Irish Journal of Agricultural and Food Research **53:** 121–131, 2014

Potential antioxidant activity of pomegranate peel and seed extracts and synergism with added phenolic antioxidants in a liposome system: a preliminary study

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This study describes the use of liposomes as biological membrane models to evaluate the potential of natural antioxidants as inhibitors of lipid peroxidation. Antioxidative effects of by-products from the pomegranate juice industry, i.e., pomegranate peel (PPE) and seeds extracts (PSE) and combined antioxidative effects of PPE or PSE with α-tocopherol (TOH), quercetin (QC) and ascorbic acid (AA) on peroxidation of L-α-phosphatidylcholine liposomes as initiated by lipophilic or hydrophilic azo-initiators were investigated. Extracts from PPE and PSE had an antioxidative effect as evidenced by a lag phase for formation of phosphatidylcholine-derived conjugated dienes. A combination of TOH or QC with PPE or PSE respectively, showed synergism in prolonging of the lag phase. Thus, the results of the present study show the possibility of utilising waste PPE or PSE as a promising source of natural antioxidants for the protection of food systems.

Keywords: AAPH; AMVN; liposome oxidation; *Punica granatum*; synergy

Introduction

Pomegranate (*Punica granatum* L.) is an important commercial fruit crop that is extensively cultivated in the Near East, India, (southeastern) Spain, Israel, and the United States (California). Pomegranate is mainly consumed fresh or used to obtain juice (Cerda *et al.* 2003). Peels and seeds are the major byproduct of pomegranate processing industries, which represent a

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major waste disposal problem for the industry. Up-grading of this by-product to value-added products is therefore of interest to the pomegranate juice industry (Kanatt, Chander and Sharma 2010).

Peels and seeds have been proposed as a source of natural antioxidants. Pomegranate peels constitute approximately 40% of the whole fruit and are rich in ellagic acid derivatives such as the ellagitannins, punicalagin, and punicalin (Negi, Jayapraska and Jena 2003). Pomegranate seed contains a range of nutraceutical components such as sterols, γ-tocopherol, punicic acid and hydroxybenzoic acids (He *et al.* 2011).

Antioxidants from natural sources, such as pomegranate peel and seeds, contain mixtures of several compounds with antioxidative properties. Addition of such extracts to foods may interact with each other and with endogenous antioxidants present in the foods. For some combinations of antioxidants the overall effect has been found to be more pronounced than the effect expected from simple addition of the individual effects. This phenomenon has been termed antioxidant synergism (Uri 1961). Several studies have shown that plant polyphenols have a synergistic effect with different antioxidant compounds (Majchrzak, Mitter and Elmafda 2004; Miller and Rice-Evans 1996; Pedrielli and Skibsted 2002).

The purpose of the present study was to determine whether antioxidants present in pomegranate peel (PPE) or seed extracts (PSE) act synergistically in combination with quercetin (QC), α-tocopherol (TOH) and ascorbic acid (AA) against lipid oxidation. Lipid oxidation was followed by the formation of conjugated dienes in a liposome system using two types of azo-initiators: the water soluble 2,2′-azobis (2-amidiopropane) dihydrochloride (AAPH) and the lipid soluble 2-2′azodi (2,4-dimethylvaleronitrile) (AMVN). Since liposomes mimic cellular structures, the feasibility of protecting lipid membranes in the presence of phenolic antioxidants can be investigated in this model system prior to further study. Such experiments are particularly interesting for lipid-containing foods, as they furnish preliminary insights with respect to lipid oxidation in relatively short time scales.

Materials and Methods

Chemicals

L-α-phosphatidylcholine (PC) from soybean (purity 99%) and QC dehydrate (purity 98%) were purchased from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany). AA and α-tocopherol and were purchased from Fluka Chemie Gmbh (Steinheim, Germany). AAPH and AMVN (99.5%) were supplied from Wako Chemicals Inc. (Neuss, Germany). Other chemicals were of analytical grade. Water was purified through a Millipore Q-plus purification train (Millipore Corp., Bedford, MA, USA).

