CHROMATOGRAPHY OF LIPIDS IN PRESENCE OF AN ANTIOXIDANT, 4-METHYL-2,6-DI-*tert.*-BUTYLPHENOL

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INTRODUCTION

The need for avoiding autoxidation in lipid research is well recognized. Numerous authors mention extraction, manipulation, and storage of lipids under inert atmospheres and addition of antioxidants such as hydroquinone[1]. Use of inert atmospheres is often tedious, and even traces of oxygen (as present in commercial, "high-purity" nitrogen) can produce enough peroxides to cause extensive degradation during storage. Antioxidants, on the other hand, are easily added and provide a useful period of stability towards oxygen if added before any autoxidation has begun. The useful antioxidants are chemically stable compounds, effective in small proportions, and readily separable from lipids.

Precautions taken against autoxidation during chromatography have mostly been limited to de-aeration of solvents and adsorbents, and use of nitrogen and carbon dioxide atmospheres (see *e.g.* refs.[2–8]). We now find that an antioxidant, 4-methyl-2,6-di-*tert.*-butylphenol (BHT), can be included at effective concentration in solvents used for lipid chromatography without affecting elution behaviour.

PROPERTIES OF THE ANTIOXIDANT

Chemical

BHT belongs to the class of "hindered phenols"[9], so called because their hydroxyl groups are nearly enveloped by *tert.*-butyl groups. Characteristic phenolic reactions are hindered[10]; strong bases and even metallic sodium detach protons very sluggishly. BHT is stable towards molecular oxygen except in basic media. There it is more stable than non-hindered phenols, but fairly readily oxidized to an unstable hydroperoxide, which tends to decompose back to BHT[11] and to products that are mostly stable and non-polar[12].

Chromatographic

BHT is very soluble in organic solvents, insoluble in water. It is not eluted from silicic acid by light petroleum or cyclohexane, but is eluted by carbon tetrachloride. Thus it behaves[8] like an aromatic hydrocarbon such as dodecylbenzene[13]. Like squalene and wax esters, it is eluted by 1 % diethyl ether in light petroleum, before long-chain fatty acid methyl and steryl esters. It moves ahead of methyl esters on Silica Gel G plates.

Spectroscopic

Hydroxyl stretching absorption[14–16] indicates remarkably little tendency to form hydrogen bonds: this explains the hydrocarbon-like chromatographic properties. Hydroxyl (3650 cm^{-1}) and other infrared absorptions[17] are useful for detecting and estimating BHT. So, too, is the ultraviolet spectrum (2 peaks near 280 mμ). Colorimetric assays have been described[18]. We find that a useful specific test is the blue colour ($\lambda_{max}^{CHCl_3}$ 635 mμ, $\varepsilon_a \sim 10$) of a BHT–tetracyanoethylene charge transfer complex.

Biochemical

In many countries BHT is an approved food additive, and it is fairly non-toxic to whole animals[19,20]. Unlike the natural antioxidants, tocopherols, it does not inhibit legume lipoxidases[21].

Stabilization of solvents

Ethers are stabilized by 0.01 % BHT[22]. To inhibit phosgene formation chloroform normally contains ethanol (0.75–2 %) which, because of its high polarity, sometimes affects elution behaviour. Ethanol can be replaced with advantage by BHT. Testing for phosgene by infrared absorption (1810 cm^{-1}) and smell, we find that BHT in concentrations as low as 0.001 % is an effective stabilizer. It would probably also stabilize other solvents such as dichloromethane.

APPLICATIONS

Column chromatography of wheat flour lipids

Some glycolipid and phospholipid fractions isolated from flour by published methods[23,24] gave ultraviolet spectra characteristic[3] of autoxidation products. Since the extracts are well protected by natural antioxidants until chromatographic separation begins, we considered adding another antioxidant in the eluent. BHT, which is not adsorbed from the eluents used (chloroform, methanol) gave satisfactory results at 0.005%, w/v, concentration. In numerous experiments we have detected no effect of BHT on gravimetric or spectrophotometric[23] elution curves.

Column chromatography of lipid pigments

When aliquots of a washed lipid extract from spinach leaves were developed with chloroform (containing ethanol) on identical silicic acid columns, in presence and absence of BHT (0.005%), there were many differences in the coloured band pattern. Thus it appears that the degradation of pigments reported by ZILL AND HARMON[25] was at least partly prevented by BHT.

Column chromatography of phosphatidylethanolamines

Hen's egg phosphatidylethanolamines are highly susceptible to autoxidation[3], which proceeds so rapidly that eluates turn brown after only a few hours' exposure to air in a fraction collector. But they remain colourless if BHT is present.

