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Food Chemistry 83 (2003) 523–529

Food Chemistry

www.elsevier.com/locate/foodchem

Antioxidant properties of annatto carotenoids

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Received 3 May 2002; received in revised form 18 March 2003; accepted 18 March 2003

Abstract

The antioxidant effects of β-carotene, oil-soluble (bixin) and water-soluble (norbixin) annatto preparations and mixtures of these carotenoids with virgin olive oil polar extract were assessed in bulk olive oil and oil-in-water emulsions stored at 60 °C. Norbixin was the only carotenoid that inhibited the oxidative deterioration of lipids in both systems. Though bixin and β-carotene did not retard autoxidation, their mixtures with the polar extract from virgin olive oil enhanced the antioxidant effect of the olive oil extract. Norbixin (2 mM) was of similar activity to δ-tocopherol (0.1 mM) in stored oil. The combination of norbixin with ascorbic acid or ascorbyl palmitate in oil showed a reduction in formation of volatile oxidation products but not in peroxide value, compared with the analogous sample lacking norbixin. In olive oil-in-water emulsions, norbixin (2 mM) reduced hydroperoxide formation to a similar extent as δ-tocopherol (0.1 mM), which in turn was a better antioxidant than α-tocopherol. A synergistic effect between norbixin and ascorbic acid or ascorbyl palmitate was observed in the emulsion systems.

Keywords: Norbixin; Antioxidant; Olive-oil; Emulsions; Oxidation

1. Introduction

Several studies have shown that, under certain conditions, carotenoids can act as chain breaking antioxidants, by scavenging and deactivating free radicals both in vitro and in vivo (Bast, Haanen, & VandenBerg, 1998). Antioxidant activity of carotenoids in organic solution is related to oxygen concentration, the chemical structure of carotenoids and the presence of other antioxidants (Krinsky, 1993). Carotenoids have been shown to maintain the stability of oils against photooxidation (Jung & Min, 1992). Pokorny and Reblova (1999) reported that oxidation of frying oil is inhibited by carotenoids, and Gutierrez, Jimenez, Ruiz, and Albi (1999) observed a very good correlation between stability of virgin olive oil and concentrations of carotenoid pigments. Most studies have focused on β-carotene, and the ability of this carotenoid to interact with free radicals, including peroxyl radicals, is well documented.

However, β-carotene did not inhibit autoxidation of oils from which tocopherols had been removed, and the compound promoted formation of lipid hydroperoxides during autoxidation in the light (Haila & Heinonen, 1994) or in the dark (Warner & Frankel, 1987).

Components of mixed antioxidant systems can contribute to the inhibition of oxidation, with the resulting antioxidant activity reflecting either additive or synergistic effects of the components. The most effective mixed antioxidant systems for foods contain antioxidants with different mechanisms of action and/or physical properties. In the presence of a sufficiently high concentration of other antioxidants, carotenoids may behave as antioxidants, even though they present a prooxidant character in the absence of other additives (Palozza, 1998). It was found that a combination of lutein and γ-tocopherol was very effective as an antioxidant, while lutein or β-carotene had no effect on the oxidation of a purified triacylglycerol fraction from rapeseed oil (Lievonen, 1996). Similarly ascorbic acid was found to enhance the retention of carotenoids and thereby increased their antioxidant potency (Sudhakar & Maini, 1994).

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doi:10.1016/S0308-8146(03)00148-1

