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# Simulated Digestion of *Vitis vinifera* Seed Powder: Polyphenolic Content and Antioxidant Properties

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There is increasing evidence that reactive oxygen species arising from several enzymatic reactions are mediators of inflammatory events. Plant preparations have the potential for scavenging such reactive oxygen species. Flavans and procyanidins are bioavailable and stable during the process of cooking. This study used conditions that mimicked digestion of *Vitis vinifera* seed powder in the stomach (acidic preparation) and small intestine (neutral preparation). The flavonoids of these two preparations were released during simulated digestion and were determined with HPLC analysis. Biochemical model reactions relevant for the formation of reactive oxygen species in vivo at inflammatory sites were used to determine the antioxidant properties of the two preparations. The inhibition of the indicator reaction for the formation of reactive oxygen species represents a potential mechanism of the physiological activity of the corresponding preparation. The results of this work show clearly that the polyphenols released during the simulated digestion of the two preparations have good scavenging potential against superoxide radicals, hydroxyl radicals, and singlet oxygen. They protect low-density lipoprotein against copper-induced oxidation due to the copper-chelating properties and their chain-breaking abilities in lipid peroxidation.

**KEYWORDS: Antioxidant properties; procyanidins; grape seed powder; ROS**

## INTRODUCTION

A diet rich in vegetables and fruits is associated with health-promoting effects. Secondary metabolites of plants, mainly polyphenols, are correlated with the reduction of certain diseases (*1*). Studies have shown that flavonoids exhibit several positive health aspects; they possess anticarcinogenic, antimutagenic, antioxidant, antiviral, immune-stimulating, and estrogen-active properties. They also inhibit lipid peroxidation and low-density lipoprotein (LDL) oxidation and chelate transition metals (*2*−*4*). Grape seed powder is a good source of polyphenols. It contains besides gallic acid and the flavans catechin and epicatechin a wide variety of procyanidins (*5*−*7*). Procyanidins are condensed polyphenols containing the monomeric flavan-3-ol units of catechin and epicatechin. A condensation degree of up to three units is reported for grape seeds (*5*). The uptake of the monomers catechin and epicatechin as well as the procyanidins B2 and B5 was shown by others (*8*−*11*).

Reactive oxygen species (ROS) are considered to play an important role in various diseases such as inflammation, atherogenesis, or age-related macula degeneration (*12*). To investigate two different preparations of *Vitis vinifera* seed powder, different biochemical assays were used to determine the antioxidant properties of the samples. The xanthine/xanthine oxidase system produces superoxide anion radicals ($O_2^{\bullet -}$) and hydrogen peroxide ($H_2O_2$), and it is generally used as a model system for ischemia/reperfusion. Xanthine dehydrogenase is transformed into xanthine oxidase (XOD) during ischemic conditions in the tissue. The degradation of adenosine triphosphate (ATP) to adenosine monophosphate (AMP) under oxygen deprivation leads to an accumulation of hypoxanthine. When the tissue is reperfused, XOD oxidizes hypoxanthine to uric acid while transferring the electron to oxygen and generating superoxide radicals (*12*). The effect of added samples on activated leukocytes can be investigated with the complex whole blood system. During degranulation of neutrophilic granulocytes induced by pathogens, the enzyme myeloperoxidase (MPO) is released into the extracellular space. Myeloperoxidase catalyzes via $H_2O_2$ and NaCl the formation of HOCl, an aggressive bactericide and strong oxidant. An overstimulation of the leukocytes and therefore the production of ROS leads to the damaging side effects of inflammation (*12*). A widely accepted method to investigate the effects of samples on LDL is the copper-induced LDL oxidation. The oxidation of LDL is the initial step for the development of atherosclerosis. The unregulated uptake of oxidized LDL by macrophages converts them into foam cells, which are the basis for atherosclerotic plaques. Copper-oxidized LDL exhibits the same properties as modified

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LDL in vivo. The formed dienes in the LDL are continuously monitored and an extension of the lag phase correlates with good protective properties of the samples under investigation (*13*−*16*). The oxidation of fatty acids results not only in the modification of LDL but also in the disintegration of membranes. The Rose Bengal assay generates singlet oxygen, which initiates lipid peroxidation of α-linolenic acid, leading to the production of ethane. With a variation of the assay, the addition of copper, the ability of a sample to chelate copper can be investigated as well (*12*, *19*).

For this study, a digestion of grape seed powder in the stomach or small intestine was mimicked with an acidic or neutral preparation, respectively. The composition of polyphenols was determined by HPLC analysis, and the two preparations were tested in four different biochemical test methods, which mimic relevant pathological processes.

## MATERIALS AND METHODS

**Materials.** 1-Aminocyclopropane-1-carboxylic acid (ACC), dextran, disodium carbonate ($Na_2CO_3$), EDTA, 2.0 N Folin−Ciocalteu's phenol reagent, gallic acid, hydroxylamine, α-keto-*S*-methylbutanoic acid (KMB), linolenic acid, *N*-(1-naphthyl)ethylenediamine (NED), potassium bromide (KBr), Rose Bengal, sodium tetraborate, sodium chloride (NaCl), sulfanilamide, Trolox, trypan blue, xanthine, and zymosan A were purchased from Sigma, Munich, Germany. Boric acid, copper(II) sulfate 5-hydrate ($CuSO_4 \cdot 5H_2O$), formic acid, methanol, 4-(dimethylamino)cinnamaldehyde (DMACA), and sulfonic acid were obtained from Merck, Darmstadt, Germany. Xanthine oxidase (XOD) was from Roche, Mannheim, Germany. The phenolic standards epicatechin and catechin were available from Roth, Karlsruhe, Germany. The procyanidins B1, B2, B3, and C1 were identified in comparison to standards previously isolated in our laboratory from *Aesculus hippocastanum*, *Malus domestica*, and *Sorbus domestica* (*18*−*20*). The gases for gas chromatography were purchased from Messer Griesheim, Darmstadt, Germany. The carrier gas was $N_2$ (type 5.0; degree of purity = 99.999%), 25 mL/min; the burning gases were $H_2$ (type 5.0; degree of purity = 99.999%), 25 mL/min, and synthetic air, 250 mL/min; ethene calibration gas (mixture of ethene and synthetic air), 1 mL = 244 pmol, 1 bar.

The grape seed powder was a gift of Vitis AG, Trittenheim, Germany. The total amount of flavans is given as 21.75 mg/g of dry weight of grape seed powder (*21*).

**Methods.** The results shown are the arithmetic means of four individual experiments ($n = 4$) except in the case of copper-induced LDL oxidation ($n = 2$). The standard deviations in the figures are given as $\delta_{n-1}$. The statistics (paired *t* test) were calculated according to the method of Precht and Kraft (*22*). All experiments were repeated on different days to guarantee reproducibility.

*Grape Seed Powder Extract Preparations.* The grape seed powder was extracted in two different ways: (a) at pH 2 to mimic gastric digestion in the stomach (referred to as acidic preparation) and (b) with Milli-Q water at pH 6 (referred to as neutral preparation). The pH was adjusted with 2 N HCl and 2 N NaOH, respectively. Briefly, 5 g of the grape seed powder was extracted in a Soxhlet extractor with 250 mL of the extracting solvent for 30 min at 80 °C under reflux. The sample was centrifuged at 2000*g* for 15 min, and the supernatant was pooled and stored as aliquots at −70 °C until required (*23*).

