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CAROTENOL ESTERS IN DEVELOPING APPLE FRUITS

MICHAEL KNEE

Institute of Horticultural Research, East Malling, Maidstone, Kent ME19 6BJ, U K

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Abstract—The carotenol esters of apple (*Malus pumila*, cv Cox's Orange Pippin) fruit peel were partially characterized, and their accumulation measured relative to changes in ethylene concentration Monoesters of violaxanthin, neoxanthin and an unknown pigment, possibly cryptoxanthin 5,6,5′,6′-epoxide, were found. Lutein, violaxanthin and neoxanthin were present as diesters. Palmitate and oleate were the main acyl substituents, with lesser amounts of laurate, myristate and stearate. There were large families of diesters, presumably including mixed fatty acyl derivatives, and these were not fully resolved by reverse phase HPLC. The pattern of acyl substitution appeared to recur among the mono- and diesters of the different carotenols. Violaxanthin mono-oleate and palmitate began to accumulate *ca* 20 days before the rise in ethylene production associated with fruit ripening, whereas the increase in the corresponding diesters was concurrent with the rise in ethylene The results suggest that carotenol esters are synthesized *de novo* in ripening apples and that synthesis may be independent of ethylene or require lower concentrations to initiate than other ripening processes The pigments could serve as early indicators of fruit maturity.

INTRODUCTION

Carotenol esters accumulate in the peel of apple fruits as they ripen [1] These were tentatively identified as mono- and diesters of lutein and violaxanthin and appeared to be present in trace quantities before the onset of ripening [1] This implies that the transition of chloroplast to chromoplast is an early event in ripening and that the appearance of the pigments could be a predictive indicator of fruit maturity [2].

Carotenol esters could be formed by random transesterification from cell membrane lipids in the course of plastid lysis [3]; this would be expected to lead to substitution with fatty acids typical of those lipids, and would be in accord with the view that fruit ripening involves disruption of cell membranes and loss of regulation [4]. Alternatively a distinct fatty acid profile would imply *de novo* synthesis, consistent with the hypothesis that ripening entails a controlled induction of new metabolic activities [5].

The objectives of this work were to characterize the carotenoid pigments of ripe apple fruits, including their acylation pattern, and to determine when carotenol ester formation begins relative to the rise in ethylene synthesis which is generally regarded as marking the onset of ripening [6].

RESULTS

Carotenols in apple peel

Cox apple fruitlets, harvested in mid-July contained carotene, lutein, violaxanthin, and neoxanthin as expected in photosynthetic, higher plant tissue; the xanthophylls were non-esterified. By contrast, in apples in mid-November the bulk of the xanthophylls occurred as esters; a saponified extract yielded 65 μg lutein, 210 μg neoxanthin and 540 μg violaxanthin from 100 g peel. Minor pigments were found of polarity intermediate between carotene and lutein, their absorption spectra in the presence and absence of acid suggested that they were cryptoxanthin and its epoxides (5,6 and 5,6,5′,6′).

Carotenol esters

After column and TLC separation of the pigments of ripe Cox apples 10 carotenoid-containing fractions were isolated Saponification of these fractions showed that six of them contained esters of carotenols which were identified by their spectral properties and mobility on TLC (Table 1) The more polar esters of the di- and trihydroxy carotenoids were assumed to be monoesters and the less polar to be diesters When pure carotenoids were acylated the mobility of the esters was observed to be unaffected by the chain length of the substituent, and the R_f values agreed with those in Table 1. Free lutein copurified with neoxanthin monoesters and was estimated by HPLC of this fraction Concentrations of esterified carotenols greatly exceeded those of the free compounds and the major pigments of the ripe apple were seen to be diesters of violaxanthin and neoxanthin and monoesters of violaxanthin (Table 1). Monoesters of lutein may have escaped detection because they were a minor fraction obscured by violaxanthin monoesters.

