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Inhibition of cholesterol oxidation in marinated foods as affected by antioxidants during heating

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Abstract

Chinese marinated foods rich in cholesterol, such as eggs and pork, can be susceptible to formation of carcinogenic cholesterol oxidation products (COPs) during prolonged heating. The objectives of this study were to compare the effects of various antioxidants on the inhibition of COPs in marinated eggs, pork and juice. The various COPs in marinated food samples were analyzed by a GC–MS technique. The incorporation of antioxidants, 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. The inhibition effect increased with increasing levels of BHA and trolox. However, vitamin E was more effective at a low level (0.02%) than at a high level (0.1%), probably because of prooxidant activity of the latter. The same phenomenon also occurred for 0.1% vitamin C in marinated eggs, but a reversed trend was observed in marinated pork and juice. The residual amounts of each antioxidant in marinated eggs, pork and juice were also determined by HPLC.

Keywords: Cholesterol oxidation products (COPs); Antioxidants; Marinated foods; GC–MS

1. Introduction

In the past decade cholesterol oxidation products (COPs) have attracted considerable attention because of their possible harmful effects on human health. Numerous studies have suggested that the consumption of COPs, in excess, could be associated with increasing risk of heart and vascular diseases (Leonarduzzi, Sottero, & Poli, 2002; Yin et al., 2000). Yin et al. (2000) reported that COPs possessed the ability to inhibit proliferation and induce apoptosis of vascular smooth muscle cells in tissue culture. In addition, COPs may occur in significant amounts in low density lipoprotein (LDL) particles, especially in hypercholesterolemic subjects, and thus contribute to the uptake of modified LDL by scavenger receptors and some of them finally accumulate in the subintimal space of major arteries (Leonarduzzi et al., 2002). Thus, it is imperative to minimize COPs formation in various food products during processing and storage.

The most common COPs found in food are 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), 5,6α-epoxycholesterol (5,6α-EP), 5,6β-epoxycholesterol (5,6β-EP), 5α-cholestane-3β,5,6β-triol (triol), 5-cholesten-3β-25-diol (25-OH) and 7-ketocholesterol (7-keto) (Raith et al., 2005; Ubhayasekera, Verleyen, & Dutta, 2004). Theoretically, COPs are formed from cholesterol during processing or storage, especially under thermal treatment in the presence of light and oxygen (Lee, Chien, & Chen, 2006). The formation mechanism of COPs during heating has been well established, and is reported to be similar to lipid oxidation (Chien, Huang, & Chen, 2004; Chien, Hsu, & Chen, 2006), which involves free radical reaction, hydroperoxide formation and degradation.

The presence of COPs in various food products has been well documented. Lercker and Rodriguez-Estrada (2000) reported a lower level of the 7-keto form in beef meat

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(⩽3.5 ppm) than in whole egg powder (⩽4.6 ppm). In dry-cured lberian hams, several COPs including 7α-OH, 7β-OH and 7-keto were detected, with 7-keto dominating at a concentration ranging from 57 to 71 μg/100 g of muscle (Petrón, García-Regueiro, Martín, Muriel, & Antequera, 2003). Seven COPs were also detected in meat-based baby foods, but the amount of 5,6β-EP was higher than that of 7-keto, indicating a strong development of both direct and indirect cholesterol oxidation pathways (Evangelisti, Zunin, Boggia, & Calcagno, 2004). Similarly, Zunin, Salvadeo, Boggia, and Evangelisti (2006) reported the presence of both 7-keto and 7-ketositosterol in meat- and fish-based homogenized baby foods containing vegetable oils, with the former (meat sample) containing 22–89 and 11–40 μg/serving, respectively, which was significantly higher than the latter (fish sample). In a recent study, Thurner, Razzazi-Fazeli, Wagner, Elmadfa, and Luf (2007) determined the formation of COPs in different processed meat samples (beef patties, braised meat, and fillets of pork); however, the most cytotoxic 25-OH and triol were not detected. Obviously the amount and variety of COPs formed in food products is mainly dependent upon processing conditions.

In addition to processing, storage conditions may have a great impact on COPs formation. Conchillo, Ansorena, and Astiasarán (2005) studied the formation of COPs in raw and cooked chicken breasts when stored at −18 °C for three months under aerobic and vacuum conditions, and the total COPs levels in grilled and roasted samples were 28.9 and 39.3 μg/g fat in aerobic packaging and 4.90 and 20.2 μg/g fat in vacuum-packaging, respectively. This result implied that vacuum-packaging was particularly efficient in slowing down cholesterol oxidation during frozen storage of cooked chicken meat. The effects of storage time on COPs formation in processed meat products manufactured by Brazilian industries were investigated by Baggio and Bragagnolo (2006), and no COPs were detected over a storage period of 120 days at 6–25 °C. This phenomenon further demonstrated that a significant amount of COPs can only be formed under drastic storage conditions.

The effect of antioxidants on inhibition of cholesterol oxidation in food systems has been studied by several authors. The incorporation of α-tocopheryl acetate was shown to retard cholesterol oxidation in cooked pork and retail packed chicken meat effectively (Kim, Ryu, Cho, & Rhee M.S., 2006; Rey et al., 2001). Likewise, the supplementation of vitamin E was demonstrated to reduce COPs formation in sausage from pigs fed soybean oil. But there is a paucity of data regarding the inhibition of cholesterol oxidation in eggs and pork as affected by some other antioxidants, e.g. BHA and vitamin C, during cooking.

Chinese marinating is a traditional Chinese cooking method which is performed by immersing the food samples in fluids containing various ingredients, e.g. sugar and soy sauce, and simmering at about 100 °C for 1 h and above (Lee et al., 2006). We used this term "Chinese marinating" to distinguish it from "Western marinating", which implies soaking of food items in a flavour-rich fluid prior to cooking. In a previous study, we reported the formation of COPs in marinated foods during heating, and an increasing trend was found for the levels of most COPs following the increase of heating time (Lee et al., 2006). However, the inhibition of COPs, by incorporation of antioxidants in marinated foods during heating, remains unknown. The objectives of this investigation were to study the inhibition of COPs by adding various antioxidants (vitamin C, vitamin E, trolox and BHA) to marinated eggs and pork during heating.

