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Antioxidant Activity of Phenolic Acids in Lipid Oxidation Catalyzed by Different Prooxidants

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The antioxidant activity of three naturally occurring phenolic acids, caffeic (CaA), ferulic (FeA), and *p*-coumaric acid (CoA), and a synthetic compound, propyl gallate (PG), was evaluated in a food-related model system, a liposome dispersion of marine polyunsaturated fatty acids. Oxidation was induced by two different prooxidants, free iron ions and bovine hemoglobin (Hb). Continuous measurement of oxygen uptake was used to quantify the rate of lipid oxidation at steady state. Free iron-induced oxidation was reduced in the following order: PG > FeA > CoA. Caffeic acid worked as a prooxidant and increased the oxidation rate by a factor of 9. For Hb-induced oxidation, the relative efficiency was PG > CaA ~ FeA ≫ CoA. The antioxidant activity was also evaluated by four antioxidant capacity assays. In the Folin−Ciocalteu, ferric reducing/antioxidant power, and 2,2-diphenyl-1-picrylhydrazyl radical scavenging assays, the antioxidant activity followed the sequence PG > CaA > FeA > CoA. The order for the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assay was found to be PG > CoA ~ FeA > CaA. The assays mainly reflected reducing abilities of the compounds. This work reports that in addition to the differences in the chemical structure of antioxidants, the antioxidant activity of phenolic compounds depends also upon the type of prooxidant.

KEYWORDS: Phenolic antioxidants; lipid oxidation; iron; hemoglobin; oxygen uptake

INTRODUCTION

Foods containing long-chain *n*-3 polyunsaturated fatty acids (LC-PUFA) are especially labile with respect to oxidation, which causes formation of undesirable flavors and rancid odors, production of potentially toxic compounds, and loss of the health beneficial and essential fatty acids. Prooxidative agents, such as transition metals (iron, copper) or heme pigments (hemoglobin, myoglobin), significantly promote quality loss and reduce the shelf life of PUFA-rich foods. Iron ions and hemoglobin can be found as endogenous constituents in both raw fish materials and a wide variety of seafood products.

One industrially acceptable technique to control oxidative instability of fatty products is the application of antioxidative compounds with different mechanisms of action, including radical scavengers, singlet oxygen quenchers, photosensitizer inactivators, and metal chelators. Conventionally used antioxidants include synthetics, such as propyl gallate (PG), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), or ethylenediaminetetraacetic acid (EDTA), and nature-identical compounds, such as L-ascorbic, citric, and tartaric acids; natural antioxidants found in rosemary and tocopherol extracts are also commercially available (*1*).

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There has been a growing trend among consumers to prefer foods without synthetic additives, which has resulted in considerable worldwide attention to replacement of fully synthetic food antioxidants with naturally occurring ones. Unfortunately, the number of antioxidative compounds of natural origin approved by authorities is very small. It is therefore challenging as well as necessary to investigate the potential usefulness of other natural compounds, both individually and in mixtures. (*2*) During the past two decades, there has been intensive research on plant phenolics as suitable protectants against oxidation. Hydroxycinnamic acid derivatives have gained special attention owing to their abundant occurrence in a wide variety of fruits, vegetables, cereals, and cocoa and coffee beans (*3*, *4*).

The evaluation of the antioxidant activity of the different phenolic compounds has lately become an important research issue, and two general approaches have been applied: (i) an indirect approach by means of so-called *antioxidant capacity* (AOC) *assays* and (ii) a direct approach by use of lipid model systems (*5*).

The direct approach utilizes a multiplicity of lipid model systems: bulk oils, biphasic systems, emulsions, membrane structures [liposomes, microsomes, low-density-lipoproteins (LDL), intact cells], as well as a variety of PUFA-rich foods. A large number of these studies have shown that phenolics can act both antioxidatively and prooxidatively depending upon the

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physicochemical nature and the composition of the lipid system as well as antioxidant structure and concentration (*3*, *6*−*12*).

To select the right antioxidant for a given application, understanding the basic factors that affect the activity of phenolics in lipid oxidation is therefore beneficial. The choice of a food-related model system for investigative purposes is somewhat problematic. Real foods and isolated membranes (microsomes, LDL) mostly contain a variety of endogenous components, which may be difficult to control and which may interfere with the effects of added antioxidants. On the other hand, the model system should not be oversimplified. It should be transparent with respect to its compositional characterization and devoid of any interfering reactions that can complicate interpretation of the experimental results and drawing conclusions (*13*).

Lipid oxidation is conventionally studied by determination of peroxide value (PV), thiobarbituric acid reactive substances (TBARS), conjugated dienes, or anisidine value (AV) or by assessing volatile compounds. Peroxides are primary products in the oxidative breakdown of lipids, and their formation is the net result of the production rate and the decomposition rate (*1*). As the decomposition rate of peroxides is a function of at least pH and temperature, the production rate is difficult to quantify. The end products of lipid oxidation are the result of several reaction pathways from peroxides; these reactions do not follow one line, but several breakdown lines are possible, giving different end products from the same peroxide molecule. Analyzing only one or a few final oxidation compounds might therefore be misleading. An alternative approach is to focus on measurements of changes in oxygen, one of the lipid oxidation substrates, which has been employed in several studies, including earlier studies in our laboratory (*14*−*16*).

An increased interest in antioxidant activity has led to the development of a wide array of indirect methods to measure the antioxidant capacity (*5*, *17*). Different results have been obtained both between individual methods and within a method itself. Moreover, many inherent drawbacks in these assays have been found and discussed in a number of works (*5*, *17*−*20*). Despite these criticisms, AOC assays are still being routinely used for the evaluation of antioxidant capacity by food laboratories (*21*), although there have been efforts to improve procedure protocols of the existing methods (*22*), as well as efforts to develop new assays utilizing and combining different approaches (*23*−*26*).

The objective of this study was to evaluate the antioxidant activity of three naturally occurring phenolic acids that have potential as food antioxidants, caffeic acid (CaA), ferulic acid (FeA), and *p*-coumaric acid (CoA), and one synthetic phenolic antioxidant, propyl gallate (PG). A food-related lipid model system, liposome dispersion of long-chain PUFAs in marine phospholipids, was chosen to avoid the complexity of real food matrices. Lipid oxidation was induced by two different dietary prooxidants, free iron (Fe^{2+}, Fe^{3+}) and bovine hemoglobin (Hb), as these prooxidants differ substantially in prooxidative mechanism (*1*, *27*). Continuous measurement of oxygen uptake was used to quantify the rate of lipid oxidation. In addition, four commonly used spectrophotometric antioxidant capacity assays were used for comparison and additional information on the antioxidant capacity of the studied compounds.