*HPLC Determination of Procyanidins.* For the determination of the procyanidins in both preparations, the method described and published previously (*24*, *25*) was used. The solvents were 5% formic acid (A) and gradient grade methanol (B) with a flow rate of 0.5 mL/min. The samples were centrifuged at 2000*g* for 10 min and filtered (0.22 μm, Millex-GP; Millipore, Schwalbach, Germany) prior to analysis. The identities of compounds were verified with relevant standards (see Materials), on the basis of their spectra and cochromatography of the preparations with the according standards. Recovery tests are routinely made in the laboratory with internal standards. The recovery rate for all compounds quantified in this study was >95%.

*Determination of Gallic Acid Equivalents (GAE) with Folin−Ciocalteu Assay.* The reducing potential is given as GAE using a calibration curve of gallic acid in the range of 50−1000 μM [equation for the linear fit of the regression line: $A_{720} = 0.0294\text{AU} + 0.000945\text{AU}/\mu\text{M} \times c$, where $A_{720}$ = absorption at 720 nm (AU) and $c$ = concentration (μM); $r = 0.9976$; data not shown]. The measurement was done as described previously (*26*), and the absorption was determined after 60 min at $\lambda = 720$ nm with a Kontron Uvikon 922 spectrophotometer.

*XOD System.* The catalyzation of xanthine (hypoxanthine) to uric acid leads to the formation of 20% superoxide anion radicals and 80% $H_2O_2$ (*29*). The resulting $H_2O_2$ forms hydroxyl radicals via Fenton chemistry. The XOD-produced ROS are detectable with two different indicator systems, (a) hydroxylamine and (b) KMB, as described below.

*(a) Hydroxylamine Oxidation.* The hydroxylamine oxidation is a photometric assay to detect specifically the produced $O_2^{\bullet -}$ via oxidation of hydroxylamine to nitrite, which is then measurable after azo-coupling with sulfanilamide, and NED (Griess reaction).

The assay is done as published previously (*27*). Briefly, 1 mL contains 0.1 M phosphate buffer, pH 7.4, 1 mM $NH_2OH$, 5 μM xanthine, 0.04 units/mL xanthine oxidase, and samples (amounts indicated). After an incubation of 30 min at 37 °C in a temperature-controlled water bath, 1% sulfanilamide and 0.02% NED are added. The level of nitrite is estimated after 15 min at $\lambda = 540$ nm with a Kontron Uvikon 922 spectrometer (*27*).

*(b) KMB Fragmentation.* KMB is fragmented by mainly $OH^{\bullet}$ into ethene, which is detectable via gas chromatography. The assay is done with and without (= control) the test substance in gastight sealed reaction tubes with known volume (13 mL). The formed ethene is measured after incubation by withdrawing 1 mL of gas out of the headspace with a gastight syringe. The ethene amount within this 1 mL is determined gas chromatographically with an Aerograph Varian Star 3400CX (retention time of ethene = 0.86 min with 0.3125 × 60 cm aluminum oxide column) and quantified with the aid of ethene calibration gas (*28*). A final volume of 2 mL contains 0.1 M phosphate buffer, pH 7.4, 1 mM KMB, 0.5 mM xanthine, 0.04 U/mL xanthine oxidase, and sample (amounts indicated). The sampling was done after an incubation of 30 min at 37 °C.

*Whole Blood System.* Zymosan A stimulates leukocytes of whole blood ex vivo for investigations of the abilities of test substances as anti-inflammatory additives. The stimulated leukocytes release MPO and $H_2O_2$, which lead to the formation of HOCl. HOCl can be determined with the fragmentation of ACC, releasing ethene, as ACC is not fragmented by Fenton-type oxidants (*30*). Briefly, blood was obtained from a healthy volunteer after written consent had been obtained. Twenty-five milligrams of EDTA per 100 mL of blood was added to prevent clotting. The blood was used immediately. An assay with a final volume of 2 mL contains 0.1 M Dulbecco phosphate-buffered saline, pH 7.2, 2.5 mM ACC, 5 mg of zymosan A, 1 mL of blood, and samples (amounts indicated). The assay is done with and without (= control) test substance in gastight sealed reaction tubes with known volume (13 mL). The formed ethene is measured after an incubation of 60 min at 37 °C by withdrawing 1 mL of gas out of the headspace with a gastight syringe. The ethene amount within this 1 mL is determined gas chromatographically on an Aerograph Varian Star 3400CX (retention time ethene = 0.86 min with 0.3125 × 60 cm aluminum oxide column) and quantified with the aid of ethene calibration gas.

*Viability Test of Leukocytes.* The viability of leukocyte cells was determined by adding 100 μL of the undiluted grape seed preparation. The assay is as follows (2 mL final volume): 0.1 M Dulbecco phosphate-buffered saline, pH 7.2, 2.5 mM ACC, 1 mL of whole blood, and sample. After an incubation of 60 min at 37 °C, 3 mL of the blood is mixed with 1 mL of dextran (1.5% in 0.9% NaCl), and the mixture is left for 45−60 min for sedimentation at room temperature in the dark. An aliquot of the clear, leukocyte-rich supernatant is diluted 1:2 with trypan blue (0.5% final concentration, in 0.9% NaCl) and a cell count of the vital cells performed.

*Copper-Induced LDL Oxidation.* Oxidized LDL plays a major role in the process of atherogenesis. Its oxidation can be continuously monitored as formed dienes according to a previously published method

**Table 1.** Concentrations and Amounts of Single Compounds of the Two Preparations of *V. vinifera* Seed Powder[a]

| | acidic preparation | | neutral preparation | |
|---|---|---|---|---|
| | concn ($\mu$M) | amount (mg/g of dw) | concn ($\mu$M) | amount (mg/g of dw) |
| gallic acid | 39.6 | 0.34 a | 45.1 | 0.38 a |
| procyanidin B3 | 7.58 | 0.22 | 7.26 | 0.21 |
| procyanidin B1 | 58.6 | 1.69 bb | 39.6 | 1.15 bb |
| catechin | 118.9 | 1.73 cc | 185.4 | 2.69 cc |
| procyanidin B2 | 55.3 | 1.59 dd | 41.0 | 1.19 dd |
| epicatechin | 101.7 | 1.48 ee | 159.8 | 2.32ee |
| unknown flavanol 1 | # | # | # | # |
| procyanidin c1 | 20.7 | 0.89f | 18.4 | 0.79 f |
| unknown flavanol 2 | # | # | – | – |
| procyanidin eb5 | 4.65 | 0.20 ggg | – | – ggg |

[a] The same letters indicate difference between the two preparations; the quantity of letters gives the level of significance: a, significant at $P < 0.05$; aa, highly significant at $P < 0.01$; aaa, very highly significant at $P < 0.001$. –, not detected; #, detected but not calculated due to the lack of standards.

(*31*). The accumulating conjugated dienes during the oxidation are monitored photometrically, and protecting properties are determined as a prolongation of the lag phase (time until rapid increase of absorbance due to oxidation onset). LDL was obtained from the pooled plasma of 15 healthy volunteers with isopycnic ultracentrifugation in a KBr gradient (*31*). A final volume of a 1 mL assay contains 0.02 M PBS, pH 7.4, LDL, 0.25 $\mu$g of protein, 1.67 $\mu$M $CuSO_4$, and the test substances in various concentrations. The measurement is started immediately with the addition of copper. The formed dienes are measured as a change in the extinction at $\lambda = 234$ nm every 10 min for 1000 min at 37 °C with a Kontron Uvikon 922 spectrophotometer with integrated tempered cuvette holder.

