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Development of a method predicting the oxidative stability of edible oils using 2,2-diphenyl-1-picrylhydrazyl (DPPH)

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Abstract

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to predict the oxidative stability of edible oils. The principle of DPPH method is to measure the free radicals generated from oxidized oils directly. The absorbance of DPPH from oils before thermal oxidation decreased proportionally to the concentration of free radical scavenging compounds such as butylated hydroxytoluene (BHT). Oxidized oils with high polyunsaturated fatty acid (PUFA) contents showed high changes in DPPH absorbance or free radical formation rates than those with low PUFA. Oxidized oils with high BHT content were more stable and showed slower pattern change of DPPH absorbance from positive to negative slopes than those with low BHT content. Oxidative stability of oils could be predicted considering the initial values of DPPH absorbance, the free radical formation rates, and the oxidation period for pattern changes. The results from DPPH method on the oxidative stability of vegetable oils agreed with conventional methods such as *p*-anisidine value (*p*-AV), conjugated dienoic acids (CDA), or total polar matter (TPM).

Keywords: DPPH; Lipid oxidation; Free radicals; BHT; Oxidative stability

1. Introduction

Lipid oxidation with triplet oxygen, or autoxidation, is a free radical chain reaction and free radicals generated from lipids are major causes for quality loss in color, flavor, texture, and nutritive values during food processing and storage (Min, 1998; Nawar, 1998). Concentration of lipid free radicals and the quantity of inherent antioxidants in lipids are important factors for predicting the stability of edible oils against oxidative stress (Choe & Min, 2005). However, instead of directly measuring the concentration of free radicals, many conventional methods including conjugated dienoic acids (CDA), *p*-anisidine value (*p*-AV), peroxide value, volatile compound analysis, total polar matter (TPM) analysis, and 2-thiobarbituric acid value determine lipid oxidation products (Alamed, Julian, & Decker, 2006; Beltran, Pla, Yuste, & Mor-Mur, 2003; Estevez & Cava, 2006; Juntachote, Berghofer, Siebenhandl, & Bauer, 2006; Rehman, Habib, & Shah, 2004; Shahidi & Wanasundara, 1998). These indirect parameters have limited validity for determining the degree of oxidation due to the principle of each method.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical and has been commonly used to screen phenolic compounds containing high free radical scavenging ability (Wettasinghe & Shahidi, 2000). When a hydrogen atom or electron was transferred to the odd electron in DPPH, the absorbance at 515–517 nm decreased proportionally to the increases of non-radical forms of DPPH (Ancerewicz et al., 1998). Conventionally, high free radical scavenging ability is regarded as high antioxidant activity and DPPH method has been used as one of basic screening steps for searching new antioxidant compounds in organic solvent extracts

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from natural resources including spices, herbs, fruits, and vegetables (Kim & Kim, 2006; Lee et al., 2004; Xu, Chen, & Hu, 2005; Yoo, Kim, Kim, & Kang, 2004).

The availability of DPPH method for predicting the stability of edible oils against oxidative stresses has not been reported to any great extent in the literature. If DPPH method is suitable for measuring the degree of lipid oxidation, it can be applied to determine the antioxidant activity of lipid soluble compounds.

The objectives of this study were to determine whether DPPH is applicable to predict the oxidative stability of thermally oxidized lipids and to test the availability of the DPPH method in thermally oxidized vegetable oils.

2. Materials and methods

2.1. Materials

Fat from a pig and vegetable oils including soybean, olive, canola, and 0.25% α-tocopherol added canola oils were purchased from local grocery market (Seoul, Korea). DPPH and 14% methanolic boron trifluoride (BF_3) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Butylated hydroxytoluene (BHT) and isooctane were purchased from Junsei Chemical Co. (Tokyo, Japan) and *p*-anisidine was procured from Kanto Chemical Co. (Tokyo, Japan). Other chemicals including sodium chloride, anhydrous sodium thiosulphate, *n*-hexane, acetic acid, and methanol were purchased from Daejung Chemical Co. (Seoul, Korea).

2.2. Lard oil preparation

Thirty grams of fat from a pig were cut into small pieces of 0.5 cm long and put into a 100-mL serum bottle sealed air-tight with a Teflon-coated rubber septum and an aluminum cap. Headspace air in the bottle was replaced with nitrogen gas and placed in a convection dry oven (Win Science, Seoul, Korea) at 80 °C for 0.5 h to melt fat, and the lard oil was filtered through a Whatman paper No. 2. Lard oil was centrifuged at 6370*g* for 10 min and collected in a 10-mL serum bottle (25 × 40 mm, 20 mm diameter from Supelco, Inc.) wrapped with aluminum foil under a flow of nitrogen gas and stored −40 °C until use.

