Flavonoids as Stabilizers of Fish Oil: An Alternative to Synthetic Antioxidants

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The antioxidant activities against fish oil oxidation of six commercially available flavonoids and of five flavonoids purified from two Chilean native plants were compared to those of *dl*-α-tocopherol and of two synthetic antioxidants, butylated hydroxytoluene and butylated hydroxyanisole. Among the commercial flavonoids, catechin, morin and quercetin showed a higher activity when fish oil oxidation (either spontaneous or Fe^{2+}-induced) was assessed from the formation of peroxides or thiobarbituric acid-reactive substances. Among the native flavonoids, the 5,3′,4′-trihydroxy-7-methoxy flavanone (designated as Pt-2) showed the highest antioxidant activity. Mixtures of quercetin or of Pt-2 with *dl*-α-tocopherol produced better inhibitory effects when compared to that of each substance assayed by itself. Also, when Pt-2 and quercetin were assayed in combination (0.3 g/kg oil and 0.7 g/kg oil, respectively), a synergistic antioxidant effect was observed. Results indicate that several flavonoids could be used as natural antioxidants as a means to replace those synthetic antioxidants, the use of which has been questioned.

KEY WORDS: *dl*-α-Tocopherol, fish oil oxidation, flavonoids as antioxidants, native flavonoids, natural antioxidants, synthetic antioxidants.

The great interest in the nutritional and pharmacological properties of marine fish oil rich in n-3 polyunsaturated fatty acids (1,2) has given rise to efforts to improve the chemical and organoleptic characteristics of these oils (3). Because fish oil is a secondary product in the manufacture of fish meal, the procedures applied in its production generally provide minimal protection against autoxidation. Therefore, in addition to its undesirable and characteristic off-odor, during its production the fish oil often attains a high peroxide content if no antioxidants are added. Although oil-refining procedures can markedly improve these oxidative indexes, the high instability of the fish oil, due to its high degree of unsaturation, leads the peroxide value back to high levels in a few days.

Among the different procedures for deodorizing fish oil, those involving distillation at high vacuum appear to be the more efficient (3), because not only do they reduce the undesirable and characteristic odor of the oil, but such methods also maintain unaltered the content of the highly polyunsaturated fatty acids (*e.g.*, eicosapentaenoic and docosahexaenoic acids) while reducing the peroxide content to minimal values (<1 meq/kg) (4). Following distillation, the refined oil becames suitable for human consumption, providing that stabilization against autoxidation by the timely addition of one or more antioxidants is accomplished.

The choice of antioxidants to stabilize fish oil for human consumption is restricted to a few substances, with *l*-α-tocopherol (or its synthetic analog, *dl*-α-tocopherol) being the most frequently used (5). Although tocopherols are good and safe antioxidants, they do not always provide effective protection, especially when the oil is contamined with trace amounts of metals (*e.g.*, Fe^{2+} or Cu^{2+}) (6).

Flavonoids constitute a large group of naturally occurring plant products that are widely distributed in the vegetable kingdom. All of them are structurally derived from the parent compound flavone (2-phenylchromone or 2-phenylbenzopirone) and are characterized by two benzene rings joined by a C_3 structure, which is condensed as a six-membered ring and changes with the nature of the flavonoid (see Table 1) (7). Some of the carbons, at any of the three rings, are often hydroxylated and some of these hydroxyl groups may have been transformed to methoxy groups. Flavonoids are ubiquitous in photosynthesizing cells, seeds, fruits and flowers. It has been estimated that the average daily Western diet may contain up to 1 g of mixed flavonoids (8).

Among other properties, flavonoids exhibit high binding affinity to biological polymers and heavy metal ions (9), they catalyze electron transport reactions (10) and may be active in scavenging free radicals (11). In view of these properties, their known liposolubility, and some preliminary observations in our laboratory on their hydroperoxide-reducing properties (12), we decided to assay them as antioxidants for polyunsaturated oils, particularly fish oil.

The present study involves some well-known flavonoids, often employed in biological studies, as well as several flavonoids isolated from native Chilean plants (13).

MATERIALS AND METHODS

Partially refined sardine oil was obtained from a local fish meal factory (Corpesca SA, Mejillones, Chile). It contained approximatively 30% n-3 fatty acids and was winterized and subjected to high vacuum distillation, as previously described (4). The oil distilled under such conditions contained less than 1 meq peroxides/kg and was kept in the dark under N_2 atmosphere at 4°C until the stabilization studies.

