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Identification of the compound in a potent cranberry juice extract that inhibits lipid oxidation in comminuted muscle ☆

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ABSTRACT

An extract prepared from cranberry juice powder using a mixture of chloroform and methanol was particularly effective at inhibiting lipid oxidation in mechanically separated turkey (MST), providing more than 3 weeks of additional stability during −4 °C storage at a usage level of 0.1% (wet weight basis). The chloroform extract was fractionated by flash chromatography (FC) and analysed using reverse-phased high performance liquid chromatography (RP-HPLC) with UV/vis diode array detection to identify the component(s) present in the fractions. One of the five fractions obtained was effective in delaying the formation of lipid peroxides and thiobarbituric acid reactive substances (TBARS) in MST, while the remaining fractions had little to no inhibitory action. Mass spectrometry (MS) analysis indicated the presence of two flavonols, quercetin and quercetin-3-*O*-(6″-benzoyl)-*β*-galactoside in the inhibitory fraction. This fraction (containing quercetin at 467 μmol/kg MST) inhibited lipid oxidation in MST similarly to pure quercetin added to MST at the same concentration. This indicated that quercetin accounted for all or nearly all of the inhibitory capacity in the chloroform extract.

1. Introduction

Quality deterioration in muscle foods caused by lipid oxidation is a major problem in the industry. Odour, flavour, texture, colour and nutritional value are negatively affected by lipid oxidation (Kanner, 1994). Conventional antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiarybutyl hydroquinone (TBHQ) and propyl gallate (PG) have been frequently used to minimise oxidative changes in meat systems. Addition of natural antioxidants is now being preferred over synthetic antioxidants due to better consumer acceptability. Phenolic compounds widely distributed in the edible and non-edible parts of plant materials are natural sources of antioxidants (Bahorun, Luximon-Ramma, Crozier, & Aruoma, 2004; Kahkonen et al., 1999; Vagi et al., 2005). A wide range of plant polyphenolics have been evaluated and tested for their antioxidant properties in unsaturated marine oils, meat and fish muscle model systems (Han & Rhee, 2005; He & Shahidi, 1997; Wanasundara & Shahidi, 1996).

American cranberry, *Vaccinium macrocarpon* Ait., has been found to possess the highest phenolic content compared to a large number of fruit species tested (Sun, Chu, Wu, & Liu, 2002). The phenolic classes identified in cranberry include phenolic acids, anthocyanins, flavonol glycosides, procyanidins and proanthocyanidins (Chen, Zuo, & Deng, 2001; Foo, Lu, Howell, & Vorsa, 2000; Hong & Wrolstad, 1990; Puski & Francis, 1967; Sun et al., 2002). The mechanisms by which the phenolic compounds influence lipid oxidation rates include free radical scavenging (Nawar, 1996) and metal ion chelation (Hudson & Lewis, 1983). Among the phenolics, flavonoids have received much attention as potential antioxidants in food lipid systems (Hudson & Lewis, 1983; Ramanathan & Das, 1993). The basic structure of a flavonoid has two benzene rings (A and B) that are linked through oxygen containing pyran ring (C) (Fig. 1). The various flavonoid classes (flavones, flavanones, flavanols, flavonols, etc.) differ in the pattern of substitution of the C-ring, whereas the compounds within a class differ in the pattern of substitution of the A- and B-rings. The free radical scavenging activity of the flavonoids have been attributed to the hydroxyl groups in the B-ring as well as the 2, 3 double bond in conjugation with a 4-oxo group in the C-ring whereas the binding sites for trace metals to flavonoids are the 3- or 5-hydroxyl- and 4-oxo groups or the hydroxyl groups in the B-ring (Hudson & Lewis, 1983; Pietta, 2000).

Flavonol aglycones like quercetin and myricetin have been reported to be present in freshly squeesed cranberry juice (Chen et al., 2001) and also in cranberry juice powder (Vvedenskaya et al., 2004). Quercetin and myricetin have been reported to be efficient antioxidants in methyl linoleate systems (Pekkarinen, Heinonen, & Hopia, 1999). Cranberry phenolics were shown to

Abbreviations: MST, mechanically separated turkey; HPLC, high performance liquid chromatograph; MS, mass spectrometry; TBARS, thiobarbituric acid reactive substances; Fr., fraction; DE, dielectric constant.

☆ Cranberry components inhibit lipid oxidation in muscle tissue.

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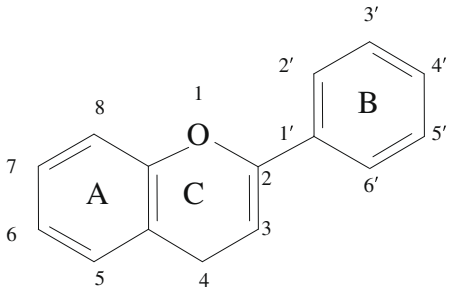

Fig. 1. Basic structure of flavonoids.

inhibit haemoglobin-mediated lipid oxidation in washed fish muscle (Lee, Krueger, Reed, & Richards, 2006), cooked pork and raw mechanically separated turkey (MST) (Lee, Reed, & Richards, 2006). These studies in muscle systems suggested that flavonol aglycones were particularly effective at inhibiting lipid oxidation; however the compound(s) responsible for inhibition were not definitively identified.

The main objective of this work was to identify the compound(s) in cranberry juice powder that are responsible for the inhibition of lipid oxidation in mechanically separated turkey (MST). MST is widely used in the formulation of non-fermented sausages such as bologna and hotdogs. MST is highly sensitive to rancidity development, the reasons being the preparation process and compositional nature of the material. MST is prepared by passing turkey frames through a mechanical device to separate tissues from skeletal frames during which the tissues are subject to aeration. The tissue is finely comminuted as it passes through small sieving. Moreover, it contains bone marrow rich in haemoglobin, lipids, and low levels of endogenous tocopherols which make MST prone to lipid oxidation (Dawson & Gartner, 1983). Adding cranberry phenolics delays the onset of oxidative rancidity in MST and identifying the primary inhibitor(s) can be used to develop procedural aspects of preparing especially potent extracts.