Preparation of pomegranate peel and seed extract

Pomegranate fruit having no visible external cuts or spoilage was purchased from the local market in Copenhagen (grown in Turkey). The fruit was washed and arils and peel were separated. The arils were then pressed to yield juice and seed. The seeds were washed with excess water for the removal of sugars and adhering materials. Peels and seeds were dried in an oven (Buch & Holm, 2730 Herlev, Denmark) at 55 °C for 3 h. Both parts were powdered in a coffee grinder (Braun AG Frankfurt/m Germany, type 4169) to get 40-mesh size powder.

To obtain water extracts (W), 25 g of dried samples were heated with 200 mL of boiling water on a magnetic stirrer/hotplate for 30 min. Then the extracts were filtered using Whatman No.4 paper. The filtrates were evaporated to dryness using a rotary vacuum evaporator at 35 °C.

To obtain methanol (M) and ethanol (E) extracts, 5 g of dried samples were mixed with 200 mL of solvent and shaken at 180 rpm (Edmund Buhler-KS-15, Johanna Otto GmbH, Hechingen, Germany) overnight at room temperature. The extracts were filtered using Whatman No.4 paper and the residue was re-extracted twice and filtered. All extracts were pooled and solvents were removed using a rotary vacuum evaporator at 45 °C. The extracts were dissolved in 8 mL methanol and the resulting solutions were covered with nitrogen and stored at -18 °C until use.

Total phenol concentration

The total amount of phenols was determined by using Folin-Ciocalteu's phenol reagent and spectrophotometric determination. Samples (200 µL, three replicates) were mixed with 1.0 mL Folin-Ciocalteu's phenol reagent (diluted 1:10 with water) and 0.8 mL of a 7.5% (w/v) sodium carbonate solution was added. The reaction mixture was stored for 30 min. at room temperature before measuring the absorbance at 765 nm on a Cintra 40, UV-Visible spectrophotometer (GBC scientific equipment, USA).

Preparation of antioxidants

The lipid-soluble antioxidants were dissolved in different solvents, depending on their solubility. QC was dissolved in methanol and α-tocopherol was dissolved in hexane. The water-soluble antioxidant AA, was dissolved in 0.01 M phosphate buffer (pH 7.4). The concentrations of TOH, QC and AA were calculated as mol % of the lipid fraction by using a molecular mass of soybean PC equal to 900 g/mol.

Preparation of liposomes

Liposomes were prepared according to Roberts and Gordon (2003). A solution (2 mL) containing 1.5 µmol soybean PC dissolved in chloroform was mixed with 1 mL pure hexane, 1 mL TOH or 1 mL QC. Eighty-six microliters of 43 mM AMVN in absolute ethanol was added to the samples when oxidation was initiated by AMVN. (Note: If the reaction is initiated with AMVN, AMVN is added at this point, when reaction is initiated with AAPH, AAPH is added just before spectrophotometric measurement in below). The solvent was subsequently removed under reduced pressure (approximately 100 mbar) on a rotary evaporator with water bath set at 30 °C. After evaporation, the atmospheric pressure was re-established by introducing N_2. The lipid residue was subsequently re-hydrated with 10 mL 0.01 M phosphate buffer (pH 7.4), vortexed for 10 min and sonicated in an ultrasonic bath for 30 s to yield a white homogeneous suspension of multilamellar liposomes. Liposomes were stored in the rotary evaporation flask protected from light by aluminum foil and kept under N at all times. Unilamellar liposomes were prepared from the suspension of multilammelar liposomes using an Avestin lipofast Basic small volume (500 µL) extrusion device (Avestin Europe Gmbh, Mannheim, Germany). The suspension was passed 21 times through a double layer of polycarbonate membranes with a pore size of 100 nm. Hydrophilic antioxidant AA, and either of the extracts was added to the liposomes with a phenol content of 1.84×10 g gallic acid equivalents (GAE)/L dissolved in a 0.040 M phosphate buffer with pH 7.4 according to Graversen *et al.* (2008).

