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Additions of caffeic acid, ascorbyl palmitate or γ-tocopherol to fish oil-enriched energy bars affect lipid oxidation differently

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ABSTRACT

The objectives of the study were to investigate the effects of caffeic acid, ascorbyl palmitate and γ-tocopherol on protection of fish oil-enriched energy bars against lipid oxidation during storage for 10 weeks at room temperature. The lipophilic γ-tocopherol reduced lipid oxidation during storage when added at a concentration above 440 μg/g fish oil. However, the best antioxidative effect was observed when it was added at a concentration of 660 μg/g fish oil. In contrast, prooxidative effects were observed when using either γ-tocopherol at concentrations below 220 μg/g fish oil, or the hydrophilic caffeic acid, or the amphiphilic ascorbyl palmitate at concentrations of 75, 150 and 300 μg/g fish oil. Prooxidative effects were observed as an increase in the formation of lipid hydroperoxides and volatile secondary oxidation products, as well as the development of rancid off-flavours. The differences in the efficacies of the three antioxidants examined are expected to be related to their different localisations and mechanisms of action.

1. Introduction

Both epidemiological and interventional studies have demonstrated that a high intake of marine *n*−3 polyunsaturated fatty acids (PUFA) reduces the risk of mortality from different cardiovascular diseases, and furthermore plays a role in brain and nerve development of growing foetuses and infants (Psota, Gebauer, & Kris-Etherton, 2006; Trautwein, 2001). Additionally, evidence has been reported, that these fatty acids also possess anti-inflammatory properties and therefore can be useful in the management of inflammatory and autoimmune diseases (Simopoulos, 2002). However, the intake of *n*−3 PUFA from fish and fish products in the Western population does not match the recommended intake required to obtain these beneficial health effects (Sanders, 2000). Efforts have therefore been made to enrich various types of food products with *n*−3 PUFA, by substituting some of the original fat with marine oils. The introduction of the healthy *n*−3 PUFA is done at the expense of the oxidative stability, as the highly polyunsaturated fatty acids are considerably more oxidative labile than are their less unsaturated counterparts, thus causing the development of unpleasant off-flavours in the fish oil-enriched food products. Hence, efficient strategies to prevent lipid oxidation in fish oil-enriched foods are necessary in order to make such kind of foods successful in the marketplace (Jacobsen & Let, 2006).

A substantial amount of research has been done on the oxidative stability of bulk oil and simple emulsion systems to gain knowledge about lipid oxidation mechanisms, understand the influence of different factors, such as pH, temperature and transition metal ions (Djordjevic, Kim, McClements, & Decker, 2004; Frankel, Satué-Gracia, Meyer, & German, 2002; Let, Jacobsen, & Meyer, 2005; Sørensen et al., 2008), and to clarify how oxidative stability can be maintained despite the introduction of highly unsaturated fatty acids to these food systems. In contrast, oxidation studies on solid matrices, such as bread and biscuits, are very scarce, and only one study has previously dealt with fish oil-enriched energy bars (Nielsen & Jacobsen, in press). In this study, the means of fish oil addition was examined by adding 5% fish oil as either, neat fish oil, as a fish oil-in-water emulsion with sodium caseinate as the emulsifier or as microencapsulated fish oil. The oxidative stability of the energy bars increased in the order neat fish oil < fish oil-in-water emulsion < microencapsulated fish oil. Moreover, the addition of the hydrophilic compound EDTA (100, 500, 1000 or 2000 ppm) to energy bars with the fish oil added as an emulsion, was studied. Despite the metal chelating properties of EDTA, the oxidative stability of the energy bars decreased, indicating a prooxidative effect of EDTA, irrespective of the concentration added.

The objective of the present study was therefore to investigate whether an improved oxidative stability could be obtained in energy bars, enriched with 5% fish oil added as an emulsion, by addition of antioxidants. The effectiveness of an antioxidant in a given food product is influenced by its mechanism of action, its polarity/

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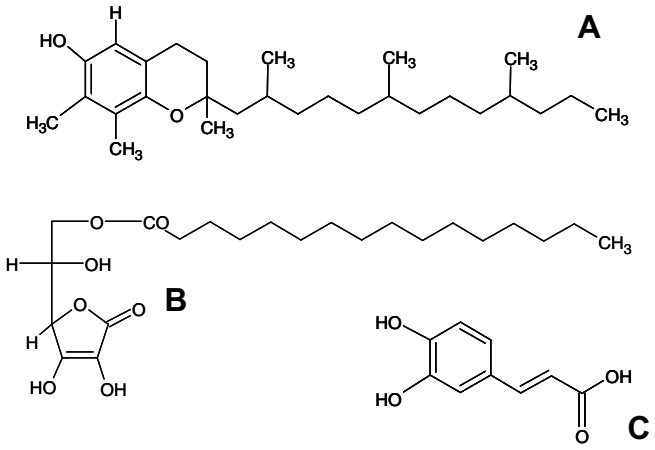

Fig. 1. Chemical structures of the three antioxidants added to energy bars. (A) The lipophilic γ-tocopherol; (B) the amphiphilic ascorbyl palmitate, with a hydrophilic ascorbic acid residue and a lipophilic palmitic tale; and (C) the hydrophilic caffeic acid.

solubility, and the concentration at which it is added. Therefore, the selection of the three antioxidants investigated in the present study was made on the basis of their differences in these properties. The selected antioxidants were the hydrophilic caffeic acid, the amphiphilic ascorbyl palmitate and the lipophilic γ-tocopherol (Fig. 1). The antioxidative properties of caffeic acid have previously been evaluated, and caffeic acid has been shown to exhibit both radical-scavenging and metal-chelating abilities (Chen & Ho, 1997; Gülçin, 2006; Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002). The same antioxidative properties have been shown for ascorbyl palmitate (Frankel, 2007; Marinova & Yanishlieva, 1992), and furthermore it has been shown to be able to regenerate other antioxidants, such as tocopherols (Beddows, Jagait, & Kelly, 2001; Cort, 1974; Niki, 1991). Tocopherols are known to have radical-scavenging properties (Buettner, 1993; Kamal-Eldin & Appelqvist, 1996). The activities of all three antioxidants have been shown to be concentration-dependent (Beddows et al., 2001; De Leonardis & Macciola, 2003; Huang, Frankel, & German, 1994).

Experiment 1 (E1) was aimed at investigating the effects of caffeic acid, ascorbyl palmitate and γ-tocopherol on the oxidative stability of fish oil-enriched energy bars, when the antioxidants were added, each at three different concentrations. Experiment 2 (E2) was set up with the purpose of making replicates of some of the sample codes from E1 and, furthermore, to investigate the effect of adding higher concentrations of γ-tocopherol (Table 1).