2. Materials and methods

2.1. Materials

About 10 kg of ground pork, 3 l of soy sauce, 1 kg of sugar (sucrose) and 100 eggs were purchased from a local supermarket in Taipei county. COPs standards, including 7α-OH, 7β-OH, 5,6 α-EP, 5,6β-EP, triol, 25-OH and 7-keto, and internal standard 5 α-cholestane were obtained from Sigma (St.Louis, MO, USA) and Steraloids Co. (Wilton, NH, USA). The purities of these standards ranged from 95 to 99%, and were used without further purification. The derivatization reagent Sylon BTZ, containing BSA, TMCS and TMSI (3:2:3), was from Supelco Co. (Bellefonte, PA, USA). The antioxidant standards, ascorbic acid and BHA (3-*t*-butyl-4-hydroxyanisole) were from Nacalai Tesque Co. (Kyoto, Japan), while α-tocopherol (vitamin E) and trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were from Sigma. The HPLC-grade solvents, methanol, acetone, isopropyl alcohol, acetonitrile, ethyl acetate, diethyl ether, *n*-hexane and chloroform, were from Mallinckrodt Co. (Paris, KY, USA). Deionized water was made using a Millipore Milli-Q purification system (Millipore Co., Bedford, MA, USA). The other reagents, anhydrous sodium sulfate and meta-phosphoric acid were from Riedel-de Haën Co. (Barcelona, Spain), and PICB (1-pentane sulfuric acid sodium salt) was from Sigma.

2.2. Instrumentation

The GC–MS instrument was composed of a gas chromatograph (model 6890) equipped with a mass spectrometer (model 5973), which was from Agilent Technologies (Palo Alto, CA, USA). A GC HP-5MS capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness, 5% diphenylpolysiloxane, 95% dimethylpolysiloxane) was used to separate the various COPs. The HPLC system was composed of a Phenomenex DG-440 degassing system (Torrance, CA, USA), a Rheodyne 7161 injector, a Jasco PU-980 pump (Tokyo, Japan) and Jasco MD-915 photodiode-array detector. An HPLC C18 column (150 × 4.6 mm I.D., particle size 5 μm) used to separate the various antioxidants was from Hypurity Co. (Runcorn, Cheshire, UK). The freeze-dryer (FD-24) was from Ching-Ming Co. (Taipei, Taiwan). The N-1 rotary evaporator was from Eyela

Co. (Tokyo, Japan). The DC-400 sonicator was from Chuan-Hua Co. (Taipei, Taiwan). The Sorvall RC5 C high-speed centrifuge was from Du Pont Co. (Wilmington, Delaware, USA). The CE 3021 spectrophotometer was from Cecil Co. (Cambridge, England).

2.3. Effect of antioxidants on COPs formation in marinated foods

Three litres of unmarinated juice was prepared by mixing 300 g of soy sauce (10%), 30 g of sugar (1%) and 2670 g of water (89%). This is the standard formula used by most restaurants in Taiwan for marinating (Lee et al., 2006). Before heating, eggs were boiled in water for 30 min and shelled. Then the juice was poured into a 4 l stainless-steel sauce pan and heated to 100 °C, after which 1 kg of ground pork or 10 shelled eggs were placed in the pan separately and marinated for 24 h with a lid on the top. At the same time, antioxidants, ascorbic acid, BHA, vitamin E and trolox, were added to the juice, separately, at levels of 0.02% and 0.1%. During marinating, the juice was replenished every hour to maintain it at the original level. Duplicate experiments were performed and, in total, 34 treatments were used, of which 2 were control treatments, containing only ground pork or shelled eggs in the juice without antioxidants. After heating, a 50 g sample of ground pork or eggs was collected and subjected to freeze-drying. Then the dried samples were ground into fine powder, and a 3 g pork or 1 g egg sample was taken for COPs analysis. For marinated juice, a 30 ml sample was taken for COPs analysis.

2.4. Analysis of COPs by GC–MS

A GC–MS method based on Lee et al. (2006) was used to determine the various COPs in marinated food samples and juice. A 1 g (of egg) or 3 g (of pork) sample was mixed with 30 ml of chloroform/methanol (2/1, v/v) and shaken for 30 min, after which the mixture was filtered through a glass filter containing 0.5 g of anhydrous sodium sulfate to remove residual moisture. The filtrate was evaporated to dryness under vacuum, dissolved in 5 ml of *n*-hexane and passed through a 0.45 μm membrane filter for purification. For marinated juice, a 30 ml sample was mixed with 30 ml of hexane and shaken for 30 min, followed by sonication for 10 min to remove excessive bubbles. The upper hexane phase was collected, evaporated to dryness, dissolved in 5 ml of hexane and passed through a 0.45 μm membrane filter for purification. Each 5 ml sample was poured into a silica cartridge (65 × 15 mm I.D.; sorbent weight, 500 mg; column volume, 6 ml; Agilent Technologies), which was prewet with 15 ml of hexane for activation. Then three solvent systems of 10 ml of hexane/diethyl ether (95/5, v/v), 25 ml of hexane/diethyl ether (90/10, v/v) and 15 ml of hexane/diethyl ether (80/20, v/v) were added successively to remove the impurities. Next, the COPs were eluted with 5 ml of acetone. The eluate was evaporated to dryness under nitrogen and dissolved in 1 ml of pyridine for derivatization. A 50 μl sample was poured into a 250 μl vial insert and mixed with 10 μl of 0.5 ppm internal standard 5 α-cholestane and 50 μl of derivatization agent. The solution was allowed to stand in the dark for 1 h for the derivatization reaction to proceed, after which 1 μl was collected and injected for GC–MS analysis.

An HP-5MS capillary column from Agilent Technologies was used to separate the various COPs, with He as carrier gas and flow rate at 0.8 ml/min and split ratio at 5:1. The injector temperature was 280 °C and the column temperature was 220 °C at the beginning, increasing to 275 °C at 15 °C/min, 280 °C at 1 °C/min, maintained for 2 min, to 290 °C at 5 °C/min and maintained for 10 min. The GC–MS interface temperature was 270 °C, with the electron multiplier voltage 70 eV and ion voltage 1360 V. Detection was carried out by selected ion monitoring (SIM) mode according to elution order, as reported by Lee et al. (2006). For the first group of compounds, 5 α-cholestane, 7α-OH and 7β-OH at retention times ranging from 0 to 13 min, six fragment ions with *m*/*z* values 217, 218, 372, 456, 457 and 458 were used for detection; the second group of compounds 5,6α-EP and 5,6β-EP had retention times of 13–14 min with 6 *m*/*z* values, 366, 384, 441, 442, 459 and 474; the compound triol had retention times of 14–15.4 min with 5 *m*/*z* values, 321, 403, 404, 456, 457; the fourth group of compounds, 25-OH and 7-keto, had retention times of 15.4–22 min with 6 *m*/*z* values, 131, 367, 457, 472, 473 and 474. Perfluorotributylamine was used for auto tune and calibration was done with an intensity of *m*/*z* at 69, 219 and 502. The various COPs were identified by comparing retention times of unknown peaks with reference standards and cochromatography with added standards. In addition, the mass spectra of unknown peaks were compared with those of COPs standards for positive identification. Quantification was carried out using 5 α-cholestane as an internal standard, and the various COPs were quantified using a formula described in a previous study (Chien et al., 2006).