2.3. Oil samples for thermally induced oxidation

Lard (0.4 g) containing 0, 500, 1000, and 2000 ppm of BHT was placed in 10-mL serum bottles, and thermally oxidized at 180 °C in the oven for 6 h. Samples were prepared in triplicate and oxidized lard oil was analyzed every 0.5 h.

Two grams of soybean, olive, canola, and 0.25% α-tocopherol added canola oils were placed in a 10-mL serum bottle, respectively, and thermally oxidized at 180 °C for 8 h. All samples were prepared in triplicate and analyzed using CDA, *p*-AV, TPM, and DPPH methods.

2.4. DPPH measurement

The conventional DPPH method uses methanol as solvent, which does not dissolve oil. Proper organic solvents for both DPPH and oil samples should be selected first. Preliminary studies showed that isooctane could dissolve both DPPH and oil samples. Five milliliters of 0.10 mM DPPH in isooctane were mixed with 56 μL oil samples in a 30-mL serum bottle and after 30 min standing in the dark, the absorbance of the sample mixture was measured at 517 nm using a UV/Vis-spectrophotometer (Model UV-1650PC, Shimadzu, Kyoto, Japan). Free radical scavenging activity from DPPH method was expressed in BHT equivalents.

2.5. Preparation of standard curve for the absorbance of DPPH against BHT concentration

BHT was dissolved in isooctane to make 0, 0.5, 1.0, 2.0, and 3.0 mM and 5 mL of 0.10 mM DPPH in isooctane were mixed with 56 μL of 0, 0.5, 1.0, 2.0, and 3.0 mM BHT. The absorbance of DPPH was determined for 96 h. Samples were prepared in triplicate.

2.6. CDA analysis

CDA of samples was measured according to AOCS (1980) method Cd 18–90. One hundred milligrams of sample were dissolved in 25 mL isooctane and stood for 10 min. Mixture was diluted with 10 fold isooctane (v/v) and absorbance at 233 nm was determined by a UV–Vis spectrophotometer. CDA was calculated using following:

$$\text{CDA } (\%) = (0.84 \times A)/(bc - K_0)$$

where A is the absorbance at 233 nm, b is the length of cell (cm), c is the gram per liter, K_0 is the absorptivity by acid groups, 0.03.

2.7. p-AV analysis

p-AV of oxidized oils was determined according to an AOCS (1990) method Ti la-64. The sample (100 mg) was dissolved in 25 mL isooctane and absorbance of this mixture was measured at 350 nm using a UV–Vis spectrophotometer. The above mixture (2.5 mL) was mixed with 0.5 mL 0.5% (w/v) *p*-anisidine in acetic acid and after 10 min standing, absorbance was read at 350 nm. Each sample was prepared in triplicate and each experiment was repeated three times. Value of *p*-AV was calculated using following:

$$p\text{-AV} = 25 \times (1.2 \times A_2 - A_1)/W$$

where A_1 is the absorbance before addition of *p*-anisidine at 350 nm, A_2 is the absorbance after addition of *p*-anisidine at 350 nm, W is the amount of sample, g.

2.8. Total polar matter (TPM) value analysis

The temperature of sample oils were maintained at 45–50 °C and TPM of samples were measured using a Testo™ 265 cooking oil tester according to the manufactures operation guide. Each sampling was done in triplicate and each experiment was repeated three times. The principles of a Testo™ 265 cooking oil tester were to measure the changes of dielectric constant in the oil samples, the concentration of which is highly correlated with the concentration of total polar matter (Bhundit, Parameswarakumar, & Joseph, 2004; Hein, Henning, & Isengard, 1998).

2.9. Fatty acid analysis

Fatty acid methyl ester (FAME) was prepared by a modified method using BF_3/MeOH (14% boron trifluoride) according to AOAC (2000) method 969.33. Samples (100 mg) were saponified with 4 mL of 0.5 N methanolic sodium hydroxide solution for 15 min with boiling. The mixture was homogenized with 4 mL of BF_3/MeOH reagent and boiled for 10 min. After cooling, 6 mL saturated NaCl solution were added to the mixture, and 3 mL of *n*-hexane were added. The upper layer of the mixture was transferred to a 20-mL bottle containing anhydrous $Na_2S_2O_3$ to absorb H_2O in the mixture. The supernatant was directly analyzed by GC.

