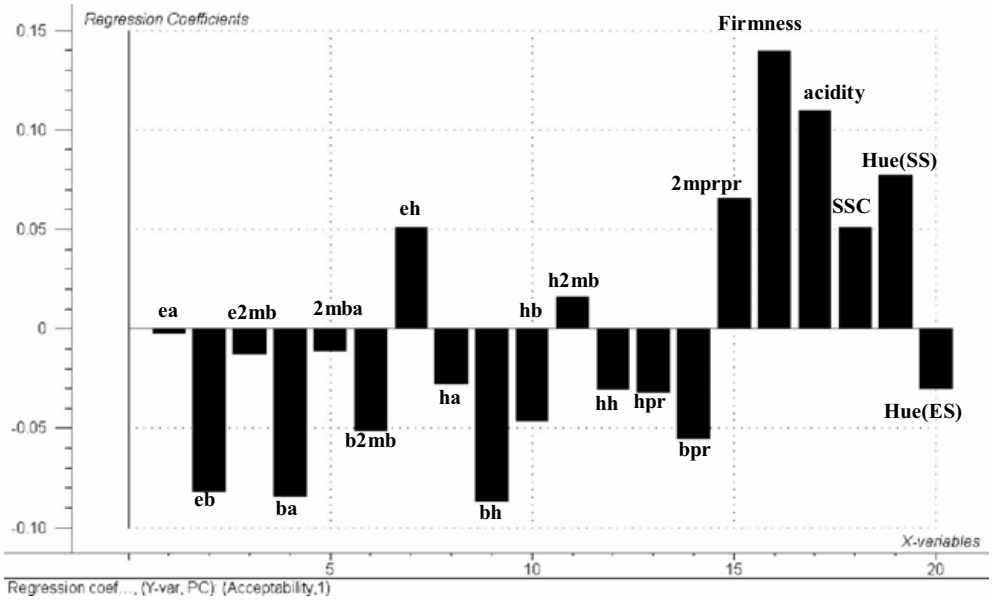

Figure 2. **Regression coefficient plot of PC1 vs. PC2 corresponding to a PLS model for acceptability. Aroma volatile compounds are coded as indicated in Table 1.**

In conclusion, CA-stored fruit displayed significantly lower emissions of most aroma volatile compounds selected in this work. In spite of these losses, and according to the present results, CA storage appears as highly advisable in order to get the best consumer acceptance of 'Pink Lady®' apples throughout cold storage period. Additionally, another group of consumers showed preference for fruit with high firmness, titratable acidity and SSC. It is important to stand out that we should taking into account possible interactions and synergisms among volatile compounds that seem to be affected by human perception of the fruit flavour and consequently the consumer's acceptability. The extra period in AIR conditions after ULO storage allowed the regeneration of the characteristics esters for 'Pink Lady®' apples, which contribute to fruity, fresh-green and apple odours.

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CAPÍTOL 6

Long-term storage of 'Pink lady®' apples modifies volatile-involved enzyme activities: Consequences on production of volatile esters.

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SUMMARY

'Pink Lady' apples were harvested at commercial maturity and stored at 1 ºC and 92% RH 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) for 27 weeks. Data on emission of volatile compounds and on activity of some related enzymes in both skin and flesh tissues were obtained during subsequent shelf life at 20 ºC. Major effects of storage atmosphere and post-storage 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 (AAT), 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 PDC 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 LOX activity and thus to lessened production of 1-hexanol and hexyl esters. Low acetaldehyde availability together with enhanced HPL and ADH levels in these fruit is 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.

1. Introduction

Apple (*Malus* × *domestica* Borkh.) fruit of the 'Pink Lady' cultivar are characterized by brilliant pink skin color, balanced sweet-tart flavor and crunchy texture (Corrigan et al., 1997; Vayesse, 2000; Castro et al., 2005). This variety originated in Australia in 1986, and has become widely accepted owing to its appealing appearance, good sensory quality ratings and potential for long-term storage (Corrigan et al., 1997).

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 (Dixon and Hewett, 2000). The precursors for the main volatile esters produced by apple fruit are derived from the metabolism of fatty acids (Rowan et al., 1999) and specific amino acids (Rowan et al., 1996). Fatty acid-derived substrates originate mainly from lipoxygenase (LOX) activity, ß-oxidation and α-oxidation (Rowan et al., 1999), and previous investigations have shown that the supply of these substrates may be a major limiting factor for the production of aroma volatiles (Song and Bangerth, 2003). 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 (Pérez et al., 1996; Dixon and Hewett, 2000; Wyllie and Fellman, 2000; Olías et al., 2002). 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 (Pérez et al., 1999; Defilippi et al., 2005; Lara et al., 2006; Lara et al., 2007).

Storage of apples under controlled atmosphere (CA) can result in both the enhancement (Mattheis et al., 1991; Yahia et al., 1991; Argenta et al., 2004) and the suppression of

particular flavor volatiles (Yahia et al., 1991; Brackmann et al., 1993; Saquet et al., 2003; Argenta et al., 2004). 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 (Lara et al., 2006; Lara et al., 2007; Fellman et al., 1993), in agreement with reports that treatment of fruit or tissue sections with deuterated flavor precursors (Rowan et al., 1996; Rowan et al., 1999; Matich i Rowan, 2007) or with the vapors of aldehydes, alcohols, and carboxylic acids (Harb et al., 1994; Dixon and Hewett, 2000) significantly enhanced concentrations of the corresponding volatile esters.

Storage of 'Pink Lady' apples under CA with low (2 kPa) or ultra-low (1 kPa) oxygen concentrations, combined with similar CO_2 levels, has been shown to extend commercial life of fruit beyond six months, and to preserve both instrumental (Drake et al., 2002; Brackmann et al., 2005) and sensory quality (Castro et al., 2007; López et al., 2007). 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 (López et al., 2007). These differences were not overcome during the post-storage period at 20 ºC, showing a permanent residual effect of CA on the capacity of fruit for biosynthesis of volatile esters. For other apple cultivars, such as 'Fuji' (Lara et al., 2006) and 'Mondial Gala' (Lara et al., 2007), similar results have been found to arise to some extent from 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.

2. Materials and methods

2.1. Plant material

Apple fruit (*Malus* × *domestica* Borkh., cv. 'Pink Lady') were hand-harvested at a commercial orchard near Lleida (NE Spain). Harvest took place in October 2005, at the usual commercial maturity in the area, corresponding to 214 days after full bloom (dafb). Fruit 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, soluble solids content was 13.9 g 100 g FW^{-1}, and titratable acidity was 5.9 g malic acid L^{-1}. Immediately after harvest, fruit were placed at 1 ºC and about 92% RH under either air or two different controlled atmosphere conditions, namely, 2 kPa O_2/2 kPa CO_2 (low oxygen; LO) and 1 kPa O_2/1 kPa CO_2 (ultra-low oxygen; ULO). The experimental chambers (20 m^3) available at the UdL-IRTA research centre 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 ºC 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).

2.2. Chemical standards and reagents

All the standards for the volatile compounds studied in this work were of analytical grade, and 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).

2.3. 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 de-adsorbed 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 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 ºC, respectively. The analysis was conducted according to the following program: 70 ºC (1 min); 70–142 ºC (3 ºC min^{-1}); 142–225 ºC (5 ºC min^{-1}); 225 ºC (10 min). A second capillary column (SGE, Milton Keynes, UK) 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 extract with authentic samples. The quantification was made using butylbenzene (assay > 99.5%, Fluka) as the internal standard. A GC–MS system

(Hewlett Packard 5890) was used for compound confirmation, in which the same FFAP 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 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}.

2.4. Analysis of acetaldehyde concentration

Juice from 20 fruit 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 ºC until analysis of acetaldehyde content as described in ref (Ke et al., 1994). Frozen juice from each fruit was thawed, and incubated at 65 ºC 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 ºC, injector temperature 180 ºC, detector temperature 220 ºC. Acetaldehyde was identified and quantified by comparison with an external standard, and results were expressed as µL L^{-1}.

2.5. Extraction and assay of volatile-related enzyme activities

Lipoxygenase (LOX), hydroperoxide lyase (HPL), pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH) and alcohol o-acyltransferase (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 (Lara et al., 2003). HPL activity was extracted and assayed according to ref (Vick, 1991). Total protein content in the enzyme extract was determined with the Bradford method (Bradford, 1976), using BSA 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}).

2.6. Statistical analyses

All data were tested by analysis of variance (GLM-ANOVA) according to standard SAS-STAT procedures (SAS, 1987), with storage atmosphere and shelf life period as the main factors. Means were separated by L.S.D. 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 the information contained in the data set was provided by means of principal component analysis (PCA). 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 7.6 software (CAMO ASA, Norway) was used for developing these models. Data were centered and weighed by the inverse of the standard deviation of each variable in order to avoid dependence on measured units (Martens and Naes, 1989), and full-cross validation was run as a validation procedure.

3. Results and discussion

3.1. 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 (Buttery, 1993). 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 its quantitative importance in the volatile fraction ($\geq$ 50µg kg^{-1}), together with ethyl acetate as an indicator of possible fermentative processes in CA-stored fruit, and acetaldehyde 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 2-methylbutanoate, butyl propanoate and 2-methylbutyl acetate) have been shown to influence positively the sensory acceptability of 'Pink Lady®' apples (López et al., 2007).

Selected volatile compounds were used to characterize samples both at harvest and after storage (8 samples × 19 variables) by means of a PCA model. The two first principal components (PC) 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 ºC 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 form ULO-stored apples, and were characterized mainly by higher levels of acetaldehyde and 2-methylbutanol. Air- and LO-stored fruit differentiated along 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.

***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
alpha-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-	1106	1016	-	1.2 a		1.6 a		
2-methyl-1-butanol	1113	667	250	8.7 a		8.8 a		2mbO
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-	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 2methylpropanoate	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 in a FFAP column (Kovats, 1958). [b] Kovats retention indices in a BPX5 column (Kovats, 1958). -, eluted with the solvent. [c] Odor thresholds as reviewed in ref (López et al., 2007), excepting (a) (Flath et al., 1967), (b) (Takeoka et al., 1990), (c) (Guadagni et al., 1966) and (d) (Burdock, 2002). -, 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 (Buttery, 1993). Only values >1 are indicated. [f] Codes used for multivariate analysis. [g] Traces (≤ 0.5 µg kg^{-1}).

As to fruit kept at 20 ºC 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 separated from air- and ULO-stored apples along PC1 (Figure 1).

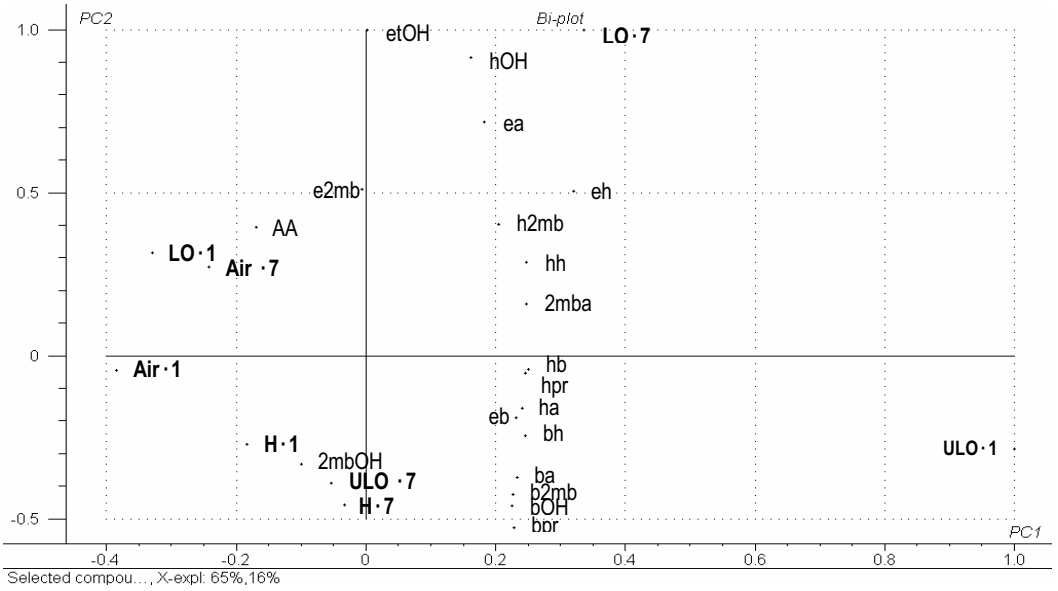

***Figure 1*. Biplot (scores and loadings) of PC1 vs. PC2 corresponding to a PCA model for volatile compounds emitted by 'Pink Lady' apple fruit at harvest (H) and after 27 weeks of storage under different conditions. For sample labels, the numerical suffix refers to the period at 20 ºC (days) following harvest or storage. Volatile compounds are coded as indicated in Table 2 (AA, acetaldehyde).**

Separation among storage conditions was not as broad as for fruit kept at 20 ºC for only 1 day, indicating partial equalization of the capacity for volatile biosynthesis along the post-storage period. The two controlled atmosphere 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 are interesting, since some of these compounds have been found to have a positive influence on the acceptability of 'Pink Lady' apples (López et al., 2007), and indeed acceptability scores of CA-stored fruit were higher than those of samples stored in air (results not shown).

In order 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 acetaldehyde 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. Non-stored 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 acetaldehyde (regression coefficients = 0.67 and -0.50, respectively). Air-stored fruit were characterized by higher levels of acetaldehyde and 2-methylbutanol, in accordance with previous reports on other apple cultivars with long-term storage potential such as 'Fuji' (Lara et al., 2006). Contrarily, preferential accumulation of acetaldehyde in CA- as compared to air-stored fruit has been observed for cultivars not as well suited for extended storage such as 'Mondial Gala' (Lara et al., 2007). Higher emission of ethanol by LO- in comparison to air- or ULO-stored fruit, particularly after 7 days at 20 ºC (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 (Lara et al., 2006). 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 (Lara et al., 2006).

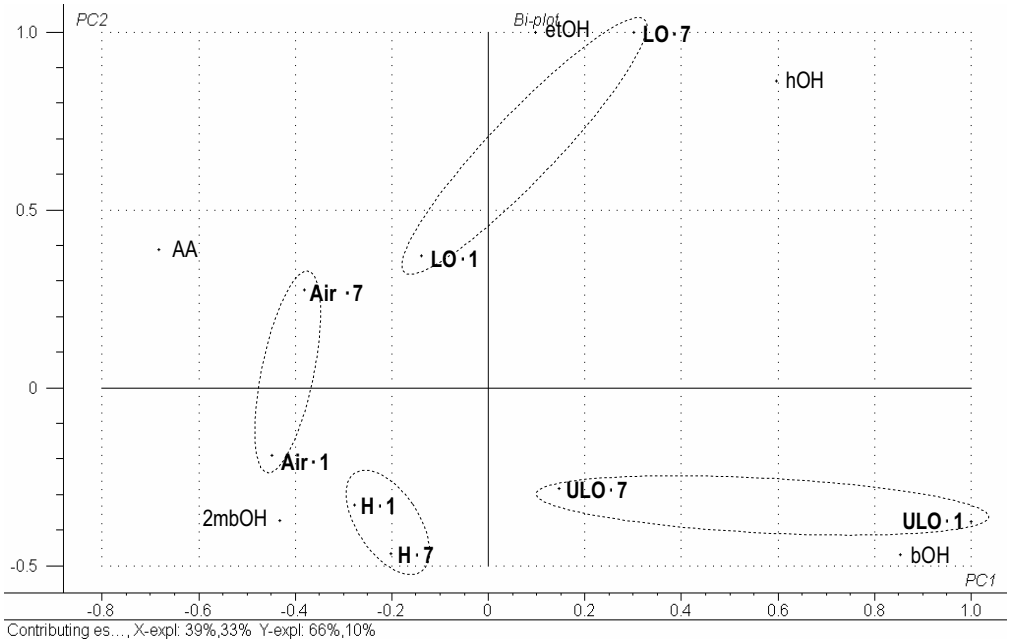

***Figure 2*. Biplot (scores and loadings) of PC1 vs. PC2 corresponding to a PLSR model of volatile compounds emitted (*Y* variables) vs. precursors available in 'Pink Lady' apple fruit at harvest (H) and after 27 weeks of storage under different conditions. For sample labels, the numerical suffix refers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

3.2. Modifications in volatile-related enzyme activities after cold storage of 'Pink Lady' apples.

The good correspondence found between 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 responsible for the production of volatile esters by fruit tissues is the enzyme 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 post-storage period at 20 ºC (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.

***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.**

Shelf life period[a]		0	1	7
Skin	**H**[b]	-	0.345 Ab	0.291 Ab
	Air	0.465 Ab	0.352 Bb	0.462 Aa
	SCA	0.588 Aa	0.431 Bb	0.532 Aa
	ULO	0.530 Aab	0.532 Aa	0.440 Aa
	H[b]	-	0.210 Aa	0.261 Aa
Flesh	**Air**	0.210 A	0.190 Aa	0.187 Ab
	SCA	0.252 A	0.245 Aa	0.251 Aab
	ULO	0.260 A	0.244 Aa	0.221 Aab

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

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' (Lara et al., 2007). 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 flesh tissues (Lara et al., 2007) 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 (Wyllie and Fellman, 2000). The products of all AAT genes isolated to date from fruit, including apple (Souleyre et al., 2005), melon (*Cucumis melo* L.) (Yahyaoui et al., 2002), cultivated strawberry (*Fragaria* × *ananassa* Duch.) (Aharoni et al., 2000), wild strawberry (*Fragaria vesca* L.), or banana (*Musa sapientum* L.) (Beekwilder et al., 2004), 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 (Souleyre et al., 2005), 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.

In order 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 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 (Lara et al., 2006; Lara et al., 2007), 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 one day thereafter both in air- and LO-stored apples, whereas a steady diminution throughout ripening at 20 ºC was noticed for ULO-stored samples, indicating that preservation under ultra-low 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 (Lara et al., 2006; Lara et al., 2007).

***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.**

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

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

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).

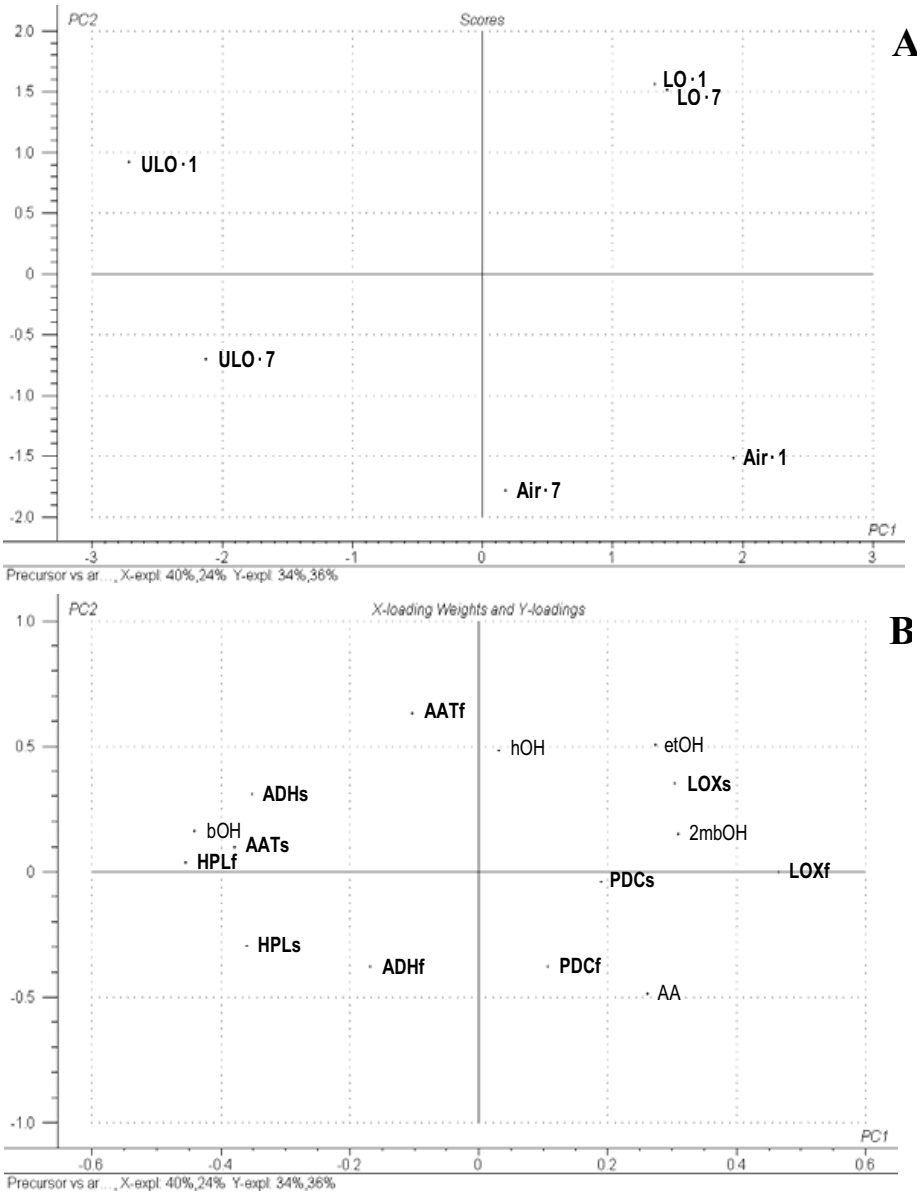

***Figure 3*. Scores (A) and loadings (B) plots of PC1 vs. PC2 corresponding to a PLSR model of precursor availability (*Y* variables) vs. volatile-related enzyme activities in 'Pink Lady' apple fruit after 27 weeks of storage under different conditions. Precursors are coded as indicated in Table 2 (AA, acetaldehyde). For sample labels, the numerical suffix refers to the period at 20 ºC (days) following harvest or storage. For enzyme labels, the suffix 's' or 'f' refers to the activity in the skin or the flesh, respectively.**

Air-stored fruit were characterized by higher PDC levels, which were associated to increased contents of acetaldehyde (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.

***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.**

Shelf life period[a]		0	1	7
Skin	**H[b]**	-	41.49 Aa	18.11 Ba
	Air	11.94 Ab	11.10 Ab	18.07 Aa
	SCA	11.44 Ab	11.48 Ab	14.26 Aab
	ULO	53.63 Aa	11.71 Bb	8.20 Bb
	H[b]	-	26.04 Aa	11.70 Ba
Flesh	**Air**	16.74 Ba	24.94 Aa	10.00 Ba
	SCA	8.69 Ab	8.38 Ab	7.69 Aa
	ULO	14.90 Aab	9.16 Ab	10.50 Aa

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

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, acetaldehyde 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 (Olías et al., 1993).