2.5. Analysis of vitamin C in marinated food samples

A method based on that of Kacem, Marshall, Matthews, and Gregory (1986) was modified. A 1 g eggor pork sample was mixed separately with 5 ml of 10% metaphosphoric acid, and the solution was shaken for 10 min. After centrifugation at 5000*g* for 10 min (4 °C), the supernatant was collected and passed through a 0.2 μm membrane filter. A 20 μl sample was injected for HPLC analysis by using a C18 column and a mobile phase of 100% 5 mM PICB solution (pH adjusted to 3.1 with glacial acetic acid), with flow rate at 0.6 ml/min and detection at 254 nm. For marinated juice, the extraction method was the same as described for egg and pork, with the exception that a 1 ml sample was used and mixed with 1 ml of 10% metaphosphoric acid.

2.6. Analysis of vitamin E in marinated food samples

A method based on Anna and Ewa (2004) was modified. A 1 g egg or pork sample was mixed separately with 5 ml of methanol, and the solution was shaken for 20 min. After centrifugation at 5000*g* for 10 min (4 °C),the supernatant was collected and passed through a 0.2 μm membrane filter. A 20 μl sample was injected for HPLC analysis by using a C18 column and an isocratic mobile phase of methanol/ethyl acetate (60/40, v/v), with flow rate at 1.0 ml/min and detection at 290 nm. For marinated juice, the extraction method was the same as that described for egg and pork, with the exception that a 1 ml sample was used and mixed with 1 ml of hexane.

2.7. Analysis of BHA in marinated food samples

A method based on that of Perrin and Meyer (2002) was modified. A 1 g egg or pork sample was mixed separately with 5 ml of methanol and the solution was shaken for 20 min. After centrifugation at 5000*g* for 10 min (4 °C),the supernatant was collected and filtered through a 0.2 μm membrane filter. A 20 μl sample was injected for HPLC analysis by using a C18 column and a mobile phase of methanol/PICB solution (pH adjusted to 3.1 with glacial acetic acid) (70/30, v/v), with flow rate at 1.0 ml/min and detection at 290 nm. For marinated juice, the extraction method was the same as that described for egg and pork, with the exception that a 1 ml sample was used and mixed with 1 ml of hexane-isopropyl alcohol (1/1, v/v).

2.8. Analysis of trolox in marinated food samples

The method used for determining trolox in marinated eggs, pork and juice was the same as that for BHA, with the exception that a mobile phase of methanol/PICB solution (pH 3.1) (60/40, v/v) was used.

2.9. Identification and quantification of antioxidants

Identification of antioxidants was performed by comparing retention times of unknown peaks with reference standards and cochromatography with added standards. In addition, the absorption spectra (200–400 nm) of unknown peaks were compared with those of reference standards. For quantification, four standard curves were prepared by using five concentrations, of 0.5, 1, 5, 10 and 25 ppm, for vitamin C, and 1, 5, 10, 25 and 50 ppm for vitamin E, BHA and trolox. Each curve was obtained by plotting concentration against area, and the linear regression equations for vitamin C, vitamin E, BHA and trolox were $y = 0.0110 \times -0.2242$, $y = 0.0498 \times -0.6191$, $y = 0.0416 \times -0.6303$ and $y = 0.0681 \times -0.8870$, respectively, with the correlation coefficient (r^2) all being higher than 0.99. The contents of each residual antioxidant were calculated as follows:

$$\text{Residual amount of antioxidant (ppm)} = (A \times a + b) \times V \times \frac{1}{\text{sample weight(g)}}$$

where A: peak area of antioxidant a: slope of the linear regression equation curve b: intercept of the linear regression equation V: final diluted volume (ml). The inhibition percentage was calculated, based on the following formula:

$$\text{Inhibition percentage} = \frac{(\text{Total COPs of control treatment}) - (\text{Total COPs of treatment with antioxidant})}{(\text{Total COPs of control treatment})} \times 100$$

2.10. Statistical analysis

Duplicate analyses were carried out and the data were subjected to analysis of variance and Duncan's multiple range test for comparison of significant difference ($p < 0.05$) using SAS. (2004).

3. Results and discussion

3.1. Effects of antioxidants on the COPs formation in marinated eggs

Table 1 shows the effect of antioxidants on the formation of COPs in marinated eggs. With 0.02% vitamin C, the amounts of 7α-OH, 7β-OH and 7-keto were decreased by 1123, 577 and 742 ng/g, respectively, compared to the standard formula (control treatment). However, no triol, 5,6α-EP, 5,6β-EP or 25-OH were detected. For 0.1% vitamin C, further declines, by 1420, 802 and 1306 ng/g were shown for 7α-OH, 7β-OH and 7-keto, respectively. This result clearly indicated that the addition of vitamin C was effective in inhibiting cholesterol oxidation. As the lipid and cholesterol portions of eggs are mainly concentrated in yolks, the cholesterol oxidation should proceed in the interior of eggs during heating. Moreover, the antioxidant vitamin C should be oriented on the surface of eggs, preventing contact of cholesterol with oxygen to slow cholesterol oxidation. Of the various COPs, a low amount of 7-keto formed in the pres-

