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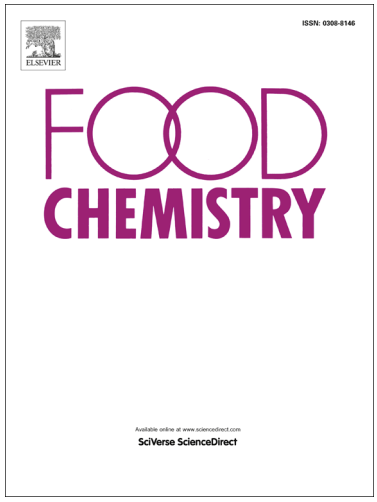
Published in:
Food Chemistry

Link to article, DOI:
10.1016/j.foodchem.2018.04.106

Publication date:
2018

Document Version
Peer reviewed version



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PII: S0308-8146(18)30746-5
DOI: https://doi.org/10.1016/j.foodchem.2018.04.106
Reference: FOCH 22801

To appear in: *Food Chemistry*

Received Date: 1 December 2017
Revised Date: 24 March 2018
Accepted Date: 23 April 2018

Please cite this article as: Qiu, X., Jacobsen, C., Sørensen, A.M., The effect of rosemary (*Rosmarinus officinalis* L.) extract on the oxidative stability of lipids in cow and soy milk enriched with fish oil, *Food Chemistry* (2018), doi: https://doi.org/10.1016/j.foodchem.2018.04.106

The effect of rosemary (*Rosmarinus officinalis* L.) extract on the oxidative stability of lipids in cow and soy milk enriched with fish oil

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ABSTRACT: Lipid oxidation of fish oil enriched cow milk and soy milk supplemented with rosemary extract stored at 2 °C was studied. Both peroxide value and volatile secondary lipid oxidation products were determined to monitor the progress of lipid oxidation. Rosemary extract inhibited lipid oxidation in fish oil enriched cow milk. In contrast, soy milk samples having much higher unsaturated fatty acid content showed higher lipid oxidation stability compared to cow milk. Reduction in the content ofchlorogenic acid during storage suggested that this compound may contribute to the lipid oxidation stability of fish oil enriched soy milk product. Total carnosic acid and carnosol concentration declined much faster in soy milk than in cow milk. It is suggested from the results that food components could have significant impact on the fate of bioactive antioxidant compounds in a specific food product during storage.

KEY WORDS: Lipid oxidation; n-3 PUFA; rosemary extract; carnosic acid; carnosol; chlorogenic acid; soy milk; cow milk

1. Introduction

Fish oil plays an important role in human neurodevelopment and vascular health as shown by numerous studies over the past decades (Costa, Losada-Barreiro, Paiva-Martins, & Bravo-Diaz, 2016； Uauy & Valenzuela, 2000). This is because it contains high concentrations of n-3 polyunsaturated fatty acids (n-3 PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which can only be obtained from the diet (Uauy & Valenzuela, 2000). Unfortunately, many consumers do not consume enough fatty fish in order to get necessary amount of essential EPA and DHA. Therefore， there is a growing interest in the food industry to enrich food products with fish oil.

Cow milk and soy milk are suitable for fish oil enrichment as they are popular and nutritious food products consumed by people worldwide. Soy milk is a water extract of soybeans. It contains significant amount of protein and unsaturated lipid and resembles cow milk in appearance (Peñalvo, Castilho, Silveira, Matallana, & Torija, 2004). It is particularly suitable for consumers with cow milk protein allergies, lactose intolerance, and galactosemia and is also a common drink for vegetarians (Sęczyk, Świeca, & Gawlik-Dziki, 2017； Xu & Chang, 2009).

However, lipid oxidation is one major concern for foods enriched with fish oil as fish oil can easily be oxidized and generate rancid off-flavors in these food products (Jacobsen, 2015). One of the strategies in the food industry is to use antioxidants to inhibit lipid oxidation. Although synthetic chemicals like tertriary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) have been widely used in various food products as efficient antioxidants, consumers are demanding antioxidants from natural sources. Rosemary extract is one of the most popular natural antioxidants on the market (Xie, VanAlstyne, & Yang, 2017). In addition, rosemary extract containing

carnosol and carnosic acid has been reported to have antiproliferative and antiinflamatory activity (González-Vallinas et al., 2013； Vicente et al., 2013). Rosemary extract would provide potential extra functional values in the food products as a natural antioxidant (Martin et al., 2017).

To the best of our knowledge, the effects of rosemary extract on the oxidative stability of fish oil enriched cow milk or soy milk have not been studied before. Compared to cow milk, soy milk contains phenolic compounds including isoflavones and phenolic acids which have been reported to have antioxidant activity (Lee et al., 2004). Thus, it is interesting to study the progress of lipid oxidation in these two enriched milk products under the same conditions (amount of fish oil and lipid content, storage temperature and time) with and without addition of rosemary extract as antioxidant. We hypothesized that: 1) rosemary extract would inhibit or reduce lipid oxidation in fish oil enriched milk products due to the antioxidative properties of the extract; 2) fish oil enriched soy milk would show higher lipid oxidative stability than cow milk as soy milk has more various phenolic compounds than cow milk. In addition, contents of antioxidant compounds such as tocopherols, carnosol, carnosic acid and other major phenolic compounds in soy milk were monitored during the storage period to investigate if lipid oxidation or food composition can have effects on the fate of these bioactive compounds during storage.