2. Materials and methods

2.1. Materials

The ingredients included soft brown sugar (Dansukker, Danisco Sugar, Kbh K, Denmark), syrup (Dansukker lys sirup, Danisco Sugar, Kbh K, Denmark), honey (Granja San Francisco vildblomst honning flydende, Nutrexpa, Barcelona, Spain), fig-date mix (Familiens fig-en-daddel smørepålæg, Castus A/S, Vadum, Denmark), wheat flour (Food line, Fællesindkøb I/S, Brøndby, Denmark), rolled oats (Food line, Fællesindkøb I/S, Brøndby, Denmark), Kelloggs rice krispies (Nordisk Kelloggs ApS, Glostrup, Denmark), raisins (Sun-maid, Kingsburg, CA, USA), and apricots (Snack ECO, Økologiske søde abrikoser, Urtekram, Mariager, Denmark). All ingredients were purchased at the local grocery store. Sodium caseinate (Miprodan® 30) was kindly donated by Arla (Arla Foods Ingredients amba, Viby J, Denmark), maltodextrin (Dry MD 01915) by Cerestar (Charlottenlund, Denmark), and fish oil (Maritex 43-01) by Maritex (Maritex, Sortland, Norway) (Table 2). Caffeic acid (purity ≥ 98%) was purchased from Sigma Aldrich, Steinheim, Germany, and γ-tocopherol (purity ≥ 95%) from Calbiochem, Darmstadt, Germany (E1) or from Sigma Aldrich, Steinheim, Germany (E2). Ascorbyl palmitate was donated from Danisco Cultor, Grindsted, Denmark. Other chemicals and solvents used were of analytical grade.

2.2. Preparation of 70% oil-in-water emulsions for energy bars

The emulsions were prepared by dissolving sodium caseinate in water (1+9). Subsequently fish oil was added to the solution (7+3) very slowly, while stirring vigorously using an Ultraturrax macerator (Ultraturrax T25, basic), with a total stirring time of approximately 3 min/100 g. Prior to emulsification, the antioxidants were dissolved in 96% ethanol to give individual standard solutions from which different amounts were taken to reach to the final concentrations stated in Table 1. The antioxidant solution was then

Table 1
Experimental design and recovery of antioxidants in fish oil-enriched energy bars

Sample code	Added antioxidant μg/g fish oil	Concentration of α-tocopherol, as analysed in energy bars μg/g total lipid ± SD			Concentration of γ -tocopherol, as analysed in energy bars μg/total lipid ± SD			Recovery of added antioxidant μg/g fish oil (%)	
		Week 0	Week 10	Δ	Week 0	Week 10	Δ		
Experiment 1 (E1)									
REF_1	–	218 ± 1	162 ± 5	56	85 ± 0	85 ± 3	0	–	–
CAF_75	75	205 ± 2	146 ± 6	59	88 ± 3	77 ± 1	11	53	71
CAF_150	150	195 ± 4	123 ± 16	72	79 ± 1	74 ± 1	5	85	57
CAF_300	300	214 ± 5	139 ± 5	75	82 ± 2	75 ± 1	7	197	66
ASC_75	75	246 ± 7	142 ± 2	104	86 ± 2	82 ± 0	4	nd	nd
ASC_150	150	211 ± 4	120 ± 5	91	86 ± 1	71 ± 5	15	nd	nd
ASC_300	300	211 ± 4	120 ± 4	91	88 ± 1	77 ± 4	11	nd	nd
TOC_110	110	276 ± 11	191 ± 7	85	150 ± 3	135 ± 3	15	75	68
TOC_220	220	233 ± 1	193 ± 5	40	234 ± 3	206 ± 1	28	181	82
TOC_440_1	440	222 ± 6	207 ± 4	15	382 ± 0	343 ± 3	39	386	88
Experiment 2 (E2)									
REF_2	–	178 ± 7	81 ± 4	97	84 ± 1	88 ± 1	−4	–	–
TOC_440_2	440	176 ± 1	126 ± 11	50	284 ± 3	256 ± 4	28	252	57
TOC_660	660	175 ± 6	124 ± 2	51	389 ± 2	329 ± 2	60	403	61
TOC_880	880	184 ± 1	136 ± 2	48	459 ± 17	418 ± 4	41	473	54
TOC_1100	1100	216 ± 4	105 ± 2	111	568 ± 3	502 ± 2	66	571	52

CAF: caffeic acid; TOC: γ-tocopherol; ASC: ascorbyl palmitate; na: no antioxidant addition; nd: not detected. Δ: The relative decrease in tocopherols from weeks 0 to 10. The recovery of γ-tocopherol was calculated as: $\frac{((\gamma\text{-tocopherol as analysed in sample})-(\gamma\text{-tocopherol as analysed in reference}))\cdot\text{total lipid content}}{\text{fish oil content}}$, where the fish oil content was 5% and the total lipid content approximately 6.2%. For the calculation of the % recovered, the recovery of γ-tocopherol was divided by the amount of added γ-tocopherol (μg/g fish oil).

Table 2
Peroxide value (PV), as determined prior to each experiment, content of tocopherols, and fatty acid composition of the fish oil used for the production of energy bars (Maritex 43-01)

E1 PV (meq/kg fish oil)	0.3 ± 0.0
E2 PV (meq/kg fish oil)	0.5 ± 0.1
α-Tocopherols (μg/g fish oil)	202 ± 1
γ-Tocopherols (μg/g fish oil)	28 ± 2

SFA (area%)		MUFA (area%)		PUFA (area%)	
Fatty acid	Av ± SD	Fatty acid	Av ± SD	Fatty acid	Av ± SD
14:0	3.6 ± 0.0	16:1 (*n*−7)	6.3 ± 0.0	18:2 (*n*−6)	4.1 ± 0.0
16:0	9.9 ± 0.0	18:1 (*n*−9)	22.4 ± 0.0	18:3 (*n*−3)	2.1 ± 0.0
18:0	2.2 ± 0.0	18:1 (*n*−7)	4.0 ± 0.0	18:4 (*n*−3)	2.2 ± 0.0
		20:1 (*n*−9)	10.6 ± 0.1	20:4 (*n*−3)	0.7 ± 0.0
		20:1 (*n*−11)	1.3 ± 0.0	20:5 (*n*−3)	7.3 ± 0.0
		22:1 (*n*−9)	0.7 ± 0.0	22:5 (*n*−3)	1.0 ± 0.0
		22:1 (*n*−11)	7.0 ± 0.1	22:6 (*n*−3)	11.1 ± 0.0
Others	1.1	Others	0.9	Others	1.5
Total	16.8	Total	53.1	Total	30.1
				Total *n*−3	24.9

Results are averages from determinations made on duplicate samples ± SD.

added to the oil, and ethanol was evaporated by nitrogen, to avoid any possible interfering effects.

