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Synergism and antagonism between quercetin and other chain-breaking antioxidants in lipid systems of increasing structural organisation

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Abstract

Antioxidative effect of quercetin was affected differently in neat sunflower oil, in methyl linoleate o/w emulsion and in phospholipid liposomes by the other chain-breaking antioxidants, α-tocopherol, rutin and astaxanthin. Quercetin was better than or comparable to α-tocopherol as an antioxidant in the three lipid systems. The presence of α-tocopherol showed a strong synergistic effect for quercetin in the emulsion, less in the liposomes and a clear antagonistic effect in the neat oil. Astaxanthin, without any effect alone in neat oil or in the liposomes, but with some effect in the emulsion, did not affect quercetin as an antioxidant. Rutin was only effective as an antioxidant in the liposomes where rutin showed clear synergism with quercetin. The interaction of quercetin with the other antioxidants is classified according to the structural organisation of the lipid substrate.

Keywords: Bulk oil; Emulsions; Liposome oxidation; Phenolic compounds; Flavonoids; Synergistic effect

1. Introduction

Lipid oxidation in biological systems, including foods, is controlled by antioxidants and the location and orientation of antioxidants within membranes or other structures are crucial for the oxidative stability of the system (McClements & Decker, 2000). Different types of antioxidants are present in nature and the choice of antioxidant for food protection depends on the actual product, and evaluation protocols for food protection and health benefits have been systematized (Becker, Nissen, & Skibsted, 2004). Antioxidants are present naturally in combinations and antioxidant interaction seems important for their effect. For some combinations of antioxidants, the over-all effect is found to be more pronounced than the effect expected from a simple addition of effects entailing what has been termed antioxidant synergism (Uri, 1961). While the mechanism behind effects of most antioxidants on lipid oxidation is fairly well understood for the chain-breaking antioxidants as scavengers of free radical intermediates, the mechanism behind antioxidant synergism and the opposite effect, antioxidant antagonism, is more speculative as conflicting observations have been reported. Antioxidant effects of combinations of α-tocopherol with plant polyphenols have been studied in homogeneous solutions (Pedrielli & Skibsted, 2002) and in heterogeneous systems, such as in o/w emulsions (Jia, Zhou, Yang, Wu, & Liu, 1998), low density-liposomes (Laranjinha & Cadenas, 1999; Laranjinha, Vieira, Madeira, & Almeida, 1995) and liposomal suspensions (Liao & Yin, 2000; Roberts & Gordon, 2003; Terao, Piskula, & Yao, 1994). Several of these studies have shown that quercetin and catechin and other plant phenols have a sparing effect on α-tocopherol, which has been ascribed to regeneration of α-tocopherol by the plant phenol, in effect leading to antioxidant synergism. In contrast, Jorgensen,

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Madsen, Thomsen, Dragsted, and Skibsted (1999) have shown that α-tocopherol in homogeneous dimethyl formamide solution may regenerate the flavonoid quercetin from its one-electron oxidized form. Carotenoids have similarly been found to be regenerated by α-tocopherol from the corresponding carotenoid radical cations (Mortensen & Skibsted, 1997). However, under other conditions α-tocopheroxyl radicals were recycled by carotenoids (Edge, Land, McGarvey, Mulroy, & Truscott, 1998). Notably, in palm oil, carotenoids have been shown to protect the tocotrienols during oxidation, despite that tocotrienols were shown to regenerate the carotenoids rather than the carotenoids regenerating the tocotrienols during oxidation (Schroeder, Becker, & Skibsted, 2006).

The conflicting results obtained for antioxidant interaction may be due to differences between the actual lipid or model systems used to study the interaction. However, at present, design of optimal protective systems for foods by combinations of antioxidants is hampered by the lack of mechanistic understanding of antioxidant interaction and of antioxidant hierarchy. Research should include (i) establishment of lipid systems and conditions where primary antioxidants show significant synergism, (ii) real-time kinetic studies of hydrogen-atom and electron transfer between antioxidants during lipid oxidation in such systems and (iii) final test of synergistic combinations, selected according to such mechanistic studies in food during processing and storage. We have embarked on such studies and, as the first part, we have explored how synergism and antagonism can be demonstrated in lipid systems of increasing structural organization for the plant phenol, quercetin, in pairwise combination with each of the three other important antioxidants shown in Fig. 1. Based on the establishment of systems for which combinations of radical-scavenging antioxidants show significant synergism, mechanistic studies of the free radical kinetics of antioxidant interactions are being planned.

The selected antioxidants had different hydrophilic/lipophilic balance and different surface activity, and the antioxidative effect of each antioxidant and of each in combination with quercetin was studied in three lipid systems of increasing structural organisation: bulk oil, o/w-emulsion and liposomal suspension. To each system, a relevant analytical method was applied: Rancimat test of induction period for the bulk oil, depletion of oxygen in myoglobin-initiated peroxidation of methyl linoleate for the o/w-emulsion, and conjugated dienes formation in the liposomal phospholipid suspension.