2.10. Gas chromatography condition for fatty acid analysis

GC Hewlett–Packard 5890-II gas chromatograph equipped with a flame ionization detector, and a 30 m × 0.32 mm ID, 0.25 μm film, HP-INNOWax, from J&W Scientific (Folsom, CA, USA) was used for fatty acid analysis. The oven temperature was held at 150 °C for 1 min, increased from 150 to 230 °C at 2.9 °C/min, and held at 230 °C for 1 min. The temperatures of both injector and detector were 260 °C. The flow rate of helium carrier gas was 1.0 mL/min, and the split ratio was 1:40.

2.11. Statistical analysis

The data were analyzed statistically by ANOVA and Duncan's multiple range test using commercially available software package SPSS software program (SPSS Inc., Chicago, IL). A p value <0.05 was considered significant.

3. Results and discussion

3.1. DPPH method for lard oil with BHT

Absorbance of DPPH from lard oil with BHT before thermal oxidation is shown in Fig. 1. Absorbance of DPPH in lard oil with 0, 500, 1000, and 2000 ppm BHT was 0.926, 0.531, 0.251, and 0.068, respectively. The absorbance values of DPPH in isooctane without lard oil and in lard without addition of BHT were 0.978 and 0.926, respectively,

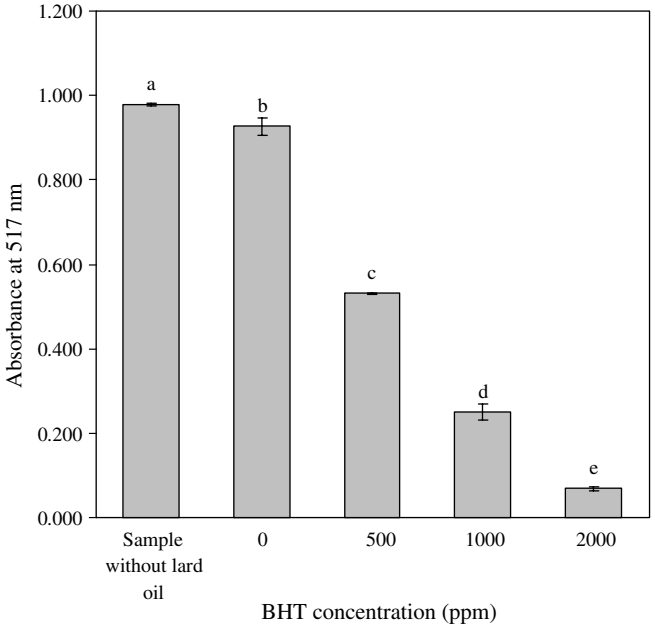

Fig. 1. Absorbance of DPPH from lard oils with 0, 500, 1000, and 2000 ppm BHT before thermal oxidation. Different letters are significant at 0.05.

which indicates that lard itself had some compounds affecting the stability of DPPH. Lard has been used as a model system for measuring antioxidant activities (Shahidi & Naczk, 2004) or for determining the effects of photosensitized oxidation (Lee et al., 2002). The presence of BHT in lard decreased the absorbance of DPPH proportionally to the BHT concentration. The results of this study clearly show that oils containing free radical scavenging compounds such as BHT can decrease the absorbance of DPPH.

Changes of DPPH absorbance from thermally oxidized lard with or without BHT for 3 h (a) and for 6 h (b) are shown in Fig. 2. The absorbance of DPPH from 0.5 h-thermally oxidized lard was significantly different from that in samples at 0 h ($p < 0.05$) irrespective of the presence of BHT (Fig. 2a). The absorbance of DPPH in lard without BHT significantly decreased during 0.5 h oxidation, while that in lard with addition of BHT increased significantly ($p < 0.05$). The absorbance changes of DPPH from lard samples were due to the free radicals generated from oxidized lard. For lard without BHT, the generated free radicals reacted with DPPH and decreased the absorbance of DPPH. However, in case of samples with BHT, free radicals from oxidized lard reacted with BHT first and the remained BHT reacted with DPPH (Fig. 2a). As oxidation time increased beyond 1.0 h, the decrease in DPPH absorbance in lard without BHT started to increase while lard oil with BHT showed a reverse pattern (Fig. 2a). Depending on the BHT concentration in lard, the oxidation period needed for the pattern changes was different (Fig. 2a). Oils with more free radical scavenging compounds need relatively long oxidation time to change