MATERIALS AND METHODS

Materials. Cod roe from Pacific cod (*Gadus macrocephalus*) caught in the North Pacific Ocean was used for extraction of phospholipids. The cod roe was frozen at −40 °C until needed.

Propyl gallate, caffeic acid, ferulic acid, *p*-coumaric acid, 2.0 M Folin−Ciocalteu phenol reagent, 2-(*N*-morpholino)ethanesulfonic acid (MES), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA), 2-methylpentane, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), sodium carbonate, sodium acetate, bovine hemoglobin, sodium sulfite, trichloroacetic acid (TCA), 1,1,3,3-tetraethoxypropane (TEP), potassium persulfate, and catalase from bovine liver were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Hydrochloric acid, acetic acid, potassium chloride, formic acid, ferric chloride tetrahydrate, ammonium thiocyanate, titrisol [$(NH_4)_2Fe(SO_4)_2$], ferrous sulfate heptahydrate, and all of the solvents used were supplied by Merck KGaA (Darmstadt, Germany). Anhydrous ferric chloride and IDRANAL II (ethylenediaminetetraacetic acid) was purchased at Riedel de Haën (Seelze, Germany). Sodium hydroxide and ferrous chloride tetrahydrate were obtained from Fluka Chemie (Buchs, Switzerland). Nitrogen (N_2) gas (99.999%) was provided by AGA AS, Oslo, Norway. All chemicals and solvents were of analytical grade.

Isolation of Phospholipids. The cod roe was allowed to thaw overnight at 4 °C. The extraction of total lipids from cod roe was performed according to the method of Bligh and Dyer (*28*). The phospholipids (PL) were isolated from the total lipids using the acetone precipitation method, as described by Kates (*29*) and modified by Mozuraityte et al. (*14*). The phospholipids dissolved in chloroform were stored in the dark at −20 °C until needed.

Purity of Phospholipids. The composition of isolated phospholipids was determined by thin layer chromatography (*30*) and detected by a Iatroscan thin layer chromatography−flame ionization detector system (TLC-FID analyzer TH-10 MK-IV, Iatron Laboratories, Inc., Tokyo, Japan) as described by Mozuraityte et al. (*14*). Three analyses were performed, and the results were expressed in area percentage as the mean value ± standard deviation (SD).

Phospholipid Classes. The classes of isolated PL were analyzed by P-31 NMR. Fifty milligrams of dried mass of phospholipids was dissolved in a 0.6 mL solution of chloroform-*d*/methanol-*d* (2:1, v/v) containing the internal standard (triethylphosphate) in 5 mm NMR tubes. NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer with QNP probe operating at a P-31 frequency of 121.49 MHz at ambient temperature (25 °C). The acquisition parameters used were as follows: spectral width, 30 ppm; 20K time domain data points; zero-filled to 64L; acquisition time, 2.8 s; relaxation delay, 50 s; 90° acquisition pulse. Chemical shifts were referred to triethylphosphate (δ = 0 ppm).

Fatty Acid Composition. The fatty acid composition of the phospholipids was determined by gas chromatography (GC) of their fatty acid methyl esters as described by Dauksas et al. (*31*) The lipids were transesterified and extracted into hexane according to AOCS method Ce 2-66 (*32*). Free fatty acid methyl esters were identified by comparison of their retention times with those of the reference solution (GLS-68D; Nu-Chek-Prep) chromatographed under identical GC conditions. Two replicate analyses were performed and the results were expressed in GC area percent as a mean value ± SD (standard deviation).

Peroxide Value (PV). PV was analyzed by the ferric thiocyanate method as described by the International Dairy Federation (*33*) and modified by Ueda et al. (*34*) and Undeland et al. (*35*). The analysis was performed in triplicate.

Analysis of Thiobarbituric Acid Reactive Substances (TBARS). TBARS values were determined according to the spectrophotometric method described by Ke et al. (*36*). The absorbance values of samples were compared to a standard curve prepared with 1,1,3,3-tetraethoxypropane for the calculation of TBARS concentrations (μM/g of fat). The analysis was performed with five parallels.

Preparation of Liposomes. The liposome dispersion was prepared according to the method of Mozuraityte et al. (*14*) fresh before experiments. A chloroform solution of phospholipids was evaporated to dryness with a stream of nitrogen gas (99.99%), and the residual solvent was completely evaporated under vacuum (2 h). The dried mass of phospholipids was dissolved in a 5 mM MES buffer, pH 5.5 or 3.0, to a concentration of 30 mg/mL, and the solution was sonicated with Vibra Cell (Sonics & Materials Inc.). MES buffer was used because it does not bind iron into complexes, has a very low solubility in nonpolar solvents, and has pK_a = 6.1, which is suitable for our oxidation experiments, as most of them were performed at pH ∼5.5. At higher concentrations of MES buffer, a higher rate of Fe^{2+} autoxidation was observed. During and after the sonication the phospholipids were kept on ice. For all of the

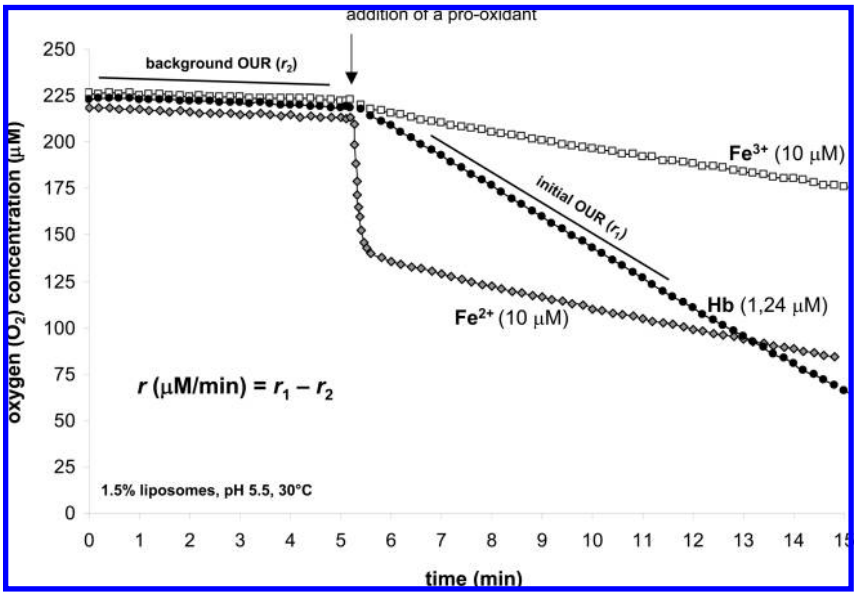

Figure 1. Representative oxygen uptake curves of oxidation of polyunsaturated fatty acids in liposomes (1.5%, pH 5.5, 30 °C) recorded before and after addition of different prooxidants (noninhibited oxidation): (◆) 10 μM Fe^{2+}; (□) 10 μM Fe^{3+}; (●) 1.24 μM Hb. Calculation of oxygen uptake rate (OUR) is schematically depicted in the curve for Hb.

experiments, the liposome solution was further diluted with a MES buffer to a concentration of 15 mg/mL.

