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Fatty acid composition of phospholipids in mesocarp of cherimoya fruit during ripening

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Abstract

Mature-green cherimoya (*Annona cherimola* Mill.) fruits were stored for 6 days at 22 °C. The analysis of the lipid classes in mesocarp tissue of mature-green and senescing fruits showed that the senescence of cherimoya was characterized by a decrease of phospholipid content. The fatty acid composition of the mesocarp phospholipids was studied in fruits at harvest, on day 3 and 6. Twenty different fatty acids were identified. The C16:0, C18:0, C18:1, C18:2$n-6$ and C18:3$n-3$ fatty acids clearly were the most abundant fatty acids and the C18 family comprised more than 50% of total fatty acids content. Major variation in the relative composition of mesocarp phospholipid fatty acids were observed at the pre-climacteric stage. These changes did not modify the unsaturation index of the membrane but increased the unsaturation level for C18 fatty acids class. In senescing fruit, a decrease in all unsaturation indexes calculated was found. The results indicated that the modification of the relative quantity of specific polyunsaturated fatty acids in phospholipids was more relevant than the total content of fatty acids for the adaptation of mesocarp membranes to ripening and senescence processes in cherimoya fruit.

Keywords: *Annona cherimola* Mill.; Cherimoya; Subtropical fruit; Phospholipids; Fatty acids; Climacteric ripening

1. Introduction

During senescence in plant tissues as well as during fruit ripening an early and progressive deterioration of membrane integrity is differentially induced (Brown, Chambers, & Thompson, 1991a, 1991b; Paliyath & Droillard, 1992). It has been described that the less resistant membranes are those from thylakoids due to the special mechanism involved in their degradation while the plasma membrane and those from the endoplasmic reticulum and vacuoles, which collectively constitute the microsomal fraction, are more resistant (Thompson, Legge, & Barber, 1987).

Increases in permeability and other functional alterations of membranes are well-described phenomena occurring during senescence and/or fruit ripening, and it has been correlated with changes in the lipid metabolism (Paliyath & Droillard, 1992; reviewed in Thompson, Froese, Madey, Smith, & Hong, 1998). It has been pointed out that the transition phase temperature of membranes rises when plant tissues are reaching senescence, producing an increase in the proportion of membrane zones having a gel-phase at physiological temperature. The resulting combination between gel-phase and liquid crystal-phase in the same lipid bilayer could lead to the membrane permeability increase reported during senescence (McKersie & Thompson, 1979; Senaratna, McKersie, & Borochov, 1987). Changes in the proportions and composition of lipids within plant cell membranes, as well as fatty acid remodelling of the phospholipid fraction may have an important role during the ripening and senescence stages by changing the physical properties of the lipid matrix and consequently the functional activity of membrane-associated proteins (Paliyath, Poovaiah, Munske, & Magnuson, 1984; Wallis & Browse, 2002).

In addition, polyunsaturated fatty acid peroxidation is initiated during the early stages of senescence and fruit ripening. This results in an increased titre of free radicals

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and later damage to membrane proteins (Duxbury, Legge, Paliyath, Barber, & Thompson, 1991). There is growing evidence that much of the metabolism of lipids during plant tissue senescence and fruit ripening is achieved through specific gene expression regulation (Dangl, Dietrich, & Thomas, 2000; Giovannoni, 2001).

Cherimoya fruit (*Annona cherimola* Mill.) has a typically climacteric ripening pattern with a burst in respiration associated with a peak in ethylene production leading to an accelerated senescence (Lahoz et al., 1993; Martínez et al., 1993; Palma, Aguilera, & Stanley, 1993). Previous work (Gutiérrez, Lahoz, Sola, Pascual, & Vargas, 1994; Sola, Gutiérrez, & Vargas, 1994) has indicated that very important metabolic changes during cherimoya ripening include starch hydrolysis with the concomitant accumulation of glucose and fructose and the degradation of cell walls. Microstructural observations of mesocarp clearly show evidence for a progressive organelle and membrane deterioration in senescing cherimoya (Gutiérrez, Sola, Pascual, Rodríguez-García, & Vargas, 1992). In the present work we studied the fatty acid composition of the phospholipid fractions isolated from white mesocarp tissues of cherimoya during ripening with the aim of correlating this result with membrane alterations leading to fruit senescence.

