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SEPARATION OF CHLOROPHYLL AND CAROTENOID PIGMENTS OF CAPSICUM CULTIVARS

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SUMMARY

A system of thin-layer chromatography on cellulose and column chromatography of mixed pigment bands on columns of magnesium oxide–Hyflo Super-Cel (1:1, w/w) and alumina was developed for the separation of chlorophyll, carotene and xanthophyll pigments in capsicum cultivars. A total of twenty six pigments were separated and identified in Large Green and Golden Californian Wonder cultivars, during maturation and ripening.

Cellulose thin layers and up to four solvent mixtures enabled chromatographic separation without degradation of chlorophylls and xanthophylls. Carotenoid hydrocarbons which travelled as one streak behind the solvent front on thin layers were separated on magnesium oxide–Hyflo Super-Cel and alumina columns.

INTRODUCTION

A rapid, inexpensive and simple chromatographic method for the separation of chlorophylls, carotenes and xanthophylls and the determination of microgram quantities of the separated pigments has long been needed to follow pigments changes in capsicum cultivars during maturation and ripening.

The separation of carotenoid pigments of capsicum cultivars previously has been achieved by column chromatography[1–4] and by countercurrent distribution followed by column chromatography[5,6]. In one study using thin-layer chromatography (TLC), Schanderl and Lynn[7] isolated chlorophylls *a* and *b* and 5 other pink fluorescent pigment derivatives from capsicum cultivars during various stages of maturation and ripening. However, Bacon[8] subsequently showed that the 5 pink fluorescent pigments isolated by Schanderl and Lynn were actually chlorophyll derivatives altered during chromatography on silica gel and not initially present in the fruit.

The purpose of the present investigation was to develop a chromatographic system permitting simultaneous separation of chlorophyll, carotene and xanthophyll pigments of capsicum cultivars at different stages of maturation and ripening.

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EXPERIMENTAL

Materials

Two capsicum cultivars, Large Green and Golden Californian Wonder, were grown at Hawkesbury Agricultural College, Richmond, Australia. Fruits were selected at the immature, mature, half-ripened and fully-ripened stages. Immature and mature stages were differentiated when fruits of the same colour and similar size of a particular colour were pressed with a finger tip. Soft fruits were graded as immature and tough fruits as mature. Half-ripened and fully-ripened stages were assessed by the changes of colour during ripening.

Phytoene was obtained from cultures of *Phycomyces blakesleeanus* (Albino 10)[9]. Phytofluene and ζ-carotene were separated from tomatoes[10,11]. β-Carotene and zeaxanthin were obtained from Hoffmann-La Roche (Nutley, N.J., U.S.A.). Lutein, neoxanthin and violaxanthin were separated from spinach using the method of Bacon and Holden[12].

Methods

Extraction of pigments. Capsicum fruits of each cultivar at one maturity level were de-stemmed, de-seeded, cored and cut into approximately 1 × 1 cm pieces. Pigments were extracted from the cut pieces of capsicums several times by homogenising with chilled acetone using an Ultra-Turrax blendor after adding a small quantity of magnesium carbonate (BDH, Poole, Great Britain), and the extracts were vacuum filtered through a sintered-glass funnel (porosity 3). The pigments in the combined filtered extracts were transferred to light petroleum (b.p. 40–60°) by adding 10% sodium chloride solution. The pigments were washed several times with distilled water to remove all traces of acetone and stored in a nitrogen atmosphere in ground-glass stoppered flasks containing sodium chloride crystals at −2° until analysed for individual pigments.

Preparation of thin layers. TLC grade cellulose powder (MN 300, Macherey, Nagel & Co., Düren, G.F.R.; 15 g) and 98 ml of distilled water were mixed for 3 min in a fast electric blendor. The slurry was immediately spread over 5 clean glass plates (20 × 20 cm) using a Desaga spreader to a layer thickness of 0.35 mm and the plates were dried overnight at room temperature.