*Rose Bengal Assay.* With the photosensitizer Rose Bengal, singlet oxygen can be generated physically with light energy. In buffered systems up to 20% $O_2^{\bullet-}$ is produced besides singlet oxygen due to the presence of electron donators. Without copper, the ethane release from lipid peroxidation of α-linolenic acid is used as indicator in this assay. The addition of copper leads to the production of ethene and ethane from lipid peroxidation of α-linolenic acid in a proportion of 1:1. With this alteration of the assay, the copper-chelating abilities of samples can be estimated (*17*).

A final volume of a 2 mL assay without $Cu^{2+}$ contains 0.1 M phosphate buffer, pH 7.4, 3.59 mM α-linolenic acid, 20 $\mu$M Rose Bengal, and sample (amounts indicated).

A final volume of a 2 mL assay with $Cu^{2+}$ contains 0. 1 M phosphate buffer, pH 7.4, 3.59 mM α-linolenic acid, 5 $\mu$M $CuSO_4$, 20 $\mu$M Rose Bengal, and sample (amounts indicated). Both assays are done with and without (= control) test substance in gastight sealed reaction tubes with known volume (13 mL). The formed ethene and ethane, respectively, are measured after incubation of 30 min at 37 °C and 500 mE/cm × s [self-made tempered water bath with four Philips Argaphoto-BM lamps of type PF 318 E/44 (220 V 500 W)] by withdrawing 1 mL of gas out of the headspace with a gastight syringe. The ethene and ethane amounts within this 1 mL are determined gas chromatographically with an Aerograph Varian Star 3400CX (retention time ethane = 1.28 min, retention time ethene = 1.88 min with 0.3125 × 120 cm aluminum oxide column) and quantified with the aid of ethene and ethane calibration gases.

## RESULTS

**Determination of Procyanidin Contents.** The concentrations and amounts per gram of dry weight are given in **Table 1**. The chromatographic pattern is the same for both preparations with catechin and epicatechin being the main compounds (example shown in **Figure 1**). Catechin, epicatechin, and gallic acid are significantly higher in the neutral preparation at the 0.05% level (**Table 1**). Statistically significantly higher amounts of the procyanidins B1, B2, C1, and EB5 were observed in the acidic preparation (0.05% level) (**Table 1**). Additionally, an unknown flavanol 2 and procyanidin EB5 were present in the acidic preparation (**Figure 1** and **Table 1**). Only procyanidin B3 had the same amounts in both preparations. With both preparation methods lower amounts per gram of grape seed powder are found than given from the producer (see Materials).

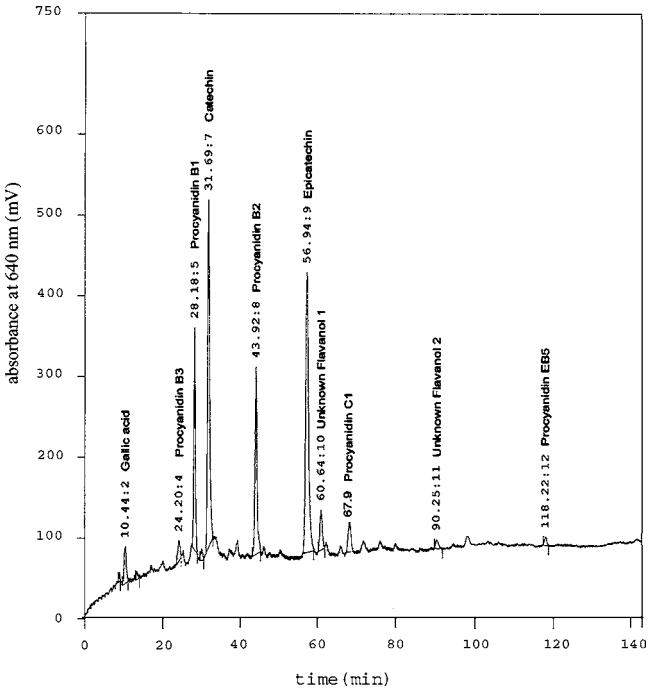


**Figure 1.** Chromatogram of acidic preparation of *V. vinifera* seed powder with postcolumn derivatization of flavanols with DMACA, recorded at 640 nm.

**Determination of GAE with Folin−Ciocalteu Assay.** The neutral preparation contained 18.23 ± 0.35 mM GAE, whereas the acidic preparation contained 17.28 ± 0.19 mM GAE. The GAE of the neutral preparation is significantly higher than the GAE of the acidic preparation ($P < 0{,}01$; paired *t* test).

As both grape seed preparations are mixtures of a variety of compounds, the concentrations of the individual compounds in the volume of the preparation required to reach $I_{50}$ are given in the corresponding tables and compared statistically (Student's *t* test).

**XOD System.** *Hydroxylamine Oxidation.* Both preparations, gallic acid alone and Trolox alone, inhibit nitrite formation in the hydroxylamine oxidation in a dose-dependent manner, giving exponential curve progressions for all samples (for equations and correlation coefficient see **Figure 2**). Hydroxylamine oxidation with Trolox gives a maximum inhibition of ≈45% with the highest concentration tested, 200 $\mu$M, not reaching $I_{50}$ (**Figure 2C**). Due to the solubility properties of Trolox, no higher concentrations can be used in this assay. With different amounts of the two preparations needed to reach $I_{50}$, the concentrations of the single compounds within these preparations vary. The individual concentrations of gallic acid, catechin, epicatechin, and EB5 present in the two preparations at the level required to reach $I_{50}$ differ in a very highly statistically significant manner ($P < 0.001$, paired *t* test; **Table 2**). The concentrations of B3 are different at the 0.01% level; the concentrations of the two procyanidins B1 and C1 vary only at the 0.05% level (**Table 2**). The B2 concentrations in the two preparations at the level required to reach $I_{50}$ were not significantly different. To reach $I_{50}$ with pure gallic acid, 2.1