2. Materials and methods

2.1. Plant material

Cherimoya fruits (*A. cherimola* Mill.) cv Fino de Jete were grown in Motril (Granada, Spain). Fruits of uniform size (300 ± 20 g) were hand harvested at the mature green stage (Gutiérrez et al., 1992). One group of six fruits was prepared for lipid analysis immediately after harvest. Twelve fruits were stored at 22 °C. Three and six days after harvesting, one lot of six fruits each were analysed.

2.2. Lipid extraction

White mesocarp tissue (1 g fresh weight) was homogenized for 1–5 min in 12 ml of $CHCl_3$:MeOH:HCl (200:100:1) containing 0.005% butylhydroxytoluene (BHT). Extraction of samples was performed according to Folch, Lees, and Sloane-Stanley (1957). Samples of lipids were dried down under N_2 and resuspended in 0.4 ml of $CHCl_3$ with 0.005% BHT to be spotted on TLC plates (see below). Aliquots were taken for determination of total content of phospholipids (see below).

2.3. Thin-layer chromatography

Lipid extracts from mesocarp were separated by Thin-layer chromatography (TLC) on silica gel G-60 developed in a solvent mixture of hexane:Et2O:HOAc (80:20:1) with 0.005% BHT according to Skipski and Barclay (1969). Lipids were located with I_2. Photo densitometry was immediately carried out. The band containing the phospholipids was removed and lipids recovered in $CHCl_3$ with 0.005% BHT.

2.4. Fatty acid methylation

Fatty acid methylation was performed according to Morrison and Smith (1964). Fatty acids from TLC phospholipid fractions were converted into their Me esters (FAMEs) by heating at 100 °C for 30 min (sealed under N_2) with 14% BF_3 in MeOH. FAMEs were extracted with *n*-heptane.

2.5. GC and GC–MS analysis

FAMEs were analysed by FID-GC using a $30\ m \times 0.5$ μm (i.d.) DB-23 semi-capillary column with an injector volume of 0.5 μl. Injector and detector heater temperature were 220 °C. N_2 at 30 ml/min was used as carrier gas. The temperature program was: 5 min at 150 °C, a ramp to 190 °C at 2 °C/min, then raised to 220 °C at 3 °C/min and then held for 4 min with a total run time of 34 min. Peak areas were recorded by integration and FAMEs were identified by co-chromatography with authentic standards and by GC–MS. GC–MS was carried out with a HP1 column ($25\ m \times 0.2$ mm) and the temperature was programmed as above with He as carrier gas and with ionisation energy of 70 eV.

2.6. Statistics

The fatty acid composition was expressed as a percentage of total content of fatty acids. The statistical significance of the results was determinated by one factor analysis of variance (ANOVA test).

3. Results and discussion

We have analysed the changes in the lipid classes composition of the white mesocarp tissue from cherimoya fruit during senescence by TLC when microstructural evidences showed high membrane deterioration (Gutiérrez et al., 1992). Two significant variations were observed in senescing fruits (Table 1): a decreased content of phospholipids and a higher percentage of free fatty acids. The diminution in the phospholipid fraction is a well-documented phenomenon in plant senescence. In cherimoya fruit this is correlated with a concomitant increase in mono- and diacylglycerol content. Growing evidence implies membrane fatty acids and derivatives as signalling molecules in several defence and developmental responses in plant

Table 1
Lipid composition of the mesocarp tissue from mature-green and senescing cherimoya fruits (represented as %)

Lipid fraction	Mature-green fruit	Senescent fruit
Phospholipids	34.65 ± 2.65	28.43 ± 1.01*
Monoacylgycerols	0.68 ± 0.17	1.50 ± 0.15*
Diacylglycerols	0.88 ± 0.12	2.54 ± 0.68**
Triacylglycerols	8.57 ± 1.06	8.15 ± 1.33
Fatty acids	15.56 ± 1.17	27.09 ± 0.56***
Sterified sterols	26.38 ± 1.19	21.96 ± 2.82
Other lipids	13.25 ± 0.90	10.20 ± 1.31

Values represent the mean of percentages ± SE of at least six fruit per each condition.
* Significant statistical difference, $P < 0.05$.
** Significant statistical difference, $P < 0.01$.
*** Significant statistical difference, $P < 0.001$.

tissues, including the promotion of fruit ripening and senescence (Weber, 2002). Since the liberation of precursor fatty acids from membrane phospholipids is the first step of different biosynthetic pathways for these regulator compounds (Farmer, 1994), it could explain the increased content of free fatty acids found in mesocarp of senescing cherimoya.

