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Inhibition of hemoglobin-mediated lipid oxidation in washed fish muscle by cranberry components

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Abstract

Fractions enriched in phenolic acids (Fraction 1), anthocyanins (Fraction 2), flavonols (Fractions 3 and 4) and proanthocyanidins (Fractions 5 and 6) were prepared from cranberry powder using Sephadex LH-20 chromatography. Fractions 2, 3, 4, and 5 had nearly equivalent reactivity in the total phenolate assay employed per mg dry weight of each fraction while Fractions 1 and 6 were less reactive. The ability of cranberry fractions to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals as well as their inhibitory effects on hemoglobin (Hb)-mediated lipid oxidation in washed cod muscle were assessed. Addition of cranberry fractions at a level of 74 μmol quercetin equivalents per kg of washed cod muscle extended the induction time of thiobarbituric acid reactive substances (TBARS) formation in the order: Fraction 1, Fraction 3, Fraction 4 > Fraction 2 > Fraction 5 > Fraction 6. This suggests that oligomeric polyphenols (e.g., proanthocyanidins) were least effective at inhibiting Hb-mediated lipid oxidation in washed cod muscle compared to the other classes of polyphenolics in cranberry. The ability of the different cranberry fractions to scavenge DPPH radicals did not reflect their relative ability to inhibit lipid oxidation in the washed cod muscle system. Quercetin was tentatively identified as a component in cranberry that was especially effective at inhibiting Hb-mediated lipid oxidation. The ability of flavonol and proanthocyanidin-enriched fractions to inhibit Hb-mediated lipid oxidation in spite of efforts to wash away the added polyphenolics prior to Hb addition indicated these classes of polyphenolics had binding affinities for insoluble components of washed cod muscle.

Keywords: Cranberry; Antioxidant; Fish; Muscle foods; Radical scavenging; Phenolic; Hemoglobin; Lipid oxidation; Heme protein

1. Introduction

Lipid oxidation is a major cause of quality deterioration in muscle foods during processing and storage. Due to the presence of highly unsaturated fatty acids, fish is more susceptible to lipid oxidation than other muscle foods such as poultry, pork, beef, and lamb (Tichivangana & Morrissey, 1985). Incorporation of antioxidants into foods can effectively retard lipid oxidation. However, due to consumer awareness of the safety and potential toxicity of synthetic antioxidants, there has been an increasing interest in exploring new antioxidants from natural origin.

Plants are the most abundant source of natural antioxidants. Phenolic compounds are widely distributed in plants and in plant-derived food and beverages such as chocolate, wine, cider, and tea (Shahidi & Naczk, 1995). Cranberry (*Vaccinium macrocarpon* Ait.) has a considerable amount of phenolic compounds including phenolic acids, flavonol glycosides, anthocyanins, and proanthocyanidins (Chen, Zuo, & Deng, 2001; Foo, Lu, Howell, & Vorsa, 2000; Hong & Wrolstad, 1990; Sun, Chu, Wu, & Liu, 2002;

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; TBARS, thiobarbituric acid reactive substances; LDL, low-density lipoprotein; SC_{50}, the concentration sufficient to obtain 50% of a maximum scavenging capacity from the regression lines with 95% confidence level at the plot of % scavenging capacity vs. concentration; LT, lag time, the extension in stability compared to control sample which was stable for around 1 day; ABTS, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid; Hb, hemoglobin; HPLC, high performance liquid chromatograph.

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Zuo, Wang, & Zhan, 2002). Studies have shown that cranberry exhibited the highest rank in the total phenolic content (Sun et al., 2002; Vinson, Su, Zubik, & Bose, 2001) as well as antioxidant activity in terms of total oxy-radical scavenging capacity (Sun et al., 2002), among 20 common fruits evaluated including orange, red grape, strawberry, apple, and banana. There was a positive relationship between oxygen radical absorbance capacity and total phenolic or anthocyanin content in cranberry (Wang & Stretch, 2001; Zheng & Wang, 2003). However, a poor correlation between total phenolic content and antioxidant activity in terms of inhibiting the oxidation of methyl linoleate was observed for cranberry wine (Heinonen, Lehtonen, & Hopia, 1998). In general, the ability of cranberry phenolic components to inhibit lipid oxidation in muscle food systems may be attributed to free radical scavenging activity (Jovanovic, Steenken, Hara, & Simic, 1996; Robak & Gryglewski, 1988), metal chelating capacity (Hider, Liu, & Khodr, 2001; Morel et al., 1993), and the deactivation of hypervalent ferryl heme pigments known to initiate lipid oxidation (Hu & Skibsted, 2002).

The health benefits of cranberry phenolic compounds have been showed in the prevention of urinary tract infections (Foo et al., 2000) and stomach ulcers (Burger et al., 2000), inhibition of low-density lipoprotein (LDL) oxidation (Porter, Krueger, Wiebe, Cunningham, & Reed, 2001; Vinson et al., 2001) and cancer chemoprevention (Ferguson et al., 2002; Kandil et al., 2002). However, there is lack of information about how these cranberry components affect lipid oxidation in food systems. A model system to assess antioxidant effectiveness in muscle foods has been described which utilizes washed fish muscle and added hemoglobin to promote oxidation of membrane phospholipids in the washed muscle (Decker, Warner, Richards, & Shahidi, 2005). The primary objective of this study was to evaluate the ability of various cranberry phenolic components to inhibit lipid oxidation processes in a muscle matrix and to investigate some of the mechanisms by which inhibition occurs.