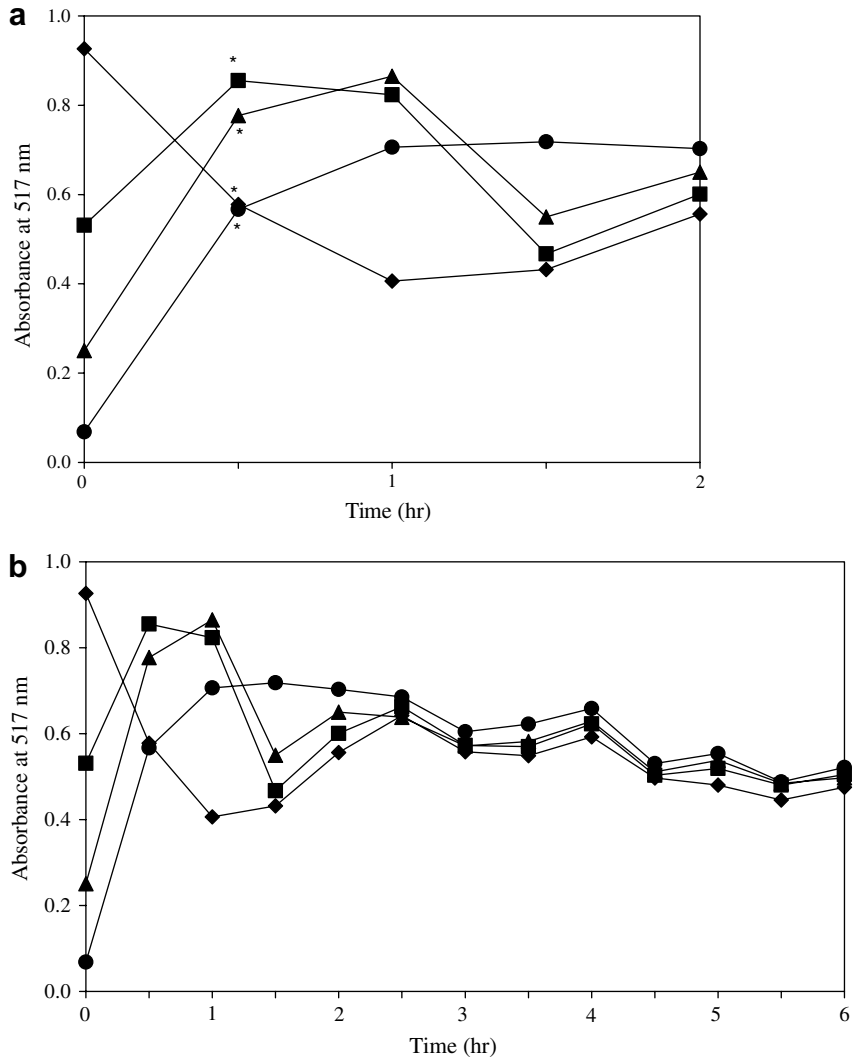

Fig. 2. Changes of DPPH stability from thermally oxidized lard oil with 0 (◆), 500 (■), 1000 (▲), and 2000 (●) ppm BHT at 180 °C for 3 h (a) and for 6 h (b). * indicates significantly different compared to 0 h samples at 0.05.

patterns of DPPH absorbance. As shown in Fig. 2a, lard with 500, 1000, and 2000 ppm BHT took 0.5, 1.0, and 1.5 h before decreasing DPPH absorbance, respectively, which could be a useful parameter for predicting the oxidative stability of edible oils. All lard samples showed similar DPPH absorbance over 3 h oxidation time irrespective of BHT concentration, which may be due to the remaining lipid free radicals after consumption of BHT (Fig. 2b).

The lipid radicals formed during autoxidation are alkoxyl (RO), peroxyl (ROO), and alkyl radicals (R) of polyunsaturated fatty acids (Decker, 1998; Min, 1998). Considering standard one-electron reduction potentials of alkoxyl (RO), peroxyl (ROO), and alkyl radicals (R) of polyunsaturated fatty acids, which are 1600, 1000, and 600 mV, respectively, peroxyl lipid radicals (ROO) have been known as major targets for hydrogen donating antioxidants (Decker, 1998). DPPH may react with either alkoxyl (RO), peroxyl (ROO), or alkyl radicals (R) of polyunsaturated fatty acids, while the major reaction substrates were not determined in this study.

