PAPER CHROMATOGRAPHIC SEPARATION OF SOME ANTIOXIDANTS

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With the increased use of antioxidants today, both as food additives and in certain types of experimental work[1], there is a definite need for methods to separate and identify these compounds. Procedures for the separation of four food approved antioxidants have been described by MITCHELL[2]; however, a distinct separation of all components in one dimension was not achieved. In addition, ZIJP[3] and DEHORITY[4] have reported the use of paper chromatography in analyses for N,N'-diphenyl-*p*-phenylenediamine in rubber and milk, respectively. This paper describes methods for the separation and identification of eight antioxidants currently used either as food additives or in experimental work.

EXPERIMENTAL

Antioxidants

The antioxidants used in this study were as follows:

a. PG: *n*-propyl gallate
b. BHA (butylated hydroxyanisole): 2-*tert.*-butyl-4-hydroxyanisole
c. BHT (butylated hydroxytoluene): 2,6-di-*tert.*-butyl-*p*-cresol
d. NDGA (nordihydroguaiaretic acid): 4,4'-(2,3-dimethyltetramethylene)-dipyrocatechol
e. DPPD: N,N'-diphenyl-*p*-phenylenediamine
f. DTBH: 2,5-di-*tert.*-butylhydroquinone
g. Santoquin: 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline
h. α-Tocopherol.

Apparatus

Chromatography was carried out in the ascending direction, with the papers suspended from glass rods near the top of Pyrex cylinders (18 in. high and 6 in. diameter) covered with glass plates. The glass rods had a rubber "policeman" on each end and were wedged into the cylinder. The developing solvent was poured directly into the bottom of the cylinder.

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Papers

For the separations described below, partially acetylated paper and cottonseed oil coated paper were utilized. These papers were prepared for use as follows:

a. Acetylated paper: Whatman No. 1 filter paper was acetylated by a modified procedure developed according to the methods of KOSTIR AND SLAVIK (as reported by BALSTON AND TALBOT[5]) and MICHEEL (as reported by BLOCK, DURRUM AND ZWEIG[6]). Sheets of Whatman No. 1 paper, 16.25 × 2 inches each (the longer side perpendicular to the machine direction of the paper), were suspended in a liter graduate cylinder (containing a glass covered magnet) from a glass rod placed across the top, and held with stainless steel chromatography clips. The papers were then covered with the acetylating mixture, which contained 400 ml of acetic anhydride, 800 ml of benzene and 1.2 ml of conc. sulfuric acid. The top of the cylinder was sealed with aluminum foil, and placed on a magnetic stirrer which was used intermittently at slow speeds throughout the acetylation process. After acetylating for 16–20 hours, the papers were removed and air dried in a hood, washed for 15 minutes in cool running tap water followed by three 5 minute washings in distilled water, washed for 15 minutes in absolute methanol, and finally air dried in the hood. To prevent curling of the papers, it was found advantageous to attach a glass rod by means of chromatography clips to the bottom edge of the papers during this final drying.

b. Cottonseed oil coated paper: Whatman No. 1 filter paper was coated by dipping into a 10% solution of cottonseed oil* in benzene. The benzene was allowed to evaporate at room temperature in a hood after which the papers were ready for use.

Solvents

a. Acetone–water (60:40).
b. Acetone–ethyl acetate–water (30:10:60).
c. Absolute methanol–water (75:25).

Chromogenic agents

The developed chromatograms were sprayed with a 0.5% solution of phosphomolybdic acid in 95% ethanol and hung in an ammonium hydroxide atmosphere as described by MITCHELL[2]. With this procedure all of the antioxidants were visible as blue spots, except DPPD which gave a brown spot.

RESULTS AND DISCUSSION

Three methods were employed for chromatographic separation of the antioxidants. The first (I) utilized partially acetylated paper with 60% acetone as the developing solvent; the second (II) cottonseed oil paper and acetone–ethyl acetate–water as the solvent mixture; and the third (III) again used cottonseed oil paper, but the solvent

* A commercially available winterized cottonseed oil (Wesson Oil), refined and distributed by the Wesson Oil and Snowdrift Sales Co., New Orleans, Louisiana, was used for this study.

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was 75% methanol. The separations obtained are given in Table I. Each value represents the mean of at least five individual separations. R_{PG} values (distance moved by antioxidant divided by the distance moved by propyl gallate) were used in preference to R_F values since the former accounted for deviations observed between different batches of acetylated paper and different cottonseed oil solutions.

Both I and III gave a distinct separation of six of the eight antioxidants. In I, BHA and Santoquin did not separate, while in III NDGA and PG were inseparable. In the report by MITCHELL[2], PG, BHA, BHT and NDGA were not completely separated by unidimensional chromatography, with PG and NDGA consistently overlapping in all the systems utilized. However, in the present study, a definite separation of PG and NDGA was accomplished by either method I or II.

TABLE I

MEAN R_{PG} VALUES OF VARIOUS ANTIOXIDANTS

Method I: Partially acetylated Whatman No. 1 paper using acetone–water (60:40) as a developing solvent. Method II: Cottonseed oil coated paper using acetone–ethyl acetate–water (30:10:60) as a developing solvent. Method III: Cottonseed oil coated paper using absolute methanol–water (75:25) as a developing solvent.

$$R_{PG} = \frac{\text{Distance moved by antioxidant}}{\text{Distance moved by propyl gallate}} \times 100$$

Antioxidant	*Method I*	*Method II*	*Method III*
PG	100	100	100
NDGA	81	74	100
BHA	65	11	75
BHT	38	2	11
DPPD	34	2	20
Santoquin	65	6	58
DTBH	60	5	84
α-Tocopherol	19	0	0

It appears that as yet, no methods are available for the quantitative determination of PG and NDGA when they occur in a mixture[7]. The results presented herein would suggest that these two antioxidants can be determined in a mixture if paper chromatography were employed as a means of separation. Method II would probably be preferable for this purpose, since it gave the widest separation of PG and NDGA while the other antioxidants remained near the origin.

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SUMMARY

Three new methods were described for the paper chromatographic separation of various antioxidants. One utilized partially acetylated filter paper while the other two employed paper coated with cottonseed oil. A simple procedure for the preparation of partially acetylated filter paper was also described. Of particular interest was the complete separation of the four food-approved antioxidants (BHA, BHT, PG and NDGA) by unidimensional chromatography.

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