Note

Reversed-phase thin-layer chromatography of carotenoids

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A variety of methods has been applied to the analytical separation of carotenoids, including column chromatography, paper chromatography, thin-layer chromatography and high-performance liquid chromatography. These techniques are discussed in recent reviews[1–3]. Thin-layer chromatography (TLC) is still, however, the most widely used technique owing to cost factors, relative reproducibility and simplicity.

A wide variety of systems, involving many different adsorbants and developers, has been used for the TLC of carotenoids[1–3]. Reversed-phase TLC (RP-TLC) of carotenoids on impregnated plates was described at an early date by Egger[4] and Randerath[5]. Later work has concentrated on this type of plate[6–7], although Sherma and Latta[8] have published a method using chemically bonded phases. This last method was not tailored specifically for carotenoids, being designed for use on all photosynthetic pigments, and yields rather broad zones. The aim of the present work was thus to develop a system of general utility for the separation of carotenoids on a chemically bonded phase.

The results obtained show that using RP-18 plates a series of compositions containing mixtures of light petroleum (b.p. 40–60°C), acetonitrile and methanol may be successfully applied to the RP-TLC of carotenoids. A system of light petroleum–acetonitrile–methanol (25:25:50) gives sharp zones and allows separation of the normal range of photosynthetic carotenoids in a single step. The results are thus fully comparable with the best obtained on impregnated plates and the method is markedly superior to normal-phase TLC, which requires serial chromatography to achieve similar separation.

EXPERIMENTAL

Thin-layer chromatography

All thin-layer chromatography was carried out using commercially coated 0.25-mm RP-18 plates (Art. 15423, Merck, Darmstadt, F.R.G.). Solvent mixtures used for chromatographic development were prepared from analytical grade solvents as received. Initial trials were carried out using 20% (v/v) mixtures of various solvents in methanol. Thereafter, a set of solvent systems having the following compositions of light petroleum (b.p. 40–60°C), acetonitrile and methanol were selected for more detailed study: system 1 (10:80:10), system 2 (10:60:30), system 3 (10:40:50), system

4 (10:20:70), system 5 (10:10:80), system 6 (20:40:40), system 7 (20:35:45), system 8 (20:30:50), system 9 (20:20:60), system 10 (20:10:70), system 11 (25:25:50) and system 12 (30:10:60). Developing tanks were lined with heavy-grade filter paper and allowed to equilibrate prior to use. Carotenoids were applied to the plates as 1-μl aliquots of solutions with an optical density of 3.0. Plates were developed to a distance of 10 cm.

Carotenoids

Canthaxanthin was available commercially (Art. 21385, Fluka, Buchs, Switzerland) and isozeaxanthin was obtained by lithium aluminium hydride reduction of canthaxanthin as described in the literature[9]. All other carotenoids were obtained by extracting to exhaustion the appropriate plant material[10–16] with portions of acetone and acetone–methanol (1:1). The combined extracts were taken to dryness and saponified by allowing the extracted material to stand overnight in a 1:1 mixture of 10% methanolic sodium hydroxide and diethyl ether. Saponified pigments were then recovered by diethyl ether extraction and, after washing to neutrality with water, the mixture was reduced to a small volume prior to preparative TLC. Individual carotenoids were isolated on Kieselgel 60 G layers (0.7 mm) prepared in the laboratory (Kieselgel 60 G, Art. 7731, Merck) using appropriate mixtures of acetone–light petroleum (b.p. 40–60°C)[2].

RESULTS AND DISCUSSION

While water is normally a component of the developing solvent in RP-TLC, carotenoids are largely of a hydrocarbon nature and preliminary work revealed that neither water–methanol mixtures nor indeed methanol alone were suitable developers for these compounds. A series of common organic solvents were thus added (20%, v/v) to methanol in an attempt to find a more suitable eluent.

Carotenoids, as commonly encountered, have between zero and four oxygen functions. The hydrocarbon β-carotene is one of the least polar and the epoxytriol neoxanthin one of the most polar compounds that are normally encountered. The R_F values for these compounds may thus be used to indicate the range of values to be expected for the carotenoids. The R_F values obtained for these compounds varied little using 20% (v/v) mixtures of the following solvents in methanol: acetone, butan-2-one, dibutyl ether, diethyl ether, ethyl acetate, light petroleum (b.p. 40–60°C), *tert.*-butanol and *tert.*-pentanol. The R_F value for β-carotene was 0.15 ± 0.03 and for neoxanthin 0.63 ± 0.06; the difference between the R_F values for β-carotene and neoxanthin was 0.50 ± 0.04, depending on the solvent. With ethanol, propanol or isopropanol in a 20% (v/v) mixture with methanol, higher R_F values than in the above cases were observed. However, these solvent mixtures were not investigated further, since only very broad zones were obtained.

Although the acetonitrile–methanol solvent pair provided the lowest R_F values recorded, concentrated zones with very little spreading were found and this advantage was sufficiently marked in comparison with the other pairs as to justify the inclusion of acetonitrile in the further development of the system. The best spread of values was obtained with light petroleum (b.p. 40–60°C) and dibutyl ether. Since dibutyl ether is considerably more difficult to remove in subsequent processes, light petro-

TABLE I

R_F VALUES FOR CAROTENOIDS

RP-TLC on 0.25-mm RP-18 layers using light petroleum (b.p. 40–60°C)–acetonitrile–methanol mixtures as developing solvent. Solvent compositions are indicated by numerals and details are given in the text. Compound sources: (a) rowan[10], (b) tomatoes[11], (c) gazania[12], (d) commercial, (e) parsley[13], (f) lithium aluminium hydride reduction[9], (g) tiger-lily[14], (h) dandelion[15] and (j) paprika[16]. The oxygenated functional groups present in each carotenoid are indicated in the column OFNG as follows: H, hydroxy; K, keto; and E, epoxy.

