

Antioxidant activity of hydroxytyrosol in frankfurters enriched with n-3 polyunsaturated fatty acids.

Susana Cofrades, Lorena Salcedo, Gonzalo Delgado-Pando, Ines Lopez-Lopez, Claudia Ruiz-Capillas, Francisco Jiménez-Colmenero*

Instituto de Ciencia y Tecnología de Alimentos y Nutrición- ICTAN (Formerly Instituto del Frío) (CSIC). Ciudad Universitaria. 28040-Madrid. Spain

* Corresponding author. Tel.: +34 91 549 23 00; fax: +34 91 549 36 27.

E-mail address: fjimenez@ictan.csic.es

Abstract

The capacity of hydroxytyrosol (HXT) to inhibit lipid oxidation in cooked pork meat batter, oil-in-water emulsions and potential functional frankfurters formulated with a healthier oil combination (as animal fat replacer) was studied during chilling storage, and its effect compared with those produced by synthetic antioxidants (BHA/BHT). Although efficiency varied, HXT was an effective antioxidant during chilling storage in the three food matrices studied. In general the order of inhibition capacity of HXT against lipid oxidation (thiobarbituric acid-reactive substances-TBARS) was cooked meat batter > oil-in-water emulsion > frankfurters, whereas in the case of BHA/BHT (with lower inhibitory activity than HXT) it was cooked meat batter > oil-in-water emulsion, and there was no antioxidative effect in frankfurters. Whereas significant correlations were established between lipid oxidation (TBARS) and antioxidative capacity measured by photochemiluminescence (PCL) in frankfurters supplemented with HXT and BHA/BHT, no significant correlations were found between ferric reducing/antioxidant power assay (FRAP) and TBARS and PCL.

Keywords: hydroxytyrosol, frankfurters, meat batters, oil-in-water emulsions, lipid oxidation, antioxidants

1. Introduction

Lipids are among the bioactive components (functional ingredients) that have received most attention with respect to the development of healthier meat products. Healthier lipid formulation based on processing strategies is one of the most important current approaches to the development of potential meat based functional foods (Jimenez-Colmenero, 2007). A number of studies have been conducted to improve the lipid profile of numerous meat products, among them frankfurters. Frankfurters are popular, frequently-consumed meat products of considerable economic importance and enjoy wide consumer acceptance in certain sectors of the population. Reformulation of frankfurters has been used to achieve better lipid compositions by reducing fat content and/or replacing the animal fat normally present in the product with another fat (of plant and/or marine origin) whose characteristics are more in line with health recommendations (WHO, 2003): i.e. contain smaller proportions of saturated fatty acids (SFA) and larger proportions of monounsaturated (MUFA) or polyunsaturated (PUFA) fatty acids, especially long chain n-3 PUFA (LC n-3 PUFA), better n-6/n-3 PUFA and PUFA/SFA ratios, and if possible cholesterol-free (Jiménez-Colmenero, 2007). In these studies the animal fat has generally been replaced by individual plant (olive, high-oleic-acid sunflower, cottonseed, soya, etc.) or marine (fish, algae) oils to improve the fatty acid profile of the meat products (Bloukas & Paneras, 1993; Jiménez-Colmenero, 2007; Muguerza, Ansorena & Astiasaran, 2004). A better approximation to optimal lipid profiles (more in line with health recommendations) can be achieved using healthier oil combinations as animal fat replacers (Delgado-Pando, Cofrades, Ruiz-Capillas & Jiménez-Colmenero, 2010a; Delgado-Pando, Cofrades, Ruiz-Capillas, Solas & Jiménez-Colmenero, 2010b), but there have been few studies on reformulation of meat products, including frankfurters, combining several types of lipid material (López-López, Cofrades

& Jiménez-Colmenero, 2009; Lopez-Lopez, Cofrades, Ruiz-Capillas & Jiménez-Colmenero, 2009; Paneras, Bloukas & Filis, 1998). In this context, recently our group (Delgado-Pando et al., 2010a) assessed the suitability of a healthier oil combination (added as oil-in-water emulsion) as a pork backfat replacer in low-fat frankfurters. The healthier oil combination used as an ingredient was formed by vegetable (olive and linseed) and fish oils in suitable amounts and proportions to produce a fatty acid profile better adjusted to healthier intake goals. These reformulated frankfurters with a low level of SFA and a high level of PUFA (including LC n-3 PUFA) are more in line with dietary recommendations (Delgado-Pando et al., 2010a). These products possess useful technological and sensory properties (Delgado-Pando et al., 2010a), although at the same time the increased in unsaturated levels could makes them more prone to lipid oxidation. One essential aspect associated with healthier lipid strategies is how the reformulation may influence the rate and extent of lipid oxidation. This type of deterioration can affect product quality characteristics, as well as having unhealthy implications, and therefore prevention of lipid oxidation is a prerequisite for successful development of foods enriched with lipids containing n-3 PUFA (Jacobsen, Let, Nielsen, & Meyer, 2008).

Synthetic and natural antioxidants have been used successfully to block or delay the oxidation process in meat (Cross, Leu, & Miller, 1987). Due to safety and toxicity problems with synthetic antioxidants, there is increasing interest in natural antioxidants (Ahn, Grun, & Fernando, 2002). Moreover, as well as increasing lipid stability, an antioxidant added to a food can act as an antioxidant in the body, thus reducing the risk of various diseases related to the production of free radicals (Bravo, 1998). Thus, different components with antioxidant activity, most of them of plant origin (fruit, vegetables, seeds, spices, etc.), have been used as functional ingredients in meat based

functional foods (Arihara, 2006; Jiménez-Colmenero, Carballo, & Cofrades, 2001; Jiménez-Colmenero, Reig, & Toldrá, 2006). The presence of phenolic compounds in olive oil as the biggest group of natural antioxidants has attracted much attention due to their known and wide-ranging biological activities as well as their health effects (Pereira-Caro et al., 2009). Particular attention has been focused on hydroxytyrosol (HXT, 3,4-dihydroxyphenylethanol), since several studies have reported that this compound has technological advantages (e.g. it enhances food lipid stability) and beneficial health properties (capacity to reduce the risk of coronary heart disease and atherosclerosis, anti-inflammatory, hypotensive and hypoglycaemic activities, inhibition of platelet aggregation, etc.) (De Leonardis, Aretini, Alfano, Macciola, & Ranalli, 2008; Fki, Allouche, & Sayadi, 2005; González-Santiago et al., 2006; Kountouri, Mylona, Kaliora, & Andrikopoulos, 2007; Medina, Lois, Alcantara, Lucas, & Morales, 2009; Pereira-Caro et al., 2009). As a powerful antioxidant with proven functional properties, HXT can be used as a potential functional food ingredient, specially in PUFA enriched muscle foods. The capacity of HXT to inhibit lipid oxidation has been demonstrated in foodstuffs rich in fish lipids (Medina et al., 2009; Pazos, Alonso, Sánchez, & Medina, 2008) and in pre-cooked beef and pork meat (DeJong & Lanari, 2009). As far as the authors are aware, there have been no studies on their use in healthier lipid meat products, where the composition changes introduced to obtain a meat based functional food (e.g. adding healthy unsaturated fatty acids) would alter the balance of oxidizable substrates, pro-oxidants and antioxidants, and this in turn would affect the rate and extent of oxidation (Jiménez-Colmenero, 2007).

