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Iron decreases the antioxidant capacity of red wine under conditions of in vitro digestion

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Abstract

The hypothesis that iron and phenolics interact in the lumen during digestion and, consequently, decrease the antioxidant capacity of phenolics, was investigated in vitro. Mixtures of red wine, iron, and three dietary factors that may reduce or chelate iron in the lumen, namely ascorbic acid, meat and casein, were subjected to a simulated gastrointestinal digestion. The process involved incubation of samples for 4.5 h at 37 °C, at different pHs, in the presence of peptic enzymes and fractionation of digests through a dialysis membrane. Antioxidant capacity (FRAP assay), iron concentration (ferrozine assay) and total phenolic content (Folin-Ciocalteau assay) were measured in the in vitro digests. Iron decreased the antioxidant capacity and the total phenolic concentration of red wine. Ascorbic acid increased, while meat and casein decreased, the antioxidant capacity of red wine. Based on these results, it was concluded that protein and iron interact with red wine phenolics during the in vitro digestion and decrease their antioxidant capacity, supporting the initial hypothesis.

Keywords: Phenolics; Wine; Antioxidants; Iron; In vitro digestion

1. Introduction

Phenolics and sources of phenolics, such as vegetables, fruits, green tea and red wine, exert antioxidant properties, repeatedly observed in various in vitro and in vivo systems (Frankel, Kanner, German, Parks, & Kinsella, 1993; Ho, Chen, Shi, Zhang, & Rosen, 1992; Ivanov, Carr, & Frei, 2001; Manach, Scalbert, Morand, Remesy, & Jimenez, 2004; Whitehead, Robinson, Allaway, Syms, & Hale, 1995; Serafini, Maiani, & Ferro-Luzzi, 1998). The antioxidant properties of phenolics may contribute to health, protecting (among others) against from cardiovascular disease and cancer (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993; Serafini, Bellocco, Wolk, & Ekstrom, 2002; Vinson, Teufel, & Wu, 2001).

Despite the interest in the health benefits of phenolics, little is known about the antioxidant capacity of phenolics in vivo in the presence of dietary factors that may interact with phenolics during digestion. Two dietary factors that have a well-known chemical affinity with phenolics are protein and iron, which form chelates with phenolics in the lumen. Information is available on the effect of protein on the antioxidant properties of phenolics (Serafini et al., 2003; Zhu, Phillipson, Greengrass, Bowery, & Cai, 1997) but not on a putative effect of iron.

Iron interactions with phenolics are of concern when evaluating iron bioavailability. Phenolics inhibit the absorption of iron when present in the same meal (Hurrell, Reddy, & Cook, 1999). To explain this effect, it has been proposed that iron–phenol chelates of low bioavailability are formed in the lumen during digestion

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(Cook, Reddy, & Hurrell, 1995; Hallberg & Hulthén, 2000). Enhancers of iron absorption, such as ascorbic acid or meat, may overcome the inhibiting effect of phenolics, presumably because they reduce iron to the ferrous state and, consequently, do not support the formation of iron–phenol chelates (Hallberg & Hulthén, 2000). Casein or other proteins or their digestion products, however, may form chelates, with iron or with both phenolics and iron that further inhibit iron absorption (Cook et al., 1995).

It is difficult, therefore, to predict whether interactions of phenolics with iron and other dietary factors, which occur in the lumen during the digestion of a mixed meal, modify the antioxidant capacity of phenolics in the gastrointestinal environment or finally in the plasma (Alexandropoulou, Komaitis, & Kapsokefalou, 2005).

The aim of this study was to investigate, for the first time, whether iron and phenolics from red wine interact and modify the antioxidant capacity of red wine. For this purpose, mixtures of iron, red wine and various dietary components (ascorbic acid, meat and casein) were subjected to a simulated gastrointestinal digestion. Subsequently, the antioxidant capacity of the digests was measured in vitro. The influence of protein and iron valence on the expressed effect of iron–phenol interactions and on the antioxidant behavior of phenolics in vitro were investigated.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich, Steinheim, Germany. Double-distilled, deionized water was used throughout the experiment. All glassware was washed, soaked overnight in 1 N HCl and rinsed with distilled deionized water. The wine used in this study was a Cabernet Sauvignon (red wine, main grape Cabernet Sauvignon, vintage 2000, Domaine Hatzimichalis, Greece). Meat was lean beef, bought from a local supermarket. The approximate fat and iron contents of meat were 8.67 g/100 g and 2.04 mg/100 g, respectively (National Nutrient Database for Standard Reference, 2004).

