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A Rapid and Simple Method to Detect Antioxidative Substances on a Thin-Layer Chromatography Plate* [1]

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A simple, rapid and sensitive method was devised to detect specifically antioxidative substances on a thin-layer chromatography (TLC) plate. After the chromatography on a silica gel plate, the plate was sprayed with a pigment solution and irradiated with ultraviolet light. Only the spots of antioxidative ability were embossed as the background pigment was bleached. Among the pigments tested, paprika pigment (mainly capsanthin), capsanthin, and canthaxanthin were excellent, and p-carotene and astaxanthin were adequate for the spray reagents. Using the paprika spray reagent, antioxidants added to olive oil and tocopherols in a brown alga *Ishige okamurae* were clearly detected on the TLC plates. Generally, a few minutes of ultraviolet light irradiation with a commercially available illuminator (2000 μW) were sufficient, and less than one μg of antioxidative substances on the silica gel plate was detectable.

INTRODUCTION

To facilitate the search for new antioxidants, a simple and rapid method for screening or monitoring of antioxidative substances is needed. Usually, after extraction and chromatographic purification, antioxidative activity of the preparations is evaluated by measuring the retardation of the oxidation of the lipids. The thiobarbituric acid or iodometric method has been used for this purpose (Palmateer et *al.*, 1960 ; Ramarathnam et al., 1989 ; kawashima et *al., 1977* ; Aoyama et *al.,* 1982). However, some of these methods are tedious and the latter method requires a relatively large sample size. The weighing method, originally developed by Olcott and Einset (1958), is simple but time consuming ; the test requires days or weeks until an expected weight gain could be obtained (Fujimoto and Kaneda, 1980 ; Ishikawa et *al., 1984).* Other methods, measuring of bleaching time of carotenoids in oil solution (Bickoff, 1951; Pratt, 1965 ; Marco, 1968), oxygen uptake of the lipid in a Warburg apparatus and the like (Quencer et *al.,* 1964 ; Niki et al., 1984), intensity of chemiluminescence (Niki et *al.,* 1982), or of amounts of specified hydroperoxides by high performance liquid chromatography (Stocker et *al.,* 1987 ; Kohen *et al.,* 1988 ; Terao, 1989), have also been used. All these methods are suitable for a quantitative and comprehensive evaluation of the antioxidant activity of the preparations. However, the methods

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described are complicated, tedious and/or time consuming for use in rapid screening or monitoring of unknown antioxidants in preparations containing large amounts of impurities.

Thin-layer chromatography (TLC) is a simple and excellent method for separation of complicated constituents in perparations. However, few detection methods universally specific for antioxidative substances are available. Seino et al. (1971) detected tocopherols and their dimers in the sludge of soybean oil using TLC and a newly-devised method : the TLC plate was sprayed with linoleic acid, then, exposed to ultraviolet (UV) light (365 nm) for 10 min, and finally sprayed with 1% N, N-dimethyl-*p*-phenylenediamine hydrochloride in ethanol. Pratt (1980) used another spray reagent made up of a-carotene and linoleic acid to detect antioxidative substances on a TLC plate or filter paper. These methods utilizing oxidation of the linoleic acid are simple and resourceful, however, they are time is required to oxidize the unsaturated acids and/or to bleach the carotene ("usually within 3 hr", described in the text). Furthermore, the resultant yellowish color of the antioxidant spots obtained by the latter method is not sensitive enough to distinguish from colored spots other than the antioxidative substances.

we describe here the development of a more rapid and sensitive method to specifically detect antioxidative substances, using a combination of TLC and subsequent bleaching of sprayed pigments by UV irradiation.

MATERIALS AND METHODS

Antioxidative substances. 2, 6-Di-*tert*-butyl-*p*-cresol (BHT), *tert*-butylhydroquinone (BHQ), 2 [3]-*tert*-butyl-4-hydroxyanisole (BHA), and ethoxyquin were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Ascorbic acid, carnosine, capsaicin, glutathione, n-mannitol, DL-α-tocopherol and uric acid were obtained from Wako Pure Chemical Ind. (Osaka, Japan). Bilirubin and indomethacin, and hydroquinone were purchased from Sigma Chemical Co. (St. Louis, MO) and Kishida Chemical Co. (Osaka, Japan), respectively.

