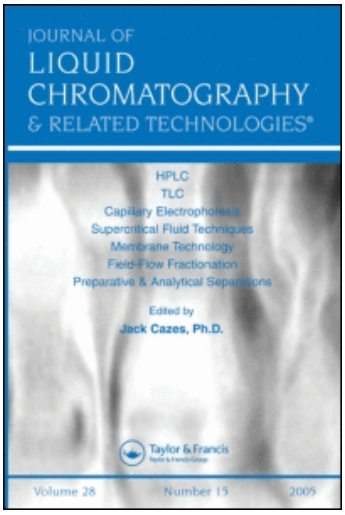

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DETERMINATION OF PHENOLIC ANTI-OXIDANTS IN EDIBLE OIL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTOR

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ABSTRACT

A high-performance liquid chromatography (HPLC) with amperometric detection was investigated for the analysis of 2- and 3-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), and *tert*-butyl-hydroquinone (TBHQ) in edible oil. The reversed-phase system developed was combined with an amperometric detector, the working electrode of which was made of glassy carbon, in order to compare the sensitivity and selectivity of ultraviolet and fluorometric detection. For the amperometric detection of HPLC, cyclic voltammetry was used to monitor the electrochemical properties of the phenolic antioxidants. A simple isolation procedure, based on the

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continuous liquid-liquid partition technique, was examined for the extraction and clean up of the antioxidants from edible oil. The recovery rates of BHA, BHT, and TBHQ added salad oil were between 90.2-107.7% in the range of 1-50 ppm of the antioxidants. By the present method, BHA, BHT, and TBHQ were well separated, identified and quantitated with a high sensitivity.

INTRODUCTION

Synthetic phenolic antioxidants are frequently used to protect foods from oxidation. In particular, BHA and BHT, registered in Japan, are added to products containing fats or oils to prevent rancidification and improve shelf-life. Although TBHQ is not registered as food additives in Japan, qualitative and quantitative determination of TBHQ are required for analysis of imported foods because TBHQ has been registered in United States and other countries.

HPLC has been shown to useful for determination of phenolic antioxidants (1-6). Detection of three compounds is mainly carried out with their ultraviolet absorption. Fluorescence, ultraviolet, and amperometric detectors connected in tandem has been used for the identification and quantitation of standard phenolic antioxidants including BHA, BHT, and TBHQ (4). Amperometric detector for HPLC are useful detection mode for the analysis of phenolic compounds, which have oxidizable character (7). Most analytical methods include distillation (8), solvent extraction (2, 9), and column (10) as separation technique. Most of these procedures are time consuming and tedious for routine analysis. Continuous liquid-liquid partition technique has been used to extract, concentrate, and purify the medicinal compounds (11, 12). In the present study, the extraction and purification of antioxidants from edible oil was investigated by using the continuous liquid-liquid partition technique as previously (12) with the two phase system composed of hexane and acetonitrile.

MATERIALS AND METHOD

Reagents

BHA, BHT, TBHQ were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). All other chemicals were of reagents grade.

Mobile phase A was prepared by mixing acetonitrile and 0.05 M sodium dihydrogen phosphate solution acidified with phosphoric acid to pH 3.0 (30+70), v/v). Mobile phase B was prepared by mixing acetonitrile and 0.1 M sodium dihydrogen phosphate solution acidified with phosphoric acid to pH 3.0 (50+50, v/v).

HPLC analysis of BHA, BHT, and TBHQ

A model LC-3A (Shimadzu Seisakusho, Ltd. Kyoto, Japan) equipped with a column oven (Shimadzu CTO-2A) was used to deliver the mobile phase at a flow rate of 1.0 ml/min. Two reversed-phase columns, Diasil CN (10 μm, 15 cm x 4.0 mm i.d., Nihon Kuromato-Kogyo, Co., Ltd. Tokyo, Japan) and Unisil Q CP (5 μm, 15 cm x 4.6 mm i.d., Gaskuro-Kogyo, Co., Ltd. Tokyo, Japan) which were cyanopropyl and dichlorophenyl bonded column, respectively, were used. The column temperature was maintained at 40°C in Diasil CN and 30°C in Unisil Q CP.

As detectors, a model E-502 (IRICA-Kogyo, Co., Ltd. Kyoto, Japan) operated at a potential setting of +1.00 V vs Ag/AgCl, a Shimadzu SPD-2A UV detector with 285 nm and a Shimadzu RF-530 spectrofluorometer with excitation at 285 nm and emission at 315 nm were used.

