

# Antioxidant Activity and Kinetics Studies of Quercetin, Epicatechin and Naringenin in Bulk Methyl Linoleate

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**Abstract** The objective of this work was to evaluate the antioxidant action of flavonoids with specific differences in chemical structure, namely, quercetin (Q), epicatechin (E) and naringenin (N), in bulk methyl linoleate (ML) under different oxidation conditions at 60 °C, in an oven and in a Rancimat apparatus. The oxidation kinetics were studied by direct and concomitant analysis of primary and secondary oxidation compounds. Addition of 200 mg/kg of E or Q to ML increased about tenfold the oxidative stability of ML. The protective effect was significantly higher for E, independent of the oxidation conditions used. However, N showed no effect. Results obtained were attributed to differences in structural features and polarity. Thus, the presence of the *ortho*-dihydroxy structure in the B ring and the 3-hydroxyl group in the C ring of E and Q seemed to be determinant and the comparatively higher polarity of E may have enhanced its antioxidant efficiency.

**Keywords** Antioxidant activity · Autoxidation kinetics · Epicatechin · Flavonoids · Quercetin · Naringenin

## Introduction

Flavonoids are a group of natural benzo- $\gamma$ -pyrane derivatives, ubiquitous in the plant kingdom, which exert beneficial effects on human health and can act as antioxidants in food lipids by means of their free-radical scavenging or metal-chelating activities [1, 2]. The essential part of the free radical scavenging activity of flavonoids is attributed to the *ortho*-dihydroxyl group in the B ring (catechol group) of their diphenylpropane structure, although the 2,3 double bond, the 4-oxo function and the 3-hydroxyl group in the C ring also appear to contribute to the antioxidant activity [3–10]. The structure–antioxidant activity relationship of flavonoids has been defined in hydrophilic systems, but the studies carried out in bulk lipid system are not conclusive [3–8]. With the exception of two studies [9, 10], the activity of flavonoids in bulk lipid systems has been investigated in oils, either in vegetable oils [11–15] or marine oils [16–18], and results have shown variable antioxidant effects for the flavonoids tested. Such differences can be expected due to the influence of compounds present in oils with antioxidant effects or prooxidant action. Additionally, difficulties in interpreting the results found are, to a large extent, due to the different analytical methods and oxidation conditions used. Specifically, application of a single analytical method which only provides a partial measurement of oxidation, such as the peroxide value or the 2-thiobarbituric acid test, is one of the main limitations found in the literature on antioxidant action of flavonoids. To obtain a complete picture of the oxidation process it is essential to apply more than one method of monitoring the formation of primary and secondary oxidation products [19].

The aim of this study was to evaluate the influence of the structural features of quercetin (flavonol structure), epicatechin (flavanol structure) and naringenin (flavanone structure)

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on their antioxidant activity in methyl linoleate, which was selected as a lipid model system without added initiators or metal-catalysts. Quercetin and epicatechin have two OH groups at the *ortho* position in the B-ring, whereas naringenin has a single OH group in the B-ring. Quercetin and epicatechin differ in the C-ring: both have three OH groups, but only quercetin has a 4-keto group and a 2,3 unsaturated bond. A method based on high-performance size-exclusion chromatography (HPSEC) was applied [20]. Thus, all non-volatile oxidation compounds formed, including primary and secondary products, can be quantified directly by HPSEC in a single analysis that provides complete and quantitative information on the oxidation state during all stages of oxidation. This method has shown high repeatability and sensitivity and was proposed as an excellent alternative to other methods for kinetic studies on fatty acid methyl esters [20, 21]. In the present study, the most commonly used accelerated oxidation conditions were applied and compared at a moderate temperature (60 °C). Assays were carried out in an oven and in a Rancimat apparatus with air bubbling. Kinetic studies on the formation of oxidized monomers, dimers and higher oligomers, quantified by HPSEC, were carried out and the remaining flavonoids were quantified.

## Materials and Methods

### Chemicals

Quercetin (Q) (>90%), (–)-epicatechin (E) (>90%), naringenin (N) (>95%), and methyl linoleate (ML) (99% purity) were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain). All other chemicals and reagents were of analytical grade and obtained from local suppliers.

### Oxidation Conditions

The addition of flavonoids to ML for oxidation assays was carried out by dissolving the flavonoids in ethanol (5 mg/mL) and mixing 200 µL of flavonoids solution/g ML. In ML samples, only 200 µL ethanol/g were added.