DPPH method may predict the oxidative stability of oils through considering initial concentration of free radical scavenging compounds and the oxidation time required for consumption of initial antioxidants.

3.2. Standard curve for the absorbance of DPPH against BHT concentration

The changes of DPPH absorbance in isooctane with BHT (a) and standard curve for absorbance of DPPH against concentration of BHT (b) are shown in Fig. 3. The reaction rate between DPPH and BHT in isooctane was low and it took more than 48 h to reach a stable state (Fig. 3a). Absorbance of DPPH without BHT addition was not changed significantly ($p > 0.05$) and the stability of DPPH decreased proportionally to the added BHT concentration. Preliminary studies showed that in case of oil

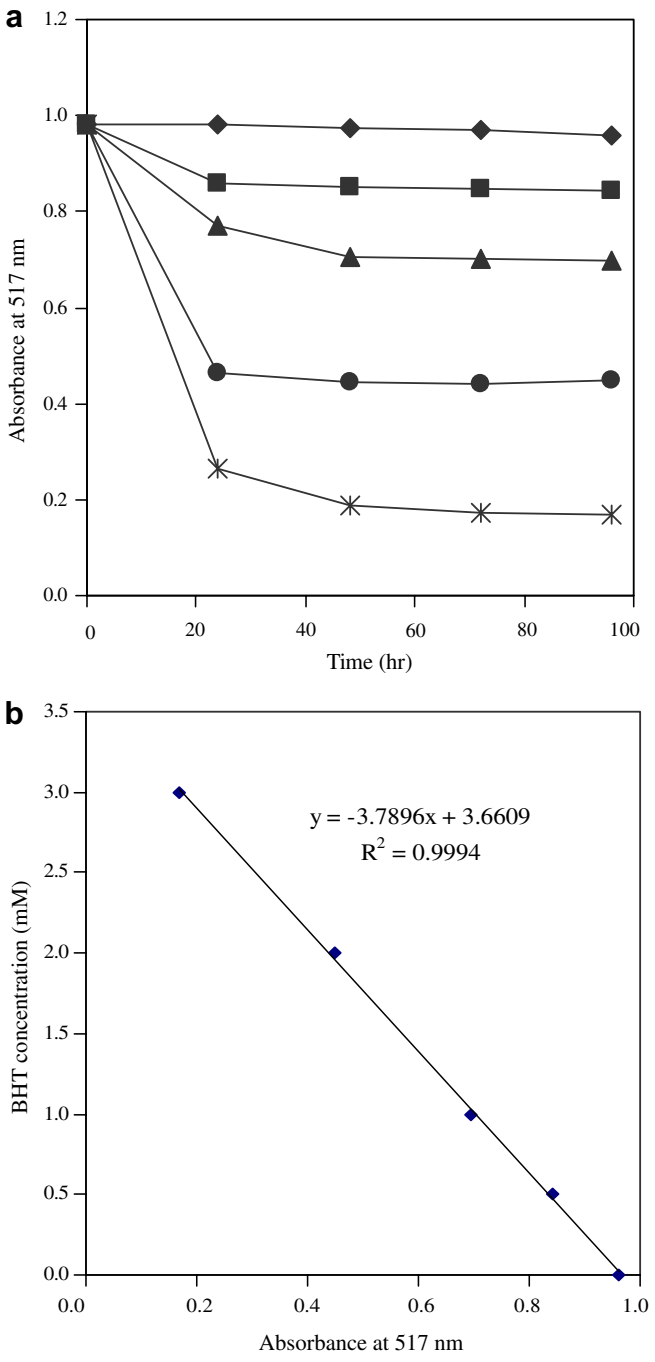

Fig. 3. The changes of DPPH absorbance in isooctane with BHT (a) and standard curve for absorbance of DPPH against concentration of BHT (b). ◆: 0 mM BHT, ■: 0.5 mM BHT, ▲: 1.0 mM BHT, ●: 2.0 mM BHT, ∗: 3.0 mM BHT.

samples, 30 min of reaction time was enough to differentiate the lard samples containing different BHT concentration using DPPH. Regression line between BHT concentration and the absorbance of DPPH in isooctane was $y = -3.7896x + 3.6609$ with 0.9994 coefficient of determination (R^2) (Fig. 3b).

