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ORIGINAL PAPER

Effect of Eleven Antioxidants in Inhibiting Thermal Oxidation of Cholesterol

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Abstract Eleven antioxidants including nine phenolic compounds (rutin, quercetin, hesperidin, hesperetin, naringin, naringenin, chlorogenic acid, caffeic acid, ferulic acid), vitamin E (α-tocopherol), and butylated hydroxytoluene (BHT) were selected to investigate their inhibitory effects on thermal oxidation of cholesterol in air and lard. The results indicated that the unoxidized cholesterol decreased with heating time whilst cholesterol oxidation products (COPs) increased with heating time. The major COPs produced were 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5,6β-epoxycholesterol, 5,6α-epoxycholesterol, and 7-ketocholesterol. When cholesterol was heated in air for an hour, rutin, quercetin, chlorogenic acid, and caffeic acid showed a strong inhibitory effect. When cholesterol was heated in lard, caffeic acid, quercetin, and chlorogenic acid demonstrated inhibitory action during the initial 0.5 h ($p < 0.05$), with caffeic acid being the best inhibitor. Hesperetin, naringenin, caffeic acid, ferulic acid, vitamin E, and BHT could decrease the peroxide value during the initial 0.5 and 1 h ($p < 0.05$) in lard. It seemed that 200 ppm antioxidant could not obviously retard the long-term oxidation of cholesterol and lard under high temperature, but caffeic acid, quercetin, and chlorogenic acid displayed great untapped potential to prevent thermal oxidation of cholesterol 0.5 h at least. For the sake of health and flavor, fast stir-frying over a high flame is recommended. If baking or deep fat frying food in oil, it is best to limit cooking time to within 0.5 h.

Keywords Antioxidant · Cholesterol · COPs · Thermal oxidation · Gas chromatography

Abbreviations

AAPH	2,2-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate)
BHT	Butylated hydroxytoluene
COPs	Cholesterol oxidation products
DPPH	2,2-Diphenyl-1-picrylhydrazyl radical
FRAP	Ferric reducing antioxidant power
POV	Peroxide value
ORAC	Oxygen radical absorbing capacity
PCL	Photochemiluminescence
TBARS	Thiobarbituric acid reactive substances
TMS	Trimethylsilyl
TPTZ	2,4,6-Tris(2-pyridyl)-*s*-triazine

Introduction

Foods of animal origin are an important part of human dietary structure, because they can provide high-quality proteins and many other beneficial elements, including cholesterol. Cholesterol is one monounsaturated component of the animal cell membrane, helping to establish proper membrane permeability and fluidity. Organisms can obtain it through diet and biosynthesis [1]. However, cholesterol is prone to oxidation during food processing and/or prolonged storage, yielding a wide range of oxysterols or cholesterol oxidation products (COPs), which are one group

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of sterols with structure similar to cholesterol except an additional hydroxyl group, epoxy group, or ketone group at the sterol nucleus or at the side chain of the molecule [2, 3]. The formation of COPs can be favored in the presence of heat, light, oxygen, and so forth [4–7]. In recent decades, special attention has been paid to them because of their various negative and undesirable biological activities. A number of papers have shown that they are likely to initiate several cardio- or cerebrovascular diseases (especially atherosclerosis), diabetes, and kidney failure and they display cytotoxic and apoptotic effects etc. [8, 9]. Hence, it is necessary to minimize the formation of COPs in food for better health in the long run. Several researchers suggested that adding antioxidants was effective in inhibiting the generation of COPs during processing and storage [3, 10–12].

Many phenolic compounds could be potentially used as natural antioxidants; this possibility has prompted interest in replacing the synthetic antioxidants, such as butylated hydroxytoluene (BHT) [3]. These phytochemicals could become effective inhibitors of COPs formation, perhaps mainly owing to their abilities to scavenge free radicals [13]. Till now, several natural phenolics have been approved as food additives in China, such as tea polyphenols and glycyrrhiza extract. In most cases, the solubility of phenolic compounds in lipids is less satisfactory than that of synthetic antioxidants, but they can be applied in edible oil as antioxidants after being dissolved in alcohol first.

Lots of studies have focused on the evaluation of antioxidant capacity of phenolic compounds via in vitro antioxidant methods, such as assays of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), ferric reducing antioxidant power (FRAP), photochemiluminescence (PCL), oxygen radical absorbing capacity (ORAC), etc. [14]. However, most of these antioxidant assays are performed at room temperature, which might be quite different from high temperature. When it comes to analyzing the relationship between the antioxidant capacity of natural phenolic compounds at mild temperature and high temperature, the topic has received less attention than it actually deserves, although thiobarbituric acid reactive substances (TBARS), peroxide value (POV), and acid value, etc., are always used to evaluate the thermal oxidation extent of lipids [5, 15].

It is noteworthy to point out that lard, rather than common vegetable oil, was selected as the lipid matrix in this study. Traditionally, lard was an important cooking and baking staple, and was once as popular as butter. By the late twentieth century, lard had begun to be considered less healthy than vegetable oils because of its high saturated fatty acid and cholesterol content. Nevertheless, lard still plays a significant role in the cuisine of many countries. For example, in southern China in particular, many people still favor lard when they are cooking, and they believe that using lard can make their foods more nutritional and delicious. Furthermore, it is also popular for many Chinese people to prepare lard at home. That said, China is the largest producer of lard and pork. Against this backdrop, there is little literature about the thermal oxidation of cholesterol in lard.

Therefore, 11 antioxidants, namely rutin, quercetin, hesperidin, hesperetin, naringin, naringenin, chlorogenic acid, caffeic acid, ferulic acid, vitamin E, and BHT, were selected to evaluate their inhibitory ability on the thermal oxidation of cholesterol in lard. The POV of the lard was measured for comparison in the presence of the antioxidants. Meanwhile, DPPH, FRAP, and ORAC assays were adopted to evaluate the antioxidant capacity of the 11 antioxidants at room temperature.