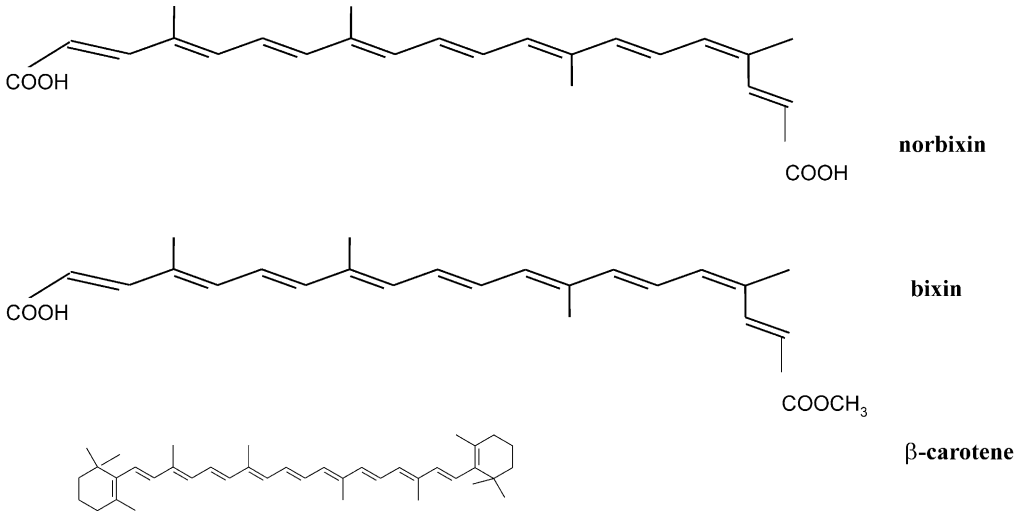

Fig. 1. Structure of the carotenoid pigments tested in the current study.

The term annatto includes a series of colouring preparations consisting of carotenoid-type pigments; all based on extracts of the seed of the tree *Bixa orellana*, which grows abundantly in the tropics (Nish, Whisman, Goetz, & Ramirez, 1991). Bixin, which is a carotenoid with two carboxylic acid groups, one of which is esterified, is the major pigment present in annatto extract (Fig. 1). Norbixin, which is derived from bixin by hydrolysis of the ester group, is also sold as a food pigment, and this molecule is water-soluble, whereas bixin is oil-soluble. As annatto is used in food as a pigment, most of the published studies about annatto pigments have concentrated on the improvement of extraction protocols or on the determination of annatto in foods (Kovary et al., 2001). There are few reports of the antioxidant properties of annatto carotenoids. Haila, Lievonen, and Heinonen (1996) reported that bixin strongly inhibited the autoxidation of rapeseed oil triglycerides, though other carotenoids, such as lutein or lycopene, had no antioxidant effect. However, there are no reports on the antioxidant activity of norbixin. The main objective of this study was to investigate the antioxidant properties of annatto carotenoids in edible oil and in oil-in-water emulsions during storage, and to investigate any synergy between these pigments and other natural antioxidants.

2. Materials and methods

2.1. Materials

Refined olive oil was provided by Leon Frenkel Ltd (UK). Spanish virgin olive oil (Filippo Berio) was purchased in a local retail outlet. *All-trans* synthetic β-carotene (purified 95%), α-, and δ-tocopherol, ascorbic acid and ascorbyl palmitate were purchased from Sigma-Aldrich Co. Ltd., Poole, UK. Bixin and norbixin samples were donated by Overseal Foods Ltd, Swadlincote, UK. Other chemicals used were of analytical grade.

2.2. Methods

Tocopherols were removed from refined olive oil by column chromatography. The antioxidant activity of *all trans* β-carotene and annatto extracts rich in bixin or norbixin (1 g l^{-1}) was tested in bulk refined olive oil with reduced tocopherol content and in 10% olive oil-in-water emulsions at 60 °C, and synergy with the polar extract (0.2 g l^{-1}) from virgin olive oil was studied. The antioxidant activity of norbixin (0.76 g l^{-1}, 2 mM) was then compared with α-tocopherol and ascorbic acid (0.1 mM) in olive oil-in-water emulsions, and synergy was investigated. Peroxide values were studied to monitor oxidation in oils, whereas conjugated dienes were determined to monitor oxidation in emulsions, since the peroxide value cannot be determined directly in emulsions without separation of the oil by the standard method. Selected samples were also analysed for volatile products, and the hexanal content was used as a measure of secondary oxidation products. Duplicate samples were stored, and each parameter was determined in triplicate on each sample.