### Rancimat Assays

ML samples were oxidized in a 679 Rancimat device (Metrohm, Switzerland). Four grams of ML alone or with 200 mg of flavonoids (Q, E or N)/kg ML were placed in normalized reaction tubes in triplicate. Sixty milliliters of deionized water was added to the vessel used to collect the volatile components. The determinations were performed in triplicate at 60 °C with an airflow of 20 L/h. Aliquots of 70 mg were withdrawn at specific time

intervals and stored in amber glass vials under a stream of nitrogen, while frozen at –20 °C until the analysis of oxidation compounds by HPSEC and flavonoids by HPLC. The oil stability index (OSI) was determined automatically by the Rancimat apparatus. Conductivity readings were first obtained throughout the test so that appropriate sampling times in the oxidation experiments could be identified. This enabled coverage of the induction period and advanced oxidation stages. Samples were withdrawn at the end of the induction period (IP), i.e. the OSI time, in order to adequately monitor changes in the oxidation rate.

### Oven Assays

Five-gram samples of ML alone or with 200 mg of flavonoids (Q, E or N)/kg ML were placed in open glass beakers (surface-to-sample volume ratio of 11.5 cm<sup>–1</sup>) in triplicate. Samples were oxidized, in the dark, in an oven heated to 60 °C (Indelab, Labolan, Spain). Aliquots of 70 mg were withdrawn at time intervals appropriate to cover the induction period and advanced oxidation stages, as determined in preliminary assays. Samples were stored in amber glass vials, under a stream of nitrogen while frozen at –20 °C until analysis of oxidation compounds by HPSEC and flavonoids by HPLC.

### Analytical Methods

#### *Quantification of Oxidized Monomers, Dimers and Higher Oligomers by HPSEC*

Samples of about 20 mg were dissolved in tetrahydrofuran (50 mg/mL) for direct analysis by HPSEC using a system equipped with a Waters 510 pump, a Waters 2414 refractive index detector, and a Rheodyne 7725i injector with 20 µL sample loop (Waters, Milford, MA, USA). Separation was performed on two 100 and 500 Å Ultrastayragel columns (25 cm × 0.77 cm I.D.) that were connected in series and packed with porous, highly cross-linked styrene divinylbenzene copolymers (film thickness 10 µm) (Hewlett–Packard, Avondale, PA, USA). Tetrahydrofuran (1 mL/min) was employed as the mobile phase. The groups of compounds separated and quantified in ML samples were non-oxidized ML, ML oxidized monomers (OxMONs), ML dimers (DIMs) and higher oligomers of ML (OLIGs). Quantification was based on peak area percentage of the total peak area. This method was developed to evaluate oxidation compounds in pure fatty acid methyl esters, and it showed excellent sensitivity and repeatability, in accord with a prior publication [20].

### Quantification of Flavonoids by HPLC

Samples of ML of about 50 mg were dissolved in water:methanol:acetonitrile (45:40:15 v/v/v) containing 1% glacial acetic acid, stirred for 2 min and analyzed directly by HPLC to determine the remaining quantity of flavonoids. Flavonoid analysis (Q, E or N) was performed by HPLC using a system equipped with a Merck Hitachi L-6200 pump, a Waters 996 photodiode-array detector and a C18 column (3  $\mu$ m, 4.6 i.d.  $\times$  150 mm, Atlantis<sup>®</sup>, Waters, Ireland). For the determination of Q or N, an isocratic mobile phase of water:methanol:acetonitrile (45:40:15 v/v/v) containing 1% glacial acetic acid was used at a flow rate of 1 mL/min; whereas for the determination of E an isocratic mobile phase A:B (80:20 v/v) was used at a flow rate of 1 mL/min. Phase A was water (Millipore) containing 2.5% glacial acetic acid and phase B was an acetonitrile:phase A mixture (80:20 v/v).

Flavonoids were quantified using calibration curves (1–100  $\mu$ g/mL,  $R^2 = 0.998, 0.999$  and  $0.998$  for Q, N and E, respectively). Q, N and E detection was at 368, 310 and 280 nm, respectively.

### Calculation of Kinetic Parameters

A compilation of data obtained during the induction period in the experiments carried out in the Rancimat device and in an oven was used to calculate kinetic parameters in order to compare rate constants and reaction orders.