Free radical scavenging activity of target compounds measured by DPPH method has been expressed in different ways, including EC_{50} (50% decrease of initial absorbance from DPPH radical), relative decrease ratio ($(OD_{control} - OD_{sample}/OD_{control}) \times 100$) or equivalent of a reference compound such as Trolox or BHT (Lee et al., 2004; Wong, Leong, & Koh, 2006; Xu et al., 2005; Yoo et al., 2004). The mechanisms of antioxidants are diverse including hydrogen donating compounds, metal chelators, singlet oxygen quenchers, oxygen scavengers, and action of antioxidant enzymes (Boff & Min, 2002; Decker, 1998; Lee, Koo, & Min, 2004). DPPH method is specifically aimed for the determination of hydrogen donating antioxidant compounds such as BHT. Free radicals formed from oxidized oils can be quantified using standard curves in BHT equivalent. Quantified results from DPPH method in BHT equivalent can be used to compare the oxidative stability of edible oils from different sources or different manufacturing procedures.

3.3. CDA, p-AV, TPM, and DPPH analysis for thermally oxidized vegetable oils

Changes of CDA, *p*-AV, TPM, and absorbance of DPPH from thermally oxidized soybean, olive, canola, and 0.25% α-tocopherol added canola oils at 180 °C are shown in Fig. 4. Initial values of CDA, *p*-AV, and TPM from 0 h vegetable oils were low indicating that all the oil samples contained little oxidation products (Fig. 4a–c). As oxidation time increased from 0 to 8 h, CDA, *p*-AV, and TPM increased with different rates depending on the type of vegetable oils. Soybean oil showed the highest increases in CDA, *p*-AV, and TPM for 8 h followed by canola, 0.25% α-tocopherol added canola, and olive oils (Fig. 4a–c).

Fatty acid compositions of lard, soybean, olive, canola, and 0.25% α-tocopherol added canola oils are shown in Table 1. Oils with high PUFA contents were more sensitive to oxidation than those with low PUFA contents (Boff & Min, 2002; Min, 1998). Soybean and olive oil had the highest and lowest PUFA contents, respectively, which can explain the low and high stability against thermal oxidation, respectively (Table 1).

Absorbance of DPPH from soybean, olive, canola, and 0.25% α-tocopherol added canola oils at 0 h were 0.368, 0.595, 0.673, and 0.032, respectively, showing that tested samples contained free radical scavenging compounds corresponding to 2.26, 1.40, 1.11, and 3.54 mM BHT equivalents, respectively. The absorbance of DPPH from soybean, olive, canola, and 0.25% α-tocopherol added canola oils increased up to 4, 5, 2, and 8 h, respectively, and decreased thereafter. Canola oil needed the shortest time for the pattern change from positive to negative slopes in DPPH absorbance while 0.25% α-tocopherol added canola oil needed the longest time. The oxidation time for the pattern changes depended on the initial concentration of free radical scavenging compounds and formation rates of lipid free radicals. Although the same canola oils were used, canola oil with 0.25% α-tocopherol took four times longer oxidation time than samples without tocoph-