Table 1
Effect of antioxidants on the formation of COPs (ng/g) in marinated eggs

COPs	Antioxidant								
	Standard formula	Trolox		Vitamin C		BHA		Vitamin E	
		0.02%	0.1%	0.02%	0.1%	0.02%	0.1%	0.02%	0.1%
7α-OH	2050 ± 197*,a	1468 ± 302^{bc}	1265 ± 251^{cd}	927 ± 97.6^{de}	630 ± 32.4^{e}	1223 ± 107^{cd}	1161 ± 97.0^{cd}	1522 ± 46.9^{bc}	1817 ± 10.3^{ab}
7β-OH	1706 ± 50.2^{bc}	1810 ± 236^{bc}	1462 ± 240^{cd}	1129 ± 203^{de+}	904 ± 167^{e}	1855 ± 16.0^{b}	1270 ± 44.7^{d}	1747 ± 3.0^{bc}	2372 ± 49.9^{a}
5,6β-EP	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
5,6α-EP	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Triol	217 ± 5.3^{a}	N.D.	N.D.	N.D.	N.D.	114 ± 6.2^{b}	N.D.	N.D.	120 ± 3.2^{b}
25-OH	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
7-Keto	1578 ± 98.5^{a}	501 ± 101^{cd}	1568 ± 55.9^{a}	836 ± 179^{b}	272 ± 63.4^{d}	289 ± 47.2^{d}	737 ± 45.0^{bc}	1294 ± 202^{a}	1373 ± 228^{a}
Total COPs	5550 ± 351^{a}	3778 ± 639^{bcd}	4295 ± 546^{bc}	2891 ± 480^{d}	1806 ± 136^{e}	3481 ± 144^{cd}	3168 ± 187^{d}	4563 ± 153^{b}	5681 ± 271^{a}

N.D.: Not detected.
Standard formula: 10% soy sauce and 1% sucrose marinated together for 24 h.
[a–e]Values within a row with different superscripts are significantly different ($p < 0.05$).
* Means of duplicate analyses ± standard deviation.

ence of vitamin C was probably due to less formation of 7-OOH and 7α-OH or 7β-OH, as both can form 7-keto, through dehydration and dehydrogenation (Chien et al., 2006). 7α-OH, showed lower stability than did 7β-OH (Smith, 1987), and thus resulted in a greater concentration change than did 7β-OH. A similar trend was found for vitamin E. With 0.02% vitamin E, 7α-OH and 7-keto were diminished by 528 and 284 ng/g, respectively, while no 5,6α-EP, 5,6β-EP, 25-OH or triol was detected. Interestingly, the level of 7β-OH was higher after heating, which may be accounted for by its higher stability than 7α-OH (Smith, 1987). In comparison with vitamin C (0.02%), vitamin E (0.02%) was less efficient in inhibiting cholesterol oxidation, as evidenced by a larger amount of total COPs generated for the latter (4563 ng/g) than for the former (2891 ng/g). Surprisingly, a higher level of COPs was formed for 0.1% vitamin E than for 0.02% vitamin E, with 7α-OH, 7β-OH and 7-keto rising by 295, 625 and 79 ng/g, respectively, and triol (120 ng/g) was detected as well. This result indicated that a high level of vitamin E may act as prooxidant; that is, it may produce a large amount of free radicals during heating, which in turn react with substrate to promote cholesterol oxidation (Terao, 1986). This phenomenon was also observed by several authors. Dougherty (1988) reported that a low concentration of vitamin E was an effective antioxidant, whereas a high concentration (500 ppm) was ineffective. Cheng (1995) further demonstrated that both levels (0.108 and 0.537%) of vitamin E possessed prooxidant activity toward lipid and cholesterol oxidation in shredded fried pork. Both 5,6α-EP and 5,6β-EP were undetected, as they can only be formed from cholesterol in the presence of cholesterol hydroperoxide (7-OOH) and oxygen (Chien et al., 2006). However, since 7-OOH can also be reduced to form 7α-OH or 7β-OH or dehydrated to form 7-keto, all of these would make the formation of cholesterol epoxides more difficult.

Like vitamin E, the addition of 0.02% trolox showed a decrease of 7α-OH and 7-keto by 582 and 1077 ng/g, respectively, compared to the control treatment. However, only a slight difference was found for 7β-OH (1810 ng/g). As explained above, a higher stability of 7β-OH than 7α-OH may account for this effect. For 0.1% trolox, sharp losses of 785 and 244 ng/g were observed for 7α-OH and 7β-OH, respectively, but there was no significant difference in 7-keto between control treatment (1578 ng/g) and 0.1% trolox treatment (1568 ng/g). This outcome implied that the incorporation of 0.1% trolox failed to prevent 7-keto formation. Theoretically, trolox may inhibit COPs formation through hydrogen donation to cholesterol hydroperoxide radicals. But, since cholesterol hydroperoxide (7-OOH) can be reduced to 7α-OH or 7β-OH and dehydrated to 7-keto simultaneously, we speculated that it is quite possible for trolox to release hydrogen to participate in the reduction reaction for 7α-OH or 7β-OH formation. This would result in 7-keto formation through dehydrogenation of 7α-OH or 7β-OH during marinating (Kim & Nawar, 1993; Nielson, Olsen, & Skibsted, 1996). Hence, with the trolox level at 0.1%, 7-keto should be more readily

formed than 7α-OH or 7β-OH as the dehydration or dehydrogenation was favoured compared to reduction.

With 0.02% BHA, the contents of 7α-OH, 7-keto and triol were reduced by 827, 1289 and 103 ng/g, respectively, compared to the control treatment. However, no significant difference was found for 7β-OH (1855 ng/g) and no 25-OH was detected. Obviously, at the same concentration (0.02%), BHA was more effective in inhibiting 7α-OH, 7β-OH and 7-keto formation than was trolox. More triol was formed for BHA than for trolox and vitamin C, which should be due to hydrolysis of 5,6α-EP or 5,6β-EP under acidic conditions (Chien et al., 2006). This may also explain why no 5,6α-EP or 5,6β-EP were detected for BHA. No significant difference was found for 7α-OH between 0.02 and 0.1% BHA, whereas 7β-OH showed a marked change by 585 ng/g. Apparently a high level (0.1%) of BHA was necessary for inhibiting 7β-OH formation and a low level (0.02%) was adequate for retarding 7α-OH formation. Conversely, 0.1% BHA failed to inhibit 7-keto formation, as shown by an increase of 448 ng/g (Table 1).