Stabilization assays were made with 20-mL aliquots of the oil placed in 100 × 20 mm Petri dishes for 48 h under air in the dark at 60°C. Samples were taken every 12 h. In addition, 250-mL sealed glass bottles containing 100 mL of oil were maintained in the dark at 25°C for 36 d and sampled every 6 d. Peroxidation was monitored as peroxide content (expressed as meq/kg oil) according to the Association of Official Analytical Chemists (14) and

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by the assessment of the thiobarbituric acid-reactive substances (TBARS) as described by Fee and Teitelbaum (15).

Flavonoids, *dl*-α-tocopherol, synthetic antioxidants and mixtures of these were dissolved in 10 μL of ethanol (99.5%)/g oil. Stabilization studies in the presence of a catalyst were performed with 250 μM $FeCl_2$ (added as an ethanolic solution, 10 μL/g oil).

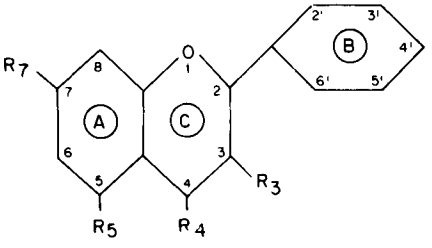
Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and the commercial flavonoids hesperidin, naringin, morin, rutin, catechin and quercetin were obtained from Sigma (St. Louis, MO). *dl*-α-Tocopherol was obtained from Roche (Productos Roche, Santiago, Chile). The flavonoids, 3,5,4′-trihydroxy-6,7,8,3′-tetramethoxy flavone (BB 14-17); 7,4′-dihydroxy-5,6,8-trimethoxy flavone (BB 21-30); 5,4′-dihydroxy-3,3′,6,7,8-penta-methoxy flavone, (BB 7-12); 5,4′-dihydroxy-6,7,8,3′-tetramethoxy flavone (BB 8-13) and 5,3′,4′-trihydroxy-7-methoxy flavanone (Pt-2), were isolated from the native chilean plants, *Baccharis boliviensis* (BB series) and *Parastrephia lucida* (Pt-2). The dried plants were ground in a mixture of petroleum ether/ethanol (1:2), and the flavonoids were separated by silica gel column chromatography. The elution of the different flavonoids was performed by different mixtures of benzene/chloroform of increasing polarity. The crystalline material obtained after drying was repeatedly recrystallized from ethyl acetate for the structural characterization, which was performed by infrared (IR) spectroscopy, H^1 and C^{13} nuclear magnetic resonance and mass spectroscopy by Morales *et al.* (13). All experiments were performed in quintuplicate or sextuplicate, and results were expressed as the means ± SD. The significance between mean values was assessed by Student's *t*-test for unpaired results. Table 1 shows the structure of the flavonoids studied.

RESULTS

The effects of the six commercial flavonoids were compared to the antioxidant effect of *dl*-α-tocopherol on sardine oil oxidation at 60°C, (Fig. 1, A-F). Three of the six tested flavonoids [catechin (Fig. 1, A and B, morin (Fig. 1, C and D) and quercetin (Fig. 1, E and F)] exerted strong antioxidant effects, which were comparable to that of *dl*-α-tocopherol. Among these, quercetin was the most effective when oxidation was measured either by peroxide formation or by TBARS. The assay of the five native flavonoids is shown in Figure 2 (A-D). The flavonoids Pt-2, BB 21-30 and BB 14-17 also exhibited antioxidant effects that were comparable to that of *dl*-α-tocopherol. From all of the flavonoids tested, quercetin (commercial) and Pt-2 (native) were selected for further studies.

TABLE 1

Chemical Structure of Flavonoids



Compounds	Substituent positions								
	Ring A		Ring C			Ring B			
	R_5	R_7	R_3	R_4	C_2-C_3 Double bond	2′	3′	4′	5′
Commercial flavonoids									
Catechin	OH	OH	OH	H	+	H	OH	OH	H
Hesperidin	OH	O-Rutinoside	OH	O	−	H	OH	O-CH_3	H
Morin	OH	OH	OH	O	−	OH	H	OH	H
Naringin	OH	OH	O-Rhamnoglucos.	O	−	H	H	OH	H
Quercetin	OH	OH	OH	O	+	H	OH	OH	H
Rutin	OH	OH	O-Rutinoside	O	−	H	OH	OH	H