Peroxidation of liposomes

A unilamellar liposome suspension (2.5 mL) was pipetted into quartz cuvettes and incubated for 10 min at 37 °C within the water-jacket cell changer. Twenty-five microliters of 75 mM AAPH in sodium phosphate buffer (pH 7.4) was added to the samples when oxidation was initiated with AAPH. The cuvettes were inverted 5 times and then sealed to avoid evaporation. Up to six samples were measured in each run with phosphate buffer as blank and liposome suspension without antioxidant as control. The absorbance was measured at 234 nm (absorption maximum of conjugated dienes) every 10 min for 900 min in total. The lag phase before onset of oxidation was measured as the time in minutes corresponding to the intercept between the tangent to the propagation phase and the tangent to the lag phase (Roberts and Gordon 2003).

Effect of extraction method on the liposome peroxidation system

Extracts of either PPE or PSE with different phenol contents were added to the liposome system. Oxidation was initiated by either AAPH or AMVN and the results were monitored as described above.

Statistical analysis

All data were analysed using SPSS (2006). Assays were carried out in triplicate and the results were expressed as mean values±standard deviations (SD). The data for the total phenolic content was subjected to one way analysis of variance. For the liposome assay, two-way analysis of variance was performed separately for the comparison of the results of PPE or PSE with antioxidants to investigate the main effects of solvents (water, ethanol and methanol) and radicals (AAPH and AMVN). A P-value < 0.05 was considered as statistically significant.

Results and Discussion

Oxidation in heterogeneous systems is suggested to occur at the interface between the lipid and the aqueous phase and the composition of the interfacial area is therefore highly important regarding oxidative stability (Schwarz *et al*. 2000). Peroxyl radicals are generated outside themembranes by AAPH, whereas AMVN generates oxidative stress within. While radicals formed by lipid soluble initiators have easier access to lipids, radicals formed by the water-soluble initiators were expected to initiate oxidation at the lipid-water interface which would propagate into the lipid substrate (Tsuchiya *et al.* 2001).

Interactions of PPE or PSE (as water, ethanol and ethanol extracts) with antioxidants of different polarity (QC, TOH and AA) were investigated by studying the ability to inhibit lipid peroxidation of liposomes made of soybean PC initiated by azo-initiators (AAPH and AMVN). The extent of oxidation was monitored by following the formation of conjugated dienes. Upon the addition of AAPH or AMVN to the liposome control (i.e., without PPE or PSE added), diene formation occurred quickly (Table 1). On the other hand, a lag phase, indicating the inhibition of lipid oxidation, was observed when PPE or PSE were present (Table 2).

Total phenolics

The total phenol content varied from 143.5±4.1 to 278.3±10.5 mg GAE/100 g in PPE and 35.1±2.3 to 76.3±3.7 mg GAE/100 g in PSE. Similarly, the phenol content was reported to be higher in pomegranate peel extracts than seeds by Pande and Akoh (2009).

Table 1. Lag phase of oxidation in soybean phosphatidyl choline liposomes after free radical initiation in the aqueous phase by AAPH or in the lipid phase by AMVN due to α-tocopherol (TOH), quercetin (QC) and ascorbic acid (AA) addition

Sample	Lag phase (min)	
	AAPH[a]	AMVN
Control	4±3[b]	4±2
TOH	100±2	54±3
QC	172±3	110±2
AA	64±4	31±1

[a]AAPH = 2,2′-azobis (2-amidiopropane) dihydrochloride, AMVN =2-2′azodi (2,4-dimethyl-valeronitrile).

[b]Mean±SD (n=3).