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 (Mir and Beaudry, 2002). 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 ºC. Furthermore, and with the exception of ethyl butanoate, fruit stored under ULO were not characterized by higher emission of either ethanol or ethyl esters (Figure 1), indicating that other factors in addition to ADH activity are involved in the production of these volatile esters, possibly including substrate supply and/or differential expression of ADH isogenes (Nanos et al., 1992). Actually, it has been reported that ADH is not the limiting factor for ethanol production in pear (*Pyrus communis* L.) fruit stored under hypoxia (Chervin and Truett, 1999).

***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.**

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

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

ULO-stored fruit were also associated to 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 (Pérez et al., 1999). It has been reported that butanal and hexanal are derived from the LOX pathway and/or ß-oxidation (Rudell et al., 2002), and partially purified extracts of apple fruit ADH have been shown to have a higher affinity for acetaldehyde than for

larger straight-chain aldehydes (Bartley and Hindley, 1980). These facts are interesting in the light of results reported herein: increased acetaldehyde contents in air-stored fruit might have out-competed butanal and hexanal for ADH-catalyzed reduction. For ULO-stored samples, in contrast, lower acetaldehyde 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 leading to higher availability of 1-butanol and thus to higher emission of butyl esters by fruit.

***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.**

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

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

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.

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CAPÍTOL 7

Cold storage conditions affect the persistence of diphenylamine, folpet and imazalil residues in 'Pink Lady®' apples.

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SUMMARY

'Pink Lady®' apples (*Malus* × *domestica*) fruit were harvested at commercial maturity treated with three different agrochemical products, and stored at 1 ºC under either air or controlled atmosphere conditions (2.5 kPa O_2 + 3 kPa CO_2 and 1 kPa O_2 + 2 kPa CO_2) for 15 and 28 weeks. Diphenylamine, folpet and imazalil contents in both skin and flesh were simultaneously determined after cold storage plus a simulated marketing period of 1 or 7 days at 20 ºC. Results showed that apples stored in 2.5 kPa O_2 + 3 kPa CO_2 retained higher contents of diphenylamine residues in comparison with those stored in 1 kPa O_2 + 2 kPa CO_2 or refrigerated air. Significant differences in imazalil skin contents were found throughout the simulated marketing period at 20 ºC after storage for 28 weeks in controlled atmospheres.

Keywords: 'Pink Lady®' apples; Residues; Diphenylamine; Fungicides and Cold storage

1. Introduction

'Pink Lady®' is a promising new apple cultivar which originated from a cross between 'Golden Delicious' and 'Lady Williams'. Since 1990, this variety has been extensively cultivated in the main apple-producing areas of the world on account of its good sensory quality (Corrigan et al., 1997). It has been recently introduced in Spain, where its production and marketing are protected by the Association Pink Lady Europe (APLE).

Lleida is the main apple-producing province in Spain, with a total of 550 t/year. Because apples are seasonal and rapidly perishable products, the time available for the commercialization of fresh fruit is limited by ripening and senescence, and the incidence of physiological disorders and postharvest decay, all of which cause important economic losses to apple producers. Storage in controlled atmospheres is a strategy that is widely used in producing areas to extend the commercial availability of fresh apples while preserving their quality and reducing the incidence of physiological disorders. The province of Lleida has a refrigerating capacity of about 2.3 million m^3, of which 70% involves the use of controlled atmospheres.
Controlled atmosphere storage with low (2 kPa) or ultra-low (1 kPa) oxygen concentrations, combined with equal CO_2 levels, extends fruit life beyond 6 months and preserves the good sensory quality of 'Pink Lady®' apples (Brackmannet et al., 2005; Drake et al., 2002; López et al., 2007).

During cold storage, apples may be attacked by a variety of infectious diseases caused by fungi (*Penicillium expansum, Botrytis cinerea and Rhizopus stolonifer*). A series of physiological disorders (e.g., flesh browning and superficial scald) may also appear. These physiological disorders and fungal diseases are important causes of losses during the storage period (Bramlage et al., 1996; Castro et al., 2005). Folpet and imazalil mixtures have proved effective for controlling these diseases (Barkai-Golan, 2001) and are the main products used by producers in Spain.

Folpet [N-(tricloromethilthio) phtalimide] is a nonsystemic fungicide with poor penetration into the epicuticular wax of the grape after treatment (Cabras et al., 2000). Previous studies of folpet residues have been concerned on analytical determination in apple (Gilvydis and Walters, 1991; Navarro et al., 2002). A previous study in 'Golden Delicious' apples suggest greater degradation of folpet in air than in controlled et atmospheres (Palazón et al., 1984).

Imazalil [(±)-1-(α-allyloxy-2,4-dichlorophenylethyl) imidazole] is a systemic fungicide used to control a wide range of fungi on fruit. Some published studies on imazalil have dealt with its feasibility as a treatment against the development of *Penicillium expansum* decay on citrus fruit (D'Aquino, Schirra, Palma, Angioni, Cabras, & Migheli, 2006). However, few studies have focussed on the effect of imazalil (IMZ) residues on apples kept in cold storage. A previous report indicated that IMZ losses declined in controlled atmospheres as compared to air conditions for 'Golden Delicious' apples (Papadopoulou-Mourkidou, 1991) and 'Blanquilla' pears (López and Riba, 1999).

Diphenylamine (DPA) is a diarylamine antioxidant used in a variety of applications, including the control of a physiological storage disorder that affects apples called superficial scald (Smock, 1955). However, in combination, DPA and low O_2 had a synergistic effect, resulting in a ninefold reduction in α-farnesene and the virtual elimination of conjugated triene production over a 28 week period (Whitaker, 2000). Moreover, DPA inhibits CO_2-induced injury (Fernández-Trujillo et al., 2001) and improves the retention of apple firmness during cold storage (DeEll et al., 2005). The persistence of DPA in treated apples, and consequently the levels of its residues in fruit during storage and subsequent marketing, greatly depends on its formulations, the dosage applied, the fruit cultivar, and the storage conditions in question (FAO, 1984). The skin DPA content of DPA-treated apple varieties generally decreased during the storage (Hanekom et al., 1976; Johnson et al.,, 1997; Kim-Kang et al., 1998; Papadopoulou-Mourkidou, 1991; Whitaker, 2000) and post-storage ripening period (Rudell et al., 2006).

As a consequence of postharvest treatments, small amounts of these compounds remain in the fruit. To ensure food safety for consumers, current Spanish legislation (Royal Decree 280/1994; Ministerial Order 1402/2008) and commission directive (08/149/EC) have established the maximum residue limits (MRLs) for fruit: these have been set at 5 mg/kg for DPA, 2 mg/kg for IMZ and 3 mg/kg for folpet. Any new information relating to these aspects will therefore be important for both producers and consumers.

The purpose of this work was to determine the effect of three different atmospheres on the persistence of diphenylamine, folpet and imazalil residues in 'Pink Lady®' apples during storage and post storage ripening in air at 20 ºC.

2. Materials and methods

2.1. Plant material

Apple fruits (*Malus domestica* cv. Pink Lady) were hand-harvested at commercial maturity (corresponding to 226 days after full bloom) from 6 year-old trees grown on M-9 EMLA rootstocks in a commercial orchard in Lleida (NE Spain). Immediately after harvest, 14 boxes each containing 50 apples were selected in accordance with norms established by Association Pink Lady Europe (diameter >70 mm; 50% diffuse or 30% intense pink colour; background colour: turning from green to yellow; starch index 5-5.8 on a 1-10 scale; flesh firmness > 80 N; and absence of defects). Fruits were placed on plastic trays and delivered to the laboratory immediately after harvest.

2.2. Postharvest treatment and storage conditions

Sampling was conducted in agreement with Spanish legislation (Royal Decree 290/2003) and European Union Directives (2002/63/EC). Accordingly, 650 apples were divided into two groups: a subsample of 50 unstored apples was analyzed 1 day after

postharvest treatment in order to assess initial contents of all three compounds, and 600 postharvest treated apples were cold stored.

Postharvest treatment was by drenching for 1 min using an aqueous emulsion of DPA, folpet and imazalil prepared from commercially available products (Productos Citrosol, S.A., Valencia, Spain; Makhteshim Agan España, S.A., Valencia, Spain; and Janssen-Cilag, S.A., Madrid, Spain, respectively). The compositions of the emulsions of the three agrochemicals were 1 g/l for DPA (310 mg/l of active ingredient (a.i.), 1 g/l for folpet (800 mg/l a.i) and 5 g/l for IMZ (375 mg/l a.i), respectively.

The apples were stored at 1 ºC and 92-93% relative humidity in commercial cold storage chambers. Storage atmospheres were air (AIR: 21 kPa O_2 + 0.03 kPa CO_2), controlled atmosphere (CA: 2.5 kPa O_2 + 3 kPa CO_2) and ultra-low oxygen controlled atmosphere (ULO: 1 kPa O_2 + 1 kPa CO_2). The capacity and volume of the commercial cold storage chamber were 180 t and 750 m^3, respectively.

Two lots of 300 fruit (two fruit boxes per atmosphere, 50 fruit per box) was removed from AIR, CA and ULO atmospheres after 15 and 28 weeks, respectively, and placed at 20 ºC to simulate commercial marketing period (SMP). Analyses were carried out one day after the application of postharvest treatments and then after 1 and 7 days at 20 ºC as in the SMP.

Storage chamber atmospheres were established within 72 h of harvest. O_2 and CO_2 concentrations were monitored and automatically corrected using N_2 supplied from a tank and by scrubbing off excess CO_2 using a charcoal system.

2.3. Extraction and quantification of residues

Skin and flesh tissue were separately collected using a potato peeler to compile five samples (three fruit/sample) for each factor (atmosphere x cold storage period x

simulated marketing period). Each skin sample was weighed and the percentage in relation to the whole fruit was calculated. Fifteen grams of skin tissue and 20 g of flesh per fruit were used to obtain each sample. Skin and flesh samples were then frozen in liquid nitrogen, lyophilized, powdered, and kept at -80 ºC until processing. For each extraction, five replicates (of 1 g) of skin and flesh of three fruit each were used. Extractions of DPA, folpet and IMZ were performed using methanol as described by López and Riba (1999) with the addition of 3-nitroaniline as an internal standard.

Identification and quantification of DPA, folpet and IMZ residues was performed in a gas liquid chromatograph (HP 5890 series II, Hewlett-Packard Co., Barcelona, Spain) equipped with a nitrogen phosphorus detector (CG-NPD) and a 5% phenyl-methyl polysiloxane (HP-5MS, 30 m x 0.25 mm i.d., x 0.25 > m film thickness) capillary column, into which a volume of 1 µl of the extract was injected in all analyses. Nitrogen was used as the carrier gas (34 cm/s), with a split ratio of 40 : 1. The injector and detector were held at 250 and 300 ºC, respectively. Analysis was conducted according to the following program: 80 ºC (1 min); 80-180 ºC (30 ºC/min); 180-200 ºC (5 ºC/min); 200-280 ºC (10 ºC/min); and 280 ºC (14 min). Compounds were identified by comparing retention times with established standards and by enriching apple extract with authentic samples. Values were corrected using the internal standard area of 3-nitroaniline (assay >98%, Fluka).

A GC-MS system (Agilent 6890N, Agilent Technologies, S.L., Madrid, Spain) was used for compound confirmation, in which the same capillary column and gradient temperature as in the GC-NPD analyses. Mass spectrometric data were collected in full-scan modes, with a scan range of 40-400 amu and a scan rate of 3.99 scans/s. Mass spectra were obtained by electron impact ionization at 70 eV. Transfer line and manifold temperatures were 300 and 250 ºC, respectively. Helium was used as the carrier gas (34 cm/s), following the same temperature gradient program as described previously. Spectrometric data were recorded (MSD Chemstation D.03.00.611) and

compared with those from the NIST NBS75K original library mass-spectra. Results were expressed as mg/kg.

2.4. Analytical standards

All solvents used for the analytical procedures were of GC grade (Merck, Germany) and were used without further purification. The standards for the identification of agrochemicals were DPA (>99% of active ingredient (a.i.)) obtained from Merck-Schuchardt (Germany), folpet (99.8% a.i.) and IMZ (99.8% a.i.) supplied by Riedel-de Haën® (Germany).

2.5. Statistical analysis

A multi-factorial design, incorporating storage atmosphere (AIR, CA and ULO), storage period (15 and 28 weeks), simulated marketing period (1 and 7 days at 20 ºC), and replication as its factors, was employed to statistically analyze results. All data were tested by analysis of variance (GLM-ANOVA) according to standard SAS-STAT procedures (1988). Means were separated by the least significant difference (LSD) test at $P \leq 0.05$.

3. Results and discussion

3.1. Evolution of diphenylamine during cold storage

The skins of air-stored apples retained lower DPA amounts than samples stored in either CA (2.5 kPa O_2) or ULO (1 kPa O_2). A previous report indicating that DPA loss was reduced in apples stored in ULO as compared to air suggested that DPA content dynamics could be affected by storage environment (Rudell et al., 2006). Somewhat surprisingly, the DPA residue content in skin from CA-stored fruit was higher than for that stored under ULO conditions (Table 1). It is presumably slowly degraded, perhaps

via oxidation (Whitaker, 2000), although in this study, DPA was slowly altered by storage under CA treatment.

The DPA content is influenced by storage period in CA and AIR conditions. Conversely, storage period in ULO conditions did not influence content of DPA. This difference may have been due to apple storage in low O_2 partial pressure reduces metabolism and enzyme-catalysed hydroxylation reactions require O_2 consistent with previous results for 'Braeburn' apples (Mattheis and Rudell, 2008).

Extending storage to 28 weeks plus 1 day at 20 ºC led to a reduction in DPA content in skin tissues (Table 1). This reduction may indicate increased adsorption or metabolism, producing 4OHDPA and smaller amounts of other metabolites (Mattheis and Rudell, 2008; Rudell et al., 2006). Whitaker (2000), observed a decline in DPA content in skin tissue of 'Empire' apples after 15 to 28 weeks of air storage. Moreover, in that study, the rate of decline in DPA was not altered by storage in a low O_2 atmosphere (1.5 kPa O_2).

Initial contents of DPA in apple skins were < 5 mg/kg. These then subsequently declined during ULO storage to 57.9% and 60% after 15 and 28 weeks (Table 1). In contrast, the initial DPA content decreased by only 2.5% in skin of CA-stored fruit after 15 weeks and by around 42% after 28 weeks of storage; this storage was therefore not very effective for the reduction of DPA. This finding contrasts with reports on 'Bramley's' apples stored under 8-10 kPa CO_2 at 4 ºC, in which the DPA content dropped to 12% and 8% of initial contents after storage for 92 and 120 days, respectively (Papadopoulou-Mourkidou, 1991). However, different CO_2 concentrations and temperatures to those considered here may have accounted for such different results. After 15 weeks of storage at 1 ºC, surface DPA contents of air-stored apples were 73.1% of those on entering cold storage (Table 1). These results are in accordance with a previous report by Whitaker (2000), in which DPA on the skin of 'Empire' apples stored in air at 20 ºC decreased by 73%. After 28 weeks of cold storage, the

reduction for air-stored fruit skin was 83.5%, consistent with previous results for 'Granny Smith' apples, in which there was a 95% reduction in initial DPA during 30 weeks of storage at 1 ºC (Papadopoulou-Mourkidou, 1991).

Table 1. **Diphenylamine (mg/kg fresh weight)[a] in skin and flesh 'Pink Lady®' apples**

	Storage (weeks)	Days at 20 ºC	Skin	Flesh
Postharvest treatment	0	1	21.89 ± 6.45	0.10 ± 0.04
Atmosphere				
ULO	15	1	9.22 ± 0.69 cd	0.05 ±0.01 de
(1 kPa O_2 + 2 kPa CO_2)		7	7.75 ± 1.61 de	0.02 ±0.01 f
	28	1	8.31 ± 2.30 d	0.05±0.004 de
		7	7.06 ± 1.49 de	0.03±0.006 ef
CA	15	1	21.52 ± 3.78 a	0.08 ± 0.01 bc
(2.5 kPa O_2 + 3 kPa CO_2)		7	19.24 ± 7.30 a	0.08 ± 0.02 bc
	28	1	12.67 ± 1.20 b	0.11 ± 0.01 a
		7	10.89 ± 1.85 bc	0.11 ± 0.01 a
AIR	15	1	5.88 ± 0.38 e	0.02±0.004 f
(21 kPa + 0.03 kPa CO_2)		7	3.11 ± 0.05 f	0.02±0.002 f
	28	1	2.53 ± 0.38 f	0.09 ± 0.02 ab
		7	1.92 ± 0.31 f	0.06 ± 0.03 cd

[a] Values are means (± SD) of five replicates. Different letters for the same skin or flesh tissue are significantly different at $P \leq 0.05$ (LSD test).

The amounts of DPA detected in flesh samples were very low (Table 1), this is in agreement with previous reports that DPA (≥ 90%) is majority localized in apple skin (Harvey and Clark, 1959; Huelin, 1968). Previous results also reported that the majority of terminal residue, which was largely confined to the skin, consisted of unmetabolized DPA (Kim-Kang et al., 1998). In the present study, the evolution of DPA in flesh was greater in CA-stored samples than for those stored in ULO and air. Extending storage to 28 weeks plus 1 day at 20 ºC led to an increase in DPA flesh content (Table 1). Investigators working with other apple cultivars have reported DPA movement through

the skin into the flesh after 24 weeks of cold storage in a low oxygen atmosphere (tSaoir et al., 2003).

The concentration of DPA in whole fruit was calculated considering the respective percentages of flesh and skin. This amount declined from 1.5 to 0.6 and 0.4 mg/kg after 15 weeks of storage at 1 ºC in ULO and air conditions, respectively (Fig. 1). Thereafter, from 15 to 28 weeks of storage, DPA content declined more gradually to a final concentration of 0.5 mg/l (ULO) and 0.2 mg/l (air). In contrast, after 15 weeks, CA-stored samples maintained higher DPA concentrations than those stored in ULO or air conditions. In all cases, residue levels were lower than the maximum residue limits (5 mg/kg).

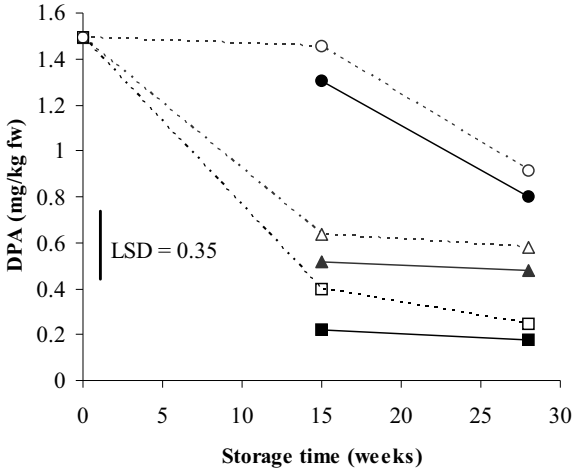

***Figure 1*. Diphenylamine (mg/kg fresh weight) in whole 'Pink Lady®' apples (ULO: 1 kPa O_2 + 2 kPa CO_2; CA: 2.5 kPa O_2 + 3 kPa CO_2) plus 1 or 7 days at 20 ºC (Δ ULO+ 1, ▲ULO+7, ○ CA+1, ● CA+ 7, □ AIR+1, ■ AIR+7). The values referred to whole fruit were calculated considering the respective percentages of flesh (93.6%) and skin (6.4%).**

3.2. Evolution of folpet during cold storage

Folpet content in the skin tissue of 'Pink Lady®' apples decreased sharply after 15 weeks of cold storage plus 1 day at 20 ºC with respect to contents in postharvest treatments, whose reductions were of 82.1, 87.7 and 80.3% in ULO, CA and air-stored samples, respectively (Table 2). Folpet should therefore be considered a non-persistent product, in line with previous report for persistence on grape surfaces (Cabras et al., 2000). In previous experiments, Akiyama, Yoshioka and Tsuji (1998) found Phthalimide as a degradation product of folpet during GC injection. Total recoveries for folpet added to kiwi fruit were 67%, while recovery of the associated degradation product ranged from 9 to 34%. In the current study, with 'Pink Lady®' apples, mean levels of folpet recovery were higher than 88% (skin) and 83% (flesh), however analysis of Phthalimide was not conducted.

Table 2. **Folpet (mg/kg fresh weight)[a] in skin and flesh 'Pink Lady®' apples**

	Storage (weeks)	Days at 20 ºC	Skin	Flesh
Postharvest treatment	0	1	3.40 ± 2.91	0.17 ± 0.10
Atmosphere				
ULO	15	1	0.61 ± 0.51 a	0.03 ± 0.01 ab
(1 kPa O_2 + 2 kPa CO_2)		7	0.45 ± 0.30 ab	0.01±0.008 c
	28	1	0.36 ± 0.13 ab	0.04±0.004 a
		7	0.16 ± 0.09 b	<0.01[b]
CA	15	1	0.42 ± 0.06 ab	0.03 ± 0.01 ab
(2.5 kPa O_2 + 3 kPa CO_2)		7	0.28 ± 0.16 ab	0.02 ± 0.01 bc
	28	1	0.29 ± 0.14 ab	0.02±0.006 bc
		7	<0.16[b]	<0.01[b]
AIR	15	1	0.67 ± 0.45 a	0.03 ± 0.01 ab
(21 kPa + 0.03 kPa CO_2)		7	0.49 ± 0.35 ab	0.02 ± 0.01 bc
	28	1	0.46 ± 0.36 ab	<0.01[b]
		7	0.16 ± 0.09 b	<0.01[b]

[a] Values are means (± SD) of five replicates. [b] Limit of detection. Different letters for the same skin or flesh tissue are significantly different at $P \leq 0.05$ (LSD test).

In skin, we did not find significant differences in folpet contents for any of the three atmospheres studied. The penetration capacities within the fruits were similar for all three cold storage atmospheres (2-7%). Regarding to fruit flesh, folpet contents for CA and air-stored were higher than those for ULO-stored samples after 15 weeks plus 7 days at 20 ºC. Higher oxygen content in the atmosphere therefore seemed to affect the disappearance of folpet from flesh. Extending storage to 28 weeks resulted in a significant decrease in the amount of folpet in flesh for CA- and air-stored samples. The folpet content also decreased during poststorage ripening (Table 2).

