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Note

Chromatography of chloroplast carotenoids on magnesium oxide thin layers

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Only one adequate selective thin-layer chromatographic (TLC) method for separation of both the major and minor chloroplast carotenoids is available[1]. However, a mixture of as many as three adsorbents and an eluent consisting of three components are required in that separation. The other methods described[2–10], on account of their low selectivity, can be applied only for resolution of a few major chloroplast carotenoids.

The purpose of the present investigation was to develop a simple and selective chromatographic system for resolution of the major, as well as minor, carotenoids from photosynthetically active tissues. Magnesium oxide was selected for these experiments because it has successfully been employed as a component of adsorbent mixtures for TLC separation of the carotenoids, primarily carotenes[1,11–18].

Alone, magnesium oxide has proved adequate for the separation of major and minor chloroplast carotenoids[19]. However, the adsorbent applied in those investigations appeared to be less selective than that used in the present work. Apart from the above example, to our knowledge, magnesium oxide has been used only for the separation of standard mixtures of carotenes (ε, α, β, δ, γ, lycopene)[20].

EXPERIMENTAL

Plant material and reagents

Leaves and cotyledons, sampled at various stages of plant ontogeny, were obtained from phytotron cultures of bean, *Phaseolus vulgaris L.* var. *Złota Saxa*. All reagents used were analytical reagent grade, except solvents of special purity for spectroscopy.

Extraction of plant tissues

The freshly collected and undamaged plant material was ground in a mortar with 2,6-di-*tert*.-butyl-4-methylphenol (antioxidant) and glass sand. The resulting homogenate was extracted repeatedly with a chilled solution of acetone–light petroleum (b.p. 40–60°C) (1:1, v/v) until all pigments were washed out. The combined extracts were filtered through a G3 sintered glass funnel and washed with distilled water to remove all traces of acetone. Any remaining water was removed by adding anhydrous sodium sulphate. The dried extract was concentrated under reduced pres-

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sure to a small volume and used for chromatography. All the isolation operations were carried out in the dark or, when this was not possible, in dim light.

Preparation of thin layers

The thin layers (0.5 mm) were formed from a slurry of magnesium oxide (International Enzymes, Great Britain) in water (1:5, w/v). Since the standard drying conditions caused the cracking of magnesium oxide layers, a special drying procedure was developed.

Following preliminary drying in the air at room temperature for about half an hour, the chromatoplates were transferred to a thermostat and kept at about 90% relative humidity. After at least 12 h of incubation the thin layers were again dried in the air until the rest of the water had been lost. The chromatoplates did not require activation.

Development of chromatogram and elution of separated carotenoids

A pigment extract was applied across the chromatoplate as a narrow linear streak with Hamilton gas-tight syringe. Development was carried out in a rectangular glass TLC tank lined with filter-paper and equilibrated for 20 min with the developing solvents prior to development. α- and β-carotene geometrical isomers, carotenes (α, β, δ, γ) and xanthophylls (with lycopene) were respectively developed with 12, 20 and 35% solutions of acetone in light petroleum. The chromatograms were run in the ascending mode until the solvent had moved about 18 cm from the origin. Resolved pigment bands were immediately scraped from the plate and then eluted with acetone, except for lycopene and ketohydroxylycopene which were eluted with acetone–benzene (1:1, v/v).

Identification of carotenoids

Identification of the separated carotenoids was mostly based on their colours, chromatographic behaviour in a number of different adsorbents, partition between two immiscible solvents, ultraviolet-visible electronic absorption spectra in different solvents and comparison of these spectra with reported values[21,22].

In most cases co-chromatography with authentic carotenoid in at least two adsorption systems was performed. The reference compounds were prepared from well characterized plant tissues or obtained from Sigma (α- and β-carotenes). The reference α- and β-carotene *cis* isomers were prepared by the photoisomerization with iodine of synthetic all-*trans* isomers according to the procedure described by Zechmeister[23]. For the identification of 5,6-epoxides, specific tests based on their acid-catalysed isomerization[24] and on their treatment with 20% hydrochloric acid[25] were carried out. Detailed methods of ketohydroxylycopene identification were as described[26].

Recovery of carotenoids

Recoveries were determined by comparing the absorbance of the original carotenoid eluted from the TLC plate with the absorbance of the same carotenoid diluted in the same solvent but not subjected to chromatography. In all recovery investigations, acetone was used both as a solvent and as the eluent for carotenoids on chromatoplates.

RESULTS AND DISCUSSION

The complete resolution of more than twenty plastidic carotenoids, nineteen of which were identified, derived from both saponified and unsaponified photosynthetic tissue extracts, was achieved. The best solvent for the separation of carotenoids on magnesium oxide thin layers was light petroleum–acetone, which was applied at various ratios depending on the polarity of the separated compounds. The separations of carotenoids from bean leaves and cotyledon extracts are given as examples of this method (Table I and II).