Acylation patterns

Reverse phase HPLC showed that each ester fraction was a complex mixture but the elution profiles of the groups of monoesters showed similarities as did the diesters (Tables 2 and 3). The fatty acid composition of violaxanthin esters was determined after preparative HPLC separations Tetramethylammonium hydroxide (TMAH) was used as a catalyst of transacylation Although 5 min may be sufficient reaction time for lipids with this reagent [7], TLC analysis of the progress of derivatization showed that for carotenol esters at least

Table 1 Carotenoids in peel of ripe Cox apples

Carotenoid	Concentration (ng/g fr wt)		
	Free	Monoester	Diester
β-Carotene	110 (2, 0 94, 0 84)	na	na
Unknown monol	nd	250 (2, 0 94, 0 69)	na
Lutein	230 (20, 0 43, 0)	nd	330 (10, 0 94, 0 74)
Violaxanthin	330 (20, 0 30, 0)	1480 (10, 0 60, 0 09)	5140 (5, 0 94, 0 50)
Neoxanthin	20 (50, 0 19, 0)	420 (20, 0 40, 0)	2010 (10, 0 87, 0 20)

na, Not applicable, nd, not detected Figures in parenthesis are % Me_2CO required for elution from MgO column, R_f on silica with petrol–Me_2CO (7 3) and R_f with petrol–Et_2O–EtOH (60 20 1)

Table 2 Carotenol monoeters in peel of ripe Cox apples

Parent carotenol						Fatty acid
Unknown monol		Violaxanthin		Neoxanthin		
R_t	%	R_t	%	R_t	%	
4.3	2.4	4.0	1.7	3.6	8.7	
6.0	2.0	4.6	3.3			
6.5	10.5	6.0	14.5	5.4	12.9	Laurate (sf).
8.0	3.9					
8.4	2.7	7.3	5.2	6.7	5.2	Myristate (sf).
9.0	17.4	8.5	17.0			Oleate (f)
				8.1	55.8	
9.5	48.7	8.8	43.6			Palmitate (sf).
11.1	12.2	10.5	14.7	9.8	12.0	Stearate (sf).

R_t in reverse phase HPLC system (see Experimental), %, percent of total peak area

The fatty acid composition was determined for the violaxanthin monoesters by co-chromatography with synthetic compounds (s) and by direct GC analysis of methyl esters (f)

Table 3 Carotenol diesters in peel of ripe Cox apples

Parent carotenol								
Lutein			Violaxanthin			Neoxanthin		
R_t	%	F	R_t	%	F	R_t	%	F
10.0	10.6	C	7.9	3.6		7.1	3.6	
11.4	2.7		9.3	1.7		8.4	1.5	
12.2	15.6	L	10.3	8.5		9.3	6.3	
12.8	17.4		10.8	4.1		9.8	3.7	
13.2	9.9		11.4	5.4		10.5	5.2	
13.9	8.4					11.2	7.0	
14.4	15.3	M	12.2	8.4	M	11.7	11.6	M
15.7	4.5		13.3	5.2		12.1	5.5	
16.5	7.5		13.9	14.9		12.8	11.1	
17.5	4.9	P	14.3	15.4	P	13.3	17.4	
18.7	1.8		14.9	11.1		14.0	11.1	
			15.8	7.3		14.7	6.8	
20.2	1.4	S	16.5	5.7	S	15.7	7.4	S

R_t (min) in reverse phase HPLC system, %, percent of total peak area F, fatty acid present in synthetic diester co-chromatographing with pigment from apple C, caproate, L, laurate, M, myristate, P, palmitate, S, stearate

24 hr was needed In the first attempts to analyse apple carotenol esters, lipid contamination gave excess fatty acids which obscured those derived from the pigments This contamination was eliminated by a five min treatment of fractions after magnesium hydroxide chromatography with TMAH, this treatment did not affect the pattern of ester peaks detected by reversed phase HPLC

Synthetic esters were prepared as a further aid to identification It was found that longer reaction times than those previously reported [8] were generally required, that lutein was more readily esterified than violaxanthin or neoxanthin, and that yields diminished as the chain length increased Attempts to prepare esters with unsaturated fatty acids were unsuccessful