Because OxMONs are comprised of hydroperoxides during the induction period and thus are, in practice, the only products formed during the early stages of oxidation [20], we can write:



Assuming that OxMONs do not participate in other side reactions during this period, the rate equation is

$$d[\text{OxMONs}]/dt = k[\text{OxMONs}]^n, \quad (2)$$

where  $k$  is the rate constant and  $n$  is the reaction order. Rearrangement and integration of (2) leads to the following equation [22]:

$$[\text{OxMONs}]^{(1-n)} = [\text{OxMONs}]_0^{(1-n)} + (1-n)kt \quad (3)$$

, which represents the relation between [OxMONs] and  $t$  (time) during the early stages of oxidation, where  $[\text{OxMONs}]_0$  is the initial concentration of OxMONs, which was 0.

As a result, Eq. (3) can be expressed as Eq. (4)

$$(1-n)\ln[\text{OxMONs}] = \ln(1-n) + \ln k + \ln t. \quad (4)$$

Rearrangement of (4) leads to the following equation:

$$\ln[\text{OxMON}] = a + b(\ln t), \quad (5)$$

where  $a = [\ln(1-n) + \ln k]/(1-n)$  and  $b = 1/1-n$ .

Plots of  $\ln[\text{OxMONs}]$  versus  $\ln t$  were fitted to a straight line, thereby enabling us to calculate the kinetic parameters from the intercept ( $a$ ) and the slope ( $b$ ) obtained by the method of least squares.

### Statistical Analysis

Determinations of OSIs provided automatically by the Rancimat apparatus were carried out in triplicate, and results are expressed as mean values with a standard deviation (SD). Results of oxidation kinetics in Rancimat and oven are expressed as mean values with the SD of three samples, each of them withdrawn from independent tubes or beakers. Relative standard deviations were lower than 8% for oxidation compounds and lower than 10% for flavonoid content. To determine the statistical differences an analysis of variance (ANOVA) was performed using Statgraphics software version 7 (Manugistics, Inc.; Statistical Graphics Corporation, 1993, Rockville, MA, USA). The significance was defined at  $p < 0.05$ .

## Results and Discussion

### Oxidation Kinetics in Rancimat Assays

Table 1 shows OSIs and protection factors for ML with and without the addition of 200 mg/kg of Q, N or E at 60 °C. OSI is the end of the induction period according to the automatic determination by Rancimat that is based on a shift in the oxidation curve due to the appearance of volatile secondary oxidation compounds. Addition of Q and E to ML significantly increased its OSI, and E showed

**Table 1** Oil stability index (OSI) and protection factor in samples of methyl linoleate alone and with flavonoids added (200 mg/kg) at 60 °C

Samples	OSI (h)	Protection factor <sup>A</sup>
ML	9.7 $\pm$ 0.7 <sup>c</sup>	–
ML–N	9.0 $\pm$ 0.3 <sup>c</sup>	0.9 <sup>c</sup>
ML–Q	85.8 $\pm$ 1.1 <sup>b</sup>	8.8 <sup>b</sup>
ML–E	116.0 $\pm$ 1.4 <sup>a</sup>	12.0 <sup>a</sup>

Values are mean  $\pm$  SD ( $n = 3$ )

OSI oil stability index, ML methyl linoleate, N naringenin, Q quercetin, E epicatechin

<sup>A</sup> Protection factor is the ratio between the OSI of ML with flavonoids and the OSI of ML alone

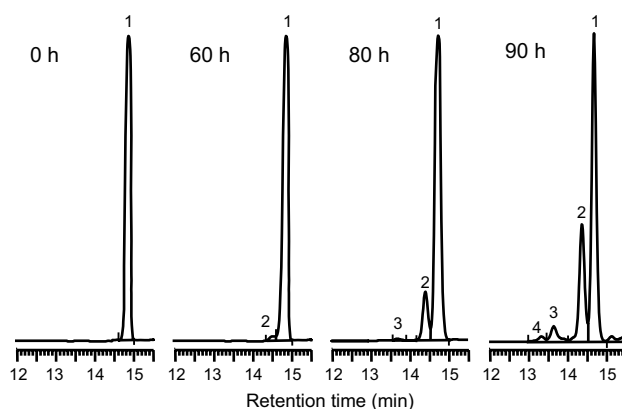
Different letters indicate significant differences ( $p \leq 0.05$ )

the greatest protection (12-fold). In contrast, no significant differences were found when N was added.