2.3. Production of energy bars and sampling

All the dry ingredients (wheat flour (11.2%), rolled oats (15.9%), maltodextrin (4.2%), rice krispies (7.0%), raisins (7.0%) and apricots chopped by hand (7.0%)) were weighed and mixed. Separately, soft brown sugar (4.2%), syrup (14.0%), honey (14.0%) and fig-date mix (8.4%) were mixed and then the fish oil emulsion (7.1%) was added. Subsequently, this mixture was poured over the dry ingredients, and mixing carried out on a Viking mixer (20PS, Seidefors Agenturer AB, Bandhagen, Sweden) for 1 min at speed 60, or on a Hobart mixer (Model N-50 g, Hobart Corporation, USA) for 3 min at speed 2. The different mixers were used to obtain a similar consistency of all sample codes, despite differences in sample volume. The sample codes REF_1, CAF_75, CAF_300, ASC_75, ASC_300, TOC_110 and $TOC440_1$ in E1 were mixed on the Viking mixer, while the three remaining sample codes in E1 (CAF_150, TOC_220 and ASC_150) and all sample codes in E2, were mixed in the Hobart mixer. The dough was oven-baked (CCM101, Rational Großküchentechnik GmbH, Landsberg, Germany) in foil trays (350 g pr tray) for 15 min at 175 °C. After cooling, the content of each foil tray, was cut into portion size pieces for chemical and sensory analyses, and packed in sealed plastic bags (NEN40 HOB/LLPDE 75, Amcore Flexibles, Horsens, Denmark). The energy bars were stored at room temperature (Av: 21.6 °C, min: 18.7 °C and max: 24.6 °C) for up to 10 weeks. In E1, samples of all codes were taken for peroxide values (PV) and GC–MS analyses in weeks 0, 3, 6, 8 and 10, for tocopherols in week 0 and 10, and for fatty acid compositions in weeks 0, 6 and 10. In E2, samples were taken at the same time points, except that samples were not taken after 3 weeks for PV and GC–MS and after 6 weeks for fatty acid compositions. For sensory analysis, samples were taken in E1 for the codes, REF_1, CAF_75, CAF_300, TOC_110, TOC_440_1, ASC_75 and ASC_300, in weeks 0, 3, 6 and 10. In week 0 the iron content was measured in a sample prepared as REF_1, but with tricaprin oil instead of fish oil. Other data for this sample are not included in the present study.

2.4. Iron content

Energy bars were crushed in a mincer (Knifetec 1095 Sample Mill, FOSS, Sweden), and 1 g was mixed with 5.0 ml of concentrated HNO_3 and 2.0 ml of H_2O_2. The samples were ashed using a microwave oven (CEM, MDS-81D, Matthews, NC, USA) and analysed according to the protocol from the Nordic Committee on Food Analysis (1991), using an atom absorption spectrophotometer (AAS 3300, Perkin Elmer, MA, USA).

2.5. Extractions of lipid, caffeic acid and ascorbyl palmitate

Energy bars were crushed in a mincer (Knifetec 1095 Sample Mill, FOSS, Sweden) prior to extractions. A lipid extract was made according to the method described by Bligh and Dyer (1959) using 10 g of energy bar for each extraction and a reduced amount of solvent (30.0 ml of methanol and chloroform). The lipid extract was evaporated under nitrogen to remove solvent, before it was used for determinations of PV, tocopherols and fatty acid compositions. Extracts were furthermore made for determinations of caffeic acid and ascorbyl palmitate, using 5 g of energy bar for each extraction. The solvents used were 15.0 ml of ethanol to extract caffeic acid or 30.0 ml of methanol to extract ascorbyl palmitate. Energy bar crumbles and solvent were mixed for 1 min and centrifuged (10 min, 1600*g*, 18 °C) (Centra GP8R, IEC, Somerville, MA, USA). Extracts were filtered through cotton wool and refiltered (Minisart SRP 15) to form the final extract. For all extractions, care was taken to avoid heat and light. For ascorbyl palmitate extracts, extra care was taken by wrapping all glassware in aluminium foil. Extractions were done in duplicate.

2.6. Determination of tocopherols, caffeic acid and ascorbyl palmitate

The contents of tocopherols, caffeic acid and ascorbyl palmitate were determined by HPLC (Agilent 1100 Series; Column: C18, Thermo Hypersil ODS (250, 4.6 mm)), by injecting each extract twice. Tocopherols were analysed according to the official AOCS Method Ce 8-89 (1998). Analyses of caffeic acid and ascorbyl palmitate were done by modifying the methods described by Que, Mao, and Pan (2006) and Perrin and Meyer (2003), respectively. Caffeic acid was analysed using a gradient elution at a flow rate of 0.7 ml/min. Solvent A was water:acetic acid, 94:6 (v/v) and solvent B was water:acetic acid:acetonitrile, 65:5:30 (v/v/v). Gradient conditions: 0–5 min, 30–100% B; 5–8 min, 100% B; 8–10 min, 100–30% B; 10–12 min, 30% B. Injection volume: 10 μl. Caffeic acid content was measured spectrophotometrically at 324 nm. Ascorbyl palmitate was analysed using a gradient elution at a flow rate of 1.0 ml/min. Solvent A was water, acidified with phosphoric acid (1% v/v) and solvent B was methanol:acetonitrile, 1:1 (v/v). Gradient conditions: 0–20 min, 80–100% B; 20–25 min, 100–80% B. Injection volume: 20 μl. Ascorbyl palmitate was measured spectrophotometrically at 250 nm.

2.7. Primary oxidation products and fatty acid compositions

Primary oxidation products were determined by measuring peroxide values on the lipid extract or directly on the oil, by colorimetric determination of iron thiocyanate at 500 nm (Shimadzu UV-160A, UV–Vis recording spectrophotometer, Struers Chem A/S, DK), as described by Shanta and Decker (1994). Fatty acid compositions were determined on the lipid extract or directly on the oil by fatty acid methylation (AOCS Official Method Ce 2-66, 1998), followed by separation through gas chromatography (HP 5890 A, Hewlett Packard, Palo Alto, CA; Column OMEGAWAX™ 320) (AOCS Official Method Ce 1b-89, 1998). All data reported are averages of determinations made on duplicate lipid extractions on the same energy bar.