When violaxanthin monoesters were analysed by HPLC they were almost fully resolved into seven peaks and it was possible to associate particular fatty acids with most of these (Table 2) The identities of these monoesters, except the oleate, were confirmed by co-chromatography with synthetic compounds The diesters were not fully resolved by HPLC but, as with the monoesters palmitate and oleate were the dominant fatty acids Synthetic compounds were available to identify some of the uniformly substituted diesters (Table 3), but most of the peaks must have been mixed esters.

Ester synthesis during fruit ripening

Cox apples were harvested at weekly intervals from mid-August, their internal ethylene concentration was measured and pigments were extracted for analysis by two methods The 'silica method' involved isocratic separation on a silica column of non-polar pigments

isolated using a reverse phase sample preparation cartridge; carotene was almost unretarded on the salica column and inclusion of a volume of petrol with the sample enhanced its separation from later peaks by causing a transient decline in the alcohol content of the eluant Purified diesters were eluted as the eluant returned to its normal composition but monoesters were eluted after about five min. Elution of the sample preparation cartridges was adjusted to discard most of the monoesters, but it was noticed that a certain amount of green pigment eluted from the cartridge with the carotene and diesters The absorption spectrum of this material suggested that it was phaeophytin, and unfortunately it was eluted from the silica column at the same time as the diesters. For this reason extracts of immature fruit showed an apparent diester peak in this system

In the 'reverse phase system' the sample cartridges were eluted to retain all of the pigments and these were analysed by gradient elution on an octadecyl silica column. Phaeophytin peaks were also observed in this system; the only monoesters which were clearly separated from these and other pigments were the palmitate and oleate of violaxanthin. The faster running diesters were also obscured so that only those violaxanthin esters with palmitate, oleate and stearate could be measured.

The internal ethylene concentration of the Cox apples fluctuated below 100 nl/l until mid September, after which it increased through three orders of magnitude (Fig 1). Less than 1 ng/cm^2 of violaxanthin mono-oleate/palmitate was present in the fruit peel through most of August These pigments increased steadily during September, beginning about 20 days ahead of the rise in ethylene (Fig. 1). The presence of monoesters was confirmed by larger scale extractions early in September; pigments were isolated having retention times in the reverse phase system similar to those of authentic violaxanthin monoesters, and saponification confirmed that they yielded the parent pigment

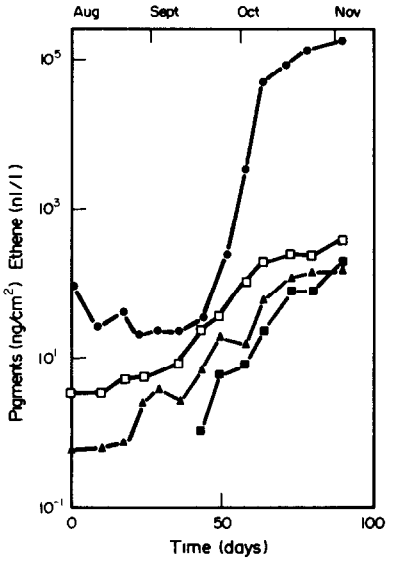

Fig 1 Carotenol esters and ethylene concentrations in ripening Cox apples ●---●, internal ethylene concentration, ▲---▲, violaxanthin mono-oleate and palmitate, ■---■, violaxanthin diesters measured by reverse phase system, □---□, diesters measured by silica system (see Experimental) Fruit samples were collected from the tree from 5 August 1986 (day 0) to 2 November 1986 and then from a bulk sample ripening at 10° in store

Violaxanthin diester peaks were first observed in the reverse phase system in mid-September (Fig. 1) Analysis of large-scale extracts of the non-polar pigments confirmed the presence of carotenoids with the characteristic elution profile of violaxanthin diesters. Interference from phaeophytin made it difficult to say when diesters first appeared in the silica system, although the peak started to increase at about the same time as diesters appeared in the reverse phase system (Fig 1). The quantitative discrepancy between the two systems could be accounted for by the presence of phaeophytin in the peak in the silica system and the non-detection of the more polar diesters in the reverse phase system However, there may also have been other unknown pigments which contributed to the diester peak in the silica system.