The objective of this paper was to examine the capacity of hydroxytyrosol to inhibit lipid oxidation in low-fat frankfurters formulated with a healthier oil combination (as animal fat replacer) specially designed (with olive, linseed and fish

oils) with fatty acids in suitable amounts and proportions in order to achieve healthier intake goals (Delgado-Pando et al., 2010a; Delgado-Pando et al., 2010b). To that end, the effects of two concentrations of hydroxytyrosol were studied (50 and 100 ppm) beforehand in cooked pork meat batter and oil (healthier lipid combination)-in-water emulsions stabilized with soy protein/sodium caseinate/microbial transglutaminase. The antioxidant capacity of hydroxytyrosol in the different food matrices during chilled storage was compared with the combination of synthetic phenolic antioxidants (BHA/BHT).

2. Materials and methods

2.1. Materials for preparation of food matrices

Post-rigor pork meat (mixture of *M. biceps femoris*, *M. semimembranosus*, *M. semitendinosus*, *M. gracilis* and *M. adductor*) and fresh pork backfat were obtained from a local meat market. The meat was trimmed of visible fat and connective tissue. Meat and backfat were passed through a grinder with a 0.6 cm plate (Mainca, Granollers, Spain). Lots of approx. 500 g were vacuum packed, frozen and stored at -20 °C until used.

Ingredients used for preparation of oil-in-water emulsions included olive oil (Carbonell Virgen Extra, SOS Cuétara SA, Madrid, Spain), linseed oil (Natursoy S.L., Alimentos Ecológicos, Castellterçol, Spain) and fish oil (Omevital 18/12 TG Gold from Cognis GmbH, Illertissen, Germany. According to supplier information the latter contained 160 mg of eicosapentenoic acid (EPA)/g and 115 mg of docosahexaenoic acid (DHA)/g plus a combination of tocopherols as antioxidants. The materials used for oil-in-water emulsion stabilization were sodium caseinate containing 86.4% protein (Julio Criado S. A., Alcorcón, Spain), soy protein isolate containing 92.1% protein (Vicoprot,

Trades S.A., Barcelona, Spain) and microbial trans glutaminase (Activa WM, Ajinomoto Europe Sales GmbH, Hamburg, Germany). According to supplier information, the enzyme was in a mixture containing 1% transglutaminase and 99% maltodextrin, with a standard transglutaminase activity of approximately 100 units/g.

Others additives used in product formulation included sodium chloride (Panreac Química, S.A. Barcelona, Spain), sodium tripolyphosphate (Manuel Riesgo, S.A. Madrid, Spain), sodium nitrite (Fulka Chemie GmbH, Buchs, Germany), flavouring (Gewürzmüller, GmbH, Münichingen, Germany), BHA and BHT (Sigma-Aldrich, Inc St Louis MO, USA) and hydroxytyrosol (40 % on dry basis, in olive extract. Genosa I+ D S. A., Málaga. Spain).

2.2. Preparation of cooked meat batter

Meat and backfat packages were thawed (approx. 18 h at 2 ± 2 °C). Four different samples were prepared (71.8% of meat, 15.1% pork backfat, 9.7% water, and 2% NaCl) as reported by Bastida et al. (2009), with the target final protein level of 17 %. Three meat batters containing 50 mg/kg (MB-50HXT) and 100 mg/kg (MB-100HXT) of hydroxytyrosol or a combination of 100 mg/kg of BHA and 100 mg/kg BHT (MB-BHA/BHT) were prepared. Control meat samples (MB-C) with no added antioxidant were also prepared.

Portions of each meat batter (approx. 35 g) were placed in tubs (diam 2.7 cm, height 11.5 cm), hermetically sealed, centrifuged (2500 g x 15 min x 3 °C) and heated to an internal temperature of 70 °C in a controlled water bath. The heat processing conditions were defined beforehand; in the definition process the internal temperature was monitored throughout heating by means of thermocouples inserted in the tub (thermal centre) and connected to a temperature recorder (Yokogawa Hokushin Electric YEW,

Mod. 3087, Tokyo, Japan). After heating, containers were cooled immediately (3 °C). The samples were stored at 2 ± 2 °C for 20 days

2.3. Preparation of oil-in-water emulsion

The oil-in-water emulsions (OWE) were prepared using a combination of soy protein, MTG and sodium caseinate to stabilize a healthier oil combination consisting of olive, linseed and fish oils in respective proportions of 44.39, 37.87 and 17.74 % . This oil combination was designed to produce a healthier lipid formulation with a small proportion of SFA, large proportions of MUFA and PUFA (including LC n-3 PUFA) and balanced n-6/n-3 PUFA and PUFA/SFA ratios as reported by Delgado-Pando et al. (2010a). Three oil-in-water emulsions containing 50 mg/kg (OWE-50HXT) and 100 mg/kg (OWE-100HXT) of hydroxytyrosol or a combination of 100 mg/kg of BHA and 100 mg/kg BHT (OWE-BHA/BHT) were prepared following the procedure reported by Delgado-Pando et al. (2010a). A control oil-in-water emulsion (OWE-C) with no added antioxidant was also prepared in a similar way. The oil-in-water emulsions were stored at 2 ± 2 °C.

2.4 Design and preparation of frankfurters

Five different frankfurters were made (Table 1): a control frankfurter (F-C) (all pork fat) and four lipid reformulated frankfurters (RF) prepared by totally replacing pork backfat with the oil-in-water emulsion described above. These frankfurters had been designed to produce a healthier fatty acid profile than that of the pork fat: with less SFA, similar MUFA levels and higher n-3 PUFA levels (including long chain n-3 PUFA). The fat level in these frankfurters is lower than normally found in such meat products. Higher levels of pork fat reduction were not considered as these would have

reduced the amount of healthy oil combination that could be incorporated in place of the pork fat (Delgado-Pando et al., 2010b). Since the antioxidant efficiency was greater with 100 mg/kg of HXT in both meat batters and oil-in-water emulsion (R & D), this concentration was used to check its behaviour in frankfurters. Lipid modified frankfurters containing 100 mg/kg of hydroxytyrosol (RF-HXT) or a combination of 100 mg/kg of BHA and 100 mg/kg BHT (RF-BHA/BHT) were prepared. Additionally a lipid reformulated frankfurter without HXT or BHA/BHT (RF-C) was formulated. In order to assess the antioxidant activity of nitrite, a modified frankfurter (RF-WN) without sodium nitrite (or any other antioxidant) added was also prepared.

