Thin-Layer Chromatographic Determination of Antioxidants by the Stable Free Radical α,α'-Diphenyl-β-picrylhydrazyl

J. GLAVIND and G. HOLMER, Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen, Denmark

Abstract

The reaction between the stable free radical α,α'-diphenyl-β-picrylhydrazyl (DPPH) and antioxidants can be used for the visualization of antioxidants in thin-layer chromatography. Amounts of tocopherols down to 0.5 μg can be detected. The identification of tocopherols in oils and other natural products and of synthetic antioxidants, added to lard, are described. Saponification is not required. Quantitative analyses of γ-tocopherol give satisfactory results.

Introduction

THE USE OF THE STABLE FREE RADICAL α,α'-diphenyl-β-picrylhydrazyl (DPPH) for the chemical determination of antioxidants was proposed by Blois (1). He found that DPPH reacts quantitatively with cysteine, glutathione, ascorbic acid, tocopherol, aromatic polyhydroxy-compounds, and certain amines.

Because of its odd electron, solutions of DPPH exhibit a deep violet color. As the odd electron is paired off through the reaction with antioxidants, the violet color vanishes and the pure yellow color caused by the picric acid group appears. The violet color corresponds to an absorption band at 517 mμ, and the decrease in optical density at this wavelength is stoichiometrically related to the amount of antioxidant.

Several investigators have fractionated antioxidants by thin-layer chromatography (TLC). Seher (2,3) used silica gel as the stationary phase and benzene and chloroform as developing solvents. He also described color reagents for the detection of the antioxidants. Methods for the determination of antioxidants by means of DPPH were worked out in this laboratory (4) and were used in nutritional and radiobiological studies on the level of biological antioxidants (5,6). Now it has been found that the novel reagent DPPH can be used for the detection of fat-soluble antioxidants on adsorbent layers.

Materials and Methods

Chemicals. Silica gel G, according to Stahl (E. Merck A. G., Darmstadt, Germany), was used as adsorbent; α,α'-diphenyl-β-picrylhydrazyl was purchased from Fluka A. G., Buchs, S. G., Switzerland. Methanol, benzene, cyclohexane, and ethyl ether were analytical-grade reagents. Before use these solvents were tested for the presence of peroxides (7). Only peroxide-free solvents were used. The α-, β-, γ-, and δ-tocopherols were prepared by saponification of the respective acetates (F. Hoffmann-La Roche and Company, Basel, Switzerland). Butylhydroxyanisol (BHA) and butylhydroxytoluene (BHT) as well as samples of lard stabilized with BHA + BHT were furnished by A/S C. E. Basts Eftf., Copenhagen, Denmark.

Lipid Extracts. These were prepared from liver by grinding with three parts of anhydrous sodium sulphate and six parts of chloroform, followed by filtration and evaporation in vacuo. Whole oats were extracted by simple mixing with chloroform, followed by filtration and evaporation. Complete extraction was not attempted.

Preparation of Plates. The procedures recommended by Stahl (8) for the preparation of layers of uniform thickness (0.25 mm) were followed. Before use, the plates were reactivated by heating at 110 C for 10 min.

Qualitative Thin-Layer Chromatography. The samples in chloroform solution were applied to the plates with micropipettes. The plates were developed by ascending technique in a plain chamber at room temperature. Benzene-methanol (99:1) was used for development. Two-step development was used in some experiments. The first solvent system was cyclohexane-diethyl ether (80:20), which was allowed to run to a height of about 15 cm. This system separates triglycerides from more polar substances, including the tocopherols. The latter were separated by the solvent system, benzene-methanol (99:1). The second development was stopped before the front reach the triglyceride fraction. The triglycerides were easily visible as a transparent zone.

Visualization of Antioxidants. The color reagent used for visualization was a solution of DPPH in methanol, about 0.1 mg/ml. This reagent will keep for several weeks if stored in a refrigerator.