Preparation of columns

Well mixed magnesium oxide and Hyflo Super-Cel (Johns-Manville, Fisher Scientific, Springfield, N.J., U.S.A.) (1:1, w/w) was added in small portions to glass columns (20 × 1.5 cm) and tamped firmly into place using a stainless-steel disc slightly smaller than the inside diameter of the column. The column was then subjected to a nitrogen pressure of 350 g/cm^2 through a Quickfit socket at the top of the column for about 5 min during which time settling of the adsorbent took place. Magnesium sulphate (AR, BDH) was added to the top of the column to a depth of 1–2 cm prior to use.

Pigments not separated on columns prepared as above were further separated on dry packed aluminium oxide (chromatographic grade, Johns-Manville) columns (15 × 1.0 cm) plugged with cotton wool. A slight vacuum was applied to the lower end of the column to facilitate packing.

Thin-layer chromatography

Quantitative application of gross pigment extracts in light petroleum to the cellulose thin layer as a 16–17 cm streak along the baseline of the plate was carried out using the method of Bacon[13]. Development of thin-layer plates was carried out in glass tanks (21 × 21 × 9 cm) lined with filter paper and equilibrated for 30 min with 100 ml of the appropriate solvent system prior to development. The chromatograms were developed over a distance of 15–16 cm for approximately 20–25 min in a dark room. The following solvent systems were used. Solvent system A, light petroleum–acetone–*n*-propanol (90:10:0.25, v/v); solvent system B, light petroleum–benzene–chloroform–acetone (50:35:10:5, v/v); solvent system C, hexane–*n*-propanol (99.9:0.1, v/v); solvent system D, hexane–*n*-propanol (99:1, v/v).

Elution and estimation of pigments

Quantitative removal of the separated bands from thin-layer plates was made with an elution tube following the technique of Jeffrey[14]. For the elution of pigments from the plates, the separated bands were first detected in daylight and/or under UV light (366 nm) in a dark room and marked with a metal scribe. Marked bands were numbered, R_F values determined, and the adsorbent sucked into the elution tube under vacuum. Pigments adsorbed on the cellulose powder deposited in the inner tube were eluted with small volumes of acetone, the acetone immediately evaporated to dryness by a stream of nitrogen, the residue dissolved in appropriate solvents and absorbances and spectra measured with a Unicam SP 800 double-beam recording spectrophotometer using 10-mm matched (Hysil) cells.

Column chromatography

The top band containing mixed carotenes of immature, mature, half- and fully-ripened fruits developed in solvent systems A, B, C and D was column chromatographed on magnesium oxide–Hyflo Super-Cel (1:1) columns and eluted with hexane containing increasing amounts of benzene. Separated pigment fractions from the column were collected, dried under nitrogen and their UV and visible spectra determined in spectroscopic-grade hexane.

Mixed fractions still unresolved after column chromatography were further purified on an alumina column developed with increasing amounts of diethyl ether in light petroleum. The separated fractions were dried and spectra recorded in hexane as above.

Identification of pigments

The criteria for identification of individual pigments were determination of R_F value on thin-layer plates, position of the band on the column, the colour of the band on the thin-layer plates and columns, and the comparison of the UV–visible spectra of the pigments with values reported in the literature[15–17]. Further confirmation of the identification of pigments was made wherever possible by co-chromatography with authentic pigments, behaviour in dilute hydrochloric acid, and by exposure to light in the presence of iodine. Table I shows the characteristics used to identify the capsicum pigments separated.