We have failed to detect any autoxidation of phosphatidylethanolamines occurring actually within silicic acid columns, but protection of eluates by BHT is valuable, as can be seen from Fig. 1. Rapid rechromatography incurred *ca.* 3 %

losses, probably accounted for by tailing. But when an eluate was exposed overnight there was an abrupt change to greater losses (*ca.* 17%) on rechromatography. BHT prevented the change.

Column chromatography of methyl esters

BHT (0.005%) does not speed or retard elution of methyl esters from silicic acid by 1 % ether in light petroleum. Even without adding BHT we have failed to detect any autoxidation of methyl esters, even when these were shaken for 3 days with silicic acid and light petroleum in air, under fluorescent strip lighting. Yet 23 % of the esters used (prepared from egg phosphatidylethanolamines) were tetra-, penta-, and hexaenoates. This contrasts sharply with the findings of earlier workers[2]. It might possibly be explained by the purity of our adsorbent, or by permeation of BHT through the laboratory. (BHT is quite volatile, see below).

Thin-layer chromatography of phosphatidylethanolamines

When unsaturated lipids are left on thin-layer plates for some time before development minor zones and streaks subsequently appear, owing to autoxidation. Rapid handling is usually practicable for single-development chromatograms but not for two-dimensional chromatograms. Incorporation of BHT in developing solvents gives the necessary protection. The photographs in Fig. 2 provide a striking if somewhat exaggerated illustration: both plates were left for 20 h between developments.

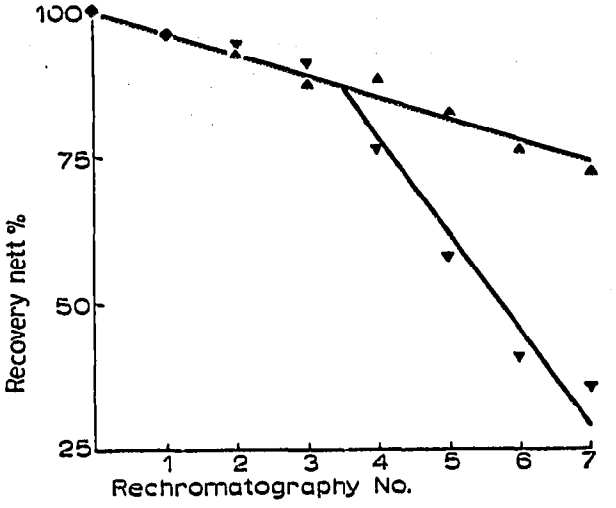

Fig. 1. Protection of phosphatidylethanolamines by BHT during silicic acid chromatography. The following series of experiments was conducted simultaneously on two identical lipid samples, with BHT (0.005 %) present in eluents for one sample (▲) but not for the other (▼). Washed, total hen's egg lipid (575 mg) was applied to a column of silicic acid (15 g, diam. 33 mm) prepared in chloroform. After washing with 10% (v/v) methanol in chloroform (100 ml), phosphatidylethanolamines were eluted with 15 % methanol (200 ml). The eluate was immediately evaporated ($< 40°$), and the residue dissolved in chloroform and made up to 25 ml. 1 ml solution was taken for measurement of ester absorption (1745 cm^{-1}). The remaining 24 ml was rechromatographed on a new column (5 g, diam. 23 mm), washed with 5 % methanol (40 ml), and eluted with 15 % methanol (50 ml). This eluate was evaporated and made up to 10 ml chloroform solution, 1 ml of which was taken for absorption measurement. The remaining 9 ml was rechromatographed on a new column (5 g), and so on. The first four measurements were made in 1 day. But then, in the third rechromatography, the eluate was collected and stood overnight. Four more measurements were made during the second day. Nett recovery includes allowance for the 1 ml samples removed.