The tocopherols and several other antioxidants react instantaneously with DPPH, but some antioxidants and certain other substances react rather sluggishly. The antioxidants show up as yellow spots on a violet background. An observation of the lapses of time

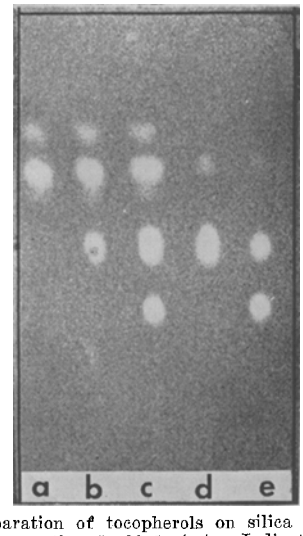

FIG. 1. Separation of tocopherols on silica gel G. Solvent system—benzene:methanol, 99:1 (v/v. Indicator: DPPH in methanol. a) α-tocopherol, 5 μg; b) α-tocopherol, 5 μg, and β-tocopherol, 4 μg; c) α-tocopherol, 5 μg; β-tocopherol, 4 μg; c) α-tocopherol, 5 μg; β-tocopherol, 4 μg; γ-tocopherol, 4 μg; and δ-tocopherol, 3 μg; d) β-tocopherol, 4 μg, and γ-tocopherol, 4 μg; e) γ-tocopherol, 4 μg, and δ-tocopherol, 3 μg. The α-tocopherol contained a small amount of a faster migrating compound, formed in the course of some weeks during the storage of the solution in the Frigidaire.

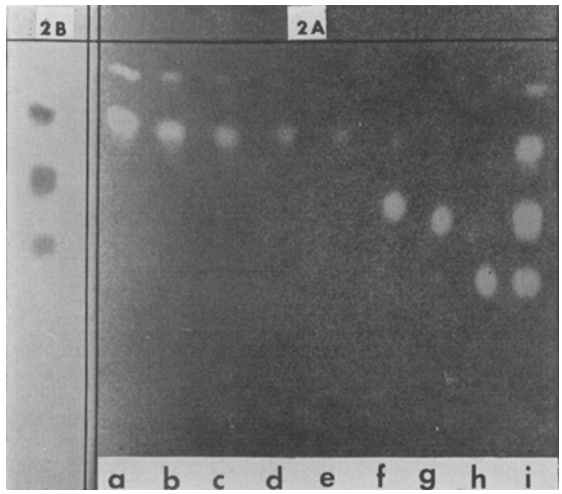

FIG. 2. A: Determination of detection limit of tocopoherols with DPPH reagent. TLC-conditions as in Figure 1. a–e) α-tocopherol, 10 μg, 5 μg, 2 μg, 1 μg, and 0.5 μg respectively; f) β-tocopherol, 4 μg; g) γ-tocopherol, 4 μg; h) δ-tocopherol, 3 μg; i) α-tocopherol, 5 μg; β-tocopherol, 4 μg; γ-tocopherol, 4 μg; and δ-tocopherol, 3 μg. B: Comparison with bipyridyl-ferric chloride reagent; amounts of tocopoherols as in i).

until the spots appear may be helpful in identifying the substances. Spraying with 50% sulfuric acid can be used after visualization with DPPH. The violet color of DPPH disappears on spraying the plates with this reagent. By subsequent heating, spots of α-tocopherol turn blue; β- and γ-tocopherols, yellow-brown; and vitamin A, black-green. Red colors are given by sterols. Later on, charring is produced by most organic substances.

Quantitative Thin-Layer Chromatography. Quantitative TLC may be carried out either by measuring the spot areas and comparing with standards, or by eluting the antioxidant spots with DPPH-reagent. The latter method of determination was carried out in the following way.