2.8. Secondary oxidation products by dynamic headspace and GC–MS

The energy bars were crushed in a mincer (Waring blender 8011EG, Waring Commercial, Connecticut, USA), and 4 g were

mixed with 25 ml of distilled water. Volatile secondary oxidation products were released from the sample by purging it with nitrogen (340 ml/min, 30 min) at 45 °C, and subsequently volatiles were trapped on Tenax GR tubes. The volatiles were desorbed from the Tenax tubes by heat (200 °C) in an Automatic Thermal Desorber (ATD-400, Perkin Elmer, Norwalk, CN), cryofocused on a cold trap (−30 °C), released again (220 °C), and led to a gas chromatograph (HP 5890 IIA, Hewlett Packard, Palo Alto, CA; Column DB-1701). The oven programme had an initial temperature of 45 °C, increased by 2.0 °C/min to 80 °C, by 3.0 °C/min to 150 °C and finally increased by 12.0 °C/min to 240 °C.

The individual compounds were analysed by mass-spectrometry (HP 5972 mass-selective detector), and quantified through calibration curves in the range of 0–1.0 mg/g. Measurements were done in triplicate. The volatiles selected for quantification were compounds that have previously been found to be markers of lipid oxidation in fish oil-enriched food products (pentanal, 1-pentanol, *t*-2-pentenal, 1-penten-3-ol, 1-penten-3-one, hexanal, *t,t*-2,4-heptadienal, 1-octen-3-ol, nonanal and *t,c*-2,6-nonadienal) (Hartvigsen, Lund, Hansen, & Hølmer, 2000; Venkateshwarlu, Let, Meyer, & Jacobsen, 2004). Calibration curves were made by dissolving the compounds in 96% ethanol, followed by injection (1 μl in triplicate) directly on the Tenax tubes with a small syringe (Hamilton syringe 7105N, Bonaduz, Switzerland). Ethanol was subsequently removed by nitrogen (purge flow 50 ml/min, 5 min).

2.9. Sensory profiling

In E1, all sample codes, except those with intermediate antioxidant concentrations (CAF_150, TOC_220 and ASC_150), were evaluated by a sensory panel consisting of eight panellists. Training of panellists and sensory analysis were generally performed according to ISO standards 6658 (1985) and 8586 (1993). In total, eight attributes were used for descriptive profiling of the fish oil-enriched energy bars (fishy odour and taste, rancid odour and taste, sweet bread/soft brown sugar odour and taste, and other odour and taste). The attributes for other odour and taste did not receive scores above 0 during profiling, and will therefore not be described further. A 90 mm line scale anchored at the ends with "none" (attribute not detectable) and "very much" (high intensity of the attribute) was used for each attribute. The energy bars were crushed prior to profiling and served at room temperature in small plastic beakers. The samples were presented in randomised order and, during the profiling, cold tap water and crisp bread were available for the panellists.

2.10. Statistical analyses

All sensory data obtained were analysed by one- and two-way analysis of variance with post tests (GraphPad Prism, version 4.03, GraphPad Software Inc.). Tukey's multiple comparison test was used for testing differences between sampling times, while Bonferroni's multiple comparison test was used to test differences between samples. All references to significant differences ($p < 0.05$) between samples or between sampling times are based on this statistical analysis of the sensory data. Standard deviations on all chemical data are calculated from 2 to 3 measurement replicates on each energy bar sample.

3. Results

3.1. Recovery of antioxidants and oxidation of the reference samples

The contents of caffeic acid, ascorbyl palmitate and γ-tocopherol in the energy bars were measured in week 0 (Table 1). For γ-tocopherol, 68–88% of the added amount was recovered in E1 and 52–61% in E2. For the three samples with caffeic acid, 57–71% of the added amount was recovered, while no ascorbyl palmitate was detected in any of the samples with this antioxidant. In spite of these results, and for the sake of convenience, the samples will still be referred to with the intended amount of added antioxidant. The content of iron was determined to be approximately 15 μg/g energy bar.

In both experiments, energy bars without additional antioxidants (REF_1 and REF_2) showed a substantial increase in PV during storage. REF_1 had a PV of 1.0 ± 0.0 meq/kg oil in week 0 and 24.1 ± 1.9 meq/kg oil in week 10 whereas REF_2 had a PV of 4.7 ± 0.2 meq/kg oil in week 0 and 23.3 ± 1.0 meq/kg oil in week 10 (Fig. 2). Simultaneously, a large increase in the content of the selected, volatile secondary oxidation products was observed for both samples, especially from weeks 6 to 10, as exemplified by 1-penten-3-ol (Fig. 3). For REF_1, these chemical findings were supported by sensory data, showing a significant increase in both rancid odour and taste during storage for 10 weeks (Table 3). This was furthermore accompanied by a significant decrease in sweet odour (data not shown).

3.2. Effect of addition of caffeic acid or ascorbyl palmitate

PV developed similarly after addition of caffeic acid or ascorbyl palmitate, as PV in all samples increased throughout storage from 1.2–2.9 meq/kg in week 0 to 27.5–32.2 meq/kg in week 10 (Fig. 2A and B). When compared to REF_1, the sample codes with ascorbyl palmitate already had markedly higher PVs in week 0, and this was also the case for the sample codes with caffeic acid from week 3. All samples with either caffeic acid or ascorbyl palmitate continued to have substantially higher PVs than had the reference in the rest of the storage period.

All the quantified volatile secondary oxidation products showed similar developments during storage, with a particularly higher increase in the later part of the storage period, from weeks 8 to 10 (Fig. 3A and B). In week 10, the order of volatile concentrations in the reference bar and in bars made with caffeic acid and ascorbyl palmitate was $REF_1 \approx ASC\_75 < CAF\_75 < ASC\_150 \approx ASC\_300 < CAF\_300 < CAF\_150$.

From determinations of fatty acid compositions, a small decrease was observed in the contents of total PUFA for both CAF_150 and CAF_300 from weeks 0 to 10 (data not shown). No considerable changes were found during storage for the fatty acid compositions of the three samples with ascorbyl palmitate. Energy bars with ascorbyl palmitate had the highest consumption (102–108 μg/g fish oil) of α- and γ-tocopherol during storage, followed by samples with caffeic acid (70–82 μg/g fish oil) (Table 1). REF_1 had the lowest consumption of total tocopherols (56 μg/g fish oil), as evaluated from the relative decreases.