Oxidation Experiments. The rate of oxidation was quantified from the rate of disappearance of the dissolved oxygen in the reaction mixture (1 mL). A polarographic oxygen electrode (Oxygraph system, Hansatech Instruments Ltd., Norfolk, U.K.) was used to continuously measure the dissolved oxygen concentration. Each oxygraphic cell was closed with a plunger with a capillary opening, equipped with a magnetic stirrer, and thermostated at 30 °C.

Stock solutions of PG, CaA, FeA, and CoA were prepared in 96% ethanol and stored in the dark at 4 °C for a maximum of 14 days. Working solutions were prepared daily by diluting an appropriate aliquot of the stock solution with ethanol. Stock solutions of Fe^{2+} ($FeSO_4 \cdot 7H_2O$) and Fe^{3+} ($FeCl_3$) in 0.5 M HCl were prepared monthly. Working solutions of Fe^{2+} and Fe^{3+} were prepared daily by diluting an appropriate aliquot of the stock solution with MES buffer; the pH was kept at 2.0 to maintain iron solubility. Working solution of bovine hemoglobin (Hb) was prepared fresh before experiments by dissolving Hb in 5 mM MES buffer (pH 5.5). The concentrations of prooxidants in the reaction mixture were 10 μM Fe^{2+}, 10 μM Fe^{3+}, and 20 μg/mL Hb, the latter corresponding to 1.24 μM iron donated via Hb.

The curves of dissolved oxygen concentration as a function of time were recorded. A background oxygen uptake rate (OUR) was measured for 2−5 min before the addition of a sample (antioxidant or ethanol). After sample addition, a background OUR (r_2) was observed again, usually for 5−10 min. When a steady background OUR was reached, a prooxidant (Fe^{2+}, Fe^{3+}, or Hb) was added into the dispersion to initiate oxidation and the initial OUR of oxidation (r_1) was measured, that is, the oxygen consumption rate between the second and fourth minutes after addition of a prooxidant. The rate of oxidation (r) was found by subtracting the initial OUR from the background OUR ($r = r_1 - r_2$), as illustrated on the Hb curve in **Figure 1**. Inhibition (%) in relation to the respective noninhibited oxidation (added ethanol) was calculated to evaluate the antioxidant effects: $I(\%) = 100 - [(r_{inh}/r_{non}) \times 100]$. Two or three parallel cells were run for each experiment. The pH of the liposome solutions was verified after each experiment by a Philips pH meter (model PW 9420, Pye Unicam, Cambridge, U.K.) coupled with a glass electrode (LIQ-GLASS 238000/08, Hamilton Co., Reno, NV).

The presence of ethanol (maximum 2% in a reaction volume) that was used to dissolve the phenolics did not have any significant influence on the oxygen uptake by liposomes themselves (data not shown) or on the prooxidative activity of Fe^{2+} and Fe^{3+} ions (6.0 ± 0.4 and 7.0 ± 0.6 μM O_2/min, respectively) and Hb (presence of ethanol, 20.8 ± 1.1; absence of ethanol, 19.4 ± 0.8 μM O_2/min).

Oxygraph software "oxyg32" and MS Excel was used for data processing and statistical analysis. The significance level was set at 95% ($p = 0.05$).

For the antioxidant capacity assays, a 10 mM methanolic stock solution of each compound was prepared and stored in the dark at 4 °C for a maximum of 14 days. Working solutions were prepared daily by dilution of suitable aliquots of a stock solution with methanol.

Folin−Ciocalteu (FC) Assay. The FC assay was performed as described by Singleton et al. (*37*) with some modifications (*38*, *39*). Briefly, a series of 0−3 mM working solutions of PG and CaA and 0−5 mM FeA and CoA were prepared. Deionized water (10 mL), antioxidant solution (1 mL), and 2.0 M Folin−Ciocalteu phenol reagent (1 mL) were transferred to a 20 mL volumetric flask, the reaction mixture was mixed by shaking, and after 3 min, 2 mL of 25% Na_2CO_3 solution (75 g/L) was added. The volume was brought up with deionized water. The absorption at 725 nm was read after 1 h of incubation at room temperature. Water was used as a blank. Five point graphs of antioxidant concentration versus absorbance values were constructed, and the FC value was taken as the slope of the linear curve derived from the constructed graphs. The assay was carried out three times with each compound, and the average slope value ± standard deviation (SD) was calculated.

FRAP Assay. The FRAP assay was performed as described by Benzie et al. (*40*) and adapted by Nenadis et al. (*38*). For the analysis, a series of 0−150 μM working solutions of PG and CaA, 0−200 μM FeA, and 0−2700 μM CoA were prepared (final dilution in the reaction mixture 1:15). Five point graphs of antioxidant concentration in the reaction mixture versus ΔA ($\Delta A = A_{AH} - A_{cont}$) were constructed. The FRAP value was taken as the slope of the linear curve derived from the constructed graphs. The assay was performed three times with each compound, and the average slope value ± SD was calculated.

DPPH Assay. The DPPH assay was performed as described by Brand-Williams et al. (*41*) with some modifications (*19*, *38*, *42*). Briefly, the day before analysis, 0.1 mM methanolic DPPH$^\bullet$ working solution was prepared and kept on a magnetic stirrer overnight at 4 °C. A series of 0−750 μM methanolic working solutions of PG, 0−1200 μM CaA, and 0−2400 μM FeA were prepared fresh from stock solutions (final dilution in the reaction mixture 1:30). An aliquot of DPPH$^\bullet$ solution (2.9 mL) was mixed with 0.1 mL of a sample or methanol (blank) and vortexed well. After 20 min of incubation at room temperature, the absorbance at 515 nm was recorded. Water was used as a blank. Five point graphs of inhibition (%) of initial absorbance of the DPPH$^\bullet$ solution [$I(\%) = (1 - A_{sample}/A_{blank}) \times 100$] versus antioxidant concentration in the reaction mixture were constructed, and EC_{50} values were calculated from the linear curves derived from the constructed graphs. The assay was performed two times with each compound, and the average EC_{50} value ± SD was calculated.