2. Materials and methods

2.1. Materials

Spray dried cranberry concentrate juice powder 90-MX was supplied by Ocean Spray Cranberries Ingredient Technologies Group (Lakeville-Middleboro, MA, USA) and was stored at −20 °C until use. Mechanically separated turkey (MST) was obtained from Kraft-Oscar Mayer (Newberry, SC, USA) and was vacuum packaged and stored at −80 °C until use. Sealable, polyethylene bags (10 cm × 15 cm) and vacuum pouches (3 mil standard barrier) were purchased from Koch Supplies (Chicago, IL, USA). Ethanol (absolute, 200 proof) was obtained from Aaper Alcohol and Chemical Co. (Shelbyville, KY, USA). 2-Thiobarbituric acid, sodium hydroxide, trifluoroacetic acid, propyl gallate, ferrous sulphate, Folin–Ciocalteu reagent, quercetin dihydrate and were procured from Sigma A/S (St. Louis, MO, USA). Silica Gel 60 RP-18, ammonium thiocyanate, barium chloride, hydrochloric acid, trichloroacetic acid and sodium carbonate were purchased from Fisher Scientific (Chicago, IL, USA). The solvents used were of HPLC grade.

2.2. Preparation of chloroform extract from cranberry juice powder

Hydrophobic constituents from 60 g cranberry concentrate juice powder were extracted with 1000 ml of chloroform:methanol (1:1, v/v) by blending with a magnetic stirrer at room temperature for 1 h. The aqueous and the organic phases were separated by the addition of 400 ml (v/v) of 0.5% NaCl (w/v) in a separatory funnel and were inverted twice for adequate mixing. The mixture was allowed to stand for 24 h and the chloroform phase was then evaporated under reduced pressure by rotary evaporation at 35 °C. Ten to fifteen millilitres of water was then added to the extract and was freeze dried (Model 4451 F, Labconco, Kansas city, MO, USA). The yield obtained for the chloroform extract ranged between 185 and 230 mg/60 g of cranberry juice powder. The final dry/powdery extract, referred to as cranberry chloroform extract was stored at −80 °C until testing or analyses.

2.3. Fractionation of chloroform extract using flash chromatography

Separation of phenolic constituents from chloroform extract was carried out by flash chromatography. For fractionation, 0.4–0.5 g of cranberry chloroform extract was dissolved in 5 ml of ethanol and mixed with 11 g of reverse phase silica gel. Ethanol was removed by rotary evaporation under reduced pressure at 35 °C. The dry silica gel with the cranberry components was then loaded on to a guard column (ID = 2.5 cm, length = 15 cm) packed with 13 g silica at the bottom. Six grams of silica were then added on top so as to form a sandwich layer. The second column (ID = 4.8 cm, length = 30 cm), loaded with reverse phase silica gel and conditioned with 30% aqueous methanol (v/v) was connected to a programmable CombiFlash Foxy Jr. fraction collector (Isco, Inc., Lincoln, NE, USA) equipped with collection tubes. Phenolic constituents were eluted from the guard column to the second column using aqueous methanol (30% v/v) until the pigmented components reached three-fourth the length of the second column after which an elution programme was used. Fractionation was carried out in a binary solvent system: solvent A, 0.1% trifluoroacetic acid in water; and solvent B, 0.1% trifluoroacetic acid in methanol. A linear gradient of 30–50% B from 0 to 64 min; an isocratic elution with 50% B from 64 to 144 min; a linear gradient of 50–70% B from 144 to 234 min; an isocratic elution with 70% B from 234 to 288 min; and a linear gradient of 70–100% B from 288 to 348 min was used. A flow rate of 7 ml/min was used for the process. The absorbance was determined at multiple wavelengths of 280, 320, 360 and 520 nm by an optical microtitre plate scanner (SpectraMax plus, Molecular Devices, Sunnyvale, CA, USA). The scan showed a separation of five peaks and were referred to as Fr. 1, Fr. 2, Fr. 3, Fr. 4 and Fr. 5 based on the order in which they were eluted. The yields obtained for the five fractions are shown in Table 1. Each fraction was concentrated under vacuum by rotary evaporation at 35 °C and lyophilisation. Subsequently, all fractions were stored at −80 °C until use.

2.4. HPLC apparatus and chromatographic conditions

HPLC analysis of chloroform extract, fractions and flavonols were performed on an Agilent 1100 HPLC system equipped with a Agilent 1100 series binary pump and photodiode array detector (Agilent, Wilmington, DE, USA). The binary solvent system used

Table 1
Yields of fractions (mg) obtained from chloroform extract (g) prepared from cranberry juice powder.

Fraction	Yields (mg)[a]
1	68–80
2	40–42
3	177–190
4	45.8–47
5	151–167

[a] Yields are expressed on a dry weight basis.

was: solvent A, 0.1% trifluoroacetic acid in water; and solvent B, 0.1% trifluoroacetic acid in methanol. Approximately 1–1.2 mg of each fraction was dissolved in 1 ml of solvent A: solvent B (1:1 v/v) and 50 μl were injected onto a Discovery®C18 reversed phase column (5 μm, 25 cm × 0.46 cm id) (Supelco, Bellefonte, PA, USA). A linear gradient of 0–100% B from 0 to 40 min was carried out at a flow rate of 1 ml/min. Multiwavelength detection was monitored at 280, 320, 360 and 520 nm. Quercetin was used as the standard.

2.5. Mass spectrometry (MS)

MS analysis and fragmentation experiments were performed on a Shimadzu LCMS-2010A mass spectrometer equipped with an electrospray ionisation (ESI) source and a single quadrupole mass analyser. The mass spectrometer was operated in the negative ion mode (Vvedenskaya et al., 2004) and under the following conditions: source voltage, 1.5 kV; and capillary temperature, 250 °C. Separations were carried out in a Discovery Bio Wide Pore C18 (5 μm packing, 25 cm × 0.21 cm id) reversed phase column protected with a guard column of the same material (Supelco, Bellefonte, PA, USA). The elution conditions were as follows: 0.200 ml/min flow rate; oven temperature, 40 °C; solvent A, water/formic acid (99.6:0.4, v/v); and solvent B, acetonitrile/formic acid (99.8:0.2, v/v). An isocratic elution with 0% B from 0 to 2 min; a linear gradient of 0–25% B from 2 to 10 min; an isocratic elution with 25% B from 10 to 18 min and a linear gradient of 25–100% B from 18 to 40 min was used. This was followed by washing and re-equilibration of the column. UV–vis spectra were recorded from 220 to 600 nm. Spectra were scanned over a mass range of m/z 100 to m/z 2000 at 1.0 s per cycle.