2. Materials and methods

2.1. Materials

Spray dried cranberry concentrate juice powder 90-MX was provided by Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA). Ethanol (absolute, 200 proof) was obtained from Aaper Alcohol and Chemical Co. (Shelbyville, Kentucky). Sephadex LH-20 was obtained from Amersham Biosciences (Uppsala, Sweden). All other chemicals and HPLC grade solvents used were obtained from Fisher Scientific (Chicago, IL) or Sigma–Aldrich Chemical Co. (St. Louis, MO).

2.2. Preparation and fractionation of cranberry crude extract

Preparation of cranberry crude extract and its fractions was done according to the method described by Porter et al. (2001) with minor modifications. 4.5 g cranberry concentrate juice powder was dissolved in 8 ml water and loaded on a Sephadex LH-20 (35 g) column (ID = 2.5 cm, length = 27 cm). 250 ml water at 1.5 ml/min was used to elute non-phenolic cranberry constituents. Subsequently, 2.5 l 80% aqueous acetone (v/v) was used to elute cranberry phenolics. A red-purple powder, which is referred to as cranberry crude extract, was obtained after removal of acetone by rotary evaporation under reduced pressure at 35 °C and removal of water by lyophilization. The cranberry crude extract (50 mg) was then dissolved in 7.5% aqueous ethanol (v/v) and subjected to a Sephadex LH-20 (23 g) column (ID = 2.2 cm, length = 25 cm) equipped with Agilent 1100 series binary pump and photodiode array detector (Agilent, Wilmington, DE) for further fractionation. Seven fractions were obtained by the use of the following solvents at a flow rate of 2.5 ml min^{-1} (1A and 1, 100% water; 2, 50% aqueous ethanol (v/v); 3, 100% ethanol; 4, ethanol:methanol (1:1,v/v); 5, 100% methanol; 6, 80% aqueous acetone (v/v)). Fraction 1A was not used in this study due to its inadequate amount. The elution profile of fractions obtained from the cranberry crude extract was monitored at 280, 320, 360 and 520 nm. Each fraction was concentrated under vacuum by rotary evaporation at 35 °C and lyophilization. Subsequently, all fractions were stored in 50% aqueous ethanol (v/v) at −80 °C until use.

Characterization of the phenolic components in each cranberry fraction was done by reverse phase high performance liquid chromatography (HPLC) equipped with a diode array detector (Krueger, Dopke, Treichel, Folts, & Reed, 2000). 50 μl of each fraction was injected onto a Rainin Dynamax C18 column (60 Å, 8 μm, 25 cm × 0.45 cm). A linear elution gradient was used; Solvent A was 0.1% trifluoracetic acid in water and solvent B was 100% methanol. A flow rate of 2 ml/min was employed and spectral data was acquired for 40 min. Multiwavelength detection was monitored at 280, 320, 360 and 520 nm.

2.3. Determination of total phenol content

Total phenol content in cranberry crude extract and its fractions were quantified by a modified spectrophotometric method described by Singleton and Rossi (1965) with quercetin as a reference standard. 2.5 ml of 10× diluted (with water) Folin–Ciocalteu reagent was added to 25 μl of cranberry fraction. After 5 min, 2 ml of 7.5% sodium carbonate was added into the resulting mixture. The absorbance was determined at 760 nm by a double-beam spectrophotometer model UV-2401 (Shimadzu Scientific Instruments Inc., Columbia, MD), after the incubation of mixture at 22–24 °C for 2 h.

2.4. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

Scavenging activities of cranberry crude extract/fractions toward DPPH radical were evaluated by spectrophotometry

(Cuendet, Hostettmann, & Potterat, 1997). 0.35 ml of cranberry crude extract/fractions was added to 0.85 ml of 43 μM DPPH in methanol. After 30 min incubation at 22–24 °C, the resultant absorbance was recorded at 515 nm by a double-beam spectrophotometer model UV-2401 (Shimadzu Scientific Instruments Inc., Columbia, MD). Ascorbate, propyl gallate, and quercetin were used as reference standards. The scavenging capacity (%) = [1 − ((Abs of cranberry crude extract/fractions − Abs of blank)/Abs control)] * 100. Control was defined as methanolic DPPH and 50% aqueous ethanol (v/v) and blank was defined as methanol and cranberry crude extract/fractions. SC_{50} is defined as the concentration required to obtain 50% of a maximum scavenging capacity from the regression lines with 95% confidence level at the plot of % scavenging capacity vs. concentration (Choi et al., 2002).

2.5. Preparation of washed cod muscle

Cod fish (*Gadus morhua*) were delivered overnight from Gloucester, MA or obtained from a local seafood store that utilizes air shipment from Boston, MA. Fillets used were considered of excellent quality based on odors that ranged from sea-like (very fresh) to minimal overall odor. Sensory panelists familiar with seafood deterioration made this assessment. All dark muscle was removed. White muscle was ground in a KS M90 mincer (Kitchen Aid Inc., St Joseph, MI) (plate diameter 5 mm). The mince was washed once in Milli-Q water at a 1:3 mince to water ratio (w:w) for 2 min. The mixture was allowed to stand for 15 min before dewatering with fiberglass screen. Mince was then washed with three volumes of 50 mM sodium phosphate (pH 6.3), dewatered and washed a final time in 50 mM sodium phosphate (pH 6.3). The slurry was then homogenized using a Polytron (Type PT 10/35, Brinkmann Instruments, Westbury, NY). It was finally centrifuged (15,000*g* for 25 min at 4 °C) using a Beckman L8-70 M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) and stored at −80 °C until use.