Table 1. Characterization of Phospholipids Isolated from Cod Roe

phospholipid classes (%)	phosphatidylcholine (PC)	69
	phosphatidylethanolamine (PE)	23
	lyso-PC	5
	lyso-PE	3
lipid classes (%)	phospholipids	97.9 ± 1.2
	free fatty acids	0.4 ± 0.1
	cholesterol	1.0 ± 0.5
	monoacylglycerol	1.0 ± 0.7
	unknown	<1.0
fatty acids (%)	saturated	28.2 ± 0.8
	monounsaturated	25.8 ± 0.3
	polyunsaturated	46.0 ± 0.5
	20:5 n-3 (EPA)	14.2 ± 0.2
	20:6 n-3 (DHA)	29.8 ± 0.3
	other PUFA	2.0 ± 0.0
PV (mequiv of peroxide/kg of fat)		6.6 ± 1.3
TBARS (μM/g of fat)		2.4 ± 0.2

ABTS Assay. The ABTS assay was performed as described by Nenadis et al. (*43*) with a few modifications: ethanol was replaced with methanol, and the amount of sample added to the $ABTS^{\bullet+}$ solution was 200 μL. For the analysis, a series of 0−55 μM working solutions of PG and 0−110 μM CaA, FeA, and CoA were prepared fresh from stock solutions (final dilution in the reaction mixture 1:11). Five point graphs of inhibition (%) of initial absorbance of the $ABTS^{\bullet+}$ solution [I (%) = $(1 - A_{sample}/A_{blank}) \times 100$] vs antioxidant concentration in the reaction mixture were constructed, and EC_{50} values were calculated from the linear curves derived from the constructed graphs. The assay was performed twice with each compound, and the average EC_{50} value ± SD was calculated.

To compare the antioxidant activities, the absolute values for each antioxidant and each assay were recalculated into propyl gallate equivalents, the absolute value for PG being equal to 1.

RESULTS AND DISCUSSION

Characterization of Cod Roe Phospholipids. The phospholipid classes, lipid classes, and fatty acid profile of the phospholipids (PL) isolated from cod roe, which were used for preparation of liposomes, are presented in **Table 1**. To evaluate the oxidation level of the material, PV and TBARS were measured after isolation (**Table 1**). Both PV and TBARS values indicated a low degree of oxidation; the PV value was consistent with the PV values for our previous isolations (*14*, *44*).

Inhibition of Iron-Catalyzed Oxidation. Both Fe^{2+} and Fe^{3+} were used as promoters of lipid oxidation. The time curves of oxygen uptake after addition of each prooxidant into liposomes (referred to as *noninhibited* oxidation) are shown in **Figure 1**. In our previous paper it has been explained that the initial fast consumption of dissolved oxygen after the addition of Fe^{2+} is due to oxidation of Fe^{2+} to Fe^{3+} by pre-existing peroxides. This process generates alkoxy and peroxy lipid radicals, which rapidly deplete dissolved oxygen by forming lipid hydroperoxides. Once the equilibrium between Fe^{2+} and Fe^{3+} is achieved, redox cycling of iron takes place, resulting in a constant rate of oxygen consumption observed after the initial drop, where Fe^{3+} reduction is the rate-limiting factor (*15*).

The relative efficiency of the tested phenolics (**Figure 2**), compared at 200 μM concentration, had the same trend regardless of the state of iron: PG was the most powerful compound, followed by FeA; CoA did not show any significant inhibitory effects. The inhibition (%) of oxidation as a function of PG, FeA, and CoA concentration is shown in **Figure 3**. Conversely, CaA strongly enhanced oxidation. The increase (%) in oxidation for different concentrations of CaA is shown in **Figure 4**. The similar values for Fe^{2+} and Fe^{3} for both inhibited and promoted oxidation clearly show that behavior of the tested phenolics is not dependent upon the initial state of iron, which supports the theory of redox cycling of iron (*15*).

In the tested concentration range of PG (1−200 μM), only the concentrations above 10 μM (PG/Fe ≥ 1) were efficient in inhibiting the oxidation rate. The concentration of 200 μM

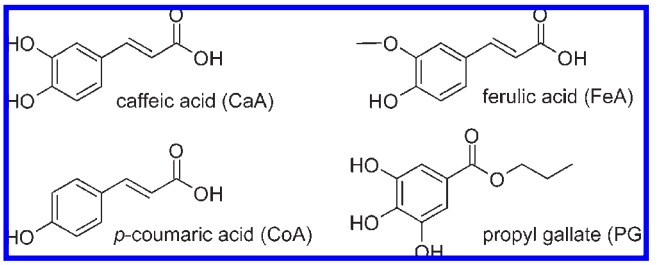

Figure 2. Molecular structures of three naturally occurring phenolic antioxidants, caffeic acid, ferulic acid, and *p*-coumaric acid, and a synthetic phenolic compound, propyl gallate.

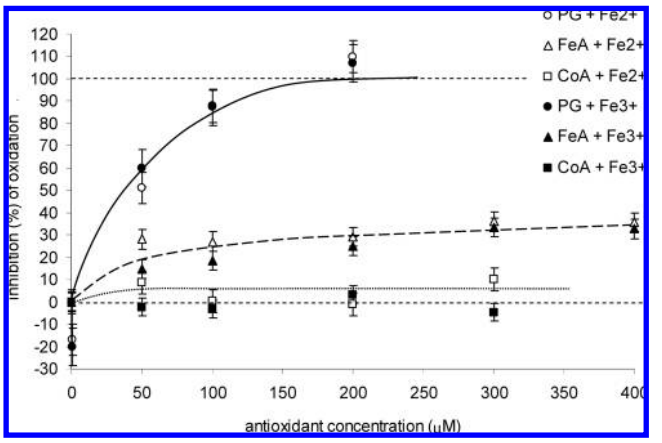

Figure 3. Inhibition (%) of Fe^{2+} (10 μM) and Fe^{3+} (10 μM) catalyzed oxidation of polyunsaturated fatty acids in liposomes (1.5%, pH 5.5, 30 °C) by different concentrations of propyl gallate (PG), ferulic acid (FeA), and *p*-coumaric acid (CoA). The values are given as the means of two to five parallel experiments ± standard error (SE). Positive values represent inhibition of oxidation, whereas negative values represent an increase in oxidation.