2.3. Preparation of oil-in-water emulsions

Tocopherols were removed from refined olive oil by open column chromatography. Aluminium oxide (14 g), dried overnight at 200 °C, was added to a glass column (wrapped in aluminium foil and connected to a Buchner flask). Olive oil (20 g) was passed through the column

by vacuum suction. This reduced the tocopherol content to about 30 mg kg^{-1}. Oil-in-water emulsion (20 g, 10% oil) was prepared by mixing a solution of sodium acetate buffer (0.1M, pH = 5.5, 18 g) containing Tween 20 (0.2 g). Olive oil (2 g), containing required antioxidants, was added dropwise, as the sample was cooled in an ice bath and sonicated with a VC-50 sonicator (Sonics and Materials Inc., Danbury, USA). Sonication was continued for 5 min after the oil had been added. Droplet size distributions of the emulsions were measured by a static light-scattering technique, with a Malvern Instruments particle and droplet sizer (Series 2600, focal length 63 mm, beam length 14.3 mm). The mean droplet diameter (μm) of the emulsions was characterised by d_{32} where

$$d_{32} = \Sigma n_i d_i^3 / \Sigma n_i d_i^2,$$

where n_i is the number of droplets with diameter d_i

It was found that the mean d_{32} value for the emulsions was 1.27 ± 0.03 μm.

2.4. Oxidation and analysis of the samples

Olive oil with reduced tocopherol content (30 mg kg^{-1}) was used in the oxidation studies. Samples of olive oil and olive oil-in-water emulsion were transferred to screw-capped sample vials and held in an oven at 60 °C. The lids were only screwed loosely on the vials, so that air could pass in and out of the headspace above the samples.

Emulsion samples were removed for analysis periodically (every 5 or 10 days), diluted in ethanol (1:100), and their oxidative state was determined by monitoring absorbance at 233 nm with a UV–vis Spectrometer (Lambda Bio 20, Perkin-Elmer, Basingstoke, UK). The amount of conjugated dienes per 100 g of sample was calculated using the relative molecular mass (280 g mol^{-1}) and the molar absorptivity of linoleic acid ($\varepsilon = 26{,}000$).

The oxidative state of olive oil samples was determined by analysis of the peroxide value (PV) (AOAS official method, 1989). Synergy between norbixin and the other additives was calculated using the equation:

$$\% \text{ Synergy} = [\text{IP mixture} - (\text{IP norbixin} + \text{IP additive})] \times 100/(\text{IP norbixin} + \text{IP additive}).$$

where IP = induction period

2.5. Headspace analysis

Solid phase microextraction (SPME) was used for analysis of the volatiles produced in the oxidised samples by a method based on that described by Zabaras and Wyllie (2002). A sample of emulsion or bulk olive oil (1g) was weighed into a vial, which was then purged with nitrogen for 2 min and sealed with a septum secured by an aluminium cap. A SPME fibre (75 nm, Carboxen-PDMS) was introduced through the septum into the headspace and was retained in the vial, held at 60 °C in the dark for 30 min. The fibre was then removed from the vial and inserted directly into the injection port of a Hewlett Packard (HP 6890) GC, equipped with a flame ionisation detector and an integrator. A WCOT CP-SIL 8CB fused silica column (60 × 0.25 mm i.d., df = 0.25 μm, Chrompack UK Ltd., London, UK), was used for GC analysis, with helium as the carrier gas. The column temperature was programmed to increase from 40 °C (2 min) to 90 °C at 4 °C/min, and then to 250 °C at 10 °C/min. 1,2 Dichlorobenzene (100 ng μl^{-1} in methanol) was used as an external standard for the quantification of volatiles.

2.6. HPLC analysis

A Hewlett Packard 1050 liquid chromatograph, equipped with diode array detector and a Chemstation 7 for data collection was used for HPLC analysis of carotenoids. Sample (20 μl) was injected onto a Spherisorb ODS-1 reversed phase column (250 × 4.6 mm i.d., 5 μm particle size), protected by a guard column (ODS-1).

For analysis of the β-carotene and bixin used as additives, isocratic elution at 1.0 ml/min with acetonitrile/methanol/tetrahydrofuran/ammonium acetate (1% w/v) in the ratio 68:22:7:3 was used with detection at 450 nm (Hart & Scott, 1995).

For analysis of the norbixin used, isocratic elution at 1 ml/min with acetonitrile:aqueous acetic acid (99.6: 0.4 v/v), with detection at 450 nm, was used, as described by Scotter, Wilson, and Appleton (1998).