2.6. Preparation of trout hemoglobin (Hb)

Anesthetized trout (0.5 g ethyl 3-aminobenzoate/l of water) were bled from the caudal vein into syringes (25 G, 1″ needle) pre-loaded with saline heparin solution (150 U/ml). Hemolysate was then obtained by repeated washing Four volumes of ice cold 1.7% NaCl in 1 mM Tris, pH 8.0, were added to heparinized blood and centrifuged (700 g for 10 min at 4 °C) in a Beckman J-6B centrifuge (Beckman Instruments Inc.; Palo Alto, CA). After removal of the plasma, the red blood cells were washed by suspending three times in 10 vol. of the above buffer (Fyhn et al., 1979). Cells were lysed in 3 vol. of 1 mM Tris, pH 8.0 for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before ultracentrifugation (28,000*g* for 15 min at 4 °C) using a Beckman L8-70 M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). Hb solutions were stored at −80 °C prior to use. SDS–gel electrophoresis indicated that the only detectable polypeptide in the Hb preparation were in the range of Hb subunits (data not shown). The method of Brown (1961) was used to quantify Hb concentration.

2.7. Lipid oxidation in washed cod muscle model system

The hemoglobin-mediated lipid oxidation system consisted of washed cod muscle, cranberry crude extract/fractions, streptomycin sulfate, and trout hemoglobin. The final concentration of hemoglobin was 12 μmol per kg washed cod muscle and streptomycin sulfate was added at a final concentration of 200 ppm. Cranberry crude extract/fractions were added at 74 μmol quercetin equivalent/kg washed cod muscle. The carrier solvent for cranberry extract/fractions was 50% aqueous ethanol (v/v) and the carrier comprised 0.3% of the final sample weight. A control sample containing no cranberry crude extract/fractions was also prepared. In trials examining the ability of quercetin and propyl gallate to inhibit Hb-mediated lipid oxidation, quercetin and propyl gallate were added at 37 and 74 μmol per kg washed cod. The carrier solvent for quercetin and propyl gallate was ethanol and the carrier comprised 0.4% of the final sample weight. A control sample containing no quercetin and propyl gallate was also prepared. For all trials, the final moisture content of washed cod muscle was adjusted to 90%. pH was adjusted to between 6.20 and 6.30 by 1 M NaOH or HCl. All samples were stored at 2 °C for 7–9 days. The progress of lipid oxidation in washed cod muscle was followed by periodically removing samples and quantifying TBARS formed by a modified method of Buege and Aust (1978) as described by (Richards, Modra, & Li, 2002).

2.8. Washing of washed cod muscle pre-treated with cranberry fractions

Washed cod muscle was premixed with a cranberry fraction (1, 3, or 5, each at a final concentration of 74 μmol quercetin equivalents/kg tissue) and subsequently subjected to one washing step with Milli-Q water (1:3, w:w), dewatered through Whatman 1 filter paper, and then two washing steps with sodium phosphate buffer (pH 6.3) (1:3, w:w). After centrifugation and removal of the supernatant from the final washing step, trout hemoglobin (a final concentration of 12 μmol per kg washed cod muscle) was added to the resulting pelleted washed cod muscle to stimulated lipid oxidation. A control washed cod muscle sample was also prepared using the same three washing steps but was not premixed with a cranberry fraction.

3. Results

3.1. Characterization of cranberry fractions

The goal of fractionating cranberry powder was to obtain fractions enriched in different classes of phenolic components. The absorbances at 280, 320, 360, and 520 nm were simultaneously obtained using a photodiode array detector during chromatographic separation with Sephadex LH-20 resin. The first elution solvent employed was water. The large majority of components in the crude extract that were eluted with water had a maximal absorbance at 320 nm. This was indicative of hydroxycinnamic acid type compounds. Continued elution with water produced no detectable peaks which prompted a change of elution solvent to ethanol:water (1:1, v/v). This caused subsequent detection of a majority of peaks with maximal absorbance at 520 nm which was indicative of anthocyanins. Changing the elution solvent then to ethanol caused the elution of peaks with maximal absorbance at 360 nm, which was indicative of flavonols. Ethanol/methanol (1:1, v/v) was then used to elute another set of components in which a dominant peak was observed that had an especially high absorbance at 360 nm (Fig. 1). When the elution solvent was switched to methanol, peaks with maximal absorbance at 280 nm dominated which indicated a majority of proanthocyanidins. As the absorbance at all the wavelengths subsided, the elution solvent was changed to acetone:water (4:1) which produced another set of peaks with maximal absorbance at 280 nm. This indicated that fractions enriched in simple phenolic acids (Fraction 1), anthocyanins (Fraction 2), flavonols (Fractions 3 and 4) and proanthocyanidins (Fractions 5 and 6) were prepared by the Sephadex LH-20 chromatography employed.

