J Amer Oil Chem Soc (2007) 84:129–136
DOI 10.1007/s11746-006-1016-2

ORIGINAL PAPER

Defining the Role of Flavonoid Structure on Cottonseed Oil Stabilization: Study of A- and C-Ring Substitution

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Received: 12 September 2005 / Accepted: 24 October 2006 / Published online: 9 January 2007

Abstract Structure—activity relationships of flavonoids against cottonseed oil oxidation are the objective of this work, which focuses on the contribution of C-ring and 5,7-di-OH A-ring. Flavonoids with a catecholic B-ring, namely quercetin, fisetin, luteolin, taxifolin, (+)-catechin and eriodictyol were added in increasing concentrations to cottonseed oil and the induced decrease in the rate constant of peroxide formation (k) and peroxide values (PV) were determined. The flavonoid depletion was monitored during the autoxidation with high-performance liquid chromatography-diode array detector (HPLC-DAD) analysis. The fully substituted C-ring was established as the most significant element for maximal antioxidant activity; quercetin even decreased k by 86% in comparison with the control and presented the highest decrease of PV per mmole of antioxidant. Flavonoids missing the 2,3-double bond or the 3-OH (taxifolin, luteolin) resulted in a smaller decrease of k, and the lack of both of the above structural elements (eriodictyol) resulted in the smallest decrease. Lack of the 4-carbonyl in the C-ring had the least negative effect on antioxidant activity since (+)-catechin presented the strongest protection after quercetin and fisetin. The activity of the latter flavonoid proved that A-ring hydroxyls, despite the unfavorable m-configuration, participate in the stabilization of the lipid substrate, although their significance was secondary.

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Keywords Peroxide formation rate · (+)-catechin · Eriodictyol · Fisetin · Luteolin · Quercetin · Taxifolin

Introduction

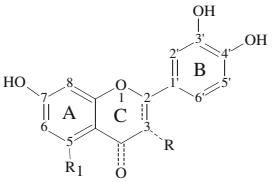
Flavonoids are a group of phenolic compounds abundant in all plants that possess in majority the 2-phenyl-chromane skeleton, while various models of substitution are encountered (Table 1). The antioxidant activity of flavonoids depends on hydroxyl substituents, which donate hydrogens to peroxyl radicals. Each free hydroxyl does not present the same donating ability; the antioxidant activity of flavonoids also presents strong variation with respect to possible substitution patterns.

The o-di-OH arrangement, usually encountered in B-ring, is active for radical scavenging. The primary produced transient semiquinone radical is stabilized through the settled resonance structures and the intramolecular hydrogen bond between the phenoxy radical and the free hydroxyl [1, 2]. The second hydrogen is susceptible to abstraction by a free radical because a stable diquinone is produced.

On the contrary the contribution of the 5,7-di-OH-A-ring, which is commonly found in natural flavonoids, is considered to be minor due to the meta configuration of the hydroxyls. The reactivity of m-hydroxyls by means of the number of scavenged radicals and reaction rates are very low or negligible according to experiments with standard radicals [3, 4]. Theoretical investigations also suggest that both the 5- and 7-hydroxyls are not important in antioxidant activity [2, 5].

A certain body of research is concerned with the effect of the structural differences of the C-ring. A fully

Table 1 The structures of the studied flavonoids

Studied compounds		2,3-double bond	4-carbonyl group	*R*	R_1
	Quercetin	√	√	OH	OH
	Fisetin	√	√	OH	H
	Luteolin	√	√	H	OH
	Taxifolin	–	√	OH	OH
	Eriodictyol	–	√	H	OH
	(+)-catechin	–	–	OH	OH

substituted C-ring seems favorable, but compounds that lack one or more structural elements presented contradictory results in different substrates and experimental conditions. Benavente-Garcia et al. [6] and Hotta et al. [7] reported a significant reduction in radical scavenging with the removal of the 3-OH, while the experiments of Silva et al. [8] did not verify this effect. In the same work of Benavente-Garcia et al. [6], further lack of the 2,3-double bond presented a minor reduction in antiradical capacity. However Hotta et al. [7] reported the opposite effect (eriodictyol presented higher activity than luteolin). The presence of a 4-carbonyl group in compounds without a 2,3-double bond has been shown to decrease activity [7] or not to affect it [8].