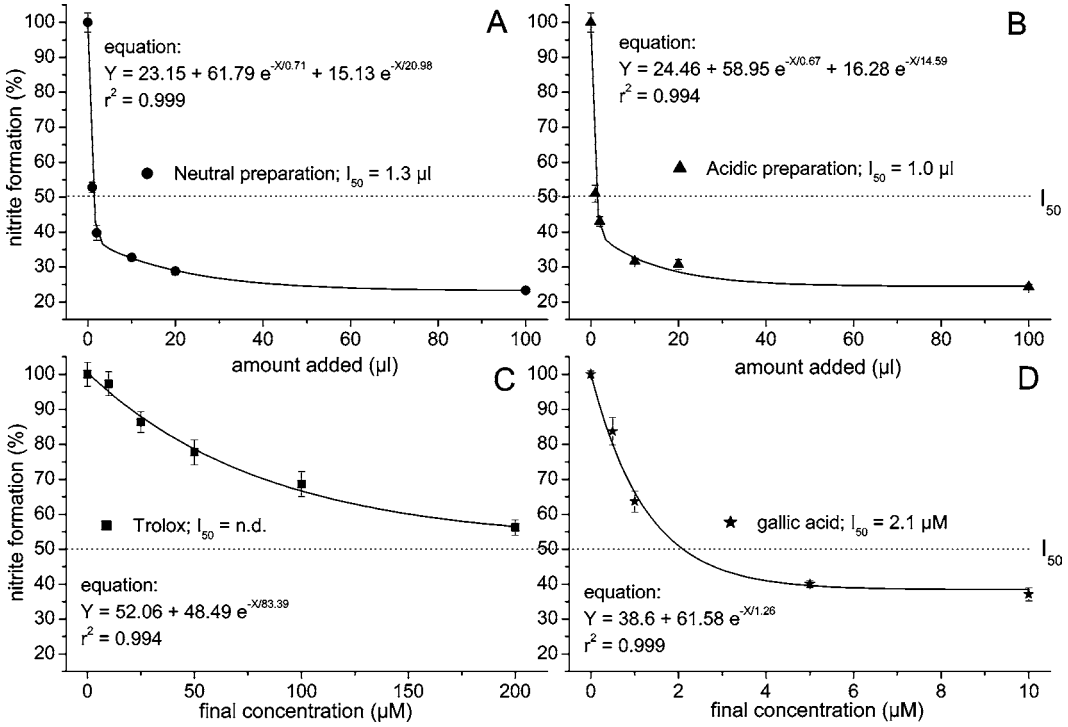


**Figure 2.** Hydroxylamine oxidation with X/XOD: (**A**) neutral preparation; (**B**) acidic preparation; (**C**) Trolox; (**D**) gallic acid. Equations and correlation coefficient ($r^2$) for fit lines as well as $I_{50}$ values are given for each sample (nd, not determinable).

**Table 2.** XOD–Hydroxylamine Assay[a]

| | gallic acid (nM) | catechin (nM) | epicatechin (nM) | B1 (nM) | B2 (nM) | B3 (nM) | C1 (nM) | EB5 (nM) | Trolox (nM) |
|---|---|---|---|---|---|---|---|---|---|
| acidic preparation (1.0 µL) | 39.6 aaa,### | 118.9 bbb | 101.7 ccc | 58.6 d | 55.3 | 9.44 ff | 20.7 g | 4.65 hhh | |
| neutral preparation (1.3 µL) | 58.6 aaa,### | 241.0 bbb | 207.7 ccc | 51.5 d | 53.3 | 7.58 ff | 23.9 g | – hhh | |
| gallic acid | 2100.0 ### | | | | | | | | |
| Trolox | | | | | | | | | nd |

[a] Concentrations of gallic acid, Trolox, and single compounds of *V. vinifera* seed powder required for the sample to reach $I_{50}$; the concentrations of the single compounds were calculated according to the added amounts of the two preparations, which are given in parentheses; due to the solubility properties of Trolox, no $I_{50}$ can be determined (nd = not determinable). The same letters indicate difference between the concentrations of single compounds of the two preparations; the quantity of letters gives the level of significance: a, significant at $P < 0.05$; aa, highly significant at $P < 0.01$; aaa, very highly significant at $P < 0.001$. #, indicates difference between gallic acid concentration of the two preparations and pure gallic acid; thequantity of number signs gives the level of significance: #, significant at $P < 0.05$; ##, highly significant at $P < 0.01$; ###, very highly significant at $P < 0.001$.

µM is needed, which is 54-fold higher than the concentration of gallic acid in the acidic preparation and 36-fold higher than that in the neutral preparation to reach $I_{50}$.

*KMB Fragmentation.* KMB fragmentation with XOD is inhibited in a dose-dependent manner, giving exponential curve progressions for all samples (for equations and correlation coefficient see **Figure 3**). The differences between the concentrations of catechin, epicatechin, B1, and EB5 in the two preparations at the level required to reach $I_{50}$ are very highly significant ($P < 0.001$, paired *t* test). The procyanidin B2 concentrations differ at the 0.01% level, whereas the concentrations of gallic acid and C1 vary significantly ($P < 0.05$, paired *t* test; **Table 3**). The procyanidin B3 concentrations in the two preparations at the level required to reach $I_{50}$ exhibit no difference. To reach $I_{50}$ with pure gallic acid, 1.8 µM is needed, which is 75-fold higher than the concentration of gallic acid in the acidic preparation and 67-fold higher than that in the neutral preparation.

**Whole Blood System.** In the whole blood system the stimulation of the natural immune response of leukocytes to zymosan is detected. $I_{25}$ values were chosen for better comparability as all four samples reached this inhibition (data not shown). The concentrations of gallic acid, catechin, and epicatechin present in the two preparations at the level required to reach $I_{25}$ are not statistically significant. The concentrations of the procyanidins B1, B2, and C1 differ at the 0.01% level, whereas the concentrations of B3 vary in a statistically significant manner ($P < 0.05$, paired *t* test; **Table 4**). The concentration of 0.5 µM pure gallic acid to reach $I_{25}$ is 20-fold higher than its concentration in the acidic preparation and 23-fold higher than that in the neutral preparation ($P < 0.001$, paired *t* test; **Table 4**).

**Viability Test of Leukocytes.** The count of the leukocytes treated with the undiluted preparations of grape seed powder gave <1% dead cells (data not shown). This percentage indicates a naturally occurring cell death caused by, for example, apoptosis or shear forces of the pipet. Both preparations have therefore no cytotoxic effect on leukocytes.

**Copper-Induced LDL Oxidation.** All tested samples prolong the lag time in a concentration-dependent manner. The two preparations exhibit the best protective abilities of all samples with a prolongation of the lag time beyond the measurable time of the spectrometer with 2 µL added (**Figure 4A**,**B**). The addition of amounts >2 µL of the preparation gives an

