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Note

Improved solvent system for plant pigment separation on silica gel thin layers

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The use of silica gels in thin-layer chromatography (TLC) of plant chlorophylls and carotenoids is hampered by the fact that these pigments are relatively labile compounds and they decompose[1–4] on these reactive adsorbents. Many studies indicate that allomerization as well as deep degradation takes place during sample application and solvent evaporation from the plates after the separation is completed[1–3]. Reversed-phase chromatography was proposed to avoid these shortcomings[2,3]. In this case, thin-layers are impregnated with hydrophobic coatings which stabilize the pigment during the separation. However, this method is limited by a low adsorbent capacity.

In this paper we report that pigment stabilization on thin silica gel plates as well as a considerable improvement in resolution are achieved by *n*-hexadecane addition to the mobile phase. The modified solvent system provides more favourable conditions for direct densitometric pigment estimation after one-dimensional separation. This solvent system can also be applied to preparative TLC.

MATERIALS AND METHODS

All solvents were analytical-reagent grade and were used without further purification.

The pigments from pea (*Pisum sativum* L.) and chestnut (*Aesculus hyppocastanum* L.) leaves and from cyanobacterium [*Synechococcus elongatus* (Strain No. 120)] were extracted (after homogenization) with chloroform–methanol (2:1, v/v). The chloroform layers were separated and dried over anhydrous Na_2SO_4. Lipid saponification and pigment identification were performed as described previously[5]. Silufol (Kavalier) precoated plates, 20 × 20 cm glass plates coated with silica gels LC 5/40 μm (Chemapol) and BDH (both containing 13% of gypsum) and also "permanent" plates prepared according to Tsydendambaev *et al.*[6] were used for chromatography in an unsaturated vertical narrow chamber. The glass plates coated with silica gel were heated at 110°C for 1.5 h. An ERJ-65 M (Carl Zeiss Jena) densitometer (filter "430", slit 0.3 mm) was used for scanning Silufol plates as was described earlier[5]. Gel chromatography on a

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Sephadex LH-20 column (100 × 1.0 cm I.D.) was performed in chloroform–methanol (1:1) with permanent registration of the fluorescence in chlorophyll emission bands. The contents of *n*-Hexadecane, butylated hydroxytoluene (4-methyl-2,6-di-*tert.*-butylphenol) and *d,l*-α-tocopherol were measured using a Chrom-5 gas chromatograph equipped with a dual flame ionization detector system and 2.5 m × 3 mm I.D. glass columns packed with 5% XE-60 on Chromaton N AW DMCS.

RESULTS AND DISCUSSION

It was found that *n*-hexane–acetone–benzene–*n*-hexadecane (1:1:1:1) as solvent provided good separation of carotenes, chlorophylls and xanthophylls on Silufol plates (Fig. 1). Comparison with chromatography in a standard system[5] shows that hexadec-