Research on the activity of different flavonoids in oils and fats is rather limited. Pratt and Hudson [9] commented that ortho-dihydroxylation of the B-ring contributes markedly to the antioxidant activity of flavonoids, while meta-5,7-hydroxylation of the A-ring has little effect, an observation in agreement with the effect of structure on activity against free radicals. Concerning the C-ring they reported that 3-OH is important and the 2,3-double bond has a minor effect, while Wanasundara and Shahidi [10] observed that the absence of the 2,3-double bond reduced the activity in canola oil.

This paper focuses on the effect of the structural differences of the flavonoid C-ring against thermal oxidation of cottonseed oil. Furthermore the effect of monohydroxy substitution of the A-ring, compared with 5,7-dihydroxy substitution, is investigated. Flavonoids with 3′,4′-o-dihydroxy substitution on the B-ring are examined because their antioxidant activity is well established. Since flavonoids are present in herbs and agro-industrial by-products a better understanding of the relation between structure and antioxidant properties could enhance the recovery of specific compounds for exploitation as natural antioxidants for oils, fats and fat-containing foods.

Experimental Procedures

Materials

The standard flavonoids were quercetin dihydrate (Fluka 99%), fisetin hydrate (Aldrich, pure), (+)-catechin (Fluka, 98%), eriodictyol (Extrasynthèse, pure), luteolin (Sigma), and taxifolin (Sigma). Hexane pro analysis, acetonitrile HPLC, isopropanol HPLC and water HPLC were used for the extraction and HPLC analysis of the flavonoids.

Addition of Antioxidants in Cottonseed Oil

The stock solutions of flavonoids were prepared in 100 mL volumetric flasks. The solvent used was ethyl acetate-ethanol, 85:15 v/v and (especially for fisetin) acetone-ethanol (85:15). Certain volumes were removed from each stock solution and added to 320 g of cottonseed oil samples under continuous stirring; the solvent was removed by purging with nitrogen. Each solvent-free oil sample, with a specific concentration of flavonoid, was transferred in portions of 50.00 g into six identical open beakers of 100 mL. The same procedure was also performed with the respective solvent of each flavonoid for the preparation of the corresponding control samples. All enriched oil samples with one flavonoid as well as the respective control samples were subjected to accelerated oxidation at 70 °C in a ventilated oven. For example, five different concentration levels of (+)-catechin were examined. Therefore during the autoxidation experiment 36 oil samples were used: six beakers of control and five sextuplets of oil samples with different (+)-catechin concentrations (i.e., thirty beakers).

Estimation of Antioxidant Activity

The oxidative process was monitored by determination of the peroxide value [American Oil Chemists' Society

(AOCS) official method, Cd 8–53]. Each determination was performed in triplicate according to random sampling from the series of beakers with the same concentration, while the results in Fig. 1 present the mean values.

Flavonoid Analysis

The remaining concentration of flavonoid was determined with a periodic removal of one beaker each time. The extraction and analysis was based on the official method of AOCS, Ce 6–86 for synthetic antioxidants with the following modifications: 10 g of oil sample was weighed into a 50 mL volumetric flask and diluted with hexane. Twenty-five milliliter of the aliquot were transferred into a 100 mL separatory funnel and extracted with three 40 mL portions of saturated acetonitrile in hexane. The extracts were combined, concentrated and diluted into a 10 mL volumetric flask with isopropanol-acetonotril (1:1). A sample of 20 μL was injected in the HPLC which consisted of an HP 1100 gradient pump and a diode array detector (Hewlett-Packard, Waldbronn, Germany). A Hypersil C_{18} column octadecyl silica (ODS) 5 μm, 250 × 4.6 mm (MZ Analysentechnik, Mainz, Germany) was used under thermostated conditions at 30 °C. The solvent system consisted of water (A), acetonitrile (B), and isopropanol (C); A and B contained 0.2% trifluroacetic acid (TFA) instead of