The relative yields of the different cranberry fractions are shown in Table 1. Fraction 5 was the most abundant fraction. Fraction 4 was the scarcest. Approximately 31% of the total phenolics determined in the crude extract were not recovered after fractionation by Sephadex LH-20 chromatography. Fractions 2–5 had similar reactivity in the total phenolate assay employed per mg of each fraction on a dry weight basis while Fractions 1 and 6 were less reactive in the assay (Table 1).

Table 1
Relative yields and quercetin equivalents in cranberry fractions

Fraction	Relative yields (%)[a]	mg quercetin equivalents per mg fraction (dry weight)
1	11.6	0.27
2	16.8	0.50
3	16.7	0.57
4	5.1	0.55
5	34.3	0.55
6	11.0	0.36
Total % recovery[b]	68.7	

[a] Relative yields are expressed as (%) individual fraction (dry weight).
[b] Total % recovery from 50 mg of crude cranberry extract loaded on the second Sephadex LH-20 column. Fraction 1A accounted for 4.3% of the recovered phenolics.

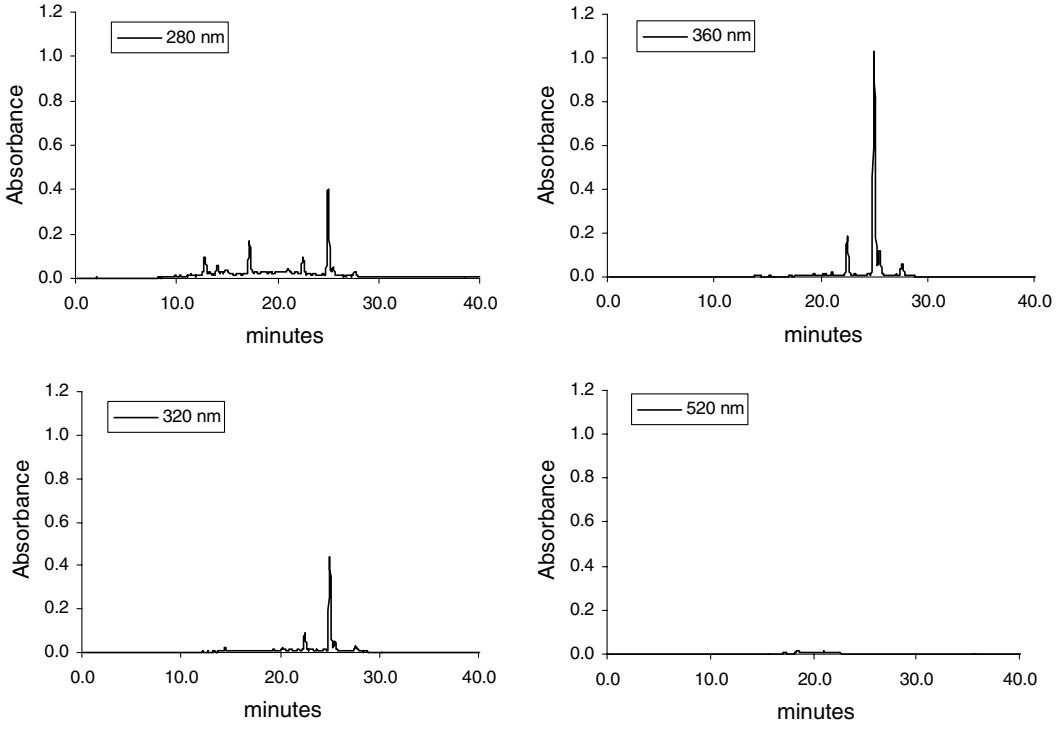

Fig. 1. Absorbance spectra obtained at 280, 320, 360, and 520 nm during sub-fractionation of Fraction 4. The chromatographic conditions are described in Section 2.

3.2. Scavenging of DPPH radicals

The cranberry crude extract and fractions were compared with ascorbic acid, propyl gallate and quercetin for free radical scavenging activity toward DPPH radicals (Tables 2 and 3). The amount of crude extract or fraction that was added to the reaction medium was standardized based on reactivity in the total phenolate assay that was used and expressed as quercetin equivalents. Each scavenger was assessed at a concentration of 1, 2.5, 5, and 10 μM. Scavenging activity was expressed as a SC_{50} value which is the concentration of component or fraction required to obtain 50% of the maximum scavenging capacity from the regression lines in the plot of % scavenging capacity vs. concentration. The scavenging activity of the crude cranberry extract ($SC_{50} = 5.09$) was similar to that of propyl gallate ($SC_{50} = 5.07$) and quercetin ($SC_{50} = 4.89$). The scavenging activity of ascorbic acid ($SC_{50} = 12.23$) was less than that of the crude extract, propyl gallate and quercetin (Table 2). Among the fractions, Fraction 6 showed the greatest scavenging activity ($SC_{50} = 4.70$), follow by Fraction 4 ($SC_{50} = 5.13$), Fraction 5 ($SC_{50} = 5.27$), Fraction 3 ($SC_{50} = 5.60$), Fraction 2 ($SC_{50} = 6.87$) and Fraction 1 ($SC_{50} = 9.47$) (Table 3).