	Ring A				Ring C			Ring B			
	R_5	R_6	R_7	R_8	R_3	R_4	C_2-C_3 Double bond	2′	3′	4′	5′
Native flavonoids											
BB 14-17	OH	O-CH_3	O-CH_3	O-CH_3	OH	O	+	H	O-CH_3	OH	H
BB 21-30	O-CH_3	O-CH_3	OH	O-CH_3	H	O	+	H	H	OH	H
BB 7-12	OH	O-CH_3	O-CH_3	O-CH_3	O-CH_3	O	+	H	O-CH_3	OH	H
BB 8-13	OH	O-CH_3	O-CH_3	O-CH_3	H	O	+	H	O-CH_3	OH	H
PT-2	OH	H	O-CH_3	H	H	O	−	H	OH	OH	H

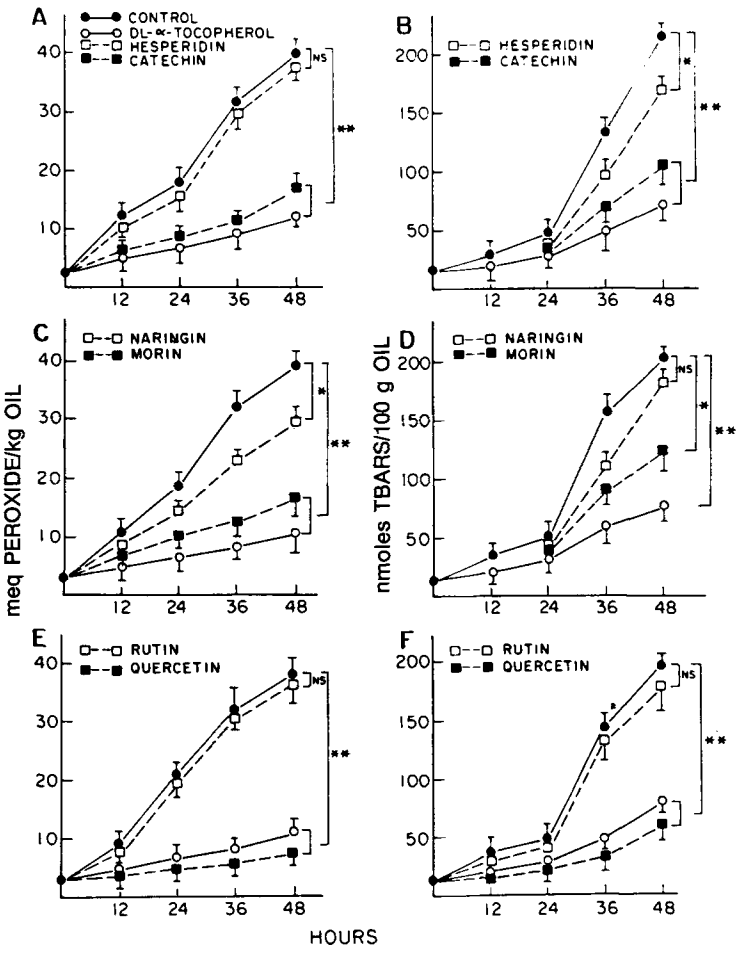


FIG. 1. Effects of six commercially available flavonoids (1 g/kg oil) on fish oil oxidation, expressed as peroxide formation (A, C, E) and as thiobarbituric acid-reactive substances (TBARS) formation (B, D, F), compared to the effect of *dl*-α-tocopherol (1 g/kg oil). Results represent the mean of six assays ± SD. *$P < 0.05$, **$P < 0.01$. NS, not significant. Other experimental conditions are in the text.

The antioxidant effects at different concentrations of *dl*-α-tocopherol, quercetin, Pt-2 and mixtures of these three substances are shown in Figure 3. Results are expressed as percentages of the control's oxidation as assessed by the peroxide content of the fish oil incubated for 48 h at 60°C. The antioxidant action of the three substances was directly related to concentration. A synergistic effect was obtained when mixtures of these antioxidants were assayed.