2. Materials and methods

2.1. Chemicals

L-α-Phosphatidylcholine (PC) from soybean (purity 99%), horse heart metmyoglobin (MMb, type II), methyl linoleate (purity 99%), astaxanthin (AX, purity 98%), rutin (RUT, purity 95%) and quercetin dihydrate (QC, purity 98%) were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). All-rac (±)-α-tocopherol (TOH, purity 98%) and Tween 20 were purchased form Fluka Chemie GmbH (Steinheim, Germany). 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) was supplied by Wako Chemicals Inc. (Richmond, VA, USA). All solvents

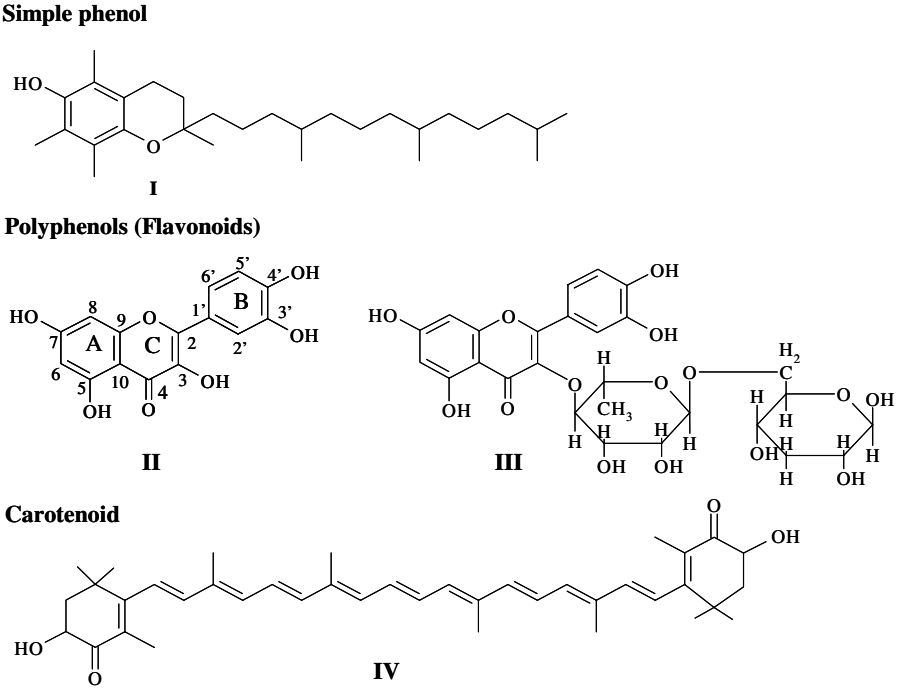

Fig. 1. Antioxidants included in the study: (**I**) α-tocopherol, (**II**) quercetin (flavon-3-ol), (**III**) rutin (flavon-3-ol rutinoside) and (**IV**) astaxanthin (xanthophyll). **II** shows flavonoid numbering.

were supplied by Lab Scan Analytical Sciences (Dublin, Ireland) and were of HPLC grade.

2.2. Analysis of tocopherols

Quantification of α-tocopherol and γ-tocopherol was carried out by reverse-phase liquid chromatography (HPLC) with fluorescence detection by using external standard solutions of known concentrations of α-tocopherol and γ-tocopherol. Oil samples (25 mg) were dissolved in 3 ml of ethanol/1-butanol (75:25, v/v) and directly analysed. An HPLC analytical column (4 mm × 125 mm) (Agilent Technologies, Karlsruhe, Germany), packed with C18 phase and containing a mean particle size of 5 μm, was used. Methanol/water (94:6, v/v) was used as mobile phase with a flow of 1 ml/min. The fluorescence detector operated with excitation wavelength set at 292 nm and emission wavelength set at 330 nm.

2.3. Fatty acid composition

Ten milligram of oil was analysed by gas-liquid chromatography after derivatisation to fatty acid methyl esters according to Jart (1997). A 5890 A-II chromatograph (Hewlett Packard Co., San Fernando, CA, USA), with a split injector operating with a 1:10 split ratio at 250 °C, a HP-FFAP capillary column, 25 m × 0.20 mm i.d. and film thickness 0.33 μm (Hewlett Packard Co., San Fernando, CA, USA), and a flame ionisation detector at 300 °C, was used. The analyses were performed using helium (0.95 ml min^{-1}) as the carrier gas, applying the following temperature programme: 50 °C held for 1 min, 15 °C min^{-1} to 180 °C, 5 °C min^{-1} to 220 °C held for 10 min.

2.4. The oil stability index (OSI) method (Rancimat test)

Food grade, high olein sunflower oil was purified by alumina column chromatography according to Fuster, Lampi, Hopia, and Kamal-Eldin (1998) in order to obtain the oil without naturally occurring tocopherol, peroxides or trace metals. The fatty acid composition, as analyzed by GC, is shown in Table 1. The purified oil was found to contain tocopherols below quantification level (<0.5 ppm). Antioxidants were dissolved in methanol (QC, TOH, RUT) or chloroform (AX). The antioxidant solutions were added to the stripped oil to obtain samples containing 0.25, 0.5, 1.0 and 2.0 μmol antioxidant/g oil. The samples were stirred for 5 min to mix the antioxidant solution with the oil. A Rancimat instrument, model 679, from Metrohm Nordic ApS (Glostrup, Denmark), was used for stability tests, which were carried out with 2.00 ± 0.01 g oil at 100 °C with an air flow of 20 l/h. The results are presented as the additional induction period (aIP in hours):

$$\mathrm{aIP} = \mathrm{IP}_{\text{with antioxidant}} - \mathrm{IP}_{\text{without antioxidant}} \quad (1)$$