Tocopherols in purified olive oil and in the carotenoid samples were determined by normal phase HPLC as described by the AOCS method Ce 8-89 (AOCS, 1989), and reverse phase HPLC (Montedoro, Sevilli, Baldioli, & Miniati, 1992) was used for determination of the phenolic components in the olive oil polar extract.

2.7. Isolation of olive oil polar extract

The polar fraction of virgin olive oil was extracted according to the procedure proposed by Satue, Huang, and Frankel (1995). The total phenolic content of the extracts was determined with Folin-Ciocalteu reagent according to Gutfinger (1981).

2.8. Statistical analysis

An ANOVA test was used to compare the mean values of each treatment for either conjugated dienes or

peroxide values at the end point of each experiment with $P < 0.05$ for significance.

Least significant differences (LSD values) were used to decide whether the antioxidant treatments differed from the control and from each other. Genstat 5.0 was the statistical program used.

3. Results and discussion

3.1. Composition of bixin and norbixin samples

HPLC analysis of the water-soluble annatto extract showed that norbixin was the major component (93.2%), and bixin was also present at 6.8% concentration. Bixin was the only component detected in the oil-soluble annatto extract. Both of the annatto extracts were free of tocopherols (<2 mg kg^{-1}).

3.2. Effect of additives on autoxidation of olive oil

The antioxidant activity of *all trans* β-carotene, bixin and norbixin (1 g l^{-1}) was tested in bulk refined olive oil, and the effect of a combination of these additives with the polar fraction of olive oil (0.2 g l^{-1}) was also studied (Fig. 2, Table 1). The polar fraction of virgin olive oil is complex including tyrosol and hydroxytyrosol and their secoiridoid derivatives together with phenolic acids and other chemical classes (Gordon, Pavia-Martins, & Almeida, 2001).

Norbixin and the olive oil polar extract inhibited hydroperoxide formation, whereas bixin did not differ significantly from the control, and β-carotene had a prooxidant character. Previous studies have shown that oxygen-containing carotenoids with polar functional groups are better antioxidants than the hydrophobic ones (Niki, Noguchi, Tsuchihashi, & Goton, 1995). β-Carotene lacks antioxidant activity in the dark at normal atmospheric pressure (Burton & Ingold, 1984), although

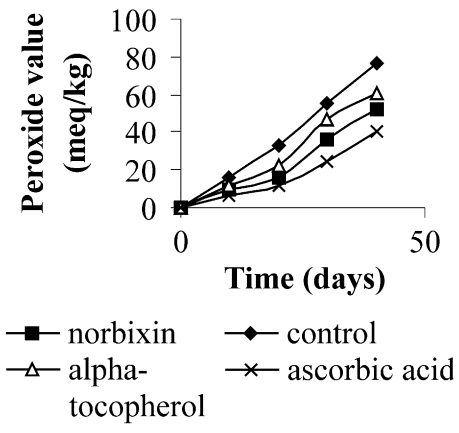

Fig. 2. Effect of selected antioxidants on oxidative stability of olive oil at 60 °C, assessed by determination of peroxide values.

bixin at a concentration of 60 mg kg^{-1}, was found to retard the autoxidation of rapeseed oil triacylglycerols (Haila et al., 1996). Henry, Gatignani, and Schwarz (1998) observed that both β-carotene and lycopene acted as prooxidants during the heat-catalysed oxidation of safflower oil. Although carotenoids readily add lipid peroxy radicals, the radical formed must not propagate further reactions, and the absence of oxygen is often important for the stability of the carotenoid derived radical, since oxygen adds to the carbon-centred radical, with the unpaired electron delocalised, to convert it to a peroxy radical in which delocalisation is impossible (Burton, 1988). The presence of the polar carboxylic acid groups in the norbixin molecule may contribute to retardation of autoxidation by chelation of prooxidant metal ions or other polar initiating species.

The polar extract from virgin olive oil contains a mixture of phenolic components and the antioxidant activity of the extract is well recognised and has been shown to be due to the presence of hydroxytyrosol and derivatives which have radical scavenging activity (Gordon et al., 2001). A combination of carotenoids with olive oil extract gave an increased antioxidant effect during the storage of refined olive oil compared with either of the components of the mixture.