By comparison, all four antioxidants were effective in retarding cholesterol oxidation, and the inhibition efficiency was mainly dependent upon concentration. For the total COPs, the inhibition effect of cholesterol oxidation increased with increasing concentrations of vitamin C and BHA. In contrast, a low level of vitamin E (0.02%) showed a better antioxidant activity than a high level of vitamin E (0.1%), which may act as a prooxidant. Trolox showed an effect similar to vitamin E, which may be attributed to its poor solubility in marinated eggs. Of the four antioxidants, vitamin C was the most efficient for inhibiting cholesterol oxidation in marinated eggs, followed by BHA, trolox and vitamin E. In a review report dealing with health hazard and the role of antioxidants in preventing cholesterol oxidation, Valenzuela, Sanhueza, and Nieto (2003) concluded that both natural and synthetic antioxidants were effective against cholesterol oxidation.

3.2. Effects of antioxidants on COPs formation in marinated pork and juice

Table 2. shows the effect of antioxidants on COPs formation in marinated pork. With 0.02% vitamin C, only 7-keto was reduced significantly with a loss of 72 ng/g, compared to the control treatment. The total COPs also declined by 128 ng/g, implying that vitamin C was slightly effective for inhibiting cholesterol oxidation in marinated pork, which may be caused by the oil distribution in ground pork being more like an oil-in-water emulsion system, and the water-soluble antioxidants should be more difficult to be oriented at the interface of this system (Frankel, Huang, Kanner, & German, 1994). Interestingly, with 0.1% vitamin C, the contents of 25-OH and 7-keto did not drop. Instead, they increased by 95 and 49 ng/g, respectively, accompanied by a rise of the total COPs to 109 ng/g (Fig. 1). It may be inferred that a high level of vitamin C (0.1%) may be oxidized and degraded to form

Table 2
Effect of antioxidants on the formation of COPs (ng/g) in marinated pork

COPs	Antioxidant							
	Standard formula	Trolox		Vitamin C		BHA		Vitamin E	
		0.02%	0.1%	0.02%	0.1%	0.02%	0.1%	0.02%	0.1%
7α-OH	$147 \pm 7.4^{*,ab}$	83.8 ± 7.1^{de}	102 ± 10.4^{cd}	133 ± 17.3^{b}	119 ± 17.7^{bc}	99.2 ± 9.8^{cd}	67.3 ± 11.0^{e}	122 ± 9.8^{bc}	163 ± 12.0^{a}
7β-OH	276 ± 27.7^{b}	132 ± 17.1^{e}	174 ± 7.6^{de}	252 ± 10.2^{bc}	212 ± 30.1^{cd}	167 ± 6.4^{de}	154 ± 49.1^{de}	246 ± 18.6^{bc}	335 ± 23.3^{a}
5,6β-EP	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
5,6α-EP	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Triol	38.2 ± 0.1^{ab}	37.4 ± 1.3^{ab}	60.4 ± 20.6^{a}	38.8 ± 12.2^{ab}	57.2 ± 16.3^{ab}	40.3 ± 0.1^{ab}	N.D.	33.8 ± 1.5^{b}	36.5 ± 2.7^{ab}
25-OH	162 ± 6.9^{cd}	329 ± 58.5^{a}	206 ± 15.5^{bc}	145 ± 6.6^{cd}	240 ± 67.5^{b}	193 ± 1.7^{bc}	115 ± 6.9^{d}	109 ± 5.8^{d}	114 ± 5.8^{d}
7-Keto	273 ± 13.4^{a}	237 ± 11.2^{ab}	135 ± 39.7^{cd}	201 ± 20.0^{b}	250 ± 9.8^{a}	232 ± 23.1^{ab}	109 ± 6.4^{d}	151 ± 0.3^{cd}	155 ± 1.3^{c}
Total COPs	897 ± 14.8^{a}	819 ± 44.1^{ab}	678 ± 57.9^{c}	769 ± 45.5^{bc}	878 ± 73.3^{a}	731 ± 8.4^{bc}	446 ± 51.3^{d}	661 ± 35.4^{c}	803 ± 30.9^{ab}

N.D.: Not detected.
Standard formula: 10% soy sauce and 1% sucrose marinated together for 24 h.
[a–e]Values within a row with different superscripts are significantly different ($p < 0.05$).
[*] Means of duplicate analyses ± standard deviation.

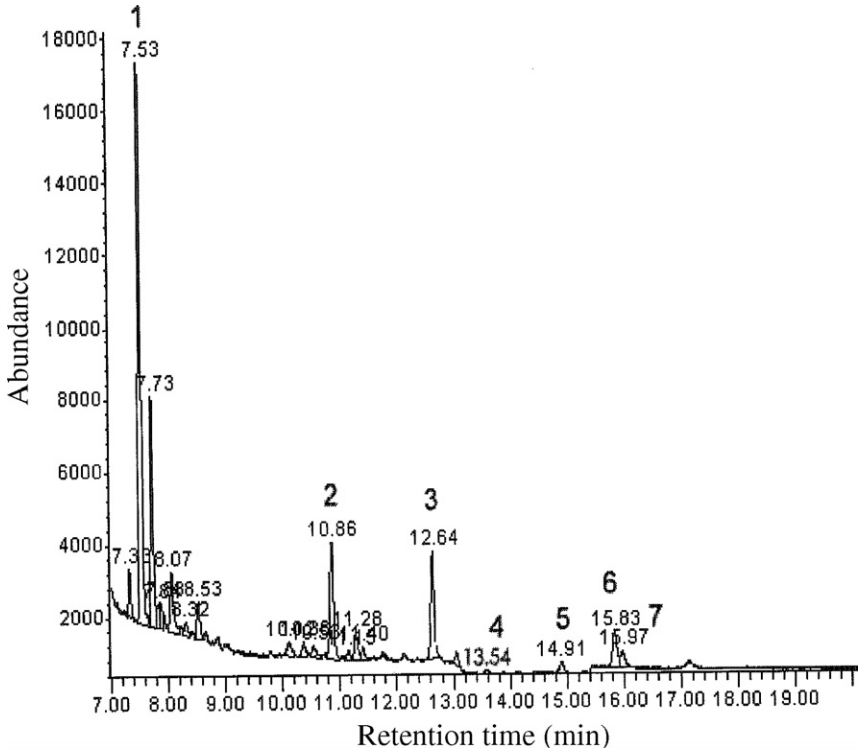

Fig. 1. GC–MS–SIM chromatogram of marinated pork in the presence of 0.1% vitamin C. Peaks: 1 = 5 α-cholestane (internal standard), 2 = 7 α-OH, 3 = 7β-OH, 4 = 5,6α-EP, 5 = triol, 6 = 25-OH, 7 = 7-keto.

