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SEPARATION OF CARROT CAROTENOIDS ON HYFLO SUPER-CEL–MAGNESIUM OXIDE–CALCIUM SULFATE THIN LAYERS

A. K. BALOCH, K. A. BUCKLE and R. A. EDWARDS

School of Food Technology, University of New South Wales, P.O. Box 1, Kensington, N.S.W. (Australia)

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SUMMARY

A Hyflo Super-Cel–magnesium oxide–calcium sulfate (8:2:0.7, w/w/w) thin-layer chromatographic adsorbent was used for the separation of carotenoid pigments of fresh and dehydrated carrots. All carrot carotenoids were separated satisfactorily in one development on thin layers treated with water, and good pigment recoveries were achieved.

The adsorbent was found to be sufficiently mild for use with carotenoid pigments, and has high resolving power for separation of the non-polar carotenes. The adsorbent containing 75% Hyflo Super-Cel is cheap and gives durable layers which are easy to handle.

INTRODUCTION

Of the various techniques reported for the separation of carotenoid pigments, thin-layer chromatography (TLC) is a comparatively inexpensive and easy method for routine examination of large numbers of samples, particularly those low in pigment content. However, pigment analyses require measures to be taken to avoid isomerisation and oxidation when polar adsorbents are used[1]. Although many adsorbents have been reported[2–6], few are non-destructive to carotenoid pigments as well as being selective in separation of non-polar carotenes.

Magnesium oxide alone or combined with Hyflo Super-Cel has been reported to be an effective adsorbent for column separation of lipids[7] and carotenoids[8–17]. However, thin layers of these adsorbents have been shown to be not entirely satisfactory for the separation of mixtures of terpene oils[18], polynuclear hydrocarbons[19] and azaaromatic compounds[20], and α- and β-carotene[21,22]. Although calcium sulfate has been used frequently with a variety of adsorbents including magnesium oxide, the role of calcium sulfate in the separation of carotenoid pigments has not been investigated in any detail.

The purposes of the present investigation was to develop a thin-layer adsorbent to resolve carrot carotenoid pigments with minimum isomerisation and oxidation.

EXPERIMENTAL

Materials

Phytofluene, and α- and β-carotene were extracted from fresh carrots (*Daucus carota*). Synthetic β-carotene (Hoffman-La Roche, Basle, Switzerland), lycopene extracted from ripe tomatoes, and lutein extracted from green leaves[23] were used as reference standards. Magnesium oxide, "heavy" (Hopkin & Williams, Chadwick Heath, Great Britain), Hyflo Super-Cel (Johns-Manville, Fisher Scientific, Springfield, N.J., U.S.A.) and calcium sulfate, pure precip. (May & Baker, Dagenham, Great Britain), were used for preparation of the adsorbent mixture. Light petroleum (b.p. 40°–60°), acetone and methanol used for pigment extraction were of analytical-reagent grade. Solvents used for development and elution of pigments were percolated through dry Hyflo Super-Cel prior to use.

Methods

Extraction of carotenoid pigments. Extractions and subsequent manipulation procedures were carried out in the dark under cool conditions. Slightly different procedures were adopted for complete extraction of carotenoid pigments from fresh and dehydrated carrots.

Fresh carrot tissue. Unblanched and blanched tissues were repeatedly extracted until almost colourless by blending in a Sorvall Omni mixer with chilled acetone containing hydroquinone (0.2 mg per 100 ml) to minimize oxidative changes[24,25], and filtered through a sintered-glass funnel (porosity 4). The residue was extracted with acetone after being ground in a mortar with methanol, until all pigment was extracted. The extracts were combined and pigments were transferred to light petroleum by addition of chilled aqueous 5% sodium chloride solution. The pigments were vacuum concentrated at room temperature and stored in a nitrogen atmosphere in ground-glass stoppered flasks at −12° until analysed for individual pigments.

Dehydrated carrot tissue. Dehydrated carrot was ground to pass 20 mesh and the powder (1.000 g) was transferred to a sintered-glass funnel and washed with 5 lots of 20 ml chilled acetone using slight suction. The residue was ground in a mortar with 10 ml of 50% methanol, and the pigments extracted with 30 ml acetone. The grinding procedure was repeated 2–3 times to complete extraction. The pigments were taken into light petroleum and stored in nitrogen at −12° until analysed.

Preparation of thin layers

Hyflo Super-Cel (32 g) and magnesium oxide (8 g) were added to 144 ml distilled water containing 2.8 g calcium sulfate, blended for 30 sec, and the slurry immediately spread over five clean glass plates (20 × 20 cm) using a Desaga spreader to a layer thickness of 0.4 mm. The plates were dried overnight at room temperature and kept in a desiccator until used within 3 days.

Preparation of graded activity thin layers

A glass plate was hung vertically behind a stainless-steel sheet containing a 30 × 3 cm hole. A 3 cm wide strip 7 cm from the lower edge of the plate was exposed to two sprays of water from a fine spraying glass nozzle 41 cm from the screen and connected to an air pressure line (0.14 kg/cm²). The plate was moved up immediately

by 2 cm, 2 cm and 1 cm, and was sprayed 4, 5 and 5 times, respectively. Uniform spraying was achieved by attaching the spray nozzle to the shaft of an oscillating fan with blades removed.