Materials and Methods

Materials and Reagents

Cholesterol, 5α-cholestane, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5,6β-epoxycholesterol, 5,6α-epoxy cholesterol, 7-ketocholesterol, lard, rutin, quercetin, hesperidin, hesperetin, naringin, naringenin, chlorogenic acid, caffeic acid, ferulic acid, vitamin E (α-tocopherol), BHT, Trolox, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), DPPH , and trimethylsilyl (TMS) reagent were purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade.

Thermal Oxidation of Cholesterol in Air

Cholesterol (0.4 mg) in 200 μL ethyl acetate mixed with 8 μg antioxidant (previously dissolved in ethanol) in a test tube, and then the solvent was removed under a gentle stream of nitrogen gas. Each test tube was then heated at 160 °C for 1 h. After oxidation, all of the samples were allowed to cool at room temperature. Cholesterol and their oxides were then converted to their TMS derivatives and subjected to GC analyses.

Thermal Oxidation of Cholesterol in Lard

Lard (20 g) was heated to 60–70 °C and then 200 mg cholesterol and 4 mg antioxidants (the phenolics must be previously dissolved in ethanol) were mixed thoroughly. Each test tube contained 1 % cholesterol and 200 ppm antioxidant, and was heated at 160 °C for 4 h. At 0.5, 1, 2, 3, and 4 h of heating, a 40-mg lard sample was taken out each time. After cooling, 80 μg 5α-cholestane was added as internal standard, and the samples were subjected to cold saponification (1 mL 2 M KOH in 90 % ethanol) overnight

at room temperature. The unsaponifiable materials were extracted after adding 1 mL H_2O and 2 mL hexane to quantify the unoxidized cholesterol and COPs. All the samples were submitted to TMS derivatization and GC analyses.

Analyses of Unoxidized Cholesterol and COPs

Cholesterol and COPs were quantified in an Agilent 7890A gas–liquid chromatograph. Briefly, 100 μL TMS reagent was added to derivatize the cholesterol and COPs after heating at 60 °C for 60 min. The TMS-ether derivatives were dissolved into 400 μL hexane and then 1 μL mixed solution was injected into a fused silica capillary column (SAC™-5, 30 m × 0.25 mm i.d.; Supelco, Inc., Bellefonte, PA, USA). Helium was employed as the carrier gas at a constant flow of 1.0 mL/min. Column temperature was programmed from 80 to 270 °C at a rate of 30 °C/min, and then held for 22 min. Both injector and detector temperatures were set at 280 °C. Cholesterol and the individual COPs were quantified according to the amount of internal standard added.

GC–MS Identification of Unoxidized Cholesterol and COPs

Identification of cholesterol and the oxides was carried out on a 6890A gas chromatograph coupled to an Agilent 5975c mass spectrometer (Agilent Technologies Inc. CA. USA) with a full scan mode (*m*/*z* 45–600). Similarly, the TMS derivatives of COPs were separated on a SAC™-5 column. Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The column temperature was programmed from 80 to 270 °C at a rate of 30 °C/min, and then held for 22 min. The MS interface temperature was 280 °C and the ion source was 230 °C. Electron ionization energy was set at 70 eV. The individual COPs were identified according to the retention time of authentic standards and specific characteristics of mass spectra ions as previously published [16–18].

POV Analyses

POV was determined as suggested by Shantha and Decker [19]. It was evaluated at 500 nm with a double-beam UV–visible 2550 spectrophotometer (Shimadzu, Japan), calculated from the absorbance, and finally expressed as milliequivalents of oxygen per kilogram of fat (meq O_2/kg). Three replicates were run per sample.

DPPH Free Radical Scavenging Assay

The DPPH free radical scavenging activity of the antioxidants was measured using the method described by Gorinstein et al. [20] with some modifications. Thus, 0.1 mmol/L solution of DPPH in methanol was prepared. An aliquot of 0.2 mL sample (100 μg/mL) was added to 2.8 mL of this solution and kept in the dark for 30 min (read at 515 nm). The ability of the antioxidant to scavenge the DPPH radical was calculated with the following equation:

$$\% \text{ Inhibition } = [(A0 - A1)/A0] \times 100$$

where A0 was the absorbance of the control, A1 was the absorbance in the presence of sample.

FRAP Assay

The ferric reducing ability of each antioxidant solution (100 μg/mL) was measured according to a modified protocol developed by Benzie and Strain [21]. To prepare the FRAP reagent, a mixture of 0.1 M acetate buffer (pH 3.6), 10 mM TPTZ, and 20 mM ferric chloride (10:1:1, v/v/v) was made. Readings at the absorption maximum (593 nm) were taken using a Shimadzu UV–visible 2501 spectrophotometer, and the reaction was monitored for 10 min. Trolox solution was used to prepare the calibration curves. Result was expressed as milligrams of Trolox equivalent antioxidant capacity (TEAC) per gram.

ORAC Assay

The procedure was based on a previous paper by Wang et al. [22]. Samples and Trolox standard were dissolved in 75 mM potassium phosphate buffer, pH 7.4, and assayed on a Bio-Tek Synergy HT plate reader with automatic injectors using a black, clear-bottomed 96-well plate. Fluorescent filters were set to pass the light with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Briefly, the final assay mixture including 25 μL sample (4 μg/mL), 25 μL fluorescein (FL) (0.504 mM), and 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) 150 μL (17.07 mM, as a peroxyl radical generator). The analyzer was programmed to record the fluorescence every 2 min after AAPH addition. Final results were calculated using the differences of areas under the quenching curves of FL between a blank and a sample, and expressed as micromoles of Trolox equivalents per gram.

Statistical Analyses

All samples were prepared and analyzed in triplicate. To verify the statistical significance of all parameters, the values of mean ± SD were calculated. To compare several groups, analysis of variance (ANOVA) was used (SPSS for Windows, release 17.0.0). Probability values of $p < 0.05$ were adopted as the criteria for significant differences.