The selection of solvent and the extraction methods also affect the concentration of total phenols. The methanolic extract of pomegranate peel and seeds contained a significantly higher quantity of polyphenols (278.3±10.5 mg GAE/100 g in PPE and 76.3±3.7 mg GAE/100 g in PSE) when compared to the ethanolic extract (196.6±2 mg GAE/100 g in PPE and 61.5±2.6 mg GAE/ 100 g in PSE) and water extracts (143.5±4.1 mg GAE/100 g in PPE and 35.1±2.3 mg GAE/100 g in PSE and variations in phenolic contents of PPE and PSE for the different solvents used are attributed to polarities of different compounds present in the PPE or PSE and such differences have been reported in the literature (Negi, Jayapraska and Jena 2003). These data are in agreement with the findings of Rojder, Skşbsted, and Andersen (2006), who found a higher phenolic content (expressed in mg GAE/L extract) in methanol (70%) extracts from cherry pomace compared with water or aqueous ethanol (70%) extracts.

Effect of extraction method on the liposome peroxidation system

Extracts of either PPE or PSE with different phenol contents were added to the liposome system and oxidation was initiated by either AAPH or AMVN (Figures 1 and 2). The total amount of radicals formed in liposomes within 30 min was 1.8×10 moles/L liposome suspension for AAPH and 2.6×10 moles/L liposome suspension for AMVN using the equations: R_i (mol/L/s) = 1.36×10 [AAPH] (Niki 1990) and R_i (mol/L/s) = 3.88×10 [AMVN] (Shi, Noguchi, and Niki 1999). These equations

Table 2. Lag phase of oxidation in soybean phosphatidyl choline liposomes after free radical initiation in the aqueous phase by AAPH or in the lipid phase by AMVN due to α-tocopherol (TOH), pomegranate peel extract (PPE) or pomegranate seed extract (PSE) addition[a]

						Lag phase (min)
Sample	AAPH[b]			AMVN[b]		
	W[c]	M[c]	E[c]	W	M	E
PPE	74±2[d]	102±5	81±4	45±2	62±4	53±3
PSE	57±2	66±6	64±4	32±3	47±5	43±2
TOH+PPE (calculated)	174±3	202±3	181±4	99±2	116±4	107±4
TOH+PPE (experimental)	183±3	215±2	191±3	108±2	122±3	117±4
TOH+PSE (calculated)	157±3	166±4	164±2	86±2	101±3	97±3
TOH+PSE (experimental)	168±2	179±4	171±1	95±5	111±4	109±2

[a]PPE or PSE corresponding to a concentration of 1.84×10^{-5} g GAE/L.

[b]AAPH = 2,2′-azobis (2-amidiopropane) dihydrochloride, AMVN = 2-2′azodi (2,4-dimethylvaleronitrile).

[c]W = water extract, M = methamolic extract, E = ethanolic extract.

[d]Mean±SD (n=3).

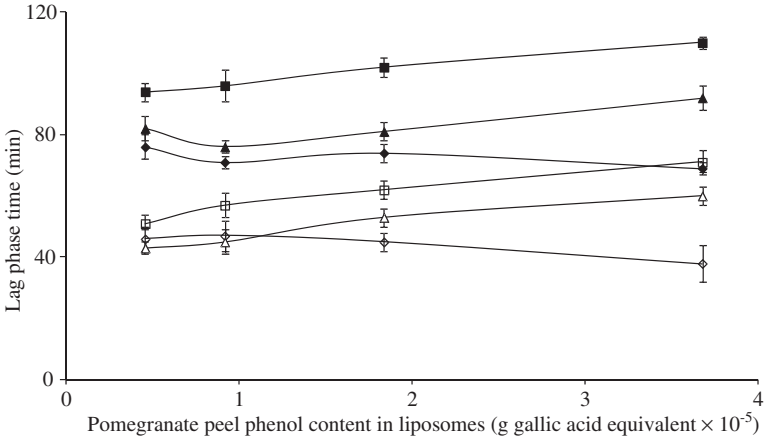

Figure 1. Lag phase of oxidation in soybean phosphatidyl choline liposomes after free radical initiation in the aqueous phase by AAPH or in the lipid phase by AMVN due to addition of extracts of pomegranate peel in different solvents.