Pigments. Paprika pigment, commercially available as a food additive, was donated by Takeda Pharmaceutical Ind. (Osaka, Japan). Astaxanthin and canthaxanthin, and shikonin were donated by Dr. Y. Tanaka (Kagoshima University, Japan) and Mitsui Petrochemical Ind. (Tokyo, Japan), respectively. β-Carotene, purpurin, cochineal pigment, monascus pigment and seven other water soluble pigments were purchased from Wako Pure Chemical Ind. (Osaka, Japan), and capsanthin was obtained from Tokyokasei Kogyo Co. (Tokyo, Japan). Forty-four artificial pigments of food additives were obtained from commercial sources.

Corn oil was purchased from Wako Chemical Ind. (Osaka, Japan). Lipids were extracted from a brown alga *Ishige okamurae* by the method using $CHCl_3$/MeOH (2: 1, v/v) (Folch *et al. 1957).* All other chemicals were of either first class grade or extra pure grade.

Detection of antioxidative substances. The pigments described were dissolved either in benzene, chloroform, ethanol or in deionezed water (1-5 mg/mL). After spraying the pigment solution uniformly onto the TLC plate and removing the solvent by air blowing with a hair dryer, the plate was exposed for an appropriate time to UV

light (254 nm) with a UV illuminator (7000 μW, model 20-TC, Atto Co. Tokyo, Japan). The optimum exposure time was determined by regular interval irradiation. TLC was carried out on a silica gel plate (Silica Gel 60 plate, 250 μm, E. Merck, Darmstadt, Germany).

RESULTS AND DISCUSSION

Selection of pigments. After spotting the antioxidants (2.5, 5, 10 μg/2.5 μL $CHCl_3$) onto silica gel plates, each of the pigment solutions was sprayed, uniformly. The plates were dried with a hair dryer and exposed to UV light, separately and for suitable time. As the background pigment was bleached by UV light, the antioxidant spots were gradually embossed. As shown in Fig. 1, paprika pigment (mainly capsanthin), capsanthin, and canthaxanthin were excellent, and p-carotene and astaxanthin were suitable as spray reagents. Although the p-carotene was able to be sprayed homogeneously, the yellowish color was less clear than the reddish colors of the others. When these carotenoid reagents were used, a few minutes of UV irradiation were sufficient to detect the antioxidant spots. Since the purpurin is more stable than the carotenoids described above, much more time was needed for the background color to fade out on the silica gel plate. The cochineal, monascus and other water soluble pigments were not suitable for detection of the lipoidal antioxidants, though some antioxidant spots were detectable. The shikonin and the artificial pigments (food additives) tested faded little within time of experiment.

Application for TLC. Olive oil (100 μg), containing authentic antioxidants (0.5 or 2 μg) was separated on a pair of silica gel plates using n-hexane/diethyl ether/acetic acid (70 : 30 : 1, v/v/v) as the developing solvent. One plate was sprayed with a pigment solution and the other was charred with 50% sulfuric acid. As shown in Fig. 2 (A), using the paprika pigment solution (5 mg/mL $CHCl_3$), even 0.5 μg of the antioxidants of reddish brown spots was clearly detected without disturbance of olive oil ; the larger amounts of triglycerides were detected only on the plate charred with the sulfuric acid. This method using the paprika pigment solution, usually was more sensitive than that using the sulfuric acid and was also applied to screen or monitor antioxidative substances in algae. As in Fig. 2 (B), three colored bands of antioxidative substances were detected in the extracted lipids of a brown alga *Ishige okamurae* ; they were isolated using this monitoring method and identified to be α-, y- and δ-tocopherols. Their total content in the extracted lipids was estimated to be 0.33% (unpublished data). Authentic antioxidants other than shown in the figures were also tested with the paprika reagent. Colored spots of ascorbic acid, capsaicin, ethoxyquin, hydroquinone and indomethacin were clearly visible. Spots of biological antioxidants, bilirubin (Stocker, 1987) and uric acid (Ames, 1981) were obscure because of their inferior solubility. However, those of other radical scavengers found in biological systems, carnosine (Kohen et al., 1988), glutathione (Tirmenstein and Reed, 1989) and mannitol (Misra and Fridovich, 1976) were not detected as colored spots. Possible reasons for this discrepancy may be due to lack of affinity between the hydrophilic antioxidants and the lipophilic spray reagent, differences of scavenging specificity to the radicals produced or to lower antioxidative activities of the biological antioxidants than that of the carotenoid in the spray reagent ; carotenoids have long been known as antiox-

idants, scavengers of singlet molecular oxygen in plants (Foote, 1976). Further studies using another pigments are in progress.