Table 1 COPs formation in air at 160 °C for 1 h with and without antioxidant

	Unoxidized cholesterol (%)	7α-Hydroxycholesterol (%)	7β-Hydroxycholesterol (%)	5,6β-Epoxycholesterol (%)	5,6α-Epoxycholesterol (%)	7-Ketocholesterol (%)	Total COPs (%)
Without	24.13 ± 4.39 e	0.46 ± 0.11	1.47 ± 0.22	2.12 ± 0.03	2.11 ± 0.06	4.47 ± 0.02	10.64 ± 0.40 a
Rutin	100.56 ± 1.43 a	ND	ND	ND	ND	ND	ND
Quercetin	97.07 ± 7.23 a	ND	ND	ND	ND	ND	ND
Hesperidin	18.16 ± 1.34 e	0.26 ± 0.05	1.04 ± 0.01	1.31 ± 0.08	1.49 ± 0.05	3.18 ± 0.17	7.27 ± 0.35 c
Hesperetin	34.36 ± 6.00 d	0.41 ± 0.01	1.55 ± 0.12	1.77 ± 0.14	1.90 ± 0.03	3.11 ± 0.03	8.75 ± 0.06 b
Naringin	17.21 ± 0.42 f	0.33 ± 0.02	1.25 ± 0.03	1.82 ± 0.04	1.73 ± 0.05	3.97 ± 0.18	9.10 ± 0.14 b
Naringenin	32.13 ± 5.80 d	0.48 ± 0.12	1.63 ± 0.12	1.59 ± 0.72	1.89 ± 0.19	3.68 ± 0.68	9.28 ± 0.46 b
Chlorogenic acid	93.05 ± 10.61 a	ND	ND	ND	ND	ND	ND
Caffeic acid	101.33 ± 2.46 a	ND	ND	ND	ND	ND	ND
Ferulic acid	43.48 ± 0.28 c	0.38 ± 0.12	1.31 ± 0.10	1.70 ± 0.03	1.42 ± 0.01	2.85 ± 0.33	7.66 ± 0.53 c
VE	54.76 ± 5.98 b	0.33 ± 0.13	0.88 ± 0.32	1.52 ± 0.91	1.12 ± 0.66	2.25 ± 1.75	6.1 ± 3.77 bc
BHT	21.04 ± 0.10 e	0.39 ± 0.04	1.14 ± 0.12	1.86 ± 0.10	1.61 ± 0.07	4.10 ± 0.47	9.10 ± 0.80 b

Values are mean ± SD, $n = 3$. Means with different letters in the same row differ significantly, $p < 0.05$

ND not detected

Results and Discussion

Thermal Oxidation of Cholesterol in Air With and Without Antioxidant

The thermal oxidation of cholesterol in air was performed at 160 °C for 1 h and the results are shown in Table 1. Five major COPs were detected by GC, namely, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5,6β-epoxycholesterol, 5,6α-epoxycholesterol, and 7-ketocholesterol. There have been several reports about cholesterol thermal oxidation in air [18, 23, 24], but much less literature about the inhibitory effect of antioxidants on it. Table 1 demonstrates that most antioxidants exhibited an inhibitory effect on the thermal oxidation of cholesterol (160 °C for 1 h). Among them, rutin, quercetin, chlorogenic acid, and caffeic acid could prevent its decomposition completely, much better than the control antioxidant vitamin E and BHT. The other antioxidants, to some extent, could also reduce the decomposition of cholesterol. On the basis of the results of total COPs generation, the antioxidant capacity sequence of 10 of the antioxidants was as follows: rutin ~ quercetin ~ chlorogenic acid ~ caffeic acid > ferulic acid ~ hesperidin > hesperetin ~ naringin ~ naringenin ~ BHT. Vitamin E deserves special attention because its antioxidant capacity was ranked between hesperidin and hesperitin, and there was no significant difference between vitamin E and hesperidin or between vitamin E and hesperitin.

Generally, cholesterol is stable at room temperature, but susceptible to thermal oxidation, and it oxidized more slowly in oils and fats [2]. In view of the popularity of lard in some cultures and the findings above, we selected lard to investigate the thermal oxidation of cholesterol after addition of antioxidants.

Thermal Oxidation of Cholesterol in Lard With and Without Antioxidant

The thermal oxidation of cholesterol in lard was performed at 160 °C for 0–4 h and the results are shown in Table 2 and Figs. 1, 2, 3. In accordance with the industrial scale, no more than 200 ppm antioxidant is added to edible oils to prevent lipids oxidation at room temperature. However, there are few reports about the inhibitory ability of antioxidants at such scale to prevent thermal oxidation of cholesterol and lard.

As shown in Table 2, without antioxidant addition, 7α-hydroxycholesterol, 5,6β-epoxycholesterol, and 5,6α-epoxycholesterol occurred after 0.5 h heating. When the 11 antioxidants were added separately, only quercetin, chlorogenic acid, and caffeic acid displayed strong

Table 2 COPs formation in lard at 160 °C with and without antioxidant as a function of heating time