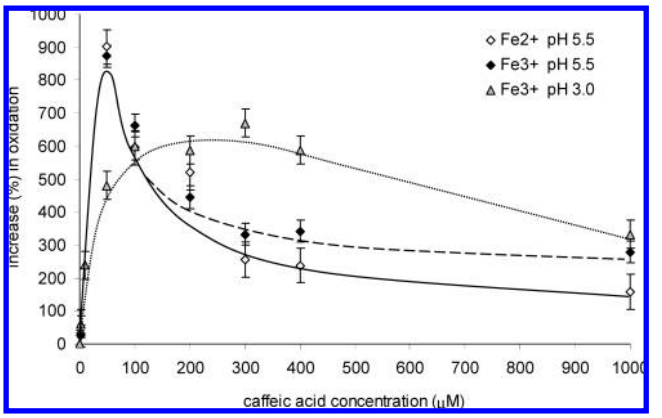

Figure 4. Increase (%) in Fe^{2+} (10 μM) and Fe^{3+} (10 μM) catalyzed oxidation of fatty acids in liposomes (1.5%, 30 °C) at pH 5.5 and 3.0 by different concentrations of caffeic acid (CaA). The values are given as the means of two to five parallel experiments ± standard error (SE).

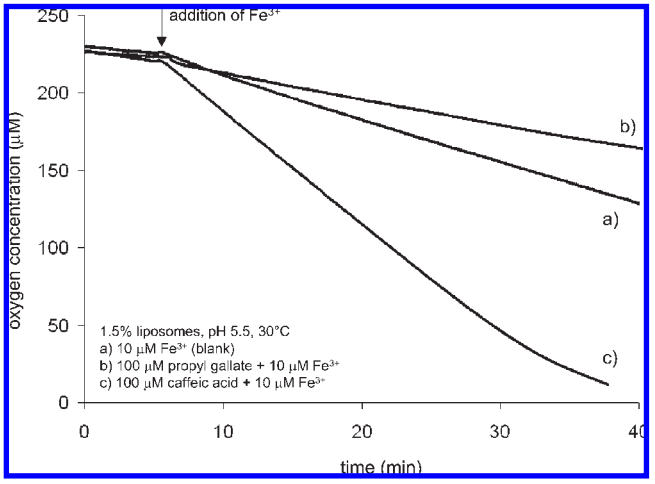

Figure 5. Time curves of oxygen consumption in Fe^{3+}-induced oxidation (line a), and Fe^{3+}-induced oxidation inhibited by propyl gallate (line b) or promoted by caffeic acid (line c).

completely inhibited oxidation. When 1 μM PG concentration was tested (PG/Fe = 0.1), an increase in oxidation rate both in Fe^{2+} and in Fe^{3+} catalyzed oxidation (20 ± 8 and 17 ± 7%, respectively) was observed.

CaA strongly enhanced oxidation at all tested concentrations except for the lower concentration (0.1 μM), at which CaA did not have any significant effect on the oxidation rate. The prooxidant effect of CaA showed a maximum at 50 μM (CaA/Fe ≥ 5) at pH 5.5 and at 200 μM (CaA/Fe ≥ 20) at pH 3.0. At higher CaA concentrations the prooxidant effect decreased. The time course of oxygen consumption for selected concentrations of PG and CaA after addition of Fe^{3+} is shown in **Figure 5**.

FeA (50−400 μM) was capable of inhibiting free iron promoted oxidation at all levels of addition. The degree of inhibition did not markedly increase with increasing concentration as was observed with PG. FeA was found to be a better antioxidant than *p*-coumaric acid; however, among the tested compounds FeA could be characterized as a less potent antioxidant.

All of our oxidation experiments were performed in the same way to observe the inhibition of the very onset of iron-induced oxidation, with the antioxidant already present in the liposome solution (**Figure 5**). However, when the order of addition of an antioxidant and a prooxidant was reversed, that is, antioxidant was added into already initiated oxidation, a significant decrease (or increase for CaA) in oxygen consumption was observed as well (data not shown). In our earlier study it was proposed that one redox cycle of iron generates a constant number of lipid alkoxy and peroxy radicals as a result of decomposing lipid hydroperoxides, which leads to a constant rate of oxygen consumption (*15*); thus, the system reaches a steady state. The experiments show that it is possible to reduce the rate of iron-catalyzed oxidation from the very beginning as well as at the later stages by eliminating the generated radicals by means of the addition of antioxidants.

Caffeic Acid and Iron-Catalyzed Oxidation. The prooxidative behavior of CaA in the free iron catalyzed oxidation is most likely the result of its ability to reduce Fe^{3+}, which was verified by the FRAP assay (shown below).

An intramolecular electron transfer (IET) within a temporary CaA−Fe^{3+} complex has been reported to be responsible for the reduction of ferrous ions (Fe^{3+}) by CaA (*45*). The reaction releases Fe^{2+} and produces *o*-semiquinone radical and possibly *o*-quinone (**Figure 6**). Superoxide anion ($O_2^{\bullet -}$) has been proposed to be generated from triplet oxygen during the IET reaction (*46*).

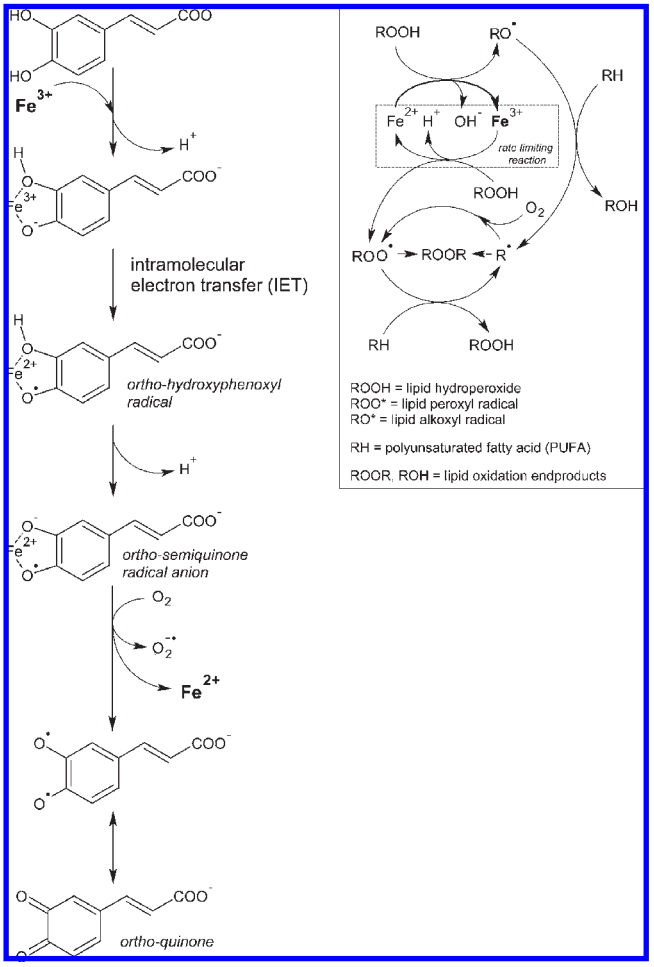

Figure 6. Reduction of ferric (Fe^{3+}) iron to ferrous (Fe^{2+}) iron by intramolecular electron transfer within a caffeic acid molecule (modified from ref *46*). (Inset) Low molecular weight (free) iron catalyzed lipid oxidation (modified from ref *14*).