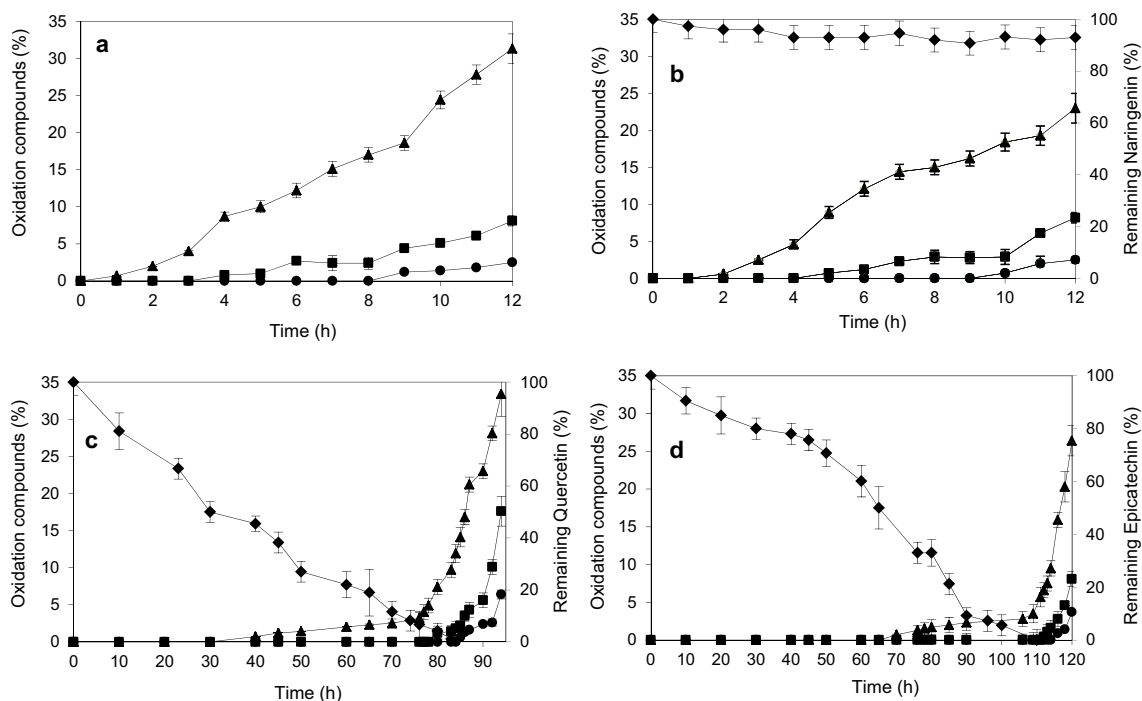
In order to obtain a complete picture of the oxidation process in Rancimat assays, samples were withdrawn periodically from initial to advanced stages of oxidation. Preliminary assays served to determine sampling time intervals and duration of the different experiments. A direct, novel HPSEC method was used to monitor oxidation via concomitant quantitation of different groups of primary and secondary oxidation products. Figure 1 shows the time course for formation of OxMONs, DIMs and OLIGs, and loss of flavonoids for ML, ML–N, ML–Q and ML–E systems (a, b, c and d, respectively), during oxidation at 60 °C in the Rancimat apparatus, up to the point at which significant polymerization occurred. The end of the induction period was characterized by the exhaustion of effective antioxidants in samples with Q or E and the initiation of polymerization, which was consistent with the OSI values provided by the Rancimat apparatus (Table 1). These results agree with those already reported on samples protected with tocopherols [20, 23]. However, initiation of polymerization occurred earlier than detection of volatile oxidation compounds in the Rancimat apparatus for ML and ML–N (4 and 5 versus 9.7 and 9 h, respectively). Besides the end of the induction period, the HPSEC method revealed that hydroperoxides, quantified as OxMONs, were first detected at 2 h in ML and ML–N

systems, 40 h in ML–Q and 70 h in Q ML–E. These results confirmed that E and Q showed high antioxidant action from the beginning of the oxidation process, while this did not occur in the case of N. In fact, the concentration of N did not decrease significantly as oxidation proceeded.