Preliminary studies showed that when fish oil oxidation is assayed at 60°C in the presence of $FeCl_2$, substantially higher peroxide values are obtained, even after short times. Therefore, the following studies were performed at 25°C instead of 60°C and for only 24 h. Figure 4 shows the antioxidant effects of *dl*-α-tocopherol, quercetin, Pt-2 and mixtures of these substances on iron-catalyzed fish oil oxidation. Relative to *dl*-α-tocopherol (Fig. 4, line B), much higher degrees of protection were achieved when both flavonoids were added, either alone (Fig. 4, lines C and D) or as a mixture (Fig. 4, line E). Under these conditions, the antioxidant effect of *dl*-α-tocopherol was negligible, although mixtures of this antioxidant with each flavonoid improved the antioxidative effect of *dl*-α-tocopherol (not shown).

Figure 5 shows the antioxidant effects of *dl*-α-tocopherol, quercetin, Pt-2 and mixtures of these substances after 36 d of incubation of the fish oil at 25°C. Results are compared to the effects obtained with BHT or BHA. Mixtures of *dl*-α-tocopherol with quercetin (Fig. 5, column D) or Pt-2 (Fig. 5, column E) provided a degree of protection comparable to that obtained with BHT (Fig. 5, column G), and significantly better than that obtained with BHA (Fig. 5, column H). Under these conditions, the mixture of both flavonoids improves the antioxidant effect of BHT and BHA (Fig. 5, column F).

DISCUSSION

Results presented here demonstrate a clear antioxidant effect of the flavonoids morin, catechin, quercetin, BB 14-17, BB 21-30 and Pt-2 on fish oil oxidation. Similar results have been obtained by Das and Pereira (16), who communicated the inhibitory effects of several flavonoids

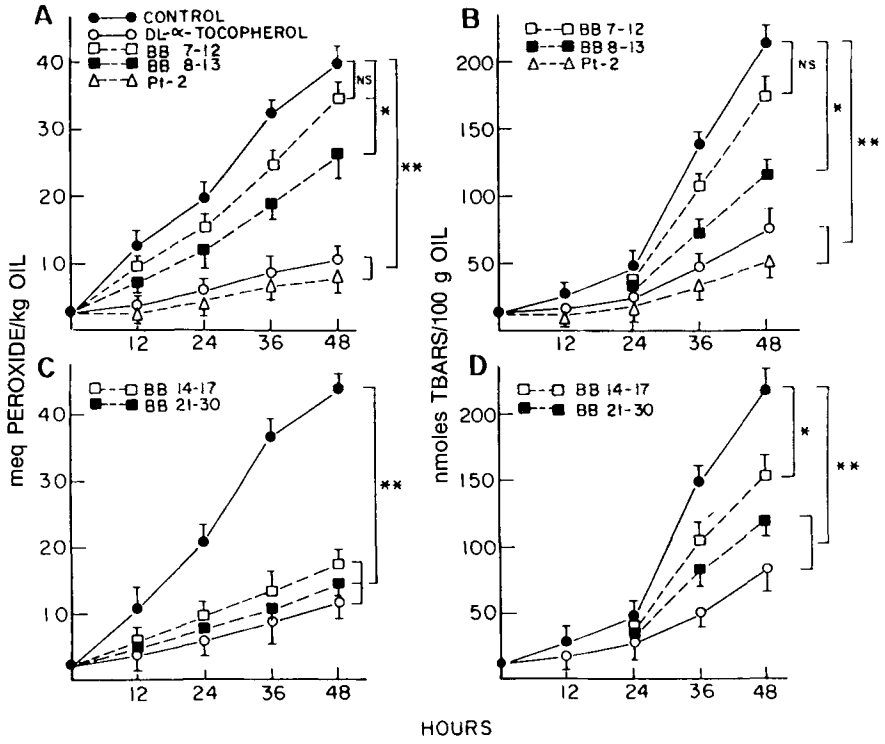


FIG. 2. Effects of five native flavonoids (1 g/kg oil) on fish oil oxidation expressed as peroxide formation (A and C) and TBARS formation (B and D) compared to the effect of *dl*-α-tocopherol (1 g/kg oil). Results represent the mean of five assays ± SD. *$P < 0.05$, **$P < 0.01$. NS, not significant. Other experimental conditions are in the text. Abbreviations as in Figure 1.

on the thermal autoxidation of refined and deodorized palm oil.