DISCUSSION

The results of this work confirm and extend those obtained earlier [1], superior chromatographic procedures showed that the carotenoid pigments of ripe apples were more complex than previously thought. The mono- and diester fractions described earlier [1] would be more accurately described as partially and fully esterified; the first of these fractions would contain neoxanthin diesters and diol monoesters and the second would contain monol (mono-) esters and diol diesters Some minor classes of pigment such as lutein monoesters probably escaped detection in the present work; the results suggest that all possible combinations of the four carotenoids and five fatty acids occur except for neoxanthin triesters

It is probably impossible to resolve all of these pigments in a single chromatographic system. Initially it was hoped that esters could be separated according to their parent carotenol and level of substitution by gradient elution of silica or alumina HPLC columns These systems were abandoned because of interference from chlorophyll degradation products and the isomerization of epoxides on the columns. The reverse phase system employed did not suffer from these problems, but it was only possible to estimate a few of the major esters routinely However the recurrent patterns of esterification observed in more detailed analyses suggest that the other esters would have increased in a similar fashion to those measured routinely.

In senescent leaves of woody plants total carotenoid levels declined and carotenol esters appeared to accumulate in the cytoplasm [3] In a more recent study of ageing beech leaves plastoglobuli were isolated from chloroplasts and shown to contain increasing quantities of carotenol esters as autumn advanced [9] The esters are said to be formed by transacylation from lipid [3, 9] which is simultaneously being degraded [9] Linolenyl esters of lutein have been reported from senescent leaves of *Acer* and *Aesculus* spp [10] and this acyl group could plausibly derive from chloroplast membrane lipid [11] However the predominant carotenol esters of *Populus* spp are palmitates [10], and the pigments of various flowers are often complex mixtures of esters of fatty acids from C_{10} to C_{18} [12, 13]; it is more likely that these are specifically synthesized than derived from cellular lipid

The predominant fatty acid of the intracellular lipid of apples is linoleate [14], which is also the major free fatty acid formed on ripening [15] Linolenate is the main fatty acid in apple galactolipid, which is likely to be the major lipid of plastid membranes in apple, as in other plant cells [14] The absence of linoleate or linolenate from apple carotenol esters is evidence that the pigments are specifically synthesized rather than derived from cellular or plastid membranes The increase in total carotenoid, which is largely ester [1] also argues for specific synthesis as an aspect of fruit ripening

The presence of oleate in a carotenol ester does not seem to have been previously reported. The absence of linoleate and linolenate suggests that the fatty acid precursor pool was formed within the plastid, which is thought to be incapable of the desaturation of oleate [16] The similarity of the esterification pattern of different pigments and the apparent formation of mixed diesters imply random esterification from a common fatty acid pool. At first the most likely substrates for esterification would be free carotenols to form monoesters, as the concentration of these built up the probability of their receiving a second fatty acid to form a diester would increase

Jeffery *et al* [17] distinguish between 'ethylene-dependent' and 'ethylene-independent' processes in ripening fruit The ethylene-independent processes seem to involve changes in the activity of existing pathways such as respiratory metabolism The synthesis of carotenol esters in apple begins before the rise in ethylene and may not be dependent on increased ethylene production; however ester synthesis involves new metabolic activity as do the ethylene-dependent processes previously described [17] The synthesis of carotenol esters in the apple is an example of a developmental change, that is associated with fruit ripening, but which precedes the rise in ethylene production usually thought to mark the onset of ripening [6] However the rise in ethylene synthesis itself requires preceding metabolic events McGlasson *et al* [18] argue that fruits become more sensitive to ethylene with time, so that the low concentrations present in the preclimacteric stage become sufficient to trigger ripening It could be that carotenol ester synthesis is more readily induced by ethylene than other ripening processes so that that it precedes the induction of rapid ethylene synthesis According to this view ester synthesis may not be an ethylene independent process, as it first appears Nevertheless, as an early process in ripening it could provide the basis for a practical indicator of maturity A feasible test requires a simple method of detection of the monoesters as a group rather than as individual pigments