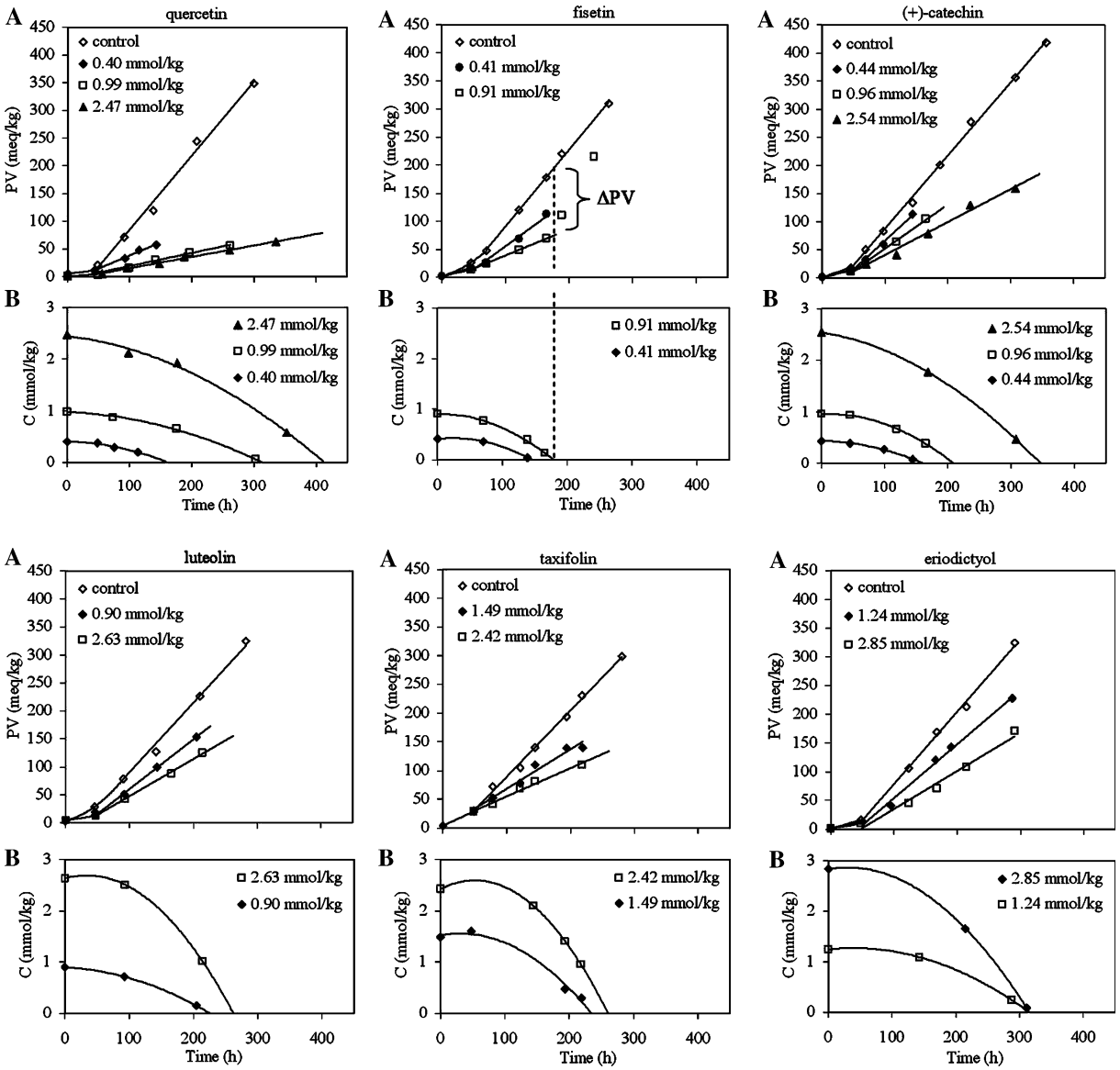


Fig. 1 **A** The PV increase of cottonseed oil in the presence of specific concentrations from the studied flavonoids compared to the corresponding control. **B** The kinetic curves of flavonoid depletion during the autoxidation experiment

acetic acid 5%, since it resulted in better reduction of peak tailing. The initial composition of the mobile phase was 70% A, 30% B and 0% C. With a linear gradient the composition was changed to 0% A, 100% B and 0% C in 10 min and 0% A, 0% B and 100% C in 15 min. The flow rate was maintained at 2 mL/min for 10 min and changed to 1 mL/min in 15 mins by following a linear gradient. In the case of taxifolin, acetonitrile-TFA (B) was replaced by methanol-TFA (D) and the conditions of the gradient were kept identical. Exceptionally for (+)-catechin a different analysis was used; the mobile phase consisted of 100% D, 0% C for 4 min and then changed with a linear gradient to 0% D, 100% C within 10 min. The flow rate was maintained at 1 mL/min.