The DPPH-reagent was a solution in n-butanol. For quantitative determination, this solvent was preferred to methanol in order to minimize solvent losses

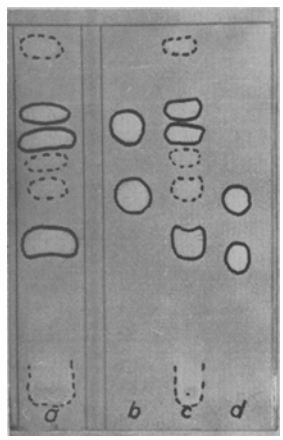

FIG. 3. Fractionation of tocopherols from oats. Solvent system—benzene:methanol, 99:1 (v/v). Indicator: DPPH in methanol. a and c) Extracts of oats, applied as a streak and as a single spot respectively; b) α-tocopherol, 5 μg; and β-tocopherol, 4 μg; d) γ-tocopherol, 4 μg, and δ-tocopherol, 3 μg.

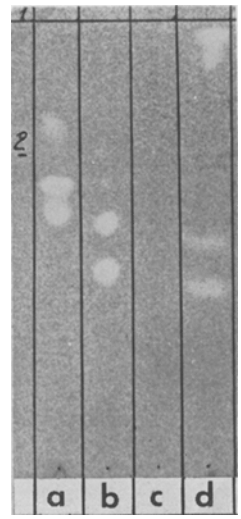

FIG. 4. Separation of tocopherols in soybean oil. Two consecutive systems—1. cyclohexane-diethyl ether, 80:20 (v/v) (front 1); 2. benzene-methanol, 99:1 (v/v) (front 2). Indicator: DPPH in methanol. a) α-tocopherol, 5 μg; and β-tocopherol, 5 μg; b) γ-tocopherol, 4 μg; and δ-tocopherol, 3 μg; c) Filtrol-treated soybean oil; d) soybean oil. The experiment was conducted to control the efficiency of the filtration of soybean oil which was dissolved in petrol ether through activated Super Filtrol as a method for the removal of tocopherols. Spots of γ- and δ-tocopherols are clearly visible on the chromatogram of the native soybean oil; none are visible on that of the Filtrol-treated oil.

because of evaporation during the following operations.

The plates were developed as described above. A standard sample, run along the edge of the plate, was sprayed with DPPH to localize the antioxidants. The bands were marked, scraped off the glass plate, and placed in centrifuge tubes which contained 1.200 ml of the DPPH-reagent. Areas of the same size as the bands were also scraped off and used for blank determinations. The remaining areas of the plate were

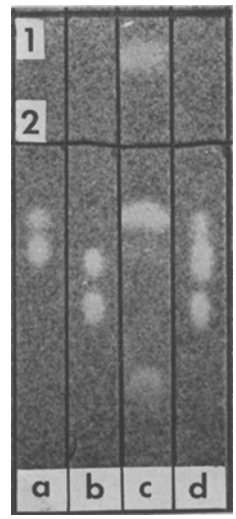

FIG. 5. TLC of rat liver extract. Development and visualization as in Figure 4. a) α-tocopherol, 5 μg; and β-tocopherol, 5 μg; b) γ-tocopherol, 4 μg; and δ-tocopherol, 3 μg; c) rat liver extract; d) α-tocopherol, 5 μg; β-tocopherol, 5 μg; γ-tocopherol, 4 μg; and δ-tocopherol, 3 μg.

sprayed with DPPH to ensure that no further antioxidant spots were present. The centrifuge tubes containing the DPPH-reagent and the scrapings from the plate were closed with aluminum foil and shaken for 20 min on a mechanical flask shaker. The tubes were then centrifuged. About 900 μl of the clear supernatant were removed with a micropipette and placed in a 10-mm Beckman semimicro cell for the spectrophotometric determination.

Results and Discussion

Separation of Tocopherols. The separation of tocopherols and visualization with DPPH is illustrated in Figure 1. The contrast between the brillant yellow spots of the antioxidants and the violet background is distinct on the plates but less satisfactorily reproduced on the photographs. Best reproductions were obtained when the plates were exposed through a yellow-green filter (Filter Number 060, Johannes Weber K. G., Wiesbaden, Germany).