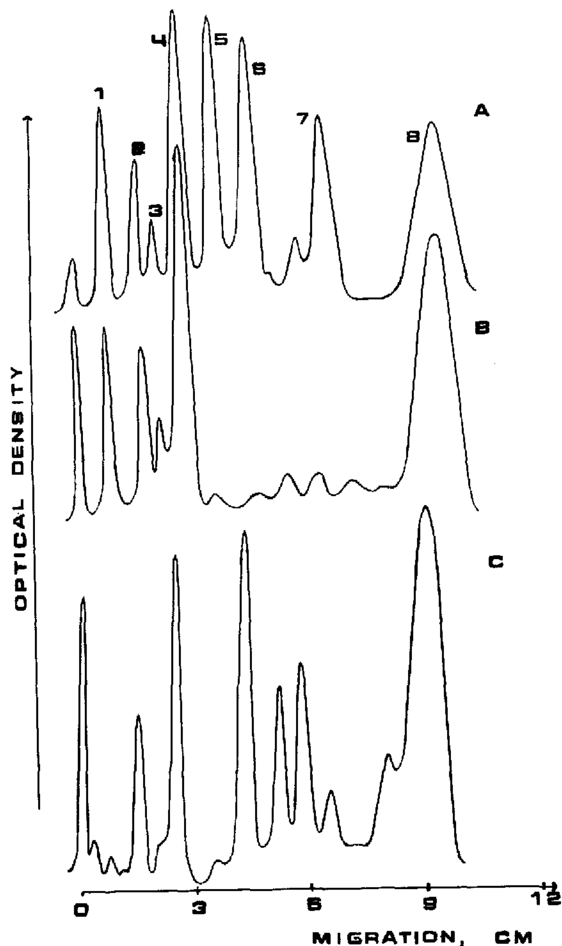

Fig. 1. Separation of plant pigments with Silufol plates in hexane–acetone–benzene–hexadecane (1:1:1:1) solvent. A, Total lipids of *P. sativum* ; B, unsaponifiable lipids of *A. hyppocastanum* ; C. total lipids of *S. elongatus*. Peaks : 1 = neoxanthin ; 2 = violaxanthin ; 3 = antheraxanthin ; 4 = lutein ; 5 = chlorophyll *b* ; 6 = chlorophyll *a* ; 7 = pheophytins and degradation chlorophyll products ; 8 = carotenes.

ane addition leads to considerable zone narrowing. A similar effect was also produced when small amounts of *n*-eicosane were introduced into the solvent system instead of hexadecane. The separation efficiency did not depend on the non-polar component since an increase in the amount of hexane (in the absence of paraffins) did not improve the resolution. This suggests that the improvement in separation is the result of partition effects due to the presence of the heavy viscous solvent component. The high separation efficiency was lost after saturation of the atmosphere in the chamber.

Application of the solvent system containing hexadecane provided good separation of the chlorophylls as well as resolution of antheraxanthin in a *P. sativum* extract. Because no protective means such as an inert gas atmosphere[3] or antioxidant addition[2,3,5] were used in these experiments (Fig. 1), the presence of chlorophyll decomposition products at the start of the chromatograms and those with high mobility were observed. Addition of butylated hydroxytoluene or α-tocopherol to the pigment solution resulted in a decrease in the amounts of these degradation products, when fresh extracts were chromatographed and the development was started immediately after sample application. After leaving the pigment solution in chloroform–methanol overnight, some allomerization products with higher mobility than the parent chlorophylls, possibly, their lactones[7], were observed on the chromatoplates. The application of the solvent system provided perfect separations of carotenoids from unsaponifiable *A. hyppocastanum* leaf lipids and the total pigments from the thermophilic cyanobacterium *S. elongatus*. In both cases the presence of a large number of minor coloured carotenoids (their identification was not performed) was observed.

It is important to note that the quality of separation depends critically on the component proportions and deteriorates rapidly when any changes in the solvent composition are made. Lowering of the chamber temperature to 3–4°C improved the separation of xanthophylls and their stability on the Silufol plates.

Satisfactory pigment resolution was also obtained after development with the same solvent system of 400–600 μm silica gel-G (LC 5/40 μm, BDH) plates and the plates with a "permanent" adsorbent layer. The visible absorption spectral characteristics of identified pigments eluted from silica gel corresponded to those in the literature. Thus, sufficient stability of plant pigments was achieved on chromatograms developed in the presence of hexadecane.

Removal of hexadecane from the samples may be carried out by crystallization from methanol at –18°C or by multiple partition (for chlorophylls and higher plant xanthophylls) between equal volumes of 80% water–methanol and hexane (partition coefficient for hexadecane, *ca.* 1:18). Complete separation of hexadecane was achieved also by gelchromatography on a Sephadex LH-20 column. It was found that addition of 0.1% butylated hydroxytoluene[2] or α-tocopherol[5] as antioxidant to a modified solvent system improved the stability of xanthophylls on the silica gel plates. Sephadex LH-20 column chromatography can be used for removal of large quantities of the antioxidants from the samples.

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