Table 2
Percent scavenging capacities[a] of cranberry crude extracts and various low molecular weight antioxidants toward DPPH radical[b]

Treatment	Concentration			
	1 μM	2.5 μM	5 μM	10 μM
Ascorbic acid	6.0 ± 0.2	11.4 ± 1.2	19.2 ± 2.9	41.6 ± 3.8
n-Propyl gallate	14.1 ± 0.3	30.3 ± 0.4	54.4 ± 0.7	90.5 ± 2.2
Quercetin	12.1 ± 0.2	29.5 ± 0.4	58.4 ± 1.2	94.1 ± 0.2
Crude extract	14.5 ± 2.9	29.9 ± 0.5	53.5 ± 4.9	90.4 ± 2.4

[a] Percent scavenging capacity = [1 − ((Abs of cranberry crude extract/fractions − Abs of blank)/Abs control)] * 100. Control was defined as methanolic DPPH and 50% aqueous ethanol (v/v) and blank was defined as methanol and cranberry crude extract/fractions. Results are presented as average ± SD from triplicate trials and in each trial duplicate determinations were done.
[b] The final concentration of DPPH radical was 43 μM.

Table 3
Percent scavenging capacities[a] of cranberry fractions toward DPPH radical[b]

Treatment	Concentration			
	1 μM	2.5 μM	5 μM	10 μM
Fraction 1	7.7 ± 0.0	15.1 ± 0.1	28.7 ± 0.1	51.8 ± 1.0
Fraction 2	10.1 ± 0.4	22.0 ± 0.6	39.1 ± 0.4	70.0 ± 0.5
Fraction 3	10.4 ± 0.2	24.2 ± 0.9	45.7 ± 0.2	87.4 ± 2.8
Fraction 4	12.0 ± 0.5	27.0 ± 0.9	53.7 ± 4.8	91.8 ± 0.8
Fraction 5	11.8 ± 0.0	26.4 ± 0.6	47.4 ± 1.7	92.8 ± 0.5
Fraction 6	14.7 ± 0.4	33.9 ± 0.7	61.9 ± 0.7	93.4 ± 0.2

[a] Percent scavenging capacity = [1 − ((Abs of cranberry crude extract/fractions − Abs of blank)/Abs control)] * 100. Control was defined as methanolic DPPH and 50% aqueous ethanol (v/v) and blank was defined as methanol and cranberry crude extract/fractions. Results are presented as average ± SD from triplicate trials and in each trial duplicate determinations were done.
[b] The final concentration of DPPH radical was 43 μM.

3.3. Inhibition of Hb-mediated lipid oxidation in washed cod muscle by cranberry fractions

The ability of the fractions to inhibit Hb-mediated lipid oxidation in washed cod was assessed at 74 μmol quercetin equivalents per kg washed cod. Formation of thiobarbituric acid reactive substances (TBARS) was used as the index of lipid oxidation in these studies. We have previously showed a good correlation between TBARS formation and onset of rancidity in washed cod muscle containing trout Hb (Richards & Hultin, 2000). Fractions 1, 3, and 4 were more effective inhibitors of TBARS formation compared to Fractions 2, 5, and 6 (Fig. 2). Lag time (LT) defined as additional days prior to onset of TBARS formation compared to control samples comprised of washed cod and Hb were as follows: fraction 1 (LT = 5 days), fraction 3 (LT = 5 days), fraction 4 (LT = 5 days), fraction 2 (LT = 2.2 days), fraction 5 (LT = 1.1 day), and fraction 6 (LT = 0.3 day). The crude extract (LT = 3.2 days) also inhibited TBARS formation. Addition of propyl gallate inhibited TBARS formation in washed cod containing added Hb throughout the entire storage period (LT ⩾ 8 days).

Since Fractions 1, 3, and 4 effectively inhibited Hb-mediated lipid oxidation, it was desired to better understand which specific components in these fractions were exerting the inhibitory effect. Fraction 4 was chosen for further consideration because a limited number of components were

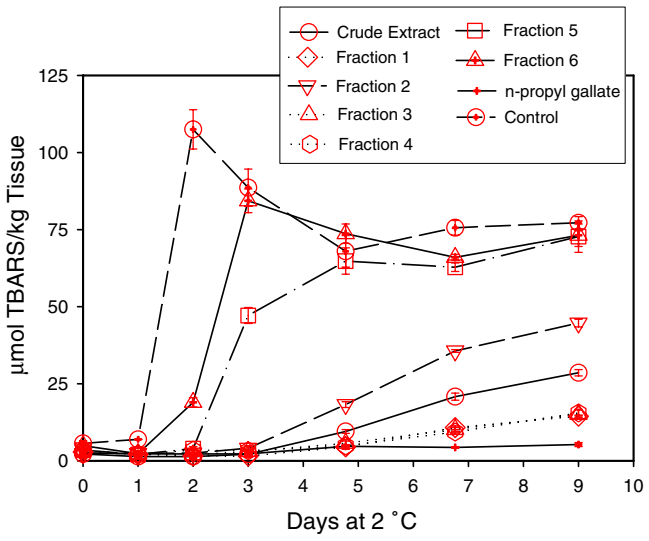

Fig. 2. Effect of cranberry fractions on the formation of thiobarbituric acid reactive substances (TBARS) in washed cod muscle. Trout hemoglobin concentration was 12 μmol per kg washed cod. Cranberry fractions were added at 74 μmol quercetin equivalent/kg washed cod. The carrier solvent for cranberry fractions was 50% aqueous ethanol (v/v) and the carrier comprised 0.3% of the final sample weight. The final pH was between 6.20 and 6.30. Results are presented as average ± SD from triplicate determinations.