TABLE I

CHARACTERISTICS USED TO IDENTIFY CAPSICUM PIGMENTS

Pigment	*Absorbance maxima (nm)*	*Solvent*	*Colour on column*	*Epoxide test (HCl treatment)*		*Cochromatography with authentic sample from*
				Hypsochromic shift	*Colour*	
Phytofluene	367, 347, 331	h	—	—	—	Tomatoes
α-Carotene	473, 443, 418	h	Yellow	—	—	—
β-Carotene	476, 451, 421	h	Orange yellow	—	—	Hoffman-La Roche
ζ-Carotene	425, 400, 380	h	Light yellow	—	—	Tomatoes
5,6-Mono-epoxy β-carotene	467, 439, 416	h	Yellow	450, 425, 400	Faint green	—
5,6-Diepoxy β-carotene	467, 439, 416	h	Yellow	426, 400, 378	Blue	—
Mutatochrome	450, 422, 397	h	Yellow	—	Blue	—
Chlorophyll *a*	622, 431	a	Green	—	—	Spinach
Chlorophyll *b*	645, 455	a	Green	—	—	Spinach
Lutein	485, 456, 429	b	Light orange	—	—	Spinach
Zeaxanthin	488, 459, 432	b	Light orange	—	—	—
Violaxanthin	482, 453, 426	b	Yellow	—	—	Spinach
Neoxanthin	477, 441, 420	b	Yellow	—	—	Spinach
Cryptoxanthin	475, 448, 424	h	Light orange	—	—	—
Mutatoxanthin	453, 433	b	Yellow	—	Blue	—
Antheraxanthin	485, 455, 431	b	Yellow	452, 426, 405	Blue	—
Luteoxanthin	460, 432, 407	b	Light yellow	—	Blue	—
Cryptocapsin	519, 486	b	Light orange	—	—	—
Capsanthin	518, 482	b	Red	—	—	—
Capsanthin isomer	516, 482	b	Red	—	—	—
Capsanthin isomer	514, 482	b	Red	—	—	—
Capsorubin	522, 481	b	Red	—	—	—
Capsorubin isomer	521, 482	b	Red	—	—	—
Cryptoxanthin 5,6-epoxide	472, 443, 422	h	Yellow	—	Blue	—
Cryptoxanthin 5,6-diepoxide	469, 438, 419	h	Yellow	—	Blue	—

h = Hexane; a = acetone; b = benzene.

RESULTS AND DISCUSSION

A rapid, quantitative method has been developed for the separation of chlorophylls and carotenoids from capsicum cultivars at four different stages of maturation and ripening.

Cellulose was selected for TLC because it is neutral in reaction and does not isomerise chlorophylls and carotenoids to the same extent as do aluminium oxide, magnesium oxide or silica adsorbents under the same conditions as has been reported by Šestak[18], Strain *et al.*[19] and Strain and Svec[20]. It has the further advantage of better mechanical adhesiveness and stronger resistance to fracture, especially when pigment solutions are applied as a streak or band as distinct from spotting. However, it is easily scraped off the plate when bands are removed for pigment elution as shown by Bacon and Holden[12].

Pigments separated on cellulose plates from the Large Green cultivar at four

different stages of maturity are presented in Table II. Lutein, neoxanthin and chlorophyll *b* were separated as pure pigment bands, violaxanthin and chlorophyll *a* travelled as one band, while the carotenes travelled with the solvent. Solvent A was used for pigment extracts of immature and mature fruit.

TABLE II

PIGMENTS FROM LARGE GREEN CULTIVAR AT DIFFERENT STAGES OF MATURITY SEPARATED ON CELLULOSE THIN-LAYERS USING FOUR SOLVENT SYSTEMS

Solvent system A, light petroleum (b.p. 40–60°)–acetone–*n*-propanol (90:10:0.25, v/v); B, light petroleum (b.p. 40–60°)–benzene–chloroform–acetone (50:35:10:5, v/v); C, hexane–*n*-propanol (99.9:0.1, v/v); D, hexane–*n*-propanol (99:1, v/v).

Band No.	R_F	*Solvent system*	*Identified pigments*	*Stage of maturity*
1	0.96	A	Carotenes	Immature, mature
2	0.75		Lutein	
3	0.62		Violaxanthin, chlorophyll *a*	
4	0.40		Chlorophyll *b*	
5	0.20		Neoxanthin	
1	0.96	B	Carotenes	Immature, mature
2	0.76		Lutein	
3	0.70		Violaxanthin	
4	0.60		Chlorophyll *a*	
5	0.35		Chlorophyll *b*, Neoxanthin	
1	0.97	C	Carotenes	Half, fully-ripened
2	0.83		Cryptoxanthin, unidentified	
3	0.51		Capsanthin	
4	0.19		Zeaxanthin	
5	0.09		Cryptocapsin	
6	Baseline		Mixed xanthophylls	
1	0.97	D	Carotenes, xanthophylls, bands 2–5 from solvent C	Half, fully-ripened
2	0.82		Mutatoxanthin	
3	0.70		Unidentified	
4	0.66		Capsorubin	
5	0.52		Capsanthin isomer	
6	0.38		Violaxanthin	
7	0.35		Antheraxanthin	
8	0.29		Luteoxanthin *a*	
9	0.25		Luteoxanthin *b*	
10	0.20		Neoxanthin	
11	0.14		Capsorubin isomer	
12	0.08		Capsanthin isomer	