2. Materials and methods

2.1. Chemicals

All solvents used were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland). Folin-Ciocalteu reagent, sodium carbonate, gallic acid, chlorogenic acid, carnosic acid, carnosol and standards for

quantification of volatile lipid oxidation products were purchased from Sigma-Aldrich (Steinheim, Germany). Daidzin and genistin were purchased from Thermo Scientific (Kandel, Germany).

2.2. Materials

Pasteurized cow milk with 1.5% and 3.5% fat content and soy milk (2.2% fat content, 7.2% solid content) were purchased from a local supermarket. Cod liver fish oil was supplied by Maritex A/S (TINE BA, Sortland, Norway). The peroxide value (PV) of the fish oil was 0.17 mequiv peroxides/kg. The fish oil had 256 mg α-tocopherol/kg, 119 mg γ-tocopherol/kg, and 46 mg δ-tocopherol/kg. The fatty acid composition of the oil was analyzed by gas chromatography with flame ionization detection (GC-FID) as: C14:0, 3.3%; C16:0, 9.2%; C16:1 (n-7), 8.7%; C18:0, 2.0%; C18:1 (n-9), 16.3%; C18:1 (n-7), 4.6%; C18:2 (n-6), 1.8%; C18:3 (n-3), 0.1%; C18:4 (n-3), 2.7%; C20:1, 13.0%; C20:5 (n-3) (EPA), 9.3%; C22:1 (n-11), 5.9%; C22:5 (n-3),1.1%;C 22:6 (n-3) (DHA), 11.6% (w/w).Rosemary extract (Type HT-125) with a content of 1.0% carnosol and 6.1% carnosic acid was provided by Kalsec Inc. (Kalamazoo, MI). The PV of this rosemary extract was 0.52 mequiv peroxides/kg. The rosemary extract also contained 956 mg α-tocopherol/kg and 203 mg γ-tocopherol/kg.

2.3. Production of fish oil enriched cow milk and soy milk with rosemary extract

Cow milk with 1.5% and 3.5% fat content were mixed (11:9, w/w) to obtain the same fat content as soy milk (2.2%). Cow milk or soy milk was then heated to 72 °C and kept for 15 sec according to a previous study (Let, Jacobsen, Sørensen, & Meyer, 2007). Fish oil (0.5%, w/w) without any addition of rosemary extract was added to one batch of cow milk or soy milk as control (Con M and Con S). Fish oil and rosemary extract at concentrations of 0.03% and 0.06% (w/w) were mixed and added to cow

milk (CR1 and CR2) and soy milk (SR1 and SR2) as treatments. Sample codes are also indicated in Table S1.

All the products were premixed by an Ultra-Turrax homogenizer (13,000 rpm for 1 min) (IKA-Werke GmbH & Co, Staufen, Germany). Then, they were homogenized by a two-valve table homogenizer (GEA Niro Soavi Spa, Parma, Italy) 3 times at a pressure of 225 bar. After homogenization, each product was separated into 5 blue cap sterilized bottles and stored at 2 °C, one for each sampling day. On sampling day 0, 3, 6, 9, 12, the product in the blue cap bottle was transferred into small brown bottles, flushed with nitrogen and stored at -40 °C for chemical analysis. On day 1 and day 12, droplet size of each treatment was measured without prior freezing.

2.4. Droplet size measurement

Triplicate samples were measured with a laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). A few drops of the sample were added to the recirculating distilled water (2800 rpm, obscuration 13-15%, method: Fraunhofer) to measure the droplet size. The surface area weighted mean droplet size, $D_{3,2,}$ was used to report the results.

2.5. Lipid oxidation analysis

2.5.1 Lipid extraction by Bligh and Dyer method

Lipids from fish oil enriched milk samples was extracted in duplicate (Bligh & Dyer, 1959). Briefly, lipid from the sample was extracted with methanol, chloroform and water with a final ratio of 1:2:2:1 (w/v/v/v). After phase separation by centrifugation, the chloroform layer containing lipid was kept for peroxide value (PV), fatty acid composition and tocopherol analysis.

2.5.2 PV determination

Lipid extracts from the samples were evaporated to dryness under nitrogen. A colorimetric ferric-thiocynanate method was performed to measure PV on the same day of lipid extraction (Shantha & Decker, 1994). Briefly, lipid was mixed with chloroform/methanol (7:3 v/v), NH_4SCN and $FeCl_2$ solutions in order. Then the mixed solution was incubated in darkness for 5 min before PV measurement at 500 nm using a spectrophotometer. Chloroform/methanol without lipid extract was used as blank sample.. Results were reported as mequiv peroxides/kg lipids.

2.5.3 Fatty acid composition analysis

Lipid extract was evaporated to dryness under nitrogen. Then it was mixed with 100 µL internal standard C23:0 in heptane, 200 µL heptane with BHT, 100 µL toluene and 1 mL boron trifluoride reagent (20%) followed by heating using a microwave (Multiwave 3000 SOLV, Anton Paar, Graz, Austria) with a 64MG5 rotor. 1 mL saturated NaCl solution and 0.7 mL heptane was then added to the solution and mixed. The top layer was used for fatty acid composition analysis by GC-FID (HP 5890A, Agilent Technology, Palo Alto, CA). GC analysis details were described by Sørensen, Lyneborg, Villeneuve, & Jacobsen (2015). Results were reported as percentages of total fatty acids.