Meat and backfat packages were thawed (approx. 18 h at 2 ± 2 °C) prior to use. Preparation of the frankfurters was as follows. Raw meat material was homogenized and ground for 1 min in a chilled cutter (2 °C) (Stephan Universal Machine UM5, Stephan u. Söhne GmbH and Co., Hameln, Germany). Half of the pork backfat or oil-in-water emulsion, NaCl, sodium tripolyphosphate and sodium nitrite (and where applicable the HXT or BHA/BHT) were added to the ground meat and mixed again for 1 min. The rest of the additives, the pork backfat and the oil-in-water emulsion were added and the whole homogenized for 1 min. Finally the whole meat batter was homogenized under vacuum for 2 min. Mixing time was standardized at 5 min. The final batter temperature was below 14 °C in all cases.

The meat batter was stuffed into 20 mm diameter Nojax cellulose casings (Viscase S.A., Bagnold Cedex, France) and hand-linked. Frankfurters were heat processed in an Eller smokehouse (model Unimatic 1000, Micro 40, Eller, Merano, Italy) until the core of the product reached 70 °C. Heat processing conditions were established beforehand, and the internal temperature was monitored throughout heating by means of thermocouples inserted in each frankfurter (thermal centre) and connected to a temperature recorder

(Yokogawa Hokuskin Electric YEM, Mod. 3087, Tokyo, Japan). Once heating was complete, the frankfurters were cooled (at room temperature), kept in a cold room (2 °C for 14 h), packed and stored at 2 °C (\pm 1 °C) and analysed periodically. Frankfurters were prepared and analysed twice.

2.5. Proximate analysis

Moisture and ash contents of the meat batters and frankfurters were determined (AOAC, 2002) in triplicate. Fat content was evaluated (in triplicate) according to Bligh and Dyer, (1959) . Protein content was measured in quadruplicate by a LECO FP-2000 Nitrogen Determinator (Leco Corporation, St Joseph, MI).

2.6. Thiobarbituric acid-reactive substances (TBARS)

Lipid oxidation, evaluated by changes in TBARS as described by Serrano, Cofrades, and Jiménez-Colmenero (2006), was determined in cooked meat batters, oil-in-water emulsion and frankfurters. Briefly, the procedure was as follows: 5 g of each sample was homogenized in 35 ml of 7.5 % trichloroacetic acid (Panreac Química, S.A. Barcelona, Spain) for 1 min at high speed in an Omnimixer blender (ES Homogenizer, OMNI International Inc., Gainesville, VA, USA). The blender sample was centrifuged (3000 g, 2 min) and 5 ml of the supernatant was mixed with 5 ml of 20 mM thiobarbituric acid; finally the solution was mixed and kept in the dark for 20 h at 20 ± 1.5 °C. The pink colour that formed was measured spectrophotometrically (Lambda 15UV/VIS spectrophotometer, Perkin-Elmer, USA) at 532 nm. A calibration curve was plotted with 1,1,3,3-tetraethoxypropane (Sigma Chemical Co., St. Louis, MO, USA) to obtain the malonaldehyde (MDA) concentration and results were expressed as mg

malonaldehyde/kg of sample. TBARS determinations for each sample were performed by triplicate.

TBARS values for each sample at a given time were used to calculate the inhibition of lipid oxidation as a percentage of control sample, which: inhibition capacity (%) = $100 \times (\text{control-treatment})/\text{control}$ (Tang, Kerry, Sheehan, Buckley, & Morrissey, 2001).

2.7. Ferric reducing/antioxidant power assay (FRAP)

The antioxidant capacity of frankfurters was evaluated in the extracts obtained following the procedure of Sayago-Ayerdi, Brenes, and Goni (2009) with slight modifications. Briefly, 5 g of sample was homogenized for 30 s (Omnimixer blender) with 20 ml HCl 16 mM in methanol/water (50:50 v/v) and shaken in an orbital shaker for 60 min. It was centrifuged at 4500 g for 10 min and the supernatant was separated. A new extraction was performed on the precipitate with 20 ml of acetone/water (70:30 v/v) and the sample was shaken in an orbital shaker for 60 min. Sample was centrifuged again at 4500 g/10 min. The two supernatants were combined and topped up to 50 ml with a 1:1 mixture of the two types of solution (acetone/water and methanol/water).

The FRAP assay (in triplicate) was performed on the extract to assess the reducing power of the sample following the method described by Pulido, Bravo and Saura-Calixto (2000). Briefly, 30 μ l of test extract was mixed with 90 μ l of distilled water and 900 μ l of FRAP reagent [containing 10 mM TPTZ (2,4,6-Tri(2-pyridil)-s-triazine, Sigma-Aldrich Co, St Louis, MO, USA)] in 40 mM HCl, 20 mM $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ and 0.3 M acetate buffer, pH 3.6), prepared daily and kept at 37 °C. Absorbance readings were taken using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan) equipped with a thermostatted auto-cell holder set at 37 °C. Reduction of

Fe^{3+} and the subsequent formation of a coloured TPTZ- Fe^{2+} complex with an absorbance maximum at 595 nm were monitored for 30 min. Solutions with known concentrations of Fe(II) in the range 100-1000 μM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were used to obtain a standard curve and the results were expressed as μmol of μmol Fe^{2+} eq per gram of frankfurter.

2.8 Antioxidative activity by photochemiluminescence (PCL)

Antioxidant activity was determined for the frankfurters in triplicate using an automated photochemiluminescent system (Photochem©, Analytik Jena Model AG; Analytic Jena USA, The Woodlands, TX, USA), which measures the capacity to quench free radicals (Popov & Lewin, 1996). This method is based on controlled photochemical generation of radicals, part of which are quenched by the antioxidant and the remaining radicals are quantified by a sensitive chemiluminescence-detection reaction. Briefly, 1 g of sample was homogenized for 30 sec in an Omnimixer with 50 ml of methanol. After mixing for 30 min, sample was filtered through Whatman No. 1 paper. 20 μL of filtrate was added to reagent kits supplied by the manufacturer and the automated PCL system measured the total antioxidant capacity. Trolox (Sigma-Aldrich, Inc St Louis MO, USA) was used as a standard, and results were expressed in Trolox equivalents (mmol TE/g sample)

2.9. Statistical analysis

The repeated measures test was used for statistical comparisons among samples. Data were analysed using SPSS Statistics 17.0 (SPSS Inc, Chicago, USA) for one-way and two-way ANOVA. Least squares differences were used for comparison of mean values among treatments and Tukey's HSD test to identify significant differences

($P < 0.05$) between formulations and storage times. Correlations were established using Microsoft Excel for Windows XP (2002; Microsoft Corp., Redmond, WA, USA).