Determination of possible synergistic effects was based on measurements of oil samples with equimolar combinations of antioxidants, each with the same concentration as the oil sample, with one antioxidant. Synergistic effects were observed when

$$\mathrm{aIP}_{\text{mixed antioxidants}(1+2)} > \mathrm{aIP}_{\text{antioxidant 1}} + \mathrm{aIP}_{\text{antioxidant 2}} \quad (2)$$

Table 1
Fatty acid composition of sunflower oil used in Rancimat test

Fatty acids[a] (%)	
C14:0	6.37 ± 1.63
C16:0	5.53 ± 0.1
C18:0	3.91 ± 0.02
C18:1	27.90 ± 0.61
C18:2	55.2 ± 0.94
C20:0	0.28 ± 0.00
C22:0	0.8 ± 0.01

[a] Results are mean values of triplicate samples.

2.5. Oxygen consumption of methyl linoleate emulsion (oxygen assay)

The oxygen consumption method was based on methyl linoleate emulsions established by evaporation of a methanol solution of methyl linoleate and Tween 20 by use of a flow of nitrogen and subsequent addition of 2.5 ml of air-saturated 50 mM phosphate buffer (pH 6.8), with metmyoglobin (MMb) as catalyst (Hu & Skibsted, 2002). Oil-soluble antioxidants were dissolved in methanol (TOH) or chloroform (AX) and 10 μl of the solution were added to the methyl linoleate-Tween 20 solution before evaporation, whereas 10 μl of water-soluble antioxidants dissolved in methanol (QC or RUT) were added directly to the emulsion. 25 μl of a 0.2 mM aqueous solution of metmyoglobin were added, and the emulsion was transferred to a thermostatted (25 °C) 70 μl measuring cell with no headspace (Chemiware, Viby J., Denmark). Oxygen consumption was measured by a microcathode Clark oxygen electrode (Stable Systems, Hederson, NEV, USA). A two point calibration [air-saturated water (25 °C) and N_2-saturated water (25 °C and added $Na_2S_2O_4$ to remove residual oxygen)] was used to calibrate the electrode and oxygen analyser before use. Oxygen consumption was recorded at time intervals of 5 s for 30 min. The concentrations in the assay samples were as follows: 2 μM MMb, 2.7 mM methyl linoleate, 0.8 mM Tween 20 and 1.0, 2.0, 3.9, 7.9 or 15.8 μM antioxidants. Thus, the molar concentration of the antioxidant relative to lipid were 0.037, 0.075, 0.15, 0.3 and 0.6 in the assay samples. The antioxidative activity was expressed as the time (in seconds) required to reduce the oxygen content from 100% to 50% O_2 in the assay samples with antioxidant compared to blank (Fig. 2):

$$t_{50\%\text{oxygen}} = t_{50\%\text{oxygen with antioxidant}} - t_{50\%\text{oxygen without antioxidant}} \quad (3)$$

Assay samples with equimolar combinations of antioxidants, each at the same concentration as in the oil samples

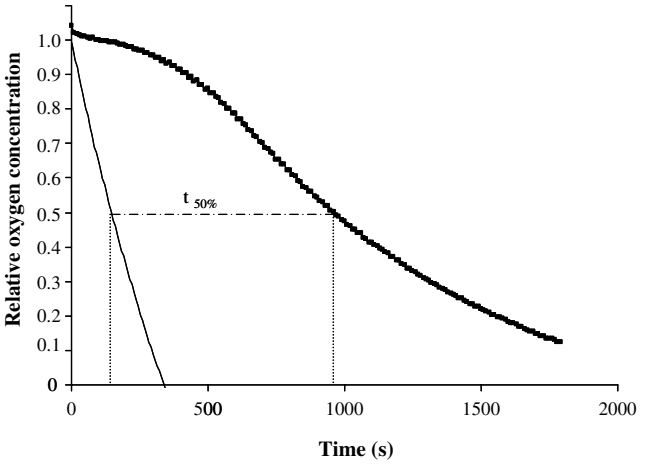

Fig. 2. Oxygen depletion in methyl linoleate o/w emulsion, as catalyzed by metmyoglobin and followed electrochemically in a closed system. ■ emulsion protected by 0.075 mol% quercetin, – emulsion without antioxidant. $t_{50\%}$ is the time in seconds where oxygen content is reduced to 50% of initial concentration.

with one antioxidant, were used to determine possible synergistic effects. Synergism was observed when

$$t_{50\%\text{oxygen of mixed antioxidant}(1+2)} > t_{50\%\text{oxygen antioxidant 1}} + t_{50\%\text{oxygen antioxidant 2}} \quad (4)$$