Further study of the interaction between norbixin and natural antioxidants (Table 2) showed that norbixin increased the antioxidant effects of α- and δ-tocopherols (0.1 mM) in bulk olive oil, when present at 2 mM concentration. Norbixin did not change the rate of hydroperoxide formation with ascorbic acid or ascorbyl palmitate (0.1 mM). The rate of hydroperoxide decomposition, assessed by formation of hexanal or total volatiles, was markedly reduced by the combination of norbixin with each tested antioxidant. The effect of norbixin, in combination with ascorbic acid or ascorbyl palmitate, was especially noteworthy since there was no evidence of a reduction in the peroxide values due to the presence of norbixin in these samples.

3.3. Effect of carotenoids on oxidation of oil-in-water emulsions

Carotenoids, olive oil polar extract and their mixtures were tested in oil-in-water emulsions prepared with refined olive oil at pH 5.5, and oxidation was monitored by measurement of conjugated dienes (CD) at 233 nm (Table 1). As in the oil system, norbixin was the only carotenoid that showed significant antioxidant action at 1 g l^{-1} (2.6 mM). Bixin did not differ significantly from the control, and β-carotene had a small prooxidant action. The polar olive oil extract was again a strong antioxidant, and its activity was not increased significantly by the presence of any of the carotenoids.

β-Carotene has been previously shown to lack antioxidant effects in oil-in-water emulsions (Keiko,

Table 1
Effect of carotenoids (1 g l^{-1}) mixed with olive oil polar extract (0.2 g l^{-1}) on oxidative stability of bulk olive oil or 10% olive oil-in-water emulsions at 60 °C after storage for 30 days

System	Bulk olive oil		10% olive oil-in-water emulsions	
Treatment	[a]Peroxide values (PV, meq kg^{-1}) at 30 days of storage	[b]Protection factor (based on time to PV = 50 meq.kg^{-1})	[b]Conjugated dienes (CD) in g 100 g^{-1} at 30 days of storage	Protection factor (based on time to CD = 0.5 g 100 g^{-1})
Control	101c ± 6.2	1.00	0.31b ± 0.02	1.00
Olive oil extract (OE)	90.5b ± 5.5	1.14	0.20a ± 0.02	1.28
β-Carotene	114d ± 7.8	0.86	0.55c ± 0.03	0.54
β-Carotene + OE	80.9a ± 6.1	1.34	0.30b ± 0.02	0.96
Bixin	106c ± 5.7	1.00	0.32b ± 0.02	0.87
Bixin + OE	84.6a ± 4.8	1.26	0.24a ± 0.02	1.22
Norbixin	57.3a ± 3.5	1.54	0.25a ± 0.02	1.19
Norbixin + OE	50.7a ± 3.1	1.71	0.19a ± 0.03	1.30

[a] For CD or PV, results are expressed as: mean ($n = 6$) ± S.D. ; Significant differences from ANOVA test indicated by different letters (a–d).
[b] Protection factors (PF) are: time for antioxidant treatment/time for control, to reach the specific oxidation level of CD or PV, respectively (PF > 1: antioxidant; PF < 1: prooxidant).

Table 2
Effect of norbixin (2 mM) and mixtures compared with antioxidants (0.1 mM) on the oxidative stability of bulk olive oil at 60 °C after 40 days of storage

Treatment	[a]Peroxide values (meq kg^{-1}) at 40 days of storage	[b]Protection factor (PF) (based on time to PV = 50 meq kg^{-1})	Volatile aldehydes (mg kg^{-1})		Synergy (%)
			Hexanal	Total volatiles	
Control	76.9d ± 4.2	1.00	0.83e ± 0.09	2.59e ± 0.35	–
Norbixin	55.6b ± 3.1	1.26	0.73d ± 0.06	2.26d ± 0.15	–
α-Tocopherol (AT)	60.7c ± 3.3	1.15	0.89e ± 0.06	2.82e ± 0.17	–
δ-Tocopherol (DT)	53.5b ± 2.7	1.27	0.75d ± 0.04	2.34d ± 0.21	–
Ascorbic acid (AS)	40.4a ± 2.1	1.62	0.16b ± 0.03	0.74c ± 0.14	–
Ascor-palmitate (AP)	47.7b ± 2.2	1.42	0.32c ± 0.04	0.85c ± 0.21	–
Norbixin + AT	39.9a ± 1.2	1.73	0.12b ± 0.02	0.53b ± 0.07	−27.4
Norbixin + DT	44.5a ± 2.8	2.93	0.10b ± 0.03	0.45b ± 0.05	−13.3
Norbixin + AS	49.3b ± 2.4	3.33	0.04a ± 0.01	0.19a ± 0.04	−17.6
Norbixin + AP	51.4b ± 3.4	3.47	0.06a ± 0.02	0.16a ± 0.04	−50.8