TLC separation of violaxanthin and chlorophyll *a* was obtained with solvent B. Carotenes, lutein, chlorophyll *b* and neoxanthin were eluted from plates developed in solvent B.

The sequence of separation on cellulose thin-layers of pigments from immature and mature stages of both capsicum cultivars was in agreement with those reported by Bacon and Holden[12] and Sherma and Lippstone[21]. However, the use of 0.25% (v/v) *n*-propanol in solvent A compared to the 0.45% (v/v) used by Bacon and Holden[12] gave better pigment separation.

No published reports are available on the TLC separation of pigments in extracts of half- or fully-ripened capsicum cultivars. Solvents C and D were subsequently developed for this purpose. Carotenes, cryptoxanthin, two unknown pigments, capsanthin and zeaxanthin were separated using solvent C, while polar xanthophylls which remained at the baseline when developed in solvent C were then separated using solvent D.

Difficulties were encountered in the separation from half-ripened fruits of the Large Green cultivar, of pigments similar to those present in both matured and fully-ripened fruits. Pigments from half-ripened fruits were separated using methods adopted for the fully-ripened stage. However, separation of chlorophyll *a* from lutein in half-ripened fruits was possible with solvent B. For routine analysis solvents B, C and D were used for the separation of pigments in half-ripened fruits.

The methods of pigment separation for the Golden Californian Wonder cultivar were the same as those used for the Large Green cultivar except that three solvent systems (A, B and C) were used instead of two (C and D) for half and fully-ripened fruit since this cultivar changes colour from green to yellow instead of to red when fully ripe. Details of pigments separated from the Golden Californian Wonder cultivar at the half and fully-ripened stages are presented in Table III.

In both cultivars, only bands of chlorophylls *a* and *b* were found, contrary to the results of Schanderl and Lynn[7] who found two fluorescent pigments in addition

TABLE III

PIGMENTS FROM GOLDEN CALIFORNIAN WONDER CULTIVAR AT HALF AND FULLY-RIPENED STAGES OF MATURITY SEPARATED ON CELLULOSE THIN-LAYERS USING THREE SOLVENT SYSTEMS*

Solvent system A, light petroleum (b.p. 40–60°)–acetone–*n*-propanol (90:10:0.25, v/v); B, light petroleum (b.p. 40–60°)–benzene–chloroform–acetone (50:35:10:5, v/v); C, hexane–*n*-propanol (99.9:0.1, v/v).

Band No.	R_F	*Solvent system*	*Identified pigment*
1	0.97	C	Carotenes
2	0.92	C	Hydroxy-α-carotene
3	0.83	C	Cryptoxanthin, cryptoxanthin 5,6-epoxide and cryptoxanthin 5,6-diepoxide**
4	0.81	C	Mutatoxanthin and unidentified**
5	0.77	C	Lutein
3***	0.70	B	Violaxanthin
4	0.62	B	Chlorophyll *a*
3§	0.50	A	Antheraxanthin
4	0.42	A	Luteoxanthin, unidentified**
5	0.40	A	Chlorophyll *b*
6	0.25	A	Auroxanthin
7	0.20	A	Neoxanthin
8	0.08	A	Unidentified

* Separation of pigments at the immature and mature stages was the same as for the Large Green cultivar shown in Table I.

** Mixed bands were column chromatographed on magnesium oxide–Hyflo Super-Cel (1:1) and eluted with hexane containing increasing amounts of ethanol.