obtained during sub-fractionation (Fig. 1). Sub-fractionation of the other cranberry fractions resulted in many more peaks and thus a larger number of components to consider (data not shown). The primary peak from Fraction 4 was observed at 360 nm, eluting at 25.0 min during sub-fractionation (Fig. 1). This component that eluted at 25.0 min also had absorbance at 280 and 320 nm, albeit to a lesser degree (Fig. 1). The UV–Vis spectrum of the component was determined between 200 and 500 nm and is presented in Fig. 3. The spectra obtained was indistinguishable from the UV–Vis spectra of pure quercetin (Fig. 3). Thus, one of the major flavonols in Fraction 4 was tentatively identified as quercetin. It has been reported that flavonol aglycons such as quercetin and myricetin were present in significant amount in processed concentrated cranberry product (Vvedenskaya et al., 2004). Up to 94% inhibition in TBARS formation was observed in washed cod muscle with 74 μmol quercetin/kg washed cod, while 83% inhibition in TBARS formation was shown in washed cod muscle with 37 μmol quercetin/kg washed cod (Fig. 4). These results suggest that quercetin in Fraction 4 was a potent inhibitor of Hb-mediated lipid oxidation in washed cod muscle. 37 μmol propyl gallate/kg washed cod was more effective in delaying the onset of TBARS formation compared to quercetin at the same concentration (Fig. 4).

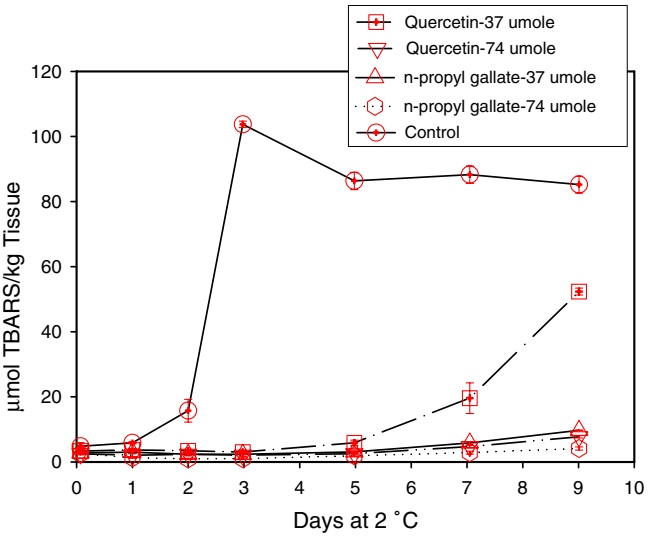

Fig. 4. Effect of quercetin and propyl gallate on lipid oxidation in washed cod muscle. Trout hemoglobin concentration was 12 μmol per kg washed cod. Quercetin and propyl gallate were added at either 37 or 74 μmol per kg washed cod. The carrier solvent for quercetin and propyl gallate was ethanol and the carrier comprised 0.4% of the final sample weight. The final pH was between 6.20 and 6.30. Results are presented as average ± SD from triplicate determinations.

3.4. Effect of washing on the ability of cranberry fractions to inhibit hemoglobin-mediated lipid oxidation

Fraction 1 (enriched in phenolic acids), Fraction 3 (enriched in flavonols) and Fraction 5 (enriched in proanthocyanidins) were added to washed cod muscle at a concentration of 74 μmol quercetin equivalents per kg washed cod which was followed by three washing steps as described in Section 2 to remove phenolics that do not bind to insoluble components of the washed cod muscle. After washing, the homogenate was centrifuged and Hb was added to the sediment to stimulate lipid oxidation

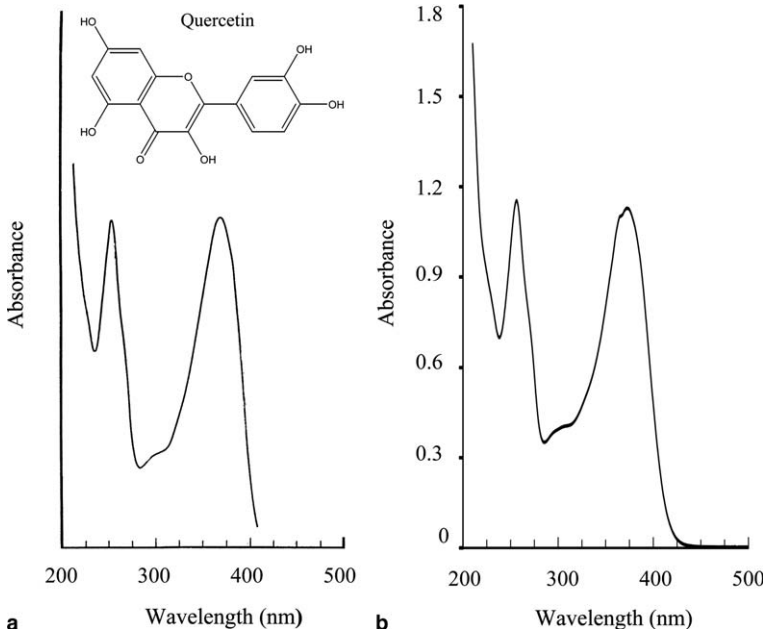

Fig. 3. UV–Vis spectra of: (a) quercetin and (b) primary peak in cranberry Fraction 4. Data for the spectrum of quercetin were taken with permission (Mabry et al. (1970)). Spectrum of primary peak in cranberry Fraction 4 was obtained by a reverse phase HPLC-diode array detector with chromatographic conditions described in Section 2.