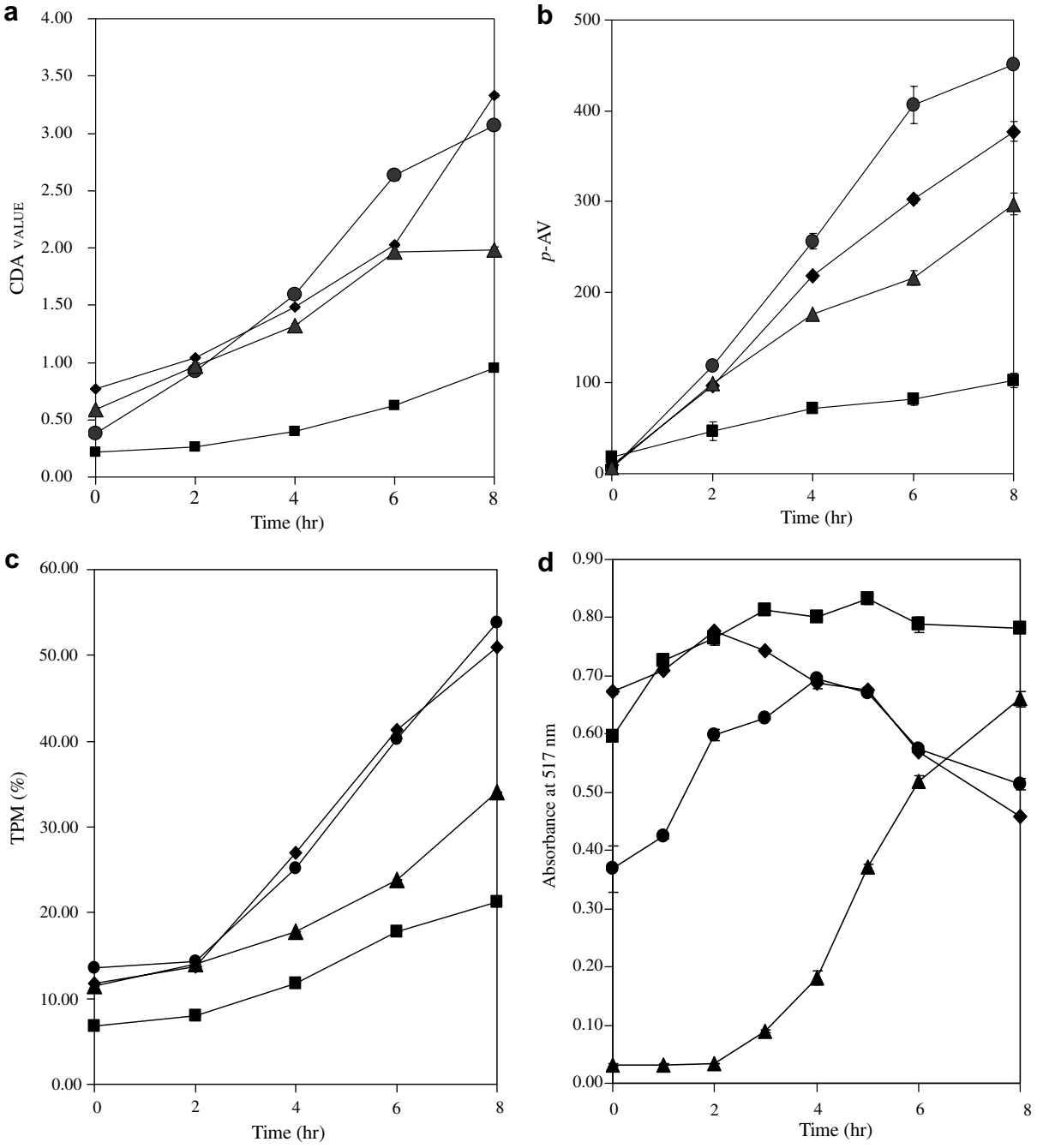

Fig. 4. Changes of CDA (a), *p*-AV (b), TPM (c), and DPPH (d) from thermally oxidized soybean (●), olive (■), canola (◆), and canola with 0.25% α-tocopherol (▲) oils at 180 °C for 8 h.

erol for the pattern changes. The absorbance of DPPH in 0.25% α-tocopherol added canola oil was not changed for 2 h, which shows that initially added tocopherol was enough to react with all the generated lipid free radicals for 2 h and can maintain the DPPH absorbance low. Therefore, the calculated BHT equivalents from 0.25% α-tocopherol added canola oil was underestimated.

Before the pattern change, the absorbance of DPPH from soybean, olive, and canola oils increased by 0.326, 0.237, and 0.104, respectively, corresponding to 1.234, 0.897, and 0.719 mM BHT, respectively. Considering the oxidation time for the pattern change and 2 g sample weight, the free radical formation rates of oxidized soybean, olive, and canola oils may be calculated as 0.154, 0.089, and 0.098 mM BHT equivalents/h/g oils, respectively. The highest formation rate of free radicals was from soybean oil, indicating that soybean oil underwent more rapid autoxidation than canola or olive oils. The free radical formation rates are closely related to the composition and concentration of PUFA. Edible oils with high PUFA generate more lipid free radicals and can give high free radical formation rates (Boff & Min, 2002; Choe