2.6. Determination of total phenol content

Total phenol content in cranberry fractions was quantified by a modified spectrophotometric method (Singleton & Rossi, 1965) with quercetin as a reference standard. A 2.5 ml aliquot of 10× diluted (with water) Folin–Ciocalteau reagent was added to 25 μl of cranberry fraction. Two millilitres of 7.5% sodium carbonate was added into the resulting mixture after 5 min. The absorbance was determined at 760 nm by a double beam spectrophotometer model UV-2401 (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) after the incubation of mixture at 22–25 °C for 2 h. Duplicate reactions were performed for each fraction. Total phenol content was expressed as μmol quercetin equivalents/g of freeze dried cranberry fraction.

2.7. Addition of chloroform extract, fractions and quercetin to MST

MST was vacuum packaged in 500 g portions and stored at −80 °C until use. The frozen packs were then thawed for 2 h at room temperature (without breaking the vacuum seal) before using them for treatments. The chloroform extract was added to MST at varied levels (0.01%, 0.05%, 0.10%, 0.20% on a final sample weight basis) while the fractions were added at a level of 0.1% (w/w). In trials on the effect of quercetin in Fr. 4, 3.16 mg of pure quercetin was added to 20 g of MST. This amount was added based on the amount of quercetin present in Fr. 4 as determined by HPLC. Fr. 4 was separately added to MST at a level of 0.1% (w/w). The chloroform extract, fractions and quercetin were mixed with MST using a plastic spatula for 3 min and transferred into zip lock bags. Samples were stored at −4 °C for a period of 53 days. The carrier solvent was 100% ethanol and was used at 1% of the final sample weight. A control sample containing 1% ethanol without the cranberry chloroform extract/fractions was also prepared for the respective trials.

2.8. Determination of thiobarbituric acid reactive substances (TBARS)

The progress of lipid oxidation in MST was followed by periodically removing samples and quantifying TBARS formed by the method described by Lemon (1975). Approximately, 1 g of the sample was mixed with 6 ml of trichloroacetic acid (TCA) solution (7.5% TCA, 0.1% disodium ethylenediamine tetraacetic acid [EDTA], 0.1% propyl gallate) and homogenised with a Polytron Type (PT) 10/35 (Brinkmann Instruments, Westbury, NY) for 30 s. The homogenate was filtered through Whatman no. 1 filter paper. One millilitre of the filtrate was then mixed with 1 ml of thiobarbituric acid (TBA) (0.02 M) and incubated at 100 °C for 40 min. After cooling the reaction mixture in cold water for 5 min and centrifuging (2000*g* for 5 min) in a Beckman J-6B centrifuge (Beckman Instruments, Inc., Palo Alto, CA), the absorbance was measured at 532 nm. TBARS value was expressed as μmol TBARS/kg of tissue. A standard curve was constructed using tetraethoxypropane as a precursor of malonaldehyde.

2.9. Determination of lipid peroxides

Lipid peroxides were determined according to the method described by Richards and Dettmann (2003). Approximately 0.3 g of the tissue was homogenised in 10 ml of chloroform:methanol (1:1 v/v). 3.08 ml of 0.5% NaCl was then added to separate the mixture into two phases. The mixture was then centrifuged at 2000*g* for 5 min and 2.0 ml of chloroform layer was removed using a glass syringe. Chloroform: methanol (1:1 v/v; 1.33 ml) was added to the removed aliquot followed by addition of 25 μl of ammonium thiocyanate and 25 μl of fresh ferrous chloride solution (made by mixing equal volumes of barium chloride (8 g/l) in 0.4 N HCl and ferrous sulphate (20 g/l)). After incubation at room temperature for 20 min, absorbance was read at 500 nm. The standard used was cumene hydroperoxide.

2.10. Statistical analysis

The MIXED procedure of the SAS system was used to analyse the data obtained from lipid oxidation. This procedure implements random effects in the statistical model and permits modelling the covariance structure of the data (Littell, Henry, & Ammerman, 1998). For the lipid oxidation studies, two or three separate reaction mixtures for each treatment were prepared and used as the source of variation for the statistical analysis. One or two indicators of lipid oxidation were measured for each treatment during the storage study. Means were separated by least significance differ-

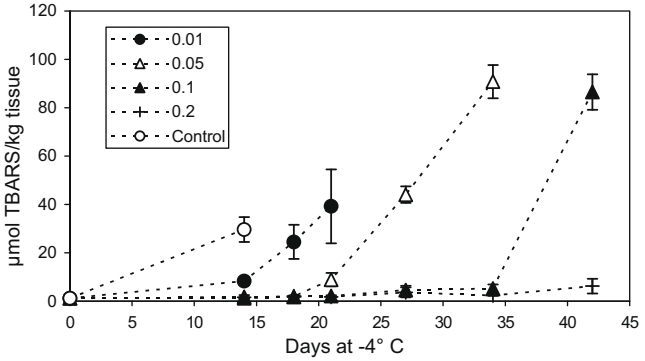

Fig. 2. Effect of varying levels (0.01%, 0.05%, 0.1% and 0.2% of muscle weight) of the chloroform extract prepared from cranberry juice powder on the formation of thiobarbituric acid reactive substances (TBARS) in MST. Ethanol was used as the carrier solvent at 1% of the final sample weight. Results are expressed as average ± standard deviation from triplicate samples.

ence test. Samples were drawn from each experimental unit at various time points and hence repeated measures were obtained for each treatment. In this model, the variation between the experimental units within a treatment is specified by the 'random' statement where as the covariation within the experimental units is specified by the 'repeated' statement. Since the repeated measures were not taken at equal intervals of time, 'sp(pow)(d)' was fitted into the model to account for this variation. Significance was defined at $p \leqslant 0.05$.

3. Results

3.1. Inhibition of lipid oxidation in MST by chloroform extract from cranberry juice powder

A fairly lipophilic extract was prepared from cranberry juice powder by extracting into chloroform. The chloroform phase was evaporated, freeze dried and used for lipid oxidation studies. Ethanol was used as the carrier solvent at 1% of the final sample weight.