2.6. Liposome oxidation test

Liposomes were prepared, following the procedure described by Roberts and Gordon (2003). Briefly, 1 ml of antioxidant solution was added for the lipophilic antioxidants to 2 ml of 0.75 mM chloroform solution of soybean PC (molecular mass of soybean PC was taken as 900). The lipophilic antioxidants were dissolved in different solvents, depending on their solubility. TOH and AX were dissolved in hexane, whereas QC and RUT were dissolved in methanol. The solvent of the combined soybean PC/antioxidant solution was evaporated on a rotatory evaporator with a water bath set at 30 °C. After evaporation, the atmospheric pressure was re-established by introducing N_2. The residue in the flask was hydrated with 10 ml of 10 mM phosphate buffer (pH 7.4), vortexed for 10 min and sonicated for 30 s. The final concentrations of the antioxidants in the liposome suspension, in mol% of the lipid fractions, were 0.25, 0.5 or 1.0. The multilamellar suspension was transferred to an Avestin Liposofast Basic (Avestin Inc., Ottawa, Canada) small volume extrusion device and passed 21 times through a double layer (100 nm pore size polycarbonate membrane) to obtain large unilamellar liposomes.

Lipid peroxidation was followed by measuring formation of conjugated diene, monitored as the change in absorbance at 234 nm (A_{234}), using a Shimadzu UV-2101PC UV–vis scanning spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The unilamellar liposome suspension (2.5 ml) was thermostatted in a quartz cuvette for 10 min at 37 °C in the spectrophotometer. A_{234} was measured immediately after addition of the free radical initiator [25 μl of 75 mM AAPH in sodium phosphate buffer (pH 7.4)] and recorded continuously for up to 10 h. A sample without soybean PC and antioxidants was used as a blank. The induction period (IP in min) was determined as the time elapsing to the point where the tangent of the propagation phase and the tangent of the lag phase intercepted and was corrected by substraction of IP obtained for a sample without antioxidant added. Interactions between antioxidants were studied in liposomes with mixtures of antioxidants, each at the same concentration as the liposomes with one antioxidant added. Synergistic effects were observed when

$$\text{aIP}_{\text{mixed antioxidants}(1+2)} > \text{aIP}_{\text{antioxidant 1}} + \text{aIP}_{\text{antioxidant 2}} \quad (5)$$

2.7. Statistical analysis

All experiments were performed at least in duplicate and the results were expressed as mean values ± standard deviation. The statistical analyses of the effects of type of antioxidant and of concentration of antioxidant were evaluated by two-way analysis of variance by a general linear model (GLM) procedure (SAS, version 8.02). The analysis was performed for the following response variables: additional induction period (aIP) for oil oxidation in Rancimat, time for reduction of oxygen content from 100% to 50% ($t_{50\%}$) for emulsions and aIP for formation of primary oxidation products as monitored by A_{234} for liposomes. Significant effects were further classified by least significant difference (LSD) ($P \leqslant 0.05$). Furthermore, aIP or $t_{50\%}$ for combinations of antioxidants in each of the test systems were compared to the sum of the aIP or $t_{50\%}$ obtained in separate experiments with each of any two antioxidant combinations by *t*-test ($P \leqslant 0.05$), in order to detect significant synergistic effects.

3. Results and discussion

Quercetin was selected for the study, since quercetin, in many studies, has been identified as the most efficient antioxidant among the non-vitamin plant phenols (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). Quercetin (Fig. 1) combines the three structural elements found important for efficient radical-scavenging and resonance stabilization of the phenoxyl radical of the one-electron oxidized compound: (i) the *o*-dihydroxyl (catechol) structure of the B ring; (ii) the 2,3-double bond in conjunction with a 4-oxo function; (iii) the additional presence of both 3- and 5-hydroxyl groups (Bors, Heller, Michel, & Saran, 1990). Quercetin is present in plants as the more hydrophilic glucoside rutin which, together with astaxanthin, is known to be effective as an antioxidant in polyunsaturated food lipids (Jensen, Birk, Jokumsen, Skibsted, & Bertelsen, 1998). Thus, quercetin (QC), rutin (RUT), astaxanthin (AX) and

also α-tocopherol (TOH), the most important lipophilic antioxidant, were selected for the study of interactions in lipid systems of increasing structural organisation.

3.1. Antioxidant interactions in bulk oil

The Rancimat test is a standard method for neat oil and the IPs for antioxidant combinations obtained are presented in Fig. 3. Antioxidant combinations showed no synergistic effects; on the contrary, significant negative effects (antagonism) were seen when combining antioxidants, QC with TOH or with AX, whereas additive effects were observed for the combination of QC with RUT. The concentrations of mixtures of antioxidants had no effect on antioxidant interaction, except for the combination of QC and AX, where a high concentration showed a high antagonistic effect, whereas low concentration showed an additive effect. In all cases, QC showed a higher antioxidative effect than did its mixture with antioxidants. Other studies have also reported a strong negative interaction of TOH with different plant extracts rich in polyphenols in lard (Banias, Oreopoulou, & Thomopoulos, 1992) and sunflower oil (Hras, Hadolin, Knez, & Bauman, 2000).