3. RESULTS AND DISCUSSION

In order to gain a clearer understanding of the antioxidative effect of HXT and achieve a more accurate assessment of the suitability of this reformulation strategy for potential functional frankfurters formulated with healthier lipid combination, the experiment was carried out both on raw materials (ingredients) ~~susceptible to lipid oxidation~~, and on the final product. Thus, the role of HXT is described in the cooked meat matrix, in healthier lipid combination oil-in-water emulsions and finally in frankfurters.

3.1. Meat batter

TBARS values were affected by meat batter formulation and storage time (Table 2), with significant interaction between both variables. Initially and over the chilling storage period, TBARS values were higher ($P < 0.05$) in MB-C sample than in samples containing antioxidants (Table 2). The high susceptibility of MB-C sample to lipid oxidation correlated closely with the meat system composition and processing. Meat batters containing relatively high fat percentages (14.7%), added NaCl (2%), with a major reduction in particle size (comminuted meat systems), without added antioxidants, and subjected to thermal treatment (cooking at 70 °C/30 min) are prone to lipid oxidation (Bastida et al., 2009). This can therefore be considered a very suitable matrix to assess the antioxidant activity of HXT.

The addition of antioxidants reduced the rate and extent of lipid oxidation in the meat batter, but the effect varied depending on the type and concentration of the

antioxidant. The initial TBARS values of the meat batters containing BHA/BHT and 50 ppm of HXT were three times lower ($P<0.05$) than those of MB-C, while in the presence of 100 ppm of HXT oxidation levels were 15 times lower ($P<0.05$) (Table 2). Variations in initial oxidation levels in meat batters can be explained as a consequence of the differences in antioxidant activity in the meat matrix during thermal processing. Heating reduces the activation energy and accelerates lipid oxidation in meat by disrupting cellular organization and protein denaturation, resulting in loss of antioxidant enzyme activity and release of protein-bound iron (Decker & Xu, 1998). Heating during meat system preparation as in the present study (70 °C) is within the optimum temperature range for release of non-haeme iron (Cross et al., 1987) favouring its pro-oxidative activity and inducing lipid oxidation.

TBARS values increased ($P<0.05$) during storage in all samples, except in those containing BHA/BHT (Table 2). Throughout the study, the greatest antioxidant efficiency was achieved by adding 100 mg/kg de HXT. 50 mg/kg of HXT and the mixture of BHA/BHT produced similar effects in thermal treatment, but during chilling storage BHA/BHA displayed more antioxidant activity, to the extent that at the end of storage time the level of activity was similar to that of 100 mg/kg de HXT (Table 2). Pazos et al. (2008) reported that HXT had an antioxidant capacity similar to that of synthetic propyl gallate in frozen fish muscle. Present results agree with those reported describing hydroxytyrosol as an effective antioxidant during chilling storage of pre-cooked beef (DeJong & Lanari, 2009).

3.2. Oil-in-water emulsion

Oil-in-water emulsions constitute a group of a complex, heterophasic food system, specially susceptible to lipid oxidation (Jacobsen et al., 2008). The oil (healthier

lipid combination of olive, linseed and fish oils)-in-water emulsions evaluated in this study had been used as pork backfat replacers in low-fat frankfurters (Delgado-Pando et al., 2010a). As a lipidic ingredient in a meat product, it is helpful to determine the antioxidant activity of HXT in those systems in order to gain a better understanding of their behaviour in finely comminuted cooked (gel/emulsion) meat products such as frankfurters. Lipid oxidation (TBARS) was affected by oil-in-water emulsion type and storage time (Table 3), with significant interaction between the two variables. Lipid oxidation increased ($P < 0.05$) during storage, although initial TBARS values were similar ($P > 0.05$) in all samples. Control emulsion (OWE-C) presented the highest lipid oxidation at the end of storage. After 6 days of storage, BHA/BHT displayed the greatest antioxidant activity; however, at 20 days lipid oxidation was most inhibited in the sample containing 100 ppm of HXT. Pazos et al. (2008) reported that higher inhibitory properties were achieved in fish oil-in-water emulsions by increasing the HXT concentration from 10 to 100 ppm. These authors also reported that HXT had similar antioxidant capacity to that of synthetic propyl gallate.

3.3. Frankfurters

Proximate composition was similar in all frankfurters, with values around 60.9%, 18.5%, 11.3% and 3.4% for moisture, protein, fat and ash contents respectively. This results, similar to those reported by Delgado-Pando et al. (2010a) using the same frankfurter formulation, are consistent with the fact that the differences among samples are only associated with changes in nitrite content and supplementation with HXT or BHA/BHT, all of them by amounts that were too small (Table 1) to modify proximate composition.

3.3.1. Lipid oxidation (TBARS)

TBARS values were affected by frankfurter formulation and storage time (Table 4), with significant interaction between the two variables. Frankfurters made with all pork fat (F-C) had the lowest TBARS values, which remained constant throughout storage. The reformulated frankfurters had higher ($P < 0.05$) TBARS values throughout storage, indicating that lipid oxidation was more extensive in reformulated frankfurters (with higher levels of unsaturated fatty acids). These results are consistent with the fact that in the experimental conditions frankfurters made with oil combinations had lower levels of saturated fatty acids (SFA) (19.3 %), similar levels of MUFA (46.9 %) and higher levels of PUFA (33.6 %) than control frankfurters (all pork fat) (39.3, 49.5 and 10.6 % respectively) (Delgado-Pando et al., 2010a).

As noted earlier, pork backfat replacement by the new oil combination made frankfurters prone to lipid oxidation, but the scale of the effect varied (Table 4) depending on the antioxidant used. Differences in lipid oxidation patterns between RF-C and RF-WN frankfurters may be attributed to the effect of the nitrite (Table 1). As compared with RF-C, the frankfurter formulated without nitrite (RF-WN) presented the highest TBARS values in the course of storage (Table 4), indicating that nitrite has a powerful antioxidant effect reducing the extent of lipid oxidation. The antioxidative activity of nitrite in comminuted meat products has been extensively reported (Igene, Yamauchi, Pearson, Gray, & Aust, 1985; Morrissey & Tichivangana, 1985).

Differences in oxidative stability between RF-C and RF-BHA/BHT and RF-HXT frankfurters in the course of the study (Table 4) may be attributed to the difference in the activities of BHA/BHT and HXT respectively. As compared with RF-C, no antioxidant activity was observed in frankfurter supplemented with synthetic BHA/BHT, but those supplemented with HXT registered a considerable inhibition

capacity (ranging between 20-40%) of lipid oxidation. Some authors classify ranges between 20-50 % of inhibition capacity as constituting an intermediate antioxidant effect (Jacobsen et al., 2008). Antioxidative activity of HXT has been compared with other commonly used food antioxidants (Pazos et al., 2008; Pereira-Caro et al., 2009), but we found no data in the literature about the use of HXT in comminuted gel/emulsion meat systems.