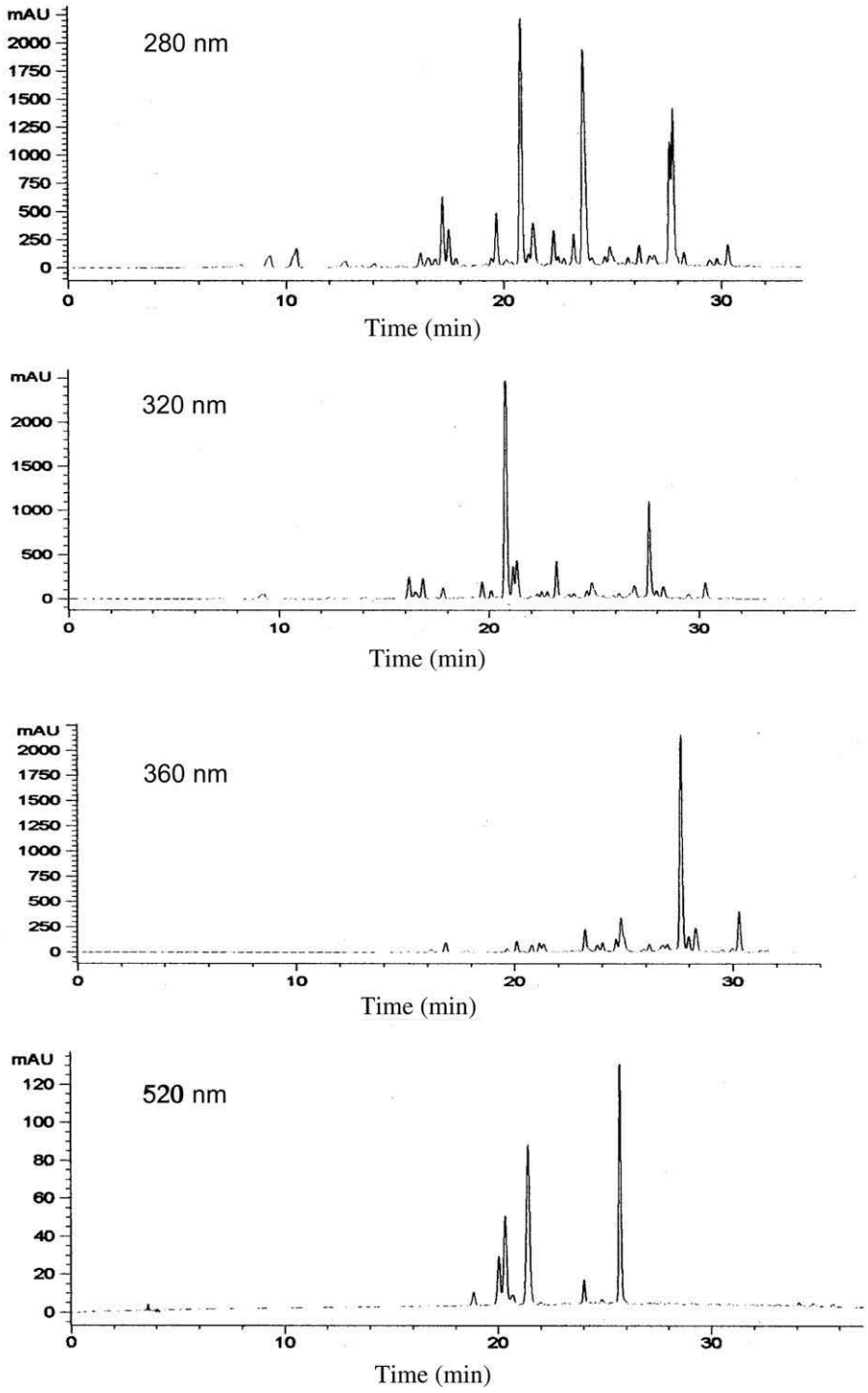

Fig. 3. HPLC chromatogram of chloroform extract prepared from cranberry juice powder. Spectra were obtained at 280, 320, 360 and 520 nm with chromatographic conditions described in Section 2.

The freeze dried extract was soluble in ethanol which facilitated addition of extract to MST. The ability of the chloroform extract to inhibit lipid oxidation in MST was assessed at four different levels (0.01%, 0.05%, 0.1% and 0.2% based on wet weight of the MST). Thiobarbituric acid reactive substances (TBARS) were used as an indicator of lipid oxidation during frozen (−4 °C) storage. The lag phase was defined as the approximate time it took for TBARS values to exceed 20 μmol/kg of MST during the storage period. TBARS reached 20 μmol/kg within a 9 day period in the control, whereas the samples containing 0.01%, 0.05% and 0.1% of extract took additional 8, 14, and 27 days, respectively, to reach the 20 μmol/kg threshold. The sample with 0.2% extract had only reached a TBARS value of 6 μmol/kg during the storage period of 43 days (Fig. 2).

3.2. Fractionation of phenolic constituents by flash chromatography and HPLC analysis

Characterisation of the chloroform extract was carried out by reverse phase high performance liquid chromatography (RP-HPLC) with a photo diode array (PDA) detector which indicated the presence of more than 20 compounds. The spectra obtained at multiple wavelengths are shown in Fig. 3. In order to obtain substantial quantities of sub-fractions, the chloroform extract was fractionated on silica gel 60 RP-18 using a non-linear methanol:water gradient. The absorbance of the eluates was determined using a plate scanner at multiple wavelengths of 280, 320, 360 and 520 nm. The scan showed a separation of five peaks and was designated as Fr. 1, Fr. 2, Fr. 3, Fr. 4 and Fr. 5. The predominant classes of cranberry phenolics in each fraction can be estimated based on absorbance values at 280 nm (proanthocyanidins), 320 nm (cinnamic acids), 360 nm (flavonols) and 520 nm (anthocyanins) (Porter, Krueger, Wiebe, Cunningham, & Reed, 2001). RP-HPLC analysis of these fractions with PDA detection indicated that Fr. 1, Fr. 2, Fr. 3 and Fr. 5 were composed primarily of phenolics with maximum absorbance at 280 and 320 nm. The chromatogram of Fr. 4 indicated the presence of two peaks with maximal absorbance at 360 nm, which is characteristic of flavonols and three other peaks with maximal absorbance at 280 nm, characteristic of proanthocyanidins (Fig. 4a). The UV/vis spectra of the two peaks (peaks F1 and F2) presented in Fig. 4b and c are characteristic of flavonols. One of the flavonols