	Heating time (h)				
	0.5	1	2	3	4
Antioxidant	Without				
7α-Hydroxycholesterol (%)	0.16 ± 0.04	0.42 ± 0.10	0.46 ± 0.07	0.23 ± 0.03	0.48 ± 0.01
7β-Hydroxycholesterol (%)	ND	0.11 ± 0.04	0.26 ± 0.02	0.16 ± 0.09	0.13 ± 0.04
5,6β-Epoxycholesterol (%)	0.17 ± 0.02	0.12 ± 0.05	0.67 ± 0.27	1.70 ± 0.02	1.53 ± 0.25
5,6α-Epoxycholesterol (%)	0.14 ± 0.02	0.36 ± 0.02	1.20 ± 0.15	2.04 ± 0.07	2.37 ± 0.15
7-Ketocholesterol (%)	ND	0.11 ± 0.04	0.32 ± 0.06	0.53 ± 0.07	1.17 ± 0.27
Antioxidant	Rutin				
7α-Hydroxycholesterol (%)	0.19 ± 0.03	0.27 ± 0.06	0.59 ± 0.06	0.30 ± 0.00	0.43 ± 0.26
7β-Hydroxycholesterol (%)	ND	0.06 ± 0.04	0.26 ± 0.10	0.26 ± 0.05	0.19 ± 0.10
5,6β-Epoxycholesterol (%)	0.09 ± 0.01	0.26 ± 0.16	0.56 ± 0.03	1.62 ± 0.77	1.09 ± 0.47
5,6α-Epoxycholesterol (%)	0.09 ± 0.01	0.29 ± 0.20	1.32 ± 0.08	1.94 ± 0.26	2.60 ± 0.36
7-Ketocholesterol (%)	ND	0.06 ± 0.04	0.48 ± 0.04	0.63 ± 0.14	1.48 ± 0.22
Antioxidant	Quercetin				
7α-Hydroxycholesterol (%)	ND	0.31 ± 0.08	0.45 ± 0.09	0.16 ± 0.05	0.27 ± 0.05
7β-Hydroxycholesterol (%)	ND	0.06 ± 0.04	0.24 ± 0.05	0.14 ± 0.03	0.21 ± 0.12
5,6β-Epoxycholesterol (%)	ND	0.34 ± 0.01	0.39 ± 0.16	2.11 ± 0.41	0.86 ± 0.29
5,6α-epoxycholesterol (%)	ND	0.32 ± 0.10	1.04 ± 0.13	2.15 ± 0.22	2.64 ± 0.11
7-Ketocholesterol (%)	ND	0.04 ± 0.02	0.31 ± 0.05	0.42 ± 0.03	1.18 ± 0.25
Antioxidant	Hesperidin				
7α-Hydroxycholesterol (%)	0.24 ± 0.03	0.33 ± 0.00	0.38 ± 0.06	0.27 ± 0.03	0.47 ± 0.09
7β-Hydroxycholesterol (%)	ND	0.08 ± 0.00	0.14 ± 0.01	0.09 ± 0.02	0.20 ± 0.05
5,6β-Epoxycholesterol (%)	0.11 ± 0.02	0.40 ± 0.09	0.30 ± 0.04	1.59 ± 0.12	0.82 ± 0.25
5,6α-Epoxycholesterol (%)	0.12 ± 0.01	0.51 ± 0.03	1.20 ± 0.07	1.87 ± 0.28	2.52 ± 0.20
7-Ketocholesterol (%)	ND	0.08 ± 0.00	0.31 ± 0.06	0.49 ± 0.27	1.38 ± 0.01
Antioxidant	Hesperetin				
7α-Hydroxycholesterol (%)	0.20 ± 0.02	0.27 ± 0.15	0.35 ± 0.01	0.23 ± 0.10	0.51 ± 0.27
7β-Hydroxycholesterol (%)	ND	0.08 ± 0.00	0.25 ± 0.06	0.16 ± 0.09	0.20 ± 0.05
5,6β-Epoxycholesterol (%)	0.09 ± 0.00	0.08 ± 0.00	0.49 ± 0.17	1.53 ± 0.32	0.53 ± 0.04
5,6α-Epoxycholesterol (%)	0.13 ± 0.04	0.25 ± 0.13	1.32 ± 0.04	1.84 ± 0.23	2.33 ± 0.01
7-Ketocholesterol (%)	ND	0.04 ± 0.01	0.46 ± 0.08	0.54 ± 0.19	1.29 ± 0.00
Antioxidant	Naringin				
7α-Hydroxycholesterol (%)	0.24 ± 0.09	0.34 ± 0.11	0.57 ± 0.06	0.44 ± 0.17	0.52 ± 0.21
7β-Hydroxycholesterol (%)	ND	0.08 ± 0.00	0.19 ± 0.08	0.21 ± 0.03	0.18 ± 0.10
5,6β-Epoxycholesterol (%)	0.10 ± 0.00	0.12 ± 0.05	0.62 ± 0.05	1.73 ± 0.12	0.87 ± 0.09
5,6α-Epoxycholesterol (%)	0.18 ± 0.01	0.32 ± 0.21	1.22 ± 0.01	2.01 ± 0.06	2.44 ± 0.07
7-Ketocholesterol (%)	ND	0.08 ± 0.00	0.47 ± 0.05	0.72 ± 0.01	1.31 ± 0.19
Antioxidant	Naringenin				
7α-Hydroxycholesterol (%)	0.23 ± 0.03	0.36 ± 0.04	0.50 ± 0.22	0.45 ± 0.02	0.46 ± 0.05
7β-Hydroxycholesterol (%)	ND	0.08 ± 0.01	0.24 ± 0.14	0.16 ± 0.03	0.14 ± 0.03
5,6β-Epoxycholesterol (%)	0.09 ± 0.01	0.08 ± 0.01	0.50 ± 0.03	1.94 ± 0.20	0.86 ± 0.07
5,6α-Epoxycholesterol (%)	0.11 ± 0.03	0.26 ± 1.08	1.08 ± 0.07	2.06 ± 0.13	2.47 ± 0.11
7-Ketocholesterol (%)	ND	0.04 ± 0.01	0.39 ± 0.02	0.67 ± 0.09	1.03 ± 0.38
Antioxidant	Chlorogenic acid				
7α-Hydroxycholesterol (%)	ND	0.23 ± 0.03	0.59 ± 0.38	0.29 ± 0.01	0.75 ± 0.09
7β-Hydroxycholesterol (%)	ND	0.08 ± 0.00	0.18 ± 0.05	0.21 ± 0.04	0.20 ± 0.02
5,6β-Epoxycholesterol (%)	ND	0.08 ± 0.00	0.66 ± 0.13	1.37 ± 0.31	0.60 ± 0.12
5,6α-Epoxycholesterol (%)	ND	0.25 ± 0.01	1.26 ± 0.07	1.89 ± 0.15	2.23 ± 0.13
7-Ketocholesterol (%)	ND	0.08 ± 0.00	0.37 ± 0.08	0.67 ± 0.04	1.42 ± 0.11