2.5.4 Volatile secondary lipid oxidation compound analysis. Volatile secondary lipid oxidation compounds were analyzed by a dynamic headspace gas chromatography (GC-MS) method described by Qiu, Jacobsen, Villeneuve, Durand, & Sørensen (2017). Briefly, 4 g of cow milk or soy milk samples with 0.5 mL of antifoam (Synperonic, 800 µL/mL water) was heated in a pear shaped bottle at 45 °C for 30 min. The volatile compounds were collected by a flow of nitrogen gas and absorbed into a Tenax GR packed tube. Volatile compounds desorbed from Tenax GR packed tubes were analyzed by GC (Agilent 6890, Palo Alto, CA) with a mass selective detector (HP 5973). The oven temperature was kept at 45 °C for 5 min, increased to 55 °C at 1.5 °C/min, and increased from 55 °C to 90 °C at 2.5

°C/min, then increased to 220 °C at 12 °C/min and finally kept at 220 °C for 4 min. Volatile compounds were quantified based on the external standard calibration curves. External standard compounds were spiked into the cow milk or soy milk samples without fish oil and analyzed in the same way. Results were reported as ng volatile/g milk (n=3).

2.6. Antioxidants and soy milk phenolic compounds analysis

2. 6.1. Tocopherol concentration determination

HPLC with fluorescence detector was used to determine the tocopherols in both cow milk and soy milk samples from the lipid extracts based on AOCS official method Ce 8-89 (1993). Tocopherols were detected with the excitation wavelength and the emission wavelength set at 290 nm and 330 nm, respectively*2.6.2. Determination of total phenolic content (TPC) in soy milk samples*

One mL cow milk or soy milk samples was mixed with 4 mL methanol. This solution was sonicated for 15 min in an ultrasonic bath at room temperature. Then the mixture was centrifuged 5400g for 10 min. An aliquot of the supernatant was transferred into 1.5 mL Eppendorf tubes and centrifuged again at 16000g for 10 min. The supernatant was used for total phenolics analysis or HPLC analysis.

TPC was analyzed by a Folin-Ciocalteu assay method (Singleton & Rossi, 1965). Briefly, 0.1 mL of the supernatant was mixed with 1.6 mL of Folin-Ciocalteau reagent solution (diluted with water 1:15). After incubation for 5 min at room temperature, 0.3 mL of 20% sodium carbonate solution was added, mixed and incubated for 2 h at room temperature. Absorbance was measured at 765 nm against a blank. Gallic acid was used to make the standard curve and results were reported as µg gallic acid equivalents (GAE)/mL sample. Both samples and standards were analyzed in triplicate.

2.6.3 Determination of carnosol and carnosic acid in fish oil enriched cow milk and soy milk samples.

Before HPLC analysis, the supernatant obtained using the same procedure as TPC analysis was filtered through a 0.2 µm polyvinylidene difluoride (PVDF) syringe filter. The quantitative analysis of carnosol and carnosic acid were based on the method described by Okamura, Fujimoto, Kuwabara, & Yagi (1994) with modification. Analysis was performed with a HPLC (Agilent 1100 series, Agilent Technology, Palo Alto, CA) equipped with a diode array detector at 210 nm. A Zorbax Eclipse XDB-C8 column (150*4.6 mm, 5µm) reversed phase column was used. The mobile phase consisted of water (0.1% phosphoric acid): acetonitrile (45:55) at a flow rate of 1 mL/min. The injection volume was 20 µL and running time was 20 min. External standard curves were made to calculate the carnosol and carnosic acid concentration. Results were reported as µg/mL sample (n=3).

2.6.4. Determination of isoflavones and phenolic acids in fish oil enriched soy milk samples

HPLC equipment and column same as above was used for this analysis based on a method by Murphy, Song, Gwen Buseman, & Barua (1998). The supernatant obtained using the same procedure as TPC analysis was also filtered through a 0.2 µm PVDF syringe filter before HPLC analysis. A linear gradient mobile phase consisting of A (water adjusted to pH 3 with acetic acid) and B (acetonitrile) was used: 15% B for 3 min, 15 to 29% B from 3 to 25 min, 29% to 35% B from 25 to 30 min, 35% back to 15% B from 30 to 31 min, and finally 15% B for 4 min. Total run time was 35 min at a flow rate of 0.6 mL min^{-1}. The injection volume was 10 µL. UV detection was performed at 260 nm and 320 nm for isoflavones and chlorogenic acid, respectively. Compounds were identified by comparing their retention times and UV spectra with those of standard compounds. Quantification was performed using calibration curves of each standard compounds. Results were reported as µg/mL sample (n=3).

2.7. Statistical analysis

All data shown represent the mean value ± standard deviation of duplicate or triplicate measurements. Data from the different quality parameters were subjected to analysis of variance using SPSS software (version 19.0; SPSS Inc., Chicago, IL). Comparison of means was performed using a least squares difference (LSD) method (P<0.05).

3. Results and discussion

3.1. Droplet size of the fish oil enriched cow milk and soy milk samples

Droplet size of the homogenized cow milk and soy milk is shown in Fig. S1. The droplet size ($D_{3,2}$) were within a range of 0.7 to 0.8 μm and did not increase significantly for the cow milk or soy milk samples during storage (P>0.05). There was no creaming or flocculation in the samples during 12 days of storage at 2 ºC. This was in agreement with our previous observation with fish oil enriched cow milk (Sørensen et al., 2015).

3.2. Fatty acid composition

Fatty acid composition of the fish oil enriched cow milk and soy milk samples is shown in Table S2. Significant differences in the fatty acid composition were found between the fish oil enriched cow milk and soy milk samples. In general, soy milk emulsion contained much higher levels of unsaturated fatty acids. It should also be mentioned that the method used in this study only detected fatty acids with a chain length above C13. Saturated short chain fatty acids present in milk were therefore not detected. The EPA and DHA content were approximately similar in these two products. During storage, the EPA and DHA amount did not decrease significantly in all the samples (data not shown), which was also observed in our previous studies (Sørensen et al., 2012; Sørensen et al., 2015).