The IP value of the purified sunflower oil with added antioxidants is shown in Fig. 4. The IP for the control, which contained no antioxidant, was 1.75 ± 0.06 h. In the antioxidant concentration range up to 0.5 μmol/g oil, the IP increased with concentration of the antioxidant for QC and TOH, whereas it was near zero for all concentrations assayed for AX and RUT. Better protection of QC than TOH was observed at concentrations above 0.5 μmol/g oil, giving the hierarchy for the antioxidant activity in bulk oil:

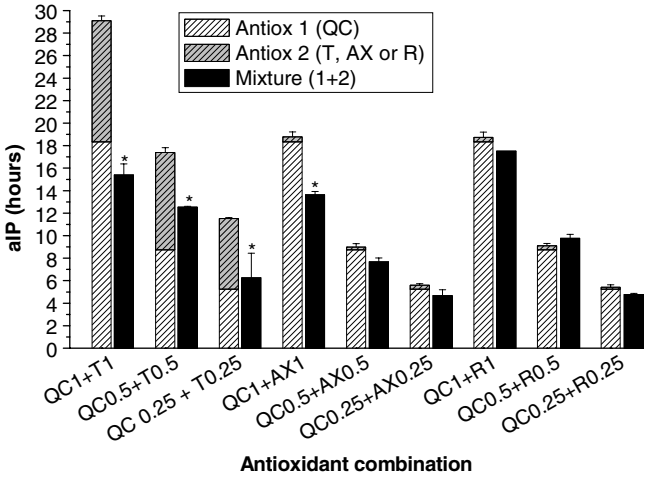

Fig. 3. Additional induction period (aIP) found experimentally for combinations of antioxidants added to bulk high oleic sunflower oil compared with single antioxidant addition by the Rancimat test. T = α-tocopherol, QC = quercetin, R = rutin, AX = astaxanthin. The number after each antioxidant abbreviation is the concentration of the antioxidant in μmol/g oil. * Indicates that aIP for antioxidant mixture is significantly different ($P \leqslant 0.05$) from sum of aIP for antioxidants 1 + 2.

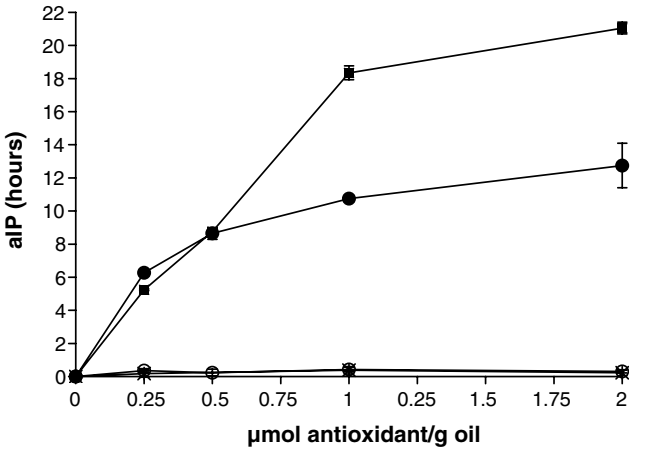

Fig. 4. Concentration-dependence of additional induction period (aIP) in high oleic sunflower oil determined in Rancimat test for ● = α-tocopherol, ■ = quercetin, × = rutin and ○ = astaxanthin.

$$QC > TOH \gg AX = RUT$$

The ranking confirms a higher efficacy of hydrophilic antioxidants in bulk lipid systems. An exception was RUT (quercetin-3-rutinoside) which is more polar than is QC, due to the sugar moiety. However, RUT has a phenolic group less than QC and the sequence, in terms of IP for the two flavonoids, is consistent with results by Bonilla, Mayen, Merida, and Medina (1999), who found the activity of QC to be higher than those of quercetin-glucosides. The results were also in good agreement with the data reported by Milovanovic, Picuric-Jovanovic, Vucelic-Radovic, and Vrbaski (1996) for lard in terms of a higher antioxidant activity of QC than for TOH and no antioxidant activity of RUT.

3.2. Antioxidant interaction in methyl linoleate emulsions

Synergistic effects were observed in emulsions using the oxygen assay with metmyoglobin initiation for combinations of QC and TOH and for combinations of 0.075 mol% of QC and RUT (Fig. 5). Synergistic effects of TOH with polyphenols have also been observed in studies of green tea catechins in AAPH-initiated oxidation of linoleic acid in *tert*-butyl alcohol–water solution (Jia et al., 1998) and for TOH in combination with QC, (+)-catechin and (−)-epicatechin in α,α′-azoisobutyronitrile initiated oxidation of methyl linoleate in *tert*-butyl alcohol or in chlorobenzene (Pedrielli & Skibsted, 2002). The effect of combining QC with AX was purely additive. The same type of interaction was observed, whether the concentration of antioxidants was high or low, except for the combination of QC and RUT, where high concentration showed a clear synergistic effect while low concentration only gave an additive effect.