(■: with AAPH for methanolic extract, ▲: with AAPH for ethanolic extract; ♦: with AAPH for water extract ; □: with AMVN for methanolic extract; △: with AMVN for ethanolic extract; ◊: with AMVN for water extract) (Mean±SD).

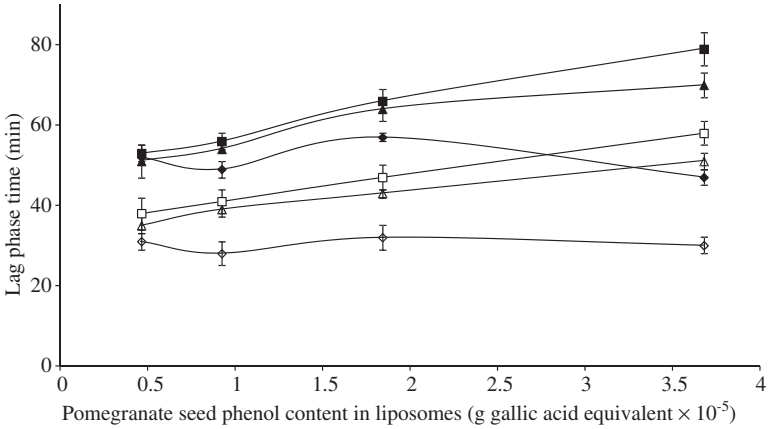

Figure 2. Lag phase of oxidation in soybean phosphatidyl choline liposomes after free radical initiation in the aqueous phase by AAPH or in the lipid phase by AMVN due to addition of extracts of pomegranate seed in different solvents.

(■: with AAPH for methanolic extract, ▲: with AAPH for ethanolic extract; ♦: with AAPH for water extract; □: with AMVN for methanolic extract; △: with AMVN for ethanolic extract; ◊: with AMVN for water extract) (Mean±SD).

are valid for use in conditions of 37 °C and neutral water (AAPH) or 37 °C and acetonitrile (AMVN), respectively. Thus, the concentration of radicals formed by AMVN was approximately 50% higher than the concentration of radicals formed by AAPH. This is in agreement with the observation that the lag phases were significantly shorter when the lipid soluble azo-initiator AMVN was used compared to the water-soluble AAPH. The better antioxidative effect on lipid oxidation observed for PPE or PSE when oxidation was initiated by AAPH can be explained by the localisation of the polyphenols in the homogeneous water phase or at the interface protecting the lipids against radicals generated in the water phase. In this respect, Faustino *et al*. (2004) reported an antioxidative effect of red wine using a water and lipid soluble peroxyl generating system and the greatest effect was seen against radicals formed in the aqueous phase.

The methanolic and ethanolic extracts of peel and seeds were better at preventing lipid oxidation compared to water extracts (Figures 1 and 2). The lower efficiency of water extracts might be due to the lack of some phenolic compounds present in methanolic or ethanolic extracts which have good antioxidant properties (or are efficient scavengers). The concentration of water extracts may not have been high enough to prevent lipid oxidation. Similarly, Farvin, Nielsen and Jacobsen (2010) reported that ethanolic extracts of potato peels in oil and in emulsions prevented lipid oxidation whereas water extracts did not.

Interaction of PPE or PSE with α-tocopherol in a liposome system

In the presence of AAPH or AMVN, a combination of TOH and either extract studied showed a lag phase that was slightly longer than the sum of lag phases of individual components (Table 2). The presence of AAPH or AMVN significantly affected the interaction between PPE or PSE with TOH. Nevertheless, the effect of solvent type (ethanol, methanol and water) on this interaction was not significant ($P>0.05$).