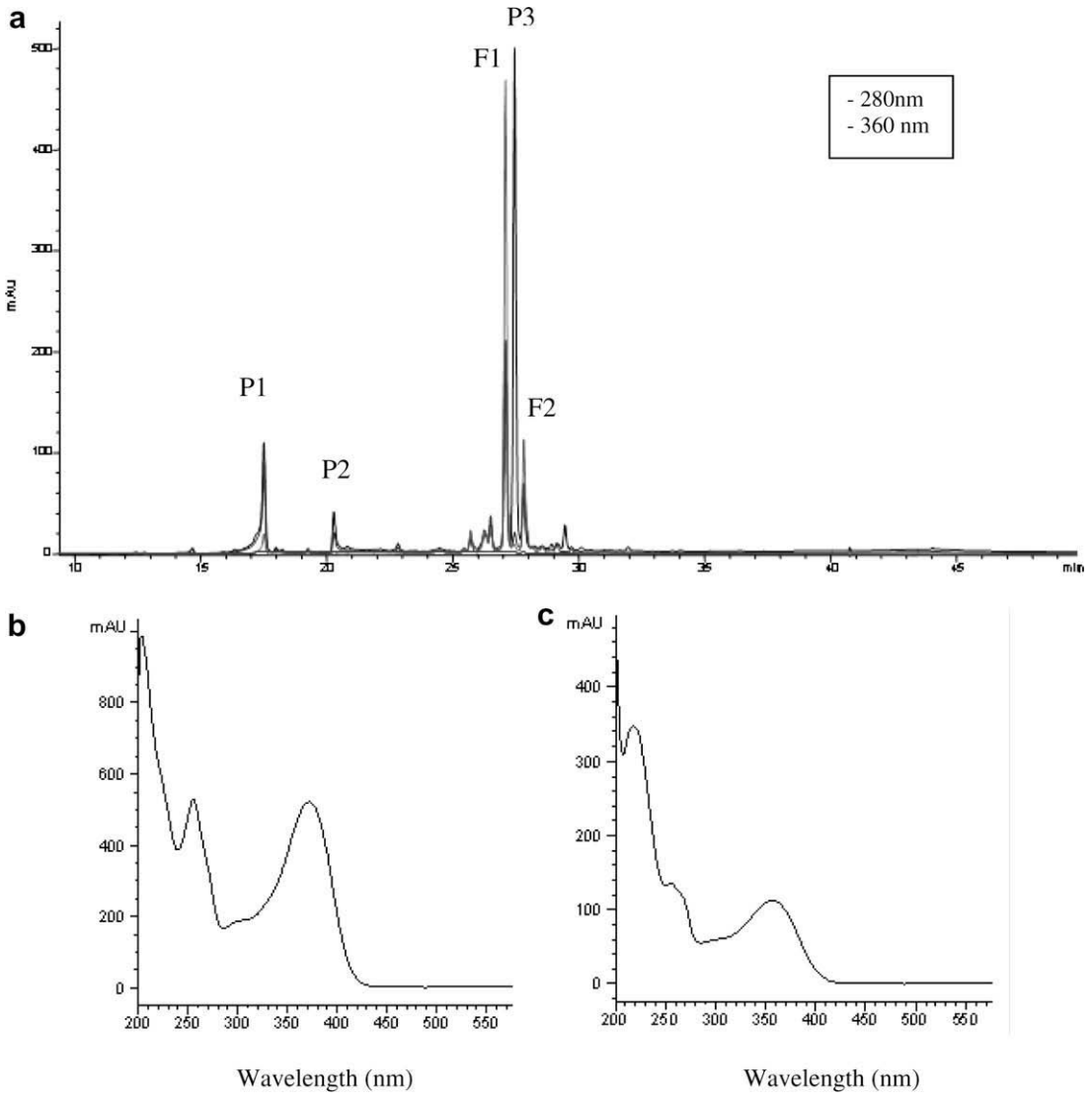

Fig. 4. HPLC chromatogram of Fr. 4 (a). Peaks P1, P2 and P3 correspond to compounds with maximal absorbance at 280 nm and peaks F1 and F2 correspond to flavonols with maximal absorbance at 360 nm. UV/vis spectra of peaks F1 (b) and F2 (c) are also shown. Spectra of peaks F1 and F2 were obtained by RP-HPLC-diode array detector with chromatographic conditions described in Section 2.

(peak F1) was tentatively identified as quercetin based on the retention time against the pure standard and spectrophotometric characteristics measured from 500 to 200 nm (Mabry, Markham, & Thomas, 1970).

3.3. Total phenolic content of fractions and their ability to inhibit lipid oxidation in MST

The fractions varied widely in the total phenolic content, with Fr. 4 containing larger amount of phenolics followed by Fr. 2, Fr. 1, Fr. 3 and Fr. 5. (Fig. 5). The fractions were tested for their ability to inhibit lipid oxidation in MST during −4 °C storage. Two indicators of lipid oxidation (thiobarbituric acid reactive substances or TBARS and lipid peroxides formation) were measured. Each fraction was added at 0.1% based on sample wet weight. Fr. 4 was the most effective of all the fractions tested, inhibiting TBARS formation for up to 40 days when compared to MST alone ($p \leqslant 0.001$), which was extensively oxidised in 13 days (Fig. 6a). Fr. 1, Fr. 3 and Fr. 5 did not inhibit TBARS formation ($p > 0.05$). Fr. 2 inhibited TBARS formation in MST when compared to the control ($p < 0.05$) but not nearly as effectively as Fr. 4. In brief, the overall effectiveness of the fractions in controlling lipid oxidation of MST in terms of TBARS measurements was: Fr. 4 ≫ Fr. 2 > Fr. 1 = Fr. 3 = Fr. 5. The content of lipid peroxides in MST treated with the fractions is shown in Fig. 6b. Similar to TBARS values, Fr. 4 was a much more potent inhibitor of lipid peroxide formation in MST compared to the other fractions. Thus, the identity of the phenolic compounds present in Fr. 4 was sought using mass spectrometry.

3.4. Mass spectrometry (MS) of fraction that effectively inhibited lipid oxidation in MST

MS analysis in the negative ion mode was carried out in order to identify the compounds in Fr. 4. Fig. 7 shows the chromatogram of Fr. 4 at 360 nm absorbance, the mass spectra of the two major peaks eluted and the chemical structures of the two flavonols identified. The first major peak (R_t 39 min) was identified as quercetin as it was found to be consistent with the retention time and UV/vis spectra of the pure standard. Moreover it exhibited a $[M–H]^-$ ion peak at m/z 301 corresponding to quercetin aglycone. The peak corresponding to m/z 611 was observed both in the first peak of Fr. 4 as well the quercetin standard. The spectrum of the second peak (R_t 39.8 min) gave $[M–H]^-$ ion peak at m/z 567 which was identified as quercetin-3-*O*-(6″-benzoyl)-*β*-galactoside, a flavonol glycoside isolated from cranberry powder (Vvedenskaya et al., 2004). None of the other compounds in Fr. 4 could be identified by mass spectrometry.

3.5. Role of quercetin in inhibiting lipid oxidation in MST

Fr. 4 was a potent inhibitor of lipid oxidation in MST. Whether the observed potency of Fr. 4 was due to the additive effect of the compounds or due to an individual compound was unknown. In order to address this issue, a direct comparison between Fr. 4 and one of the flavonols identified in Fr. 4 in terms of inhibiting lipid oxidation in MST was carried out.