Measurement of cyclic voltammogram

The cyclic voltammograms were obtained employing a IRICA EC-2000 with WX 1000 X-Y recorder (Grathtec Ltd. Tokyo, Japan). A three electrode cell, which a glassy carbon working electrode, a platinum wire counter electrode, and a Ag/AgCl reference electrode are housed, was used to record cyclic voltammogram. BHA, BHT, and TBHQ were dissolved in 10 ml of mobile phase B at 5 mM concentration and deoxygenated by purging with purified N_2 for 10 min and the voltammograms were measured at the scan rate of 200 mV/sec.

Continuous liquid-liquid partition technique

A device for the continuous liquid-liquid extraction was constructed similarly as described (12). The extraction system was composed of three fluoro ethyl propylene (FEP) tubings of 19.5 cm x 10 mm i.d., which were connected each other with Teflon tubing (27 cm x 1 mm i.d.). The capacity for stationaly phase and sample loop were about totally 45 ml and 20 ml, respectively.

After acetonitrile and hexane were allowed equilibrate in a separating funnel, the upper layer was loaded into FEP tubing as the stationary phase and the lower layer was used as the mobile phase. By using the present device, the following procedure for preparation of sample solution was used.

The solution for continuous extraction were prepared by dissolving one gram of salad oil in hexane-acetonitrile (50+50,v/v) and made the final volume to 10 ml. The solution was loaded into the sample loop of the extraction device with glass syringe and the lower phase was immediately pumped at 5 ml/min through the sample loop into the FEP tubings with a model 635 A solvent delivery system (Hitachi Seisakusho, Ltd. Tokyo, Japan) as the mobile phase. Two hundred fifty ml of eluate from the tubings was collected and concentrated on rotary vacuum evaporator at 40°C. The concentrate was transfered into volumetric flask and made volume into 10 ml with acetonitrile. Five microliter of the solution was injected into the HPLC columns. The quantitation was carried out by measurement of peak height.

RESULTS AND DISCUSSION

The cyclic voltammetry was used in order to set up optimum applied voltage for amperometric detection of HPLC. Figure 1 shows the typical voltammograms of BHA, BHT, and TBHQ in 0.1 M sodium phosphate buffer (pH 3.0)-acetonitrile (50+50, v/v). The cathodic peak of BHA, BHT, and TBHQ were obtained at +0.78, +1.38, and +0.54 V vs Ag/AgCl, respectively. Although TBHQ and BHA are easily oxidized less than 1.0 V vs Ag/AgCl, the applied potential is required to set at nearly 1.4 V vs Ag/AgCl for simultaneous determination of three compounds with high sensitivity. However themeasurement of the oxidative potential by the cyclic voltammetry was carried out under static field using solid electrode, whereas amperometric detection was achieved under dynamic field as liquid flow. That is to say, the peak potential obtained by cyclic voltammetry is not compatible with the optimum applied voltage of amperometric detector.

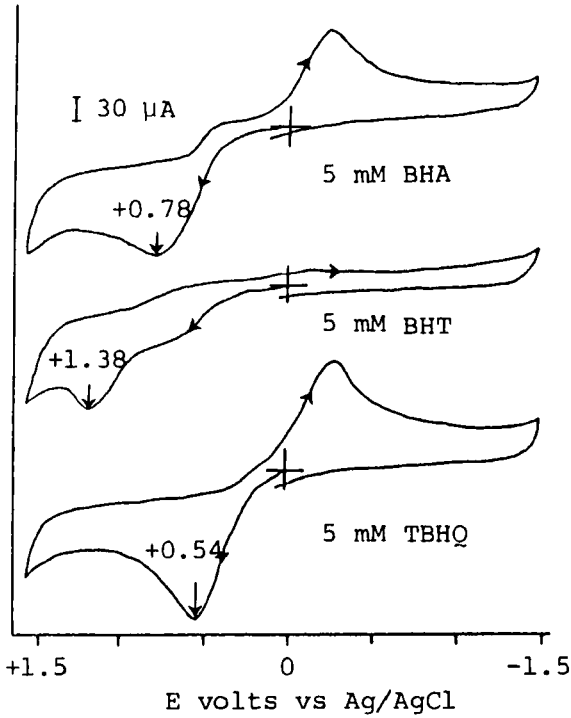

FIGURE 1: Cyclic voltammograms of BHA, BHT, and TBHQ

To determine the optimum applied voltage for the amperometric detector, the peak heights of antioxidants was measured at the various potential in the range from +0.80 to +1.25 V. Figure 2 shows that the peak height of TBHQ increased to +0.90 V vs Ag/AgCl and then became constant. On the other hand, these of BHA and BHT increased with applied potential. In consideration of the intensity of dark current, the stability of base line, and the interference of the other oxidizable components from the extract, the applied potential of amperometric detector was set at +1.00 V vs Ag/AgCl.