3.3. Effect of rosemary extract on primary lipid oxidation in fish oil enriched cow milk and soy milk samples

Primary lipid oxidation results analyzed by PV are presented in Figure 1. For Con C sample, PV increased dramatically from day 3 to day 9 and declined, suggesting that lipid in the sample was significantly oxidized (Fig. 1A). In contrast to Con C, both CR1 and CR2 had a general trend of reducing the development of PV from day 3 to day 12. Thus, addition of the rosemary extract exerted a significant reduction of PV in the cow milk compared to control starting from day 6 to the end of storage (P<0.05). Interestingly, CR2 with higher content of rosemary extract had significantly higher PV than CR1 from day 3 to day 12 (P<0.05).

In contrast, PV in both soy milk control and soy milk with rosemary extract maintained low value (Fig. 1B). PV in Con S did not increase as dramatically as Con C. SR1 and SR2 had significantly higher PV than Con S on day 0. At the end of storage, SR1 had the lowest PV (P<0.05). The initial higher PV of both cow and soy milk samples with rosemary extract compared to control samples may be explained by the oil matrix of the rosemary extract, which had higher PV than fish oil.

PV is a measure of the primary lipid oxidation products, lipid hydroperoxides, in the samples. During the lipid oxidation process, hydroperoxides are formed but could also decompose into secondary compounds such as aldehydes, ketones, hydrocarbons, alcohols, etc. at the same time. In order to get a better understanding of the lipid oxidation of the product during the storage period, the determination of secondary lipid oxidation products is necessary and important. In addition, the formation of volatile compounds could have significant sensory impact as these volatile compounds can impart an off-flavor at very low concentrations (Jacobsen, 2015).

3.4. Effect of rosemary extract on secondary lipid oxidation in fish oil enriched cow milk and soy milk samples

Fig. 2 shows the changes of hexanal, 1-penten-3-one, 1-penten-3-ol and 2,4-heptadienal, which are important lipid oxidation markers for fish oil enriched cow milk products (Benedetti, Drusch, & Mannino, 2009; Sørensen et al., 2012). Hexanal content in the control cow milk sample increased dramatically after day 3 and decreased after day 9 (Fig. 2A). The increase of both hexanal and PV in Con C indicated that lipid oxidation progressed significantly in fish oil enriched cow milk after day 3. The decrease after day 9 suggested that lipid oxidation could enter into termination stage. Samples with rosemary extracts had significantly lower hexanal content from day 6 to day 12 compared to the control (P<0.05). Although CR2 contained a higher amount of rosemary extract than CR1, this did not lead to a higher reduction of hexanal content than for CR1. On day 12, it even had a higher hexanal concentration than CR1 (P<0.05), which was in agreement with the relatively higher PV in CR2. The higher hexanal content in CR1 and CR2 at day 0 than Con C could stem from the oil matrix of the rosemary extract, which consists of canola oil.

1-penten-3-one in Con C increased dramatically from day 3 to day 6 to the peak amount and then declined (Fig. 2B). 1-penten-3-one is a highly reactive α,ß-unsaturated ketone that could react further with other compounds like protein, which caused its reduction during the later stage of the storage period (Koo, Waisbourd-Zinman, Wells, Pack, & Porter, 2016). This was in agreement with our previous studies on the lipid oxidation progress of fish oil enriched cow milk (Alemán et al., 2015; Sørensen et al., 2015). The change of 1-penten-3-one also indicated that care should be exercised when it is solely used to evaluate the lipid oxidation status of fish oil enriched products as a marker compound. For all the products containing rosemary extract, 1-penten-3-one concentration was very

low and did not increase during the whole storage period, suggesting a strong inhibition effect exerted by the rosemary extract.

1-penten-3-ol in Con C increased significantly from day 3 and reached a plateau after day 9 (Fig. 2C). This trend was also observed in our previous studies (Alemán et al., 2015; Sørensen et al., 2015). Rosemary extract also showed a strong inhibition effect with no increase of 1-penten-3-ol during the whole storage period, which was in agreement with the observation for 1-penten-3-one with no changes in concentration in the samples containing rosemary extract.

Although 2,4-heptadienal was significantly formed after day 3 in Con C, no 2,4-heptadienal was detected in the CR1 and CR2 samples (Fig. 2D). In addition, 2,6-nonadienal was also formed significantly in Con C, but was not detected in CR1 and CR2 samples (data not shown). 2,4-heptadienal and 2,6-nonadienal are mainly derived from the oxidation of n-3 long chain polyunsaturated fatty acids (PUFAs) and could contribute to the off-flavor of oxidized fish oil (Karahadian & Lindsay, 1989). In the present study, nonanal and heptanal were also detected in cow milk samples. Rosemary extract also showed antioxidant effects by significant inhibition of the formation of nonanal and heptanal (data not shown). These results demonstrated that rosemary extract could inhibit the formation of important secondary lipid oxidation products. However, the highest concentration used in this study (CR2) did not show additional antioxidant effect compared with the lower concentration (CR1) applied. This is partially because the lipid oxidation was relatively mild due to the low temperature storage condition and also the high quality of the fish oil (low PV). Nevertheless, it shows the importance of optimization of the dosage of rosemary extract in each specific fish oil enriched food product to get the desired effects at the most economical cost.