Table 3
Effect of antioxidants on the formation of COPs (ng/g) in marinated pork juice

COPs	Antioxidant								
	Standard formula	Trolox		Vitamin C		BHA		Vitamin E	
		0.02%	0.1%	0.02%	0.1%	0.02%	0.1%	0.02%	0.1%
7α-OH	$13.5 \pm 0.5^{*,a}$	4.6 ± 0.3^{de}	4.5 ± 0.1^{e}	4.9 ± 0.4^{de}	7.4 ± 0.1^{c}	3.8 ± 0.1^{f}	3.6 ± 0.1^{f}	5.2 ± 0.0^{d}	10.0 ± 0.4^{b}
7β-OH	20.8 ± 1.4^{a}	5.8 ± 1.1^{def}	6.0 ± 0.3^{def}	7.1 ± 0.8^{d}	12.8 ± 1.1^{c}	5.0 ± 0.4^{ef}	4.5 ± 0.1^{f}	7.0 ± 0.2^{de}	15.4 ± 1.1^{b}
5,6β-EP	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
5,6α-EP	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Triol	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
25-OH	14.4 ± 1.4^{a}	12.3 ± 1.1^{ab}	10.4 ± 0.2^{bc}	6.0 ± 0.8^{d}	8.1 ± 0.6^{cd}	10.1 ± 1.1^{bc}	9.4 ± 1.3^{c}	9.3 ± 1.1^{c}	12.7 ± 1.7^{ab}
7-Keto	29.1 ± 1.2^{a}	17.2 ± 1.0^{bc}	5.7 ± 0.8^{e}	13.7 ± 2.3^{d}	15.0 ± 1.0^{cd}	5.7 ± 1.1^{e}	N.D.	17.6 ± 1.1^{bc}	18.9 ± 0.8^{b}
Total COPs	77.8 ± 2.2^{a}	39.9 ± 3.4^{c}	26.6 ± 0.9^{de}	31.7 ± 4.2^{d}	43.3 ± 1.6^{c}	24.6 ± 1.8^{e}	17.5 ± 1.5^{f}	39.1 ± 2.1^{c}	57.0 ± 4.0^{b}

N.D.: Not detected.
Standard formula: 10% soy sauce and 1% sucrose marinated together for 24 h.
[a–f]Values within a row with different superscripts are significantly different ($p < 0.05$).
* Means of duplicate analyses ± standard deviation.

dehydroascorbic acid during heating, which in turn promotes cholesterol oxidation through the intermediate semi-dehydroascorbic acid (Buettner & Jurkiewics, 1993). A similar tendency was shown for the effect of antioxidants on the COPs formation in marinated pork juice (Table 3). Compared to the control treatment, a low level of 0.02% vitamin C was adequate to reduce 7α-OH, 7β-OH, 25-OH and 7-keto by 8.6, 13.7, 8.4 and 15.4 ng/g, respectively. Like marinated pork, the addition of 0.1% vitamin C resulted in higher amounts of 7α-OH, 7β-OH, 25-OH and 7-keto by 2.5, 5.7, 2.1 and 1.3 ng/g, respectively, than of 0.02% vitamin C. This outcome clearly demonstrated that the cholesterol oxidation could be accelerated in the presence of 0.1% vitamin C.

In marinated pork with 0.02% vitamin E, all the COPs showed a declining trend with 25-OH and 7-keto being inhibited most, as indicated by a sharp loss of 53 and 122 ng/g, respectively, compared to the control treatment (Table 2). This antioxidant activity was much lower than that in eggs, as a decrease of total COPs by 26.2% was shown for pork and 17.8% for eggs. This is probably because of greater solubility of vitamin E in marinated pork. Likewise, marinated pork juice showed a higher loss of total COPs by 49.7% (Table 3). The same phenomenon