The effect of the phosphate buffer on the peak heights and the capacity factors of these antioxidants was investigated at the

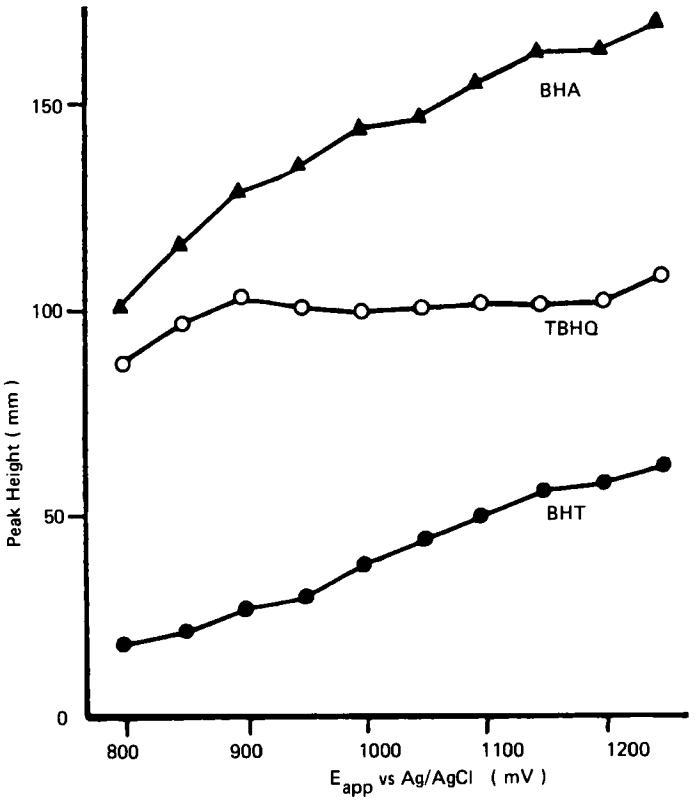

FIGURE 2: Hydrodynamic voltammograms for antioxidants (25 ng) dissolved in the mobile phase
Conditions: column, Diasil CN (10 μm, 15 cm x 4.0 mm i.d.); mobile phase, A; flow rate, 1.0 ml/min; column temp., 40°C

different concentration of the phosphate in the mobile phase. Figure 3-A shows that the peak heights of BHA and BHT were maximum at 0.25 M and 0.10 M, and then became constant. The peak height of TBHQ increased with the concentration of the phosphate. Figure 3-B shows that the capacity factors of BHA and TBHQ increased slightly, but that of BHT increased remarkably with the concentration of the phosphate. As the result, the concentration of the phosphate was determined at 0.10 M to gain maximum peak height of BHT. The effect