A high antioxidative activity was observed for QC and TOH (Fig. 6). The ranking of the antioxidant was:

$$QC > TOH \gg AX > RUT$$

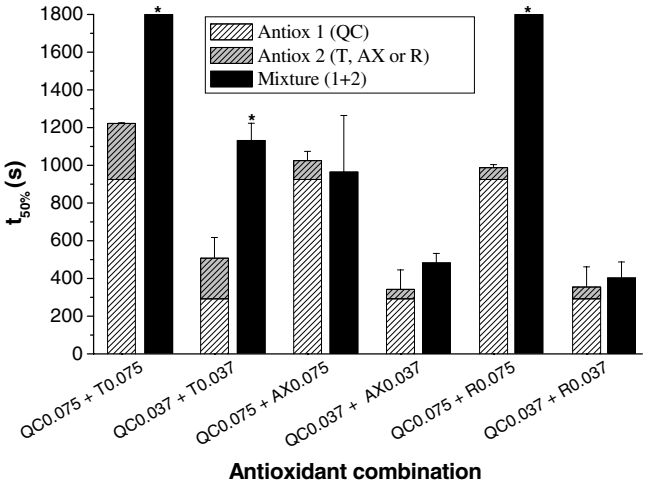

Fig. 5. Time elapsed until oxygen content decreased to 50% of initial concentration ($t_{50\%}$) for mixtures of antioxidants added to methyl linoleate o/w emulsions with metmyoglobin as initiator compared to sum of $t_{50\%}$ as determined in experiments with single antioxidants. T = α-tocopherol, QC = quercetin, R = rutin, AX = astaxanthin. The number after each antioxidant abbreviation is the concentration of the antioxidant in mol% of lipid. ∗ Indicate that $t_{50\%}$ for antioxidant mixture is significantly different ($P \leqslant 0.05$) from the sum of $t_{50\%}$ for antioxidants 1 + 2.

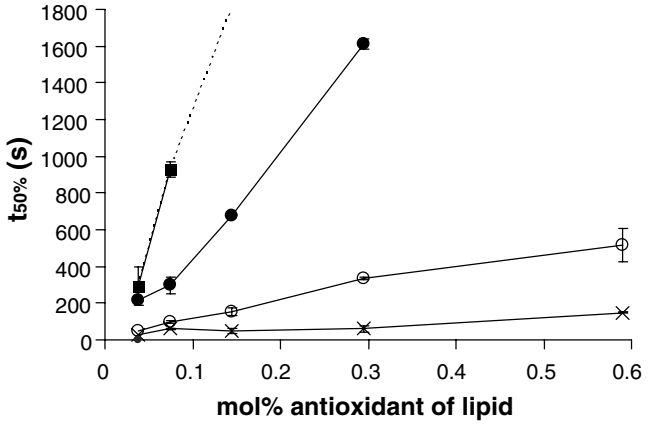

Fig. 6. Time elapsed until oxygen content decreased to 50% of initial concentration ($t_{50\%}$) in a methyl linoleate o/w emulsion with metmyoglobin as initiator. ● = α-tocopherol, ■ = quercetin, × = rutin and ○ = astaxanthin. The dotted line indicates that the $t_{50\%}$ was longer than the recorded time of 30 min (1800 s).

QC was clearly a stronger antioxidant than was the more hydrophilic RUT. In linoleic acid emulsions, where oxidation was initiated by AAPH, the activity of the aglycones has also been reported to be higher than that of glycosides, although RUT still displayed a clear antioxidative activity (Azuma, Ippoushi, Ito, Higashio, & Terao, 1999). In the present study, a concentration of about 6 mol% of RUT would have been necessary to achieve the same antioxidative effect as 0.075 mol% of QC (results not shown); thus QC was about 80 times more effective than RUT. A similar effect of increased hydrophilicity has been noted for TOH and the water-soluble analogue Trolox. Although the results found in the literature are sometimes contradictory, there is a general agreement for the better effectiveness of the more lipophilic antioxidant, TOH, in comparison to that of the water-soluble Trolox in methyl linoleate emulsions (Frankel, Huang, Kanner, & German, 1994; Huang, Hopia, Schwarz, Frankel, & German, 1996; Taylor, Richardson, & Jasensky, 1981). AX was clearly less effective than were QC or TOH although AX, a dihydroxy carotenoid, was expected to orientate close to the hydrophobic-hydrophilic interface of the oil droplet due to the polar hydroxyl groups. Even though AX is suggested to be properly localized, its reactivity and protective ability in the system studied was low but significant.

3.3. Antioxidant interaction in PC-liposomes

In PC liposomes, oxidation was initiated by peroxyl radicals generated from the water-soluble azo-initiator AAPH. The interactions of antioxidants are shown in Fig. 7. The combination of QC and RUT showed a clear synergistic effect in retarding lipid oxidation. The combination of QC and TOH only had a synergistic effect at high concentrations of QC and low concentrations of TOH; for the other combinations, the effects were additive. The combinations of QC and AX exerted additive or slightly synergistic effects ($P \geqslant 0.05$). No synergy was shown for mixtures of β-carotene and TOH in a study performed by Roberts and Gordon (2003) using practically the same liposome peroxidation method. Several studies of carotenoids and tocopherols have shown that the carotenoid concentration should be considerably lower than that of the tocopherol in order to observe synergy (Palozza & Krinsky, 1992; Schroeder et al., 2006; Wrona, Korytowski, Roznowska,