Change of hexanal, hexanol, pentanal, 1-penten-3-ol in fish oil enriched soy milk samples is shown in Fig. 3. In general, hexanal in all samples had some variation during the whole storage period. An increase of hexanal concentration in SR2 was observed, but not with SR1 (Fig. 3A). At the end of storage, SR1 had the lowest concentration of hexanal, compared to Con S and SR2 ($P<0.05$). The relatively lower concentrations of hexanal and other volatiles on day 6 for all the samples compared to the other sampling days were possibly due to the instrumental error. Hexanal had a higher initial concentration in soy milk than cow milk. Hexanal is mainly derived from the oxidation of C18:2 (n-6) (Choe & Min, 2006). Correspondingly, the amount of C18:2 (n-6) (44.4%) in soy milk was dramatically higher than in cow milk (3%) (Table S1). In contrast, cow milk had a significantly higher initial concentration of nonanal (32.5 ng/g) than soy milk (1.5 ng/g). Nonanal is mainly formed by the oxidation of C18:1 (n-9) (Choe & Min, 2006). Although both soy milk and cow milk had a relatively high amount of C18:1 (n-9), the dramatically lower concentration of nonanal in fish oil enriched soy milk could be due to the natural antioxidants in the soy milk.

There was no obvious trend of increase or decrease for hexanol in the soy milk samples (Fig. 3B). Pentanal increased only in SR2 sample, whereas no increase was observed in SR1 and Con S (Fig. 3C). No difference was found between SR1 and Con S ($P>0.05$). The concentration of 1-penten-3-ol in all samples was relatively very low (less than 4 ng/g) and probably did not have any impact on the change of the flavor of this product (Fig. 3D). Nonanal concentration was also very low (less than 4 ng/g) for all the soy milk samples during the whole storage period. Decane and dodecane had a relatively higher concentration, but did not show any trend in both the control and treated samples (data not shown). In particular, 2,4-heptadienal and 2,6-nonadienal, which were found in Con C did not appear in any of the soy milk samples. These two compounds were mainly formed from the lipid oxidation process of n-3

PUFA in the fish oil (Karahadian & Lindsay, 1989; Peinado, Miles, & Koutsidis, 2016). Both the primary and secondary lipid oxidation results clearly showed that lipid oxidation was insignificant for the fish oil enriched soy milk stored at 2 °C. Although soy milk had significantly higher unsaturated fatty acid composition than cow milk, lipid in soy milk samples was not as oxidized as cow milk samples. To further understand the lipid oxidation mechanism, change of major bioactive antioxidant compounds in these two types of milk (i.e. tocopherols in cow and soy milks and natural antioxidants in soy milk, antioxidants from rosemary extract addition) was analyzed by HPLC during storage.

3.5. Change of tocopherol in cow milk and soy milk samples

Change of α-tocopherol in cow milk and soy milk samples is shown in Fig. 4. The higher concentration of α-tocopherol in treatment sample was due to α-tocopherol content in the canola oil based rosemary extract. After day 3, α-tocopherol in Con C decreased significantly, corresponding to the increase of PV (Fig. 4A, Fig. 1A). The correlation of decrease of α-tocopherol and increase of PV in cow milk samples enriched with fish oil was in agreement with our previous studies (Alemán et al., 2015; Sørensen et al., 2015). In contrast, α-tocopherol in CR1 and CR2 only decreased slightly and maintained a steady level after day 3. Carnosic acid and carnosol, which are compounds with antioxidant properties in the rosemary extract ,may regenerate the α-tocopherol that was consumed as antioxidant in the fish oil enriched cow milk samples. Alternatively, α-tocopherol could be protected at the expense of carnosic acid and carnosol. For the soy milk samples, the initial concentration of α-tocopherol was 2.9 μg/g, which was higher than in cow milk (1.3 μg/g). α-tocopherol levels decreased only slightly in Con S compared to Con C. No significant reduction of α-tocopherol in SR1 and SR2 during the whole storage period was found (P>0.05) (Fig. 4B). Due to the high oxidative stability of this soy milk product, α-tocopherol was not consumed as antioxidant or could be regenerated by other

antioxidants such as phenolics in soy milk or carnosic acid, carnosol in the rosemary extract. β-tocopherol and ϒ-tocopherol in both cow milk and soy milk samples did not decrease significantly (data not shown).

3.6. Change of carnosic acid and carnosol in fish oil enriched cow milk and soy milk samples

In both cow milk and soy milk samples, carnosic acid concentration was reduced during storage (P<0.05) (Fig. 5). In cow milk samples, the reduction started after day 6, while carnosic acid concentration was reduced significantly from day 3 in soy milk samples (P<0.05). The concentration of carnosic acid was even lower on day 0 in soy milk samples than in cow milk samples. Both cow milk and soy milk samples treated with rosemary extract showed high oxidative stability during storage, but carnosic acid was less stable in soy milk compared to cow milk. In contrast, Martin et al. (2017) did not find significant reduction of carnosic acid in a cooked meat product with partial replacement of animal fat by alkylglycerols-rich oil and inclusion of rosemary extract although lipid oxidation did occur in the control sample.