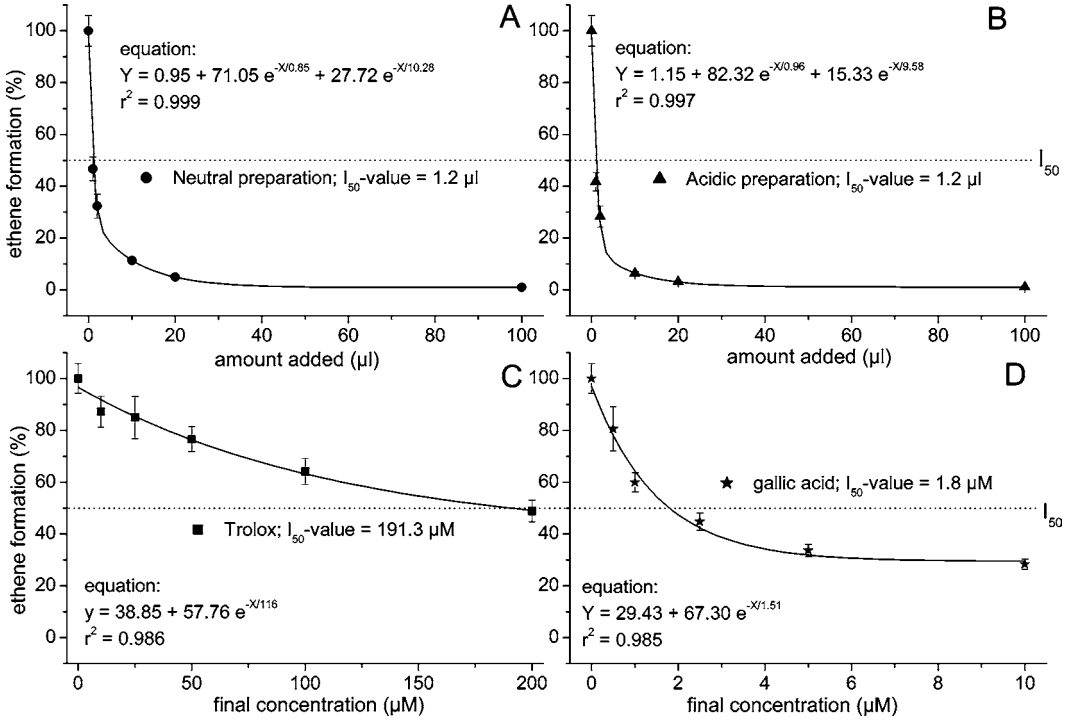


**Figure 3.** KMB fragmentation with X/XOD: (**A**) neutral preparation; (**B**) acidic preparation; (**C**) Trolox; (**D**) gallic acid. Equations and correlation coefficient ($r^2$) for fit lines as well as $I_{50}$ values are given for each sample.

**Table 3.** XOD–KMB Assay[a]

| | gallic acid (nM) | catechin (nM) | epicatechin (nM) | B1 (nM) | B2 (nM) | B3 (nM) | C1 (nM) | EB5 (nM) | Trolox (nM) |
|---|---|---|---|---|---|---|---|---|---|
| acidic preparation (1.2 $\mu$L) | 23.8 a,### | 71.4 bbb | 61.0 ccc | 35.2 ddd | 33.2 ee | 4.55 | 12.4 f | 2.79 ggg | |
| neutral preparation (1.2 $\mu$L) | 27.1 a,### | 111.2 bbb | 95.9 ccc | 23.8 ddd | 24.6 ee | 4.36 | 11.1 f | – ggg | |
| gallic acid | 1800.0 ### | | | | | | | | |
| Trolox | | | | | | | | | $1.91 \times 10^5$ |

[a] Concentrations of gallic acid, Trolox, and single compounds of *V. vinifera* seed powder required for the sample to reach $I_{50}$; the concentrations of the single compounds were calculated according to the added amounts of the two preparations, which are given in parentheses. The same letters indicate difference between the concentrations of single compounds of the two preparations; the quantity of letters gives the level of significance: a, significant at $P < 0.05$; aa, highly significant at $P < 0.01$; aaa, very highly significant at $P < 0.001$. #, indicates difference between gallic acid concentration of the two preparations and pure gallic acid; the quantity of numbers signs gives the level of significance: #, significant at $P < 0.05$; ##, highly significant at $P < 0.01$; ###, very highly significant at $P < 0.001$.

**Table 4.** Whole Blood Assay

| | gallic acid (nM) | catechin (nM) | epicatechin (nM) | B1 (nM) | B2 (nM) | B3 (nM) | C1 (nM) | EB5 (nM) | Trolox (nM) |
|---|---|---|---|---|---|---|---|---|---|
| acidic preparation (1.3 $\mu$L) | 25.7 ### | 77.3 | 66.1 | 38.1 aa | 35.9 bb | 4.93 qc | 13.5 dd | 3.02 eee | |
| neutral preparation (0.9 $\mu$L) | 21.4 ### | 88.1 | 75.9 | 18.8 aa | 19.5 bb | 3.45 c | 8.75 dd | – eee | |
| gallic acid | 500.0 ### | | | | | | | | |
| Trolox | | | | | | | | | 7700.0 |

[a] Concentrations of gallic acid, Trolox, and single compounds of *V. vinifera* seed powder required for the sample to reach $I_{25}$; the concentrations of the single compounds were calculated according to the added amounts of the two preparations, which are given in parentheses. The same letters indicate difference between the concentrations of single compounds of the two preparations; the quantity of letters gives the level of significance: a, significant at $P < 0.05$; aa, highly significant at $P < 0.01$; aaa, very highly significant at $P < 0.001$. #, indicates difference between gallic acid concentration of the two preparations and pure gallic acid; the quantity of number signs gives the level of significance: #, significant at $P < 0.05$; ##, highly significant at $P < 0.01$ (paired $t$ test); ###, very highly significant at $P < 0.001$.

absorbance above the linear range of the instrument (absorbance > 2, data not shown). None of the samples had pro-oxidant abilities in this assay; this was shown with the highest concentration or volume added to LDL solely (**Figure 4**). For comparison of all samples, the longest lag time prolongation was chosen. The concentrations of the individual compounds of the two preparations in the volume required to reach longest lag time prolongation are given in **Table 5**. All concentrations of the individual compounds differ at the 0.05% level for the two preparations, except the concentrations of C1, which are not significantly different (**Table 5**). The concentration of 10 $\mu$M pure gallic acid is 253-fold higher than its concentration in the acidic preparation and 222-fold higher than that in the neutral preparation ($P < 0.001$, paired $t$ test; **Table 5**).

**Rose Bengal Assay.** *Rose Bengal without* $Cu^{2+}$. All samples inhibit the reaction in a concentration-dependent manner, with the two preparations exhibiting an exponential curve progression (for equations and correlation coefficients, see **Figure 5A**,**B**),