The materials used for the in vitro digestion experiment were the following: pepsin was a porcine pepsin preparation, suspended in 0.1 M HCl at 4 g/100 ml in 0.1 M HCl. Pancreatin/bile mixture was porcine pancreatin (0.2 g) and a crude bile extract (1.2 g) suspended in 100 ml of 0.1 M $NaHCO_3$. Pipes buffer, 0.15 M Pipes (piperazine-*N*,*N*′-bis[2-ethane-sulfonic acid] disodium salt), was adjusted to pH 6.3 using concentrated HCl. Hepes buffer, 0.3 M Hepes (*N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid sodium salt), pH 9.9, was used without pH adjustment. Protein precipitant solution (reducing) was 100 g trichloroacetic acid, 50 g hydroxylamine monohydrochloride and 100 mL concentrated HCl per 1 l of water. Protein precipitant solution (non-reducing) was prepared as the reducing solution, except that the hydroxylamine monohydrochloride was not added. Ferrozine chromogen solution (5 mg/mL) was ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4 triazine, disodium salt). Sprectapore I dialysis tubing, with a molecular weight cut-off of 6000–8000 (Spectrum Laboratories, Rancho Dominguez, CA, USA), was cut into 25 cm lengths and soaked in water for at least 1 h prior to use and stored in 0.15 M Pipes, pH 6.3, prior to use.

2.2. Sample preparation

The wine was de-alcoholized in a rotary evaporator at 25 °C for 4 h (Serafini et al., 1998). The alcohol-free red wine was then stripped with nitrogen and kept at −20 °C for 1–2 days prior to further use. The meat was minced, shaped into patties, about $2 \times 2 \times 1$ cm and of 10 g each and kept at −20 °C prior to further use. On the day of the experiment, the patties to be used for testing were thawed and cooked in a microwave oven at 620 W for 15 s. Samples of 50 ml were prepared by mixing iron with red wine and with ascorbic acid (wine + AA), casein (wine + casein) and meat (wine + meat) at the following concentrations: meat and casein samples provided 4 g of protein/100 ml sample. The sample concentration of ascorbic acid samples was 5 mM. The sample concentration of iron was 0.25 or 1 mM. The iron source was a standard solution of $FeCl_3$, 1010 ppm in 1 M HCl, from Sigma–Aldrich, Germany. The amount of iron added to the meat sample was adjusted, allowing for the iron content of meat (2.04 mg/100 g) according to the published data (National Nutrient Database for Standard Reference, 2004). Samples were homogenized and digested in vitro as described below.

2.3. In vitro digestion

The digestion process is described in detail by Kapsokefalou and Miller (1991). This in vitro model simulates the gastrointestinal digestion by subjecting samples to incubation for 4.5 h at 37 °C, at different pHs, in the presence of peptic enzymes and by fractionating digests through a dialysis membrane. Briefly, samples of 10 ml from the homogenized sample of 50 ml were pH adjusted to 2.3, with concentrated HCl to mimic gastric pH following the ingestion of a meal. The samples were transferred in 120 ml screw-cap vials and placed in a shaking water bath maintained at 37 °C. The samples were incubated for 2 h in the presence of 0.5 ml pepsin suspension, added to each sample. At the end of this incubation, the pH of the samples was

adjusted gradually from 2.3 to around 6 with the aid of a dialysis sac, filled with 10 ml of a 0.15 M Pipes buffer, pH 6.3. The dialysis sac was immersed in the incubating samples. This process allows gradual pH adjustment, mimicking intestinal conditions. After 30 min, 2.5 ml of a pancreatin-bile salt mixture (0.5 and 3 g, respectively, in 250 ml of 0.1 M $NaHCO_3$) was added to the samples and the incubation continued for a further 2 h. At the end of this incubation period, the dialysis sac was removed. The dialysate (fraction inside the dialysis sac), consisting of soluble compounds of low molecular weight, and the retentate (fraction outside the dialysis sac), consisting of soluble and insoluble compounds of low and high molecular weight, were collected. The dialysate and the retentate were centrifuged at 10,000*g* for 10 min. The supernatant was removed and was analyzed immediately after collection.

2.4. Analysis

Measurement of antioxidant capacity and determination of soluble ferrous and total iron and of phenolics were performed on the supernatant of the centrifuged dialysates and retentates, respectively.

Antioxidant capacity was estimated from the reducing power of the samples (FRAP assay) according to Benzie and Strain (1996). Results were expressed as increase in absorbance 4 min after the chromogen addition.