Carotenoid (source)	OFNG	Solvent system											
		1	2	3	4	5	6	7	8	9	10	11	12
β-Carotene (a)	None	0.07	0.09	0.10	0.11	0.09	0.13	0.16	0.13	0.13	0.16	0.16	0.20
Lycopene (b)	None	–	0.16	0.17	0.15	0.13	0.23	0.27	0.21	0.21	0.21	0.25	0.27
β-Cryptoxanthin (a)	H	–	0.17	0.22	0.23	0.21	0.31	0.37	0.31	0.31	0.33	0.37	0.41
Gazaniaxanthin (c)	H	–	0.21	0.26	0.26	0.23	0.38	0.42	0.37	0.34	0.37	0.43	0.45
Canthaxanthin (d)	KK	–	0.29	0.32	0.31	0.30	0.51	0.52	0.45	0.45	0.46	0.55	0.57
Lutein (e)	HH	0.18	0.30	0.37	0.41	0.37	0.55	0.56	0.52	0.52	0.54	0.59	0.63
Izozeaxanthin (f)	HH	–	0.31	0.38	0.43	0.38	0.57	0.58	0.52	0.54	0.55	0.62	0.66
Antheraxanthin (g)	HHE	–	0.36	0.44	0.46	0.43	0.60	0.63	0.58	0.57	0.57	0.63	0.66
Taraxanthin (h)	HHE	–	0.38	0.47	0.47	0.44	0.62	0.64	0.60	0.58	0.59	0.64	0.67
Capsanthin (j)	HHK	–	0.40	0.48	0.49	0.45	0.64	0.65	0.62	0.60	0.62	0.66	0.69
Violaxanthin (e)	HHEE	–	0.43	0.55	0.56	0.52	0.68	0.69	0.65	0.63	0.66	0.68	0.73
Neoxanthin (e)	HHHE	0.31	0.51	0.63	0.64	0.58	0.72	0.75	0.70	0.67	0.71	0.74	0.77

leum (b.p. 40–60°C) was preferred. Further experiments were thus directed towards obtaining a solvent containing a mixture of acetonitrile–light petroleum (b.p. 40–60°C)–methanol, which could separate as wide a variety of carotenoids as possible in a single operation.

A partial approximate phase diagram for the solvent trio was obtained on an experimental basis. This diagram, reproduced in Fig. 1, is similar to the exact diagram recently published[17] for hexane–acetonitrile–methanol, although in the latter case the area of miscibility is reduced.

A series of compositions in the bulk of the miscibility area were tested using representative carotenoids. Solvent compositions are given in the experimental part and R_F values and carotenoid sources in Table I. It is apparent that, in general terms, R_F values increase as acetonitrile is replaced by petroleum ether. The same result is obtained by replacing methanol by light petroleum. The situation along the third axis is different: maximum R_F values are observed at 30–40% acetonitrile, with a rapid fall as more acetonitrile is added, while virtually no change is observed on reduction of the acetonitrile content to the 10% level.

The R_F values recorded for the individual carotenoids follow the order expected on the basis of polarity considerations. The hydrocarbon carotenes have the lowest values and increases are observed for each functional group added, the effect of a hydroxy group being greater than that of a keto group, which in turn has a greater effect than an epoxy group.

Detailed examination of the R_F values recorded here shows that the various individual compounds do not vary greatly in their response to solvent changes. The most important criteria for judging the various systems are thus the effective spread of R_F values and the extent of spot diffusion. Systems 6, 7, 8, 11 and 12 have R_F values which differ by 0.58 ± 0.01 for β-carotene and neoxanthin, and are thus the best from the point of view of optimal use of the plate surface. The smallest spots were obtained on development with system 11, although solvent systems 6, 7 and 8 gave only slightly more diffuse spots. System 1 was alone in giving spots which were too diffuse for normal use.

While solvent systems 2–12 are all useable, system 11 appears to be most suited for RP-TLC of the type of carotenoid mixture normally encountered in practice.
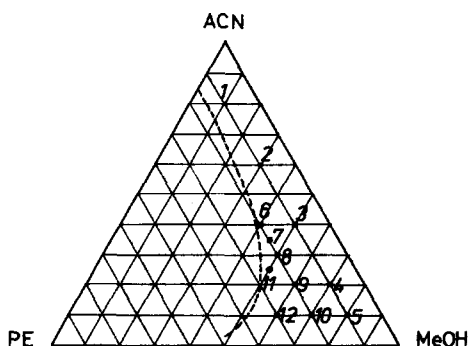

Fig. 1. Solvent compositions (by volume) for the light petroleum (b.p. 40–60°C)–acetonitrile–methanol (PE–ACN–MeOH) system. The miscibility limit (at 22°C) is indicated by a broken line. Developing solvents for the RP-TLC of carotenoids are shown by numerals.

When the carotenoid mixture consists largely of less polar compounds, system 12 might be preferred; whereas when only more polar compounds are present, system 3 is probably advantageous.

In conclusion, a wide variety of solvent compositions consisting of light petroleum (b.p. 40–60°C), acetonitrile and methanol may be successfully applied to the RP-TLC of the normal range of carotenoids on RP-18 thin layers. The serial chromatography usually required in normal-phase TLC of carotenoid mixtures is obviated.

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