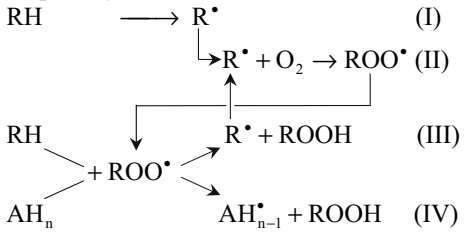
The detection of the compounds was performed at the following wavelengths—quercetin: 256, 280, 300 and 372 nm, fisetin: 248, 280, 320 and 360 nm, luteolin: 254, 266, 282 and 348 nm, eriodictyol: 280, 288 and 336 nm, taxifolin and (+)-catechin at 280 nm. The main reason that multiple wavelengths were used for most compounds was to eliminate any interference by co-extracted traces of oxidation products that might elute at the same time as the analyzed flavonoid. Each set of wavelengths could screen a wide range of the UV-vis spectrum of the relevant flavonoid and furthermore corresponded to specific spectral maxima and minima. In the case of taxifolin and (+)-catechin the use of additional wavelengths was not feasible due to the low absorbance of the compounds above 300 nm and the lack of sensitivity below 250 nm. Reference curves at each wavelength were constructed for all flavonoids and used for the calculation of flavonoid concentration; the values obtained by each wavelength reference curve were averaged to obtain the concentration of the specific compound.

Statistical Analysis

Peroxide Values were determined in triplicate samples and the results were averaged ($n = 3$). Microsoft Excel was used for linear regression analysis of PV versus time curves, after the induction period, to obtain the rate constants (k) ± standard error (SD) The SD values of Protection parameters were calculated accordingly and the results are presented as $P \pm SD$ Furthermore all data were statistically analyzed with one-way ANOVA test ($p < 0.05$).

Results and Discussion

Flavonoids, acting as chain-breaking antioxidants, interfere in the free-radical route and reduce the rate of the propagation stage (equations I—IV). In fact, according to equations (III) and (IV), they are competitors to unsaturated fatty acids (FA) for the donation of hydrogen atoms to the peroxyl radicals and thus retard the rate of peroxyl-induced oxidation of FA (III). The higher the availability of a flavonoid for hydrogen donation, the more predominant becomes reaction (IV) compared to (III). Therefore less FA participate in propagation reactions to produce by turns new peroxyl radicals.



Consequently, the activity of a flavonoid at a specific concentration in oil can be evaluated by the decrease in the rate of peroxide formation. The difference in the rate of peroxide formation in the presence of antioxidant, compared to the respective rate of control, represents the overall degree of competition between antioxidant hydrogens and the allylic, bis-allylic hydrogens of fatty acids to be transferred to peroxyl radicals.

Additionally, the total peroxide radicals scavenged by the flavonoid until it is exhausted defines its antioxidant potential and can be estimated by the difference in peroxide value (ΔPV) between the blank lipid substrate and that enriched with antioxidant, at the total consumption of the antioxidant. The amplitude of this difference represents the non-formed peroxides due to the added concentration of antioxidant. ΔPV depends on the overall stoichiometry of the antioxidant versus free radicals and could provide a direct, stoichiometry-dependent estimation of the antioxidant activity. In the same manner EC_{50} values and the stoichiometric factors have already been used in order to describe the antioxidant activity of pure compounds or extracts against standard free radicals in rather simple reaction systems, compared to the complex oil autoxidation [11, 12].

The structural differences of the studied flavonoids are presented in Table 1. The results of their addition to cottonseed oil at representative concentrations are presented in Fig. 1. All control samples possessed identical characteristics during the oxidation experiments: an induction period during the first two days, with low peroxide formation and a subsequent PV increase at a high rate. In the latter period of oxidation, PV was linearly correlated with time ($R^2 > 0.9900$).