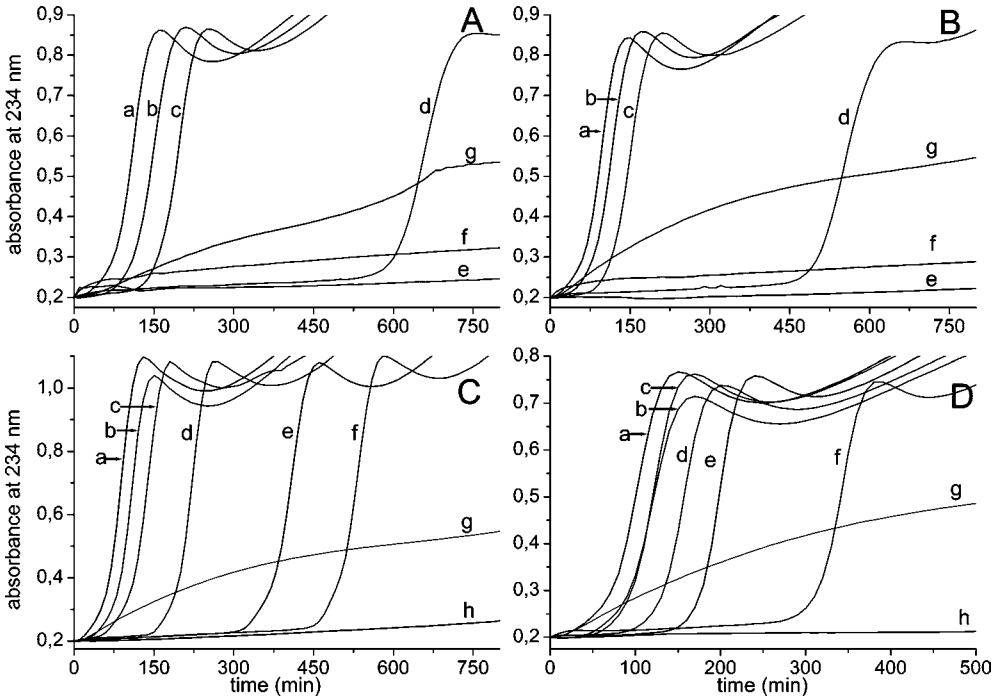


**Figure 4.** Copper-induced LDL oxidation: (**A**) neutral preparation [a, control (lag time = 76 min); b, 0.1 $\mu$L sample (lag time = 107 min); c, 0.2 $\mu$L sample (lag time = 160 min); d, 1.0 $\mu$L sample (lag time = 603 min); e, 2.0 $\mu$L sample (lag time > 1000 min); f, LDL without $Cu^{2+}$ + 2.0 $\mu$L sample; g, LDL without $Cu^{2+}$; absorbance LDL + 10 $\mu$L sample = 2.3]; (**B**) acidic preparation [a, control (lag time = 63 min); b, 0.1 $\mu$L sample (lag time = 80 min); c, 0.2 $\mu$L sample (lag time = 110 min); d, 1.0 $\mu$L sample (lag time = 500 min); e, 2.0 $\mu$L sample (lag time > 1000 min); f, LDL without $Cu^{2+}$ + 2.0 $\mu$L sample; g, LDL without $Cu^{2+}$; absorbance LDL + 10 $\mu$L sample = 2.5]; (**C**) Trolox [a, control (lag time = 62 min); b, 0.5 $\mu$M Trolox (lag time = 81 min); c, 1.0 $\mu$M Trolox (lag time = 107 min); d, 2.5 $\mu$M Trolox (lag time = 188 min); e, 5.0 $\mu$M Trolox (lag time = 370 min); f, 10.0 $\mu$M Trolox (lag time = 490 min); g, LDL without $Cu^{2+}$; h, LDL without $Cu^{2+}$ + 10.0 $\mu$M Trolox]; (**D**) gallic acid [a, control (lag time = 70 min); b, 0.5 $\mu$M gallic acid (lag time = 91 min); c, 1.0 $\mu$M gallic acid (lag time = 91 min); d, 2.5 $\mu$M gallic acid (lag time = 132 min); e, 5.0 $\mu$M gallic acid (lag time = 174 min); f, 10.0 $\mu$M gallic acid (lag time = 313 min); g, LDL without $Cu^{2+}$; h, LDL without $Cu^{2+}$ + 10.0 $\mu$M gallic acid].

**Table 5.** Copper-Induced LDL Oxidation[a]

| | gallic acid (nM) | catechin (nM) | epicatechin (nM) | B1 (nM) | B2 (nM) | B3 (nM) | C1 (nM) | EB5 (nM) | Trolox (nM) |
|---|---|---|---|---|---|---|---|---|---|
| acidic preparation (500 min) | 39.6 a,### | 118.9 b | 101.7 c | 58.6 d | 55.3 e | 9.44 f | 20.7 | 4.65 ggg | |
| neutral preparation (603 min) | 45.1 a,### | 185.4 b | 159.8 c | 39.6 d | 41.0 e | 7.26 f | 18.4 | – ggg | |
| gallic acid (313 min) | 10000 ### | | | | | | | | |
| Trolox (490 min) | | | | | | | | | 10000 |

[a] Concentrations of gallic acid, Trolox, and single compounds of *V. vinifera* seed powder required for the sample to reach the maximum lag time, which are given in parentheses; the concentrations of the single compounds were calculated according to the added amount of the two preparations giving the longest lag-time prolongation. The same letters indicate difference between the concentrations of single compounds of the two preparations; the quantity of letters gives the level of significance: a, significant at $P < 0.05$; aa, highly significant at $P < 0.01$; aaa, very highly significant at $P < 0.001$. #, indicates difference between gallic acid concentration of the two preparations and pure gallic acid; the quantity of number signs gives the level of significance: #, significant at $P < 0.05$; ##, highly significant at $P < 0.01$; ###, very highly significant at $P < 0.001$.

whereas Trolox has a linear curve progression (for equation and correlation coefficients, see **Figure 5C**) and gallic acid shows a hyperbolic curve progression (for equation and correlation coefficient, see **Figure 5D**). Gallic acid inhibits the reaction maximally ≈20%; a 50% inhibition cannot be reached with the used concentrations of gallic acid (**Figure 5D**). The concentrations of catechin, epicatechin, and EB3 present in both preparations at the level to reach $I_{50}$ are very highly statistically significant ($P < 0.001$, paired $t$ test). The concentrations of gallic acid present in both preparations at the level to reach $I_{50}$ differ at the 0.01% level, whereas the concentrations of the procyanidins B1, B2, B3, and C1 are not significantly different (**Table 6**).

*Rose Bengal with $Cu^{2+}$.* In the Rose Bengal assay with copper, the copper-chelating abilities of a sample can be tested. The addition of copper to the assay results in the production of ethane and ethene from linolenic acid. A copper-chelating sample results in a gap of the two graphs, with less ethene production than ethane. An additional antioxidant effect also decreases the ethane production. The two preparations inhibit both ethene and ethane formation in concentration-dependent manner, giving exponential curve progressions. The gaping of the ethane and ethene curves indicates a copper-chelating ability for both preparations (**Figure 6A**,**B**). Trolox also decreases ethane and ethene formation in a concentration-dependent manner but gives a linear curve progression. This indicates clearly the interference of Trolox in the chain reaction of lipid peroxidation with a protection of the fatty acid (**Figure 6C**). The addition of gallic acid results in a hyperbolic curve progression for ethane and ethene formation with no gap. Concentrations >5 $\mu$M seem to have no protective effect,