EXPERIMENTAL

Source of fruit Apple (*Malus pumila*) fruits were harvested from trees of Cox's Orange Pippin on M9 rootstock from 5 September 1986 (day 0) at weekly intervals, for analysis on the day of harvest On 2 October 1986 a bulk sample of fruit was taken and kept at 10° for later analyses Three samples of 10 fruit were taken on each occasion for routine analysis

C_2H_4 *analysis* A gas sample was taken through a hypodermic needle inserted in the core cavity of each apple and analysed for C_2H_4 content by GC on an alumina column (50 × 0 4 cm) at 100° with N_2 carrier at 20 ml/min^2 and FID C_2H_4 concentrations were transformed logarithmically before calculation of means

Pigment extraction Apple peel was disintegrated in Me_2CO (4 ml/g fr wt) For routine extractions discs with a total area of 68 cm^2 were processed using an Ultra Turrax and for large scale extractions 100 g of peel was homogenised in a blender Routine extracts were centrifuged and the supernatant was adjusted to *ca* 30% H_2O and applied to a C_{18} sample preparation cartridge ('Sep-pak', Waters Ass, Milford, MA 01757) The cartridge was eluted with Me_2 CO–H_2O (17 3 for the silica system and 7 3 for the reverse phase system) before the pigments eluted in Me_2CO were collected Large scale extractions were filtered and partitioned into petrol (bp 60–80°), residual Me_2CO was washed out with H_2O and the extract was dried with Na_2SO_4 before loading onto a MgO column (10 × 2 5 cm), which was eluted by stepwise increments of Me_2CO in petrol

Chromatography For qualitative and preparative TLC, silica gel plates impregnated with KOH and activated 1 hr at 105° were developed with petrol–Et_2O–EtOH (60 20 1) for esters and petrol–Me_2CO for free carotenols If required, silica was scraped into funnels and eluted with Me_2CO

Most HPLC separations used a column (25 × 0 4 cm) of Whatman Partisil ODS (10 μm) eluted at 2 ml/min with a gradient from 12 5 to 0% H_2O in MeCN–MeOH (3 1) in 10 min Peaks were detected by absorbance at 440 nm and quantified by peak area with reference to synthetic standards Some separations used a 5 μm silica column (10 × 0 4 cm) eluted with petrol–2-MePrOH (200 1) at 2 ml/min, a 0 5 ml sample loop was flushed with petrol prior to loading a 0 1 ml sample for each injection

Identification of carotenol esters Fractions isolated by MgO, TLC and reverse phase HPLC were evapd and dissolved in 50 μl Et_2O After addition of 5 μl tetramethylammonium hydroxide in MeOH (Aldrich) [7] they were held 48 hr at room temp The Me esters formed were analysed by GC on 170 × 0 4 cm columns of 5% SE30 on Chromosorb G (isothermal, 220°) and 10% Silar 10C on Gas Chrom Q (programmed 4°/min, 110–200°) Identifications were based on the R_ts of dominant peaks on the two columns The carotenols liberated were identified by TLC with authentic pigments isolated from nettle (*Urtica dioica*) leaves and by comparison of absorption spectra with published data [19], the size of hypsochromic shift in the presence of HCl was used to characterise epoxides

To confirm the identity of certain pigments they were co-chromatographed with synthetic esters These were prepared from 0 1–0 5 mg of carotenols isolated from nettle leaves incubated in 1 ml pyridine with 25 μl acyl chloride for 24 hr at room temp, the products were purified using C_{18} cartridges and TLC as described above

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