Some of the flavonoids assayed here (quercetin and Pt-2) exhibited synergism when assayed as mixtures or when combined with *dl*-α-tocopherol. Although the antioxidant capacity of some flavonoids has been demonstrated, both in *in vivo* (17) and in *in vitro* (18) models, their mechanism of action is still a matter of speculation. All flavonoids

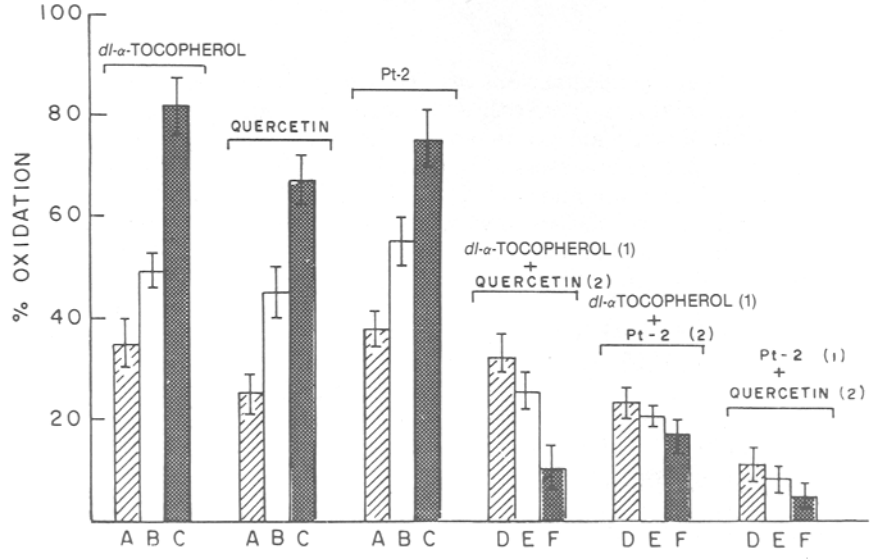


FIG. 3. Effects of different concentrations of *dl*-α-tocopherol, quercetin and Pt-2 flavonoid on fish oil oxidation. A, 1.0 g/kg oil; B, 0.7 g/kg oil; C, 0.3 g/kg oil; D, 0.7 g/kg oil (1) + 0.3 g/kg oil (2); E, 0.5 g/kg oil (1) + 0.5 g/kg oil (2); F, 0.3 g/kg oil (1) + 0.7 g/kg oil (2). Results represent the mean of six assays ± SD. Other experimental conditions are in the text.

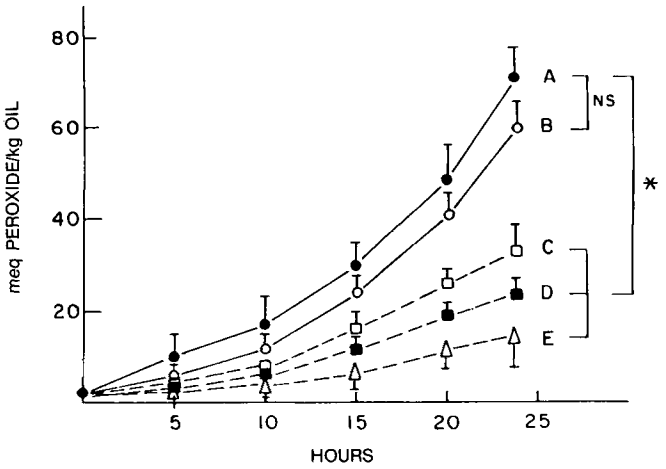


FIG. 4. Effects of *dl*-α-tocopherol, quercetin, Pt-2 flavonoid and the mixture of quercetin + Pt-2 flavonoid on fish oil oxidation catalyzed by $FeCl_2$. A, control; B, *dl*-α-tocopherol (1 g/kg oil); C, Pt-2 (1 g/kg oil); D, quercetin (1 g/kg oil); E, Pt-2 (0.3 g/kg oil) + quercetin (0.7 g/kg oil). Results represent the mean of five assays ± SD. $*P < 0.05$. NS, not significant. Other experimental conditions are in the text.

assayed as antioxidants have in common one or two hydroxyl groups (free or substituted) attached to the B ring of their structure, and their potency appears to be affected by the level of substitution and the position of these groups on the ring (16). It has been proposed that