Folpet concentration in whole fruit was calculated considering the respective percentages of flesh and skin. This amount declined from 0.38 to 0.05 mg/kg after 15 weeks of cold storage regardless of atmospheres conditions (Fig. 2). In all cases, residue levels were less than the maximum residue limits (3 mg/kg). Palazón et al. (1984) showed a decrease of folpet content in 'Golden Delicious' apples after 6 months of cold stored in air although this decrease was not obtained in controlled atmosphere fruit.

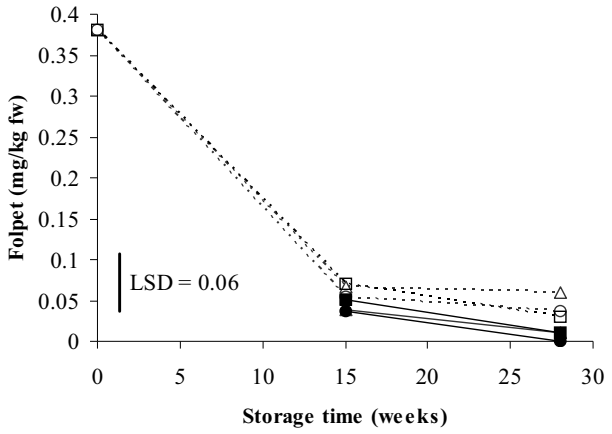

***Figure 2*. Folpet (mg/kg fresh weight) in whole 'Pink Lady®' apples (ULO: 1 kPa O_2 + 2 kPa CO_2; CA: 2.5 kPa O_2 + 3 kPa CO_2) plus 1 or 7 days at 20 ºC (Δ ULO+ 1, ▲ULO+7, ○ CA+1, ● CA+ 7, □ AIR+1, ■ AIR+7). The values referred to whole fruit were calculated considering the respective percentages of flesh (93.6%) and skin (6.4%).**

3.3. Evolution of imazalil during cold storage

Significant changes in IMZ contents were noted in both apple skin and flesh for the different atmosphere, storage period and SMP. After 15 weeks of storage plus 1 day at 20 ºC, the skin of ULO-stored apples retained higher amounts of IMZ than samples stored in CA- or air-stored apples (Table 3). In contrast, IMZ content in apple skin after 28 weeks of cold storage plus 1 day at 20 ºC were not different for the three atmospheres studied. Extending storage to 28 weeks and 1 day at 20 ºC resulted in a significant drop in the amount of IMZ in skin for ULO- and air-stored samples with respect to after 15 weeks. Conversely, samples stored in CA were not affected by extending the storage period from 15 to 28 weeks (Table 3).

Table 3. **Imazalil (mg/kg fresh weight)[a] in skin and flesh 'Pink Lady®' apples**

	Storage (weeks)	Days at 20 ºC	Skin	Flesh
Postharvest treatment	0	1	7.30 ± 1.16	0.20 ± 0.10
Atmosphere				
ULO	15	1	3.72 ± 0.82 a	0.18 ± 0.07 a
(1 kPa O_2 + 2 kPa CO_2)		7	2.50 ± 0.75 bcd	0.12 ± 0.07 bc
	28	1	2.62 ± 0.73 bc	0.20±0.02 a
		7	0.73 ± 0.31 e	0.08 ± 0.04 c
CA	15	1	2.32 ± 0.44 bcd	0.10 ± 0.01 c
(2.5 kPa O_2 + 3 kPa CO_2)		7	1.74 ± 0.85 cd	0.11 ± 0.04 bc
	28	1	1.85 ± 0.46 cd	0.16 ± 0.02 ab
		7	0.36 ± 0.21 e	0.09 ± 0.01 c
AIR	15	1	2.88 ± 0.64 ab	0.12 ± 0.02 bc
(21 kPa + 0.03 kPa CO_2)		7	2.36 ± 0.36 bcd	0.02 ± 0.01 d
	28	1	1.98 ± 1.17 bcd	0.21 ± 0.03 a
		7	1.63 ± 0.54 d	0.09 ± 0.02 c

[a] Values are means (± SD) of five replicates. Different letter for the same skin or flesh tissue are significantly different at $P \leq 0.05$ (LSD test).

Imazalil content in apple skin after drenching with 375 mg/l at 20 ºC decreased to 49% under ULO conditions after 15 weeks plus 1 day at 20 ºC. In contrast, the respective

percentages for samples stored in CA and air were 68.2% and 60.6%. At the end of conservation (28 weeks), IMZ content of surface were 64%, 75%, and 73% on ULO, CA and air-stored apples, respectively (Table 3). Papadopoulou-Mourkidou (1991) reported the residual persistence of IMZ for 'Golden Delicious' apples dipped in a 500 mg/l solution of IMZ and stored under CA conditions: over 80% of total IMZ applied was recovered even after 7 months of storage. Moreover, it has been suggested that low IMZ reduction rates for ULO-stored pears relate to low O_2 levels in the storage atmosphere (López and Riba, 1999).

With regard to fruit flesh, IMZ contents were around 0.02-0.21 mg/kg and were significantly higher after 15 weeks plus 1 day at 20 ºC in ULO-stored samples than for those kept in CA or air (Table 3). Imazalil content in flesh increased after 28 weeks in comparison with 15 weeks of storage plus 1 day at 20 ºC for CA- and air-stored samples. There may have been absorption between the two tissues during storage, as there were decreases in IMZ skin concentrations under the same storage conditions (Table 3). In contrast, fruit flesh stored in ULO was not affected by extending the storage period from 15 to 28 weeks, may be because of apples respond to ULO storage by slowing down the metabolism rate. Simulated marketing period at 20 ºC had a significant effect on IMZ contents in both skin and flesh after storage. IMZ content in skin dropped during SMP in ULO-stored samples at 15 weeks of storage. Additionally, a significant decrease was observed in CA-stored fruit during SMP at 28 weeks of storage. In the case of air-stored apples, no significant differences in IMZ contents were found in skin throughout SMP. In fruit flesh, IMZ contents in ULO and air-stored samples declined during SMP throughout the cold storage period, while no significant differences were noted after 15 weeks for samples stored in CA (Table 3).

Imazalil concentration in whole fruit was calculated considering the respective percentages of flesh and skin. This amount displayed the same trend regardless of atmosphere, with IMZ contents decreasing significantly after 15 weeks of cold storage. No changes in IMZ contents were observed after 28 weeks for any of the atmospheres

(Fig. 3). Likewise, IMZ content was significantly lower after 7 days than for 1 day at 20 ºC, regardless of the atmosphere conditions. In all cases, residue levels were less than maximum residue limits (2 mg/kg).

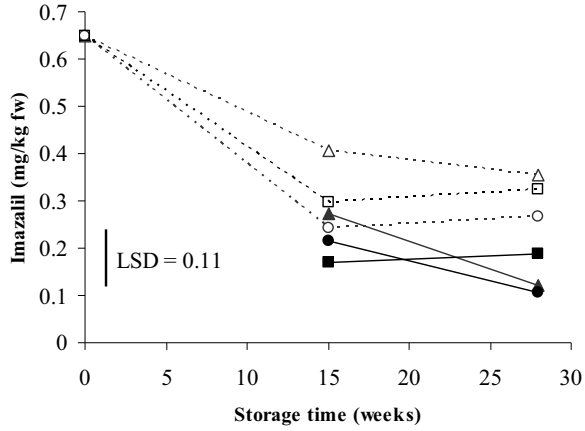

***Figure 3*. Imazalil (mg/kg fresh weight) in whole 'Pink Lady®' apples (ULO: 1 kPa O_2 + 2 kPa CO_2; CA: 2.5 kPa O_2 + 3 kPa CO_2) plus 1 or 7 days at 20 ºC (Δ ULO+ 1, ▲ULO+7, ○ CA+1, ● CA+ 7, □ AIR+1, ■ AIR+7). The values referred to whole fruit were calculated considering the respective percentages of flesh (93.6%) and skin (6.4%).**

4. Conclusions

DPA, folpet and IMZ were mainly retained by the fruit skin, with values for fruit flesh ranging from 0.01 to 0.21 mg/kg. The results of this study show that the O_2 and CO_2 concentrations in the storage period had a significant effect on the persistence of DPA. DPA content in CA-stored apples was higher than in fruit stored in ULO or air. In general, folpet content decreased during storage and simulated marketing period. IMZ content was affected by atmosphere conditions, storage period and simulated marketing period in air at 20 ºC. Moreover, for short storage periods plus one day at 20 ºC, ULO-stored apples retained the highest content of IMZ. Further studies revealing the effect of

different controlled atmospheres within different seasons and cultivars would be useful in order to better understand the DPA, folpet and imazalil persistence of apples.

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CAPÍTOL 8

Influence of the combination of different atmospheres on diphenylamine, folpet and imazalil content in cold-stored 'Pink Lady®' apples.

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SUMMARY

'Pink Lady®' apples were harvested at commercial maturity, treated with three different agrochemical products, and stored at 1 ºC 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 13 and 27 weeks, followed by 4-week storage in air at 1 ºC. Diphenylamine, folpet and imazalil contents in both the skin and flesh were simultaneously determined after cold storage plus simulated marketing periods of 1 and 7 d at 20 ºC. After 27 weeks plus 7 d, diphenylamine and folpet levels in apple skin were lower for fruit stored in low (2 kPa) or air than for those kept under ultra-low (1 kPa) O_2. An additional storage period of 4 weeks in air reduced diphenylamine and folpet contents in whole apples stored for 13 weeks in low O_2 controlled atmosphere. For imazalil, the same result was obtained in apple skins stored for 27 weeks under ultra-low O_2 controlled atmosphere. Differences in diphenylamine and folpet contents were found for skin and flesh samples throughout the simulated marketing period, but there were observable differences in imazalil contents only for flesh samples.

Keywords: 'Pink Lady®' apple; Diphenylamine; Imazalil; Folpet; Controlled atmosphere.

1. Introduction

'Pink Lady®' is a new, late-maturing apple cultivar that maintains high quality after cold storage in air atmospheres (Saftner et al., 2005). It is appreciated for its brilliant pink colour, sweet-tart taste and crunchy texture (Castro et al., 2005) and has become widely accepted due to its high quality flavour characteristics (Corrigan et al., 1997; James et al., 2005).

Controlled atmosphere storage with low (2 kPa) or ultra-low (1 kPa) oxygen concentrations, combined with equal CO_2 levels, extends fruit life beyond 6 months and preserves the good sensory quality of 'Pink Lady®' apples (Brackmann et al., 2005; Drake et al., 2002; López et al., 2007).

During cold-storage, apples may be attacked by a variety of infectious diseases caused by fungi (*Penicillium expansum, Botrytis cinerea, and Rhizopus stolonifer*). The physiological disorders (e.g., flesh browning and superficial scald) may also appear. These are the most important causes of losses during storage (Bramlage et al., 1996; Castro et al., 2005). Folpet and imazalil mixtures have proved effective for controlling these diseases (Barkai-Golan, 2001) and are the main products used by producers in Spain.

Folpet is a contact fungicide that belongs to the phtalimide family and whose penetration of the epicuticular wax of the grape is poor after treatment (Cabras et al., 2000). Similarly, only a small amount of this product appears to enter the cuticle layer of cold-stored tomatoes (El-Zemaity, 1988). A previous study in 'Golden Delicious' apples suggest greater degradation of folpet in air than in controlled atmospheres (Palazón et at., 1984).

Imazalil (IMZ) is a broad-spectrum systemic imidazole fungicide with protective and curative actions against *Gloeosporium* spp. and *Penicillium expansum*. Some published

studies on IMZ have dealt with its feasibility as a treatment against the development of *Penicillium* decay on citrus fruit (Schirra et al., 2005; D'Aquino et al., 2006; Ghosoph et al., 2007). However, few studies have addressed on the effect of imazalil (IMZ) contents on apples kept in cold storage. A previous report indicated that imazalil (IMZ) losses declined in controlled atmospheres as compared to air conditions for 'Golden Delicious' apples (Papadopoulou-Mourkidou, 1991) and 'Blanquilla' pears (López and Riba, 1999).

Diphenylamine (DPA) is a diarylamine antioxidant used in a variety of applications, including the control of superficial scald in apples (Rudell et al., 2005). DPA also inhibits CO_2-induced injury (Fernández et al., 2001) and improves the retention of apple firmness during cold storage (DeEll et al., 2005). The persistence of DPA in treated apples, and consequently the levels of its residues in fruit during storage and subsequent marketing, greatly depends on its formulation, the dosage applied, the fruit cultivar, and the storage conditions in question (FAO, 1984).

As a consequence of postharvest treatments, small amounts of these compounds remain in the fruit. To ensure food safety for consumers, current Spanish legislation (Royal Decree 280/1994) and European Council Directive (08/148/EC) have established maximum residue limits (MRLs) for whole fruit. These have been set at 5 mg kg^{-1} for DPA and IMZ, and 3 mg kg^{-1} for folpet. Any new information relating to these aspects will therefore be important for both producers and consumers.

In this work, we assessed the concentrations of diphenylamine, folpet and imazalil in 'Pink Lady®' apples under different cold storage conditions, and the effect of an additional storage period in an air atmosphere at 1 ºC after controlled atmosphere storages, on the persistence of these compounds.

2. Materials and methods

2.1. Plant material

Apples (*Malus domestica* cv. 'Pink Lady®') were hand-harvested at the commercial maturity (27th October 2005, corresponding to 214 d after full bloom) from 7 year-old trees grown on M-9 EMLA rootstock in a commercial orchard in Lleida (NE Spain). Immediately after harvest, 14 boxes each containing 50 apples were selected in accordance with norms established by the Association Pink Lady Europe (diameter >70 mm; 50 % diffuse pink or 30 % intense pink; background colour: turning from green to yellow; starch index 5 - 5.8 on a 1 - 10 scale; flesh firmness > 80 N; and absence of defects). The fruits were placed in plastic trays and delivered to the laboratory immediately after harvest.

2.2. Postharvest treatment and storage conditions

Sampling was conducted in agreement with current Spanish and EU legislation (Royal Decree 290/2003 and Directive 2002/63/EC). Accordingly, 700 apples were divided into three groups: a subsample of 50 apples was used as control, 50 unstored apples were also analyzed 1 d after postharvest treatment in order to assess initial levels of all three compounds, and 600 postharvest treated apples were cold stored.

Postharvest treatment was by dipping of apples for 1 min in an aqueous solution of DPA, folpet and IMZ prepared from commercially available products (Productos Citrosol, S.A., Valencia, Spain; Makhteshim Agan España, S.A., Valencia, Spain; and Janssen-Cilag, S.A., Madrid, Spain, respectively). The compositions of the emulsions of the three agrochemicals were 1 g L^{-1} for DPA (31 % w/v), 5 g L^{-1} for folpet (80 % w/v) and 1 g L^{-1} for IMZ (7.5 % w/v), respectively.

Fruit samples were stored at 1 ºC and 92-93 % relative humidity in three experimental chambers located at the UdL-IRTA centre. Previously, the cold stores were cleaned using a commercial detergent plus 2 % sodium hypochlorite for 20 min and they were then washed with water. Storage atmospheres were air (AIR: 21 kPa O_2 + 0.03 kPa) and two different controlled atmospheres (CA): low oxygen (LO: 2 kPa O_2 + 2 kPa CO_2) and ultra-low oxygen (ULO: 1 kPa O_2 + 1 kPa CO_2). The capacity and volume of the cold stored chambers were 4000 kg and 22 m^3, respectively.

A first lot of 300 fruit was removed from AIR, LO and ULO atmospheres after 13 and 27 weeks, and placed at 20 ºC to simulate commercial marketing period. A second lot of 350 fruit was kept 13 and 27 weeks in theses atmospheres following by 4 weeks in AIR. Analyses were carried at 1 and 7 d at 20 ºC, thereafter. The control sample of 50 fruit was analysed after 27 + 4 weeks of cold storage plus 1 d at 20 ºC.

Storage under CA began 30 h after harvest, and the target atmospheres were established within 48 h of harvest. O_2 and CO_2 concentrations were monitored continuously and corrected automatically using N_2 from a tank and by scrubbing off excess CO_2 using a charcoal system. The controlled atmospheres were obtained by mixing CO_2 and O_2. All gas mixtures were analysed using an Oxysat 2002 gas analyser type 770 produced by David Bishop (Heathfield, East Sussex, UK).

2.3. Extraction and quantification of diphenylamine, folpet and imazalil contents

Skin and flesh tissue were separately collected using a fruit peeler to compile five samples (three fruits/sample) for each factor (atmosphere x cold storage period x simulated marketing period).

Each skin tissue was weighed and the percentage in relation to the whole fruit was calculated. All the skin tissue and 20 g of flesh per fruit were used to obtain the samples. Skin and flesh samples were then frozen in liquid nitrogen, lyophilized,

powdered, and kept at -80 ºC until processing. Extractions of DPA, folpet and IMZ were performed by methanol as described by López and Riba (1999) with the addition of 3-nitroaniline as an internal standard. For all analysis, five replicates of three fruits each were used.

Identification and quantification of DPA, folpet and IMZ compounds was performed in a gas-liquid chromatograph (HP 5890 series II, Hewlett - Packard Co., Barcelona, Spain) equipped with a nitrogen-phosphorus detector (CG-NPD) and a 5 % phenyl-methyl polysiloxane (HP-5MS, 30 m x 0.25 mm i.d., x 0.25 μm film thickness) capillary column, into which a volume of 1μL of the extract was injected in all analyses. Nitrogen was used as the carrier gas (34 cm s^{-1}), with a split ratio of 40 : 1. The injector and detector were held at 250 and 300 ºC, respectively. Analysis was conducted according to the following program: 80 ºC (1 min); 80-180 ºC (30 ºC min^{-1}); 180-200 ºC (5 ºC min^{-1}); 200-280 ºC (10 ºC min^{-1}); and 280 ºC (14 min). Compounds were identified by comparing retention times with established standards and by enriching apple extract with authentic samples. Quantification was carried out using 3-nitroaniline (assay > 98 %, Fluka) as the internal standard.

A GC-MS system (Agilent 6890N, Agilent Technologies, S.L., Madrid, Spain) was used for compound confirmation, using the same capillary column and gradient temperature as in the (CG-NPD) analyses. Mass spectrometric data were collected in full-scan modes, with a scan range of 40-400 amu and a scan rate of 3.99 scans s^{-1}. Mass spectra were obtained by electron impact ionization at 70 eV. The transfer line and manifold temperatures were 300 and 250 ºC, respectively. The Single Ion Monitoring (SIM) technique was used for MS identification of compounds, and the ions selected for each compound were: m z^{-1} 167, 168, 170 (for DPA), m z^{-1} 76, 104, 147 (for folpet), and m z^{-1} 173, 215, 249 (for imazalil). Helium was used as the carrier gas (34 cm s^{-1}), following the same temperature gradient program as previously described.

2.4. Reagents and analytical standards

All solvents used for the analytical procedures were of GC grade (Merck, Germany) and were used without further purification. The standards for the identification of agrochemicals were DPA (> 99 % of active ingredient (a.i.)) obtained from Merck-Schuchardt (Germany), folpet (99.8 % a.i.) and IMZ (99.8 % a.i.) supplied by Riedel-de Haën® (Germany).

2.5. Statistical analysis

A multi-factorial design, with storage atmosphere, storage period, simulated marketing period, and replication as factors, was employed to statistically analyze the results. All data were tested by analysis of variance (GLM-ANOVA) according to standard SAS-STAT procedures (1988). Means were separated by a L.S.D. test at $P \leq 0.05$.

3. Results and discussion

3.1. Diphenylamine contents after storage

After 13 weeks of storage in air plus 1 d at 20 ºC, the skin of air-stored 'Pink Lady®' apples retained lower amounts of DPA than samples stored in either LO or ULO (Table 1). Extending cold storage to 27 weeks, the skins of air-stored apples only retained less DPA than samples stored in the ULO atmosphere. A previous report indicating that DPA losses were reduced in 'Granny Smith' apples stored in ULO as compared to air suggested that DPA content dynamics could be affected by storage environment (Rudell et al., 2006). Furthermore, in other studies, skin DPA content in 'Empire' apples were not altered by storage under low O_2 (1.5 kPa O_2) as opposed to air conditions, because DPA is only slowly degraded via oxidation after 28 weeks (Whitaker, 2000).

DPA concentration was influenced by storage period in all atmospheres after 1 d at 20ºC. Extending storage to 27 weeks produced a reduction in DPA content in skin tissues (Table 1). Skin DPA contents only decreased during the simulated marketing period at 20 ºC when the fruits were stored in CA for 13 weeks and in CA combined with air for 13 + 4 weeks. Another report indicated that DPA content decreased during post-storage ripening (Rudell et al., 2006) in 'Granny Smith' apples stored for up 6 months in air and ULO atmospheres plus 14 d at 22 ºC. The difference in results between the two studies may have been due to the use of different cultivars, storage conditions, and/or durations of storage.

The amounts of DPA detected in flesh samples were very low (Table 1), this is in agreement with previous reports (Huelin, 1968; Kim-Kang et al., 1998) that DPA residue is majority localized in apple skin. In the present study after one day at 20 ºC of simulated marketing period, the DPA content in fruit flesh decreased during cold storage for all atmospheres.

Initial level of DPA in apple skins was 5.35 mg kg^{-1}. These then subsequently declined during ULO storage to 46 % and 68 % after 13 and 27 weeks (Table 1). In contrast, the initial DPA level decreased by only 32 % in the skin of LO fruit after 13 weeks of storage and by around 76 % after 28 weeks. This finding contrasts with reports on 'Bramley's' apples stored under 8-10 kPa CO_2 at 4 ºC, in which DPA contents dropped to 12 % and 8 % of initial levels after storage for 92 and 120 d, respectively (Papadopoulou-Mourkidou, 1991). However, different CO_2 concentrations and temperatures to those considered here may have been the reason for such different results. Combining CA storage with 4 weeks in air at 1ºC, DPA skin contents respectively dropped to 52 % and 59 % of initial levels after storage for 13 weeks under ULO and LO conditions.