Development of chromatogram

A carotenoid solution in an Agla micrometer syringe was delivered as a streak onto one edge of a thin-layer plate (the treated or wet edge in the case of a graded activity thin layer). After pigment application, the adsorbent on either side of the plate at right angles to the pigment streak was scraped away leaving a straight edge 2 mm from the side of the plate. 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 solvent prior to development. During development for 25 min the tanks were covered with black polythene sheet to avoid light-induced decomposition of pigments.

Two solvent systems were developed for the separation of non-polar carotenes and polar carotenoids using untreated thin layers. For the separation of non-polar carotenes, including phytofluene, and α-, β-, ζ- and γ-carotenes, 0.3% ethanol in light petroleum was used. Resolution of polar carotenoids was made with 1% ethanol in light petroleum. Graded activity thin layers were used for the separation of the majority of carotenoids of carrot. A developing solvent of 0.5% acetone in light petroleum was used.

Elution and estimation of pigments

Separated pigment bands detected in day light, or fluorescent streaks under a UV lamp (*e.g.* phytofluene) were marked and scraped from the plate into a small sintered-glass funnel attached to a side-arm test tube. Pigments were eluted with acetone under slight suction. The acetone was evaporated under a stream of nitrogen and

TABLE I

R_F VALUES OF CAROTENOIDS SEPARATED BY TLC OF CARROT EXTRACTS

Solvents in light petroleum (b.p. 40°–60°): A = 0.3% ethanol, B = 1.0% ethanol, C = 0.5% acetone.

Carotenoids	*Untreated thin layer*				*Graded-activity thin layer*
	Fresh carrot extract		*Dehydrated carrot extract*		*Fresh carrot extract*
	A	*B*	*A*	*B*	*C*
Phytofluene	0.95	0.98	0.95	0.98	0.99
α-Carotene	0.92	0.98	0.92	0.98	0.95
β-Carotene	0.69	0.98	0.75	0.98	0.83
ζ-Carotene	0.50	0.98	0.51	0.98	0.67
γ-Carotene	0.14	0.98	0.14	0.98	0.42
cis-Neurosporene	0.06	0.78	0.06	0.78	0.31
Neurosporene	0.06	0.35	0.06	0.36	0.22
Lycopene	0.06	0.21	0.05	0.20	0.17
Lutein	0.06	0.11	0.06	0.11	0.10
Unidentified 1	0.06	0.06	0.06	0.06	0.07
2	0.06	0.01	0.06	0.01	0.02

TABLE II

CHARACTERISTICS USED TO IDENTIFY CARROT CAROTENOIDS

Carotenoid	*Absorbance maxima in light petroleum (nm)*	*Criteria for identification in addition to $\lambda_{max.}$*
Phytofluene	328, 345, 364	Green fluorescence in UV light
α-Carotene	420, 445, 474	First coloured band after phytofluene on magnesium oxide–Hyflo Super-Cel (1:1, w/w) column
β-Carotene	423, 450, 476	Co-chromatography with authentic β-carotene
ζ-Carotene	379, 400, 424	Light yellow band on magnesium oxide–Hyflo Super-Cel (1:1, w/w) column after β-carotene band
γ-Carotene	436, 460, 490	Red colour
cis-Neurosporene	414, 437, 466	Characteristic shift to 415, 440, 490 nm on iodine treatment
Neurosporene	415, 440, 469	Bright yellow band ahead of lycopene on magnesium oxide–Hyflo Super-Cel (1:1, w/w) column
Lycopene	446, 470, 502	Co-chromatography with lycopene from red tomatoes
Lutein	420, 445, 471	Co-chromatography with lutein from green leaves

the pigments were dissolved in light petroleum. Absorbances and spectra were measured with a Unicam SP800 spectrophotometer using matched 1-cm silica cells.

Identification of carotenoids

Individual carotenoids were characterised by comparing their chromatographic behaviour (Table I) and ultraviolet–visible absorption spectra (Table II) with values reported in the literature[2,26]. For co-chromatographic tests, synthetic β-carotene, and purified lycopene and lutein were used as reference standards. *Cis–trans* pigment configurations were determined by the iodine isomerisation test reported by Goodwin[3].

Recovery of carotenoids on graded activity plates

A mixture of known carotenoids (10–13 ml) was streaked onto a graded activity plate, developed with 0.5% acetone in light petroleum, and the separated bands scraped from the plate. Pigments were eluted with acetone, the acetone evaporated, and the pigments dissolved in light petroleum. Pigment recoveries are reported in Table III.