At the three stages monitored during ripening of cherimoya fruit (i.e., 0, 3 and 6 days after harvesting), we have studied the fatty acid composition of the phospholipid fraction isolated by TLC from the methanol extracts of the mesocarp of cherimoya fruit (Table 2). We have been able to clearly detect and identify 20 different fatty acids. Adding the weight percentage of the five most abundant fatty acids resulted in a proportion higher than 80% of the total content, reaching 95% at the climacteric stage. These fatty acids are C16:0, C18:0, C18:1, C18:2$n-6$ and C18:3$n-3$. The C16:0 was the most abundant saturated fatty acid and the C18:2$n-6$ was the most abundant of the unsaturated fatty acids. The C18 family comprised more than 50% of total fatty acid content during ripening. Other fatty acids of different carbon lengths were found in lesser quantity with percentages smaller than 3% in any case. This relative composition resembles that described for other fruits, and is indicative of the global fatty acid metabolism in plant tissues (Harwood, 1988; Izzo et al., 1995).

During ripening important changes in the fatty acid composition of mesocarp phospholipids occur (Table 2). However, the changes observed were not linear since changes at the pre-climacteric stage were generally opposite to those found at the post-climacteric period. Three days after harvesting, when ethylene production was initiated (Lahoz et al., 1993), the content of the major fatty acids in phospholipids changed variably with respect to mature-green fruit. That is, the percentage of C18:0 and C18:1 decreased significantly while those for C18:2$n-6$ and C18:3$n-3$ fatty acids increased. These results could be interpreted as indicative of a higher desaturase activity during this period. The relative content of C16:0 also increased between mature-green and climacteric stages, therefore showing a different profile to another major saturated fatty acid (C18:0) suggesting that there two fatty acids are metabolised differently during ripening. Individually and as a whole, the content of all other minor fatty acids

Table 2
Fatty acid content of phospholipids from the mesocarp of cherimoya fruits at different days post-harvest (represented as %)

Fatty acid	Day 0	Day 3	Day 6
16:0	28.80 ± 2.10	36.22 ± 8.91	29.80 ± 2.41
16:1	1.26 ± 0.33	0.99 ± 0.30	1.54 ± 0.54
17:0	1.50 ± 0.31	1.33 ± 0.34	2.31 ± 0.36
17:1	2.67 ± 0.63	0.30 ± 0.24**	3.27 ± 0.67*
18:0	5.21 ± 0.68	2.61 ± 0.11**	7.52 ± 0.67*
18:1	9.33 ± 1.04	3.31 ± 0.49*	11.76 ± 0.79
18:2$n-6$	24.18 ± 1.87	29.29 ± 4.51	15.91 ± 1.27**
18:3$n-6$	2.10 ± 0.72	0.28 ± 0.21*	2.35 ± 0.38
18:3$n-3$	15.35 ± 1.24	23.37 ± 2.54*	15.44 ± 1.36
18:4$n-3$	0.71 ± 0.30	0.01 ± 0.01*	0.28 ± 0.07
20:0	1.35 ± 0.29	0.18 ± 0.03**	1.10 ± 0.23
20:1	0.13 ± 0.06	0.02 ± 0.02	0.06 ± 0.02
20:3$n-6$	0.18 ± 0.05	0.04 ± 0.02*	0.14 ± 0.03
20:4$n-6$	0.54 ± 0.09	0.03 ± 0.02***	0.61 ± 0.12
20:5$n-3$	0.12 ± 0.02	0.03 ± 0.02**	0.33 ± 0.11
22:0	1.58 ± 0.17	1.02 ± 0.40	1.85 ± 0.29
22:3$n-6$	0.60 ± 0.10	0.23 ± 0.12*	0.62 ± 0.08
22:4$n-6$	0.87 ± 0.17	0.07 ± 0.04***	0.73 ± 0.01
22:6$n-3$	1.24 ± 0.17	0.54 ± 0.17*	1.41 ± 0.17
24:1	2.26 ± 0.52	0.11 ± 0.11**	2.95 ± 0.47

Values represent the mean of percentages ± SE of at least six fruit per each condition. Determinations were done in triplicate.
* Significant statistical difference regarding the value for day 0, $P < 0.05$.
** Significant statistical difference regarding the value for day 0, $P < 0.01$.
*** Significant statistical difference regarding the value for day 0, $P < 0.001$.

dropped at the pre-climacteric stage. For example, there was a diminution in the percentage of the C20 saturated, the C17:1 and C24:1 monounsaturated and the C18:3$n-6$, C20:4$n-6$ and C22:4$n-6$ polyunsaturated fatty acids.