Table 1. **Diphenylamine (mg kg^{-1} fresh weight)[a] in 'Pink Lady®' apples after postharvest treatment and cold storage in different atmospheres plus 1 and 7 d at 20 ºC**

	Storage (weeks)	Days at 20 ºC	Skin	Flesh	Whole fruit[b]
Postharvest treatment	0	1	5.35	0.04	0.44
Atmosphere[c]					
ULO	13	1	2.90 aB	0.03 aA	0.25 aA
	13	7	2.10 aAB	0.02 aA	0.18 aAB
ULO + AIR	13 + 4	1	2.54 aA	0.02 abB	0.21 abA
	13 + 4	7	1.82 aA	0.02 aA	0.16 aA
ULO	27	1	1.73 bA	0.01 bA	0.14 cA
	27	7	1.51 aA	0.01 aA	0.12 aA
ULO + AIR	27 + 4	1	1.82 bA	0.01 bA	0.15 bcA
	27 + 4	7	1.84 aA	0.01 aA	0.15 aA
LO	13	1	3.65 aA	0.03 aA	0.30 aA
	13	7	2.57 aA	0.03 aA	0.22 aA
LO + AIR	13 + 4	1	2.18 bA	0.03 aAB	0.19 bAB
	13 + 4	7	1.37 bA	0.02 abA	0.12 bA
LO	27	1	1.26 cAB	0.01 bA	0.10 cA
	27	7	1.22 bAB	0.01 bA	0.10 bA
LO + AIR	27 + 4	1	1.24 cAB	0.01 bA	0.10 cAB
	27 + 4	7	1.14 bAB	0.01 bA	0.09 bAB
AIR	13	1	1.64 aC	0.03 aA	0.15 aB
	13	7	1.55 aB	0.02 aA	0.13 aB
AIR	13 + 4	1	1.41 aB	0.04 aA	0.14 aB
	13 + 4	7	1.39 abA	0.02 aA	0.12 aA
AIR	27	1	1.11 bB	0.01 bA	0.09 abA
	27	7	0.81 bcB	0.01 aA	0.07 aA
AIR	27 + 4	1	0.75 bB	0.01 bA	0.07 bB
	27 + 4	7	0.62 cB	0.02 aA	0.07 aB

[a] Values are means of five replicates. [b] Values were calculated considering percentages of 92.5 % and 7.5 % in fruit flesh and skin, respectively. [c] ULO: 1 kPa O_2 + 1 kPa CO_2; LO (2 kPa O_2 + 2 kPa CO_2); AIR (21 kPa O_2 + 0.03 kPaCO_2. ULO + AIR: ULO + 4 weeks in AIR. LO + AIR: LO + 4 weeks in AIR. Different small letters for the same tissue and the same atmosphere and day at 20 ºC are significantly different at $P \leq 0.05$ (LSD test). Different capital letters for the same tissue and the same storage period and day at 20 ºC are significantly different at $P \leq 0.05$ (LSD test). $LSD_{skin} = 0.63$, $LSD_{flesh} = 0.02$ and $LSD_{whole\ fruit} = 0.07$.

After 13 weeks of storage at 1 ºC, the levels of surface DPA on apples stored in air were 69 % of those on entering cold storage (Table 1). These results are in accordance

with a previous report by Whitaker (2000), in which DPA contents on the skin of 'Empire' apples stored 15 weeks in air at 0 ºC decreased by 73 %. In our work, after 27 weeks of storage, the reduction for air-stored fruit skins was 79 %.

It is apparent that the uptake and persistence of DPA contents during storage are greatly dependent on cultivar and storage conditions (Johnson et al., 1997; Papadopoulou-Mourkidou, 1991). Similarly stored 'Red Delicious' and 'Granny Smith' apples (1.5 kPa O_2 + 1.9 kPa CO_2 and 1.3 kPa O_2 + 1.5 kPa CO_2, respectively) retained very different levels of DPA after 36 weeks of cold storage (more than 50 % and 30 % of their respective initial contents) (Johnson et al., 1997).

The concentration of DPA in whole apples dipped in a 310 mg L^{-1} DPA solution after harvest declined from 0.44 to 0.15, 0.10 and 0.07 mg kg^{-1} after 27 + 4 weeks of storage under ULO, LO and air conditions, respectively. However, we did not find any significant differences in the levels of DPA for any of the three atmospheres studied when the storage period was 27 weeks (Table 1).

An additional 4 weeks of storage in air at 1 ºC reduced the DPA content in skin tissues and whole fruits stored for 13 weeks under LO atmosphere conditions (Table 1). The DPA concentration in whole apples stored 13 weeks in LO plus 7 d at 20 ºC was lower than in those kept at 1 d at 20 ºC of simulated marketing period. Air atmosphere produced apples containing lower amounts of DPA than in those stored in an ULO atmosphere. These results showed a favourable effect of an extra period of storage in air at 1 ºC after LO storage, with a decrease in DPA content for whole fruit over short storage periods. Twenty-seven weeks of cold storage followed by 4 weeks stored in air at 1 ºC led to a reduction in DPA in air-stored apples with respect to those only stored under ULO conditions.

3.2. Folpet contents after storage

The levels of folpet in 'Pink Lady®' apples after dipping in 800 mg L^{-1} followed by one day of storage at 20 ºC were very low (Table 2). Cabras et al. (2000) reported the presence of folpet in grape epicuticular wax at very low levels (0.02 mg kg^{-1}) 6 d after dip treatment.

Significant changes in folpet concentrations were noted in both apple skin and flesh for different storage atmospheres and periods. After 13 weeks of cold storage plus 1 d at 20 ºC (Table 2), folpet contents in fruit skins were significantly higher in fruit samples stored in ULO than those stored in LO or air. Concentrations of folpet in flesh were higher after 13 weeks plus 7 d at 20 ºC for ULO-stored samples than for those kept in LO or air.

After extending cold storage to 27 weeks plus one day at 20 ºC, folpet contents in fruit skins were not different. Furthermore, when apples were stored under LO and air plus 7 d at 20 ºC, folpet was not detectable in the skins of fruit samples (Table 2). Flesh folpet contents registered in fruit decreased during storage in controlled atmospheres (ULO and LO) and CA following 4 weeks in an air atmosphere.

After 13 weeks of ULO storage, the levels of surface folpet in apples were 40 % of those on entering cold storage (Table 2). In contrast, the respective content for samples stored under LO and air conditions were 70 % and 65 %. Combining CA storage with 4 weeks in air at 1ºC, folpet contents dropped to 70 % and 88 % of initial levels after storage for 13 weeks under ULO and LO conditions, respectively. After 27 weeks, an extra 4 weeks of storage in air at 1 ºC increased these percentages to 88 % (for ULO) and over 99 % (for LO). The greater oxygen content in the atmosphere seemed to influence the observed decrease in this compound in the outer surface of the fruit.

Table 2. **Folpet (mg kg^{-1} fresh weight)[a] in 'Pink Lady®' apples after postharvest treatment and cold storage in different atmospheres plus 1 and 7 d at 20 ºC**

	Storage (weeks)	Days at 20 ºC	Skin	Flesh	Whole fruit[b]
Postharvest treatment	0	1	0.60	0.07	0.11
Atmosphere					
ULO	13	1	0.36 aA	0.03 aA	0.05 aA
	13	7	0.18 aA	0.02 aA	0.03 aA
ULO + AIR	13 + 4	1	0.14 bA	0.03 aA	0.04 bA
	13 + 4	7	0.10 aA	0.02 aA	0.03 aA
ULO	27	1	0.10 bA	0.01 bB	0.02 cA
	27	7	0.08 aA	ND	0.01 bA
ULO + AIR	27 + 4	1	0.10 bA	ND	0.01 dA
	27 + 4	7	0.10 aA	ND	0.01 bA
LO	13	1	0.14 aC	0.03 aA	0.04 aB
	13	7	0.10 aA	0.01 aB	0.02 aB
LO + AIR	13 + 4	1	0.07 aA	0.02 bB	0.02 bC
	13+4	7	0.07 aA	0.01 aB	0.01 bC
LO	27	1	0.06 aA	0.01 cB	0.01 cB
	27	7	ND	ND	ND
LO + AIR	27 + 4	1	ND	ND	ND
	27 + 4	7	ND	ND	ND
AIR	13	1	0.21 aBC	0.02 aB	0.03 aC
	13	7	0.18 aA	0.01 bB	0.02 aB
AIR	13 + 4	1	0.09 bA	0.02 aB	0.03 aB
	13 + 4	7	0.05 bA	0.02 aA	0.02 aB
AIR	27	1	0.08 bA	0.02 aA	0.02 bA
	27	7	ND	0.01 bA	0.01 bA
AIR	27 + 4	1	ND	ND	ND
	27 + 4	7	ND	ND	ND

[a] Values are means of five replicates (ND: not detected). [b] Values were calculated considering percentages of 92.5 % and 7.5 % in fruit flesh and skin, respectively. [c] ULO: 1 kPa O_2 + 1 kPa CO_2; LO (2 kPa O_2 + 2 kPa CO_2); AIR (21 kPa O_2 + 0.03 kPa CO_2. ULO + AIR: ULO + 4 weeks in AIR. LO + AIR: LO + 4 weeks in AIR. Different small letters for the same tissue and the same atmosphere and day at 20 ºC are significantly different at $P \leq 0.05$ (LSD test). Different capital letters for the same tissue and the same storage period and day at 20 ºC are significantly different at $P \leq 0.05$ (LSD test). $LSD_{skin} = 0.11$, $LSD_{flesh} = 0.01$ and $LSD_{whole\ fruit} = 0.01$.

Concentrations of folpet in whole apples subjected to postharvest treatments with a 310 mg L^{-1} solution declined from 1.0 to 0.01 mg kg^{-1} after 27 + 4 weeks of storage under ULO + AIR. Apples that have been stored in ULO atmosphere showed the highest

amounts of folpet after 13 weeks of storage. An additional period of 4 weeks in air at 1 ºC reduced the folpet content in fruits that were subjected to cold storage and then kept for 1 d at 20 ºC (Table 2). When this cold storage period was followed by 7 d at 20 ºC there was a decrease in the folpet content of whole fruits under LO and air atmospheres, but this decrease was not significant under ULO conditions. Palazón et al., (1984) showed a decrease of folpet content in Golden Delicious apples after 6 months of cold stored in air although this decrease was not obtained in controlled atmosphere fruit.

After 27 weeks of cold storage, the lowest folpet content was associated with the LO atmosphere, but extending cold storage to 4 weeks in air at 1 ºC led to a reduction in folpet content in apples stored under LO and air conditions with respect to fruits stored in an ULO atmosphere (Table 2). These results showed the favourable effect of an extra period of air storage at 1 ºC after LO-storage, which produced a reduction in folpet content in whole fruits.

3.3. Imazalil contents after storage

IMZ was more persistent during storage than folpet (Tables 2 and 3). After 13 weeks plus 1 d at 20 ºC, the level of IMZ in 'Pink Lady®' apples only decreased by 10 % with respect to the postharvest treatment in the skin of fruit stored under ULO conditions (Table 3). In contrast, the respective decreases for samples stored in LO and air atmospheres were 20 % and 26 %. Extending cold storage to 27 weeks produced a reduction in IMZ contents of surface to 22 %, 33 %, and 44 % for ULO, LO and air-stored fruits, respectively. This trend is in agreement with previous reports (Cabras et al., 1999; Schirra et al., 2000) which showed that IMZ demonstrated great persistence during the storage of oranges and grapefruits.

Significant changes in IMZ concentrations were noted in both apple skin and flesh for the different storage periods and atmospheres. The results also showed that an extra period of 4 weeks in air at 1 ºC after 27 weeks under ULO atmosphere conditions led to

a reduction in IMZ contents in skin tissues. After 27 weeks plus 1 d at 20 ºC, the skins of air-stored apples retained lower amounts of IMZ than samples stored in ULO (Table 3). Simulated marketing period at 20 ºC did not have a significant effect on levels of IMZ residue in skin after storage.

With regard to fruit flesh, levels of IMZ ranged from 0.14 - 0.44 mg kg^{-1} and were significantly higher after 13 weeks in air-stored samples than for those kept in an ULO atmosphere (Table 3). No significant differences in IMZ concentrations were detected between different periods of storage under controlled atmospheres (ULO and LO). Only the flesh of air-stored apples after 27 weeks plus 1 d at 20 ºC retained lower levels of IMZ than the samples stored for 13 weeks.

Imazalil content in apple flesh after air-storage plus 7 d was lower than those kept at 1 d at 20 ºC, with the exception of fruits stored for 27 weeks.
In whole fruit, we did not find any significant differences in imazalil concentrations for any of three atmospheres studied (Table 3). IMZ content was significantly lower after 27 weeks plus 1 d at 20 ºC than for 13-week storage in an air atmosphere.

Our results indicate that diphenylamine, folpet and imazalil were mainly retained by the fruit skin, with values for fruit flesh ranging from 0.01 to 0.52 mg kg^{-1}. Long periods of cold storage followed by 7 d at 20 ºC produced lower diphenylamine and folpet apple skin contents in low O_2 and air atmospheres than in those kept in ultra-low O_2 atmosphere. However, the same storage period followed by 1 d at 20 ºC produced lower levels of imazalil in the skin of air-stored apples than in samples stored in ultra-low O_2 atmosphere. An extra storage period of 4 weeks in air at 1 ºC after low O_2 atmosphere led to a favourable effect on the reduction of diphenylamine and folpet contents for short storage periods. The imazalil losses in controlled atmospheres are equal as compared to air conditions.

***Table 3*. Imazalil (mg kg^{-1} fresh weight)[a] in 'Pink Lady®' apples after postharvest treatment and cold storage in different atmospheres plus 1 and 7 d at 20 ºC**

	Storage (weeks)	Days at 20 ºC	Skin	Flesh	Whole fruit[b]
Postharvest treatment	0	1	9.10	0.52	1.16
Atmosphere					
ULO	13	1	8.15 aA	0.19 aB	0.79 abA
	13	7	6.93 abA	0.14 aB	0.65 aA
ULO + AIR	13 + 4	1	8.47 aA	0.24 aB	0.86 aA
	13 + 4	7	7.99 aA	0.17 aA	0.76 aA
ULO	27	1	7.10 abA	0.18 aA	0.70 abA
	27	7	6.08 bA	0.15 aA	0.59 aA
ULO + AIR	27 + 4	1	5.70 bA	0.21 aB	0.62 bA
	27 + 4	7	5.34 bA	0.19 aA	0.58 aA
LO	13	1	7.23 aA	0.22 aB	0.75 aA
	13	7	6.66 aA	0.18 aAB	0.67 aA
LO + AIR	13 + 4	1	6.61 aA	0.27 aB	0.75 aA
	13 + 4	7	5.97 aB	0.25 aA	0.68 aA
LO	27	1	6.07 aAB	0.23 aA	0.67 aA
	27	7	5.29 aA	0.21 aA	0.59 aA
LO + AIR	27 + 4	1	5.91 aA	0.26 aAB	0.68 aA
	27 + 4	7	5.63 aA	0.17 aA	0.58 aA
AIR	13	1	6.76 aA	0.44 aA	0.91 aA
	13	7	5.99 aA	0.26 aA	0.69 aA
AIR	13 + 4	1	6.74 aA	0.41 aA	0.88 abA
	13 + 4	7	6.81 aAB	0.24 aA	0.73 aA
AIR	27	1	5.06 aB	0.29 bA	0.65 bA
	27	7	6.10 aA	0.24 aA	0.68 aA
AIR	27 + 4	1	6.11 aA	0.35 abA	0.78 abA
	27 + 4	7	6.27 aA	0.24 aA	0.69 aA

[a] Values are means of five replicates. [b] Values were calculated considering percentages of 92.5 % and 7.5 % in fruit flesh and skin, respectively. [c] ULO: 1 kPa O_2 + 1 kPa CO_2; LO (2 kPa O_2 + 2 kPa CO_2); AIR (21 kPa O_2 + 0.03 kPa CO_2. ULO + AIR: ULO + 4 weeks in AIR. LO + AIR: LO + 4 weeks in AIR. Different small letters for the same tissue and the same atmosphere and day at 20 ºC are significantly different at $P \leq 0.05$ (LSD test). Different capital letters for the same tissue and the same storage period and day at 20 ºC are significantly different at $P \leq 0.05$ (LSD test). $LSD_{skin} = 1.89$, $LSD_{flesh} = 0.10$ and $LSD_{whole\ fruit} = 0.24$.

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CAPÍTOL 9

Influencia del método de tratamiento postcosecha sobre el contenido de difenilamina, folpet e imazalil en manzanas 'Pink Lady®' frigoconservadas: estudio de los desórdenes fisiológicos externos e internos.

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Resumen

Se pretende conocer cómo afecta el método de aplicación postcosecha en el contenido de difenilamina (DPA), folpet e imazalil en manzanas de la variedad 'Pink Lady®' conservadas a 1 ºC en frío normal (21% O_2 + 0.03% CO_2) o en ultra bajo oxígeno (ULO: 1% O_2 + 2% CO_2) durante 13 y 27 semanas. Las concentraciones tanto en piel como en fruto fresco de DPA, folpet e imazalil se determinaron simultáneamente por cromatografía de gases después del tratamiento post-cosecha y tras el almacenamiento frigorífico más 1 y 7 días a 20 ºC, para simular el periodo de comercialización de los frutos. Los niveles de DPA tanto en piel como en fruto fresco fueron significativamente mayores con el método de aspersión respecto al de inmersión durante toda la frigoconservación en ULO y tras 13 y 27 semanas y 1 día a 20 ºC en frío normal. Por el contrario, los niveles de imazalil tanto en piel como en fruto fresco fueron significativamente mayores con el método de inmersión comparado con aspersión durante toda la frigoconservación. El folpet mostró niveles mayores con el método de aspersión en los frutos de frío normal sobretodo tras cortos almacenamientos (13 semanas). La incidencia al escaldado superficial disminuyó significativamente en los frutos conservados en atmósfera controlada en comparación con el frío normal. Para los frutos tratados con difenilamina, sólo los frutos conservados en frío normal mostraron escaldado superficial. No se encontró presencia de pardeamiento interno en ninguna de las campañas estudiadas.

Palabras clave: Antioxidante; Aspersión; Atmósfera controlada; Fungicidas; Inmersión; 'Pink Lady®'.

1. Introducción

La manzana 'Pink Lady®' es una nueva variedad de maduración tardía que se obtiene del cruzamiento entre 'Lady Williams' y 'Golden Delicious' y destaca por su color inconfundible y aroma (James, 2007). La pulpa del fruto es blanca, densa y moderadamente jugosa (Cripps y col., 1993). El análisis sensorial ha indicado que la manzana 'Pink Lady®' tuvo el nivel más elevado de aceptabilidad comparado con otras variedades y que los consumidores estarían dispuestos a pagar más por esta variedad (Corrigan y col., 1997).

Debido al carácter estacional y perecedero de la fruta, el almacenamiento frigorífico permite alargar su período de comercialización, limitando las pérdidas debidas a la senescencia. Lleida es la provincia con mayor producción en manzanas del estado español (550 t/año, en promedio) y posee una capacidad de refrigeración de 2.2 millones de m^3 de los cuales el 70% corresponden a atmósfera controlada. La conservación frigorífica en atmósfera controlada con bajos (2%) o ultra bajos (1%) contenidos en oxígeno, permite extender a 6 meses el periodo de comercialización de esta variedad (Drake y col., 2002; Vayesse y Laudry, 2004; Brackmann y col., 2005), preservando su alta calidad sensorial (López y col., 2007).

Durante el almacenamiento frigorífico, las manzanas pueden ser atacadas por una variedad de podredumbres causadas principalmente por *Penicillium expansum*, *Botrytis cinerea* y *Rhizopus stolonifer*. Además pueden aparecer una serie de desórdenes fisiológicos (descomposición interna, escaldado superficial, etc.). Todo ello ocasiona las mayores pérdidas durante el período de conservación frigorífica (Bramlage y col., 1996; Castro y col., 2005). La combinación de folpet e imazalil se ha mostrado muy efectiva para el control de estas podredumbres (Barkai-Golan, 2001) y por ello su aplicación mezclada está muy extendida entre los productores frigoristas.

La mayoría de los trabajos publicados respecto al contenido de fungicidas en manzanas se centran en la metodología analítica y pocos han estudiado su persistencia durante la conservación frigorífica. Palazón y col. (1984) indicaron que el contenido en folpet en manzanas 'Golden Delicious' disminuye de forma más marcada en condiciones de frío normal y de manera insignificante en atmósfera controlada tras 6 meses de almacenamiento. Lo contrario se obtuvo para el imazalil donde las concentraciones fueron mayores en atmósfera controlada respecto al frío normal (Papadopoulou-Mourkidou, 1991). La difenilamina (DPA) es un antioxidante utilizado en gran variedad de aplicaciones, incluyendo el control del escaldado superficial (Curry y Kupferman, 1993; Rudell y col., 2005). También la DPA inhibe los daños inducidos por el CO_2 (Fernández-Trujillo y col., 2001) y contribuye a retener la firmeza de las manzanas durante su almacenamiento frigorífico (DeEll y col., 2005).

La persistencia de la DPA en manzanas tratadas, y consecuentemente sus concentraciones en la fruta durante su almacenamiento y posterior comercialización, están influidas por el tipo de aplicación (aspersión o inmersión), formulación, dosis, variedad y las condiciones de almacenamiento (FAO, 1984; revisado en Papadopoulou-Mourkidou y col., 1991). La concentración de DPA generalmente disminuye durante la conservación (Hanekom y col., 1976; Papadopoulou-Mourkidou, 1991; Johnson y col., 1997; Kim-Kang y col., 1998; Whitaker, 2000) y durante la maduración post-almacenamiento (Rudell y col., 2006).

Como consecuencia del tratamiento post-cosecha, pequeñas cantidades de éstos compuestos son retenidas en el fruto. Para asegurar la seguridad sanitaria por los consumidores, tanto la legislación española (Real Decreto 280/1994) y comunitaria (Directiva 08/149/CEE; 07/73/CEE) han establecido el límite máximo de residuos (LMRs) en fruta entera (fresca o conservada). Estos LMRs son 5 mg kg^{-1} para la DPA, 3 mg kg^{-1} para el folpet y 2 mg kg^{-1} para el imazalil.

En el presente trabajo se pretende conocer cómo afecta el contenido de DPA, folpet e imazalil en manzanas de la variedad 'Pink Lady®' durante la frigoconservación en frío normal o ultra-bajo oxígeno según el método de aplicación postcosecha y estudiar la incidencia a los desórdenes fisiológicos tras largos periodos de almacenamiento.

2. Material y métodos

2.1. Material vegetal

Las manzanas (*Malus domestica* cv. 'Pink Lady') fueron recolectadas dentro del periodo de recolección comercial en una finca del término municipal de Lleida. Inmediatamente después de la cosecha, 14 cajas de manzanas (con 50 frutos/caja) fueron seleccionados de acuerdo con los estándares de madurez que exige la Asociación Pink Lady Europa (APLE) para poder comercializar la variedad como tal (diámetro > 70 mm; 50% de color rosa difuso o 30% de rosa intenso; color de fondo virando de verde a amarillo; índice de almidón: 5-5.8 (en la escala de 1-10); firmeza de la pulpa > 80 N; y ausencia de defectos).