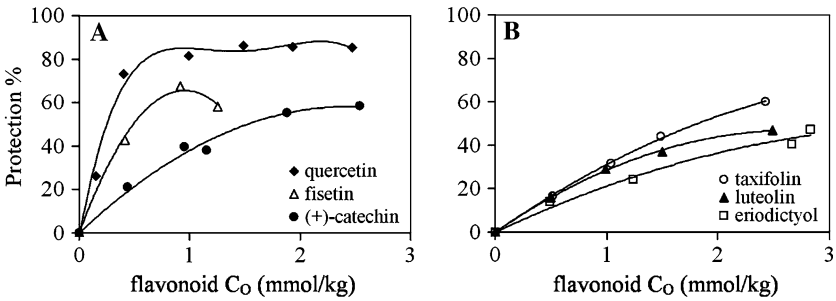


Fig. 2 The increase of the protection parameter with concentration (mmol/kg) of the studied compounds

Flavonoids affected the rate of PV formation, with a pronounced decrease being observed in the second period while linearity was maintained.

In order to quantify the effect of each flavonoid concentration on the rate of the second period a protection parameter (P) was calculated; it represents the decrease in the rate constant of peroxide formation obtained by the addition of antioxidant (k_i) compared to that of the blank oil (k_c), after the induction period, and is expressed by Eq. V:

$$P = \left(1 - \frac{k_i}{k_c}\right) \cdot 100\% \qquad \text{(V)}$$

Figure 2 presents the increase of the protection parameter with the initial concentration of each flavonoid and the corresponding values are shown in Table 2. The most effective stabilization of cottonseed oil was achieved with quercetin. More specifically this flavonoid reduced the rate of oxidation by as much as 86%. The P parameter increased with quercetin concentration up to 1 mmol/kg, which reduced the rate of oxidation by 81 ± 5% and remained almost constant at higher concentrations. The flavonol fisetin also exhibited significant protection values: the highest P value was 67 ± 2% and was achieved by a concentration of 0.91 mmol/kg. At increased concentrations of fisetin, solubility problems were encountered and the P parameter tended to decrease, as shown in Fig. 2. These results indicate that lack of 5-OH results in a decrease of the rate of peroxyl radicals scavenging, nevertheless the maximum effectiveness, estimated by the protection parameter requires the 3-OH group attached to the 2,3-double bond and adjacent to the 4-carbonyl group in the C-ring. The same result was reached through studies of flavonoid activity against free radicals in various systems [7, 8, 12] and against autoxidation of canola oil [10]. Cook and Samman [13] also reported that all three structural elements are important for antioxidant activity.

The 2,3-double bond results in an increase of the resonance structures and consequently the intermediate phenoxy radical is more stable and is formed more easily. Indeed, in experiments we performed with the free radical diphenyl picryl hydrazyl (DPPH) the rate constants of luteolin and rutin during the initial, rapid stage of the reaction have been determined to be much higher than the respective constants of taxifolin, eriodictyol, (+)-catechin and (–)-epicatechin. Despite the fact that all the compounds scavenged two radicals during the initial, rapid stage of the reaction, the reaction rates of those with conjugated C-ring were higher [14]. The lack of the 2,3-double bond (taxifolin)