Figure 2 illustrates the resolution obtained between the peaks corresponding to the four groups of compounds



**Fig. 2** High-performance size-exclusion chromatograms of methyl linoleate (ML) samples with added quercetin, withdrawn at 0 h, 60 h, 80 h and 90 h during oxidation at 60 °C in Rancimat. Peaks: 1 non-oxidized ML, 2 oxidized monomers of ML, 3 dimers of ML and 4 higher oligomers of ML



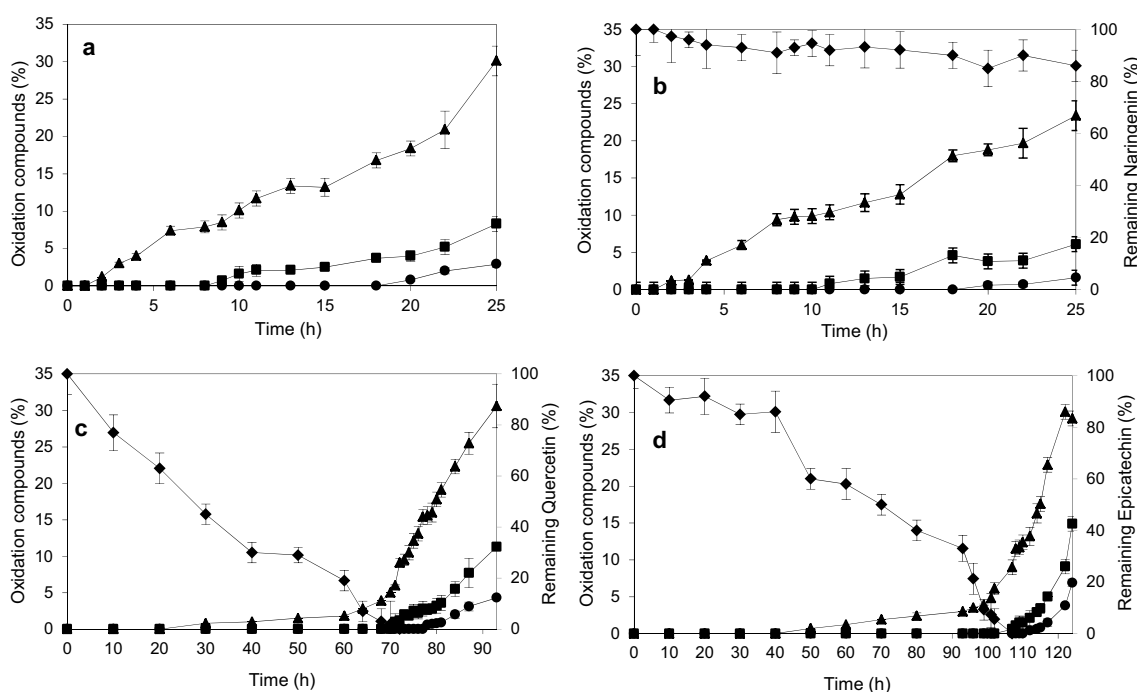
**Fig. 1** Time-course of formation of oxidized monomers (*triangle*), dimers (*square*) higher oligomers (*circle*) and retention of flavonoids (*diamond*) during oxidation of methyl linoleate alone (**a**) and with

200 mg/kg of added naringenin (**b**), quercetin (**c**) or epicatechin (**d**) at 60 °C in Rancimat. Values correspond to means of triplicate samples and *error bars* to standard deviations

quantified by HPSEC in ML samples oxidized at 60 °C in Rancimat: (1) initial, non-oxidized substrate (2) OxMONs including primary oxidation compounds, and polymerization products as (3) DIMs and (4) OLIGs. Chromatograms correspond to samples with Q added. The initial sample (0 h) contained exclusively non-oxidized ML. The sample withdrawn after 60 h, during the early stage of oxidation, before rancidity was detected, already showed an additional peak corresponding to 1.8% OxMONs. The peak of OxMONs comprises a large number of monomeric ML molecules containing one or more oxidized functions that may be hydroperoxide groups or any other oxygenated functions, such as epoxy, keto, hydroxy, etc. At this stage, OxMONs formed are virtually all hydroperoxides, hence an excellent correlation has been found between levels of OxMONs and peroxide values during the induction period [20, 22, 24]. The sample withdrawn at 80 h corresponds to the end of the induction period, denoted by the initiation of polymer formation and exhaustion of antioxidants. This sample contained 7.4% OxMONs and 1.2% DIMs. The last chromatogram (90 h) corresponds to a sample withdrawn after the induction period. This sample contained 22.7% OxMONs, 5.6% DIMs and 2.4% OLIGs. Polymerization compounds, here separated into dimers and higher oligomers, are characteristic of advanced oxidation.