Quercetin was one of the major flavonols identified in Fr. 4 and was chosen for the comparative study due to its ready availability. Standard for quercetin-3-*O*-(6″-benzoyl)-*β*-galactoside could not be obtained. Quantitative determination of quercetin in Fr. 4 was performed by RP-HPLC using quercetin as a standard. Quercetin content was estimated to be 0.158 mg/mg of Fr. 4. Fr. 4 was added to MST at 0.1% which indicates that the fraction contained 467 μmol quercetin/kg of MST. An equivalent concentration of pure quercetin was added to separate samples of MST and the storage study was conducted at −4 °C. TBARS and lipid peroxides were measured and the data are presented in Fig. 8. There was no significant difference ($p \geqslant 0.05$) between pure quercetin and Fr. 4 in inhibiting lipid oxidation. This indicated that quercetin was responsible for the total amount of inhibition incurred by Fr. 4 in MST. This also suggests that quercetin present in the chloroform extract was the compound responsible for the majority of the inhibitory activity in MST since Fr. 4 inhibited lipid oxidation much more effectively than each of the other fractions.

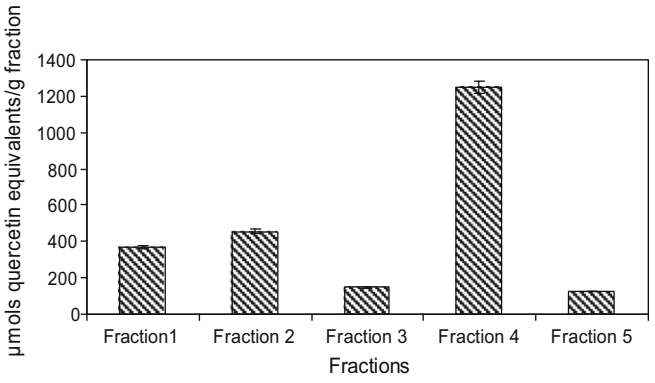

Fig. 5. Total polyphenol content of cranberry fractions as determined by the Folin–Ciocalteu method as described in the methods section expressed as μmols quercetin equivalents/g fraction (dry weight). Results are expressed as average ± standard deviation from duplicate samples.

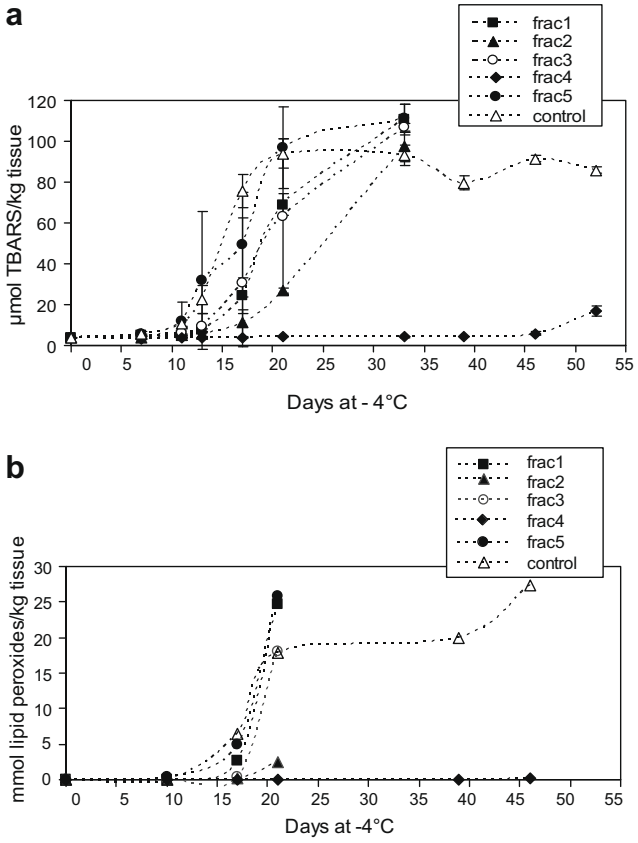

Fig. 6. Effect of cranberry fractions on the formation of (a) TBARS and (b) lipid peroxides in MST. Cranberry fractions were added at 0.1% (dry basis) of the weight of MST and ethanol was used as the carrier solvent at 1% of the final sample weight. Results are expressed as average ± standard deviation from duplicate samples.