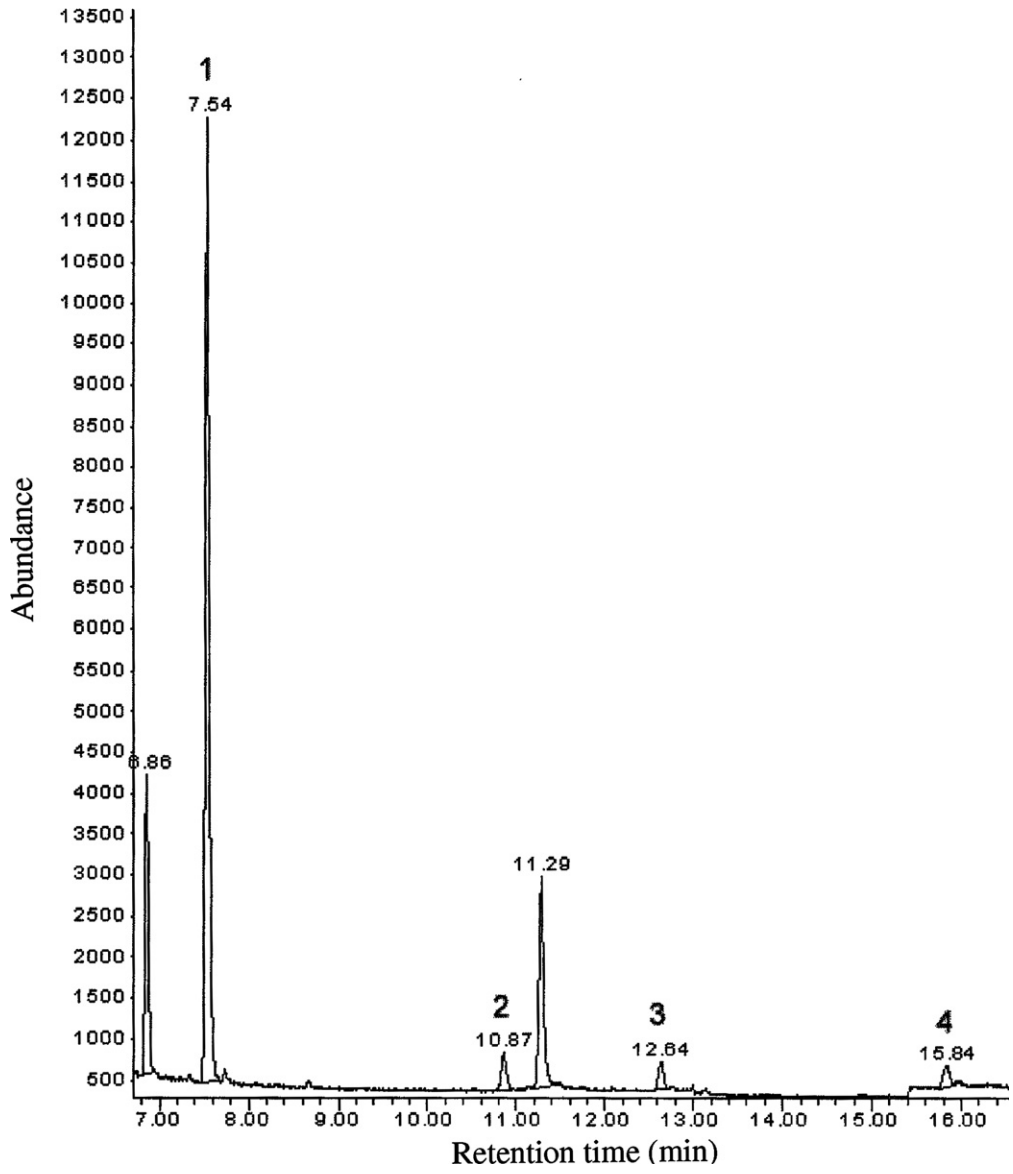

Fig. 2. GC–MS-SIM chromatogram of COPs in marinated pork juice in the presence of 0.1% BHA. Peaks: 1 = 5α-cholestane (internal standard), 2 = 7α-OH, 3 = 7β -OH, 4 = 25-OH.

also occurred for 0.1% vitamin E and BHA (Fig. 2). By contrast, both water-soluble antioxidants, trolox and vitamin C, exhibited a reversed trend, as revealed by a larger loss of total COPs in marinated eggs, which equalled 31.9% for 0.02% trolox and 67.5% for 0.1% vitamin C.

In a study dealing with the effect of dietary oils and α-tocopheryl acetate supplementation on cholesterol oxidation in cooked pork, Rey et al. (2001) also reported that the incorporation of α-tocopheryl acetate could significantly decrease total COPs production across the dietary groups. Similarly, a sausage from pigs fed soybean oil was shown to possess a higher concentration of oxysterols than that fed palm oil, but the addition of vitamin E could reduce oxysterol formation substantially in sausage from pigs fed soybean oil (Eder, Müller, Kluge, Hirche, & Brandsch, 2005). Kim et al. (2006) further demonstrated that supplementation with 200 or 400 IU of α-tocopheryl acetate was effective in reducing total COPs in retail packed chicken meat during refrigerated storage. Obviously both oil-soluble and water-soluble vitamin E should be protective against cholesterol oxidation, depending on concentration.

In marinated pork juice, a level of 0.02% vitamin E was found to reduce 7α-OH, 7β-OH, 25-OH and 7-keto by 8.3, 13.8, 5.1 and 11.5 ng/g, respectively, compared to control treatment (Table 3). Interestingly, higher increases of 7α-OH, 7β-OH, 25-OH and 7-keto, by 4.8, 8.4, 3.4 and 1.3 ng/g, respectively, were shown for 0.1% vitamin E than for 0.02% vitamin E. As mentioned before, the prooxidant activity of the former should account for this effect. For marinated pork with 0.02% trolox, a similar change was shown, with the contents of 7α-OH, 7β-OH and 7-keto

being lowered by 63.2, 144 and 36 ng/g, respectively, compared to the control treatment (Table 2). Surprisingly, a sharp increase of 167 ng/g occurred for 25-OH, implying that trolox failed to protect cholesterol from side-chain oxidation. However, a level of 0.1% trolox exhibited a different trend, as revealed by increases of 7α-OH, 7β-OH and triol, and decreases of 25-OH and 7-keto. The increase of 7α-OH or 7β-OH is probably due to reduction of cholesterol hydroperoxide, while the decrease of 7-keto is probably because of further degradation to 3,5-cholestadiene during heating (Peng, Taylor, Tham, Werthessen, & Mikkelson, 1979).

For 0.02% BHA, the contents of 7α-OH, 7β-OH and 7-keto were decreased by 47.8, 109 and 41 ng/g in marinated pork, respectively, compared to the control treatment (Table 2). However, no significant differences were found for triol and 25-OH. Declines of 31.9, 13.0, 78 and 123 ng/g for 7α-OH, 7β-OH, 25-OH and 7-keto, respectively, were shown for 0.1% BHA. Apparently the inhibition effect of cholesterol oxidation increased with increasing level of BHA. In marinated juice, only small amounts of 7α-OH, 7β-OH and 25-OH were formed for both 0.02% and 0.1% BHA, with 7-keto being undetected for the latter (Table 3). Comparatively, BHA was more effective towards cholesterol oxidation in marinated pork and juice than were the other antioxidants.

In addition to commercial antioxidants, the effects of crackling hydrolysates on cholesterol oxidation in model emulsions and pork meatballs were investigated by several authors. Cholesteryl linoleate was used as a model system and both acid and enzymatic hydrolysates prepared from cracklings were added and compared with BHT, with the protection of BHT against cholesterol oxidation shown to be less than that of enzymatic hydrolysate (Flaczyk, Rudzińska, Korczak, & Amarowicz, 2006a). The same phenomenon was also observed for the meatball system when stored under refrigerated conditions for 7 days and frozen conditions at −18 °C for 360 days (Flaczyk, Rudzińska, Wąsowicz, Korczak, & Amarowicz, 2006b; Rudzińska, Flaczyk, Amarowicz, Wąsowicz, & Korczak, 2007). The presence of some more functional components possessing antioxidant activity in hydrolysates may account for this effect.

3.3. Residual amounts of antioxidants in marinated eggs and pork as well as juice

Table 4 shows the residual amount of antioxidants in marinated eggs and pork as well as juice. The residual contents for 0.1% vitamin E in marinated egg juice and pork juice were 719 and 344 μg/g, respectively, whereas low levels of 26.5 and 150 μg/g were present in marinated eggs and pork. A similar result also occurred for 0.02% vitamin E. As explained before, the less effectiveness of vitamin E in inhibiting cholesterol oxidation in egg juice can be attributed to its poor solubility in the water phase. In contrast, vitamin E could be evenly distributed in the oil phase of marinated pork juice and, because of its orientation in the oil-in-water interface (Frankel et al., 1994), vitamin E could be more protective against cholesterol oxidation. For 0.1% vitamin C, the residual amounts were 335 and 266 μg/g in marinated eggs and pork, respectively. However, no vitamin C was detected in marinated egg juice, which may be due to complete degradation after prolonged heating for 24 h. Nonetheless, a residual level of 85.4 μg/g of vitamin C was shown in marinated pork juice, which may be caused by its leaching from ground pork into juice during heating. This phenomenon would be difficult to occur in marinated eggs because of presence in the solid form. Only slight amounts (4.1 and 3.1 μg/g) of vitamin C (0.02%) were detected in marinated eggs and pork, respectively, demonstrating that vitamin C is more susceptible to heat loss than are the other antioxidants.