2.2. Tratamiento postcosecha y condiciones de almacenamiento

La toma de muestras se ha realizado según legislación vigente española (R.D 290/2003) y europea (Directiva 2002/63/CEE). Un total de 500 frutos fueron recolectados y separados en: un control de 50 frutos no tratados, 50 frutos tratados con los fungicidas y el antioxidante, para ser analizados tras permanecer 24 h a 20 ºC y 400 frutos repartidos en tres grupos (cuatro cajas por atmósfera, 50 frutos por caja) para su análisis después de la conservación frigorífica. El tratamiento post-cosecha se realizó por aspersión la primera campaña y por inmersión la segunda durante 1 minuto en una solución acuosa de DPA, folpet e imazalil preparada a partir de los productos comerciales (Productos Citrosol, S.A., Makhteshim Agan España, S.A.y Janssen-Cilag, S.A., respectivamente). Las composiciones de la emulsión fueron de 1 g L^{-1} en DPA (31 % w/v), 5 g L^{-1} en

folpet (80 % w/v) y 1 g L^{-1} en imazalil (7.5 % w/v), respectivamente. El tratamiento se realizó en las instalaciones de la central hortofrutícola FRUILAR (Lleida).
Los frutos se conservaron en cámaras de frigoríficas industriales con una capacidad de 180 t y un volumen de 750 m^3, la primera campaña y en cámaras semicomerciales del centro UdL-IRTA de Lleida con una capacidad de 4 t y un volumen de 22 m^3, la segunda campaña. Las atmósferas ensayadas fueron frío normal (FN: 21% O_2+ 0.03% CO_2) y ultra bajo contenido en oxígeno (ULO: 1% O_2 + 1-2% CO_2). Las muestras se conservaron durante 13 y 27 semanas. Después los frutos fueron trasladados a una cámara climatizada a 20 ºC dónde permanecieron 1 y 7 días, tras los cuales se analizaron simultáneamente las concentraciones en DPA, folpet e imazalil de las muestras.

2.3. Extracción y cuantificación de DPA, folpet e imazalil

Se ha seguido la metodología de extracción descrita por López y Riba (1999). Muestras de 15 manzanas por tratamiento (atmósfera de conservación x periodo de almacenamiento x estancia a 20 ºC) fueron peladas manualmente. La piel de cada fruto y la pulpa han sido pesadas para obtener el porcentaje de piel y pulpa respecto al fruto entero. Toda la piel y 20 g de pulpa fueron congeladas con nitrógeno líquido, liofilizadas, trituradas y conservadas a -80 ºC, separando 5 repeticiones (3 frutos cada una) de piel y pulpa por tratamiento. Cada una de las réplicas, previa adición de 3-nitroanilina como patrón interno, fueron sometidas a una triple extracción con metanol, lavado con agua ultra pura (miliQ), extracción con éter dietílico, separación por decantación, purificación con NaCl y Mg_2SO_4, filtración y evaporación al vacío (15 mbar, 30 ºC). El residuo se recupera con tolueno para proceder a su análisis por cromatografía de gases.

Las identificaciones y cuantificaciones DPA, folpet e imazalil se han realizado en un cromatógrafo de gases (HP 5890 series II, Hewlett-Packard Co., Barcelona) equipado con un detector de nitrógeno-fósforo (GC-NPD) y una columna capilar con 5% fenil-

metil polysiloxano (HP5-MS, 30 m x 0.25 mm (d.i) x 0.25 μm). Se utiliza nitrógeno como gas portador (34 cm s^{-1}) y una relación de 'split' de 40:1. El inyector y el detector se han mantenido a 250 ºC y 300 ºC, respectivamente. Los análisis se han realizado con la siguiente programación de temperaturas: 80 ºC (1 min); 80-180 ºC (30 ºC min^{-1}), 180-200 ºC (5 ºC min^{-1}) y 200-280 ºC (10 ºC min^{-1}) durante 14 minutos. Los compuestos fueron identificados por comparación con los tiempos de retención de patrones analíticos y por enriquecimiento de los extractos con muestras auténticas. La cuantificación se ha realizado por el método del patrón interno (3-nitroanilina, de pureza > 98 %, Fluka).

La confirmación de los compuestos se realizó por espectrometría de masa (GC-EM) (Agilent 6890N, Agilent Technologies, S.L., Madrid) utilizando la misma columna capilar y gradiente de temperaturas que las usadas en GC-NPD. Los espectros de masas se han obtenido por ionización de impacto eléctrico de 70 eV. Se utilizó la técnica de monitorización del ión simple (SIM) para la identificación de los 3 compuestos seleccionando para cada uno de ellos las siguientes masas: m/z 167, 168, 170 (DPA), m/z 76, 104, 147 (folpet) y m/z 173, 215, 249 (imazalil). Se ha empleado helio como gas portador (34 cm s^{-1}).

Todos los reactivos utilizados han sido con un grado de pureza de cromatografía de gases (Merck, Alemania). Las materias activas han sido, difenilamina (>99% de ingrediente activo (i.a)) procedente de Merck-Schuchardt (Alemania), folpet (99.8% i.a) e imazalil (99.8% i.a) suministrado por Riedel-de Haën® (Alemania).

Para evaluar si las concentraciones de difenilamina, folpet e imazalil en manzana se encuentran dentro de los límites máximos de residuos (LMRs) establecidos por la legislación (estatal, europea y de producción integrada) se han referido sus concentraciones a nivel de fruto fresco entero. Para ello, a partir de las medidas de las muestras de piel y pulpa liofilizadas, extraídas directamente del análisis cromatográfico,

se han aplicado los correspondientes coeficientes de reducción de peso y las proporciones de piel y de pulpa calculados en la preparación de las muestras.

2.4. Análisis de los desórdenes fisiológicos externos e internos

Los desórdenes fisiológicos externos (escaldado superficial) e internos (pardeamiento) se evaluaron visualmente después de 25-27 semanas de conservación más 7 días a 20 ºC durante 3 campañas consecutivas en diferentes condiciones de atmósfera controlada (CA: 2.5% O_2 + 3 % CO_2, LO: 2% O_2 + 2% CO_2 y ULO: 1% O_2 + 1% CO_2) y frío normal (21% O_2 + 0.03% CO_2). La incidencia fue determinada como porcentaje (%) de fruto afectado. Para cada tratamiento se evaluó el porcentaje de superficie afectada de 30 frutos, donde leve = 1 a 25%, moderado = 26 a 50%, y severo = 51 a 100% El índice de escaldado se calculó según describe Zanella (2003a).

2.5. Análisis estadístico

Un diseño multifactorial utilizando la atmósfera de conservación, el periodo de almacenamiento, el periodo de estancia a 20 ºC, el tipo de tratamiento postcosecha y la repetición como factores se empleó en el análisis estadístico de los resultados. Para poder analizar los efectos de los factores sobre los resultados obtenidos, se han sometido éstos al análisis de varianza (GLM-ANOVA) según el procedimiento estándar SAS-STAT (1988). Las concentraciones medias se han separado según el test de la mínima diferencia significativa (MDS, $P \leq 0.05$).

3. Resultados y discusión

3.1. Control de los desórdenes fisiológicos externos e internos

La incidencia al escaldado superficial disminuyó significativamente en los frutos conservados en atmósfera controlada en comparación con el frío normal, tal y como

muestran otros estudios en la 'Granny Smith' (Soria y col., 1999; Zanella y col., 2003). Así, el porcentaje de escaldado en la epidermis de la 'Pink Lady®' tuvo una incidencia elevada en los frutos no tratados con DPA y conservados en frío normal durante largos periodos de frigoconservación (25-27 semanas), más 7 días a 20 ºC, siendo del 15 al 47% .En cambio para los frutos conservados en atmosfera controlada, la incidencia al escaldado fue como máximo del 7% (Figura 1). El porcentaje de escaldado superficial fue mayor para los frutos almacenados en frío normal respecto a la atmosfera controlada, debido a que los altos niveles de O_2 favorecieron la peroxidación del α-farneseno (Whitaker, 2000). Para los frutos tratados con DPA, sólo los frutos conservados en frío normal mostraron un 10% de los frutos afectados (Figura 1). Algunos autores recomiendan tratar con DPA con la finalidad de disminuir la elevada incidencia al escaldado superficial (Crouch, 2003; Calvo i col., 2008).

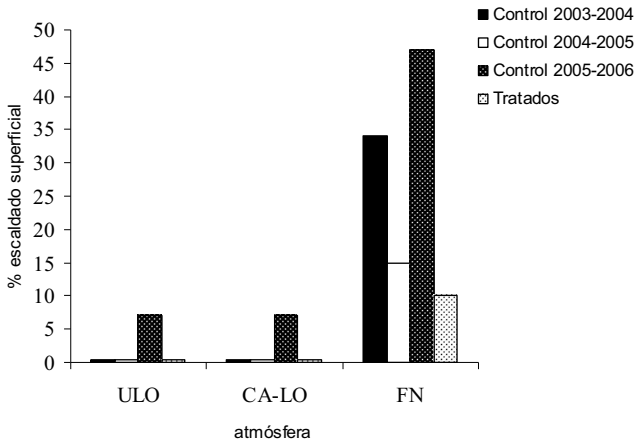

Figura 1. **Incidencia al escaldado superficial de 'Pink Lady®' frigoconservada en diferentes condiciones de atmosfera controlada (CA: 2.5% O_2 + 3% CO_2, LO: 2% O_2 + 2% CO_2 y ULO: 1% O_2 + 1% CO_2) y frío normal (21% O_2 + 0.03% CO_2) después de 25-27 semanas de conservación más 7 días a 20 ºC durante 3 campañas consecutivas.**

Esta incidencia incrementó al alargar el periodo de conservación y disminuyó en cosechas tardías. Cripps y col. (1993) y Zanella y col. (2003b) observó hasta un 50%

del fruto afectado por escaldado superficial en cosechas prematuras y la incidencia aumentó un 20% al pasar de 160 a 180 días de conservación en frío normal (East, 2006).

Los síntomas no se presentaron inmediatamente después de la salida de cámara de los frutos, pero apareció a 1 día a 20 ºC y fue incrementando su severidad después de 7 días a 20 ºC. Mir y Beaudry (1999) afirmaron que la incidencia al escaldado en manzanas 'Cortland' se aceleró después de un período de 5 días a 22 ºC. Según Burmeister y col. (2001), la aplicación de 300 ppm de DPA fue suficiente para el control del escaldado superficial durante un periodo de 16 semanas de conservación.

No se encontró presencia de pardeamiento interno en ninguna de las campañas estudiadas. Este comportamiento fue bastante diferente al encontrado en la 'Pink Lady®' cultivada en otras zonas geográficas. La severidad al pardeamiento interno estuvo favorecida por las zonas de cultivo con condiciones frescas y húmedas durante el periodo de precosecha (Moggia y Pereira, 2003).

Conforme aumenta la madurez del fruto, así como la concentración de CO_2, el periodo de conservación y los días de shelf life a 20 ºC aumenta la incidencia al pardeamiento interno (Burmeister y col., 2001; Zanella y col., 2003b; De Castro y col., 2007). Según Folchi y col. (2003) y Mazollier (2003), los frutos con fecha de cosecha tardía aumentó el riesgo de pardeamiento interno hasta un 50% de los frutos afectados después de 7 meses en atmósfera controlada (1-3% O_2 y 1-3% CO_2).

3.2. Nivel de DPA, folpet e imazalil en relación al método de tratamiento post-cosecha

Se compararon dos métodos de tratamiento (immersión o aspersión) en dos campañas sucesivas, con la finalidad de determinar el efecto de la aplicación. La concentración de DPA fue significativamente más baja en los frutos tratados por immersión respecto a

los de aspersión durante toda la frigoconservación tanto en ULO (1% O_2 i 1-2% CO_2) como en frío normal. En general, los niveles de DPA en piel fueron mayores en ULO respecto al frío normal durante toda la frigoconservación independientemente del método de aplicación (Tabla 1). Estudios anteriores mostraron que la formulación comercial de DPA, la temperatura, el tiempo y la dosis del método de tratamiento influyó en la concentración de DPA en el fruto (Little y col., 1984). Al contrario, se observó que la concentración de DPA en 'Red Delicious' después de 281 días de conservación en atmósfera controlada fue superior con el método de inmersión comparado con el de aspersión aplicado en manzanas 'Granny Smith' (FAO, 1984). Además, según Harvey y Clark (1959), la concentración de DPA después de la conservación con aspersión fue de 2-6 mg kg^{-1}; en cambio, la concentración de DPA obtenido por inmersión fue superior (entre 8 y 12 mg kg^{-1}).

Los niveles de imazalil fueron significativamente superiores con el método de inmersión comparado con el de aspersión durante toda la frigoconservación (Tabla 2). El folpet sólo mostró niveles superiores con el método de aspersión para los frutos de frío normal después de cortos (13 y 15 semanas) almacenamientos (Tabla 3). Durante el periodo de maduración a 20 ºC se encontraron diferencias en el contenido de imazalil por aspersión en ULO y sólo hubo algunas diferencias para la DPA y el folpet en frutos almacenados por inmersión tras 13 semanas en ULO (Tabla 2 y 3).

Como conclusión, el método de aplicación postcosecha influye en la concentración de difenilamina, folpet e imazalil en manzana 'Pink Lady®'. La atmósfera controlada disminuye la incidencia al escaldado de forma significativa. Para los frutos conservados en frío normal sería recomendable tratar con difenilamina con el fin de evitar elevada incidencia al escaldado sobretodo en largos periodos de almacenamiento.

Tabla 1. **Difenilamina (mg kg^{-1} peso fresco)[a] en manzanas 'Pink Lady®' después del tratamiento postcosecha y frigoconservación en frío normal y ultra-bajo oxígeno más 1 y 7 días a 20 ºC**

	Periodo (semanas)	Días a 20 ºC	Piel			
			Aspersión	Inmersión	Aspersión	Inmersión
Tratamiento post-cosecha	0	1	21.89	5.35	1.49	0.44
Atmósfera[c]						
ULO	13	1	9.22 Aa	2.90 Ba	0.64 Aa	0.25 Ba
	13	7	7.75 Aab	2.10 Bb	0.51 Aab	0.18 Bb
ULO	27	1	8.31 Aa	1.73 Bbc	0.58 Aab	0.14 Bbc
	27	7	7.06 Abc	1.51 Bcd	0.48 Aab	0.12 Bbcd
FN	13	1	5.88 Acd	1.64 Bc	0.40 Abc	0.15 Bbc
	13	7	3.11 Ade	1.55 Bc	0.22 Acd	0.13 Abcd
FN	27	1	2.53 Ae	1.11 Bde	0.25 Acd	0.09 Bcd
	27	7	1.92 Ae	0.81 Be	0.18 Ad	0.07 Ad

[a] Los valores son medias de 5 repeticiones. [b] Calculados considerando porcentajes de 93.6% y 6.4 % para los frutos tratados por aspersión y 92.5 % y 7.5 % para los frutos tratados por inmersión de pulpa y piel fresca, respectivamente. [c] ULO: 1% O_2 + 1-2% CO_2; FN: 21% O_2 + 0.03% CO_2. Medias dentro de la misma fila seguidas de diferente letra mayúscula es significativamente diferente con $P \leq 0.05$ (test LSD). Medias dentro de la misma columna seguidas de diferente letra minúscula es significativamente diferente con $P \leq 0.05$ (test MDS).

Tabla 2. **Imazalil (mg kg^{-1} peso fresco)[a] en manzanas 'Pink Lady®' después del tratamiento postcosecha y frigoconservación en frío normal y ultra-bajo oxígeno más 1 y 7 días a 20 ºC**

	Periodo (semanas)	Días a 20 ºC	Piel		Fruto entero[b]	
			Aspersión	Inmersión	Aspersión	Inmersión
Tratamiento post-cosecha	0	1	7.30	9.10	0.65	1.16
Atmósfera[c]						
ULO	13	1	3.72 Ba	8.15 Aa	0.41 Ba	0.79 Aab
	13	7	2.50 Bcd	6.93 Aab	0.27 Bbc	0.65 Ab
ULO	27	1	2.62 Bb	7.10 Aab	0.35 Bab	0.70 Aab
	27	7	0.73 Bd	6.08 Abc	0.12 Bd	0.59 Ab
FN	13	1	2.88 Bab	6.76 Ab	0.30 Bb	0.91 Aa
	13	7	2.36 Bbc	5.99 Abc	0.17 Bcd	0.69 Aab
FN	27	1	1.98 Bbc	5.06 Ac	0.32 Bab	0.65 Ab
	27	7	1.63 Bc	6.10 Abc	0.19 Bcd	0.68 Aab

[a] Los valores son medias de 5 repeticiones. [b] Calculados considerando porcentajes de 93.6% y 6.4% para los frutos tratados por aspersión y 92.5% y 7.5% para los frutos tratados por inmersión de pulpa y piel fresca, respectivamente. [c] ULO: 1% O_2 + 1-2% CO_2; FN: 21% O_2 + 0.03% CO_2. Medias dentro de la misma fila seguidas de diferente letra mayúscula es significativamente diferente con $P \leq 0.05$ (test LSD). Medias dentro de la misma columna seguidas de diferente letra minúscula es significativamente diferente con $P \leq 0.05$ (test MDS).

***Tabla 3*. Folpet (mg kg^{-1} peso fresco)[a] en manzanas 'Pink Lady®' después del tratamiento postcosecha y frigoconservación en frío normal y ultra-bajo oxígeno más 1 y 7 días a 20 ºC**

	Periodo	Días a	Piel		Fruto entero[b]	
	(semanas)	20 ºC	Aspersión	Inmersión	Aspersión	Inmersión
Tratamiento post-cosecha	0	1	3.40	0.60	0.38	0.11
Atmósfera[c]						
ULO	13	1	0.61 Aab	0.36 Aa	0.07 Aa	0.05 Ba
	13	7	0.45 Aab	0.18 Abc	0.04 Ab	0.03 Ab
ULO	27	1	0.36 Aab	0.10 Abc	0.06 Aab	0.02 Bbc
	27	7	0.16 Ab	0.08 Ac	0.01 Ac	0.01 Ac
FN	13	1	0.67 Aa	0.21 Bb	0.07 Aa	0.03 Bb
	13	7	0.49 Aab	0.18 Bbc	0.05 Aab	0.02 Bbc
FN	27	1	0.46 Aab	0.08 Bc	0.03 Abc	0.02 Abc
	27	7	0.16 Ab	nd	0.01 Ac	0.01 Ac

[a] Los valores son medias de 5 repeticiones (nd: no detectado). [b] Calculados considerando porcentajes de 93.6 % y 6.4 % para los frutos tratados por aspersión y 92.5% y 7.5% para los frutos tratados por inmersión de pulpa y piel fresca, respectivamente. [c] ULO: 1% O_2 + 1-2% CO_2; FN: 21% O_2 + 0.03% CO_2. Medias dentro de la misma fila seguidas de diferente letra mayúscula es significativamente diferente con $P \leq 0.05$ (test LSD). Medias dentro de la misma columna seguidas de diferente letra minúscula es significativamente diferente con $P \leq 0.05$ (test MDS).

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DISCUSSIÓ GENERAL

DISCUSSIÓ GENERAL:

La discussió dels resultats presentats en els anteriors punts s'ha estructurat en els següents apartats:

1. Producció de compostos volàtils aromàtics.
- **1.1.** Maduració en camp.
- **1.2.** Frigoconservació.

2. Qualitat estàndard, sensorial i sanitària.
- **2.1.** Qualitat estàndard.
- **2.2.** Acceptació sensorial.
- **2.3.** Nivells de difenilamina, folpet i imazalil.
  - **2.3.1.** Persistència dels productes aplicats.
  - **2.3.2.**. Incidència de desordres fisiològics.

1. PRODUCCIÓ DE COMPOSTOS VOLÀTILS AROMÀTICS

1.1. Maduració en camp

En aquesta tesi s'ha constatat que la producció dels compostos volàtils aromàtics emesos per la poma 'Pink Lady®' va incrementar gradualment en 23 dels 28 compostos volàtils que defineixen el seu perfil aromàtic de les pomes 'Pink Lady®'estudiades (capítol 1). Es van observar certes variacions per a la resta de campanyes estudiades van mostrar un increment progressiu de 20 dels 25 (1ª campanya) i 25 dels 43 (3ª campanya) compostos volàtils que defineixen el perfil aromàtic de la poma 'Pink Lady®' (dades no mostrades).

La concentració total dels compostos volàtils aromàtics es va mantenir baixa i constant fins als 199 ddpf, augmentant després amb l'inici del procés de maduració. L'emissió de la majoria de compostos volàtils va incrementar durant el procés de maduració

arribant al màxim d'emissió el dia de maduresa comercial (226 ddpf) (Taula 2, capítol 1). Els ésters d'hexil van ser els més importants quantitativament, aportant al perfil de la poma 'Pink Lady' un aroma a 'poma' característic (Plotto i col., 1999; 2000). Aquest grup d'ésters també ha estat majoritari en altres varietats de poma bicolor com ara 'McIntosh' i 'Cortland' (Yahia i col., 1990a) i 'Delicious' (Fellman i col., 2003).

Els ésters volàtils acetat d'hexil, 2-metilbutanoat d'hexil, hexanoat d'hexil, butanoat d'hexil, acetat de 2-metilbutil, acetat de butil (52-74% respecte al total depenent de la data de collita), butanoat d'etil i hexanoat d'etil van ser els ésters volàtils més destacats dels produïts pel fruit durant la maduració en camp (Taula 2, capítol 1). Aquests ésters van tenir una influència quantitativa molt elevada en la diferenciació dels estadis de maduresa (Fig.1, capítol 1), indicant que l'emissió de compostos volàtils és un factor important per definir l'estat fisiològic del fruit i qua la producció d'aquests ésters es podria utilitzar com a índex de maduresa. Això és interessant, ja que les unitats d'olor del butanoat d'etil, l'hexanoat d'etil, l'acetat d'hexil, el 2-metilbutanoat d'hexil, l'acetat de 2-metilbutil i l'acetat de butil van ser positives i, per tant, van tenir impacte en el perfil aromàtic de la poma 'Pink Lady®' en el moment de maduresa comercial. Alguns autors han recomanat l'acetat de 2-metilbutil com a indicador no destructiu de l'estat de maduresa dels fruits per a la fixació de la data de recol·lecció comercial en 'Bisbee Delicious' (Mattheis i col., 1991). L'éster 2-metilbutanoat d'etil va mostrar un patró irregular durant la maduració, amb un increment significatiu 3 setmanes abans de la collita (Taula 2, capítol 1), en contrast amb observacions prèvies en 'Fuji' (Echeverría i col., 2004a) o 'Mondial Gala' (Lara i col., 2008), on la concentració del 2-metilbutanoat d'etil va disminuir al llarg de la maduració en camp. Malgrat la baixa producció observada durant el mostreig en camp, el seu llindar olfactiu és el més baix (0.006 μg L^{-1}; segons Takeoka i col., 1992), i per tant, va tenir un gran impacte en el perfil aromàtic de la poma 'Pink Lady®'.