In cow milk samples, carnosol increased significantly after day 6, corresponding to the reduction of carnosic acid. Carnosic acid can be converted to carnosol and then to other compounds (Wijeratne & Cuppett, 2006). The increase of carnosol in cow milk samples indicated that such conversion took place. Total carnosic acid and carnosol concentration in CR1 and CR2 only decreased approximately 11% during the storage period, while 60% and 74% reduction of total carnosic acid and carnosol concentration in SR1 and SR2 was observed, respectively. Carnosic acid and carnosol in the soy milk samples could be consumed as antioxidant during storage to protect the high amount of unsaturated fatty acids. However, as Con S also showed high oxidative stability, food components other than unsaturated lipid could interact with carnosic acid and carnosol and cause the dramatic loss in the soy

milk sample. Studies on the change of bioactive carnosic acid and carnosol during storage in real food samples are scarce. As carnosic acid and carnosol were found to have various health benefits, care should be taken to keep these bioactive antioxidant compounds stable when using these compounds in various food products because different foods could influence their stability significantly as shown in this study. To further explore the lipid oxidation mechanism, it is interesting to investigate the change of potential antioxidants in fish oil enriched soy milk with and without rosemary extract.

3.7. Change of TPC in soy milk samples

TPC results are presented in Fig. 6A. SR1 and SR2 samples containing rosemary extract had higher phenolic content than Con S (P<0.05). Various phenolic compounds are reported to have antioxidant activity due to their ability to donate their phenolic hydrogen atoms to scavenge free radicals and interrupt free radical chain reaction (Maqsood, Benjakul, Abushelaibi, & Alam, 2014). However, during the whole storage period, phenolics were not significantly reduced. As TPC analysis is not enough to provide the information for the change of individual phenolic compounds, HPLC analysis of major phenolic compounds in soy milk were conducted.

3.8. Change of isoflavones and chlorogenic acid in fish oil enriched soy milk samples

Major isoflavones found in this soy milk sample was daidzin, genistin, malonylgenistin. Daidzin and genistin were quantified in this study. Isoflavones have been reported to have antioxidant activity but were found to be less effective than α-tocopherol by human low-density lipoprotein oxidation, the ferric reducing-antioxidant power (FRAP) and the anti-DPPH free radical assays (Lee et al., 2005). Daidzin and genistin did not decrease significantly during the whole storage period in the control and treated samples (P>0.05) (Fig. 6B and 6C). As lipid oxidation was relatively mild in fish oil enriched

soy milk samples stored at low temperature, it cannot preclude the possibility of the antioxidant activity of these compounds. In addition, various antioxidant compounds such as α-tocopherol, isoflavone, and phenolic acids could act synergistically so each compound may only decrease slightly. Further study is required to clarify this.

Chlorogenic acid was also determined in this soy milk product. Chlorogenic acid has been reported to be the major phenolic acid in soy milk and have the highest concentration among all the phenolic acids in the soy products (Xu & Chang, 2009). The concentration of chlorogenic acid was reduced during storage in both control and treated samples ($P<0.05$) (Fig. 6D). Chlorogenic acid has been reported to have antioxidant activity in various studies (Kandasamy et al., 2014; Rossato, Haas, Raseira, Moreira, & Zuanazzi, 2009). In the present study only reduction in the concentration of chlorogenic acid was found, which could indicate that it was used as an antioxidant and thereby contributed to the lipid oxidation stability in fish oil enriched soy milk. Further studies are required to confirm this.

4. Conclusions

The present study demonstrated that rosemary extract inhibited lipid oxidation and preserved the content of α-tocopherol in fish oil enriched cow milk stored at 2 °C. In addition, fish oil enriched soy milk showed higher lipid oxidative stability compared to cow milk. Our hypotheses that addition of rosemary extract will reduce lipid oxidation and that soy milk is more oxidatively stable than cow milk, were thus confirmed. Reduction of chlorogenic acid during storage suggested this compound may contribute to the oxidative stability of fish oil enriched soy milk. A higher reduction in the concentrations of total carnosic acid and carnosol was observed in fish oil enriched soy milk supplemented with rosemary extract, compared to cow milk. Food components other than lipids could

also have a major impact on the fate of bioactive antioxidants in a complex food system such as soy milk.

Acknowledgements

The financial support provided by Natural Science Foundation of Fujian Province (2016J01708) and China Scholarship Council (201608350049) is acknowledged. We thank Maritex (subsidiary of TINE BA, Norway) and Kalsec (Kalamazoo, MI, USA) for providing fish oil and rosemary extract to our research, respectively.

Conflict of interest statement

The authors declare no competing financial interest.

References

Alemán, M., Bou, R., Guardiola, F., Durand, E., Villeneuve, P., Jacobsen, C., & Sørensen, A. D. M. (2015). Antioxidative effect of lipophilized caffeic acid in fish oil enriched mayonnaise and milk. *Food Chemistry*, *167*, 236–244.

Benedetti, S., Drusch, S., & Mannino, S. (2009). Monitoring of autoxidation in LCPUFA-enriched lipid microparticles by electronic nose and SPME-GCMS. *Talanta*, *78*(4–5), 1266–1271.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917.

AOCS. (1993). Method Ce 8-89. Determination of Tocopherols and Tocotrienols in Vegetable Oils and Fats by HPLC. Urbana, IL, USA: American Oil Chemists' Society.

Choe, E., & Min, D. B. (2006). Mechanisms and factors for edible oil oxidation. *Comprehensive Reviews in Food Science and Food Safety*, *5*(4), 169–186.

Costa, M., Losada-Barreiro, S., Paiva-Martins, F., & Bravo-Diaz, C. (2016). Optimizing the efficiency of antioxidants in emulsions by lipophilization: tuning interfacial concentrations. *RSC Advances*, *6*(94), 91483–91493.