There are several possible pathways for its further reactions. In an aqueous medium, $O_2^{\bullet -}$ is capable of Fe^{3+} reduction, forming hydrogen peroxide (H_2O_2) or triplet oxygen ($^3O_2$) (*1*). Measurable amounts of hydrogen peroxide were not found when catalase (40 μM), an enzyme decomposing hydrogen peroxide into water and oxygen molecules, was added into liposomes containing iron and CaA, as dissolved oxygen concentration did not increase significantly after the addition of catalase (data not shown).

In experiments with a ratio of CaA/Fe ≤ 1, the reaction pathways of CaA−Fe complex formation and decomposition may differ. When iron is present in great abundance, a total breakdown of CaA rather than formation of quinones was reported to follow complex formation (*45*). This could explain the markedly lower prooxidative activity at 1 μM CaA concentration and no effect at 0.1 μM CaA concentration.

Interestingly, the prooxidative activity of CaA was significantly lowered also at 1000 μM CaA concentration, at both pH 5.5 and 3.0. This could be attributed to the radical scavenging abilities of the proportion of CaA that was associated with the phospholipid bilayers or to a low solubility of CaA in aqueous phase.

In an aqueous dispersion of liposome, the stability of hydrated iron ions is sensitive to the pH of the surrounding environment. In aqueous solutions the solubility of iron is maintained by low pH (≤2.0); an increase in pH leads to precipitation of iron as iron

Table 2. Antioxidant Activities of the Studied Phenolic Compounds Tested with Different Antioxidant Capacity Assays Expressed in Propyl Gallate Equivalents[a]

antioxidant	FC assay (slope ± SD)	FRAP (slope ± SD)	DPPH (EC_{50} ± SD)	ABTS (EC_{50} ± SD)
propyl gallate *absolute values*	0.34 ± 0.02	0.119 ± 0.005	10.6 ± 0.3	20.0 ± 0.4
propyl gallate	1.00 a	1.00 a	1.00 a	1.00 a
caffeic acid	0.96 a	0.65 b	0.47 b	0.32 b
ferulic acid	0.30 b	0.54 c	0.22 c	0.43 c
p-coumaric acid	0.11 c	0.04 d	< 0.02 d	0.44 c

[a] Folin−Ciocalteu (FC) assay; ferric reducing/antioxidant power (FRAP) assay; 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH) assay; 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. The absolute values are given as means ± SD (standard deviation) of two (DPPH and ABTS) or three (FC and FRAP) parallel measurements; slope = slope value of a linear curve derived from the dependence absorbance = *f*(antioxidant concentration in the reaction mixture); EC_{50} = efficient antioxidant concentration (μM) for scavenging 50% of the radical. Values within the same column with different letters are significantly different at $p < 0.05$.

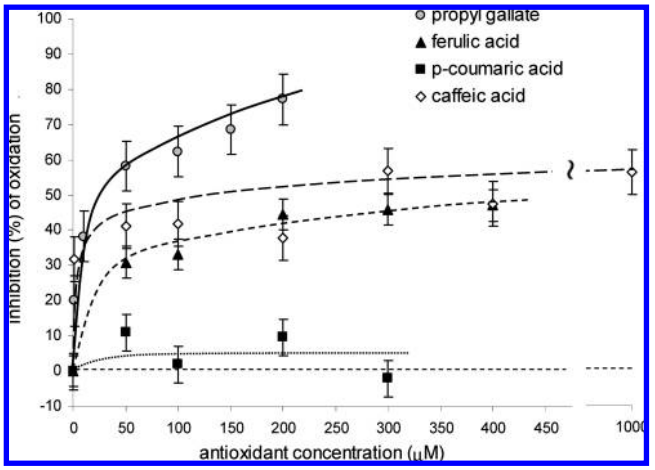

Figure 7. Inhibition (%) of Hb (1.24 μM) catalyzed oxidation of liposomes (1.5%, pH 5.5, 30 °C) by different concentrations of the tested phenolics. The values are given as the means of two to five parallel experiments ± standard error (SE).

hydroxides (*47*). The solubility product constants for Fe^{2+} and Fe^{3+} hydroxides are 5×10^{-17} and 3×10^{-39}, respectively (*15*). Observations in our earlier study (*15*) indicate that in working solutions of iron with a higher pH, formation of iron hydroxides leads to changes in concentration of active iron resulting in somewhat lowered and different OURs between Fe^{2+} and Fe^{3+} mediated oxidation. Thus, both the Fe^{2+} and Fe^{3+} working solutions were prepared at pH 2.0 to prevent hydroxide formation prior to the addition of iron into the liposome solution. We cannot exclude that precipitation of iron occurs in the liposome solution. However, the low concentration of iron (10 μM) and not significantly different OUR values measured in Fe^{2+} and Fe^{3+} initiated oxidation, (7.2 ± 0.4 and 7.0 ± 0.4 μM O_2/min, respectively) suggest that phospholipids attract iron in the way that prevents hydroxide precipitation, as the proportion of active iron remains equal for both Fe^{2+} and Fe^{3+} after redox cycle equilibrium is achieved. The addition of an acidic working solution of iron (10 μL) into liposomes had only a minor effect on the pH value of the resulting reaction mixture, being in the range of 5.3−5.5.

Inhibition of Hb-Induced Oxidation. Except for CoA, all of the tested phenolics inhibited Hb-induced oxidation (**Figure 7**). The relative efficacy, compared at 100 μM concentration, followed the sequence PG > CaA ∼ FeA.