[a] Mean ($n = 6$) ± S.D.; Significant differences from ANOVA test indicated by different letters (a–e).
[b] PF > 1: antioxidant effect, PF < 1: prooxidant effect.

Higashio, & Terao, 1999), or even to be prooxidant (Heinonen, Haila, Lampi, & Piironen, 1997). However, the antioxidant activity of norbixin in emulsions has not previously been reported.

3.4. Antioxidant activity of combinations of norbixin with vitamins

The antioxidant properties of norbixin (2 mM) were compared with those of α- and δ-tocopherols, ascorbic acid and ascorbyl palmitate (0.1 mM) in refined olive oil at 60 °C. All additives showed antioxidant activity, when oil deterioration was assessed by the peroxide value (Fig. 2) and the order of activity was: ascorbic acid > ascorbyl palmitate, δ-tocopherol > α-tocopherol. The activity of norbixin (2 mM) was similar to ascorbyl palmitate or δ-tocopherol (0.1 mM). The effect of the antioxidants was confirmed by analysis of the volatiles formed during lipid oxidation (Table 2), with ascorbic acid again being very effective. α-Tocopherol did not inhibit formation of volatiles. When added in the same concentration (0.1 mM) to oil-in-water emulsions at pH 5.5, the antioxidants retarded the formation of hydroperoxides, but the order of antioxidant activity was: δ-tocopherol > ascorbyl palmitate, α-tocopherol > ascorbic acid (Table 3). Norbixin, at 2 mM concentration, was similar in antioxidant activity to δ-tocopherol (0.1 mM) in the emulsion system.

The change in activity of ascorbic acid from being the strongest antioxidant in oil to the weakest in the emulsion has been previously reported (Frankel, Huang, Kanner, & German, 1994) and the change in order of

Table 3
Antioxidant effects of norbixin (2 mM) and mixtures compared with other antioxidants (0.1 mM) on the oxidative stability of 10% olive oil-in-water emulsions at 60 °C after 40 days of storage

Treatment	[a]Conjugated dienes (g 100 g^{-1}) at 40 days of storage	[b]Protection factors (PF) based on time to CD = 0.5 g 100 g^{-1}	Synergism (%)
Control	2.41d ± 0.02	1.00	–
Norbixin	1.49a ± 0.03	1.33	–
α-Tocopherol (AT)	1.94b ± 0.05	1.14	–
δ-Tocopherol (DT)	1.48a ± 0.03	1.37	–
Ascorbic acid (AS)	2.08c ± 0.05	1.17	–
Ascor-palmitate (AP)	1.98b ± 0.07	1.33	–
Norbixin + AT	1.31b ± 0.04	1.73	+ 8.3
Norbixin + DT	0.91a ± 0.03	2.93	+ 13.5
Norbixin + AS	1.23b ± 0.04	2.33	+ 23.5
Norbixin + AP	0.92a ± 0.03	2.97	+ 21.4

[a] Mean (n = 6) ± S.D.; significant differences indicated by different letters (a–d).
[b] PF > 1: antioxidant effect, PF < 1: prooxidant effect.