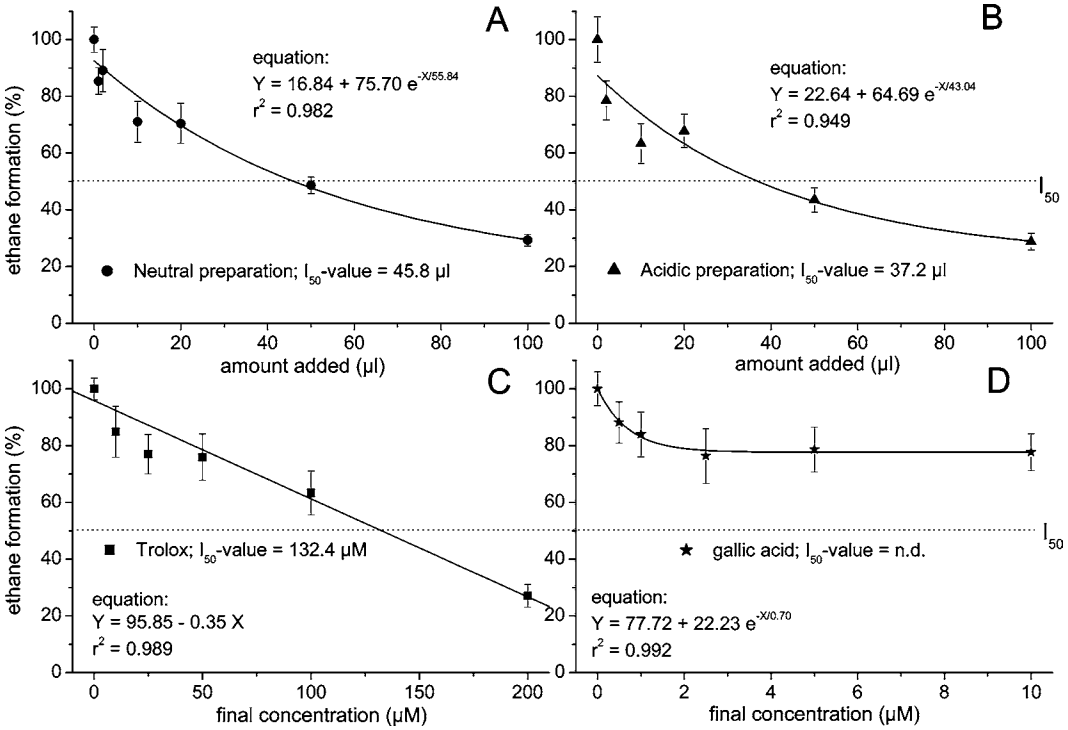


**Figure 5.** Rose Bengal assay without copper: (**A**) neutral preparation; (**B**) acidic preparation; (**C**) Trolox; (**D**) gallic acid. Equations and correlation coefficient ($r^2$) for fit lines as well as the $I_{50}$ values are given for each sample (nd, not determinable).

**Table 6.** Rose Bengal Assay without $Cu^{2+}$ [a]

| | gallic acid (nM) | catechin (nM) | epicatechin (nM) | B1 (nM) | B2 (nM) | B3 (nM) | C1 (nM) | EB5 (nM) | Trolox (nM) |
|---|---|---|---|---|---|---|---|---|---|
| acidic preparation (37.2 µL) | 736.8 aa | 2212.3 bbb | 1892.0 ccc | 1089.9 | 1028.2 | 140.9 | 384.8 | 86.5 ddd | |
| neutral preparation (45.8 µL) | 1032.6 aa | 4245.7 bbb | 3659.4 ccc | 907.1 | 939.4 | 166.3 | 422.1 | – ddd | |
| gallic acid | nd | | | | | | | | |
| Trolox | | | | | | | | | $1.32 \times 10^5$ |

[a] Concentrations of gallic acid, Trolox, and single compounds of *V. vinifera* seed powder required for the sample to reach $I_{50}$; the concentrations of the single compounds were calculated according to the added amounts of preparations, which are given in parentheses. nd, not determinable. The same letters indicate difference between the concentrations of single compounds of the two preparations; the quantity of letters gives the level of significance: a, significant at $P < 0.05$; aa, highly significant at $P < 0.01$; aaa, very highly significant at $P < 0.001$.

leading to values of ethane and ethene formation of the basic reaction (= 100%) (**Figure 6D**).

## DISCUSSION

Grape seed powder is a procyanidin-rich neutraceutical. To investigate the release of procyanidins from grape seed powder during digestion in the stomach or small intestine, we chose two extraction methods published earlier by Record and Lane (*23*). As flavonoids are stable during heating (*32*, *33*), this method is an appropriate simulation of the digestive track. The information provided by the producer states the total amount of flavans as 2.175% (w/w). However, both extracting methods released <40% of this value: for the acidic preparation, 0.78% (w/w); for the neutral preparation, 0.84% (w/w). An acidic digestion of the grape seed powder leads to a release of procyanidins. At neutral pH, as occurring in the small intestine (e.g., by intake of stomach-stable capsules), more catechin and epicatechin is released in comparison to a digestion in the stomach. The pattern of the flavan composition and amounts agree well with investigations of grape seed compounds of other *Vitis vinifera* cultivars (*5*, *6*). The higher polymers of epigallocatechin and epicatechin gallate are only minor components of grape seed and are laborious to quantify. The focus of this paper is therefore on the main compounds, the procyanidins and gallic acid (*5*, *6*, *10*, *11*, *34*, *35*).

**Xanthine Oxidase System.** With the X/XOD system the procedures in ischemia/reperfusion are mimicked. The XOD-generated ROS lead to the damaging effects of this pathological condition. In the two XOD assays used in this study, samples can exert activity via enzyme inhibition, iron chelation, and/or scavenging of the produced radicals. Interestingly, in the hydroxylamine oxidation the neutral preparation achieves $I_{50}$ values with 1.3 µL, whereas the acidic preparation needs only 1.0 µL. The smaller amount needed for the acidic preparation to reach 50% inhibition is probably due to higher concentrations of procyanidins in this preparation (**Table 2**). In the KMB assay both preparations achieve the $I_{50}$ value with 1.2 µL. With the same amounts added the two preparations show differences in the concentrations of the single compounds (**Table 3**). However, this distinction can be explained with the difference of the generated ROS; in the hydroxylamine assay the main ROS are superoxide radicals, whereas in the KMB assay hydroxyl radicals are the detectable species (*36*, *37*). Flavans scavenge superoxide radicals as well as hydroxyl radicals. The rate constant for hydroxyl radical scavenging is within the range of $K = (1-7.1) \times 10^9\ M^{-1}\ s^{-1}$; for the flavan-rich mixture of

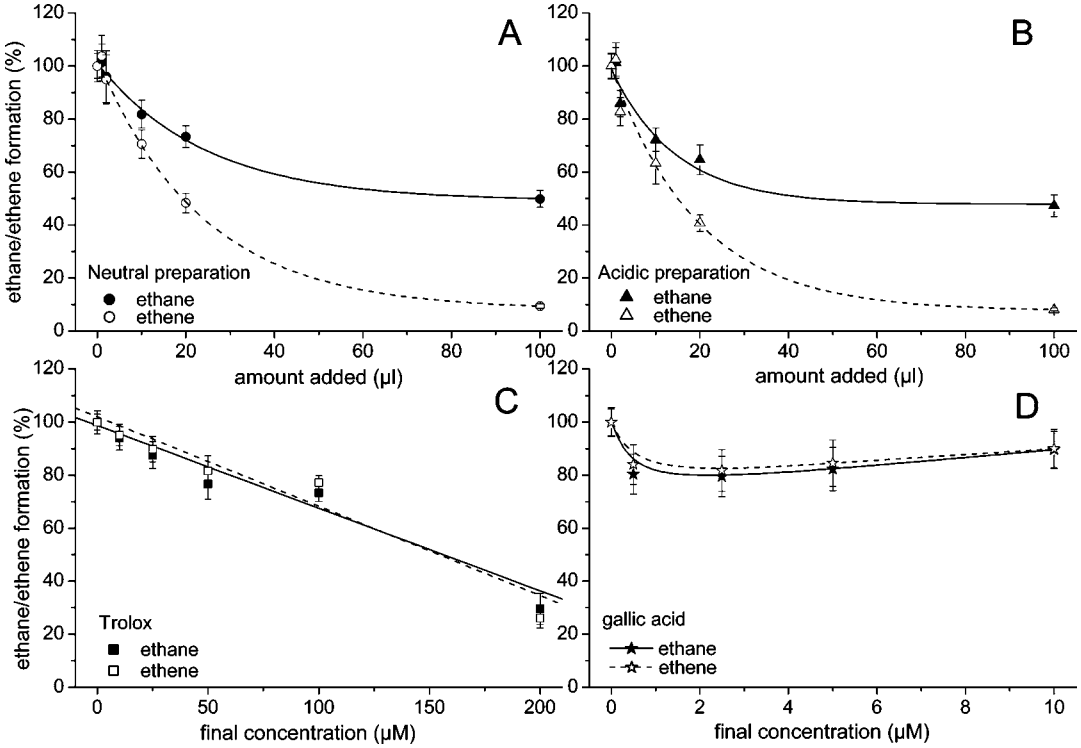


**Figure 6.** Rose Bengal assay with copper: (solid symbols and compact line, ethane; hollow symbols and dotted line, ethene); (**A**) neutral preparation; (**B**) acidic preparation; (**C**) Trolox; (**D**) gallic acid.