González-Vallinas, M., Molina, S., Vicente, G., de la Cueva, A., Vargas, T., Santoyo, S., García-Risco, M.R., Fornari, T., Reglero, G. Ramírez de Molina, A. (2013). Antitumor effect of 5-fluorouracil is enhanced by rosemary extract in both drug sensitive and resistant colon cancer cells. *Pharmacological Research*, *72*, 61–68.

Jacobsen, C. (2015). Some strategies for the stabilization of long chain n-3 PUFA-enriched foods: A review. *European Journal of Lipid Science and Technology*, *117*(11), 1853–1866.

Kandasamy, S., Baggu, C., Javagal, M. R., Lingamallu, J. R., Yenamandra, V., & Aradhya, S. M. (2014). Antioxidant Properties of Isolated Compounds from Banana Rhizome. *Journal of Food Science*, *79*(5), 988–1001.

Karahadian, C., & Lindsay, R. C. (1989). Evaluation of compounds contributing characterizing fishy flavors in fish oils. *Journal of the American Oil Chemists' Society*, *66*(7), 953–960.

Koo, K. A., Waisbourd-Zinman, O., Wells, R. G., Pack, M., & Porter, J. R. (2016). Reactivity of biliatresone, a natural biliary toxin, with glutathione, histamine, and amino Acids. *Chemical Research in Toxicology*, 29(2), 142–149.

Lee, C. H., Yang, L., Xu, J. Z., Yeung, S. Y. V., Huang, Y., & Chen, Z. Y. (2005). Relative antioxidant activity of soybean isoflavones and their glycosides. *Food Chemistry*, *90*(4), 735–741.

Lee, J. H., Renita, M., Fioritto, R. J., MArtin, S. K., Schwartz, S. J., & Vodovotz, Y. (2004). Isoflavone Characterisation and Antioxidant Activity of Ohio Soybean. *Journal of Agricultural and Food Chemistry*, *52*(9), 2647–2651.

Let, M. B., Jacobsen, C., Sørensen, A.-D. M., & Meyer, A. S. (2007). Homogenization Conditions Affect the Oxidative Stability of Fish Oil Enriched Milk Emulsions: Lipid Oxidation. *Journal of Agricultural and Food Chemistry*, *55*(5), 1773–1780.

Maqsood, S., Benjakul, S., Abushelaibi, A., & Alam, A. (2014). Phenolic Compounds and Plant Phenolic Extracts as Natural Antioxidants in Prevention of Lipid Oxidation in Seafood: A Detailed Review. *Comprehensive Reviews in Food Science and Food Safety*, *13*(6), 1125–1140.

Martin, D., Salas-Perez, L., Villalva, M., Vázquez, L., Garcia-Risco, M. R., Jaime, L., & Reglero, G. (2017). Effect of alkylglycerol-rich oil and rosemary extract on oxidative stability and antioxidant properties of a cooked meat product. *European Journal of Lipid Science and Technology*. *118*, 1600412.

Murphy, P. A., Song, T., Gwen Buseman, A., & Barua, K. (1998). Isoflavones in soy-based infant formulas. *Journal of Agricultural & Food Chemistry*, *46(8)*, 4635-4638.

Okamura, N., Fujimoto, Y., Kuwabara, S., & Yagi, A. (1994). High-performance liquid chromatographic determination of carnosic acid and carnosol in *Rosmarinus officinalis* and *Salvia officinalis*. *Journal of Chromatography A*, *679*(2), 381–386.

Peinado, I., Miles, W., & Koutsidis, G. (2016). Odour characteristics of seafood flavour formulations produced with fish by-products incorporating EPA, DHA and fish oil. *Food Chemistry*, *212*, 612–619.

Peñalvo, J. L., Castilho, M. C., Silveira, M. I. N., Matallana, M. C., & Torija, M. E. (2004). Fatty acid profile of traditional soymilk. *European Food Research and Technology*, *219*(3), 251–253.

Qiu, X., Jacobsen, C., Villeneuve, P., Durand, E., & Sørensen, A.-D. M. (2017). Effects of Different Lipophilized Ferulate Esters in Fish Oil-Enriched Milk: Partitioning, Interaction, Protein, and Lipid Oxidation. *Journal of Agricultural and Food Chemistry*, *65*(43):9496-9505.

Rossato, S. B., Haas, C., Raseira, M. do C. B., Moreira, J. C. F., & Zuanazzi, J. A. S. (2009). Antioxidant potential of peels and fleshes of peaches from different cultivars. *Journal of Medicinal Food*, *12*(5), 1119–1126.

Sęczyk, Ł., Świeca, M., & Gawlik-Dziki, U. (2017). Soymilk enriched with green coffee phenolics – Antioxidant and nutritional properties in the light of phenolics-food matrix interactions. *Food Chemistry*, *223*, 1–7.

Shantha, N. C., & Decker, E. A. (1994). Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. *Journal of AOAC International*, *77*(2), 421–424.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture*, *16*(3), 144 -158.

Sørensen, A.-D. M., Lyneborg, K. S., Villeneuve, P., & Jacobsen, C. (2015). Alkyl chain length impacts the antioxidative effect of lipophilized ferulic acid in fish oil enriched milk. *Journal of Functional Foods*, *18*, 959–967.

Sørensen, A.-D. M., Petersen, L. K., de Diego, S., Nielsen, N. S., Lue, B.-M., Yang, Z., … Jacobsen, C. (2012). The antioxidative effect of lipophilized rutin and dihydrocaffeic acid in fish oil enriched milk. *European Journal of Lipid Science and Technology*, *114*(4), 434–445.