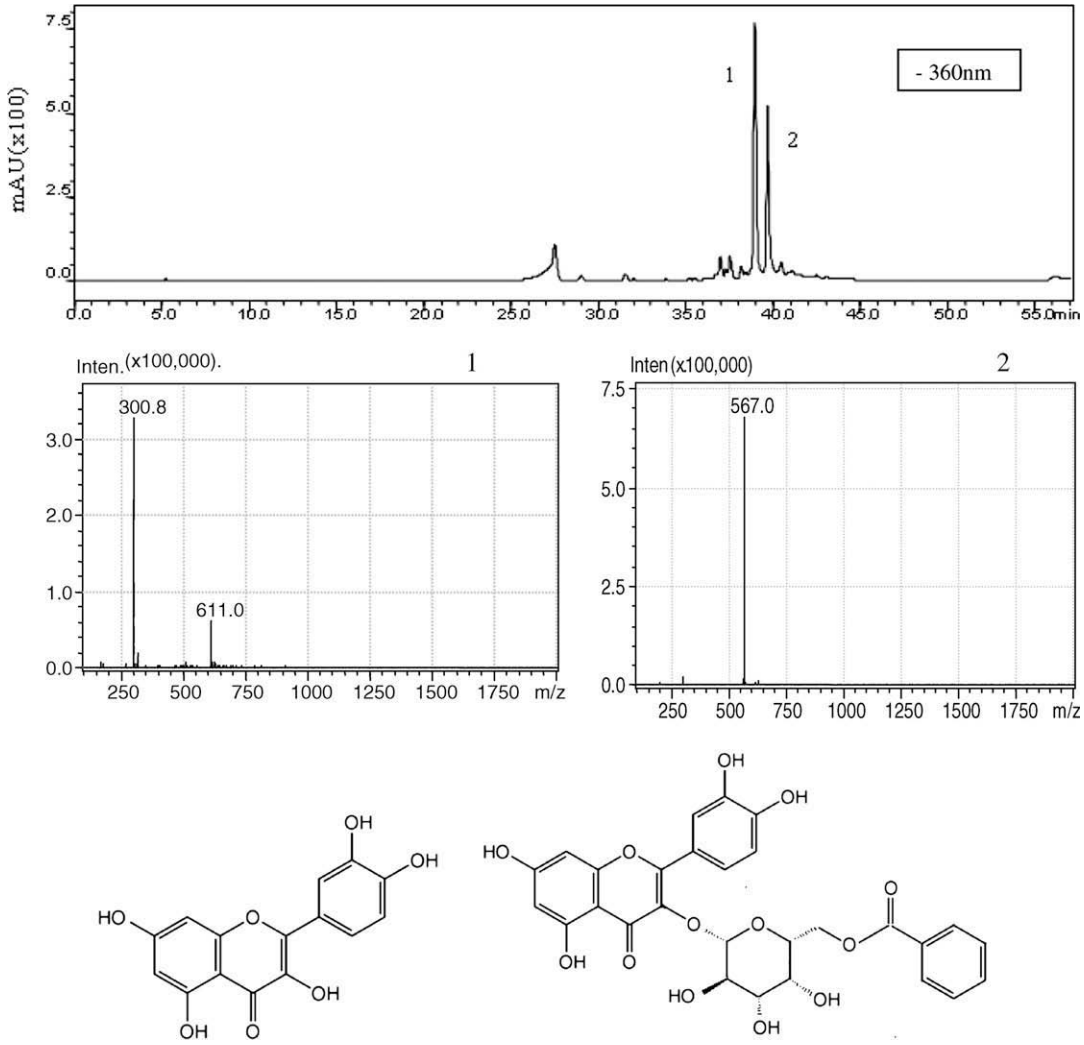

Fig. 7. LC–MS data of Fr. 4 indicating the masses of two flavonols: (1) quercetin and (2) quercetin-3-*O*-(6″-benzoyl)-*β*-galactoside. Chemical structures of both the compounds are given below their corresponding mass spectra.

4. Discussion

Extracts from various plant tissues have been examined as inhibitors of lipid oxidation in muscle foods (Han & Rhee, 2005; He & Shahidi, 1997). Cranberries are a good source of phenolic compounds including flavonols, anthocyanins and proanthocyanidins (Chen et al., 2001; Foo et al., 2000; Sun et al., 2002). Cranberry juice powder is one of the products prepared from cranberry juice and is extensively used in the formulation of wide variety of food and pharmaceutical products. Few studies have been carried out with cranberry juice powder as a source of antioxidants in muscle food systems, and the compound(s) in cranberry that most effectively inhibit lipid oxidation in muscle systems has not been previously determined. Fractionation of spray-dried cranberry juice using LH-20 chromatography and multiple solvent-step elutions were previously done to determine which classes of compounds in cranberry were most effective at inhibiting lipid oxidation in muscle-based systems (Lee, Krueger, et al., 2006). Flavonol aglycones were found to be most inhibitory and possessed lower polarity compared to other classes of phenolics present (Lee, Krueger, et al., 2006). This prompted the examination of a one step chloroform extraction of spray-dried cranberry juice to rapidly obtain the compounds which effectively inhibit lipid oxidation in muscle food systems. Flavonol aglycones can be described as amphiphilic molecules having intermediate polarity (i.e., both polar and non-polar segments are present in each molecule). Chloroform was used as an extractant in our study as it is classically used to extract phospholipids which are also amphiphilic in nature.

The chloroform extract was highly inhibitory to lipid oxidation in MST at low levels of addition (Fig. 2). This made it desirable to identify the compounds in the extract that were inhibitory. The overwhelming evidence indicated that quercetin was largely responsible for the inhibitory potency of the chloroform extract. This was based on the fact that the fraction rich in quercetin (identified by mass spectrometry) was able to inhibit lipid oxidation much better compared to other fractions (Fig. 6). Further, addition of pure quercetin to MST was equally inhibitory compared to addition of Fr. 4 containing an equivalent amount of quercetin (Fig. 8). This made it clear that other compounds in Fr. 4 including glycosylated quercetin were weakly inhibitory in muscle tissue.

Apart from quercetin and glycosylated quercetin, the HPLC chromatogram of Fr. 4 had spectra indicative of proanthoanthocyanidins (PA) (Fig. 4a). The PA in Fr. 4 could not be identified by MS analysis. Cranberry PA have been shown to inhibit Cu^{2+} induced oxidation in low density lipoproteins (LDL) (Porter et al., 2001). The PA were shown to bind to the lipoprotein which may explain the extended lag time prior to Cu^{2+}-induced LDL oxidation. Phenolic binding can physically orient the compound for efficient free radical scavenging or block sites on LDL where copper needs to bind. On the other hand, a PA rich fraction prepared from cranberry

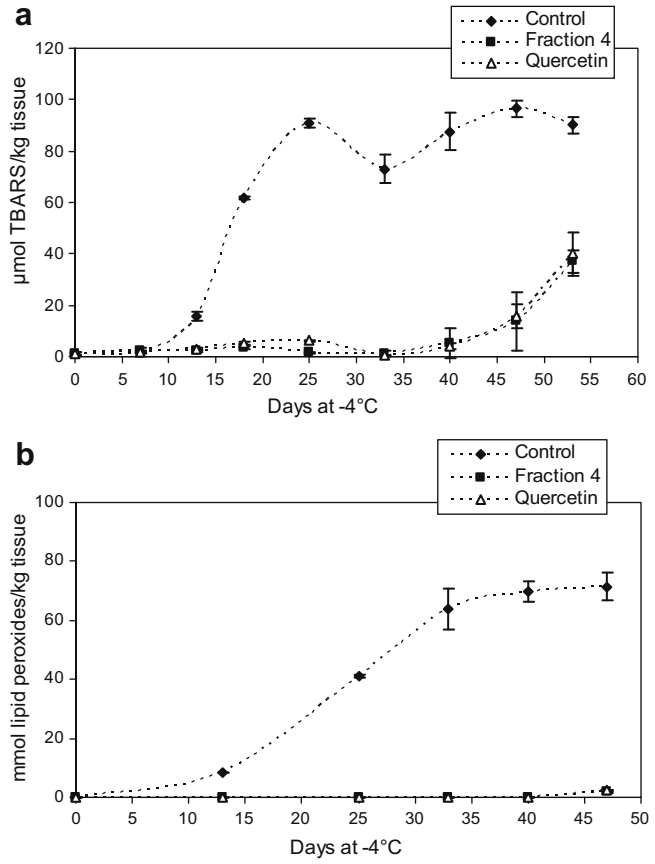

Fig. 8. Effect of Fr. 4 and quercetin on the formation of (a) TBARS and (b) lipid peroxides in MST. Fr. 4 was added at 0.1% of sample weight. This resulted in 467 µmol quercetin/kg MST based on the amount of quercetin determined in Fr. 4 by HPLC. Pure quercetin was added separately at 467 µmol/kg MST. Ethanol was used as the carrier solvent at 1% of the final sample weight. Results are expressed as average ± standard deviation from duplicate samples.