The accumulation of polyunsaturated octadecanoid acids (C18:2$n-6$ and C18:3$n-3$) in cherimoya mesocarp at the pre-climacteric period is in accordance with their function as precursors of several compounds generically named oxylipins (reviewed in Weber, 2002). Specifically, jasmonates are a family of oxylipin compounds derived from linoleic or α-linolenic acids of phospholipids in the biosynthetic pathway known as octadecanoid pathway (Gundlach & Zenk, 1998; Hamberg & Gardner, 1992; Schaller, 2001) that play important signalling roles in a number of processes including fruit ripening in higher plants (Creelman & Mullet, 1997). It has been described for apple and tomato fruit that the endogenous concentration of jasmonates increases transiently prior to the climacteric increase in ethylene biosynthesis during the onset of ripening (Fan, Mattheis, & Fellman, 1998). It could be postulated that the higher content of linoleic and α-linolenic acids in mesocarp phospholipids of pre-climacteric cherimoya is necessitated, partly at least, for the peroxidative reactions leading to the synthesis of jasmonates required for climacteric fruit ripening. Hydroperoxides, products of the peroxidation of polyunsaturated fatty acids mediated by the lipoxygenase activity, also promote deterioration of cell membranes increasing permeability (Duxbury et al., 1991). However, an increase in lipoxygenase activity is not a common characteristic in all senescing plant tissues allowing thinking that a role of lipoxygenase in the degradation of senescent membranes is less relevant than its implication in the biosynthesis of jasmonates (Feussner & Wasternack, 2002).

All the described modifications in the fatty acid composition of mesocarp phospholipids on day 3 post-harvest reverted at the end of experimental period (day 6). The only statistically significant variations found in the composition of senescent fruits with respect to the mature-green fruits were the fall in the relative content of the abundant fatty acid C18:2$n-6$ and the increase of the C18:0 and the C17:1 acids (Table 2).

When the changes in fatty acid composition of mesocarp phospholipids from pre- and post-climacteric cherimoya were studied by means of calculating unsaturation and saturation indexes (Table 3), it was observed that in spite of the individual described variations there were no modification in the total content of saturated or unsaturated fatty acids. The most important differences were found amongst unsaturated fatty acid classes, with the content of monoenes changing opposite to that of the polyenes. Percentual content of total monounsaturated fatty acids significantly diminished on day 3 after harvesting, its value reverting at the end of experimental period to an initial value. A greater desaturase activity at the pre-climacteric than the mature-green stage and a later diminution at senescence could explain that the measured polyenes content rose on day 3 and significantly fall on day 6. These results indicate that during ripening of cherimoya, variations in individual fatty acids content of phospholipids could be a factor contributing more to the membrane permeability modifications associated with fruit senescence than variations in the total content of saturated or unsaturated fatty acids. In fact, it has been described that the different fatty acid composition of membrane lipids is determinant for their different hydrolysis susceptibility, thus different phospholipids show distinctive patterns of catabolism during fruit ripening or tissue senescence (Brown et al., 1991a, 1991b; Whitaker, 1988).

The different patterns of changes for individual fatty acids was made more convenient to study the unsaturation characteristics of phospholipids expressed by taking the number of double bond into account than as

Table 3
Lipid indexes of the fatty acid composition of the phospholipids in mesocarp of cherimoya during ripening calculated from the results showed in Table 2

	Day 0	Day 3	Day 6
$\sum$ Saturates	38.4 ± 2.2	41.4 ± 8.9	42.6 ± 2.5
$\sum$ Unsaturates	61.5 ± 2.7	58.6 ± 5.2	57.4 ± 2.4
$\sum$ Monoenes	15.6 ± 1.4	4.7 ± 0.6**	19.6 ± 1.3
$\sum$ Polyenes	45.9 ± 2.4	53.9 ± 5.2	37.8 ± 1.9*
UI	135 ± 4	139 ± 8	124 ± 3*
UIC18	113 ± 4	133 ± 8*	98 ± 3**
USI	3.5 ± 0.2	3.4 ± 0.8	2.9 ± 0.2*
USIC18	21.7 ± 2.9	51.0 ± 3.7***	13.0 ± 1.2*

UI (Unsaturation index) = $\sum m_i r_i$, where m_i is the number of double bonds and r_i is the relative content for each fatty acid. USI (unsaturation/saturation index) = UI/$\sum$ saturates. UIC18 and USIC18 have been calculated for C18 fatty acids as UI and USI.

* Significant statistical difference regarding the value for day 0, $P < 0.05$.