Pycnogenol they give a rate constant well within diffusion limits of $K = 0.52 \times 10^9$ $M^{-1}$ $s^{-1}$ (*38*, *39*). Facinó et al. (*40*) even calculated a hydroxyl radical entrapping capacity of $10^{12}$ $M^{-1}$ $s^{-1}$ for procyanidins of *V. vinifera* seeds in an ischemic reperfusion test in isolated rabbit hearts. Contrary to these high rate constants for hydroxyl radicals of procyanidins and flavans, respectively, are the published constants for superoxide radical scavenging. They lie in the intermediary range of $10^2-10^6$ $M^{-1}$ $s^{-1}$ (*38*−*41*). The iron-chelating properties also play a role in the difference between the results of the preparations in the two assays. The acidic preparation has a higher total procyanidin concentration than the neutral one (**Table 1**). The stoichiometric ratio for catechin with iron is given as 1:1 (*42*), whereas the ratio for procyanidins with iron is 1:2 (*40*). The higher total procyanidin concentration can result in a lower hydroxyl radical production in the KMB assay. The more iron is chelated, the less hydroxyl radicals can occur via Fenton/Haber Weiss reaction, which leads to a lesser KMB fragmentation and therefore less ethene. The high total procyanidin concentration in the acidic preparation contributes to both the radical scavenging and the transition metal chelating, which explains the slightly better result of the acidic preparation in the XOD assays. Both preparations are better than pure gallic acid and Trolox, although the sum of the concentrations of the single compounds is much lower than the concentrations of gallic acid and Trolox required to give the same effect (difference very highly significant, $P < 0.001$, paired $t$ test). The mixture probably exerts synergistic properties, which achieves better results than the two pure substances used and is consistent with the results of the Folin−Ciocalteu assay. In this assay, the two preparations have much higher GAE than the sum of the concentrations of the single compounds. According to our experience with other natural products, this high reducing potential should be due to the mixture of the single compounds and their synergistic abilities.

**Whole Blood System.** The neutral preparation inhibits stimulated leukocytes in the same manner as Trolox. Both samples show a concentration-dependent effect with a maximum inhibition of 40% of the reaction. The concentration of procyanidins is higher in the acidic than in the neutral preparation; the observed effect is therefore probably due to these procyanidins (**Tables 1** and **4**).

**Copper-Induced LDL Oxidation.** Shafiee et al. (*43*) showed good protective capacities in the copper-induced LDL oxidation for procyanidin-rich grape seed preparations; however, there are no concentrations of single compounds of the sample published. **Table 5** shows the concentrations of the single compounds in the whole preparation, yielding a distinct lag phase. Procyanidins and flavans are known to chelate copper, with catechin having a 1:1 (*42*) and procyanidins a 4:1 $Cu^{2+}$/procyanidin stoichiometry (*40*). Their protective potential in this assay is likely due at least in part to the chelating of copper to prevent the onset of the oxidation of the fatty acids in the LDL particle. Furthermore, the C4−C8 dimers (procyanidins B1, B2, and B3) as well as the trimer (procyanidin C1) are more hydrophobic than the monomers and can interact more easily on the surface of the LDL particle with arising peroxyl radicals (*44*). The augmentation of free hydroxyl groups as hydrogen donors increases their antioxidant potential as well (*2*, *5*, *44*, *45*). The addition of exogenous antioxidant results in an exponential prolongation of the lag phase (*46*). This also occurs with the two preparations and is surely amplified with higher endogenous antioxidant status. Nevertheless, it can be concluded that procyanidins are able to protect LDL from oxidation, which has been also shown in vivo by Fuhrman et al. (*47*).

**Rose Bengal Assay.** The main generated ROS in the Rose Bengal assay is singlet oxygen (80%), but in buffered systems up to 20% of superoxide radicals are arising due the presence of electron donators (*12*). Flavonoids are efficient quenchers of singlet oxygen with determined rate constants of $10^4-10^9$ $M^{-1}$ $s^{-1}$ (*41*) and have, as already discussed for the XOD system, rate constants in the intermediary range for superoxide radicals. The two preparations should give similar results in the

sum of compound concentrations with the Rose Bengal assay without copper, yet the acidic preparation has a much lesser sum of compound concentration than the neutral one (**Table 6**) to reach 50% inhibition of the assay. The hydrophobic character of the procyanidins B1, B2, and B3 as proposed in ref *44* explains this difference very well. The stronger hydrophobic procyanidins in the acidic preparation are able to interact with the α-linolenic acid and the arising fatty acid radicals. The combination of singlet oxygen quenching and interaction within the chain reaction of the lipid peroxidation leads to an exponential curve progression in the assay as seen for the two preparations (**Figure 5A**,**B**). In the assay variation with copper, both grape seed preparations exhibit copper-chelating properties as well as protective properties (**Figure 6A**,**B**). These results meet well with the expectations of the mentioned copper-chelating abilities of procyanidins and flavans as well as of their antioxidant properties (*5*, *41*, *44*, *45*).

Tocopherols are mainly chain breakers in lipid peroxidation as well as scavengers/quenchers of ROS (*12*). The linear curve progression (**Figure 5C**) infers stoichiometric interactions. Trolox is lacking the phytyl side chain, which makes it the water-soluble analogue for tocopherols, but it has the same antioxidant properties as α-tocopherol (*48*). Reacting 1000 times faster with occurring fatty acid hydroperoxides than these do with fatty acids, Trolox is therefore able to disrupt the chain propagation before its onset (*49*). The direct reaction with the fatty acid hydroperoxides causes the linearity seen in **Figure 5C**. Supporting the conclusion of the stoichiometric interaction between Trolox and occurring hydroperoxides is the result of Trolox in the Rose Bengal assay with copper. In this variation of the assay, Trolox also shows a linear curve progression (**Figure 6C**) with no copper chelation.

It can be concluded that a digestion of *V. vinifera* seed powder in the stomach (the acidic preparation in this study) or small intestine (the neutral preparation) releases flavans and procyanidins. According to several publications (*8*, *10*, *47*, *50*), flavans and procyanidins are bioavailable. The results of this work show clearly that the single compounds released during the simulated digestion occurring either in the stomach or in the small intestine have good scavenging potential against superoxide radicals, hydroxyl radicals, and singlet oxygen. They protect LDL against copper-induced oxidation due to the copper-chelating properties and their chain-breaking abilities in lipid peroxidation. The influence of a combined acidic and neutral extraction (mimicking the physiological situation of digestion in the stomach followed by digestion in the small intestine) on the composition of the released procyanidins has to be investigated in the future. In addition, the impact of other food materials such as proteins and fat on the release of the procyanidins from grape seed powder needs to be determined.

## ABBREVIATIONS USED

ACC, 1-aminocyclopropane-1-carboxylic acid; MPO, myeloperoxidase; LDL, low-density lipoprotein; ROS, reactive oxygen species; XOD, xanthine oxidase.

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