### Oxidation Kinetics in Oven Assays

Figure 3 shows the time course for formation of OxMONs, DIMs and OLIGs and loss of flavonoids for ML, ML–N, ML–Q and ML–E systems (a, b, c and d, respectively) during oxidation at 60 °C in an oven up to the point at which significant polymerization occurred. The observed oxidation kinetics and protection order among the flavonoids tested were very similar to those found in Rancimat assays. However, it is important to note that oxidation of ML and ML–N was delayed in the oven as compared to the Rancimat. Thus, even though OxMONs appeared at about 2 h in both cases, polymerization started at 9 and 11 h for ML and ML–N, respectively, later than in Rancimat, 4 and 5 h, respectively (Fig. 1). In contrast, samples containing the effective flavonoids, ML–Q and ML–E, oxidized slightly earlier in the oven than in the Rancimat; OxMONs were detected at 30 h for ML–Q and at 50 h for ML–E. These apparently contradictory results can be explained considering that oxygen availability is a very important factor influencing oxidation. Hence the development of oxidation was expected to be faster with bubbling air in the Rancimat than in the oven, despite the large surface-to-volume ratio used in the latter; this occurred with ML and ML–N. However, in the case of the effective flavonoids Q and E, the bubbling air in the Rancimat may have favored homogenization and access



**Fig. 3** Time-course of formation of oxidized monomers (*triangle*), dimers (*square*), higher oligomers (*circle*) and retention of flavonoids (*diamond*) during oxidation of methyl linoleate alone (**a**) and with

200 mg/kg of added naringenin (**b**), quercetin (**c**) or epicatechin (**d**) at 60 °C in an oven. Values correspond to means of triplicate samples and *error bars* to standard deviations

of flavonoids to the active sites of oxidation, thus slightly enhancing their antioxidant effects.

### Comparison of Kinetic Parameters Obtained in Rancimat and Oven Assays

Table 2 lists the values corresponding to linear regression parameters ( $a$ ,  $b$ , and  $R$ ), the rate constants for OxMONs formation ( $k$ ) and the reaction order ( $n$ ) for all the samples assayed under different conditions and within the induction period, that is, before substantial formation of advanced oxidation products (dimers and higher oligomers). Values for the reaction order, which may differ depending on the mechanism of OxMONs formation, were found to be similar and in the range of those previously reported for methyl linoleate and trilinolein samples oxidized at 60 °C [20, 22]. However, it is interesting to note the remarkable decrease in the oxidation rate of ML when adding Q or E independently of the conditions used (Rancimat or oven).

### Global Assessment of the Antioxidant Effectiveness

The results obtained in this study showed the high antioxidant efficiency of E, and to a lesser extent, Q, in a model lipid system. In contrast N had practically no effect. In previous reports, it was found that the structural features of flavonoids affected the antioxidant capacity in lipids [6, 8, 11, 13, 15, 16, 25, 26]; however, the results were inconclusive. Pereira and Das [25] reported that flavonoids with OH groups in the 3,4 *ortho*-position in the B-ring (as in quercetin) displayed a reduced antioxidant effect, whereas when the OH groups were in 2,4 *meta*-position in the B-ring (as in morin) the antioxidant effect was enhanced. Conversely, Tsimogiannis and Oreopoulou found that the *ortho*-dihydroxy arrangement in the B-ring is active for radical scavenging. The primarily produced transient semiquinone radical is stabilized through the settled resonance structures and the intramolecular hydrogen bond between the phenoxyl radical and the free hydroxyl radical. The second hydrogen is