The inhibitory effect of PG (1−200 μM) increased strongly with increasing concentration. Contrary to free iron catalyzed oxidation, CaA did not show any prooxidant activity and was able to inhibit oxidation at concentrations above 0.1 μM to 1000 μM; at 0.1 μM CaA was inactive. Slightly increasing inhibitory effect was observed with increasing concentration. FeA was tested in the concentration range from 50 to 400 μM. The degree of inhibition did not markedly increase with increasing concentration, and the inhibitory effect was approximately equal to that of CaA. CoA was inactive toward Hb-induced oxidation at all tested concentrations (50−300 μM) as well as being inactive toward free iron induced oxidation.

Both prooxidants, free iron and Hb, are dispersed in the aqueous phase of the liposome solution. This shows that there has to be attractive interactions between the prooxidants and charged liposome particles, which locate the prooxidants to the very proximity of the phospholipid bilayers. The aqueous environment also allows the prooxidants to be in direct contact with the phenolic molecules distributed in the water phase.

In most of our experiments the molar concentrations of phenolics were >10 times higher than the concentration of iron in Hb (1.24 μM). However, at molar PG-to-Hb ratio close to 1, PG was efficient in inhibiting oxidation (the inhibition reached 20 ± 4%) (**Figure 7**), which is in contrast with Fe-induced oxidation, for which at the same ratio a prooxidative tendency of PG was observed (**Figure 3**).

So far, the mechanism for Hb-initiated peroxidation has not been fully clarified. Forms of heme-proteins (Hb, Mb) containing the oxoferyll complex (Fe^{4+}=O) are believed to be the main driving force of heme-iron-initiated lipid oxidation (*27*, *48*). The mechanism of phenolics for inhibiting lipid oxidation promoted by Hb has not been elucidated either. Pazos et al. suggested that the mechanism did not seem to be related to a direct effect of phenolics on Hb autoxidation ($Hb-Fe^{2+} \Leftrightarrow Hb-Fe^{3+}$) (*8*).

The studied phenolics are capable of noncovalent binding to proteins (*49*). Binding of chlorogenate to ferrylmyoglobin ($Mb-Fe^{4+}$) led to reduction of the oxoferryl moiety to a less prooxidative metmyoglobin ($Mb-Fe^{3+}$) (*50*). Metmyoglobin/H_2O_2-dependent oxidation of LDL also resulted in reduction of ferrylmyoglobin to metmyoglobin by phenolic acids (*51*).

It could be assumed that in the aqueous phase of the liposome solution the phenolic acids can easily enter the heme crevice of Hb and/or bind to ferrylhemoglobin ($Hb-Fe^{4+}$), where they can quickly reduce the oxoferryl moiety, which is observed as the inhibition of Hb-catalyzed oxidation. Indeed, the studied phenolics with a higher reduction potential inhibited the oxidation more strongly that those with a lower reduction potential.

Due to low polarity and relatively high affinity of PG toward phospholipid membranes (discussed below), PG probably functions both as an efficient free lipid radical scavenger and as a powerful reductant of ferrylhemoglobin, providing the highest inhibitory effects. The inhibitory effect of FeA on Hb-promoted oxidation was approximately equal to that of CaA, although the standard reduction potential of FeA has been reported to be half that of CaA (*52*). Apart from the ability of phenolics to reduce the prooxidative initiator, the accessibility of antioxidant molecules to the heme crevice or the strength of noncovalent bonds between proteins and the phenolics may play an important role.

Antioxidant Capacity Assays. The results from the antioxidant capacity assays are summarized in **Table 2**. In the FC, FRAP, and

DPPH assays, PG showed the highest antioxidant activity followed by CaA, FeA, and CoA. In the ABTS assay, PG was also found to have the highest activity, followed by CoA and FeA, whereas CaA was found to have the lowest antioxidant activity. The obtained orders are in agreement with data reported in the literature (*10*−*12*, *19*, *43*, *53*, *54*). Although the orders determined by the FC, FRAP, and DPPH assays are almost identical, indirect expression by means of propyl gallate equivalents revealed considerable differences in the degree of activity.

The inconsistencies in the orders and degrees could be attributed to a number of factors, involving some specific reactions between the different assay reagents and the antioxidants, unrelated reactions of phenolics (dimerization, polymerization) that probably occur in the reaction mixtures, and drawbacks and limitations in the chemistry and methodology of the assays (*5*, *17*−*20*).

Good correlations were found between the orders established in FC, FRAP, and DPPH assays and the order established in Hb-induced oxidation. The order found in the ABTS assay correlated neither with free iron catalyzed oxidation nor with Hb-catalyzed oxidation.

Results from the FC and FRAP assays show that PG and CaA possess a high ability to donate an electron. When related to the catalytic role of metals in lipid oxidation, a positive reducing capacity signals possible redox reactions with transition metals (Fe, Cu) and their reduction into a more prooxidative valence status. The FRAP assays also showed that all of the compounds possess an electron-donating ability in an acidic (pH 3.6) aqueous solution. The oxidative potentials (E_{pa}) for CaA, FeA, and CoA were reported to be 0.212, 0.430, and 0.583 (V vs Ag/AgCl), respectively (*52*), which is in agreement with the orders found in the two assays.

The results support the concepts of other authors that the AOC determined by these assays cannot universally predict the antioxidant activity and should, therefore, serve as a tentative or preliminary estimation of antioxidant capacity; any predictions regarding protection of lipid systems (foods) are uncertain or could even be misleading (*17*). From the reaction mechanism point of view, the assays only provided information on the reducing potentials (ability to donate an electron) of the compounds or the ability to scavenge synthetic free radicals in an aqueous environment via single electron transfer.

Factors Affecting the Antioxidant Activity. The polarity of phenolic molecules has a strong influence on their location in lipid systems (*1*). In emulsion type systems, the affinity of antioxidants toward the interface, represented by phospholipids in our system, is a key parameter, as well.

When PG was added to the liposome dispersion not containing a prooxidant, a total inhibition of the background oxygen uptake by liposomes was observed (data not shown), which clearly indicated that PG was active in the phospholipid bilayers. It should be noted that none of the tested phenolic acids reduced the background oxygen uptake after addition. Another laboratory reported high partitioning of PG in the emulsion interface consisting of egg yolk phospholipids (*55*); microsomal phospholipid membranes incorporated 52.1% of added PG (*8*), whereas 10% of PG was found in liposome bilayers (*56*). On the basis of our observations and these studies, it could be assumed that a substantial amount of PG would be located within the phospholipid bilayers, where it can act as a potent radical scavenger.