Sensory data complimented chemical findings, and showed a significant increase in both rancid odour and taste in bars from weeks 6 to 10 for REF_1, CAF_75, CAF_300 and ASC_300, whereas ASC_75 showed no significant changes during storage (Table 3). The scores in the rancid attributes between samples were significantly different only in week 10, where CAF_75 and ASC_300 both had a more rancid odour than had REF_1 and ASC_75. The only significant change in the fishy attributes over time was seen for ASC_300, which had a significant increase in fishy taste from weeks 6 to 10 (data not shown). No significant differences were seen for the fishy attributes when comparing the individual samples. The intensity of sweet odour decreased significantly in the caffeic acid and ascorbyl palmitate samples and also in the reference during storage. For the attribute sweet taste, significant decreases were seen for CAF_75, CAF_300 and ASC_300 (data not shown). The only significant difference between samples for the sweet attributes

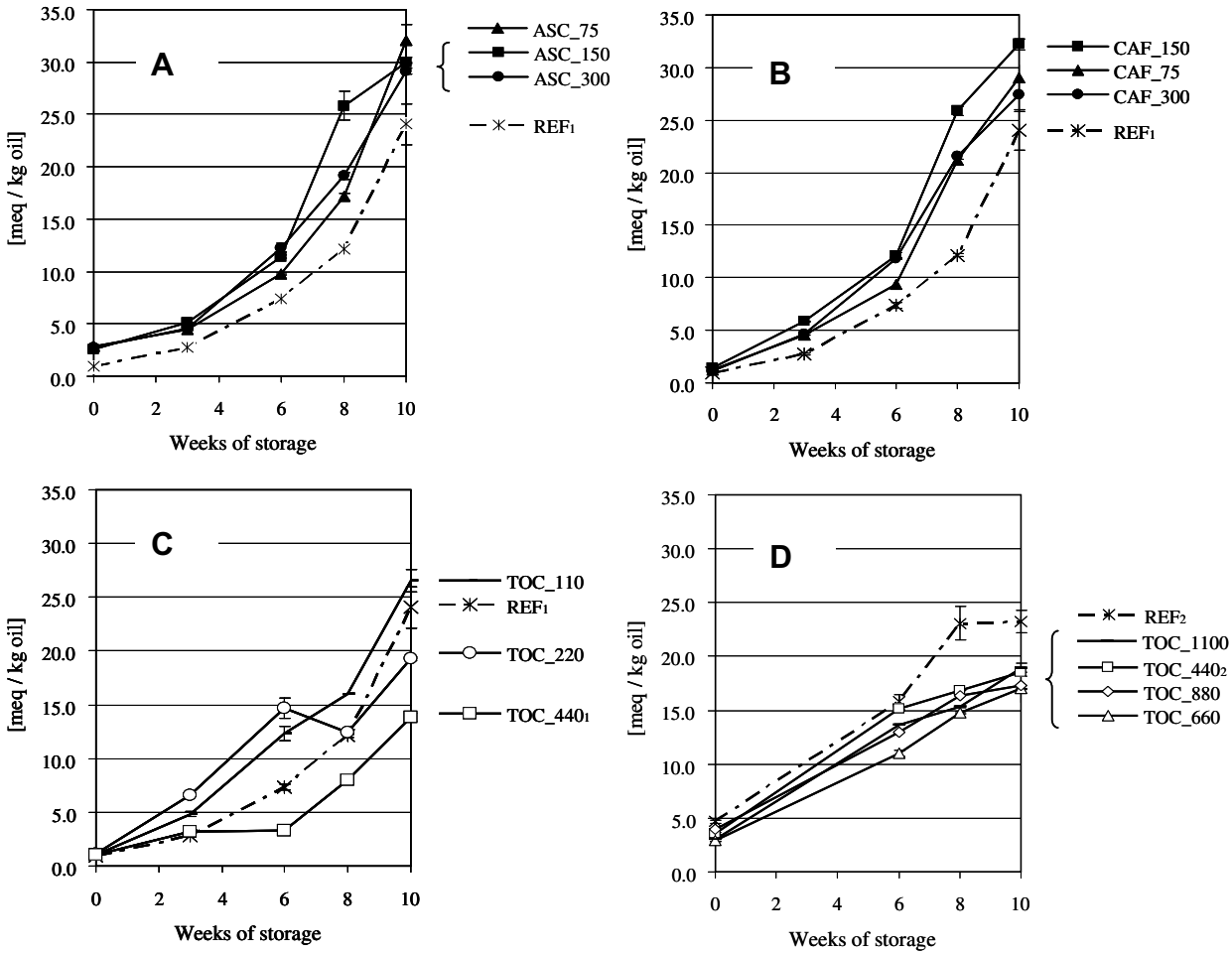

Fig. 2. Peroxide values (meq/kg oil) of energy bars with ascorbyl palmitate (A), caffeic acid (B) or γ-tocopherol (E1:C; E2:D) stored for 10 weeks at room temperature. Data points are averages of duplicate measurements ± SD. For sample names refer to Table 1.

was seen in week 10, where ASC_300 had a less sweet taste than REF_1.

3.3. Effect of addition of γ-tocopherol

In E1, TOC_110 had a substantially higher PV than had REF_1 in week 3–10, whereas TOC_440_1 had markedly lower PVs in weeks 6–10 (Fig. 2C). The intermediate concentration (TOC_220) had higher PVs in weeks 3 and 6 than REF_1, and a lower PV in week 10. In week 8, TOC_220 and REF_1 were not considerably different from each other. In E2 the development of PV was similar in all samples with γ-tocopherol, irrespective of the concentration (Fig. 2D). REF_2 had a higher PV than had the samples with γ-tocopherol throughout the storage period, being markedly higher than all other sample codes in weeks 8 and 10. TOC_660 had the lowest PV throughout the storage period.

The developments of all the quantified volatile compounds were, in each experiment, almost the same, despite small differences at the initial stages of lipid oxidation. In E1, the content of all the quantified volatiles was in the order $TOC\_110 > TOC\_220 \geq REF_1 > TOC\_440_1$ in week 10 (Fig. 3C). In E2, REF_2 generally had a much higher content of all the quantified volatiles than had the rest of the samples. Furthermore, TOC_440_2 in general had the highest content of the 10 quantified volatiles in week 10, compared to the other samples containing γ-tocopherol.

The lipid oxidation of the samples with γ-tocopherol in E1 was not sufficiently strong to cause reduction in total PUFA. However, lipid oxidation in REF_2 resulted in a reduction in total PUFA from weeks 0 to 10, partly due to a decrease in the contents of EPA and DHA (data not shown).