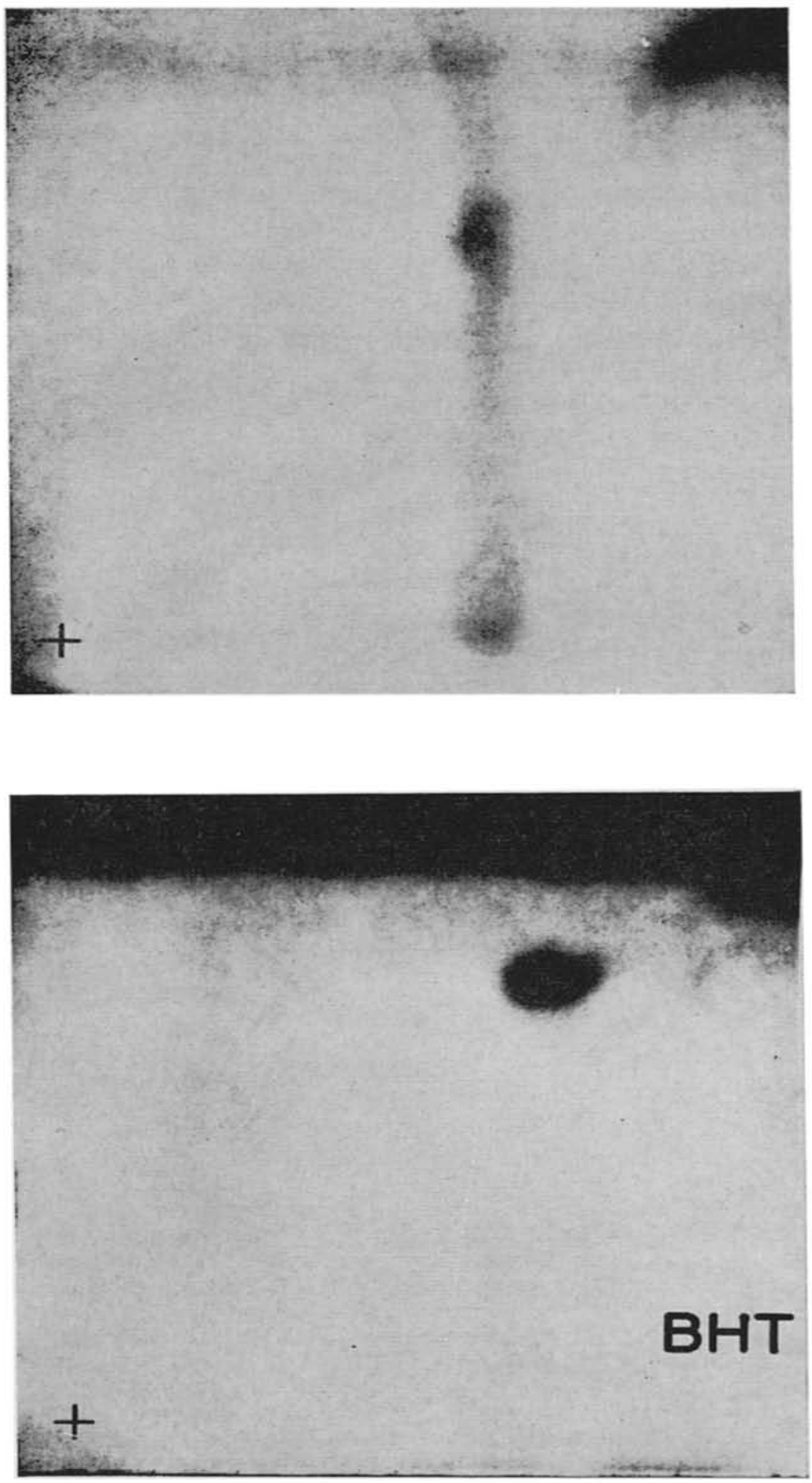

Fig. 2. Thin-layer chromatograms of egg phosphatidylethanolamines (170 μg aliquots) without and with BHT (0.1%, w/v) in the solvent. Plates: Silica Gel G (250 μ), non-activated. Solvent: Chloroform–methanol–water (65:35:4, v/v/v) for both developments. First development, left to right. Plates left 20 h to dry. Second development, upwards. Plates dried 1 h at 80°. Detection, H_2SO_4.

METHODS FOR REMOVING THE ANTIOXIDANT

(1) *Vacuum oven.* When fractions are evaporated at 40° in a vacuum oven (15 mm Hg) BHT is lost with the eluents.

(2) *Vacuum desiccator.* Over silica gel (0.05 mm Hg) crystals of BHT disappear in a few hours, and solutions in lipids are completely purged within 24 h.

(3) *Chromatography.* A weak eluent such as carbon tetrachloride can be used to remove BHT from lipids on silicic acid. Small, wide columns permit rapid separations.

(4) *Solvent fractionation.*

(5) *Sublimation in a cold-finger apparatus.*

(6) *Steam distillation*[18].

(7) *Heating (thin-layer plates).*

PURIFICATION

Food grade BHT is purified before use by passage in carbon tetrachloride solution through activated silicic acid. M.p. 70.5–71°.

DISCUSSION

We now routinely add BHT to chromatographic solvents, usually at the 0.005% level. It is easily detected and removed, although its presence is neglected for many purposes, *e.g.* deacylation[27] and acidic hydrolysis. It permits safe storage of lipid solutions almost indefinitely at —20°.

Use of BHT should greatly facilitate the application of preparative thin-layer chromatography to lipids.

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SUMMARY

Inclusion of small quantities of 4-methyl-2,6-di-*tert.*-butylphenol in solvents protects lipids from autoxidation during chromatography, manipulation, and storage. Since this compound has a higher chromatographic mobility than most lipids, it does not affect their separation. It is easily detected and removed when necessary.

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