On the contrary, partitioning of polar compounds in the oil phase of both biphasic systems and emulsions has been reported to be low in general (*12*, *55*, *57*). According to Medina et al. (*11*), the polarity of the phenolic acids determined by their partitioning in the oil phase of an oil−water mixture (1:1, w/w) decreases in the following sequence (values in the parentheses give percent in the oil phase): FeA (49.6) ∼ PG (49.4) > CoA (22.6) > CaA (0.30). The affinity of FeA for incorporation into microsomal membranes was reported to be 5% (*9*). In these works, the pH of the systems could affect both the partitioning pattern and the properties of the antioxidants. Nevertheless, on the basis of these reports, distribution of CaA, CoA, and FeA in the phospholipid bilayers at pH above the pK_a of the acidic group of the antioxidant molecules is expected to be lower than in the aqueous phase. Consequently, the radical scavenging abilities of these compounds are likely to be less involved in the inhibition of oxidation.

However, in free iron catalyzed oxidation a prooxidative effect was observed at 1 μM PG concentration (PG/Fe = 0.1). At low concentrations and in an abundance of iron, the capacity of PG to scavenge free radicals seems to be insufficient. Moreover, PG possesses a strong metal reducing power as verified in this study by the FRAP assay. The proportion of PG that is active as a free radical scavenger may be rapidly depleted and, at the same time, the proportion remaining in the aqueous phase may reduce ferric iron, resulting in an overall promotion of lipid oxidation.

Liposomes are charged particles (*13*). The charge of the particles may significantly affect the oxidation processes (*2*). The zeta potential, characterizing the electric potential difference between the particle surface and the surrounding aqueous phase, of cod roe phospholipid liposomes at pH 5.5 is strongly negative (∼−20 mV) (*14*). At pH 5.5, the molecules of CaA, FeA, and CoA exist mostly as anions because of the ionized acidic group [pK_a ∼ 4.4 (*58*)]. Due to this, the accessibility of the negatively charged molecules toward the phospholipid interface may be hindered because of electrostatic repulsion into the aqueous phase by the negatively charged outer surface of liposomes. This may decrease the amount of molecules that can act as radical scavengers in the phospholipid interface and increase the proportion available for interactions with iron in the aqueous phase.

In additional experiments at pH 3.0, the prooxidative maximum of CaA was shifted to higher concentrations (∼300 μM) and the maximum of oxidation was significantly lower compared to pH 5.5 (**Figure 4**), indicating the importance of pH. At pH below the pK_a value of the acidic group, the CaA molecules are mostly uncharged as the acidic group is protonated (*58*). Due to this, the polarity of the molecules and consequently the solubility of CaA in the water phase should decrease. Moreover, at pH 3.0 the zeta potential of liposomes is approximately zero, and a weaker catalytic activity of iron relative to pH 5.5 was observed in our earlier studies (*14*, *15*). A combination of these factors might facilitate a better accessibility of CaA molecules into the phospholipid bilayers, where they could act as radical scavengers. Both the shift in maximum and the overall lower degree of prooxidative activity at pH 3.0 could be attributed to these aspects because higher concentrations of CaA were needed to reach the maximum prooxidative effect.

A number of studies reported on the chelating properties of phenolics bearing catechol or pyrogallol moieties (*8*, *38*, *45*, *46*, *59*, *60*) as a mechanism that inactivates the redox cycling of metals by the formation of stable metal−antioxidant complexes (*1*). These studies are rather inconsistent regarding the degree of chelation; moreover, environmental conditions may also play a significant role.

Due to the strong pro-oxidative behavior of CaA, we observed that interactions between CaA and low molecular (free) iron are clear and apparently do not seem to produce any stable complexes which would reduce the concentration of free iron. In other words, the complex between iron and CaA would have reduced the concentration of free iron in solution, thereby shifting the

Table 3. Overview of the Results and Their Relative Comparison with Different Properties of the Studied Phenolics[a]

	inhibition of oxidation		antioxidant capacity assays				antioxidant properties[b]		
antioxidant (OH groups)	Fe	Hb	FC	FRAP	DPPH	ABTS	partitioning in oil[c](1)	chelating ability (2)	redox potential (3)
PG (3)	++++	++++	++++	++++	++++	++++	++++	++++	++++
CaA (2)	−	+++	++++	+++	+++	+	+	+++	+++
FeA (1)	++	++	++	++	++	+++	++++	−	++
CoA (1)	−	−	+	+	−	+++	++	−	+

[a] PG, propyl gallate; CaA, caffeic acid; FeA, ferulic acid; CoA, *p*-coumaric acid; + and − signs refer to the positivity or negativity/absence of the feature, respectively; the number of + signs refers to the strength of the feature within the group of studied phenolics, ++++ being the strongest. [b] Data based on literature findings: 1 = ref (*11*); 2 = ref (*11*, *59*, *60*); 3 = ref (*11*). [c] Partitioning in an oil phase of an oil−water (1:1) biphasic system.

equilibrium between free iron and iron bound to phospholipids, reducing the iron concentration on the lipid/water interphase, and finally reducing the rate of lipid oxidation. In this way, CaA would have shown an antioxidative effect. If there is such a complex binding between iron and CaA, the effect is clearly overshadowed by another reaction, intramolecular electron transfer, that increases the oxidation rate (**Figure 6**).

A schematic comparison between the experimental results and a correlation between the results and the antioxidants' properties that are discussed above is shown in **Table 3**. Good agreement was found between inhibition of Hb-catalyzed oxidation and the redox potential of the phenolics. The antioxidant capacity assays based on single electron transfer, that is, the FC and FRAP assays, also correlated well with the redox potentials. The findings of other laboratories on positive chelating ability of CaA toward iron were in great contrast to our observations in the iron-mediated oxidation.

In conclusion, the present study proves that the type of prooxidant, free iron versus hemoglobin, and the antioxidant-to-prooxidant ratio are factors of high importance for the efficiency of the studied phenolics in the inhibition of catalyzed oxidation of LC-PUFAs in liposomes. Therefore, when a particular phenolic compound is selected for an application in LC-PUFA-rich food emulsions, the type and content of different prooxidative agents should be known. Caffeic acid acted as a good antioxidant in Hb-promoted oxidation, whereas in free iron induced oxidation it was found to be a potent prooxidant, which indicates that different reactions are involved in interactions between free iron, Hb, and phenolic compounds. We assume reducing abilities of caffeic acid are responsible both for the promotion of free iron catalyzed oxidation and for the inhibition of Hb-catalyzed oxidation. Among the tested phenolics, only propyl gallate, a synthetic antioxidant, fulfilled the requirement for high efficacy both in free iron catalyzed and in Hb-catalyzed oxidation.

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