La baixa capacitat de biosíntesi d'ésters volàtils a fruits immadurs és deguda principalment a un baix subministre de precursors derivats d'àcids grassos (Song i

Bangerth, 1994, 2003; Mattheis i col., 1995). Per tant, l'augment observat al llarg de la maduració en camp en l'emissió dels ésters predominants com ara l'acetat de 2-metilbutil, l'acetat de butil i l'acetat d'hexil va resultar probablement de l'increment a les produccions dels seus respectius alcohols precursors (2-metil-1-butanol, 1-butanol i 1-hexanol, respectivament) (Taula 2, capítol 1). De fet, els resultats obtinguts al capítol 1 indiquen que un 93% de la variabilitat en la producció d'ésters volàtils es va explicar per la disponibilitat dels precursors (Fig. 4).

L'activitat de l'enzim AAT (a la pell i polpa) no va experimentar canvis siginificatius durant la maduració en camp, malgrat l'augment en l'emissió d'ésters. Altres varietats de poma, com ara 'Fuji' (Echeverría i col., 2004a) i 'Mondial Gala' (Lara i col., 2008) van mostrar també activitat AAT constant durant la seva maduració en camp, tot i que es va trobar un augment de l'activitat AAT durant la maduració de pomes 'Gala' (Fellman i col., 2000). Aquestes dades suggereixen que l'activitat AAT és necessària però no suficient per a la biosíntesi d'aquests compostos volàtils i que l'especificitat de substrat i/o la disponibilitat dels precursors necessaris per l'activitat AAT juga un paper molt important en la determinació de la concentració i la identitat dels ésters emesos pel fruit. Diversos autors han observat l'àmplia varietat de substrats acceptats per les AAT caracteritzades en poma (Defilippi i col., 2005; Souleyre i col., 2005), concluint que les preferències de substrat no necessàriament es van reflectir en els ésters produïts, mostrant que l'emissió d'ésters concrets depèn de la identitat dels precursors subministrats.

Sis dels dotze gens *AAT* estudiats a la pell, i només 3 dels 12 gens estudiats a la polpa de la poma 'Royal Gala', van mostrar un patró de regulació depenent de l'etilè, segons es descriu al capítol 2. Aquests resultats van confirmar que la producció d'ésters volàtils en poma és un procés depenent de l'etilè, d'acord amb les investigacions realitzades en 'Royal Gala' (Souleyre i col., 2005), 'Greensleeves' (Defilippi i col., 2005) i 'Golden Delicious' (Li i col., 2006). Addicionalment, els gens putatius *MpAT2*, *MpAT5*, *MpAT9* i *MpAT11* van mostrar un patró d'expressió gènica similar, amb

increments a partir d'estadis mitjans de maduració seguits d'una disminució en fruit madur. Estudis realitzats per Holland i col. (2005), Souleyre i col. (2005) i Li i col. (2006) van observar que els nivells d'activitat AAT també van incrementar amb la maduració i el desenvolupament del fruit. Altres isogens d'AAT van mostrar patrons d'expressió diferents, tal i com descriu el capítol 2. Aquestes dades suggereixen que més d'un gen *AAT* està involucrat en la biosíntesi d'ésters volàtils en poma 'Royal Gala'. Els diversos patrons d'expressió d'aquests isogens al llarg de la maduració del fruit podrien explicar per què els nivells d'activitat AAT es mantenen aproximadament constants durant el desenvolupament del fruit a 'Pink Lady®' (Capítol 1, Fig. 2), 'Fuji' (Echeverría i col., 2004a) i 'Mondial Gala' (Lara i col., 2008).

L'activitat LOX va incrementar-se de forma pronunciada en estadis de maduració avançats, tant a la polpa com a la pell (capítol 1, Fig. 5A). Ja que aquest augment pronunciat va coincidir cronològicament amb l'increment en la producció de la majoria d'ésters volàtils, és probable que LOX va influís en l'increment de la capacitat del fruit per a la biosíntesi de compostos volàtils aromàtics. Les activitats ADH i HPL van incrementar un mes abans de la data de collita comercial tant a la pell com a la polpa, paral·lelament a la producció d'acetaldehid. L'augment a l'activitat HPL es va produir una setmana abans de l'increment en l'activitat LOX, suggerint que LOX es va activar com a mecanisme per restablir la reserva d'hidroperòxids consumits per HPL. En canvi, l'activitat PDC tant a la pell com a la polpa no va mostrar increments significatius, suggerint que l'augment als nivells d'acetaldehid no va resultar de l'activitat d'aquest enzim.

En el moment de la collita, el principal compost volàtil que es va emetre a totes tres campanyes estudiades va ser l'acetat d'hexil (19% i 27% després d'1 i 7 dies a 20 ºC, respectivament), el qual va ser l'éster quantitativament predominant en el perfil aromàtic en poma 'Pink Lady®', proporcionant un aroma afruitat (Dimick i Hoskin, 1982). Els següents ésters en importància quantitativa van ser l'acetat de 2-metilbutil, el 2-metilbutanoat d'hexil, l'acetat de butil, el butanoat d'hexil, el propanoat d'hexil,

l'hexanoat de butil, i l'hexanoat d'hexil. No es van trobar diferències en la concentració dels compostos volàtils aromàtics durant la collita entre les 3 campanyes estudiades. Junts, aquests vuit ésters van contribuir al 84% i 86% del total de compostos volàtils aromàtics després d'1 i 7 dies a 20 ºC, respectivament, a les tres campanyes estudiades (capítols 3, 4 i 5). Els ésters d'hexil van ser els predominants quantitativament en el perfil aromàtic de 'Pink Lady®' les tres campanyes estudiades, representant el 56% del total de compostos volàtils aromàtics emesos pel fruit.

Un 46% (mitjana de les 3 campanyes) dels compostos volàtils aromàtics van incrementar la seva emissió després de 7 dies de maduració a 20 ºC (capítols 3, 4 i 5). L'estímul de la producció d'ésters durant el període a 20 ºC després de la collita també ha estat observat en poma 'Delicious' (Kondo i col., 2005). Aquest increment va ser facilitat probablement per la disponibilitat dels alcohols precursors necessaris, com ara 1-propanol, 1-butanol i 1-hexanol, tal i com s'ha observat en poma 'Gala' (Fellman i col., 2000), 'Greensleeves' (Defilippi i col., 2005) i 'Fuji' (Lara i col., 2006).

Si tenim en compte que la varietat 'Pink Lady®' és el resultat d'un encreuament entre 'Golden Delicious' i 'Lady Williams' sembla lògic pensar que el perfil aromàtic d'aquesta varietat vindrà influenciat pel perfil aromàtic dels seus parentals. D'acord amb diversos autors, l'acetat de butil i l'acetat d'hexil són els compostos predominants i característics del perfil aromàtic en poma 'Golden Delicious' (Song i Bangerth, 1996; López i col., 1998a). Lo Bianco i col. (2008) van trobat que l'acetat d'hexil era el compost volàtil més abundant, seguit del acetat de 2-metilbutil, l'acetat de butil, el 2-metilbutanoat d'hexil i l'acetat d'heptil, tant a la polpa com a la pell de poma 'Pink Lady®' en el moment de la collita.

A més de la importància quantitativa dels ésters, també s'ha de tenir en compte el seu llindar olfactiu. Així, cinc dels ésters majoritaris citats anteriorment, l'acetat d'hexil, el 2-metilbutanoat d'hexil, el propanoat d'hexil, l'acetat de 2-metilbutil i l'acetat de butil, amb llindars de 2, 6, 8, 11 i 66 μg L^{-1} respectivament (Takeoka i col., 1992; Buttery,

1993; Takeoka i col., 1996), són els que més contribueixen al perfil aromàtic d'aquesta varietat, aportant un aroma característic a 'poma' degut fonamentalment a la presència de l'acetat de butil i l'acetat d'hexil. Es va observar també un aroma afruitat amb notes 'verdes', associat amb l'acetat d'hexil i el 2-metilbutanoat d'hexil, i un aroma a banana degut l'acetat de 2-metilbutil (Dimick i Hoskin, 1982; Young i col., 1996; Plotto, 1998) (capítol 3, 4 i 5). Cal fer esment també dels compostos 2-metilbutanoat d'etil i 2-metilpropanoat de propil que, tot i les baixes concentracions obtingudes en la collita comercial (2.8-11.95 μg kg^{-1} i 3.5-6.8 μg kg^{-1}, respectivament), degut als seus baixos llindars olfactius (0.006 μg L^{-1} i 0.086 μg L^{-1}; Burdock, 2002 i Takeoka i col., 1992) van tenir un gran impacte en el perfil aromàtic de la 'Pink Lady®'.

En un altre estudi en poma 'Pink Lady®', els compostos amb més unitats d'olor (quocient entre la concentració i el llindar olfactiu) i, per tant, els que més van contribuir al moment de la collita, van ser l'acetat d'hexil, el 2-metilbutanoat d'etil, el 2-metilbutanoat d'hexil, l'acetat de 2-metilbutil, el butanoat d'etil i l'hexanoat d'etil (Lo Bianco i col., 2008). No obstant, aquests mateixos autors van apuntar que quan la concentració de volàtils es converteix a unitats d'olor, els valors elevats dels llindars olfactius d'alguns compostos impedeixen la seva contribució a l'aroma del fruit. Aquest fet és cert solament en teoria perquè les unitats d'olor individuals no tenen en compte possibles interaccions i sinèrgies entre els compostos volàtils amb la matriu del fruit, els quals podrien canviar la percepció olfactiva de l'aroma del fruit (Lo Bianco i col., 2008).

1.2. Frigoconservació

L'éster predominant quantitativament entre els emessos durant la frigoconservació va ser l'acetat d'hexil (27%, 30% i 32%, la 1ª, 2ª i 3ª campanya, respectivament). Els següents ésters en importància quantitativa, igual com al moment de la collita, van ser l'acetat de 2-metilbutil, el 2-metilbutanoat d'hexil, l'acetat de butil, el butanoat d'hexil, el propanoat d'hexil, l'hexanoat de butil i l'hexanoat d'hexil. Junts, aquests vuit ésters

van representar més del 80% del total de compostos volàtils aromàtics emessos (capítols 3, 4 i 5). L'acetat de butil, l'acetat d'hexil i l'acetat de 2-metilbutil han estat identificats com ara els principals responsables de l'aroma després de la frigoconservació en altres varietats de poma com 'Golden Delicious' (un dels parentals de 'Pink Lady®') (Brackmann i col., 1993; López i col., 1998a; 1999; 2000), juntament amb el butanoat d'hexil, l'hexanoat de butil i el 2-metilbutanoat d'hexil trobats per altres autors en poma 'Pink Lady®' (Young i col., 2004; Saftner i col., 2005).

Els ésters d'hexil van ser els predominants en el perfil aromàtic de la poma 'Pink Lady®' durant la frigoconservació, representant el 66%, 54% i 53% del total de compostos volàtils aromàtics la 1ª, 2ª i 3ª campanya, respectivament. Els ésters d'hexil també són importants en la fracció aromàtica emesa per altres varietats de poma bicolor com 'McIntosh' i 'Cortland', on l'acetat d'hexil és l'éster més important quantitativament (Yahia i col., 1990a).

La concentració d'aromes totals va ser superior pel cas de la 2ª i 3ª campanya respecte a la 1ª. Aquestes diferències van ser degudes al major vigor de l'arbre, així com les diferents condicions climàtiques durant el creixement del fruit, tal i com també es va observar en pomes de la varietat 'Aroma' (Tahir i col., 2007) i 'Fuji' (López i col., 2008).

És difícil determinar quina és la tecnologia de frigoconservació més adecuada per a maximitzar la producció d'aromes en 'Pink Lady®', ja que són molts els factors que hi influeixen, com ara l'estat de maduresa, el període i l'amosfera d'emmagatzemament i el període de maduració a 20 ºC. De totes maneres, segons el nostre estudi, la poma 'Pink Lady®' frigoconservada amb fred normal, especialment després d'un període de 13 a 15 setmanes, va mostrar una emissió d'aquests ésters majoritaris significativament major que amb atmosfera controlada (AC) estàndard (2.5% O_2 i 3% CO_2), LO (2% O_2 i 2% CO_2), ULO (1% O_2 i 1% CO_2 o 1% O_2 i 2% CO_2) (capítols 3 i 4). Els resultats del capítol 5 mostren com a l'atmosfera amb baix oxigen (2%), l'emissió dels compostos

volàtils aromàtics va ser major que amb molt baix oxigen (1%), sobretot després de llargs períodes de conservació (27 setmanes) i 7 dies a 20 ºC.

Diversos autors han senyalat que les atmosferes controlades poden reduir la producció total de compostos volàtils aromàtics en diferents varietats de poma com ara 'Golden Delicious' (Streif i Bangerth, 1988; Brackmann i col., 1993), 'Bisbee Delicious' (Mattheis i col., 1995), 'Gala' (Saftner i col., 2002; Lo Scalzo i col., 2003; Moya-León i col., 2007), 'Red Delicious' (Fellman i col., 2003) i 'Fuji' (Argenta i col., 2004; Echeverría i col., 2004b). Aquesta acció negativa s'accentúa amb nivells molt baixos d'O_2 i/o alts de CO_2 juntament amb períodes d'emmagatzemament prolongats (Yahia i col., 1990b). Aquesta disminució produeix una disminució de l'acceptació per part del consumidor en 'Golden Delicious' (López i col., 2000). Contràriament, altres estudis en 'Fuji' (Echeverría i col., 2003), 'Starking Delicious' (López i col., 1998b) i 'Golden Delicious' (López i col., 2000), van trobar la màxima producció aromàtica després de 5 mesos en atmosfera controlada (2% O_2 i 2% CO_2).

Com es mostra als capítols 3, 4 i 5, els resultats confirmen que la producció d'ésters ramificats com ara l'acetat de 2-metilbutil o el 2-metilbutanoat d'hexil, no es va veure supresa pel baix O_2, en concondància amb els resultats obtinguts per Dirinck (1990) i Fellman i col. (2000). Respecte als ésters lineals com ara els ésters de butil o els ésters d'hexil, es va observar una disminució en la seva producció per les pomes emmagatzemades en atmosfera controlada respecte al fred normal. Els precursors d'aquests ésters de cadena lineal es produeixen majoritàriament a partir d'àcids grassos, per ß-oxidació i/o per acció de la LOX. Ambdues vies requereixen O_2 i per tant, són inhibides durant la conservació amb baix i/o molt baix oxigen (Brackmann i col., 1993).

En general, allargar el període de conservació de 13-15 setmanes a 25-28 setmanes va disminuir l'emissió dels compostos volàtils aromàtics (capítol 3, 4 i 5), a excepció del 2-metilbutanoat d'etil (capítol 4), que va incrementar-se indenpendentment de les condicions d'atmosfera. Aquest increment probablement va induïr canvis en la qualitat

sensorial dels fruits a causa de l'augment considerable del valor de les unitats d'olor contribuint amb notes a 'poma madura' (Flath i col., 1967). El 2-metilbutanoat d'etil també ha estat identificat com a un dels principals contribuïdors de l'aroma en altres varietats de poma com ara 'Fuji' (Echeverría i col., 2004a), 'Starking Delicious' (López i col., 1998b) i 'Delicious' (Flath i col., 1967). La reducció dels compostos volàtils aromàtics durant el període de conservació ha estat també estudiada en 'Golden Delicious' (López i col., 2000), un dels parentals de la 'Pink Lady®'. Alguns autors han mostrat que, tot i la disminució de la producció dels compostos volàtils aromàtics en poma 'Pink Lady®' durant conservacions prolongades en fred normal, el fruit va tenir un bon aroma fins i tot després de 12 mesos (Saftner i col., 2005).

En relació als compostos volàtils aromàtics que més van contribuir a l'aroma característic de la poma 'Pink Lady®' al llarg de la frigoconservació durant totes tres campanyes estudiades, de major a menor quantitat d'unitats d'olor van ser el 2-metilbutanoat d'etil, l'acetat d'hexil, l'acetat de 2-metilbutil i el 2-metilbutanoat d'hexil (capítols 3, 4 i 5). Tots aquests ésters van contribuir al perfil aromàtic de 'Pink Lady®' amb descriptors olfactius a 'poma madura', 'afruitat', 'afruitat' amb notes 'verdes' i a 'banana', respectivament. Així, la màxima concentració de 2-metilbutanoat d'etil, l'éster volàtil que més va influir en l'aroma d'aquesta varietat, es va produir als fruits conservats en fred normal independentment dels períodes de conservació i de maduració a 20 ºC (capítols 4 i 5). Aquest fet podria ser el causant d'una menor acceptació per part dels consumidors degut a l'excés d'olor a poma madura propi d'aquests éster.

El fet d'allargar 4 setmanes la frigoconservació en condiciones de fred normal després de 27 setmanes amb atmosfera controlada ULO (1% O_2 i 1% CO_2) més 1 dia a 20 ºC va produir un increment dels ésters volàtils acetat d'hexil, hexanoat d'hexil i 2-metilbutanoat d'hexil, i després de 7 dies a 20 ºC del 2-metilbutanoat d'etil, hexanoat de butil, propanoat d'hexil i butanoat d'hexil, mostrant una regeneració d'aquests compostos volàtils aromàtics (capítol 5). Degut a que el llindar d'olor de l'acetat d'hexil (2 μg L^{-1}), el 2-metilbutanoat d'hexil (6 μg L^{-1}) i el propanoat d'hexil (8 μg L^{-1})

són baixos, és probable que contribueixin al perfil aromàtic de la poma 'Pink Lady®' aportant un aroma característic afruitat amb notes 'verdes' i a poma (Dimick i Hoskin, 1982; Plotto, 1998). Aquest increment dels compostos volàtils després d'un període de 4 setmanes en fred normal també va ser observat a la varietat 'Jonagold' (Hansen i col., 1992), 'Delicious' (Fellman i col., 2003), 'Royal Gala' (Young i col., 2004) i 'Fuji', on Altisent i col. (2008) i López i col. (2008) van revelar que una conservació en ULO més un període addicional de 4 setmanes en fred normal va aconseguir regenerar la majoria dels compostos volàtils que contribueixen a l'aroma d'aquesta varietat en ambdues campanyes consecutives.

Respecte al període de maduració a 20 ºC fins a 7 dies, es va observar un efecte reductor sobre el contingut d'ésters volàtils en condicions de fred normal (capítols 3 i 4). Les pomes conservades en les tres atmósferes controlades AC-estàndard, LO i ULO, en general no van mostrar canvis notables durant la maduració a 20 ºC, a excepció dels ésters ramificats com el 2-metilbutanoat d'hexil i l'acetat de 2-metilbutil, que van augmentar significativament durant tota la frigoconservació, tal i com descriuen els capítols 3 i 4. Aquest efecte durant la maduració a 20 ºC es podria explicar per la insuficient disponibilitat de substrat necessari per a la biosíntesi de compostos volàtils aromàtics. En canvi, els resultats del capítol 5 mostren una estimulació de l'emissió dels compostos volàtils en fruits conservats en LO a llarg de tot el període de conservació, i cap variació pels fruits conservats en ULO, a excepció dels ésters ramificats 2-metilbutanoat de butil, 2-metilbutanoat d'hexil i l'acetat de 2-metilbutil, que van mostrar un augment de la seva concentració. Aquests resultats confirmen que la producció d'ésters de cadena ramificada no es va veure supresa pel baix contingut d'O_2 (Brackmann i col., 1993). Altres estudis previs van demostrar que el nombre d'ésters detectats incrementa significativament durant 10-14 dies de maduració a 20 ºC en pomes 'Bisbee Delicious' (Mattheis i col., 1995), 'Royal Gala' (Young i col., 2004) i 'Jonagold' (Róth et al., 2007).

En estudiar un període de permanència a 20 ºC fins 50 dies, es va observar un augment significatiu de la majoria dels compostos volàtils aromàtics (capítol 4), amb un màxim entre 10 i 17 dies a 20 ºC, i una significativa disminució fins al final dels 50 dies pels fruits conservats en fred normal i de AC-estàndard (2.5% O_2 i 3% de CO_2). Pel que fa a l'atmosfera controlada ULO (1% O_2 i 2% de CO_2), tant els ésters de butil com d'hexil van arribar al seu màxim d'emissió als 17 dies a 20 ºC, sense diferències significiatives respecte als 24 o 50 dies, suggerint una possible regeneració dels ésters volàtils. Als 10 i 17 dies a 20 ºC, l'emissió total dels compostos volàtils als fruits conservats en AC-estàndard va ser major comparada amb els fruits procedents d'ULO. Els fruits conservats en atmosfera controlada AC-estàndard va resultar en les màximes concentracions de 2-metilbutanoat d'etil i l'acetat de 2-metilbutil, dos dels ésters que més van contribuir a l'aroma d'aquesta varietat, als 17 dies a 20 ºC. L'efecte residual de la conservació en atmosfera controlada en la producció de compostos volàtils aromàtics depén de la varietat, de les condicions d'atmosfera i d'altres factors. Lo Scalzo i col. (2003) va trobar una disminució dels ésters volàtils en poma 'Gala' després d'un període de 17 dies a 20 ºC en condicions d'ULO (1.2% O_2 + 1% CO_2). Aquest fet, ens indica que la poma 'Pink Lady®' és una varietat que manté una bona qualitat aromàtica durant un període comercial llarg. Les màximes emissions d'alcohols totals es van obtenir després de 50 dies a 20 ºC, especialment per a l'1-etanol, l'1-propanol, l'1-butanol, l'1-pentanol i el 2-metil-1-butanol, coincidint amb resultats previs obtinguts en poma 'Golden Delicious' per Kondo i col. (2005), on els alcohols van incrementar al llarg de la senescència. El 1-butanol va experimentar un augment molt elevat als 17 dies després de la conservació en totes tres atmosferes estudiades, suggerint que aquest compost podria ser indicatiu d'una sobremaduració del fruit.