In E1, sensory data, furthermore, showed that in the later part of the storage period, both REF_1 and TOC_110 developed a significantly more rancid odour than did TOC_440_1. Moreover, TOC_110 had a significantly more rancid taste than had TOC_440_1 (Table 3). For the attribute sweet odour, TOC_440_1 had a significantly higher score than had TOC_110 in week 10 (data not shown).

Increasing the amount of added γ-tocopherols in E1 resulted in a correspondingly lower consumption of α-tocopherols and a higher consumption of γ-tocopherols (Table 1). Furthermore, the order in consumption of total tocopherols was $TOC\_110 > TOC\_220 > REF_1 > TOC\_440_1$. In contrast to E1, no relationship was observed between the concentrations of γ-tocopherol added and the consumption of the two tocopherol homologues, α, and γ, during storage in E2 (Table 1). The total consumption of α-, and γ-tocopherols was in the order $TOC\_440_2 < TOC\_880 < REF_2 < TOC\_660 < TOC\_1100$. This order corresponded to the order of consumption of γ-tocopherol alone, except for the reference that had no γ-tocopherol consumption. In contrast, the consumption of α-tocopherol were almost the same for TOC_440_2, TOC_660 and TOC_880 (approximately 50 μg/g total lipid), whereas the

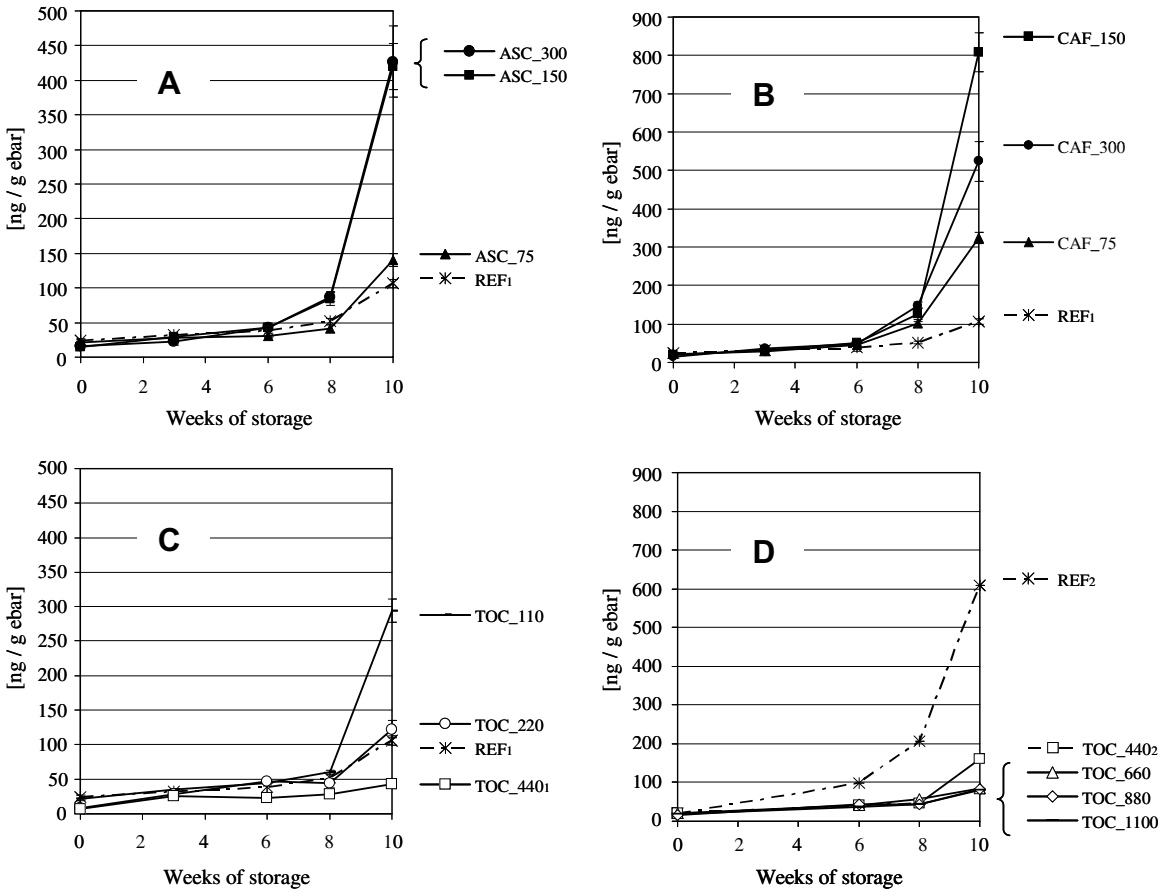

Fig. 3. Development of volatile secondary oxidation products in energy bars with ascorbyl palmitate (A), caffeic acid (B) or γ-tocopherol (E1:C; E2:D), as exemplified by 1-penten-3-ol (ng/g energy bar) during 10 weeks of storage at room temperature. Data points are averages of triplicate measurements ± SD. For sample names refer to Table 1.

Table 3
Sensory evaluation of fish oil-enriched energy bars (from E1) during storage for 10 weeks at room temperature

Sample code name	Week 0	Week 3	Week 6	Week 10
Rancid odour				
REF_1	$6 \pm 8^{a;x}$	$6 \pm 7^{a,b;x}$	$10 \pm 11^{a;x}$	$34 \pm 19^{b;y}$
CAF_75	$15 \pm 17^{a;x}$	$21 \pm 17^{b;x}$	$18 \pm 15^{a;x}$	$53 \pm 15^{c,d;y}$
CAF_300	$9 \pm 15^{a;x}$	$12 \pm 10^{a,b;x}$	$24 \pm 13^{a;x}$	$44 \pm 17^{b,c,d;y}$
ASC_75	$14 \pm 15^{a;x}$	$18 \pm 18^{a,b;x}$	$15 \pm 14^{a;x}$	$35 \pm 20^{b;x}$
ASC_300	$8 \pm 11^{a;x}$	$12 \pm 14^{a,b;x}$	$15 \pm 15^{a;x}$	$55 \pm 11^{d;y}$
TOC_110	$14 \pm 11^{a;x}$	$12 \pm 13^{a,b;x}$	$16 \pm 15^{a;x}$	$39 \pm 18^{b,c,d;y}$
TOC_440_1	$7 \pm 11^{a;x}$	$4 \pm 5^{a;x}$	$14 \pm 12^{a;x}$	$15 \pm 12^{a;x}$
Rancid taste				
REF_1	$7 \pm 10^{a;x}$	$9 \pm 12^{a;x}$	$17 \pm 12^{a;x,y}$	$35 \pm 18^{a,b;y}$
CAF_75	$17 \pm 17^{a;x}$	$22 \pm 10^{a;x}$	$19 \pm 15^{a;x}$	$48 \pm 11^{b;y}$
CAF_300	$8 \pm 15^{a;x}$	$19 \pm 13^{a;x,y}$	$30 \pm 13^{a;y,z}$	$43 \pm 16^{b;z}$
ASC_75	$14 \pm 15^{a;x}$	$20 \pm 17^{a;x}$	$23 \pm 20^{a;x}$	$37 \pm 20^{b;x}$
ASC_300	$5 \pm 6^{a;x}$	$15 \pm 12^{a;x}$	$20 \pm 21^{a;x}$	$52 \pm 15^{b;y}$
TOC_110	$9 \pm 8^{a;x}$	$16 \pm 15^{a;x}$	$26 \pm 15^{a;x,y}$	$43 \pm 17^{b;y}$
TOC_440_1	$9 \pm 11^{a;x}$	$6 \pm 6^{a;x}$	$18 \pm 16^{a;x}$	$18 \pm 15^{a;x}$