The observation of these synergistic effects (Figure 3), which is in agreement with previous reports on the combination of TOH and polyphenols in heterogeneous systems (Graversen *et al.* 2008), probably depends on different solubilities of antioxidants in the two phases of the

Table 3. Lag phase of oxidation in soybean phosphatidyl choline liposomes after free radical initiation in the aqueous phase by AAPH or in the lipid phase by AMVN due to quercetin (QC), pomegranate peel extract (PPE) or pomegranate seed extract (PSE) addition[a]

						Lag phase (min)
Sample	AAPH[b]			AMVN[b]		
	W[c]	M[c]	E[c]	W	M	E
QC+PPE (calculated)	246±4[d]	274±2	253±4	155±3	172±2	163±4
QC+PPE (experimental)	266±2	291±2	269±3	169±2	187±1	177±4
QC+PSE (calculated)	229±3	238±4	236±2	142±2	157±3	153±3
QC+PSE (experimental)	241±2	253±4	251±1	153±5	172±4	169±2

[a]PPE or PSE corresponding to a concentration of 1.84×10^{-5} g GAE/L.

[b]AAPH = 2,2′-azobis (2-amidiopropane) dihydrochloride, AMVN = 2-2′azodi (2,4-dimethylvaleronitrile).

[c]W = water extract, M = methamolic extract, E = ethanolic extract.

[d]Mean±SD (n=3).

heterogeneous systems. Indeed, TOH, a major natural chain-breaking antioxidant in the cell membranes, provides protection against early events in lipid oxidation at the site of radical initiation (Becker, Ntouma and Skibsted 2007). Plant extracts on the other hand proved effective in blocking the peroxidation process in both phases by scavenging free radicals, by initiating catalysis by iron, and through chain-breaking activity (Becker, Nissen and Skibsted 2004). In this respect, Zhu *et al.* (1999) reported that a mixture of TOH and green tea catechins in low density lipoproteins exerted a stronger antioxidant effect than either TOH or green tea catechins alone. They proposed that the synergistic action of green tea catechins is related to the regeneration of TOH through the donation of a hydrogen atom to the tocopheroxyl radical.

Interaction of PPE or PSE with quercetin in a liposome system

Incorporation of 1 mol % QC in the liposomes after initiation of oxidation with AAPH or AMVN caused a lag phase of 172±3 and 110±2 min, which was extended by the addition of PPE or PSE (Table 3). The interaction between PPE or PSE with QC was significantly affected by the presence of AAPH or AMVN. The type of solvent did not affect this interaction (>0.05). Moreover, since the lag phase of the combination of QC with respective extracts was found to be significantly longer than the sum of lag phases of each component, synergism occurred.

Quercetin is one of the most abundant flavonoids and occurs in food, glycosylated (attached to a sugar molecule) (Ratty, Sunamoto and Das 1988). Quercetin could both act as a metal chelator and a scavenger of chain-initiating peroxyl radicals on the lipid-aqueous interphase in heterogeneous systems (Beer *et al.* 2005). Thus, localisation of flavonoids within membranes may modify lipid peroxidation. Similar effects were observed by Becker *et al*. (2007) who found that TOH acted synergistically with QC in a liposome assay when oxidation was initiated by metmyoglobin.

Interaction of PPE or PSE with ascorbic acid in a liposome system

The combination of AA with PPE or PSE showed a lag phase that was approximately

Table 4. Lag phase of oxidation in soybean phosphatidyl choline liposomes after free radical initiation in the aqueous phase by AAPH or in the lipid phase by AMVN due to ascorbic acid (AA), pomegranate peel extract (PPE) or pomegranate seed extract (PSE) addition[a]

	Lag phase time(min)					
Sample	AAPH[b]			AMVN[b]		
	W[c]	M[c]	E[c]	W	M	E
AA+PPE (calculated)	138±3	166±2	145±4	76±2	93±2	84±4
AA+PPE (experimental)	131±2	162±2	139±3	70±1	89±3	71±4
AA+PSE (calculated)	121±3	130±4	128±2	63±2	78±3	74±3
AA+PSE (experimental)	115±2	129±4	121±1	55±5	69±4	74±2

[a]PPE or PSE corresponding to a concentration of 1.84×10^{-5} g GAE/L.

[b]AAPH = 2,2′-azobis (2-amidiopropane) dihydrochloride, AMVN = 2-2′azodi (2,4-dimethylvaleronitrile).