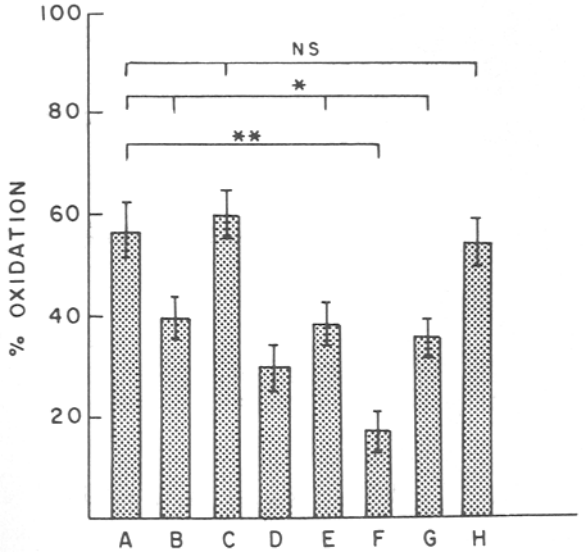


FIG. 5. Antioxidant effects of *dl*-α-tocopherol, quercetin, Pt-2 flavonoid, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) on fish oil oxidation after 36 d of incubation at 25°C. A, *dl*-α-tocopherol (1 g/kg oil); B, quercetin (1 g/kg oil); C, Pt-2 (1 g/kg oil); D, *dl*-α-tocopherol (0.5 g/kg oil) + quercetin (0.5 g/kg oil); E, *dl*-α-tocopherol (0.5 g/kg oil) + Pt-2 (0.5 g/kg oil); F, quercetin (0.7 g/kg oil) + Pt-2 (0.3 g/kg oil); G, BHT (1 g/kg oil); H, BHA (1 g/kg oil). Results represent the mean of five assays ± SD. $*P < 0.05$, $**P < 0.01$. NS, not significant. Other experimental conditions are in the text.

flavonoids having free hydroxyl groups at the *ortho* position (3′,4′) should exhibit a reduced antioxidative effect, whereas those containing hydroxyl groups in the *para* position (2′,5′) should be the most favored (16). However, in our experimental model, two flavonoids bearing free hydroxyl groups at the *ortho* position (quercetin and Pt-2) showed the best antioxidative effects.

Our results show that a glycoside of a flavonoid (R-3 substituted), such as rutin, does not exhibit any antioxidative properties in fish oil oxidation. A similar conclusion was suggested by Das and Pereira (16) in studies conducted on palm oil. Furthermore, a free R-3 position at ring C (as in Pt-2) or substituted by a hydroxyl group (as in quercetin) appears to be desirable to obtain a good antioxidative response. On the other hand, the double bond between C-2 and C-3 appears not to be a determinant in obtaining an antioxidative effect. In fact, Pt-2, which lacks this double bond, shows a potency similar to that of quercetin, which is unsaturated between the C-2 and C-3 positions.

Flavonoids have been defined as "high-level" antioxidants (11). That is, they act by scavenging those free radicals or exited forms of oxygen involved in the first stages of oil oxidation, such as the singlet oxygen, the superoxide free radical or the hydroxyl free radical (19). On the other hand, *dl*-α-tocopherol, defined as a "low-level" antioxidant (11), acts at the later stages of oxidation by stabilizing those free radicals formed at the structure of the polyunsaturated fatty acid (methylene, alcohoxy or peroxy free radicals). These different sites of action in the oxidation chain may relate to the synergistic effect observed between *dl*-α-tocopherol and the flavonoids.

Additional antioxidative action of most flavonoids may be resulting from their metal-chelating properties (20). As a matter of fact, quercetin may form strong binding complexes, particularly with copper and iron (9). Although a metal-complexing property of Pt-2 has not yet been established, it is not far-fetched to postulate a metal-chelating action in its antioxidant effect. The strong antioxidative response obtained for both flavonoids when fish oil oxidation was catalyzed by Fe^{2+} reinforces this possibility.

In conclusion, several of the commercial and native flavonoids assayed, added as single species, as a mixture or in combination with other antioxidants, such as *dl*-α-tocopherol, have been shown to act as stabilizers of fish oil against spontaneous or metal-induced oxidation. The results indicate that some of these flavonoids could be used as natural antioxidants and might substitute for those synthetic antioxidants whose use has been questioned due to their potential undesirable secondary effects (21,22). This subject is controversial because several beneficial effects attributed to synthetic antioxidants in experimental animals have been described (23,24). However, it is important to consider that BHA was ruled out as a GRAS (generally regarded as safe) substance by the Food and Drug Administration of the United States, and that other synthetic antioxidants structurally related to BHA, such as BHT, may also be subjected to the same determination. However, further physicochemical and toxicological evaluations are required to assess the effectiveness and the future of flavonoids as antioxidants for fish oils or other oils rich in polyunsaturated fatty acids.

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