Table 2 The P and ΔPV values corresponding to specific flavonoid concentrations

Flavonoid	Initial concentration (mmol/kg)	P (% ± SD)	ΔPV (meq/kg)
Quercetin	2.47 ± 0.00	85 ± 5[a]	434
	1.93 ± 0.02	86 ± 9[a]	405
	1.49 ± 0.01	86 ± 11[a]	380
	0.99 ± 0.02	81 ± 5[a]	306
	0.40 ± 0.02	73 ± 3	102
	0.15 ± 0.03	27 ± 4	33
Fisetin	1.26 ± 0.04	58 ± 4	196
	0.91 ± 0.08	67 ± 2	146
	0.41 ± 0.01	43 ± 3	76
(+)-catechin	2.54	58 ± 4[b]	262
	1.88	55 ± 4[b]	237
	1.15	38 ± 3[c]	96
	0.96	40 ± 2[c]	91
	0.44	21 ± 1	38
Luteolin	2.49 ± 0.01	47 ± 3	126
	1.50 ± 0.02	37 ± 2	93
	0.99 ± 0.01	29 ± 2	62
	0.50 ± 0.01	16 ± 1	33
Taxifolin	2.42	60 ± 7	164
	1.49	44 ± 4	93
	1.04	32 ± 2	57
	0.52	17 ± 1	7
Eriodictyol	2.85 ± 0.03	47 ± 4[d]	172
	2.68 ± 0.02	41 ± 3[d]	161
	1.24 ± 0.06	24 ± 1	87
	0.49 ± 0.01	14 ± 1	26

Values in the same column concerning specific flavonoid are significantly different ($p < 0.05$) apart from those bearing the same superscript

eliminates the means of delocalization of electrons from the aryloxyl radical on the B-ring to the C-ring, resulting in a lower rate of reaction with the peroxyl radical.

However, if C-ring unsaturation was the main factor that influenced the radical scavenging kinetics and capacity, luteolin should have been the most effective competitor to fatty acids for the donation of H-atoms to peroxyl radicals between the non-fully substituted C-ring members. This was not proved by our results, since taxifolin (lacking the 2,3-double bond) presented similar protection values to luteolin at low concentrations. Furthermore it is noted that taxifolin tended to become more effective than expected with increasing concentration. For example, luteolin and taxifolin at the addition level of 1 mmol/kg presented protection values of 29 ± 2% and 32 ± 2%, respectively. With an increased concentration of 1.5 mmol/kg, luteolin protected the lipid substrate by 37 ± 2% while taxifolin did so by 44 ± 4%, and the difference increased at higher concentration, as can be seen from Fig. 2. Pratt and Hudson [9] reported a much lower rate in PV formation of corn oil enriched with taxifolin, comparable to that of quercetin; however comparison is difficult as they measured the time to reach a PV of 50, and not the rate constant after the induction period.

The lack of a second structural element in the case of (+)-catechin and eriodictyol did not induce an equivalent reduction in protection. In fact (+)-catechin possessed the highest protection values among the non-fully substituted C-ring catecholic members, while eriodictyol the lowest. Consequently the loss of C-ring structural elements resulted in a decrease of protection values as far as the flavonoids with 4-carbonyl group (chromanone-type) are concerned: $P_{\text{quercetin}} > P_{\text{taxifolin}} \geq P_{\text{luteolin}} > P_{\text{eriodictyol}}$.

(+)-Catechin that lacks the 4-carbonyl group (chromane-type flavonoid) possessed the highest activity among the non-fully substituted C-ring compounds. According to Lemańska et al. [14] a strong hydrogen bond of 5-OH is settled with the oxygen atom of C_4. The bond dissociation energy (BDE) of the 5-O-H bond in flavones was calculated to be 105.7 kcal/mol (while the corresponding BDE values of catecholic hydroxyls are 80.1 and 81.8 kcal/mol). Consequently the H-bond stabilizes the hydrogen atom and may prevent donation to peroxyl radicals. The lack of this H-bond in (+)-catechin results in a decrease of the 5-O-H BDE, thereby increasing the susceptibility of that hydrogen to abstraction.

This is strong evidence that the observed kinetic activity during autoxidation is due not only to the catechol moiety but also to the A-ring hydroxyls. This was also verified by comparison of fisetin and quercetin. It is noted however that the influence of A-ring substitution on the rate of peroxyl-radical scavenging is secondary since the loss of one A-hydroxyl from quercetin to fisetin did not result in a dramatic decrease of the protection parameter as in the case of the 3-OH (quercetin versus luteolin). A minor effect of di- or monohydroxy substitution of the A-ring was observed by Pratt and Hudson [9] through experiments with quercetin and fisetin in corn oil.