susceptible to abstraction by a free radical because a stable diquinone is formed [5]. Also, they reported that the presence of the 3-hydroxyl group, the 2,3-unsaturated bond and the 4-keto group in the C-ring had a protective effect against lipid oxidation [6]. However, the effect of the 2,3-unsaturated bond in the C-ring is controversial. Michotte and coworkers suggested that the 2,3-unsaturated bond in myricetin contributed to increasing its antioxidant capacity, when comparing different flavonoids [15]. In contrast, Nieto *et al.* reported that the 2,3-unsaturated bond had no effect on antioxidant activity when flavonoids with differences in the C-ring were compared [16]. Furthermore, similar antioxidant activity was reported for luteolin and taxifolin, which contain and lack the 2,3-unsaturated bond, respectively [6]. For the flavonoids tested in this study, the flavonol Q, the flavanol E and the flavanone N, the differences found in their antioxidant activity could be, to a large extent, attributed to differences in their structural features. Q and E have two OH groups at the *ortho*-position in the B-ring, whereas N has a single OH group in the B-ring. The *ortho*-dihydroxy structure in the B ring is crucial for the antioxidant capacity, in accord with previous reports [8, 10, 11]. This could account for the high efficiency of Q and E in the present work. Although N has shown significant potential for scavenging radical oxygen species in hydrophilic media [27], the present study is the first to report that such a protective effect was not found in lipidic media. In turn, Q and E present differences in the structural features of the C-ring, both have the 3-OH group but only Q has a 4-keto group and a 2,3 unsaturated bond. These three components together may contribute to increasing the antioxidant capacity, as was reported in hydrophilic media when comparing Q and E [5, 28]. Also in methyl linoleate, the greater efficiency of Q as compared to catechin was attributed to the structural differences in the C-ring mentioned above [10]. However, in the present study, even though both E and Q showed high antioxidant capacity, significantly better results were obtained for E. These results suggest that additional factors besides the structural features of flavonoids, such as solubility and

**Table 2** Oxidation of methyl linoleate alone and with flavonoids added (200 mg/kg) in Rancimat and oven at 60 °C: linear regressions [ $\ln r(h) = a + b \ln \text{OxMONs} (\%)$ ] and kinetic parameters

Oxidation conditions	Sample	$a$	$b$	$R$	$n$	$k \times 10^3$
Rancimat	ML	-0.68	1.90	0.9982	0.47	1400
	ML-N	-1.41	2.12	0.9991	0.53	1310
	ML-Q	-9.15	2.41	0.9501	0.58	54
	ML-E	-12.68	3.22	0.9459	0.59	42
Oven	ML	-0.10	1.50	0.9855	0.33	1096
	ML-N	0.03	1.03	0.9701	0.18	1063
	ML-Q	-5.95	1.74	0.9558	0.42	57
	ML-E	-9.33	2.33	0.9644	0.57	46

$a$  interception,  $b$  slope,  $R$  correlation coefficient,  $n$  reaction order,  $k$  rate constant, *ML* methyl linoleate, *N* naringenin, *Q* quercetin, *E* epicatechin

polarity, could have contributed to the differences in antioxidant efficiency found between E and Q in the present study.

According to the polar paradox theory [29], polar antioxidants are more effective in less polar media, such as bulk oils, while nonpolar antioxidants are more effective in relatively more polar media, such as oil-in-water emulsions [30]. Polar antioxidants are thought to locate at the oil–water interface of association colloids, including reverse micelles and lamellar structures, formed by the self-assembly of amphiphilic minor components present naturally in oils, e.g. phospholipids and others, or produced by lipid oxidation, e.g. hydroperoxides and other secondary oxidation compounds [31]. The trace amounts of water present in oils from atmospheric moisture constitute the inner part of these structural groupings. The amphiphilic molecules position their polar groups towards the water phase. At present, there is a considerable body of evidence that points out that these association colloids are the active sites of lipid oxidation in bulk oils. The preferential location of polar antioxidants in the oil–water interface of reverse micelles would explain their larger antioxidant effectiveness compared to lipophilic or nonpolar antioxidants, which would be homogeneously distributed in the lipid phase [30, 31]. Cuvelier and coworkers reported apolar factors of 5, 19 and 53 for E, Q and N, respectively [32]. The fact that E is more polar than Q could support the higher antioxidant effect found for E as compared to Q in the lipidic media used in the present study. The comparatively low polarity of N could have also contributed to its lack of antioxidant efficacy in this study.

## Conclusions

By applying a fast, simple and direct analytical method which enables complete and quantitative analysis of the oxidation progress in methyl linoleate samples, the kinetics and antioxidant efficiency of flavonoids have been determined and compared under different oxidation conditions. The present study highlights the high antioxidant action of epicatechin and quercetin at low concentrations in a bulk lipid system. In contrast, naringenin was found to be inefficient. On the one hand, the presence of the *ortho*-dihydroxy structure in the B ring and the 3-hydroxyl group in the C ring, in epicatechin and quercetin, seemed to be essential for the antioxidant activity. On the other, the higher polarity of epicatechin as compared to quercetin, and hence its preferential location at the active sites of lipid oxidation, may have contributed to their differential efficiency.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare no conflict of interest.

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