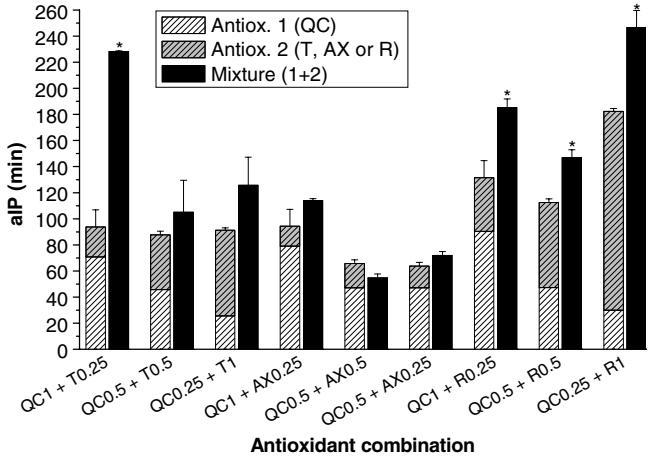

Fig. 7. Additional induction period (aIP) found by spectrophotometric measurement of conjugated dienes for combinations of antioxidants added to soybean phosphatidyl choline liposomes with free radical initiator of oxidation compared to aIP calculated as sum of IP found for liposomes with single antioxidant addition. T = α-tocopherol, QC = quercetin, R = rutin, AX = astaxanthin. The number after each antioxidant abbreviation is the concentration of the antioxidant in mol% of soybean PC. ★ Indicates that aIP for antioxidant mixtures is significantly different ($P \leqslant 0.05$) from the sum of aIP for antioxidants 1 + 2.

Sarna, & Truscott, 2003). In the study by Roberts and Gordon (2003), the relative concentrations of TOH and β-carotene were 1:1 or 2:1 while, in the present study, the relative concentrations were adjusted accordingly and were QC/AX 4:1 and 2:1. However, no clear tendency was observed by changing the ratio of the antioxidants. Small synergistic effects were observed by Liao and Yin (2000) for equimolar mixtures of TOH and QC and for QC and β-carotene in delaying Fe^{2+}-initiated liposome peroxidation. Terao et al. (1994) also suggested positive interactions of flavonoids with TOH through a repair of tocopherol from tocopheroxyl radicals by flavonoids.

The ranking of the antioxidants was as follows (Fig. 8):

$$\text{RUT} > \text{TOH} = \text{QC} > \text{AX}$$

TOH showed a concentration-dependent effect at concentrations as high as 10 mol%, where the IP reached 451 ± 28 min (result not shown). The higher antioxidant activity of RUT, compared to TOH, is, however, in agreement with a number of studies. Several catechins and QC displayed much higher antioxidative effects than did TOH in AAPH-initiated liposome oxidation (Terao et al., 1994) or metal ion-induced liposome oxidation assay at pH 5.4 (Gordon & Roedig-Penman, 1998), whereas TOH was found to exert the best antioxidative effect in a metal ion-induced liposome oxidation system at pH 7.4 compared to other phenolic compounds (Liao & Yin, 2000). Several studies have also shown that catechins and RUT display a higher antioxidative activity than does QC (Liao & Yin, 2000; Terao et al., 1994), which is consistent with the present results. The study performed by Saija et al. (1995) showed a greater protection by RUT than by QC in inhibiting Fe^{2+}-induced linoleate peroxidation, whereas the opposite was the case for autoxidation of rat cerebral membranes, thus indicating that the effect of the antioxidant depends on the substrate and the method used to induce lipid oxidation. Studies by Liao and Yin (2000) showed highest antioxidant activity for TOH, followed by (+)-catechin, (−)-epicatechin, RUT, QC while β-carotene had little effect. For these four flavonoids, the ranking negatively correlated with their partition coefficients, where a high partition coefficient means a high lipophilicity, measured as ratio between concentration of flavonoid in *n*-octanol and water. Liao and Yin (2000) suggested that flavonoids with high partition coefficients, favouring the lipid phase, such as QC, penetrate deeper into the membrane bilayer, thus displaying less interaction with free radicals present in the aqueous phase.

AX, incorporated into liposomes, did show a low protective effect against AAPH-initiated oxidation. AX is expected to orientate close to the interface of the lipid bilayer, but did not protect the lipid membrane efficiently. Other studies have analyzed the effect of several carotenoids on PC peroxidation, initiated by AAPH following the inhibition of phospholipids hydroperoxide formation (Woodall, Britton, & Jackson, 1997) or conjugated dienes (Roberts & Gordon, 2003). These studies showed that TOH gave more protection than did β-carotene, whereas AX did not inhibit peroxidation to any extent. However, it was observed that other hydroxycarotenoids, such as zeaxanthin, lutein and β-cryptoxanthin, gave more protection against lipid peroxidation than did β-carotene. The relatively high effectiveness of diketo-compounds, including AX, in preventing lipid peroxidation, has also been corroborated by other studies (Palozza & Krinsky, 1992; Lim et al., 1992; Miki, 1991). Thus, another explanation for the low antioxidative activity of AX in the present study is a possible oxidative deterioration of AX. Chen and Djuric (2001) observed a substantial depletion of carotenoids after incubation with free radicals generated from Fe^{2+} or AAPH. Other experiments in this study involved substantial percentages of carotenoids present in the liposomes after incubation with AAPH but still without any protective effect of the carotenoids against lipid oxidation, suggesting that carotenoids do not provide any protection against lipid peroxidation in unilamellar liposomes.