The results of this study show that the effect of BHA/BHT on lipid oxidation varied with the matrix (Tables 2-4). BHA/BHT can be effective in enhancing the oxidative stability of cooked meat batters and oil-in-water emulsions, but such effect has been not observed in frankfurters, where oxidation levels were even higher than in the control product (RF-C), with similar formulation but without synthetic compounds added). On the other hand, HXT demonstrated antioxidative capacity in the three matrices studied, although the efficiency varied. In general, the order of HXT's capacity to inhibit lipid oxidation was cooked meat batter > oil-in-water emulsion > frankfurters, whereas in the case of BHA/BHT the order was cooked meat batter > oil-in-water emulsion (it had no antioxidative effect in frankfurters). Antioxidant efficacy in heterophasic systems like the ones evaluated in this study depends on many factors, but particularly on the chemical properties of the antioxidant (antioxidant partitioning, electron/hydrogen-donating ability, capacity to regenerate other antioxidants, etc.) and on the nature of the matrix (physical structure, composition: phospholipids, PUFA and/or free iron contents, hydrophobic/hydrophilic balance, presence of antioxidants such as nitrite, etc.) which affect the mechanisms of oxidation and antioxidant activity. Because of this variability, the effectiveness of antioxidants varies in different types of emulsions and the same antioxidant exerts different effects in different systems

(Jacobsen et al., 2008). Taking all the matrixes studied here, HXT showed greater inhibitory capacity than BHA/BHT.

3.3.2. Antioxidant capacity by FRAP assay

The antioxidant capacity of samples, determined as their reducing power by the FRAP assay, is reported in Table 5. This parameter was affected by frankfurter formulation and storage time, with significant interaction between the two variables. Although heating produces a decrease of reducing agents, heat-stable ferric ion-reducing compounds are present in cooked meat (Min, Nam, Cordray, & Ahn, 2008). Except in RF-WN frankfurter, the initial antioxidant activity was lower ($P<0.05$) at day 7 of storage, while there were no appreciable changes during the remainder of the storage period (Table 5). The effects of the type of lipidic material contained (F-C versus RF-C) and the presence of nitrite (RF-C versus RF-WN) on FRAP values were not evident (Table 5), but it was observed in the course of storage that supplementation with HXT and BHA/BHT increased ($P<0.05$) antioxidant activity to similar extents. These results are consistent with a report on purified sunflower oil where HXT and BHT had similar ferric reducing powers (Pereira-Caro et al., 2009).

3.3.3. Antioxidative activity by photochemiluminescence

The PCL inhibition assay measured the superoxide scavenging capacity of the meat matrix. PCL was affected by frankfurter formulation and storage time (Table 6), with significant interaction between the two variables. In general the antioxidative capacity (antiradical efficiency) in frankfurters decreased ($P<0.05$) during storage, but there were two clearly observable patterns of behaviour (Table 6). From the outset of storage, the PCL of the sample supplemented with HXT (RF-HXT) was significantly

higher (around 5 times) than in all the other samples, where the range of values was very similar. The decrease of PCL over time entails a reduction of the system's ability to scavenge radicals as lipid oxidation progresses, and it is therefore hard to account for the absence of any effect on PCL associated with the changes in the lipid profile and the presence of BHA/BHT and nitrite in the frankfurters. It is important to note that the increase in unsaturated fatty acids caused a rise in lipid oxidation levels (Table 4), and radical production plays an important role in the mechanics of this. Also, considering the strong electron donating effect and reactivity of phenolic antioxidants (BHA/BHT) with lipid radicals (Gordon, 1990), their presence might plausibly be expected to have a greater effect on the antiradical efficiency of the meat matrix. One would also expect to see an effect associated with the antioxidative power of nitrite, based mainly on the stabilization of the muscle pigment myoglobin (preventing release of Fe^{2+} during cooking process), which cannot act as a catalyst of lipid peroxidation (Igene et al., 1985; Morrissey & Tichivangana, 1985).

The PCL inhibition assay has been used with various different vegetable products (Balogh, Hegedus, & Stefanovits-Banyai, 2010; NetZel, NetZel, Tian, Schwartz, & Konczak, 2007; Wall, 2010), but hitherto there have been no reports on its use with meat products. Again, chemiluminescence assay has been used only on scant occasions to study lipid oxidation in muscle foods, and then sometimes with conflicting results. Harms, Fuhrmann, Nowak, Wenzel, & Sallmann (2003) reported that reinforced radical production in cured pork sausage resulted in high chemiluminescence, while the opposite effect was reported by Olsen et al. (2005). In fact these authors reported that their chemiluminescence method was not directly applicable to measurement of lipid oxidation in pork backfat or mechanically recovered poultry meat. Studies conducted to evaluate the activity of antioxidants in liver microsomal membranes showed reductions

of chemiluminescence by BHA and BHT (Kahl, Weimann, Weinke, & Hildebrandt, 1987). Our results were in agreement with other authors (Fki et al., 2005; Pereira-Caro et al., 2009), who reported a higher free radical scavenging capacity (DPPH) with HXT than with BHA/BHT. Hydroxytyrosol is an excellent free radical scavenger through H atom donation (Leopoldini, Russo, & Toscano, 2011).

3.3.4. Correlation between lipid oxidation (TBARS) and protective capacity against oxidation (FRAP and PCL)

TBARS, FRAP and PCL are methods that furnish information on the state of the system and the progress of lipid oxidation in frankfurters, and the authors believe it is useful to determine the level of correlation among them. No significant correlations were found (TBARS/FRAP, TBARS/PCL, FRAP/PCL) when all experimental data (irrespective of formulations and storage time) were used. However, when those correlations were established for each of the frankfurter formulations (Table 1), they were significant for TBARS/PCL in the case of samples RF-C (-0.560, $P < 0.05$), RF-BHA/BHT (-0.829, $P < 0.0002$) and RF-HXT (-0.929, $P < 0.0001$). This indicates that there is an inverse relationship between the progress of lipid oxidation and the radical quenching capacity of the system. No significant correlations were found between FRAP and the other two parameters (TBARS and PCL). There are several possible explanations for the differences in correlations among the parameters evaluated on the basis of frankfurter formulations. The three methods are based on the measurement of different properties of the system, which vary according to the resistance to and progress of oxidation in the product; while TBARS (as a measure of secondary lipid oxidation) is a more direct measurement of oxidation, FRAP and PCL, each in a different way, measure the system's antioxidant capacity. Noll et al. (1987) showed that

during microsomal lipid peroxidation, there is a significant delay in the appearance of changes in chemiluminescence with respect to TBARS values. They attributed this to the fact that MDA formation and the generation of electronically excited species proceed via different pathways. Another factor to be borne in mind is that in some cases there is little difference in antioxidant activity and lipid oxidation with respect to different product formulations and/or storage times (Tables 4-6). Olsen et al. (2005) reported that no relationship was found between chemiluminescence measurement and other methods of measuring either primary or secondary lipid oxidation products. FRAP assay showed a significant inverse correlation with TBARS in fresh meat (Descalzo et al., 2008) and fish muscle (Medina, Gallardo, Gonzalez, Lois, & Hedges, 2007), although these authors did not consider this kind of relationship when there was little difference in the antioxidant activity and lipid oxidation. Unlike the products analysed in this paper, those other studies were conducted on raw biological systems, but nonetheless heat-stable ferric ion reducing compounds have been reported to be primarily responsible for the regeneration of ferrous ion increasing TBARS in cooked meat during storage (Min et al., 2008).