Ferrous and total (ferrous + ferric) iron were measured using a modification of the ferrozine method proposed by Reddy, Chidambaram, Foneca, and Bates (1986). Briefly, for total iron determination, reducing protein precipitant solution (0.5 ml) was added to a 1 ml aliquot of each supernatant of centrifuged dialysate and retentate. The samples were held overnight at room temperature. Subsequently they were centrifuged at 5000*g* for 10 min. Aliquots of the supernatants (0.5 ml in duplicate) were transferred to separate tubes. Ferrozine solution (0.25 ml) and Hepes buffer (1.0 ml) were added to each tube. Absorbance was measured at 562 nm immediately after chromogen addition, for ferrous iron determination, or one hour after addition, for total iron determination. Sample iron concentrations were calculated from the absorbance readings, using a regression equation derived from data generated from standards of $FeCl_3$ in the presence of protein precipitant solution.

Percent ferrous dialyzable iron was used as an indicator for the prediction of iron bioavailability (Kapsokefalou & Miller, 1991) and was calculated as [(concentration of ferrous iron in dialysate in mg/ml) × (total volume of treatment in ml) × 100/(amount of iron in original sample in mg)].

Total phenolic content was determined in all samples and in the in vitro digests according to a modification of the Folin-Ciocalteu method (Kähkönen et al., 1999; Singleton & Rossi, 1965). The total phenolic content was expressed as mg of gallic acid equivalents per ml digest (mg GAE/ml).

2.5. Data analysis

Samples were run in duplicate and each experiment was repeated three times. Differences among samples were tested with one-way ANOVA, LSD test at 95% confidence interval, after testing for normality (Zar, 1999). Data were also analyzed according to a 4×3 factorial design, for the factors dietary factor and iron, and according to a 2×3 factorial design, for the factors protein and iron. Analysis of data was carried out with the programme Statistica, version 5.1 (StatSoft, OK, USA).

3. Results

3.1. Antioxidant capacity

The antioxidant capacity of the dialysates (fraction containing soluble compounds of molecular weight less than 6000) is presented in Fig. 1A. In the absence of iron, the antioxidant capacity of the dialysates of red wine was high; however, that of the dialysates of the wine + AA was higher ($P < 0.05$). The addition of casein and of meat to wine before digestion decreased the antioxidant capacity of the dialysates of wine digests ($P < 0.05$). There was no difference between the effects of meat and casein ($P > 0.05$). The addition of iron at 0.25 mM and at 1 mM, before the in vitro digestion, to wine and to wine + AA, decreased the antioxidant capacity of the dialysates of all digests. This decrease was statistically significant when iron was added at 1 mM ($P < 0.05$) for the wine sample and at both iron concentrations for the wine + AA sample. On the contrary, there was no statistically significant change in the antioxidant capacity of the wine + casein and wine + meat samples when iron was added ($P > 0.05$).

Differences in the antioxidant capacities of the dialysates of the various digestion treatments noted in the absence of iron were also observed in the presence of iron, at both concentration levels. In particular, the antioxidant capacities of the dialysates of the wine digests were high, while those of the dialysates of wine + AA were higher and those of dialysates of wine + casein and wine + meat digests were low at both concentration levels of iron (Fig. 1).

The factorial statistical analysis showed that iron or protein alone, added before digestion, to the various wine treatments, modulates the antioxidant capacity of the dialysates of the red wine digests ($P < 0.05$) (Fig. 1) but iron and protein do not interact statistically ($P > 0.05$). However, iron and the various dietary