Table 1
Fatty acid compositions of lard and vegetable oils used in this study

Fatty acids	Fatty acids compositions (%)					
	Lard	Soybean oil	Corn oil	Olive oil	Canola oil	Canola oil with tocopherol
C12:0	0.22	–	–	–	–	–
C14:0	1.71	–	–	–	–	–
C16:0	24.35	10.47	11.94	12.66	4.47	5.75
C16:1	1.8	0.11	0.09	1.17	0.23	0.2
C17:0	0.91	–	–	–	–	–
C17:1	0.51	–	–	–	–	–
C18:0	14.24	4.64	2.03	3.06	1.83	2.44
C18:1	37.62	24.29	29.45	71.63	57.31	50.13
C18:2	13.25	51.19	53.69	9.7	20.89	27.92
C18:3	0.9	5.83	0.68	0.65	9.25	10.44
C20:0	0.22	0.39	0.48	0.45	0.68	0.64
C20:1	0.9	0.22	0.37	0	1.33	1.28
C22:0	–	0.45	0.15	0.16	0.42	0.45

& Min, 2005). Therefore, free radical formation rates of oxidized oils from DPPH absorbance could be another indicator predicting the stability of oils against thermal oxidation.

Although the absorbance of DPPH at 0 and 5 h oxidation of canola oil were the same as 0.67, the main causes for the DPPH absorbance were completely different. At 0 h, the presence of free radical scavenging compounds in canola oil decreased the stability of DPPH while at 5 h oxidation, alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), or alkyl radicals ($R^{\bullet}$) from oxidized lipids reduced the absorbance of DPPH. Therefore, it is highly recommend to use also one or more conventional method such as CDA, *p*-AV, or TPM for comparison with the results of DPPH method.

The developed DPPH method generates three valuable parameters for judging the oxidative stability of oils; the initial values of DPPH absorbance, the increase rate of DPPH absorbance or free radical formation rate, and the oxidation time for pattern change of DPPH absorbance from a positive to negative slope. The initial values indicate the concentration of hydrogen donating antioxidant compounds in oils before thermal oxidation. The increase rate of DPPH absorbance is related to the amount of alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), and alkyl radicals ($R^{\bullet}$) generated from oxidized lipids. The oxidation time for pattern change indicates the period for complete consumption of inherent hydrogen donating antioxidant compounds by lipid free radicals. Among the three parameters, overall oxidative stability of vegetable oils could be predicted considering the oxidation time for pattern changes and free radical formation rate. The oxidation time for pattern changes is influenced by both initial concentration of antioxidants and the concentration of lipid free radicals. If the same type of edible oils tested, the free radical formation rates will not be different much and initial concentration of antioxidants will determine the oxidative stability. For example, canola oil with 0.25% α-tocopherol needed longer oxidation time for pattern changes than canola oil without tocopherol addition (Fig. 4d). After the oxidation time for pattern change, free radical formation rate can predict the oxidation rate. Although soybean oil needed twice oxidation time for pattern change than canola oil due to the high inherent antioxidants, the higher free radical formation rate of soybean oil gave more oxidation products than canola oil in *p*-AV and TPM analyses. Olive oil needed 5 h oxidation time for the pattern change of DPPH absorbance and also had the lowest free radical formation rate, giving the highest oxidative stability. Although canola oil with 0.25% α-tocopherol needed 8 h oxidation time to consume the inherent tocopherol, considering high free radical formation rate, the sample would be oxidized rapidly in the prolonged oxidation time.

Many conventional methods measuring the degree of oil oxidation such as CDA, *p*-AV, and TPM determine the oxidation products (Hein et al., 1998; Shahidi & Wanasundara, 1998). CDA measures primary oxidation products (Shaker, 2006) while *p*-AV and TPM determine secondary oxidation products (Andrikopoulos, Antonopoulou, & Kaliora, 2002; Naz, Sheikh, Siddiqi, & Sayeed, 2004). However, DPPH method does not represent the concentration of oxidation products but measure the concentration of inherent hydrogen donating antioxidants or generated lipid radicals such as alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), or alkyl radicals ($R^{\bullet}$).

The characteristic of the DPPH method are the determination of the initial concentration of inherent hydrogen donating antioxidants and the free radical formation rate from oxidized oils, which can not be provided by other conventional methods. Small sampling size, relatively simple steps of sample preparation, and the easy quantitative comparisons among different sources are other features of DPPH method. However, there are some limitations in DPPH method. DPPH method is valid for the prediction of oxidative stability of fresh oils because highly oxidized oils can not be differentiated from unoxidized samples.

The result of this study can give important information for the researches screening new antioxidant compounds from natural sources through DPPH method. The interpretation of the results of studies using DPPH method should be carefully discussed because not only free radical scav-

enging compounds but also free radicals themselves can decrease the stability of DPPH.

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