TABLE III

RECOVERY OF CAROTENOIDS FROM A GRADED-ACTIVITY THIN-LAYER OF HYFLO SUPER-CEL–MAGNESIUM OXIDE–CALCIUM SULFATE (8:2:0.7, w/w/w)

Carotenoids	*Pigment applied (μg)*	*Pigment recovered (μg)*	*Recovery (%)*
Phytofluene	10.0	9.9	99.0
α-Carotene	12.0	11.9	99.2
β-Carotene	13.0	12.9	99.2
ζ-Carotene	13.0	12.8	98.5
Lycopene	13.0	12.9	99.2
Lutein	12.0	12.0	100.0

RESULTS AND DISCUSSION

Separation of carotenoid pigments by thin-layer chromatography

During an investigation of the apparent increase in carotenoid pigment content during the processing of carrots[27], it was necessary to separate carotenoids pigments of processed carrots without the pigments being damaged or changed during separation. Most of the TLC adsorbents available, such as silica gel G, magnesium oxide and calcium hydroxide, which have been used for the separation of α- and β-carotenes, were found to be either moderately destructive to carotenoids or the separation took a long time with substantial losses in carotenoid pigments. During preliminary trials, it was of great interest to note that satisfactory separation of α-, β- and ζ-carotenes on thin layers was achieved with BDH (Poole, Great Britain) magnesium oxide, which was found to contain calcium sulfate. Since BDH magnesium oxide was not readily available in sufficient quantity during these studies, calcium sulfate was added to Hopkin & Williams magnesium oxide adsorbent resulting in a satisfactory separation of the carotenes. To determine if this effect was due to the binding power of calcium sulfate, it was replaced by the same amount of starch. In this case the adsorbent did not separate α-, β- and ζ-carotenes, indicating that the mode of action of calcium sulfate in the adsorbent was not entirely due to its adhesive property.

Although α-, β- and ζ-carotenes were separated by using magnesium oxide and Hyflo Super-Cel in a ratio as low as 1:7, a level of magnesium oxide lower than 1:5 was progressively less effective in yielding consistent results. Hyflo Super-Cel–magnesium oxide (4:1) containing 7% calcium sulfate was finally adopted as the thin-layer adsorbent for the separation of carrot carotenoids.

Separation of carotenoids of fresh and dehydrated carrots

Raw and blanched carrots. Separation of pigments from raw and blanched carrots was carried out on untreated thin layers and also on graded activity thin layers, both layers producing satisfactory results. There was no appreciable difference in the separation pattern or the R_F values of the pigments in raw and blanched carrot extracts. The R_F value of phytofluene, α-, β-, ζ- and γ-carotenes separated on untreated thin layers using 0.3% ethanol in light petroleum as developing solvent are shown in Table I. An over-lapping of the phytofluene and α-carotene bands was observed although this did not interfere with estimations of these pigments as the major adsorption maxima were at 345 and 445 nm, respectively (Table I).

However, a clear separation was achieved for the other pigments of the group, while the relatively polar carotenes and xanthophylls remained unresolved near the baseline. These unresolved pigments were separated on the same layer using 1.0% ethanol in light petroleum as developing solvent. The less polar carotenes such as phytofluene, α-, β-, ζ- and γ-carotenes travelled as a group with the solvent, whereas the other carotenes and xanthophylls were separated as indicated in Table I.

All of the carotenoids in a carrot extract were separated in one development with 0.5% acetone in light petroleum using graded activity thin layers. Difficulties occurred in the detection of some of the pigments due to their presence in low concentrations in the carrot extract. α- and β-carotenes constituted about 18 and 68%, respectively, of the total pigments of the carrot, whereas the amounts of the relatively polar carotenes and xanthophylls were considerably lower (Table IV).

TABLE IV

CAROTENOIDS OF RAW CARROT

Carotenoids	*Proportion of total pigments (%)*
α-Carotene	17.8
β-Carotene	68.4
ζ-Carotene	5.6
γ-Carotene	2.3
cis-Neurosporene	0.7
Neurosporene	1.7
Lycopene	0.5
Lutein	2.9
Unidentified carotenoids	0.2

Dehydrated carrot

Pigments in extracts of dehydrated carrots were analysed on untreated thin layers. Using 0.3% ethanol in light petroleum as developing solvent, the only noticeable difference in pigments pattern between raw and blanched carrots and dehydrated carrots was in the β-carotene fraction of the latter, which was diffused with a higher R_F value. Nevertheless, there was a clear separation of α-, β-, ζ- and γ-carotenes (Table I). The diffused band of β-carotene was probably due to the presence of isomers of β-carotene. There was no detectable difference in R_F values of the relatively polar carotenes and xanthophylls of fresh and dehydrated carrots when 1.0% ethanol in light petroleum was employed as the developing solvent.

Recovery of separated carotenoids from treated thin layers

A mixture of known amounts of pigments including phytofluene, α-, β- and ζ-carotenes, lycopene and lutein was streaked onto a graded activity thin layer, and the pigments separated using 0.5% acetone in petroleum ether. The recovery of individual pigments is reported in Table III. The high recoveries are attributed mainly to the low concentration of magnesium oxide used in the adsorbent mixture. Moreover, the water content of the graded activity thin layer kept the layer moist during development resulting in higher pigment stability. A greater recovery of anthracene on magnesium oxide using pentane saturated with water as a solvent has also been reported by Snyder[28].

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