Thus, with the combined use of TLC and UV light bleaching of sprayed carotenoids of less than one μg of antioxidative substances led to a clear detection on the TLC plate, with few exceptions. The paprika pigment used in this study is a low priced food additive commercially available in Japan. The UV illuminator is a popular equipment used to detect fluorescent substances on chromatograms or electrophoresis patterns.

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LEGENDS

FIG. 1. Screening of pigments to detect antioxidative substances on a TLC plate. Antioxidants, 2.5, 5 and 10 μg in 2.5 μL chloroform, were spotted onto a silica gel plate. After spraying the pigment solution and drying with a hair dryer, each plate was exposed to UV light for a suitable time. BHA : 2[3]-*tert*-butyl-4-hydroxyanisole, BHT : 2, 6-di-*tert*-butyl-*p*-cresol, BHQ : *tert*-butyl-hydroquinone, Toc : a-tocopherol

FIG. 2. Separation and detection of antioxidants by TLC and by the combined use of pigment-spraying and UV light bleaching. (A) : Olive oils (100 μg) containing 0.5 and 2 μg antioxidants, (B) : Lipids of a brown alga *Ishige Okamurae*. Both were developed with n-hexane/ethyl ether/acetic acid (70 : 30 : 1, v/v/v) on duplicate silica gel plates (Silicagel 60, 250 μm) respectively. After removing the solvent, one plate was sprayed with 50% H_2SO_4 and charred, while the other was sprayed with a paprika pigment solution (5 mg/mL $CHCl_3$) and exposed to UV light. Abbreviations used in the figures are the same as in FIG. 1. TG : triglycerides

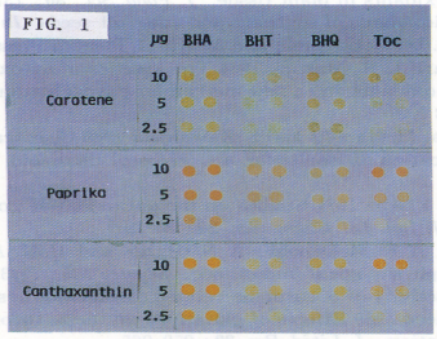
FIG. 1
μg BHA BHT BHQ Toc
Carotene
Paprika
Canthaxanthin
10
5
2.5

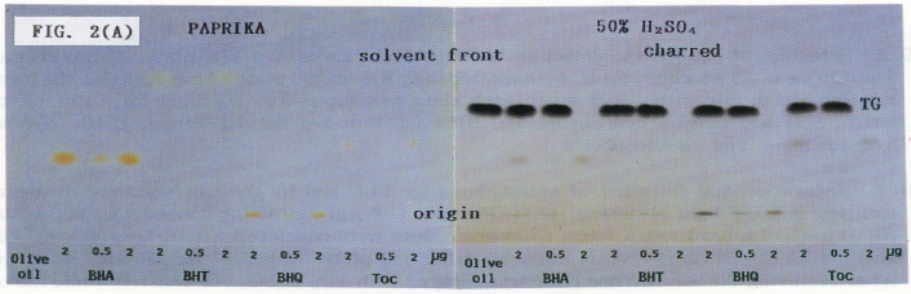
FIG. 2(A)
PAPRIKA
solvent front
50% H2SO4
charred
TG
origin
Olive oil
BHA
BHT
BHQ
Toc
μg

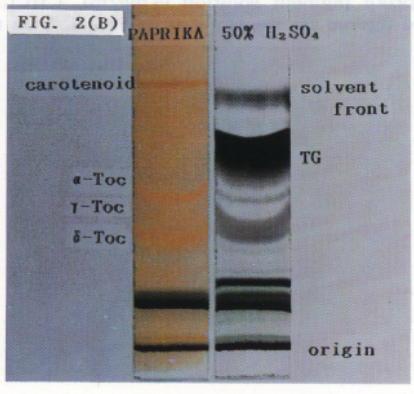
FIG. 2(B)
PAPRIKA
50% H2SO4
carotenoid
α-Toc
γ-Toc
δ-Toc
solvent front
TG
origin