Table 2 continued

	Heating time (h)				
	0.5	1	2	3	4
Antioxidant	Caffeic acid				
7α-Hydroxycholesterol (%)	ND	0.10 ± 0.00	0.34 ± 0.05	0.44 ± 0.12	0.40 ± 0.12
7β-Hydroxycholesterol (%)	ND	ND	0.08 ± 0.00	0.12 ± 0.00	0.25 ± 0.01
5,6β-Epoxycholesterol (%)	ND	ND	0.50 ± 0.13	0.47 ± 0.16	1.62 ± 0.14
5,6α-Epoxycholesterol (%)	ND	ND	0.98 ± 0.09	1.94 ± 0.11	1.73 ± 0.12
7-Ketocholesterol (%)	ND	ND	0.23 ± 0.11	0.94 ± 0.17	0.55 ± 0.08
Antioxidant	Ferulic acid				
7α-Hydroxycholesterol (%)	0.16 ± 0.02	0.24 ± 0.10	0.56 ± 0.04	0.32 ± 0.03	0.31 ± 0.01
7β-Hydroxycholesterol (%)	ND	0.13 ± 0.06	0.19 ± 0.01	0.18 ± 0.01	0.13 ± 0.04
5,6β-Epoxycholesterol (%)	0.12 ± 0.03	0.13 ± 0.06	0.72 ± 0.08	2.05 ± 0.17	0.80 ± 0.44
5,6α-Epoxycholesterol (%)	0.11 ± 0.03	0.29 ± 0.06	1.22 ± 0.04	2.13 ± 0.15	2.39 ± 0.36
7-Ketocholesterol (%)	ND	0.09 ± 0.00	0.48 ± 0.02	0.63 ± 0.05	1.34 ± 0.18
Antioxidant	Vitamin E				
7α-Hydroxycholesterol (%)	0.22 ± 0.04	0.41 ± 0.08	0.55 ± 0.15	0.27 ± 0.04	0.39 ± 0.20
7β-Hydroxycholesterol (%)	ND	ND	0.25 ± 0.05	0.22 ± 0.04	0.24 ± 0.03
5,6β-Epoxycholesterol (%)	0.18 ± 0.10	0.18 ± 0.00	0.89 ± 0.02	1.68 ± 0.11	1.17 ± 0.10
5,6α-Epoxycholesterol (%)	0.18 ± 0.10	0.18 ± 0.00	1.39 ± 0.07	2.09 ± 0.11	2.72 ± 0.14
7-Ketocholesterol (%)	ND	ND	0.42 ± 0.08	0.49 ± 0.05	1.53 ± 0.12
Antioxidant	BHT				
7α-Hydroxycholesterol (%)	0.23 ± 0.01	0.66 ± 0.10	0.56 ± 0.19	0.18 ± 0.07	0.52 ± 0.12
7β-Hydroxycholesterol (%)	ND	0.08 ± 0.00	0.16 ± 0.08	0.12 ± 0.05	0.18 ± 0.11
5,6β-Epoxycholesterol (%)	0.20 ± 0.16	0.08 ± 0.00	1.13 ± 0.16	1.68 ± 0.22	1.44 ± 0.32
5,6α-Epoxycholesterol (%)	0.23 ± 0.20	0.13 ± 0.06	1.17 ± 0.18	1.83 ± 0.26	2.69 ± 0.09
7-Ketocholesterol (%)	ND	0.04 ± 0.02	0.31 ± 0.03	0.41 ± 0.09	1.40 ± 0.05

Values are mean ± SD, $n = 3$

ND not detected

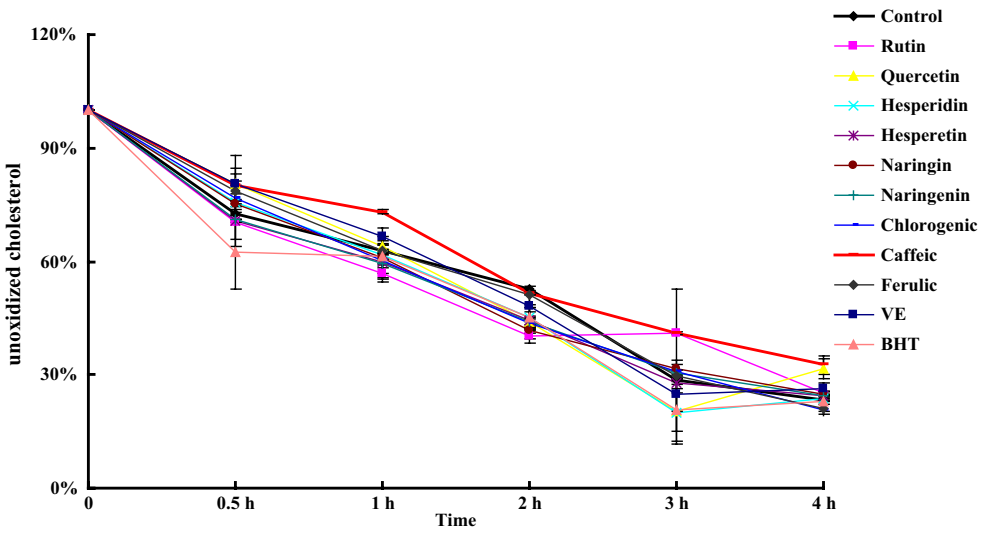

Fig. 1 Time course of unoxidized cholesterol in lard at 160 °C with and without antioxidant (values are mean ± SD, $n = 3$)

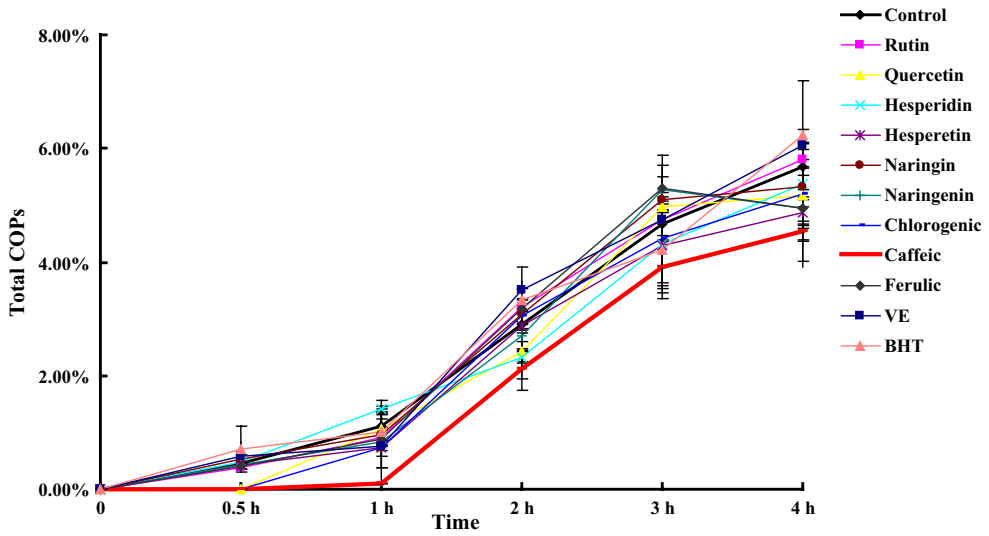

Fig. 2 Time course of total COPs in lard at 160 °C with and without antioxidant (values are mean ± SD, $n = 3$)