Uauy, R., & Valenzuela, A. (2000). Marine oils: the health benefits of n-3 fatty acids. *Nutrition*, *16*(7–8), 680–684.

Vicente, G., Molina, S., González-Vallinas, M., García-Risco, M. R., Fornari, T., Reglero, G., & de Molina, A. R. (2013). Supercritical rosemary extracts, their antioxidant activity and effect on hepatic tumor progression. *The Journal of Supercritical Fluids*, *79*, 101–108.

Wijeratne, S. S. K., & Cuppett, S. L. (2006). Antioxidant activities of carnosol and carnosic acid in food and biological model systems - A review Part 1. *Agro Food Industry Hi-Tech*, *17*(6), 7–10.

Xie, J., VanAlstyne, P., & Yang, X. (2017). A review on rosemary as a natural antioxidation solution. *European Journal of Lipid Science and Technology*, *119*, 1600439.

Xu, B., & Chang, S. K. C. (2009). Isoflavones, flavan-3-ols, phenolic acids, total phenolic profiles, and antioxidant capacities of soy milk as affected by ultrahigh-temperature and traditional processing methods. *Journal of Agricultural and Food Chemistry*. *57*, 4706–4717

Figure captions:

Fig. 1. Development of PV in fish oil enriched cow milk and soy milk samples without and with rosemary extract during 12 days storage at 2 °C. Data points and error bars represent means ± standard deviations (n=2). Con C: cow milk sample with 0.5% (w/w) fish oil and without rosemary extract; CR1: cow milk sample with 0.5% (w/w) fish oil and 0.03% (w/w) rosemary extract; CR2: cow milk sample with 0.5% (w/w) fish oil and 0.06% (w/w) rosemary extract; Con S: soy milk sample with 0.5% (w/w) fish oil and without rosemary extract; SR1: soy milk sample with 0.5% (w/w) fish oil and 0.03% (w/w) rosemary extract; SR2: soy milk sample with 0.5% (w/w) fish oil and 0.06% (w/w) rosemary extract.

Fig. 2. Development of hexanal (A), 1-penten-3-one (B), 1-penten-3-ol (C), and 2,4-hepatadienal (D) in fish oil enriched cow milk samples without and with rosemary extract during 12 days storage at 2 °C. Data points and error bars represent means ± standard deviations (n=3). Con C: cow milk sample with 0.5% (w/w) fish oil and without rosemary extract; CR1: cow milk sample with 0.5% (w/w) fish oil and 0.03% (w/w) rosemary extract; CR2: cow milk sample with 0.5% (w/w) fish oil and 0.06% (w/w) rosemary extract. Fig. 3. Development of hexanal (A), hexanol (B), pentanal (C), and 1-penten-3-one (D) in fish oil enriched soy milk samples without and with rosemary extract during 12 days storage at 2 °C. Data points and error bars represent means ± standard deviations (n=3). Con S: soy milk sample with 0.5% (w/w) fish oil and without rosemary extract; SR1: soy milk sample with 0.5% (w/w) fish oil and 0.03% (w/w) rosemary extract; SR2: soy milk sample with 0.5% (w/w) fish oil and 0.06% (w/w) rosemary extract. Fig. 4. Change of α-tocopherol concentration in fish oil enriched cow milk (A) and soy milk (B) samples without and with rosemary extract during 12 days storage at 2 °C. Data points and error bars represent means ± standard deviations (n=2). Con C: cow milk sample with 0.5% (w/w)

fish oil and without rosemary extract; CR1: cow milk sample with 0.5% (w/w) fish oil and 0.03% (w/w) rosemary extract; CR2: cow milk sample with 0.5% (w/w) fish oil and 0.06% (w/w) rosemary extract; Con S: soy milk sample with 0.5% (w/w) fish oil and without rosemary extract; SR1: soy milk sample with 0.5% (w/w) fish oil and 0.03% (w/w) rosemary extract; SR2: soy milk sample with 0.5% (w/w) fish oil and 0.06% (w/w) rosemary extract.

Fig. 5. Change of carnosic acid and carnosol concentrations in fish oil enriched cow milk and soy milk samples with rosemary extract during 12 days storage at 2 °C. Data points and error bars represent means ± standard deviations (n=3). CR1: cow milk sample with 0.5% (w/w) fish oil and 0.03% (w/w) rosemary extract; CR2: cow milk sample with 0.5% (w/w) fish oil and 0.06% (w/w) rosemary extract; SR1: soy milk sample with 0.5% (w/w) fish oil and 0.03% (w/w) rosemary extract; SR2: soy milk sample with 0.5% (w/w) fish oil and 0.06% (w/w) rosemary extract. Fig. 6. Change of TPC (A), daidzin (B), genistin (C) and chlorogenic acid (D) concentrations in fish oil enriched soy milk samples without and with rosemary extract during 12 days storage at 2 °C. Data points and error bars represent means ± standard deviations (n=3). TPC: total phenolic content. Con S: soy milk sample with 0.5% (w/w) fish oil and without rosemary extract; SR1: soy milk sample with 0.5% (w/w) fish oil and 0.03% (w/w) rosemary extract; SR2: soy milk sample with 0.5% (w/w) fish oil and 0.06% (w/w) rosemary extract.

Highlights

- Rosemary extract inhibited lipid oxidation in fish oil enriched milk
- enriched soy milk showed higher lipid oxidative stability compared to cow milk Total carnosic acid and carnosol decreased differently in cow and soy milk