In conclusion, HXT demonstrated antioxidative capacity, although with varying degrees of efficiency, in cooked meat batter, oil-in-water emulsion and frankfurters (formulated with healthier oil combination) and showed greater inhibitory capacity than BHA/BHT. Factors associated with the complexity of the matrix, the nature of the compounds evaluated or the type of substrate to be oxidized, among others, limit the possibilities of establishing correlations among parameters of lipid oxidation (levels reached) and antioxidant capacity (protective capacity against oxidation) of the gel/emulsion matrix. Consequently, the measurement of antioxidant potential was not directly applicable to lipid oxidation in frankfurters. In order to develop healthier lipid

meat based functional foods, it is essential to gain a better understanding of lipid oxidation and antioxidative strategies and to evaluate them quantitatively.

Acknowledgment

This research was supported by the projects AGL2008-04892-CO3-01 and Consolider Ingenio 2010: CARNISENUSA (CSD2007-00016). The authors would like to thank Genosa I+D for supplying the hydroxytyrosol.

References

- Ahn, J., Grun, I. U., & Fernando, L. N. (2002). Antioxidant properties of natural plant extracts containing polyphenolic compounds in cooked ground beef. *Journal of Food Science*, 67(4), 1364-1369.
- AOAC (2002). Official methods of analysis (17th ed). Maryland, USA: Association of Official Analytical Chemistry.
- Arihara, K. (2006). Strategies for designing novel functional meat products. *Meat Science*, 74(1), 219-229.
- Balogh, E., Hegedus, A., & Stefanovits-Banyai, E. (2010). Application of and correlation among antioxidant and antiradical assays for characterizing antioxidant capacity of berries. *Scientia Horticulturae*, 125(3), 332-336.
- Bastida, S., Sanchez-Muniz, F. J., Olivero, R., Perez-Olleros, L., Ruiz-Roso, B., & Jimenez-Colmenero, F. (2009). Antioxidant activity of Carob fruit extracts in cooked pork meat systems during chilled and frozen storage. *Food Chemistry*, 116(3), 748-754.

- Bloukas, J. G., & Paneras, E. D. (1993). Substituting olive oil for pork backfat affects quality of low-fat frankfurters. *Journal of Food Science*, 58(4), 705-709.
- Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews*, 56(11), 317-333.
- Cross, H. R., Leu, R., & Miller, M. F. (1987). Scope of warmed-over-flavor and its importance to the meat industry. In: A. J. S. Angelo, & M. E. Bailey, *Warmer-over-flavor of meat* (pp. 1-18). Orlando: Academic Press.
- De Leonardis, A., Aretini, A., Alfano, G., Macciola, V., & Ranalli, G. (2008). Isolation of a hydroxytyrosol-rich extract from olive leaves (*Olea Europaea* L.) and evaluation of its antioxidant properties and bioactivity. *European Food Research and Technology*, 226(4), 653-659.
- Decker, E. A., & Xu, Z. M. (1998). Minimizing rancidity in muscle foods. *Food Technology*, 52(10), 54-59.
- DeJong, S., & Lanari, M. C. (2009). Extracts of olive polyphenols improve lipid stability in cooked beef and pork: Contribution of individual phenolics to the antioxidant activity of the extract. *Food Chemistry*, 116(4), 892-897.
- Delgado-Pando, G., Cofrades, S., Ruiz-Capillas, C., & Jimenez-Colmenero, F. (2010a). Healthier lipid combination as functional ingredient influencing sensory and technological properties of low-fat frankfurters. *European Journal of Lipid Science and Technology*, 112(8), 859-870.
- Delgado-Pando, G., Cofrades, S., Ruiz-Capillas, C., Solas, M. T., & Jimenez-Colmenero, F. (2010b). Healthier lipid combination oil-in-water emulsions prepared with various protein systems: an approach for development of functional meat products. *European Journal of Lipid Science and Technology*, 112(7), 791-801.

- Descalzo, A. M., Rossetti, L., Sancho, A. M., Garcia, P. T., Biolatto, A., Carduza, F., & Grigioni, G. M. (2008). Antioxidant consumption and development of oxidation during ageing of buffalo meat produced in Argentina. *Meat Science*, 79(3), 582-588.
- Fki, I., Allouche, N., & Sayadi, S. (2005). The use of polyphenolic extract, purified hydroxytyrosol and 3,4-dihydroxyphenyl acetic acid from olive mill wastewater for the stabilization of refined oils: a potential alternative to synthetic antioxidants. *Food Chemistry*, 93(2), 197-204.
- Gonzalez-Santiago, M., Martin-Bautista, E., Carrero, J. J., Fonolla, J., Baro, L., Bartolome, M. V., Gil-Loyzaga, P., & Lopez-Huertas, E. (2006). One-month administration of hydroxytyrosol, a phenolic antioxidant present in olive oil, to hyperlipemic rabbits improves blood lipid profile, antioxidant status and reduces atherosclerosis development. *Atherosclerosis*, 188(1), 35-42.
- Gordon, M.H. (1990). The mechanism of antioxidant action in vitro. In B. J. F. Hudson (Ed). *Food antioxidants*. (pp. 1-18). London: Elsevier Applied Science.
- Harms, C., Fuhrmann, H., Nowak, B., Wenzel, S., & Sallmann, H. P. (2003). Effect of dietary vitamin E supplementation on the shelf life of cured pork sausage. *Meat Science*, 63(1), 101-105.
- Igene, J. O., Yamauchi, K., Pearson, A. M., Gray, J. I., & Aust, S. D. (1985). Mechanisms by which nitrite inhibits the development of warmed-over flavor (WOF) in cured meat. *Food Chemistry*, 18(1), 1-18.
- Jacobsen, C., Let, M. B., Nielsen, N. S., & Meyer, A. S. (2008). Antioxidant strategies for preventing oxidative flavour deterioration of foods enriched with n-3 polyunsaturated lipids: a comparative evaluation. *Trends in Food Science & Technology*, 19(2), 76-93.