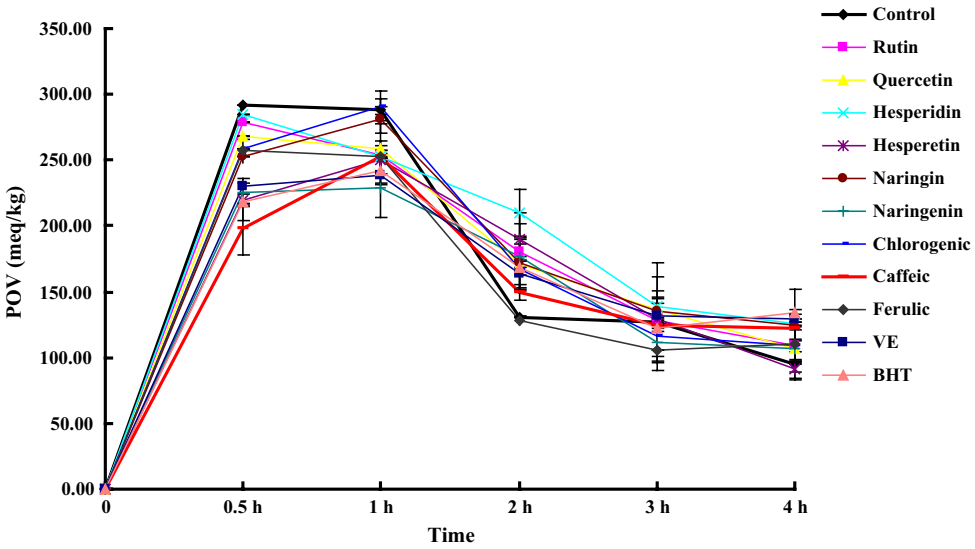

Fig. 3 Changes of POV in lard at 160 °C with and without antioxidant (values are mean ± SD, $n = 3$)

inhibitory effect and no COPs were found. We tentatively suggest that these three antioxidants were effective in inhibiting thermal oxidation of cholesterol during th first 0.5 h. However, after 1 h heating, quercetin and chlorogenic acid lost the power to inhibit COPs formation. In contrast, no 7β-hydroxycholesterol, 5,6β-epoxycholesterol, 5,6α-epoxycholesterol, and 7-ketocholesterol were detected after caffeic acid addition. But after 2 h heating, the five COPs were found in all samples. During the 4 h heating, among the five COPs, 7α-hydroxycholesterol and 7β-hydroxycholesterol appeared in lower amounts; meanwhile, the content of 5,6α-epoxycholesterol and 7-ketocholesterol increased with heating time, and 5,6β-epoxycholesterol achieved the highest content after 3 h heating, suggesting that each COP occurrence followed its own oxidation pathway [24]. Additionally, we observed that sometimes after heating, individual COPs were higher with antioxidant than without antioxidant even though the differences were generally minimal, and we supposed that it was mainly due to experimental error. Meanwhile, prooxidant activity of some selected antioxidants might also play a role [25].

One critical review summarized the inhibition of cholesterol oxidation by antioxidants [3]. BHT, *tert*-butylhydroquinone (TBHQ), vitamin E, rosemary oleoresin extract, and quercetin showed strong inhibitory action against cholesterol oxidation. Previously, we investigated the effect of green tea catechins (GTC), α-tocopherol, quercetin, and BHT in the prevention of cholesterol and β-sitosterol oxidation at 180 °C, and found that natural

antioxidants were more effective than BHT [2]. Both studies [2, 3] agreed with our results to some extent. Valenzuela et al. compared several synthetic antioxidants and reported that BHT and TBHQ were the most effective inhibitors of cholesterol oxidation; butylated hydroxyanisole (BHA) and ethoxyquin (EQ) were less effective; propyl gallate(PG) was unable to prevent cholesterol oxidation [26]. Recent research investigated the capacities of 15 vitamins to inhibit the formation of COPs in beef patties, indicating that L-ascorbic acid, retinoic acid, and α-tocopherol were the best ones [27]. Similarly, the effect of natural and synthetic antioxidants in protecting phytosterols during heating at 180 °C was reported along with the following sequence about the effectiveness of antioxidants: synthetic tocopherols > green tea extract > natural tocopherols from rapeseed oil > rosemary extract > phenolic compounds extracted from rapeseed meal > sinapic acid > BHT [28]. Lee reported that vitamin C, vitamin E, BHA, and Trolox were all effective in inhibiting COPs formation, with vitamin C being the most pronounced in marinated eggs, and BHA in marinated pork and juice [11].

When our results were compared with the above observations, in most cases, the thermal antioxidant capacity of the antioxidants selected was much weaker in terms of COPs generation and cholesterol retaining, probably as a result of the small amount added (200 ppm). Meanwhile, the temperature, time, and model system selected could also have affected the result;, therefore, more further research on this area is needed.

Changes of Unoxidized Cholesterol in Lard With and Without Antioxidant

As shown in Fig. 1, during the 4 h heating, whether adding antioxidants or not, cholesterol continuously degraded in lard. After 0.5 h heating, among the 11 antioxidants, quercetin (80.66 %), caffeic acid (79.97 %), and vitamin E (80.34 %) could inhibit the decrease of cholesterol compared with the control (72.44 %) with a statistically significant difference ($p < 0.05$). After 1 h heating, only caffeic acid retained the inhibitory capacity (caffeic acid, 73.09 %; control, 63.00 %). After 2 h heating, all antioxidants lost the ability to inhibit the loss of cholesterol. However, after 3 and 4 h heating, caffeic acid still led to a higher cholesterol content compared with the control (caffeic acid, 3 h (41.01 %), 4 h (32.54 %); control, 3 h (28.47 %), 4 h (23.18 %), $p < 0.05$). Generally, quercetin, caffeic acid, and vitamin E could inhibit cholesterol loss during the initial 0.5 h; and among these antioxidants, caffeic acid demonstrated a significant effect almost all the time (except 2 h heating).