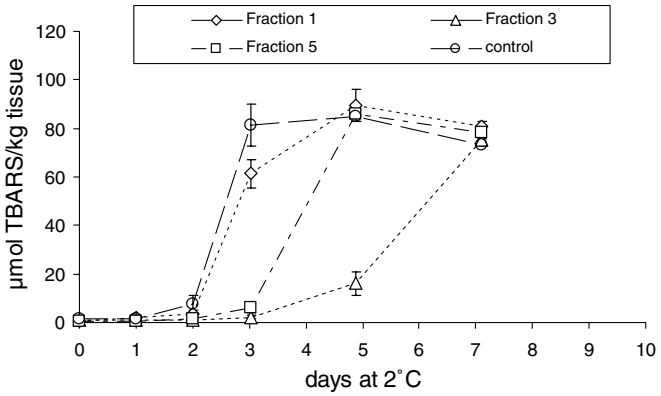

Fig. 5. Effect of three washing steps on the ability of cranberry fractions to inhibit hemoglobin-mediated thiobarbituric acid reactive substances (TBARS) formation. Trout hemoglobin was added into the system at the final concentration of 12 µmol per kg washed cod. Fractions were added at 74 µmol quercetin equivalents per kg washed cod.

processes. After the washing steps, Fraction 1 did not inhibit Hb-mediated lipid oxidation, Fraction 5 extended the lag time around 1 day while Fraction 3 extended the lag time around 3 days (Fig. 5). Without the washing step, Fractions 1, 3, and 5 extended the lag phase 5, 5, and 1 day, respectively (Fig. 2).

4. Discussion

Our results showed different degree of DPPH radical scavenging activity when comparing fractions enriched in phenolic acids, anthocyanins, flavonols, and proanthocyanidins (Table 3). The ability of components to scavenge DPPH radicals depends on the structural arrangement of the molecule such as the quantity and location of phenolate groups as well as the degree of glycosylation (Brandwilliams, Cuvelier, & Berset, 1995; Fukumoto & Mazza, 2000; Nenadis, Zhang, & Tsimidou, 2003). Increasing the number of phenolate groups increased the radical scavenging activities of benzoic and cinnamic acid derivatives, anthocyanidins and flavonols. Glycosylation reduced the radical scavenging activities of quercetin, cyanidin, pelargonidin and peonidin.

It is tempting to speculate that the ability of a fraction to scavenge DPPH radicals could be used to predict the ability of that fraction to inhibit lipid oxidation in a food system. Our results indicate this was not the case. For example, Fraction 6 was the most efficient scavenger of DPPH radical compared to other fractions (Table 3) but was weakly inhibitory to Hb-mediated lipid oxidation compared to other fractions (Fig. 2). When using pure components, quercetin and propyl gallate had nearly equal DPPH scavenging activity but propyl gallate was the superior inhibitor of Hb-mediated lipid oxidation (Table 2 and Fig. 4). Thus, it appears that the ability of components to scavenge DPPH radicals does not adequately predict their ability to inhibit Hb-mediated lipid oxidation in washed cod muscle.

An important consideration when examining fractions from a heterogeneous starting material such as cranberry powder is the basis on which to add the fractions to the system under investigation, in our case washed cod muscle or DPPH radicals. One method is to add the fractions on a dry weight basis. The advantage of adding on a weight basis is that the utility of all the extracted materials in each fraction are assessed relative to the extracted materials in other fractions. The disadvantage is that a fraction which contains a particular potent inhibitor of lipid oxidation may not be realized due to dilution from an excess of non-inhibitory components. A second approach is to add the fractions based on the use of a colorimetric assay that estimates the number of phenolate (–OH) groups in each fraction. In the assay we employed, phenolate groups are oxidized by metal-containing heteropoly acids that become partially reduced from the +6 to a mixture of +6 and +5 valence states resulting in the production of a chromophore (Singleton & Rossi, 1965). We decided to add the fractions based on their reactivity in the colorimetric assay to provide a better opportunity of discovering the most active inhibitors of a phenolic nature in each fraction. Fraction 1 had 0.27 mg phenolate equivalents per mg of the fraction on a dry weight basis while Fraction 5 possessed 0.57 mg phenolate equivalents/mg dry weight (Table 1). This indicates that more total phenolate groups are present in Fraction 5 per mg dry weight compared to Fraction 1. By adding Fractions 1 and 5 to the model systems on a phenolate basis, the total amount of phenolates is standardized but 2.1 times more of Fraction 1 was added on a dry weight basis (Table 1).