The method gives an easy separation of α-tocopherol (highest R_f-value) from a double-spot of β- and γ-tocopherols and from δ-tocopherol (lowest R_f). Thus the separation takes place according to the number of methyl groups on the chroman nucleus. In the case of the position-isomeric β- and γ-tocopherols only a tendency to a separation is observed. The results are in accordance with the experiments of Seher, who used chloroform as an eluent (2). Amounts of 1 to 5 μg of tocopherols give clearly visible spots, and 0.5 μg can still be discerned (Figure 2A).

Figure 2B shows a comparison with the most commonly used reagent for tocopherols, ferric chloride-bipyridyl. The same mixture of the four tocopherols in the same amounts, as was visualized with DPPH, was applied to another part of the plate and, after development, sprayed with a solution of 0.2% $FeCl_3$ and 0.5% α,α'-bipyridyl in ethanol. Spots occurred in the same places but were less conspicuous than when visualization was made with DPPH.

Identification of Tocopherols in Natural Products. The use of the method for the separation and identification of tocopherols in natural products is illustrated in Figure 3.

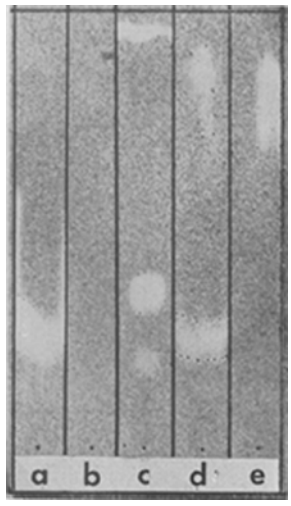

FIG. 6. TLC of synthetic antioxidants in lard. Solvent system—benzene-methanol, 99:1 (v/v). Indicator: DPPH in methanol. a) lard + 10 μg BHA; b) unstabilized lard; c) BHT, 5 μg; and BHA, 5 μg; d) stabilized lard, commercial sample; e) lard + 10 μg BHT. The standard BHA shows two spots, probably 2- + 3-tert.-butyl-4-hydroxyanisol and 4-tert.-butoxyanisol (Seher, 1959).

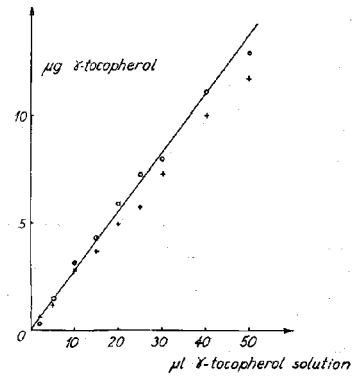

FIG. 7. Quantitative TLC of γ-tocopherol. Comparisons between results obtained by direct estimation of tocopherol with DPPH (o) and recoveries from chromatographic spots by elution with DPPH in butanol (+). Abscissa: graded amounts of standard γ-tocopherol-solution. Ordinate: estimate of tocopherol.

It is seen in Figure 3 that the method allows an easy separation of the tocopherols in a product like a chloroform extract of whole oats, which has a relatively high content of tocopherols in proportion to total lipids. An adequate amount of tocopherols for TLC was present in less than one mg of total lipids, and the separation could be accomplished in one solvent system.

It is well known that, besides α-, γ-, and δ-tocopherols, also ϵ- and ζ-tocopherols occur in oats (9). The tocopherols are probably found in the five bands in the area between the bonds indicated by the α- and δ-tocopherol reference spots. A slowly migrating spot is probably not caused by a tocopherol but by some other, unknown substances. These substances may be identical with the ferric chloride-bipyridyl reducing compounds separated from the tocopherols by chromatography on $ZnCO_3$-treated paper by earlier authors (9).