Changes of Total COPs in Lard With and Without Antioxidant

As indicated in Fig. 2, with and without antioxidant the total COPs in lard always increased with heating time. After 0.5 h heating, among the 11 antioxidants, quercetin (0.00 %), chlorogenic acid (0.00 %), and caffeic acid (0.00 %) could inhibit the overall increase of total COPs compared with the control (0.46 %) with a statistically significant difference ($p < 0.05$). After 1 h heating, only caffeic acid could prevent the increase of total COPs, which had lower COPs throughout the 4 h heating compared with the control (caffeic acid, 1 h (0.1 %), 2 h (2.11 %), 3 h (3.91 %), 4 h (4.55 %); control, 1 h (1.11 %), 2 h (2.90 %), 3 h (4.66 %), 4 h (5.68 %), $p < 0.05$). Accordingly, quercetin, chlorogenic acid, and caffeic acid displayed the desirable inhibitory capacity of suppressing total COPs increase during the initial 0.5 h, with caffeic acid being the best one all the time (0–4 h).

It should be pointed out that the molecular weight of the selected antioxidants played an important role here. Antioxidants with lower molecular weight had higher molar concentration, and tended to demonstrate higher antioxidant capacity. This could partly explain why caffeic acid and quercetin achieved better results. Though BHT had the lowest molecular weight, it was volatile under the high temperature conditions; therefore, BHT was not more effective than other antioxidants. Meanwhile, the number of hydroxyl groups in the polyphenols was always supposed to be related to their antioxidant capacity when determined by certain antioxidant assays, such as FRAP [14]. However, it was hard to get this conclusion from our results because we employed ppm not molar units, making the comparison difficult. The complicated cause-and-effect relationship between them deserves further study.

Changes of POV in Lard With and Without Antioxidant

POV was always used to evaluate the extent of lipid oxidation. As shown in Fig. 3, POV in lard with and without antioxidant achieved the highest result within 1 h, and decreased rapidly thereafter. After 0.5 h heating, among the 11 antioxidants, hesperetin (219.86 meq/kg), naringin (252.31 meq/kg), naringenin (225.85 meq/kg), caffeic acid (197.71 meq/kg), chlorogenic acid (258.07 meq/kg), ferulic acid (257.17 meq/kg), vitamin E (230.49 meq/kg), and BHT (218.05 meq/kg) could inhibit the increase of POV compared with the control (291.42 meq/kg) at a statistically significant difference level ($p < 0.05$). After 1 h heating, quercetin (258.52 meq/kg), caffeic acid (253.04 meq/kg), ferulic acid (252.42 meq/kg), hesperetin (250.15 meq/

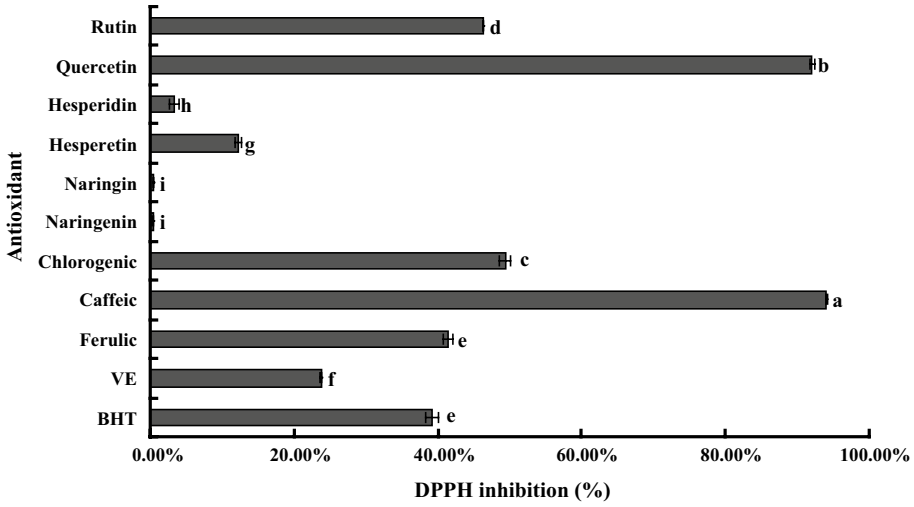

Fig. 4 Antioxidant capacity of 11 selected antioxidants determined by DPPH method (values are mean ± SD, $n = 3$. Means with different letters differ significantly, $p < 0.05$)

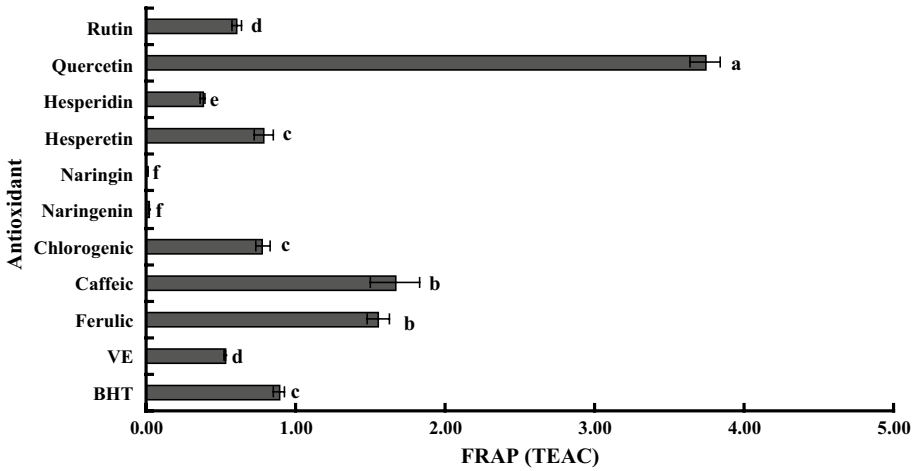

Fig. 5 Antioxidant capacity of 11 selected antioxidants determined by FRAP assay (values are mean ± SD, $n = 3$. Means with different letters differ significantly, $p < 0.05$)

kg), naringenin (229.02 meq/kg), vitamin E (238.18 meq/kg), and BHT (242.24 meq/kg) were able to inhibit the increase of POV compared with the control (288.02 meq/kg) with a statistically significant difference ($p < 0.05$). However, after 2 h heating, all antioxidants could not decrease the POV of the lard. To conclude, hesperetin, naringenin, caffeic acid, ferulic acid, vitamin E, and BHT could inhibit POV increase during the initial 0.5 h and 1 h, but all antioxidants ceased to be effective when being heated over 2 h.