*** Bands 3 and 4 only were eluted.

§ Bands 1 and 2 were not eluted

to chlorophylls *a* and *b* in an unripe capsicum cultivar when analysed on silica gel G thin-layers. At the half-ripened stage, both the Large Green and Golden Californian Wonder cultivars contained only chlorophylls *a* and *b*. These results also conflict with those of Schanderl and Lynn who separated fluorescent chlorophyll degradation products in addition to chlorophylls *a* and *b* by two dimensional silica gel TLC. The present results support the conclusions of Bacon[8] that the chlorophyll degradation products isolated by Schanderl and Lynn were probably artifacts resulting from pigment separations on silica gel instead of cellulose.

Carotenes from fruits of each maturity stage which travelled just below the solvent front were subsequently separated on a magnesium oxide–Hyflo Super-Cel column. Such columns were also used for the separation of mixed xanthophyll streaks (Table I). The columns were developed with hexane containing increasing amounts of benzene for carotenes, and hexane containing increasing amounts of ethanol for xanthophylls[22].

In summary, cellulose thin layers and a variety of solvent mixtures provide a chromatographic system for the rapid separation without degradation of chlorophylls and xanthophylls of capsicum cultivars at different stages of maturation and ripening. Carotenoid hydrocarbons (α-, β- and ζ-carotenes, epoxides of β-carotene and mutatoxanthin) which travel as one band just behind the solvent front were separated on magnesium oxide–Hyflo Super-Cel and alumina columns. By judicious selection of various solvent systems, qualitative and quantitative separation of chlorophyll and carotenoid pigments is possible.

REFERENCES

1 L. Cholnoky, K. Gyorgyfy, E. Nagy and M. Panczel, *Acta Chim. Acad. Sci. Hung.*, 6 (1955) 143.
2 L. Cholnoky, K. Gyorgyfy, E. Nagy and M. Panczel, *Nature (London)*, 178 (1956) 410.
3 L. Cholnoky, K. Gyorgyfy, E. Nagy and M. Panczel, *Acta Chim. Acad. Sci. Hung.*, 16 (1958) 227.
4 B. H. Davies, S. Mathews and J. T. O. Kirk, *Phytochem.*, 9 (1970) 797.
5 A. L. Curl, *J. Agr. Food Chem.*, 10 (1962) 405.
6 A. L. Curl, *J. Agr. Food Chem.*, 12 (1964) 522.
7 S. H. Schanderl and D. Y. C. Lynn, *J. Food Sci.*, 31 (1966) 141.
8 M. F. Bacon, *Biochem. J.*, 101 (1966) 34c.
9 T. C. Lee, *Ph.D. Thesis*, University of California, Davis, 1970.
10 B. K. Koe and L. Zechmeister, *Arch. Biochem. Biophys.*, 46 (1953) 100.
11 R. A. Edwards, *Ph.D. Thesis*, University of New South Wales, Kensington, 1963.
12 M. F. Bacon and M. Holden, *Phytochem.*, 6 (1967) 193.
13 M. F. Bacon, *J. Chromatogr.*, 16 (1964) 552.
14 S. W. Jeffrey, *Biochem. Biophys. Acta.*, 162 (1968) 271.
15 L. P. Vernon and G. R. Seely (Editors), *The Chlorophylls*, Academic Press, New York, London, 1966.
16 B. H. Davies, in T. W. Goodwin (Editor), *Chemistry and Biochemistry of Plant Pigments*, Academic Press, New York, 1965, p. 489.
17 F. H. Foppen, *Chromatogr. Rev.*, 14 (1971) 133.
18 Z. Sestak, *Photosynthetica*, 1 (1967) 269.
19 H. H. Strain, J. Sherma and M. Grandolfo, *Anal. Biochem.*, 24 (1968) 54.
20 H. H. Strain and W. A. Svec, *Advan. Chromatogr.*, 8 (1969) 119.
21 J. Sherma and G. S. Lippstone, *J. Chromatogr.*, 41 (1969) 220.
22 R. R. DelaMar and F. J. Francis, *J. Food Sci.*, 34 (1969) 287.