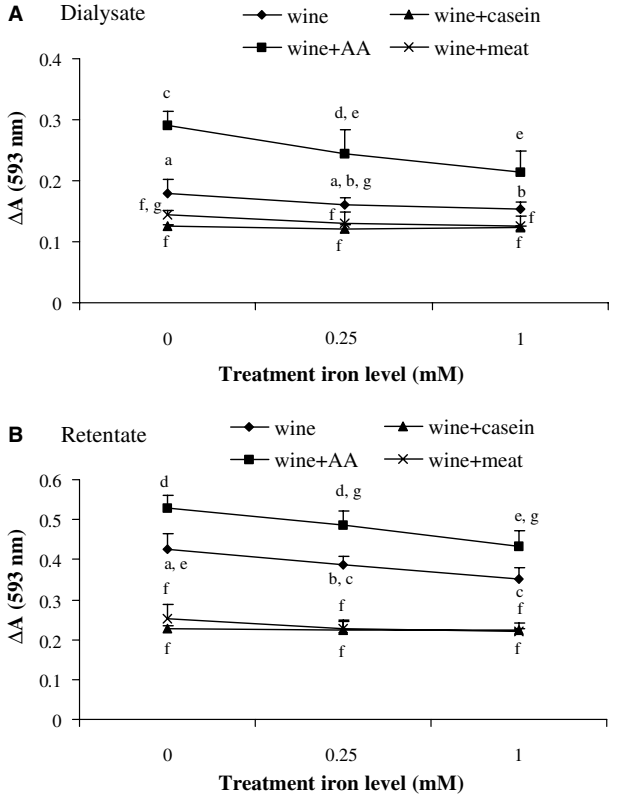

Fig. 1. Antioxidant capacity of red wine digested in vitro in the presence of iron and of ascorbic acid, casein or meat. The antioxidant capacity was measured in the dialysate (fraction containing soluble compounds of molecular weight smaller than 6000) and the retentate (fraction containing soluble and insoluble compounds of molecular weight greater than 6000) of the in vitro digests (A and B, respectively) with the FRAP assay and was expressed as change in absorbance monitored at 593 nm, 4 min after the addition of the chromogen mixture. Means with different letters are significantly different ($P < 0.05$).

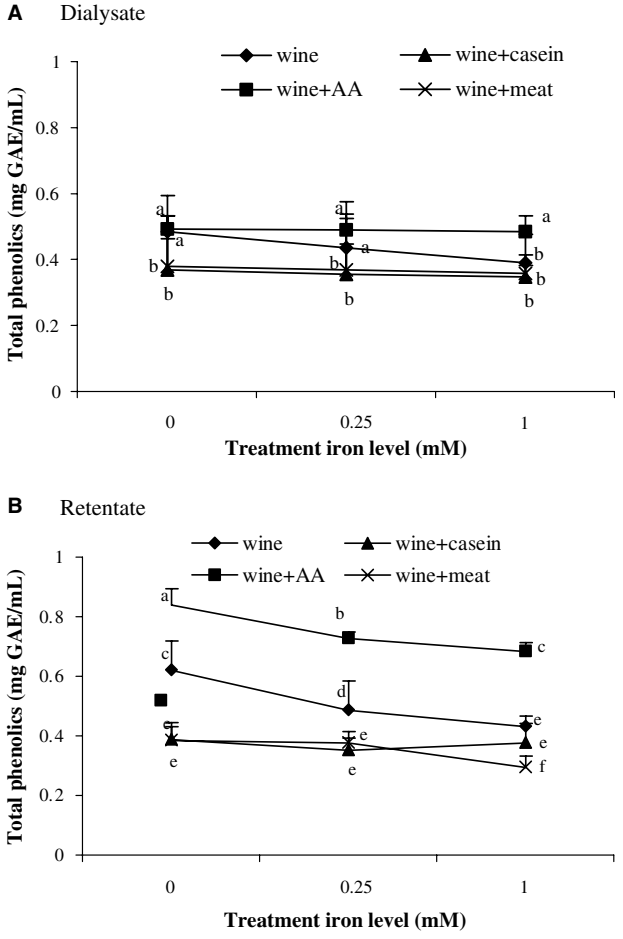

Fig. 2. Total phenolics content of red wine digested in vitro in the presence of iron and of ascorbic acid, casein or meat. Phenolic content was determined in the dialysate (fraction containing soluble compounds of molecular weight smaller than 6000) and the retentate (fraction containing soluble and insoluble compounds of molecular weight greater than 6000) of the in vitro digests (A and B, respectively) with the Folin-Ciocalteau assay and was expressed as milligrammes of gallic acid equivalents per gramme dry weight (mg GAE/gdw). Means with different letters are significantly different ($P < 0.05$).

factors interact statistically and modulate the antioxidant capacity of the digests ($P < 0.05$).

The antioxidant capacity of the retentates (fraction containing soluble compounds of molecular weight above 6000) is presented in Fig. 1B. The results follow the same trends as observed for the dialysates of the digests. The results of the statistical comparisons were similar to those reported for the respective dialysates, with the exception of the wine and wine + AA samples. In the wine sample, a decrease in antioxidant capacity was observed at both iron levels ($P < 0.05$). In the wine + AA sample, the effect was significant when iron was added at 1 mM ($P < 0.05$).