The results provide evidence that the magnesium oxide thin layers are extremely selective media for chloroplast carotenoid resolution, especially for double-bond positional isomers, lutein and zeaxanthin, δ- and γ-carotenes or α- and β-carotenes, as well as for geometrical isomers of α- and β-carotenes, which are usually difficult to separate[6,27–29].

In addition, these layers also proved selective for the separation of some ketocarotenoids originating from extraplastidic sources[30].

The separations were relatively rapid and the adsorbent need not be mixed with any filter aid to facilitate the flow of wash liquids. Ascending development over a distance of 18 cm requires 40 min, but weakly adsorbed carotenoids such as α- and β-carotenes could be separated in only 5 min.

As result of the chromatography, homogeneous and sharp bands without any tailing have been obtained. The homogeneity was confirmed by rechromatography on other adsorbents of various polarities such as cellulose, Celite, Kieselguhr, silica gel and aluminium oxide. Good quantitative recovery of some labile carotenoids from magnesium oxide thin layers testifies to the low degree of isomerization, oxidation or decomposition of carotenoids during chromatography on this adsorbent (Table III). The high adsorption capacity of this adsorbent should be emphasized. On magnesium oxide layers (0.5 mm) the optimum loading was about 60 μg of bean leaf carotenoids in any volume of photosynthetic tissue extract. Otherwise, about 300 mg of an extract of the fresh leaves could be applied to a single chromatoplate as a narrow stripe, 15 cm long and 0.3 cm wide, and yielded high resolution.

TABLE I

R_F VALUES OF GEOMETRICAL ISOMERS OF α- AND β-CAROTENES FROM PRIMARY BEAN LEAF EXTRACTS SEPARATED ON MAGNESIUM OXIDE THIN LAYERS

Solvent: light petroleum–acetone (88:12, v/v). Identification of the *cis* isomers was performed on the basis of their chromatographic behaviour and spectral effects of *trans cis* isomerization in the visible and ultraviolet regions[23].

Isomer	R_F
Neo-α-carotene W	0.90
All-*trans*-α-carotene	0.84
Neo-α-carotene A	0.78
Neo-α-carotene B	0.70
Neo-β-carotene U	0.64
All-*trans*-β-carotene	0.59
Neo-β-carotene B	0.51
Neo-β-carotene E	0.44

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Book Review

Essential oils analysis by capillary gas chromatography and carbon-13 NMR spectroscopy, by V. Formáček and K.-H. Kubeczka, Wiley, Chichester, New York, 1982, XIV + 373 pp., price £ 53.50, ISBN 0-471-26218-8.

This book is intended to serve as a guide for students and professionals concerned with essential oils, perfumes and flavours. Its first and largest part (307 pp.) consists of an excellent description of some 50 essential oils of industrial importance (*e.g.*, Bergamot, Lavender, Lemon, Lemongrass, Mint, Orange, Pine needle, and other oils). The data provided for each essential oil include a brief introductory section, a quantitative listing of components, and good pictures of the capillary gas chromatogram and ^{13}C nuclear magnetic resonance (NMR) spectrum. The latter, in which characteristic signals are labelled by a substance code, is given complete for a total chemical shift range from −8 to 232 ppm, with appropriate parts expanded, and with a tabulated presentation of shift and intensity data.

To facilitate their identification in essential oils, the ^{13}C NMR spectra of 134 reference compounds of common natural occurrence (mainly terpenes and aromatic substances) have been included in the second part (49 pp.) of the book. There is no doubt that such a set of reference spectra, although very limited in size, will be highly appreciated by analytical chemists. The book also contains a ^{13}C NMR data table of reference compounds (in the order of decreasing ppm values) (Appendix A), and a short, very general bibliography (Appendix B).

The illustrations (gas chromatograms, ^{13}C NMR spectra), tables, and formulae are well set out, the printing is clear, an excellent quality grade paper was used, and there is no subject index problem (however, the farnesol formula on p. 332 is not correct, while, strangely enough, lavandulol is described as *cis* and *trans* isomers on p. 336). Unfortunately, the good general appearance of the book does not counterbalance two serious weak points. First, there are no specific literature references provided for any of the essential oils dealt with. This deliberate omission seems unexplainable inasmuch as plenty of free space was left on most pages of the book (for an example of a well-referenced, similar book, see *Analysis of essential oils by gas chromatography and mass spectrometry*, by Yoshiro Masada, Wiley, 1976). Second, a number of pages were either mixed up or lost, at least in the reviewer's copy. The reader is thus surprised to go from p. 18 directly to p. 211, and then back from p. 242 to p. 51, while pages 19 to 50 are simply missing.

A comment is also in order about the authors' intent to demonstrate that essential oils can be directly and safely analyzed by ^{13}C NMR spectrometry in a way requiring no preliminary separation of their individual components. In spite of the 50 examples or so provided in the book, the reviewer is not fully convinced that this direct analytical approach will be largely accepted and applied in the near future, merely because most analysts are reluctant to deal with needlessly complicated prob-

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