Es van observar mínimes diferències (pell) o cap diferència (polpa) en l'activitat AAT després d'un període de frigoconservació curt de 15 setmanes (dades no mostrades) o llarg de 27 setmanes. L'activitat AAT després de la frigoconservació va ser major pels fruits d'atmosfera controlada respecte als fruits de fred normal (capítol 6), en concordància amb resultats anteriors en 'Mondial Gala' (Lara i col., 2007). En canvi, en

poma 'Fuji' l'activitat AAT no va permetre diferenciar entre mostres (Lara i col., 2006). L'increment en l'activitat AAT als fruits conservats en atmosfera controlada podria contribuir a les diferències observades en l'emissió d'ésters després de la frigoconservació, tot i que diferents autors han afirmat que la disminució en la producció de compostos volàtils podria resultar d'una disponibilitat limitada dels precursors més que de la degradació o inactivació de l'enzim (Fellman i col., 1993; Wyllie i Fellman, 2000). Per tant, la concentració d'àcids grassos i dels seus derivats al fruit, juntament amb l'especificat de substrat dels enzims implicats, podria ser un factor limitant per a la producció de compostos volàtils aromàtics (Sanz i col., 1997; Aharoni i col., 2000; Fellman i col., 2000; Song i Bangerth, 2003). Així, Souleyre i col. (2005) van assegurar que el subministrament del substrat alcohol és més limitant que el d'acil CoA, per a l'activitat AAT i que la preferència de l'enzim per l'alcohol precursor és depenent de la seva concentració als teixits, la qual determina el perfil aromàtic final. I, efectivament, els resultats obtinguts al capítol 6 indiquen que el 76% de la variabilitat en la producció d'ésters volàtils a poma 'Pink Lady®' després de la frigoconservació depenen de la disponibilitat dels precursors.

Les mostres de fred normal, AC-estàndard (2.5% O_2 i 3% CO_2) i LO (2% O_2 i 2% CO_2) es van caracteritzar per alts nivells d'activitat LOX i PDC tant a la pell com a la polpa; aquest increment en l'activitat possiblement va estar associat amb els elevats nivells d'acetaldehid, mentre que als fruits conservats en ULO va disminuir significativament l'activitat LOX i, per tant, la producció d'1-hexanol i dels ésters d'hexil. En canvi, la baixa disponibilitat d'acetaldehid i l'increment en l'activitat de l'HPL i l'ADH pels fruits frigoconservats en ULO durant llargs períodes (27 setmanes) van contribuir a l'increment en l'emissó de l'1-butanol i els ésters de butil (capítol 6).

Els nivells de LOX a la polpa van ser majors als fruits emmagatzemats en fred normal i LO (2% O_2 i 2% CO_2) comparat amb els fruits d'ULO. El fet que tant els fruits conservats en fred normal com els conservats en LO (2% O_2 i 2% CO_2) tinguessin uns nivells d'activitat LOX similars suggereix que es requereix una forta disminució en la

concentració d'O_2 per inhibir de forma significativa l'activitat LOX a 'Pink Lady®', a diferència de resultats previs en 'Fuji' i 'Mondial Gala' (Lara i col., 2006; 2007), on es va mostrar una inhibivció de l'activitat LOX, i per tant una disminució de la biosíntesi d'ésters volàtils, després de la frigoconservació en atmosfera controlada amb 3% d'O_2. Aquests resultats suggereixen que l'atmosfera controlada condueix a una inhibició parcial de l'expressió gènica o de l'activitat del producte gènic.

2. QUALITAT ESTÀNDARD, SENSORIAL I SANITÀRIA

2.1. Qualitat estàndard

En relació als paràmetres de qualitat estàndard de la 'Pink Lady®' durant la maduració en camp, els fruits collits en estadis de maduració prematurs van mostrar una elevada acidesa, fermesa i valor del to, i un baix contingut en sòlids solubles i índex de midó comparat amb els fruits collits a la data comercial (226 ddpf). La fermesa, l'índex de midó, el contingut en sòlids solubles i el to van ser els paràmetres amb més influència per diferenciar la maduresa entre mostres. L'acidesa va mostrar un patró irregular (capítol 1). La mateixa variació en els paràmetres de qualitat estàndard durant la maduració en camp es va observar en uns altres estudis realitzats en poma 'Pink Lady®' cultivada a Califòrnia (De Castro i col., 2007) i a Itàlia (Gualanduzzi i col., 2005).

En el moment de la collita, aquesta varietat de poma va presentar uns nivells d'acidesa alts amb valors mitjans (les tres campanyes) de 6.4 g àcid màlic L^{-1}. El mateix va succeir amb el sòlids solubles on el valor es va situar a 14.2%. De la fermesa es pot dir que va ser bastant alta en el moment de la seva recol·lecció, situant-se de mitjana les tres campanyes estudiades al voltant de 86 N (capítol 3, 4 i 5). Aquesta fermesa s'intenta retenir al llarg de tota la seva evolució comercial perquè és una característica important a destacar de la varietat. Aquests paràmetres de qualitat van ser molt similars als obtinguts en altres zones geogràfiques, on la 'Pink Lady®' va obtenir valors de 92 N de fermesa i 14.4% de als Estats Units (Drake i col., 2002), 90 N de fermesa, 14.6% de

sòlids solubles i una acidesa molt més baixa de 2.0 g àcid màlic L^{-1} a Itàlia (Lo Bianco i col., 2008) i 83 N de fermesa, 13% de sòlids solubles i 7.3 g àcid màlic L^{-1} a Austràlia (Cripps i col., 1993).

El resultat dels estudis realitzats al llarg de les 3 campanyes consecutives, mostren que la poma 'Pink Lady®' va presentar un excel·lent manteniment de la qualitat estàndard després d'un període de 13 a 15 setmanes i 25 a 28 setmanes d'emmagatzemament tant en atmosfera controlada com en fred normal i 7 dies de maduració a 20 ºC, amb algunes variacions dels paràmetres de fermesa, acidesa, contingut en sòlids solubles i color (capítols 3, 4 i 5). Altres autors com Saftner i col. (2005), van revelar que la poma 'Pink Lady®' conservada en fred normal va tenir bones característiques de qualitat durant al menys 8 mesos. A més, la poma 'Pink Lady®' va tenir una bona qualitat fins 14 dies a 20 ºC sense mostrar cap símptoma de deshidratació (Cripps i col., 1993; Gualanduzzi i col., 2005; Guzmán, 2006). En canvi, els estudis realitzats a Nova Zelanda per East (2006) van afirmar que a 20 ºC hi havia una pèrdua de la qualitat de la 'Pink Lady®'. Drake i col. (2002), van estudiar la influència sobre la qualitat de la poma 'Pink Lady®', en funció de diversos factors com l'estat de maduresa a la collita i la frigoconservació en diferents tecnologies durant tres anys consecutius, aconseguint fruita comercialment acceptable en qualsevol data de collita, així com en qualsevol tipus de frigoconservació aplicada. D'aquest manera, la poma 'Pink Lady®' es descriu com una varietat amb un bon potencial de frigoconservació.

En relació a la fermesa, els fruits conservats en AC-estàndard (2.5% O_2 i 3% CO_2), LO (2% O_2 i 2% CO_2) i l'ULO (1% O_2 i 1-2% CO_2) van mantenir valors de fermesa superiors en comparació amb els fruits conservats en fred normal, independentment del període de conservació i després de 7 dies de maduració a 20 ºC, d'acord amb estudis similars en poma 'Pink Lady®' (Burmeister i col., 2001; Drake i col., 2002; Folchi i col., 2003; Hurndall, 2003; East, 2006; De Castro i col., 2007). Altres estudis van demostrar que la fermesa es va mantenir constant després de 7 i 9 mesos en atmosfera controlada (1.5% O_2 i 1% CO_2) i 7 dies a 20 ºC (Hurndall, 2003; Zanella i col., 2003).

Així, les pomes conservades en fred normal van presentar una caiguda significativa de la fermesa, sobretot en emmagatzematges llargs (25-28 setmanes) i després de 7 dies a 20 ºC. Tot i la disminució de la fermesa, cal destacar valors alts de 55 N a 75 N de fermesa depenent de la campanya (capítol 3, 4 i 5). Per tant, la 'Pink Lady®' és una varietat que reté molt bé la fermesa. Altres autors ens indiquen el bon potencial de retenció de fermesa (68 N) d'aquesta varietat inclús amb llargues conservacions (10 mesos) en fred normal (Saftner i col., 2005). Pel contrari, Gualanduzzi i col. (2005) van afirmar que la fermesa de la 'Pink Lady®' és inacceptable per sota de 59 N i suggereixen que la 'Pink Lady®' té una vida potencial d'emmagatzemament de 21 a 25 setmanes en aire a 0 ºC.

L'acidesa va disminuir amb el temps de conservació, però no va variar entre condicions d'atmosfera controlada. De forma generalitzada, les pomes frigoconservades en AC-estàndard, LO i ULO mantenen un nivell d'acidesa superior tant en emmagatzematges curts (13-15 setmanes) com llargs (25-28 setmanes), fins i tot després de 7 dies de maduració a 20 ºC respecte a la tecnologia de fred normal. Aquest efecte de l'atmosfera controlada sobre l'acidesa és degut a una reducció de la respiració i una menor degradació de la paret cel·lular. Una elevada acidesa dels fruits d'atmosfera controlada és deguda també a una inhibició de l'activitat enzimàtica de l'àcid màlic. Cal destacar que la conservació en fred normal assoleix en la situació més desfavorable (25-28 setmanes i 7 dies a 20 ºC) un valor d'acidesa de 3.6 g àcid màlic L^{-1}, a les 3 campanyes estudiades (capítols 3, 4 i 5). Aquest valor d'acidesa va contribuir a una disminució de l'acceptació sensorial del consumidor pels fruits de fred normal respecte als d'atmosfera controlada, tal i com es mostra al l'apartat 2.2. Els resultats obtinguts en altres estudis van mostrar com l'acidesa es va reduir tant en fred normal (Drake i col., 2002; Saftner i col., 2005) com en atmosfera controlada (Drake i col., 2002; Kupferman, 2003; Hurndall, 2003) al llarg de la frigoconservació. Segons Hurndall (2003), l'acidesa va ser major a l'atmosfera controlada respecte al fred normal tant a 3, 6 i 9 mesos després de 7 dies a 20 ºC i va disminuir significativament amb el temps de frigoconservació.

Pel que fa al contingut en sòlids solubles, es va observar que després de llargs emmagatzemaments (25-28 setmanes) i 7 dies a 20 ºC, els sòlids solubles van ser significativament més alts en els fruits conservats amb atmosfera controlada respecte al fred normal. El període de maduració a 20 ºC va suposar un augment del contingut en sòlids solubles després de curts emmagatzemaments (13-15 setmanes) i una disminució després de llargs emmagatzemaments (25-28 setmanes) (capítol 3, 4 i 5). Això segurament és degut a la major ralentització del metabolisme respiratori provocat pels nivells d'O_2 baixos i/o nivells de CO_2 alts propis de l'atmosfera controlada. Segons East (2006), no es va observar cap canvi significatiu en el contingut dels sòlids solubles de la 'Pink Lady®' independenment de la data de collita o el temps de frigoconservació, coincidint amb els resultats de Drake i col. (2002) i Kupferman (2003) i en contradicció amb la reducció constant del sòlids solubles conservats en fred normal observada per Burmeister i col. (2001), Hurndall (2003) i Saftner i col. (2005). A més, Brackmann i col. (2005) van trobar que el contingut de sòlids solubles en pomes 'Pink Lady®' després de 9 mesos en els diferents tipus d'atmosfera controlada i un període de 7 dies a 20 ºC van ser superiors als dels fruits conservats en fred normal, tanmateix, no hi va haver diferències entre els fruits d'atmosfera controlada.

El color superficial de l'epidermis de la 'Pink Lady®' no va presentar diferències significatives entre condicions de frigoconservació ni durant el període de conservació frigorífica i posterior maduració a 20 ºC (capítol 4 i 5). De totes maneres, es va observar uns valors del to del color superficial més elevats després de curts emmagatzemaments (13-15 setmanes) en fred normal i LO respecte a l'ULO, la qual cosa indicaria una pèrdua de coloració roja (capítol 3). Segons Drake i col. (2002), la poma 'Pink Lady®' adquireix més color rosat al cap de 6 mesos en fred normal. A més, aquests autors obtenen que l'atmosfera 1% O_2 i 3% CO_2 permet un color superficial més rosat (és a dir, un to més baix) que l'atmosfera 1% O_2 i 1% CO_2. En el nostre cas els resultats no coincideixen exactament amb aquests, possiblement perquè la diferència de percentatge de CO_2 entre les dues cambres d'AC-estàndard (2.5% O_2 i 3% CO_2) i ULO (1% O_2 i

2% CO_2) en aquest treball és molt menor en comparació amb l'estudi realitzat per Drake i col. (2002).

El manteniment d'una coloració de fons més verda en pomes 'Pink Lady®' es va manifestar de forma general pels fruits conservats en atmosfera controlada respecte als fruits conservats en fred normal, durant tot el període de conservació tant a 1 com a 7 dies a 20 ºC. El període de maduració a 20 ºC va mostrar poques diferències en la coloració de fons (capítols 3, 4 i 5). Diversos autors van trobar un canvi gradual en el color de fons de la 'Pink Lady®' de verd a groc pels fruits conservats en fred normal (Brackmann i col., 2005). En canvi els fruits conservats amb 3% de CO_2 van retardar l'aparició del color groc a la pell més que amb 1% de CO_2, especialment després de 4 mesos i 9 mesos (De Castro i col., 2007).

2.2. Acceptació sensorial

L'acceptació sensorial evaluada mitjançant un panell de consumidors de les pomes 'Pink Lady®' depèn de molts factors que hi influeixen, com ara el període i l'amosfera d'emmagatzemament, el període de maduració a 20 ºC i la campanya, tenint tots ells un efecte siginificatiu. Així, els resultats de l'anàlisi sensorial realitzat a la 1ª campanya estudiada formada per un panell de 100 consumidors habituals de pomes, es va trobar que les pomes més acceptades pels consumidors van ser les conservades en atmosfera LO (2% O_2 i 2% CO_2) i ULO (1% O_2 i 1% CO_2) després de 25 setmanes i 7 dies a 20 ºC. Els paràmetres positivament més influenciats per l'acceptabilitat del consumidor de la varietat 'Pink Lady®' la 1ª campanya van ser el contingut de sòlids solubles, l'acidesa, i els ésters volàtils 2-metilbutilbutanoat d'hexil, l'hexanoat d'hexil, el propanoat d'hexil i el 2-metilbutanoat de butil (capítol 3). Aquests ésters són els responsables d'aportar un aroma característic a 'poma' i 'afruitat'amb notes 'verdes'.

Els resultats de la 2ª campanya formada per un panell de 61 consumidors habituals de pomes, van obtenir que les mostres més aceptades pels fruits conservats en fred normal

després de 15 setmanes tant a 1 com a 7 dies a 20 ºC. Els paràmetres amb més influència sobre la major acceptació de les pomes per part del consumidor la 2ª campanya van ser el contingut en sòlids solubles, la fermesa, l'acidesa, l'hexanoat d'hexil, l'acetat de 2-metilbutil, el propanoat d'hexil, el 2-metilbutilbutanoat d'hexil, el 2-metilbutanoat de butil i el butanoat d'hexil (capítol 4). Aquests ésters són els responsables d'aportar un aroma característic a 'banana', a 'poma vermella' i 'afruitat' amb notes 'verdes'.

La 3ª campanya estudiada amb un panell de 40 consumidors habituals de pomes, va concluir que les pomes més acceptades pels consumidors van ser les d'atmosfera controlada LO (2% O_2 i 2% CO_2) i ULO (1% O_2 i 1% CO_2) després de 13 i 27 setmanes i 7 dies a 20 ºC. Les mostres provinents de LO després de 13 i 1 dia a 20 ºC van mostrar les majors quantitats d'acidesa i sòlids solubles. Els fruits menys acceptats pels consumidors van ser els de fred normal després de 27 setmanes tant a 1 com a 7 dies a 20 ºC; es creu que la causa sigui la gran diferència de fermesa d'aquest fruits (54.9 N) respecte als fruits conservats en atmosfera controlada (62-74.8 N), juntament amb la baixa acidesa (3.6 g àcid màlic L^{-1}) obtinguda. Les variables que més van influir en l'acceptabilitat dels fruits pels consumidors van ser la fermesa, l'acidesa, el contingut en sòlids solubles, el color de fons, l'hexanoat d'etil i el 2-metilpropanoat de propil (capítol 5). L'hexanoat d'etil és el responsable de donar un aroma 'afruitat' característic.

Els fruits amb major acceptació sensorial durant la 2ª campanya van correspondre amb els fruits conservats en fred normal i que mostraven una emissió més elevada de compostos volàtils aromàtics. En canvi la 1ª i 3ª campanyes, els fruits més acceptats van ser els d'atmosfera controlada LO i ULO, coindicint amb resultats obtinguts en 'Mondial Gala' (Cliff i col., 1998; Graell i col., 2008). Alguns autors han especulat que la millor acceptabilitat sensorial de la poma 'Gala' conservada en atmosfera controlada està relacionada amb l'elevada fermesa de la polpa, l'acidesa i el contigut en sòlids solubles (Lau, 1998; Boylston i col., 1994). Per aquesta raó, no sempre els fruits més apreciats

pels consumidors són els que mostren una emissió de compostos volàtils aromàtics més elevada, com també van demostrar altres autors (Aaby i col., 2002; Echevarría i col., 2004b). Es creu que la concentració d'alguns compostos volàtils aromàtics és més important que l'emissió total d'aromes a l'hora de determinar l'acceptació sensorial del fruit. En conseqüència, la contribució específica de cada compost volàtil al perfil aromàtic de la 'Pink Lady®' depèn del seu llindar olfactiu (Buttery, 1993) i altres factors que poden produir diferències en l'acceptació sensorial com són els canvis en els altres atributs de qualitat, com la fermesa, el contingut en sòlids solubles i l'acidesa en les diferents condicions d'atmosfera.

Per tant, alguns compostos volàtils aromàtics van permetre diferenciar entre pomes 'Pink Lady®' ben acceptades i poc acceptades organolèpticament; en concret, els ésters volàtils amb més influència sobre l'acceptabilitat i que van coincidir la 1ª i 2ª campanyes estudiades van ser el propanoat d'hexil (8 μg L^{-1}), l'hexanoat d'hexil, el 2-metilbutanoat de butil (17 μg L^{-1}) i el 2-metilbutanoat d'hexil (6 μg L^{-1}). La 3ª campanya cal destacar l'elevada influència de l'hexanoat d'etil i del 2-propilpropanoat de propil sobre l'acceptació sensorial del consumidor.

La fermesa, l'acidesa i els sòlids solubles van ser els paràmetres de qualitat estàndard més influenciats per l'acceptació de les pones 'Pink Lady®' pel consumidor (capítol 3, 4 i 5). Aquests resultats confirmen els trobats per Alavoine i col. (1990) en 'Golden Delicious' i 'Granny Smith' i Echeverría i col. (2004b) en poma 'Fuji'. No obstant, els resultats de la 1ª campanya van correlacionar negativament la fermesa amb l'acceptabilitat; aquesta observació podria ser deguda al petit efecte de les condicions de conservació en la fermesa d'aquest varietat.

Investigacions prèvies van resaltar la importància de l'acidesa en la qualitat organolèptica de la 'Pink Lady®', ja que podia explicar que un 70% dels fruits van ser aptes amb valors de fermesa entre 39 i 49 N (Calvo i col., 2008). Aquest alt percentatge per valors de fermesa tan baixos pot atribuir-se a que l'acidesa de la 'Pink Lady®' pot

mantenir la qualitat de la fruita amb baixa fermesa (Candan i Calvo, 2004). Altres autors van trobar que els atributs de textura i aroma de la 'Pink Lady®' per sota de valors menors a 59 N, 15% de sòlids solubles i 7 g L^{-1} d'acidesa no van ser ben apreciats per un panell entrenat de consumidors (Gualanduzzi i col., 2005). Diversos autors han trobat que la 'Pink Lady®' va ser millor valorada en atmosfera controlada (1.2% O_2 i 0.8% CO_2 i 2% O_2 i 3% CO_2) respecte al fred normal (Testoni i col., 2002). Els estudis realitzats a Argentina van concloure que l'acceptabilitat de la 'Pink Lady®' es reduïa dràsticament després de 4 a 5 mesos (Calvo i Candan, 2006) o després de 6 mesos de conservació amb fred normal més 21 dies de maduració a 20 ºC (Calvo i col., 2008). A més, l'acceptabilitat va ser del 100% fins a 10 dies a 20 ºC. Després de 21 dies a 20 ºC, només el 33% dels fruits eren aptes pel consum, el qual significava que la vida útil de la 'Pink Lady®' era de 14 dies.

A Nova Zelanda s'han realitzat anàlisis sensorials amb panelistes entrenats, panell de consumidors i anàlisis físico-químics per caracteritzar la qualitat gustativa de la 'Pink Lady®'. Aquests resultats van indicar que la poma 'Pink Lady®' es vendria bé inicialment degut a la seva aparença i la bona qualitat organolèptica. Els valors dels panelistes entrenats van indicar que la 'Pink Lady®' és una poma cruixent, dura, de sucositat mitjana i amb un bon balanç àcid-dolç (Corrigan i col., 1997). Aquesta valoració va coincidir amb els resultats obtinguts a Itàlia per Neri i col. (2003), on la 'Pink Lady®' va ser molt bona en cruixicitat, fermesa i d'aspecte atractiu mantenint-se durant el període de frigoconservació.

Resumint, els fruits conservats en atmosfera controlada amb baix O_2 (2%) o molt baix O_2 (1%) combinat amb nivell de CO_2 similars van mostrar una millor conservació tant de la qualitat estàndard com sensorial respecte als fruits conservats en fred normal després de 25-28 setmanes de frigoconservació.

2.3. Nivell de difenilamina, folpet i imazalil

2.3.1. Persistència dels productes aplicats

En general la concentració de difenilamina (DPA) a la pell dels fruits conservades en fred normal va ser menor que les mostres d'AC-estàndard (2.5% O_2 i 3% CO_2), LO (2% O_2 i 2% CO_2) i ULO (1% O_2 i 1-2% CO_2). Els nivells de DPA a la pell van ser superiors en AC-estàndard respecte als fruits d'ULO o fred normal al llarg de tota la frigoconservació (capítol 7). En canvi, els nivells de DPA a la pell obtinguts la 2ª campanya van ser superiors en ULO respecte als fruits conservats en LO o fred normal després de 13 setmanes de conservació (capítol 8). Això podria ser degut a que la conservació amb baix O_2 redueix el metabolisme i les reaccions d'hidroxilació enzimàtiques, ja que aquestes necessiten O_2, tal i com van mostrar els resultats obtinguts en poma 'Granny Smith' o 'Braeburn' (Rudell i col., 2006; Mattheis i Rudell, 2008). És evident que la persistència de la DPA durant la conservació depén de la varietat i de les condicions de conservació (Papadopoulou-Mourkidou, 1991; Johnson i col., 1997).