As catecholic flavonoids react with peroxyl radicals they are transformed to less-polar quinones and further degradation products that elute at much longer retention times than the corresponding parent molecules. Therefore HPLC analysis was applicable for the quantification of the remaining catecholic form of each flavonoid during the autoxidation experiments. Figure 3 presents the produced chromatograms from extracted quercetin at 284 nm during different cottonseed oil autoxidation levels. It is apparent that the elution of quercetin was not significantly interfered by co-extracted compounds such as fatty acids, tocopherols or oxidation products. The same chromatograms were produced for the other flavonoids, without significant evidence for interference.

In order to determine the value of ΔPV, the remaining concentration values of each flavonoid, determined by HPLC analysis, were plotted against the oxidation time. At the time of complete exhaustion of the flavonoid the difference in PV was calculated between the control and the sample, as presented in Fig. 1. The respective values of ΔPV plotted versus the initial flavonoid concentrations (Table 2) produced the characteristic diagrams of Fig. 4a,b.

Quercetin induced the highest reduction of peroxides, until it was exhausted, followed by fisetin and catechin. At a concentration of 1 mmol/kg, quercetin inhibited the formation of 306 meq peroxides per mmol of the flavonoid, fisetin 146 meq and catechin 91. Though linearity was not observed as concentration increased, quercetin maintained the highest capacity to reduce peroxide formation. Taxifolin, luteolin and eriodictyol presented lower values of reduced peroxides, which amounted to approximately 60 meq/mmol flavonoid. This fact indicated that the loss of the 2,3-double bond, the 3-OH group, or even both resulted in a significant decrease of ΔPV and, by contrast to the aforementioned differentiation of P parameters, the classification of chromanone-type flavonoids according to ΔPV emerges as follows: quercetin > >luteolin ≈ taxifolin ≈ eriodictyol. Therefore the stoichiometry of chromanone-type catecholic flavonoids (Table 1) is highly dependent on the full substitution of the C-ring;

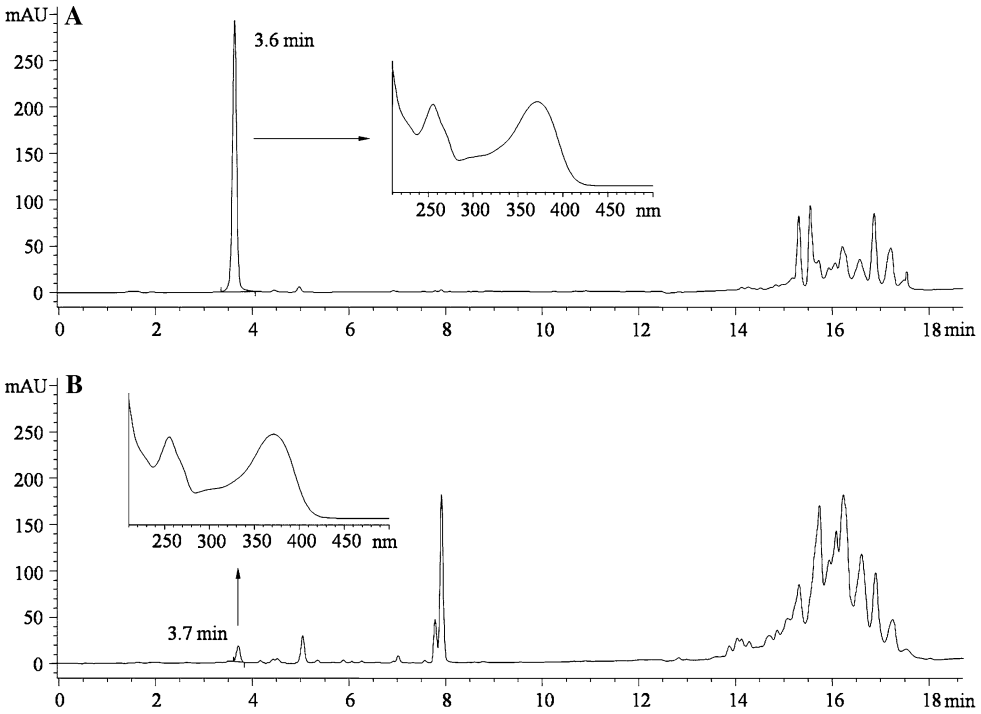


Fig. 3 Two comparative chromatograms for the extracted quercetin with an initial concentration 0.99 mmol/kg detected at 284 nm. **A** before autoxidation of cottonseed oil **B** after 302.4 h of oxidation. The UV-vis spectra detected by DAD are also presented