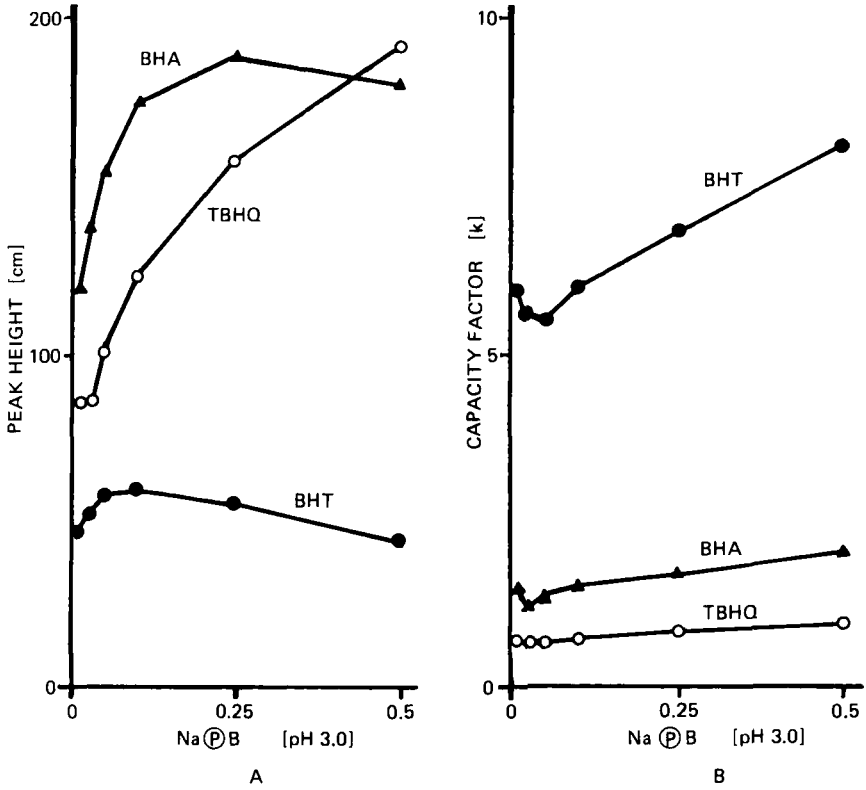

FIGURE 3: Relationship between peak height or capacity factor of antioxidants and concentration of sodium dihydrogen phosphate solution
Conditions:column, Diasil CN;mobile phase, acetonitrile -NaH_2PO_4 (pH 3.0) (30+70, v/v);flow rate, 1.0 ml/min; column temp., 40°C;applied voltage, +1.00 V vs Ag/AgCl

of the pH of the phosphate on the peak heights and the capacity factors of three antioxidants was investigated in the range from 3 to 8. Each peak height and capacity factors was constant in studied range of pH, and the pH of the phosphate was set at 3.0 in consideration of the stability of antioxidants.

The effect of the concentration of acetonitrile on the base-line separation of BHA, BHT, and TBHQ was investigated in two

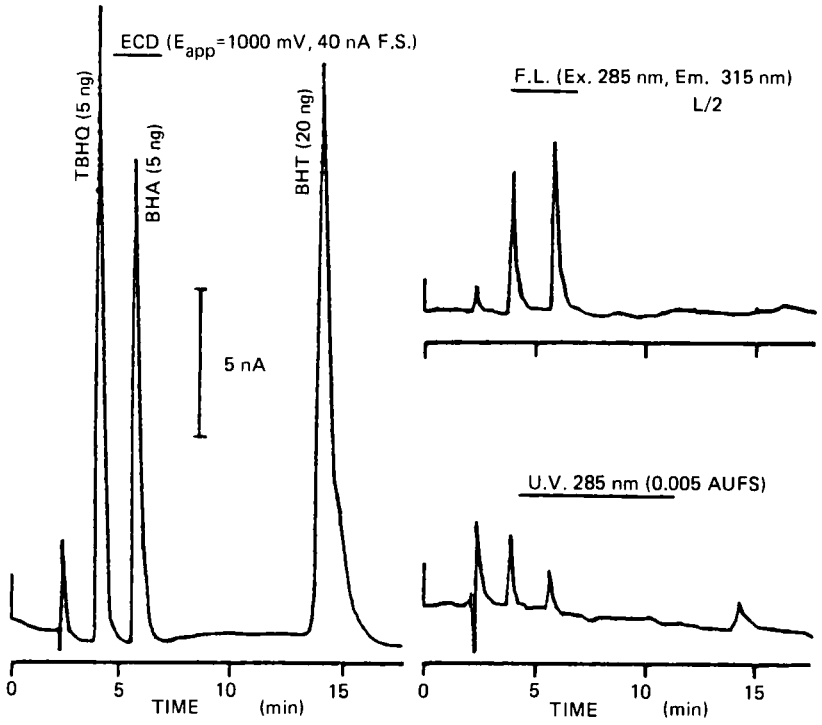

FIGURE 4: Comparison of sensitivity with different detectors
Detectors: EC, electrochemical; FL, fluorescence; UV, ultraviolet
Conditions: column, Diasil CN (10 μm, 15 cm x 4.0 mm i.d.); mobile phase, A; flow rate, 1.0 ml/min; column temp., 40°C

reversed-phase columns. Good baseline separation of these antioxidants was obtained at 30 v/v% in Diasil CN and 50 v/v% in Unisil Q CP. BHA, BHT, and TBHQ were sufficiently detected at levels of 1.0, 4.0, and 1.0 ng, respectively. The complete elution time of these antioxidants was within 20 min and these column was effectively useful for daily routine analysis. As is shown in Figure 4, the detector response of each antioxidant was highest in ECD by a comparison with UV and FLD in sensitivity. Especially, the ECD response of BHT was highly sensitive.