El fet d'allargar la frigoconservació fins a 27-28 setmanes va disminuir el contingut de DPA, especialment a les condicions de fred normal i AC-estàndard. Altres resultats similars van revelar que la concentració de DPA en diferents varietats de poma com 'Granny Smith' (Denmead i col., 1961; Papadopoulou-Mourkidou, 1991), 'Red Delicious' (Johnson i col., 1997; Kim-Kang i col., 1998) o 'Empire' (Whitaker, 2000) van disminuir durant la conservació i posterior maduració a 20 ºC (Rudell i col., 2006). Aquesta reducció podria indicar una adsorció o metabolisme, produint derivats de la DPA com el 4-hidroxidifenilamina (4OHDPA) i quantitats petites d'altres metabolits (Kim-Kang, 1998; Rudell i col., 2006; Mattheis i Rudell, 2008).

L'efecte d'un període addicional de 4 setmanes en fred normal només va reduir la concentració de DPA a la pell i al fruit sencer pels fruits conservats en LO després de

13 setmanes. Després de 27 setmanes de conservació més 4 setmanes en fred normal, la concentració de DPA pels fruits conservats en fred normal va ser menor respecte als fruits d'ULO, tal i com es descriu al capítol 8.

El contingut de DPA a la pell va disminuir durant la maduració a 20 ºC pels fruits conservats en fred normal després de 15 setmanes (capítol 7) i pels fruits conservats en LO i ULO durant 13 setmanes i durant 13 + 4 setmanes addicionals en fred normal (capítol 8). En un altre estudi en poma 'Granny Smith' van observar una disminució del contingut de DPA durant la maduració a 20 ºC després de 6 mesos en fred normal i ULO més 14 dies a 22 ºC (Rudell i col., 2006).

La quantitat de DPA detectada a la polpa va ser molt baixa, en relació amb resultats previs on la DPA (>90%) es va trobar localitzada a la pell (Harvey i Clark, 1959; Huelin, 1968; Kim-Kang i col., 1998). De forma general, el contingut de DPA a la polpa no es va veure afectat pels dies de maduració a 20 ºC en cap de les atmosferes de conservació estudiades, tal i com es mostren als capítols 7 i 8. Al allargar la conservació a 28 setmanes es va produir un augment en el contingut de DPA a la polpa pels fruits conservats en fred normal (capítol 7), suggerint una possible migració de la DPA de la pell a la polpa. Aquest resultat es va confirmar pels resultats trobats en poma 'Granny Smith' o 'Bramley's' on es va mostrar un moviment de la DPA de la pell a la polpa després de 24 setmanes de conservació amb baix oxigen (Hall i col., 1961; tSaoir i col., 2003), així com, després d'un període a 15-20 ºC durant 1 setmana (Ginsburg, 1962).

La concentració de folpet a la pell va disminuir de forma marcada després de 15 setmanes de conservació més 1 dia a 20 ºC en totes les atmosferes de conservació estudiades amb una reducció del 80% (capítol 7). En canvi, els nivells de folpet a la pell van ser superiors en ULO respecte als fruits LO i fred normal després de 13 setmanes i 1 dia a 20 ºC (capítol 8). Un estudi previ publicat per Palazón i col. (1984) en poma 'Golden Delicious' va mostrar una degradació de folpet major en fred normal que amb

atmosfera controlada després de 6 mesos de conservació en trataments fets en precollita.

Al allargar la conservació a 27 setmanes la concentració de folpet a la pell només va disminuir per les condicions ULO. A més, al afegir un període de 4 setmanes en fred normal després de curts períodes de conservació (13 setmanes), la concentració de folpet a la pell va reduir-se als fruits conservats amb ULO i fred normal. En canvi, després de 27 + 4 setmanes en fred normal, el contingut de folpet als fruits conservats en LO i fred normal es va reduir totalment. Durant el període de maduració a 20 ºC el contingut de folpet a la pell només va disminuir al fruits conservats en ULO després de 13 setmanes (capítol 8).

La concentració de folpet a la polpa, en general no va mostrar diferències significatives entre atmosferes i períodes de conservació i dies a 20 ºC , ja que el contingut de folpet obtingut a la polpa va ser molt baix (capítol 7 i 8). Resultats previs van trobar un producte de degradació del folpet al kiwi, suggerint que el folpet podria no eliminar-se i convertir-se en altres compostos (Akiyama i col., 1998).

L'imazalil va ser més persistent que el folpet durant la conservació frigorífica. Això ho demostra els percentatges de disminució del folpet de 40%, 77% i 65% després de curts períodes de conservació (13 setmanes) en ULO, LO i fred normal, respectivament, en comparació amb els obtinguts per l'imazalil de 10%, 20% i 26% en ULO, LO i fred normal.

La concentració d'imazalil a la pell va ser major pels fruits d'ULO respecte als fruits d'AC-estàndard o fred normal, després de 15 i 27 setmanes de conservació més 1 dia a 20 ºC (capítols 7 i 8). Resultats previs similars van indicar que el contingut d'imazalil en poma 'Golden Delicious' en atmosfera controlada va ser major comparat amb els fruits de fred normal (Papadopoulou-Mourkidou, 1991).

A la polpa, el contingut d'imazalil va ser significativament major als fruits d'ULO després de 15 setmanes més 1 dia a 20 ºC, respecte als fruits d'AC-estàndard o fred normal (capítol 7), totalment contrari al que va succeir la campanya següent on el contingut d'imazalil va ser significativament major als fruits de fred normal després de 13 setmanes més 1 dia a 20 ºC respecte als fruits d'atmosfera controlada (capítol 8); no obstant cal remarcar que la concentració de gasos de l'atmosfera va variar entre campanyes.

En allargar el període de conservació, el contingut d'imazalil a la pell va disminuir pels fruits d'AC-estàndard i ULO més 7 dies a 20 ºC. En canvi, a la polpa es va observar un augment en el contingut d'imazalil pels fruits conservats en fred normal possiblement degut a una migració d'imazalil de la pell a la polpa, segons mostra el capítol 7. L'efecte d'un període addicional de 4 setmanes en fred normal només va reduir la concentració d'imazalil a la pell després de 27 setmanes amb ULO (1% O_2 i 1% CO_2) (capítol 8).

Durant el període de maduració a 20 ºC, el contingut d'imazalil va disminuir tant a la pell com al polpa pels fruits conservats amb ULO al llarg de tota la frigoconservació i pels fruits conservats en AC-estàndard després de 28 setmanes (capítol 7). En canvi, si considerem els resultats del capítol 8, no es va trobar un efecte significatiu en els nivells d'imazalil degut al periode de maduració a 20 ºC, a excepció del fruits conservats amb fred normal, on la concentració d'imazalil a la polpa va disminuir durant aquest període.

Estudis preliminars van mostrar que l'imazalil és fàcilment metabolitzat a altres productes de degradació (1-(2,4-dichlorofenill)-2-imidazol-1-letanol) en pomes. No obstant, el producte de degradació de l'imazalil va ser evident a partir de 4 mesos representant el 10% del residu total (Woestenborghs i col., 1988).
Els nivells de residus en fruit fresc sencer procedents dels tractaments postcollita utilitzats en aquesta tesi van respectar els límits màxims fixats per la legislació.

És difícil determinar quin és el mètode d'aplicació postcollita (aspersió o immersió) que millor funciona en poma 'Pink Lady®', ja que com es va observar depén del antifúngic i antiescaldant utilitzat. Comparant els resultats es van trobar concentracions de DPA en fruit sencer superiors amb el mètode d'aspersió respecte al d'inmersió durant tota la frigoconservació tant amb ULO (1% O_2 i 1-2% CO_2) com amb fred normal. Contràriament, els nivells d'imazalil van ser significativament superiors amb el mètode d'inmersió comparat amb l'aspersió durant tota la frigoconservació tant amb ULO com en fred normal. El folpet només va mostrar nivells superiors amb el mètode d'aspersió pels fruits de fred normal després de curts (13 i 15 setmanes) emmagatzemamements (capítol 9). Moggia i col. (2003) van suggerir que la eficàcia del tractament es basa en una concentració adequada de la solució al tanc, i una distribució uniforme dels productes químics a la superfície del fruit. Estudis anteriors van observar que la concentració de DPA en 'Red Delicious' amb el mètode d'inmersió després d'una conservació en atmosfera va ser superior respecte al mètode d'aspersió aplicat en pomes 'Granny Smith' (FAO, 1984). A més, segons Harvey i Clark (1959), la concentració de DPA va ser de 2-6 mg kg^{-1} amb aspersió i superior quan es va aplicar el tractament per inmersió (entre 8 i 12 mg kg^{-1}).

2.3.2. Incidència de desordres fisiològics.

El percentatge de pomes 'Pink Lady®' amb escaldat superficial a l'epidermis va ser elevat pels fruits no tractats amb DPA i conservats en fred normal durant llargs períodes de frigoconservació (25-28 setmanes) més 7 dies a 20 ºC. Mir i Beaudry (1999) van afirmar que la incidencia a l'escaldat en pomes 'Cortland' es va accelerar després d'un període de 5 dies a 22 ºC. El percentatge d'escaldat superficial va ser major per les pomes emmagatzemades en fred normal respecte a les d'atmosfera controlada, degut a que els alts nivells d'O_2 afavoreixen la peroxidació del α-farnesè (Whitaker, 2000). Pels fruits tractats amb DPA, només els fruits conservats en fred normal van mostrar escaldat superficial (capítol 9). Alguns autors recomanen tractar amb DPA amb la finalitat d'aminorar l'elevada incidència a l'escaldat (Crouch, 2003; Calvo i col., 2008).

En altres estudis en poma 'Pink Lady®' els principals factors que van influir en la incidència a l'escaldat superficial van ser les condicions climàtiques, la data de collita, el període i la tecnologia de conservació. Temperatures per sota de 10 ºC un mes abans de la collita va reduir el risc a l'escaldat (Kupferman, 2001). A més, les pomes 'Pink Lady®' collides en la data comercial van prevenir l'escaldat superficial quasi totalment en fred normal (només un 2% mostren escaldat) i totalment en atmosfera controlada (2-2.5% O_2-2-2.5% CO_2) i ULO (1% O_2-1% CO_2) (Cripps i col.,1993; Folchi i col., 2003; Zanella i col., 2003). Altres estudis realitzats a Argentina van observar que el període mínim pel desenvolupament de l'escaldat als fruits va ser de 6 mesos en fred normal (Calvo i col., 2008). En canvi, els resultats obtinguts per Burmeister i col. (2001) i East (2006) a Nova Zelanda van mostrar que l'escaldat superficial de la 'Pink Lady®' tenia una incidència major als fruits conservats en fred normal després de 3 i 4 mesos de conservació arribant fins al 73% dels fruits afectats. Segons De Castro i col. (2007), l'escaldat superficial de la 'Pink Lady®' cultivada a Califòrnia va afectar més del 80% del fruit després de 6 mesos en fred normal.

En aquesta tesi no es va trobar presència d'embruniment intern (capítol 9). Aquest comportament va ser bastant diferent del mostrat en altres estudis de la mateixa varietat cultivades en determinades zones del món com Xile o Austràlia. La severitat de l'embruniment intern va variar conforme augmentava la maduresa, en funció de la zona geogràfica i segons la campanya (James, 2007), afavorint-se a les zones de cultiu amb predominància de condicions fresques i humides durant el període de precollita (Moggia i Pereira, 2003; Tanner i col., 2004; Brown i col., 2005), ja que és possible que les condicions climàtiques tinguin influència en la estructura i estabilitat de les membranes cel·lulars del fruit (Schotsmans i col., 2004). A més, segons Folchi i col. (2003) i Mazollier (2003), els fruits amb data de collita tardana van augmentar els risc d'embruniment intern fins un 50% dels fruits afectats després de 7 mesos en atmosfera controlada (1-3% O_2 i 1-3% CO_2). La severitat de l'embruniment intern també va variar segons les condicions de l'atmosfera. Per tant, a mesura que s'incrementava la concentració de CO_2 a la cambra mantenint la concentració d'O_2 constant, augmentava el

percentatge de fruits afectats (Zanella i col., 2003; De Castro i col., 2007). No obstant, la concentració òptima de gasos a l'atmosfera va ser variable en funció de la zona geogràfica. Així, Brackmann i col. (2005) van recomanar atmosferes controlades per la 'Pink Lady®' de 1.5% O_2 amb 1% o 2% de CO_2 a 0.5 ºC; en canvi, altres resultats realitzats a la 'Pink Lady®' cultivada a Califòrnia van mostrar que concentracions de CO_2 majors d'1% podien causar l'embruniment intern (De Castro i col., 2007). A més, Kupferman (2003) i East (2006) van observar que la incidència a l'embruniment intern va augmentar amb el temps de conservació. Aquesta incidència també va incrementar durant el període de maduració a 20 ºC (Burmeister i col., 2001).

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CONCLUSIONS

1. **L'emissió de la majoria de compostos volàtils va incrementar durant la maduració, arribant al màxim d'emissió 226 dies després de plena floració. Els ésters volàtils acetat d'hexil, 2-metilbutanoat d'hexil, hexanoat d'hexil, butanoat d'hexil, acetat 2-metilbutil i acetat de butil, van ser els ésters volàtils més importants quantitativament emessos pel fruit durant la maduració en camp.**

2. **L'activitat AAT (a la pell i polpa) no va experimentar canvis significatius durant la maduració en camp, malgrat l'augment en l'emissió d'ésters. Aquestes dades suggereixen que l'activitat AAT és necessària però no suficient per a la biosíntesi d'aquests compostos volàtils i que la disponibilitat de precursors necessaris i/o l'especificitat de substrat de l'enzim juguen un paper molt important en la determinació de la concentració i la identitat específica dels ésters emessos pel fruit.**

3. **Sis dels dotze gens *AAT* estudiats a la pell i només 3 dels 12 gens estudiats a la polpa de la poma 'Royal Gala' van mostrar un patró de regulació depenent de l'etilè, suggerint que la capacitat de biosíntesi d'ésters volàtils és en part constitutiva.**

4. **Els gens putatius *MpAT2*, *MpAT5*, *MpAT9* i *MpAT11* aïllats en poma 'Royal Gala' van mostrar un patró d'expressió genètica similar, amb increments a partir d'estadis de maduració mitjana seguits d'una disminució en poma madura. Altres isoformes d'AAT van mostrar patrons d'expressió diferents durant el desenvolupament en camp del fruit. Aquestes dades suggereixen que més d'un gen *AAT* està involucrat en la biosíntesi d'ésters volàtils en poma 'Royal Gala'.**

5. **L'activitat LOX va incrementar de forma pronunciada a partir d'estadis de maduració avançats, tant a la polpa com a la pell, suggerint que LOX és un factor important en l'increment de la capacitat del fruit per a la biosíntesi de compostos volàtils aromàtics.**

6. **Les activitats ADH i HPL van incrementar un mes abans de la data de collita comercial, tant a la pell com a la polpa. La producció d'acetaldehid també va mostrar un increment el mateix període. En canvi, l'activitat PDC no va mostrar increments significatius, cosa que indica que l'acetaldehid produït durant la maduració no resulta d'aquesta activitat enzimàtica.**

7. **L'éster predominant en el perfil aromàtic de la poma 'Pink Lady®', tant en el moment de la collita com durant la frigoconservació en les tres campanyes estudiades, va ser l'acetat d'hexil, representant entre un 27 i un 32% de la concentració total, segons la campanya. Els següents ésters en importància quantitativa van ser l'acetat de 2-metilbutil, el 2-metilbutanoat d'hexil, l'acetat de butil, el butanoat d'hexil, el propanoat d'hexil, l'hexanoat de butil i l'hexanoat d'hexil.**

8. **La poma 'Pink Lady®' frigoconservada en fred normal, especialment després d'un període de 13 a 15 setmanes, va obtenir una emissió de compostos volàtils aromàtics significativament major en comparació amb els fruits conservats en atmosfera controlada estàndard (2.5% O_2 i 3% CO_2), atmosfera controlada amb baix oxigen (2% O_2 i 2% CO_2) i amb molt baix oxigen (1% O_2 i 1-2% CO_2).**

9. **Un període addicional de 4 setmanes en fred normal després de 27 setmanes amb atmosfera controlada amb molt baix oxigen (1% O_2 i 1% CO_2) va produir un increment de les concentracions dels ésters volàtils acetat d'hexil, hexanoat d'hexil i 2-metilbutanoat d'hexil després d'un dia a 20 ºC, i de 2-metilbutanoat d'etil, hexanoat de butil, propanoat d'hexil i butanoat d'hexil després de 7 dies a 20 ºC.**

10. **Després d'un període de maduració de set dies a 20 ºC es va observar una disminució en l'emissió dels compostos volàtils en els fruits conservats en fred normal, especialment després de llargs períodes de conservació frigorífica. Els fruits procedents de les tres condicions d'atmósfera controlada considerades en aquesta Tesi no van mostrar canvis notables durant aquest període, a excepció dels ésters ramificats com el 2-metilbutanoat de butil, el 2-metilbutanoat d'hexil i acetat de 2-metilbutil, que van augmentar significativament.**

11. **Allargar el període de maduració a 20 ºC va suposar un augment significatiu de la majoria dels compostos volàtils aromàtics, mostrant el seu màxim entre 10 i 17 dies a 20 ºC, després de llargs períodes de conservació frigorífica.**

12. **Durant els períodes de frigoconservació i posterior maduració a 20 ºC, es van observar mínimes diferències en l'activitat AAT, tant a la pell com a la polpa. L'activitat AAT va ser major pels fruits conservats en atmosfera controlada respecte als fruits procedents de fred normal.**

13. **Es va observar una elevada activitat PDC als fruits procedents de fred normal, possiblement associada amb alts nivells d'acetaldehid. En canvi, als fruits conservats en molt baix oxigen (ULO) va disminuir significativament l'activitat LOX i, per tant, la producció d'1-hexanol i**

d'ésters d'hexil. La baixa disponibilitat d'acetaldehid i l'increment en l'activitat de l'HPL i l'ADH als fruits frigoconservats en ULO durant 27 setmanes van contribuir probablement a l'increment en l'emissió d'1-butanol i d'ésters de butil respecte de les mostres frigoconservades en les altres condicions considerades.

14. **La biosíntesi de compostos volàtils aromàtics al llarg de la maduració en camp i durant la frigoconservació va estar condicionada fonamentalment per la disponibilitat dels precursors dels compostos volàtils, i també, probablement, pel nombre d'isoformes d'AAT presents i la seva regulació, més que per l'activitat AAT.**

15. **De forma generalitzada, les pomes frigoconservades en atmosfera controlada van mantenir un nivell de fermesa, acidesa, contingut en sòlids solubles i color de fons superiors respecte de les conservades en fred normal, tant en emmagatzematges curts com llargs, fins i tot després de 7 dies de maduració a 20 ºC. En canvi, el color superficial de l'epidermis no va presentar diferències significatives entre condicions d'atmosfera ni durant els períodes de conservació frigorífica i posterior maduració a 20 ºC.**

16. **Els resultats de l'anàlisi sensorial realitzat durant 3 campanyes consecutives van mostrar que a la 1ª i 3ª campanya de les estudiades les pomes més acceptades van ser les conservades en atmosfera controlada amb baix oxigen (2% O_2 i 2% CO_2) i molt baix oxigen (1% O_2 i 1-2% CO_2) després de 13 setmanes + 1 dia a 20 ºC i 25 setmanes + 7 dies a 20 ºC. En canvi, a la 2ª campanya les mostres més aceptades es van correspondre amb els fruits conservats en fred normal i que mostraven una emissió més elevada de compostos volàtils aromàtics totals.**

17. Els compostos volàtils aromàtics que caracteritzaven les pomes 'Pink Lady®' més acceptades van ser el propanoat d'hexil, l'hexanoat d'hexil, el 2-metilbutanoat de butil i el 2-metilbutanoat d'hexil (1ª i 2ª campanyes), i l'hexanoat d'etil i 2-metilpropanoat de propil (3ª campanya).

18. La concentració de difenilamina a la pell dels fruits mantinguts en fred normal va ser menor que a les mostres conservades en atmosfera controlada. La concentració de difenilamina va disminuir després dels períodes de conservació en fred independentment de la tecnologia i posterior maduració a 20 ºC. Un període addicional de 4 setmanes en fred normal només va reduir la concentració de DPA a la pell i al fruit sencer als fruits conservats en LO durant de 13 setmanes.

19. La concentració de folpet a la pell va disminuir de forma marcada després de 13-15 setmanes de conservació més 1 dia a 20 ºC, en totes les atmosferes de conservació estudiades. La reducció va ser del 80%, sense diferències notables entre les condicions d'atmosfera considerades. No es van observar diferències significatives durant la maduració a 20 ºC, ni després d'un període addicional de 4 setmanes en fred normal, després de l`emmagatzematge en atmosfera controlada.

20. La concentració d'imazalil, tant a la pell com a la polpa, va ser major pels fruits emmagatzemats en ULO respecte de les mostres conservades en atmosfera controlada estàndard o fred normal durant períodes curts. En general, l'imazalil va disminuir durant la conservació i posterior maduració a 20 ºC, sobretot en atmosfera controlada. Un període addicional de 4 setmanes en fred normal només va reduir la concentració d'imazalil a la pell després de 27 setmanes en ULO.

21. Es van observar diferències en els nivells de residus estudiats en pomes 'Pink Lady®' segons el mètode utilitzat per a l'aplicació. El mètode d'aspersió va causar concentracions de difenilamina superiors respecte als fruits on l'aplicació s'havia fet per immersió durant tota la frigoconservació, tant en atmosfera controlada amb molt baix oxigen (1% O_2 i 1-2% CO_2) com en fred normal. El folpet només va mostrar nivells superiors pels fruits tractats per aspersió i conservats en fred normal, i després de períodes d'emmagatzematge curts. Contràriament, els nivells d'imazalil van ser significativament superiors, durant tota la frigoconservació, als fruits tractats amb el mètode d'inmersió .

22. La conservació en atmosfera controlada no va ser suficient per evitar totalment la incidencia de l'escaldat superficial. No es va trobar embruniment intern als fruits en cap de les condicions estudiades en poma 'Pink Lady®'.