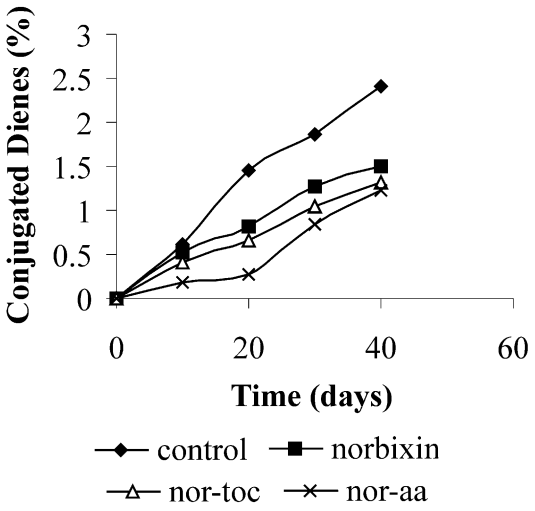

Fig. 3. Effect of norbixin (2 mM) mixtures on oxidative stability of 10% olive oil-in-water emulsions at 60 °C, evaluated by measurement of conjugated dienes at 233 nm. [nor-toc = norbixin (2mM) + α-tocopherol (0.1 mM); nor-aa = norbixin (2 mM) + ascorbic acid (0.1 mM)].

activity is consistent with the "polar paradox", whereby a hydrophilic antioxidant is more active in oil whereas lipophilic antioxidants, such as tocopherols and ascorbyl palmitate, are more effective in the emulsion system, since they are concentrated at the oil–water interface, where they are effective in quenching radicals generated in the aqueous phase. Norbixin molecules are soluble in water, probably as aggregates, and insoluble in oil but the carotenoid is probably oriented at the oil–water interface in the emulsion due to its extensive hydrocarbon backbone. This would allow it to show the strong antioxidant activity observed in the emulsion system.

In refined olive oil, combinations of norbixin (2 mM) with α- and δ-tocopherols (0.1 mM) showed enhanced activity compared with each antioxidant, although the effects were additive rather than synergistic, but combinations of norbixin with ascorbic acid or ascorbyl palmitate did not lead to increased protection against lipid autoxidation compared with the individual additives when oil deterioration was assessed by peroxide values (Table 2).

In the emulsion system, combinations of norbixin with the other additives reduced the oxidative deterioration of the samples to a greater extent than the individual additives (Fig. 3). The protective effect, calculated by induction times to conjugated diene values of 0.1%, was synergistic for each combination (Table 3) with synergy being greatest between norbixin and ascorbyl palmitate (21.4%).

There are no previous reports describing interactions of norbixin with other natural antioxidants. However, tocopherols have been found to enhance the antioxidant activity of carotenoids, and the effect has been ascribed to reduction of the degradation of the carotenoids. Palozza and Krinsky (1992) found that a combination of β-carotene with α-tocopherol inhibited lipid peroxidation significantly better than the sum of the effects of the individual antioxidants. Similarly, Henry et al. (1998) found that a mixture of β-carotene with α- or δ-tocopherols exhibited a stronger antioxidant effect than each single compound did. Palozza, Luberto, and Bartoli (1995) reported that carotenoids exerted their radical trapping activity more efficiently when acting cooperatively with tocopherols in microsomal membranes. Several in vitro studies have investigated interactions between carotenoids and vitamin C. Ascorbic acid inhibited β-carotene degradation in a linoleate model system and thereby reinforced its antioxidant activity, probably by regenerating the carotenoid (Bohm, Edge, Lange, McGarvey, & Truscott, 1997). Mortensen and Skibsted (1998) found that dietary carotenoids react with a wide range of radical species to produce radical cations by electron transfer, and the radical cations react with vitamin C, which regenerates the carotenoid.

4. Conclusions

Norbixin, which is the water-soluble annatto derivative, was found to retard oxidative deterioration both of oils and oil-in-water emulsions, but it was particularly effective in the emulsion where it was more effective than other carotenoids, including bixin and β-carotene at 2.6 mM concentration, as well as being more effective at 2 mM than ascorbyl palmitate, α-tocopherol and ascorbic acid (added at 0.1 mM). Mixtures of norbixin

with ascorbic acid, ascorbyl palmitate and δ- or α-tocopherols enhanced the antioxidant effect beyond that of the phenolic antioxidants in both oils and emulsions, and synergistic effects were observed with the tocopherols and ascorbic acid in the emulsion system.

Acknowledgements

We thank Overseal Foods Ltd. for providing the bixin and norbixin samples and the Greek State Scholarships Foundation for funding this research project.

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