Results are given as a score on a 90 mm line scale, with 0 indicating that the attribute is not detectable, and 90 that the attribute has a very high intensity. The scores are given as averages ± SD over profiling results from eight panellists.
For each attribute, significant differences between samples are marked with the letters a–d, while significant differences over time are marked with the letters x–z (i.e. scores that are not significantly different are marked with the same letters).

consumption of this homologue was almost double for REF_2 and TOC_1100.

4. Discussion

4.1. The extent of lipid oxidation in experiment 1 vs. experiment 2

Comparison of two reference codes, in E1 and E2, showed that REF_2 oxidised faster than REF_1. The difference in PV between the oils used in the two experiments was small (Δ: 0.2 meq/kg oil). However, marked differences in PV appeared between REF_1 and REF_2 after dough making and baking. Thus, in week 0, the PV of REF_2 was almost five times greater than the PV of REF_1. As it is known that lipid autoxidation is a chain reaction which can propagate faster when more lipid hydroperoxides are available for the reaction with O_2 (Frankel, 2005), it is hypothesised that the greater lipid oxidation in E2 was caused by the higher PV of the oil used in E2 compared with the oil used in E1. Previous studies on freshly produced fish oil-enriched milk have shown that an emulsion made with an oil having a PV of 0.1 meq/kg was less oxidised than an emulsion made with an oil with PV 0.5 and 1.0 meq/kg (Let, Jacobsen, Frankel, & Meyer, 2003; Let et al., 2005). These data thus confirm our hypothesis that even small quality differences of the oil can have an impact on the extent of lipid oxidation in the energy bars.

4.2. Recovery of the added antioxidants and consumption of tocopherols

In E1, the percentage of the intended addition of γ-tocopherol and caffeic acid that was recovered was in the range of 57–88%. In E2, the recovery of γ-tocopherol was slightly lower than that

in E1 and hence probably resulted in smaller differences in lipid oxidation rates between samples than would otherwise be expected. For ascorbyl palmitate, no antioxidant was recovered. The observation, that no ascorbyl palmitate could be detected, could either result from a defective extraction related to the amphiphilic nature of ascorbyl palmitate, or a possible cleavage of ascorbyl palmitate into ascorbate and palmitate during production of the energy bars. However, it must be assumed that the antioxidant, to some extent, had been incorporated into the energy bars, since the samples with ascorbyl palmitate were more oxidised than the reference without added antioxidant, indicating a prooxidative effect of ascorbyl palmitate addition. Such a prooxidative effect would only be observed if ascorbyl palmitate were present.

All samples with caffeic acid had the same total consumption of tocopherols, irrespective of the caffeic acid concentration. This was also the case for all samples with ascorbyl palmitate. Furthermore, the total consumption of tocopherols was lower in the samples with caffeic acid than in the ascorbyl palmitate samples. However, it is not possible to explain these observations from the present data.

When the addition level of γ-tocopherol was increased among samples in E1, a great decrease in the consumption of α-tocopherol was observed, together with only a minor increase in the consumption of γ-tocopherol. Consequently, a higher addition level of γ-tocopherol led to a decrease in the total consumption of tocopherols. This pattern indicated that γ-tocopherol displayed a higher antioxidative activity in the current system than did α-tocopherol. Different activities of the two tocopherol homologues have been demonstrated in a previous study on the kinetics of their antioxidant action in sunflower and soybean triacylglycerols (Yanishlieva, Kamal-Eldin, Marinova, & Toneva, 2002). The pattern shown to exist for the consumption of tocopherols in E1 was not confirmed by the results in E2, where TOC_1100 had a much higher consumption of especially α-tocopherol than had the rest of the samples with γ-tocopherol. It has previously been proposed that tocopherols, particularly α-tocopherol, can lose efficiency or even act as prooxidants by participating in side reactions (Huang et al., 1994; Jacobsen, Hartvigsen, Lund et al., 2001; Kamal-Eldin & Appelqvist, 1996). A competition between the antioxidative radical-scavenging effect of tocopherol and the prooxidative mechanisms may lead to higher tocopherol consumption, simultaneously with some antioxidative effect and may at least partly explain the higher consumption of α-tocopherol in sample TOC_1100.

4.3. Prooxidative effects of caffeic acid

All chemical results, as well as sensory data indicated a prooxidative effect of caffeic acid. The prooxidative effect of caffeic acid is possibly due to its ability to reduce transition metal ions, and thereby generate very potent prooxidants, as described by Andersen, Lauridsen, and Skibsted (2003). In other types of systems, the reducing capacity of caffeic acid on transition metal ions has been shown to depend on a low pH (Deiana, Gessa, Marchetti, & Usai, 1995). However, a prooxidative effect of caffeic acid has also been observed in fish oil-in-water emulsions at pH 6 (Sørensen et al., 2008) and it is therefore likely that caffeic acid can exert a similar prooxidative mechanism at the higher pH in energy bars.