3.4. Inhibition percentage of various antioxidants on the COPs formation in marinated eggs and pork as well as juice

Fig. 3 shows the inhibition percentage of various antioxidants on the COPs formation in marinated eggs and pork, as well as juice. High inhibitions of 67.5 and 59.4 percent, respectively, were shown for 0.1 and 0.02% vitamin C, whereas low retardations of 2.2 and 14.3 percent were found for marinated pork. As indicated above, vitamin C in ground pork should be more susceptible to heat loss than in eggs. In contrast, a distinct inhibition occurred for 0.02% vitamin E in marinated pork and juice, which amounted to 26.3 and 49.8%, respectively, and a moderate

Table 4
Residual amount (μg/g) of antioxidants in marinated eggs and pork, as well as juice

Sample	Antioxidant							
	Vitamin E		Vitamin C		BHA		Trolox	
	0.10%	0.02%	0.10%	0.02%	0.10%	0.02%	0.10%	0.02%
Marinated eggs	$26.5 \pm 0.6^{*,dCD}$	8.2 ± 0.01^{cD}	335 ± 15.8^{aB}	4.1 ± 0.2^{aD}	374 ± 7.6^{bB}	110 ± 0.8^{bC}	2345 ± 105^{aA}	352 ± 5.8^{aB}
Marinated egg juice	719 ± 20.0^{aB}	45.8 ± 3.5^{aD}	N.D.	N.D.	6.2 ± 0.2^{dE}	N.D.	828 ± 7.9^{cA}	138 ± 0.1^{bC}
Marinated pork	150 ± 10.2^{cD}	27.8 ± 1.5^{bE}	266 ± 6.7^{bC}	3.1 ± 0.2^{bE}	532 ± 13.9^{aB}	174 ± 0.1^{aCD}	1977 ± 119^{bA}	N.D.
Marinated pork juice	344 ± 12.4^{bB}	40.0 ± 3.2^{aE}	85.4 ± 1.3^{cD}	N.D.	192 ± 13.8^{cC}	N.D.	590 ± 3.79^{dA}	N.D.

N.D.: Not detected.
[a–d]Values within a column with different superscripts are significantly different ($p < 0.05$).
[A–E] Values within a row with different superscripts are significantly different ($p < 0.05$).
[*] Means of duplicate analyses ± standard deviation.

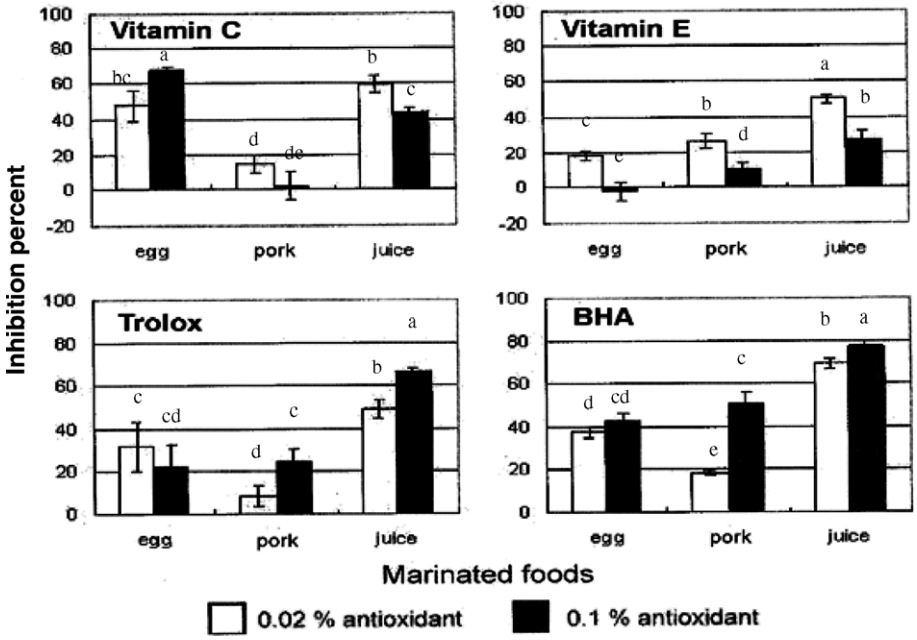

Fig. 3. Inhibition percent of antioxidants on the COPs formation in marinated eggs, pork and juice.

inhibition of 17.8% in marinated eggs. The greater solubility of vitamin E in ground pork and juice may account for this phenomenon. Nevertheless, 0.1% vitamin E showed a poor inhibition, implying a possible prooxidant activity (Dougherty, 1988; Terao, 1986). Like vitamin C, both BHA and trolox were more protective against cholesterol oxidation in marinated pork juice, with the inhibition being 68.5 and 77.6 percent for the former (0.02 and 0.1% BHA), and 48.8 and 66.0 percent (0.02 and 0.1% trolox) for the latter. Conversely, in marinated pork, low inhibitions of 18.5 and 8.7 percent were observed for 0.02% BHA and trolox, respectively, but with 0.1% BHA and trolox, the inhibitions increased by 31.8 and 15.8 percent. Interestingly, a reversed trend did occur for 0.1% trolox in marinated eggs, as evidenced by a 9.3 percent drop in inhibition.

In conclusion, additions of all antioxidants, trolox, BHA, vitamin C and vitamin E, were effective in retarding cholesterol oxidation, and the inhibition efficiency was dependent on the variety of food sample and the concentration of antioxidants. Among the various antioxidants, 0.1% vitamin C was the most effective in preventing COPs formation in marinated eggs, whereas 0.1% BHA showed profound inhibition of COPs in marinated pork and juice. The inhibition effect increased with increasing levels of BHA and trolox; however, both vitamin E and vitamin C showed a reversed trend.

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