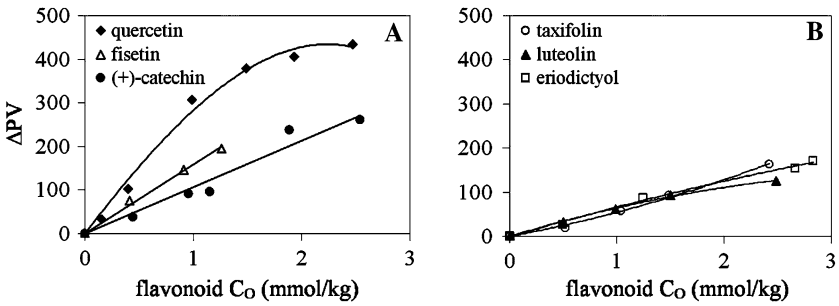


Fig. 4 The ΔPV values of the studied compounds at different concentrations (mmol/kg)

losing one structural element the stoichiometry decreases to a standard level that is independent of further loss of a structural element. Das and Pereira [15] also observed a decrease in activity with the lack of either the 3-OH or the 2,3-double bond; luteolin and taxifolin inhibited the formation of malonyldialdehyde during autoxidation of palm oil (the former being slightly more effective than the latter) but both presented approximately half of the percentage inhibition of quercetin.

As indicated by the results obtained by (+)-catechin, lack of the 4-carbonyl group affected not only the protection parameter but also the overall stoichiometry of the reaction with peroxyl radicals. (+)-Catechin does not belong to the chromanone-type flavonoids and presented higher ΔPV than luteolin, taxifolin and eriodictyol. According to the data of Nieto et al. [16], catechin presented a high antioxidant capacity in fish oil too, though lower than that of quercetin. Cook and Samman [13] also presented results that indicated that catechin has a lower hydroxyl radical-scavenging potency than quercetin but they also commented that the 4-carbonyl group is necessary for antiperoxidant activity in some studies but not others. This observation might be explained by the differences in the rest of the structural elements of the flavonoids that were used in these studies.

Similarly to the protection parameter, the ΔPV value of fisetin verified that A-ring hydroxyls definitely participate in the observed antioxidant activity. The m-A-ring hydroxyls have been treated so far as minor elements for antioxidant activity. We observed that even the lack of the hydrogen-bonded 5-OH, in fisetin, lowered the protection parameter of cottonseed oil as well as the overall non-formed peroxides compared with quercetin, which possesses the 5-OH.

The results for ΔPV at exhaustion of the flavonoids are in good agreement with the stoichiometric factors

or EC_{50} found in our previous research with DPPH [11] with the exception of eriodictyol. Also they are more or less in agreement with the results of Rice-Evans et al. [12] and of Benavente-Garcia et al. [6] with ABTS.

The present study indicated that the structure of flavonoids affects both the stoichiometry of reactions with peroxyl radicals and the rate of peroxide formation with a different structure-dependent effect.

Taking into account both the protection parameters and the ΔPV of the studied flavonoids we can conclude that quercetin presented the highest number of peroxyl radical reductions per mol of flavonoid consumed, and the highest rate of reaction, followed by fisetin. Thus the flavonoids that exhibit superior activity also present a fully substituted C-ring. Catechin presented the third most significant activity; consequently the general statement that fewer substituents on C-ring leads to a lower antioxidant activity is not always accurate since luteolin and taxifolin possess more structural elements on the C-ring than catechin but are less active. The latter compounds and eriodictyol presented about the same stoichiometry but the reaction rate decreased from taxifolin to eriodictyol; thus at any time before exhaustion of the antioxidant the oil presented lower PV and consequently better quality when taxifolin was added, followed by luteolin and eriodictyol. Comparing these compounds with the flavonols and (+)-catechin it can be deduced that 4-carbonyl when not accompanied by 2,3-bouble bond and 3-OH is a rather unfavorable substituent for antioxidant activity.

Acknowledgments This project was co-funded by the European Social Fund (75%) and National Resources (25%)-(EPEAEK II)–PYTHAGORAS.

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