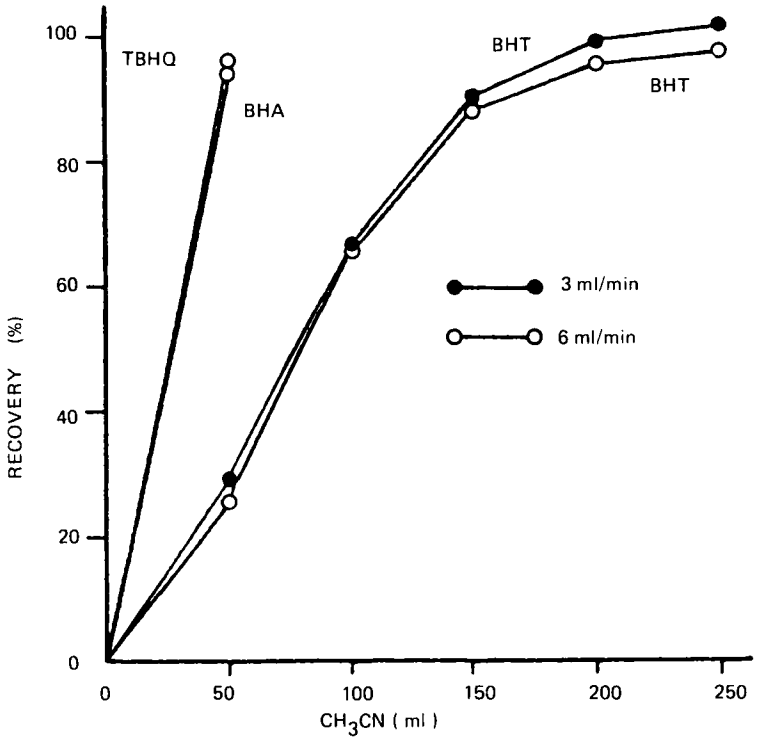

FIGURE 5 Recovery of added BHA, BHT, and TBHQ from salad oil by the continuous liquid-liquid partition technique with acetonitrile

To analyze antioxidants in oily food, it is usually required to separate lipid material from antioxidants. Liquid-liquid extraction procedure was widely employed (2). The continuous liquid-liquid partition technique was used by the two phase system composed of hexane and acetonitrile. In the method, the effect of the number of FEP tubings (3 to 5) and the flow rate of the mobile phase (3, 4, 5, and 6 ml/min) were investigated. A recovery test was carried out using 1 g of salad oil added BHA, BHT, and TBHQ at levels of 1.0, 4.0, and 1.0 ppm, respectively. As shown in Figure 5, more than 90 % of BHA and TBHQ were recovered in the first 50 ml of eluate and more than 90 % of BHT was recovered in

TABLE 1

Recovery of added BHA, BHT, and TBHQ from salad oil

Antioxidants	Level of addn. (μg/g)	Recovery (%) 1	2	3	Average
BHA	1	100.0	105.4	100.0	101.8
	5	98.8	100.0	90.2	96.3
	50	98.9	99.9	102.9	100.6
BHT	4	95.8	90.9	95.8	94.2
	20	96.2	96.2	92.3	94.9
	50	94.1	93.0	100.0	95.7
TBHQ	1	106.3	97.7	106.3	103.4
	5	105.1	107.7	97.4	103.4
	50	99.7	97.1	104.9	100.6

The transfer rate of oil to acetonitrile layer; 3.5%

250 ml of eluate in three tubings. In five tubings, 300 ml of eluate was necessary in order to recover more than 90 % of BHT. Therefore, three FEP tubings were employed and 250 ml of eluate was collected. The flow rate of the mobile phase had no effect on the recoveries in three tubings. The average recovery rates of three antioxidants were more than 94 % and 3.5 % of lipid material transfered to eluate by weight (Table 1).

The determination of BHA, BHT, and TBHQ in edible oil by HPLC was investigated. As the result, these compounds were effectively extracted and purified from edible oil by using the continuous liquid-liquid partition technique, and separated by two reversed-phase chromatography, which used cyanopropyl and dichlorophenyl bonded columns, respectively. By using amperometric detector, these compounds were sensitively assayed in comparison with UVD and FLD. Moreover, the measurement of electro-chemical character by using cyclic voltammeter was the effective means with respect to determination of applied potential of ECD.

The method described here was simple, rapid, and sensitive, and sufficiently used in a regulatory laboratory.

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