- Jimenez-Colmenero, F. (2007). Healthier lipid formulation approaches in meat-based functional foods. Technological options for replacement of meat fats by non-meat fats. *Trends in Food Science & Technology*, *18*, 567-578.
- Jimenez-Colmenero, F., Carballo, J., & Cofrades, S. (2001). Healthier meat and meat products: their role as functional foods. *Meat Science*, *59*(1), 5-13.
- Jiménez-Colmenero, F., Reig, M., & Toldrá, F. (2006). New approaches for the development of functional meat products. In: F. Mollet, & F. Toldrá, *Advanced technologies for meat processing* (pp. 275-308). Boca Raton, London, New York: Taylor & Francis Group.
- Kahl, R., Weimann, A., Weinke, S., & Hildebrandt, A. G. (1987). Detection of oxygen activation and determination of the activity of antioxidants towards reactive oxygen species by use of the chemiluminogenic probes luminol and lucigenin. *Archives of Toxicology*, *60*(1-3), 158-162.
- Kountouri, A. M., Mylona, A., Kaliora, A. C., & Andrikopoulos, N. K. (2007). Bioavailability of the phenolic compounds of the fruits (drupes) of *Olea europaea* (olives): Impact on plasma antioxidant status in humans. *Phytomedicine*, *14*(10), 659-667.
- Leopoldini, M., Russo, N., & Toscano, M. (2011). The molecular basis of working mechanism of natural polyphenolic antioxidants. *Food Chemistry*, *125*(2), 288-306.
- Lopez-Lopez, I., Cofrades, S., & Jimenez-Colmenero, F. (2009). Low-fat frankfurters enriched with n-3 PUFA and edible seaweed: Effects of olive oil and chilled storage on physicochemical, sensory and microbial characteristics. *Meat Science*, *83*(1), 148-154.

- Lopez-Lopez, I., Cofrades, S., Ruiz-Capillas, C., & Jimenez-Colmenero, F. (2009). Design and nutritional properties of potential functional frankfurters based on lipid formulation, added seaweed and low salt content. *Meat Science*, 83(2), 255-262.
- Medina, I., Gallardo, J. M., Gonzalez, M. J., Lois, S., & Hedges, N. (2007). Effect of molecular structure of phenolic families as hydroxycinnamic acids and catechins on their antioxidant effectiveness in minced fish muscle. *Journal of Agricultural and Food Chemistry*, 55(10), 3889-3895.
- Medina, I., Lois, S., Alcantara, D., Lucas, R., & Morales, J. C. (2009). Effect of lipophilization of hydroxytyrosol on its antioxidant activity in fish oils and fish oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 57(20), 9773-9779.
- Min, B., Nam, K. C., Cordray, J., & Ahn, D. U. (2008). Endogenous factors affecting oxidative stability of beef loin, pork loin, and chicken breast and thigh meats. *Journal of Food Science*, 73(6), C439-C446.
- Morrissey, P. A., & Tichivangana, J. Z. (1985). The antioxidant activities of nitrite and nitrosylmyoglobin in cooked meats. *Meat Science*, 14(3), 175-190.
- Muguerza, E., Ansorena, D., & Astiasaran, I. (2004). Functional dry fermented sausages manufactured with high levels of n-3 fatty acids: nutritional benefits and evaluation of oxidation. *Journal of the Science of Food and Agriculture*, 84(9), 1061-1068.
- NetZel, M., NetZel, G., Tian, Q. G., Schwartz, S., & Konczak, I. (2007). Native Australian fruits - a novel source of antioxidants for food. *Innovative Food Science and Emerging Technologies*, 8(3), 339-346.
- Noll, T., De Groot, H., & Sies, H. (1987). Distinct temporal relation among oxygen uptake, malondialdehyde formation, and low-level chemiluminescence during

- microsomal lipid peroxidation. *Archives of Biochemistry and Biophysics*, 252(1), 284-291.
- Olsen, E., Vogt, G., Ekeberg, D., Sandbakk, M., Pettersen, J., & Nilsson, A. (2005). Analysis of the early stages of lipid oxidation in freeze-stored pork back fat and mechanically recovered poultry meat. *Journal of Agricultural and Food Chemistry*, 53(2), 338-348.
- Paneras, E. D., Bloukas, J. G., & Filis, D. G. (1998). Production of low-fat frankfurters with vegetable oils following the dietary guidelines for fatty acids. *Journal of Muscle Foods*, 9(2), 111-126.
- Pazos, M., Alonso, A., Sanchez, I., & Medina, I. (2008). Hydroxytyrosol prevents oxidative deterioration in foodstuffs rich in fish lipids. *Journal of Agricultural and Food Chemistry*, 56(9), 3334-3340.
- Pereira-Caro, G., Madrona, A., Bravo, L., Espartero, J. L., Alcudia, F., Cert, A., & Mateos, R. (2009). Antioxidant activity evaluation of alkyl hydroxytyrosyl ethers, a new class of hydroxytyrosol derivatives. *Food Chemistry*, 115(1), 86-91.
- Popov, I. N., & Lewin, G. (1996). Photochemiluminescent detection of antiradical activity; IV: testing of lipid-soluble antioxidants. *Journal of Biochemical and Biophysical Methods*, 31(1-2), 1-8.
- Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry*, 48(8), 3396-3402.
- Sayago-Ayerdi, S. G., Brenes, A., & Goni, I. (2009). Effect of grape antioxidant dietary fiber on the lipid oxidation of raw and cooked chicken hamburgers. *LWT-Food Science and Technology*, 42(5), 971-976.

- Serrano, A., Cofrades, S., & Jimenez-Colmenero, F. (2006). Characteristics of restructured beef steak with different proportions of walnut during frozen storage. *Meat Science*, 72(1), 108-115.
- Tang, S., Kerry, J. P., Sheehan, D., Buckley, D. J., & Morrissey, P. A. (2001). Antioxidative effect of added tea catechins on susceptibility of cooked red meat, poultry and fish patties to lipid oxidation. *Food Research International*, 34(8), 651-657.
- Wall, M. M. (2010). Functional lipid characteristics, oxidative stability, and antioxidant activity of macadamia nut (*Macadamia integrifolia*) cultivars. *Food Chemistry*, 121(4), 1103-1108.
- WHO (2003). Diet, Nutrition and the prevention of chronic diseases. *WHO Technical report series* 916. Geneva (Switzerland).

Table 1. Components (g) in frankfurter formulation.

	Meat	Pork backfat	Oil-in-water emulsion	Water	Nitrite	BHA/BHT	HXT
F-C	963.5	179.0	0	315.2	0.18	0	0
RF-C	963.5	0	301.9	192.4	0.18	0	0
RF-WN	963.5	0	301.9	192.5	0.00	0	0
RF-BHA/BHT	963.5	0	301.9	192.1	0.18	0.3	0
RF-HXT	963.5	0	301.9	192.0	0.18	0	0.38

All our samples also contain: 7.5 g flavour enhancer, 30 g NaCl and 4.5 g TPP.