On the basis of the above observations, the 11 selected antioxidants could not obviously retard the thermal oxidation of cholesterol and lipids, although caffeic acid was the best one. According to the industrial standard, generally, no more than 200 ppm antioxidant can be added to edible oils, which is even lower than many inherent antioxidants in vegetable oils, such as vitamin E. Therefore, we suggested that 200 ppm antioxidant could hardly inhibit the oxidation of cholesterol and lipids under high temperature conditions.

Antioxidant Capacity of the 11 Antioxidants Determined by DPPH, FRAP, and ORAC Assays

Three frequently used antioxidant methods, namely, DPPH, FRAP, and ORAC assays, were used to evaluate the antioxidant capacity of the selected antioxidants and the results are illustrated in Figs. 4, 5, 6. According to the results of the DPPH free radical scavenging assay, the antioxidant capacity sequence of the 11 antioxidants was as follows: caffeic acid > quercetin > chlorogenic acid > rutin > ferulic acid ~ BHT > VE > hesperetin > hesperidin > naringin ~ naringenin ($p < 0.05$). On the basis of the results of the FRAP assay, the antioxidant capacity sequence of was quercetin > caffeic acid ~ ferulic acid > BHT ~ hesperetin ~ chlorogenic acid > rutin ~ vitamin E > hesperidin > naringin ~ naringenin ($p < 0.05$). The result of the ORAC assay obtained the following sequence: quercetin ~ hesperetin ~ caffeic acid ~ naringenin > rutin ~ ferulic acid ~ chlorogenic acid ~ naringin ~ hesperidin > BHT ~ vitamin E (rutin ~ ferulic acid > hesperidin,

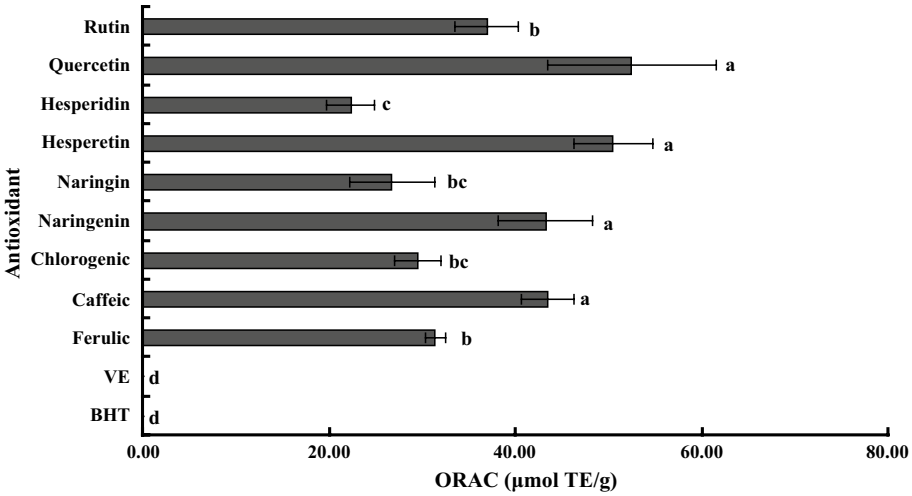

Fig. 6 Antioxidant capacity of 11 selected antioxidants determined by ORAC assay (values are mean ± SD, $n = 5$. Means with different letters differ significantly, $p < 0.05$)

$p < 0.05$). It should be pointed out that the data compared here was based on the weight of each antioxidant. If molecular weight was considered, the sequences would change accordingly.

DPPH, FRAP, and ORAC assays were all performed at room temperature, and several of the 11 antioxidants exhibited rather high antioxidant capacity, which was very different from the result achieved at high temperature. Therefore, we suggest that antioxidant capacity obtained at mild temperature may be very different from that at high temperature.

In this study, the capacities of 11 antioxidants to inhibit the formation of COPs in air and lard at 160 °C were investigated systematically. Rutin, quercetin, chlorogenic acid, and caffeic acid were effective in inhibiting the thermal oxidation of cholesterol in air. Caffeic acid, quercetin, chlorogenic acid, and vitamin E were effective in inhibiting the thermal oxidation of cholesterol in lard, especially during the initial 0.5 h, and caffeic acid appeared to be the best of these antioxidants. As for POV, hesperetin, naringenin, caffeic acid, ferulic acid, vitamin E, and BHT could significantly decrease the POV of lard during the initial 0.5 h and 1 h. Antioxidant capacity determined by DPPH, FRAP, and ORAC assays afforded different sequences as a result of their different antioxidant mechanisms. Finally, it seemed that 200 ppm antioxidant could hardly avoid the oxidation of cholesterol and lard under high temperature conditions.

Nowadays, lard is still popular for cuisine uses in many countries such as Britain, Central Europe, Mexico, and China. The 1990s and early 2000s even witnessed its partially rehabilitation for the unique culinary properties valued by chefs and bakers. When foods rich in cholesterol (such as egg, meat, and seafood) are cooked by frying in oil over a high flame, fast stir-frying is recommended for the sake of health and flavor. If baking or deep fat frying food in oil, it is best to limit the cooking time to within 0.5 h. According to the present research, cholesterol oxidation was difficult to avoid, even in the presence of antioxidants, at high temperatures. Therefore, further research is needed to understand the levels of COPs which are harmful to human health, as well as to develop strategies for controlling COP_s production in thermally processed foods.

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