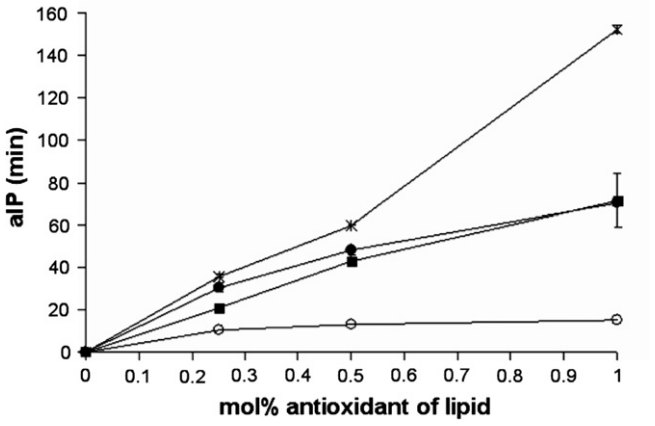

Fig. 8. Concentration-dependence of additional induction period (aIP) found by spectrophotometric measurement of conjugated dienes in soybean phosphatidyl choline liposomes with free radical initiation of oxidation. ● = α-tocopherol, ■ = quercetin, × = rutin and ○ = astaxanthin.

3.4. Comparison of the three oxidation systems

The mechanism that explains the performance of antioxidants in multiphase systems differs from that in bulk oils, due to the more complex solvation and interfacial phenomena that are expected to affect interaction and activity (McClements & Decker, 2000). The antagonism observed in bulk oil, we suggest, is caused by intermediates formed by antioxidants other than quercetin and which may be chain-carrying. Under the high temperature conditions of the oil, the antioxidants may thus act as an easily oxidizable substrate. Clearly, any influence of a less effective antioxidant regenerating the most effective, i.e. QC, is not seen. Such a mechanism has been suggested for TOH/QC interaction at lower temperatures in *tert*-butyl alcohol (Pedrielli & Skibsted, 2002). Synergistic interactions were, in contrast, observed in the biphasic systems for combinations

of phenolic antioxidants, both for QC with TOH and for QC with RUT.

For the organized lipid systems, differences in antioxidative activity compared to the homogeneous system may be explained by the partitioning of antioxidants into different phases, but the relationship between partition of antioxidants and their effectiveness in multiphase systems is not very clear. The synergism observed most significantly for QC and TOH in the emulsions and, to a lesser degree, in the liposomes, may be explained by a binding of QC to the surface of the oil/water interface where they protect TOH against radicals generated by metmyoglobin in the homogeneous aqueous phase of the emulsion. RUT is freely soluble in the aqueous phase and is to a lesser degree, available at the interface to protect TOH which, in turn, protects the lipids. In the liposome, the PC is more exposed to the aqueous solvent and while QC protects the lipids from attack of radicals generated in the aqueous phase by the initiator and TOH protects against radicals generated in the lipid layer, respectively, the interaction apparently becomes less important compared to the emulsion, and the two types of antioxidants serve as two independent pools. In the liposomes, the synergism only appears when QC is in large excess compared to TOH. This observation could be explained by a double-sided exposure of the lipids to water, requiring presence of QC at both interfaces, when oxidation is initiated in the water phase. With respect to the activity of flavonoids, Ratty, Sunamoto, and Das (1988) have demonstrated that flavonoids interact in the polar surface region of the phospholipid bilayers. It is likely that QC and other flavonoids are mostly localized near the surface of membranes where aqueous peroxyl radicals are easily trapped and are more readily accessible to peroxyl radicals than is TOH, thus explaining the better antioxidative effect of QC in liposomes and emulsions, as seen in the present study with initiation of oxidation by a hydrophilic initiator. The hydrophobicity of AX could also explain the scarce efficacy of this carotenoid in bulk oils, due to its localization in the oil and not in the air–oil interface where lipid oxidation is initiated. In multiphasic systems, however, it was expected that AX due to the diketo-group was close to the interface and thus to peroxyl radicals. This was clearly observed by the protection of methyl linoleate in emulsions but not in liposomes, in analogy with the less significant synergism seen for QC and TOH in the liposomes.

4. Conclusions

Synergistic effects between antioxidants were only observed in multiphasic systems and especially in the o/w emulsion and in the liposomes for combinations of quercetin with α-tocopherol and of quercetin and rutin. No synergism was observed with the carotenoid astaxanthin. Synergism is suggested to be mainly caused by differences in solubility, and differences in phase distributions near or at the interface. Results of antioxidant activity in bulk oil correlated with the polarity of the antioxidants, with a higher effectiveness of hydrophilic antioxidants in bulk lipid systems. An exception was rutin, which is relatively hydrophilic but had the lowest activity in the oil, indicating that it is not only polarity that has an influence on effectiveness. The poor solubility of rutin in the bulk oil or degradation of the glycoside at high temperature may explain the decrease in efficiency. In emulsions, the more lipophilic antioxidants protected the lipid phase better, indicating that a proper localization of the antioxidant at the oil–water interface, where oxidation takes place, is important. Scavenging of AAPH-generated lipid peroxyl radicals in liposomes was more efficient for the hydrophilic rutin, than for the more hydrophobic α-tocopherol and quercetin. The difference in synergism observed for quercetin and α-tocopherol between the liposomes and the emulsion, is important, and we are currently exploring the phase-transfer kinetics in more detail.

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