Sample denomination: F-C, control frankfurter (all pork fat); RF-C reformulated frankfurter formulated by replacing pork backfat by oil-in-water emulsion; RF-WN, reformulated frankfurter as RF-C but without sodium nitrite added; RF-BHA/BHT, modified frankfurters as RF-C but adding a combination of 100 mg/kg BHA and 100 mg/kg BHT; RF-HXT, reformulated frankfurters as RF-C but containing 100 mg/kg of hydroxytyrosol (HXT).

Table 2. Thiobarbituric acid-reactive substances (TBARS, mg MDA/kg sample) in cooked meat batters.

Sample	Storage (days at 2 °C)		
	1	6	20
MB-C	1.212±0.110 ^{cA}	1.390±0.171 ^{cA}	1.659±0.087 ^{cB}
MB-BHA/BHT	0.420±0.097 ^{bA}	0.492±0.255 ^{aA}	0.415±0.096 ^{aA}
MB-50HXT	0.458±0.078 ^{bA}	0.730±0.004 ^{bB}	0.927±0.236 ^{bB}
MB-100HXT	0.079±0.008 ^{aA}	0.294±0.012 ^{aB}	0.384±0.023 ^{aB}

Mean ± SD. Different letters in the same column (a,b,c...) and in the same row (A,B,C,...) indicate significant differences (P<0.05). Sample denomination: MB-C, meat batter control; MB-BHT/BHA, meat batter as MB-C with 100 mg/kg of BHA and 100 mg/kg of BHT added; MB-50HXT and MB-100HXT meat batter as MB-C with 50 and 100 mg/kg HXT added respectively. n = 6

Table 3. Thiobarbituric acid-reactive substances (TBARS, mg MDA/kg of sample) in oil-in-water emulsion

Sample	Storage (days at 2 °C)		
	0	6	20
OWE-C	0.705±0.038 ^{aA}	1.142±0.099 ^{bcB}	2.067±0.180 ^{cC}
OWE-BHA/BHT	0.834±0.247 ^{aA}	0.789±0.037 ^{aA}	1.389±0.099 ^{bB}
OWE-50HXT	0.665±0.260 ^{aA}	1.298±0.083 ^{cB}	1.231±0.131 ^{bB}
OWE-100HXTH	0.750±0.052 ^{aA}	1.084±0.069 ^{bB}	0.983±0.086 ^{aB}

Mean ± SD. Different letters in the same column (a,b,c...) and in the same row (A,B,C,..) indicate significant differences (P<0.05). Sample denomination: OWE-C, control oil-in-water emulsion with no added antioxidant; OWE-BHA/BHT, oil-in-water emulsion with 100 mg/kg of BHA and 100 mg/kg BHT added; OWE-50HXT and OWE-100HXT, oil-in-water emulsions with 50 and 100 mg/kg hydroxytyrosol, respectively. n = 6

Table 4. Thiobarbituric acid-reactive substances (TBARS, mg MDA/kg product) in frankfurters.

Sample	Storage (days at 2 °C)				
	1	7	14	28	56
F-C	0.064±0.010 ^{aA}	0.069±0.013 ^{aA}	0.096±0.019 ^{aA}	0.111±0.014 ^{aA}	0.066±0.011 ^{aA}
RF-C	0.974±0.061 ^{eA}	1.243±0.061 ^{eB}	1.202±0.033 ^{eB}	1.212±0.014 ^{dB}	1.542±0.043 ^{eC}
RF-WN	0.376±0.023 ^{cA}	0.411±0.009 ^{cA}	0.411±0.021 ^{cA}	0.644±0.052 ^{cC}	0.527±0.013 ^{cB}
RF-BHA/BHT	0.507±0.016 ^{dA}	0.614±0.051 ^{dB}	0.556±0.013 ^{dAB}	0.677±0.033 ^{cC}	0.622±0.034 ^{dC}
RF-HXT	0.302±0.025 ^{bA}	0.327±0.040 ^{bA}	0.313±0.026 ^{bA}	0.432±0.019 ^{bB}	0.310±0.014 ^{bA}

For sample denomination see table 1. Mean ± SD. Different letters in the same column (a,b,c...) and in the same row (A,B,C,..) indicate significant differences (P<0.05). n = 6

Table 5. Ferric reducing/antioxidant power assay (FRAP, $\mu\text{mol Fe}^{2+}$ eq/g) in frankfurters.

	Storage (days at 2 °C)				
	1	7	14	28	56
F-C	4.15±0.78 ^{bbB}	1.91±0.27 ^{aA}	1.94±0.48 ^{aA}	2.39±0.16 ^{aA}	2.00±0.21 ^{aA}
RF-C	3.92±0.47 ^{abB}	2.86±0.63 ^{abA}	2.53±0.63 ^{aA}	2.88±0.45 ^{aA}	2.70±0.37 ^{aA}
RF-WN	3.06±0.65 ^{aA}	3.00±0.64 ^{bA}	2.95±0.45 ^{aA}	2.87±0.38 ^{aA}	3.06±0.70 ^{aA}
RF-BHA/BHT	9.04±0.66 ^{cB}	6.27±0.24 ^{cA}	6.15±0.11 ^{bA}	5.76±0.38 ^{bA}	6.39±0.22 ^{bA}
RF-HXT	8.41±0.82 ^{cC}	6.96±0.57 ^{cA}	7.28±0.74 ^{cAB}	8.05±0.36 ^{cB}	7.00±0.32 ^{bAB}

For sample denomination see table 1. Mean \pm SD. Different letters in the same column (a,b,c...) and in the same row (A,B,C,..) indicate significant differences ($P < 0.05$). n = 6

Table 6. Antioxidative capacity (Trolox–equivalents mmol/g) of frankfurters

Sample	Storage (days at 2 °C)				
	1	7	14	28	56
F-C	1.165±0.103 ^{aBC}	1.362±0.123 ^{aC}	0.942±0.018 ^{aB}	0.505±0.001 ^{aA}	0.454±0.030 ^{aA}
RF-C	1.155±0.153 ^{aC}	1.293±0.050 ^{aC}	0.907±0.056 ^{aB}	0.511±0.005 ^{aA}	0.547±0.068 ^{abA}
RF-WN	1.433±0.165 ^{bC}	1.630±0.020 ^{bC}	1.004±0.051 ^{aB}	0.630±0.035 ^{abA}	0.533±0.003 ^{abA}
RF-BHA/BHT	1.581±0.058 ^{bC}	1.765±0.019 ^{bC}	1.317±0.033 ^{bB}	0.786±0.062 ^{bA}	0.729±0.027 ^{bA}
RF-HXT	6.364±0.168 ^{cC}	7.168±0.155 ^{cD}	6.083±0.026 ^{cB}	6.053±0.026 ^{cB}	5.278±0.010 ^{cA}

For sample denomination see table 1. Mean ± SD. Different letters in the same column (a,b,c...) and in the same row (A,B,C,..) indicate significant differences (P<0.05). n = 6