The hydrophilic nature of caffeic acid could contribute to its prooxidative effect in this study. Thus, it is expected that, after emulsification, caffeic acid is mainly partitioning into the water phase, although it to some extent may adsorb to the oil–water interface. When the emulsion is poured into the energy bar dough, caffeic acid is expected to be located outside the oil droplets, and thus be in close proximity to the transition metal ions present in the other ingredients. These metal ions will subsequently catalyze oxidation. Since the content of iron was found to be relatively high in the energy bars, this could also support the hypothesis, that a prooxidative effect of caffeic acid is related to the reduction of transition metal ions. Moreover, if caffeic acid becomes oxidised itself, other endogenous phenolic compounds may use their antioxidative capacity for regenerating caffeic acid instead of lipid radicals. This will reduce the antioxidative effect of the phenolic acids present in ingredients such as rolled oats and raisins (Adom & Liu, 2002; Yeung, Glahn, Wu, Liu, & Miller, 2003). However, the actual availability of iron in the energy bars, and the actual location of iron and caffeic acid in the energy bars, deserve further investigation.

4.4. Prooxidative effects of ascorbyl palmitate

Ascorbyl palmitate addition promoted oxidation of fish oil-enriched energy bars. A prooxidative effect of ascorbyl palmitate or ascorbic acid has previously been observed in other food products (Jacobsen, Adler-Nissen, & Meyer, 1999; Let, Jacobsen, & Meyer, 2007) and is suggested to be caused by the ability of the ascorbic acid residue to reduce transition metal ions, such as Fe^{3+} and Cu^{2+} to their more active reduced forms (Kanner, Mendel, & Budowski, 1977). A previous study on fish oil-enriched salad dressing showed that a high concentration (300 μg/g fish oil) of ascorbyl palmitate had a prooxidative effect, whereas a low concentration (50 μg/g fish oil) had a slightly antioxidative effect (Let et al., 2007). As the lowest concentration of 75 μg ascorbyl palmitate/g fish oil in the present study is shown to be less prooxidative than the higher concentrations, it may be hypothesised that an even lower concentration could lead to an antioxidative effect, as was seen in the salad dressing. It is proposed that this concentration-dependence exists because the antioxidative mechanism of ascorbyl palmitate is overridden by its ability to reduce transition metal ions when it is present in a high concentration. However, the two highest concentrations (150 and 300 μg/g fish oil) appeared equally prooxidative, and it seems that a maximum level of the prooxidative effect was reached in this experiment.

Energy bars with ascorbyl palmitate had higher PVs in week 0 (2.6–2.8 meq/kg fish oil) as compared to all other sample codes (1.0–1.4 meq/kg fish oil). Thus, it seems that ascorbyl palmitate triggered the oxidation during the emulsion production or during dough making or baking of the energy bars. As mentioned before, lipid autoxidation is a chain reaction process, which can propagate faster when more lipid hydroperoxides are available, as was the case for the ascorbyl palmitate samples in week 0. Therefore, the prooxidative effect of ascorbyl palmitate during storage may, at least partly, be due to the increased oxidation level at time 0. Regarding the localisation of ascorbyl palmitate, it is assumed that, if it was purely located in the oil phase where the metal ion concentration is very low, an antioxidative effect of ascorbyl palmitate would be expected. The observed prooxidative effect of ascorbyl palmitate therefore suggests that it is in contact with transition metal ions outside the oil droplets.

4.5. Pro- and anti-oxidative effects of γ-tocopherol

All data showed that addition of more than 440 μg γ-tocopherol/g fish oil resulted in antioxidant behaviour, which is likely due to the ability of γ-tocopherol to work as a hydrogen-donor, and thereby scavenge free radicals (Kamal-Eldin & Appelqvist, 1996; Mäkinen & Hopia, 2000; Yoshida, Niki, & Noguchi, 2003). The lipophilic nature of γ-tocopherol is also expected to lead to an optimal localisation of the antioxidant in order to carry out its antioxidative effect in energy bars. It is anticipated that γ-tocopherol, after emulsification, will be located at the oil–water interface or inside the oil droplets, and thereby close to the site of lipid oxidation and close to where it is expected to exert its function.

With regard to the concentration-dependence, it was observed from PV that the best antioxidative effect was obtained at a concentration above 660 μg γ-tocopherol/g oil. However, the content of volatiles in the bars made with 660–1100 μg γ-tocopherol/g fish oil were not markedly different from each other. The prooxidative effect of low γ-tocopherol concentrations is contradictory to previous studies of tocopherols, where prooxidative effects have only been observed with high concentrations, while lower concentrations were either ineffective or antioxidative (Huang et al., 1994; Jung & Min, 1992). Prooxidative effects have been shown to rely on the ability of tocopherols to participate in side reactions in some food systems (Huang et al., 1994; Kamal-Eldin & Appelqvist, 1996; Yanishlieva et al., 2002), and such side reactions may, to some extent, explain the present findings.

4.6. Antioxidant and prooxidant mechanisms

If the proposed hypothesis that caffeic acid and ascorbyl palmitate were prooxidative because they are able to reduce transition metals to their most active state is true, this indicates that metal catalysis of oxidation to some extent is an important factor in energy bars. On the other hand, EDTA was previously shown to be prooxidative in this system (Nielsen & Jacobsen, in press), suggesting that the metal-catalyzing mechanism may be different from what has been observed in systems such as dressing and mayonnaise (Jacobsen, Hartvigsen, Thomsen et al., 2001; Let et al., 2007). In the present study, the observed strong antioxidant activity of γ-tocopherol suggests that free radical-scavengers can reduce lipid oxidation in our system, which means that initiation of lipid oxidation, by already existing free radicals, may be an important factor. Taken together, the data indicate that it might be possible to further reduce oxidation by a combination of antioxidants with both free radical-scavenging and metal-chelating properties.

5. Conclusions

The current investigations on the oxidative stability of fish oil-enriched energy bars, showed that addition of caffeic acid or ascorbyl palmitate, in the range 75–300 μg/g fish oil, had a prooxidative effect compared to energy bars without antioxidants, as measured both chemically and through sensory analyses. However, the addition of γ-tocopherol, in concentrations above 440 μg/g fish oil, improved the oxidative stability of the energy bars considerably, with the best effect at 660 μg/g fish oil. Thus, both the type of antioxidant used, and the concentration at which it is applied are of great importance when the purpose is to reduce lipid oxidation in fish oil-enriched energy bars. The differences in the efficacy of the three antioxidants examined in the current study are expected to be related to the different localisations and mechanisms of action executed by them, but it cannot be ruled out that interactions between the antioxidants and ingredients in the energy bars may have affected their efficacy, either positively or negatively. Since this is one of the first studies concerning lipid oxidation in a fish oil-enriched bread-like product, further studies are needed in order to increase our knowledge about lipid oxidation, antioxidant reactions and their localisation in solid matrices such as the present one.

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