Soybean oil was taken as a typical vegetable oil containing a mixture of tocopherols in moderate amounts (Figure 4). Five to 10 mg of the oil were applied to the plate in order to obtain spots of a suitable size. Two-step development was the most appropriate method in this case. The spots of γ- and δ-tocopherols are clearly visible on the plate. The method allows the determination of tocopherols directly in oils, without saponification. Obviously the separation can also be made on the unsaponifiable fraction of oils. The isolation of this fraction without losses of tocopherols is however quite a cumbersome procedure.

Determination of tocopherols in rat liver was also carried out with the two-step method. Only one spot, probably of α-tocopherol, appeared immediately. The spot corresponded to about 5 μg of α-tocopherol in a total amount of lipid applied to the plate of 10 mg. In the course of some minutes a large yellow spot appeared (Figure 5). The spot was attributable to vitamin A ester, which migrates faster than α-tocopherol.

The content of tocopherols in cod liver oil is still smaller than in soybean oil, and the application of about 50 mg of oil would be required to obtain the optimal amount of tocopherols (about 5 μg) for separation and visualization of the tocopherols. The

tocopherols appear only as a yellow zone at the lower border of the triglyceride zone after development with the same solvent as was used for soybean oil. When only 10 mg of cod liver oil are used, separation from triglycerides is satisfactory, and a clearly visible spot of α-tocopherol is seen. DPPH reacts not only with antioxidants but also with certain substances having easily dissociable π-electrons, e.g., vitamin A, which occurs in large amounts in cod liver oil. This reaction however is slower than that of the tocopherols, which makes the identification of vitamin A in cod liver oil rather easy.

The Identification of Synthetic Antioxidants Added to Lard. The DPPH-reagent reacts with synthetic antioxidants, such as BHA (a mixture of 2- and 3-tert.-butyl-4-hydroxyanisol) and BHT (3,5-di-tert.-butyl-4-hydroxytoluene). An example of the use of the method for the identification of the small amounts of BHA and BHT (0.01% of each) which are used for the stabilization of lard is shown in Figure 6.

Although the identification of small amounts of tocopherols in great amounts of triglycerides requires the consecutive use of two solvent systems, the identification of BHA and BHT in lard is feasible with only one solvent system (benzene-methanol, 99:1, v/v). BHT moves faster, BHA more slowly than triglycerides; although the chromatogram is overloaded with triglycerides, the zones of the two antioxidants are clearly visible in front of and just behind the broad band of triglycerides. The overloading of the chromatogram somewhat distorts the position of the antioxidant spots, but still they are easily identifiable. When sprayed with DPPH, the yellow color appears more rapidly on the BHA spot than on that of BHT.

Quantitative Determination of γ-Tocopherol. The quantitative determination of antioxidants is exemplified in Figure 7. Two series of graded amounts of (2–50 μl) of a solution of γ-tocopherol in hexane (0.32 mg/ml, calculated from the ultraviolet absorption about 295 mμ) were carefully pipetted, one series in test tubes, the other on a chromatographic plate. The first series was used for the determination of the tocopherol content before chromatography by direct addition of the DPPH-reagent; the second was used for quantitative TLC as described earlier.

It can be seen that the recovery after chromatography is about 90% of the amount of tocopherol which was added. The control chromatogram revealed that the γ-tocopherol used was not homogenous but contained a more polar substance. When this fraction was scraped off and eluted separately with the DPPH-reagent, it was found to account for about 8% of the added γ-tocopherol. This meant that the recoveries were almost complete when the results of the direct determinations were corrected for the presence of the contaminant.

It is therefore concluded that quantitative TLC, as described above, can be carried out with satisfactory recovery. It may appear a useful method, not only for control of tocopherol concentrates but also, especially since the presence of triglycerides does not interfere and since saponification is not required, for the analysis of fats and oils. The method may also be used for synthetic antioxidants, but the reactions between the individual antioxidants and DPPH in solution must be studied beforehand. Both BHA and BHT react more slowly with DPPH than does α-tocopherol, and a quantitation by measuring the spot areas may be preferable.

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