** Significant statistical difference regarding the value for day 0, $P < 0.01$.

*** Significant statistical difference regarding the value for day 0, $P < 0.001$.

Table 4
Ratios among relative content of the major C18 fatty acids of the phospholipids from mesocarp of cherimoya during ripening

Ratio	Day 0	Day 3	Day 6
C18:1/C18:0	1.79 ± 0.30	1.27 ± 0.19	1.56 ± 0.17
C18:2$n-6$/C18:1	2.60 ± 0.35	$8.85 \pm 1.89^{**}$	$1.35 \pm 0.14^{**}$
C18:3$n-6$/C18:2$n-6$	0.087 ± 0.028	$0.009 \pm 0.006^{*}$	0.147 ± 0.026
C18:3$n-3$/C18:2$n-6$	0.64 ± 0.07	0.80 ± 0.15	$0.97 \pm 0.11^{*}$

* Significant statistical difference regarding the value for day 0, $P < 0.05$.
** Significant statistical difference regarding the value for day 0, $P < 0.01$.

the total content of each type of unsaturated fatty acid (Table 3). No changes at the climacteric stage were observed in both the total number of double bond (unsaturation index, UI) and in its the ratio to the total content of saturates (unsaturation/saturation index, USI) suggesting that for climacteric ripening the changes in phospholipid composition for specific polyunsaturated fatty acids were more relevant that the unsaturation level of the membrane. However, in senescing cherimoya both unsaturation indexes decreased significantly, in accordance with the well-documented membrane deterioration of plant tissues at senescence.

UI and USI were also calculated for the results of the C18 family fatty acids (Table 3), since this was the most abundant class of fatty acids in cherimoya phospholipids and the unsaturated C18 fatty acids summed up 90% of total content of unsaturated. Both indexes for C18 fatty acids like for all fatty acids were lower in senescent than in mature-green cherimoya. However, at the pre-climacteric period both indexes for the C18 fatty acids raised because of the greater increase of C18:2$n-6$ and C18:3$n-3$ content at this stage than in the diminution of the other C18 unsaturates (Table 2). Interestingly, this result supports the importance of specific composition of phospholipids in the ripening of cherimoya. This result corroborated the fact that the variations in fatty acid composition of cherimoya phospholipids were very different at the pre- and post-climacteric period, probably responding to different physiological requirements of membrane fatty acids for fruit ripening or senescence.

Ratios among products and substrates of C18 series for different fatty acid desaturases are shown in Table 4. Comparing the changes in these ratios, we can to obtain a convenient approach to infer the relative activity of each specific desaturase during ripening. No significant changes were observed for the C18:1/C18:0 ratio suggesting that in cherimoya the activity of Δ^9-desaturase is not variable during ripening. By contrast, different profiles were found for variations of ratios reflecting desaturase activities generating polyunsaturated fatty acids. For example, the C18:2$n-6$/C18:1 ratio significantly rose at the pre-climacteric stage and decreased at senescence. Inversely, the C18:3$n-6$/C18:2$n-6$ ratio fell drastically at the climacteric stage and turned up again at senescence, to yield a value similar to the one for mature-green fruit. Only at senescence was a change in the C18:3$n-3$/C18:2$n-6$ ratio observed with an increase on day 6. Taking into account the individual content of C18 fatty acids (Table 1), the above-described variations in the different ratios suggest that the Δ^{12}-desaturase is the main desaturase activity contributing to the specific fatty acids composition of mesocarp phospholipids in climacteric cherimoya fruit. Furthermore, Δ^{15}-desaturase (ω-3 desaturase) would be active as well in this period in contrast to the very little activity of the Δ^6-desaturase. In mesocarp of senescent cherimoya, when the unsaturation index of membranes diminished (Table 3), Δ^{12}-desaturase would become less active whereas Δ^{15}- and Δ^6-desaturase activities would likely increase for the recovery of C18:3$n-3$ and C18:3$n-6$ contents.

4. Conclusion

The results of our analysis of the fatty acid composition of the phospholipids from the mesocarp of cherimoya during ripening revealed a different profile at the pre- and post-climacteric periods. However, the changes observed in the fatty acid content on different days indicated that the variation of the quantity of specific fatty acids is more important for the adaptation of mesocarp membranes to ripening and senescence processes than that of the total content of fatty acid classes. Therefore, more work is needed to identify the individual phospholipids as well as the possible positional changes of fatty acids and the effects in the membrane alterations during ripening of cherimoya fruit.

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