It must also be kept in mind that the quantity of phenolate groups can relate poorly to the degree of inhibition of lipid oxidation processes. For example catechin, epicatechin and quercetin each contain 5 phenolate groups per molecule, but their ability to inhibit Fe^{2+}-mediated lipid oxidation in liposomes varied widely (Liao & Yin, 2000). Quercetin partitioned more efficiently into the lipid phase compared to catechin (6.4-fold difference) and epicatechin (4.1-fold). Consistent with the partitioning, catechin more effectively inhibited Fe^{2+}-mediated lipid oxidation compared to quercetin (2.2-fold difference) and epicatechin (1.2-fold). Thus the physical location of the inhibitory components was more important in dictating inhibitory efficacy than the number of phenolate groups that were present.

The binding affinity of phenolics to insoluble muscle components (e.g., myofibrillar proteins and membrane phospholipids) could influence the ability of phenolics to inhibit lipid oxidation processes. Previously, cranberry fractions were added to serum prior to isolating low density lipoproteins (LDL) and it was found that the proanthocyanidin-enriched fractions (Fractions 5 and 6) most effectively increased the lag time of Cu^{2+}-induced LDL oxidation compared to Fractions 1–4 (Porter et al., 2001). Apparently the proanthocyanidins remained associated with the LDL during purification whereas the other fractions were washed away. We found that Fraction 3

was more effective than Fractions 1 and 5 in inhibiting TBARS formation in washed cod when a washing step was employed to wash away loosely bound phenolic components prior to adding Hb catalyst to the sediment after washing (Fig. 5). Apparently some flavonols in Fraction 3 were removed by the washing step because their ability to inhibit Hb-mediated lipid oxidation was diminished compared to when the components were added and not exposed to a washing treatment (Figs. 2 and 5). Fraction 5 was weakly inhibitory to Hb-mediated lipid oxidation whether a washing step was employed or not; the lag phase was extended around 1 day with or without the washing step (Figs. 2 and 5). These results suggest that Fraction 5 has a higher binding affinity for insoluble components in washed cod muscle while Fraction 3 retained some binding affinity and was a better inhibitor of Hb-mediated lipid oxidation.

It is interesting that Fraction 5 better inhibited Cu^{2+}-stimulated lipid oxidation in the LDL system than Fraction 3 (Porter et al., 2001) while Fraction 3 was a better inhibitor of Hb-mediated lipid oxidation in the washed cod muscle systems (Figs. 2 and 5). It may be that proanthocyanidins inactivate Cu^{2+}-mediated lipid oxidation better than flavonols while flavonols are more potent inhibitors of Hb-mediated lipid oxidation. A grape seed extract rich in oligomeric proanthocyanidins was more effective at inhibiting lipid oxidation in algal oil-in-water emulsions than catechin, a flavonol (Hu, McClements, & Decker, 2004). Lipid oxidation in oil-in-water emulsions is attributed to the action of metals such as iron and copper present in the aqueous phase (Diaz, Dunn, McClements, & Decker, 2003). This begs the question as to which form of Hb (e.g., the water-soluble ferryl heme protein catalyst, released heme, or released iron) stimulates lipid oxidation processes. The ability of flavonols to orient in the membrane bilayer has been shown (van Acker, Schouten, Haenen, van der Vijgh, & Bast, 2000). Having the antioxidant molecule located in the lipophilic membrane would be especially useful in the scavenging of lipophilic hemin radicals and alkoxyl radicals that formed during the reaction of released heme with preformed lipid peroxides (Tappel, 1955). The more bulky proanthocyanidins are less likely to orient in the membrane bilayer which can explain the poor ability of proanthocyanidin-enriched fractions to inhibit Hb-mediated lipid oxidation.

There are numerous factors that can explain why cranberry fractions that most effectively scavenged DPPH radicals were least effective in inhibiting lipid oxidation processes in washed cod (Table 3 and Fig. 2). The DPPH system was conducted in a polar medium (water:ethanol:methanol, 1:1:5, v/v/v) at ambient temperature and without any additional catalyst. In washed cod muscle, hemoglobin promoted oxidation of the lipids while DPPH radicals are never produced. Further, in a heterogeneous system such as washed cod muscle, the physical location and orientation of cranberry components in the membrane phospholipids is likely relevant. The ability of the flavonol fractions to effectively inhibit lipid oxidation in washed cod might be due to the ability of flavonols to modulate the biomembrane fluidity in such a way that limits the diffusion of free radicals (Arora, Byrem, Nair, & Strasburg, 2000; Caturla, Vera-Samper, Villalain, Mateo, & Micol, 2003). Myofibrillar proteins of washed cod could also decrease the free radical scavenging ability of certain cranberry phenolics. The interaction between tea flavonoids and milk proteins diminished radical scavenging toward 2,2′azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical (Arts et al., 2002).

Preparing isolated fractions from a crude cranberry extract was more effective at inhibiting Hb-mediated lipid oxidation than the crude extract itself (Fig. 2). This demonstrates the potential utility of isolating certain classes of polyphenolics from a crude plant extract to increase efficacy. At the same time propyl gallate, a synthetic antioxidant, was more effective at inhibiting Hb-mediated lipid oxidation than any of the cranberry fractions (Fig. 2). These results indicate the difficulties that will likely be encountered when attempting to produce natural plant extracts that are competitive with synthetic antioxidants.

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