[c]W = water extract, M = methamolic extract, E = ethanolic extract.

[d]Mean±SD (n=3).

the same as when AA was present alone (Table 4). Hence a synergistic effect could not be demonstrated for PPE or PSE combined with AA in any of the experimental conditions. Although AA can react with radicals generated in the water phase by AAPH disintegration or it can regenerate polyphenols from their oxidised forms due to its low oxidation potential (Becker *et al*. 2004) further effects were inhibited in the liposome system. A pronounced synergism was previously observed between ascorbyl palmitate, a lipophilic ascorbate derivative, and oregano extract (to preserve TOH in sunflower oil); suggesting solubility issues hamper synergy in the present experiments (Beddows, Jagait and Kelly 2001).

Comparison of combinations of antioxidants with PPE or PSE in a liposome system

The interaction between PPE or PSE with TOH, QC or AA in the liposome system was investigated. When the initiation occurred in the water phase by AAPH, all combinations of PPE or PSE showed a better antioxidative effect against lipid oxidation than when initiation occurred in the lipid phase with AMVN. Since some flavanoids localise near the surface of the membrane, they could easily trap the aqueous peroxyl radical place in the liposome (Becker *et al*. 2007).

Synergistic effects were observed for combinations of either PPE or PSE and TOH or QC in inhibiting lipid oxidation from the water phase with AAPH and the lipid phase with AMVN. The effect was greatest for QC. In contrast, combinations with AA showed antagonism. Since α-tocopherol is located in the lipid phase, it efficiently scavenges AAPH and AMVN derived peroxyl radicals in the lipid phase near the water/lipid interphase. However, QC is mostly localized near the surface of membranes where these radicals are easily trapped and is more readily accessible to the radicals than is TOH, thus explaining the better antioxidative effect of QC in liposomes, as seen in the present study (Ratty *et al*. 1988). The absence of synergy between AA and PPE or PSE with respect to lipid oxidation can be explained by the localisation of AA in the homogeneous water phase and not at the interface where lipid oxidation takes place. Interestingly, AA showed antioxidative effects against AMVN-derived radicals, even though it is localised in the homogeneous water phase.

Synergistic interactions between mixtures of antioxidants can be explained as follows: 1. Regeneration of the more effective antioxidant in the system by other less effective antioxidants or oxidation retarders, 2. Metal chelation by one antioxidant sparing a chain breaking antioxidant, 3. Interaction of antioxidants with different solubility (Becker *et al*. 2004). A combination of these different mechanisms is a possible explanation of synergy between TOH or QC and polyphenols, thus protecting each other against reactive oxygen species (Yin *et al*. 2012). On the other hand, AA did not show synergistic effects in combination with PPE or PSE in liposomes but rather antagonistic effects. An antagonistic effect was also shown between thyme or rosemary extracts and AA in liposomes, which was explained by kinetic factors (Nieto, Huvaere and Skibsted 2011).

Conclusion

Pomegranate peel and seed extracts were found to be a rich source of antioxidants as shown by the inhibition of the formation of conjugated dienes in a liposome system. Comparing the by-products, the major concentration of phenolic compounds was found in peels. Generally aqueous extracts had a lower phenolic content than extracts

using other solvents (i.e., methanol or ethanol) and methanolic and ethanolic extracts of peel and seeds were better at preventing lipid oxidation compared to water extracts. Compounds in extracts worked synergistically in combination with α-tocopherol and quercetin, but not with ascorbic acid. The degree of lipid oxidation in the liposomes in the presence of α-tocopherol, quercetin, AA and the extracts was significantly lower when oxidation was initiated by free radicals formed in the water phase compared to initiation in the lipid phase. Based on this study, it can be concluded that pomegranate peel and seeds, as by-products of the pomegranate juice industry, are a readily accessible source of natural antioxidants, which possibly provides a good alternative to synthetic antioxidants in the protection of food systems.

Acknowledgements

The author would like to thank Prof. Dr. Leif H. Skibsted for supplying laboratory facilities for this study.

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Received 15 May 2013