juice powder did not effectively inhibit haemoglobin-mediated lipid oxidation in washed cod (Lee, Krueger, et al., 2006). In order for PA to inhibit lipid oxidation in washed cod, it may be necessary for the compounds to partition into the membrane. The reduced lipophilicity and bulky size of PA may have limited their ability to orient into the cod membranes which can explain their weak inhibitory action in washed cod. A significant correlation between the affinity of phenolic compounds for cellular membranes and inhibition of TBARS generation in fish microsomes activated by haemoglobin and iron have been reported (Pazos, Lois, Torres, & Medina, 2006). These findings are supported by the fact that PAs present in Fr. 4 did not inhibit lipid oxidation in MST.

Since quercetin in the chloroform extract was highly effective at inhibiting lipid oxidation in MST, the mechanism of inhibition is of interest. Membrane phospholipids are believed to be the most susceptible lipid fraction to lipid oxidation compared to neutral lipids (e.g., triacylglycerols) mainly due to the higher degree of fatty acid unsaturation and increased surface area of the membranes (Pikul, Leszczynski, & Kummerow, 1984). The amphiphilic nature of quercetin should allow some of the quercetin to partition more effectively into the membrane phospholipids compared to less amphiphilic components in the extract. A likely pathway by which lipid oxidation in muscle foods occurs is by breakdown of pre-existing lipid hydroperoxides by trace metals, haeme proteins and released haemin (Harel & Kanner, 1985; Svingen, Buege, O'Neal & Aust, 1979; Tappel, 1955). The lipid hydroperoxides are converted to alkoxyl and peroxyl radicals that can abstract hydrogen atoms from a neighbouring polyunsaturated fatty acid which propagates lipid oxidation. It is likely that having quercetin at the location where the amphiphilic radical formation occurs will limit lipid oxidation more effectively than cranberry-derived phenols that reside away from the membrane. Since membrane phospholipids are the most susceptible lipid fraction to lipid oxidation processes, the presence of quercetin in the membranes should inhibit the membrane lipids from oxidising and hence less lipid oxidation in MST treated with Fr. 4. Quercetin was found to be a better antioxidant than catechins in unilamellar liposomes composed of egg yolk phosphatidyicholine (Terao, Piskula, & Yao, 1994) and was found to be more effective than α-tocopherol in inhibiting the formation of hydroperoxides in the methyl linoleate system (Pekkarinen et al., 1999).

Quercetin-3-*O*-(6″-benzoyl)-*β*-galactoside present in Fr. 4 did not contribute to the overall inhibitory activity of the fraction in MST. This could be due to the sugar moiety conferring more hydrophilicity to the compound and hence allowing it to partition into the aqueous phase rather than into the membrane phospholipids. The octanol–water partition coefficient (log*P*) of quercetin glucosides have been reported to be lower compared to quercetin (Rothwell, Day, & Morgan, 2005). Octanol–water partition coefficient best represents the ability of a compound to partition between non-polar and polar phases. Lower log*P* of quercein glucosides indicates that they are more hydrophilic compared to the aglycone suggesting lesser affinity for the membrane lipid phase. The substitution of hydroxyl group at C3 position with a sugar moiety has been shown to decrease the antioxidant efficacy of rutin, a flavonol glycoside in methyl lineolate system compared to quercetin (Pekkarinen et al., 1999). The decreased efficacy of rutin in inhibiting the formation of hydroperoxides was attributed to the absence of hydroxyl group at C3 position for free radical scavenging and metal chelation.

Although Fr. 4 was by far the most inhibitory fraction, Fr. 2 slightly but significantly inhibited lipid oxidation in MST compared to MST with no added antioxidant (Fig. 6). If Fr. 2 was more abundant than Fr. 4, then Fr. 2 would have contributed appreciably to the inhibition incurred by the chloroform extract. However, the yield of Fr. 2 (dry weight) was nearly equivalent to Fr. 4; each was 40–50 mg/g of freeze dried chloroform extract (Table 1).

Carrier solvent has an important role in delivering antioxidants into muscle tissue. It regulates antioxidant partitioning between lipid and aqueous phases. For our studies, we used ethanol as the carrier solvent. Ethanol was a more effective carrier solvent than propylene glycol in inhibiting TBARS formation in MST by cranberry juice powder (Lee, Reed, et al., 2006). The efficacy of ethanol as a carrier has been attributed to its optimal dielectric constant (ε). Ethanol has a ε of 24 compared to propylene glycol ($\varepsilon = 32$). Another study using cod muscle and canola oil, suggested the use of carrier solvents having dielectric constants in the range of 17–27 for maximal uptake of amphiphilic antioxidants by membrane lipids (Raghavan & Hultin, 2005). The decreased incorporation of antioxidant into membrane lipids when carrier solvents of low ($\varepsilon = 3$) or high dielectric constants ($\varepsilon = 32$) were used was either due to the inability of the antioxidant to leave the hydrophobic carrier or due to the rapid exposure of the antioxidant to the aqueous phase before coming in contact with the membrane lipids. This indicates that a carrier solvent of intermediate polarity is necessary for selective incorporation of amphiphilic antioxidants into membrane lipids. Based on these studies, the use of propylene glycol as a carrier solvent was ruled out and ethanol having a dielectric constant of 24 was chosen for our studies. Moreover the chloroform extract and fractions were completely soluble in ethanol.

In conclusion, the flavonol aglycone, quercetin has been identified as the compound responsible for the inhibitory activity of a chloroform extract prepared from cranberry juice powder in

MST. There was no synergy between the compounds in Fr. 4 in terms of inhibiting lipid oxidation in MST. The reason why quercetin-3-*O*-(6″-benzoyl)-*β*-galactoside was not effective in MST might be due to its polar nature which orients it more towards the aqueous phase. Future work should compare the ability of quercetin and quercetin glucoside to inhibit lipid oxidation in muscle systems. Moreover the mechanisms by which inhibition occurs should be examined including free radical scavenging capacity, metal chelation ability, partitioning of the antioxidant between the different phases in muscle tissue, and haeme protein–flavonoid interactions.

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