3.2. Total phenolic content

The total phenolic content of the dialysates is presented in Fig. 2A. Protein and iron affected the phenolic content of the dialysates of the various treatments in a way similar to the way that they affected their antioxidant capacities. However, the observed differences were not statistically significant ($P > 0.05$) in most treatments. The factorial statistical analysis showed that iron, added before digestion to the various tea treatments, did not modulate the phenolic content of the dialysates of red wine digests. It also showed that protein, added before digestion to the various wine treatments, modulates the phenolic content of the dialysates of red wine digests. However, there was no statistical interaction between iron and protein for the expression of this effect.

The phenolic content of the retentates is presented in Fig. 2B. In the absence of iron, the phenolic content of the retentates of wine was high. However, that of the retentates of the wine + AA was higher ($P < 0.05$), while that of casein and meat was lower ($P < 0.05$). There were no differences between the effects of meat and of casein

on the phenolic content of the retentates of the wine digests ($P > 0.05$).

The addition of iron, at 0.25 mM and at 1 mM, before the in vitro digestion in wine, wine + AA and wine + meat, decreased the phenolic content in the retentates of the digests. This reduction was statistically significant ($P < 0.05$) in wine digests at both iron levels, but, in wine + AA and wine + meat digests, only when iron was added at 1 mM ($P < 0.05$). There were no statistically important differences between the phenolic contents of the wine digests in the presence of casein at the two iron levels.

Differences in the phenolic contents of the retentates of the various treatments digested in vitro were observed in the presence of iron at the two levels. In particular, the phenolic contents of the retentates from the wine digests were high; those from the wine + AA digests were higher and those of retentates from wine + casein and wine + meat digests were different at both iron levels.

3.3. Iron content

The soluble iron content was measured in the dialysate and the retentate of the in vitro digests of all treatments (Figs. 3 and 4). Most of the soluble iron in the dialysate or the retentate was in the ferrous form (Table 1). Ascorbic acid increased ferrous and total iron content in both the dialysate and the retentate. In contrast with observations of the antioxidant capacity and phenolic content, meat and casein affected iron content differently. In particular, meat increased the content of ferrous and soluble iron in both the dialysate and the retentate compared to casein. These effects are reflected in the predicted iron bioavailability for each of the treatments (Table 1). The index employed herein for the prediction of iron bioavailability is percent ferrous dialyzable iron. This index has been evaluated as the most reliable when the in vitro digestion approach is employed, because it corresponds with published data on human iron absorption better than percent dialyzable iron (Kapsokefalou & Miller, 1991). Casein decreased, while meat and ascorbate enhanced, iron bioavailability compared to the wine treatment at both iron levels.

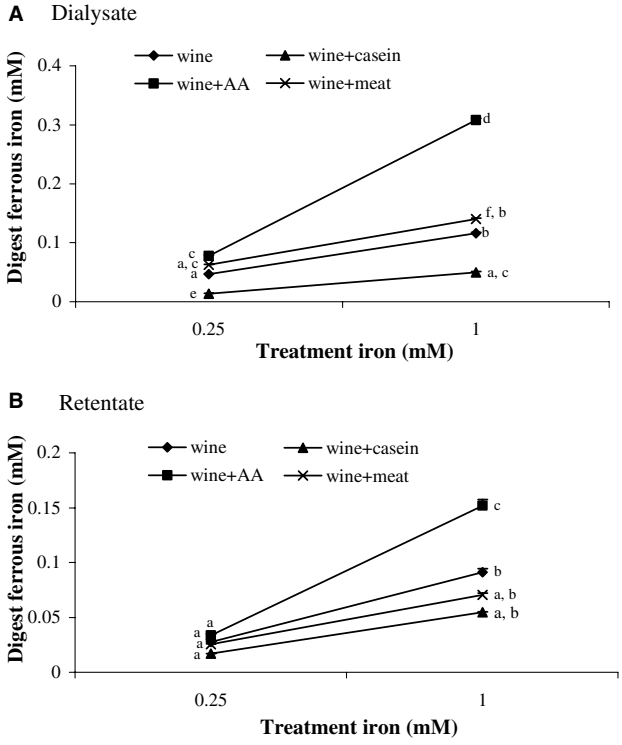

Fig. 3. Ferrous iron content of red wine digested in vitro in the presence of iron and of ascorbic acid, casein or meat. Iron was measured in the dialysate (fraction containing soluble compounds of molecular weight smaller than 6000) and the retentate (fraction containing soluble and insoluble compounds of molecular weight greater than 6000) of the in vitro digests (A and B, respectively) with the ferrozine assay. Means with different letters are significantly different ($P < 0.05$).

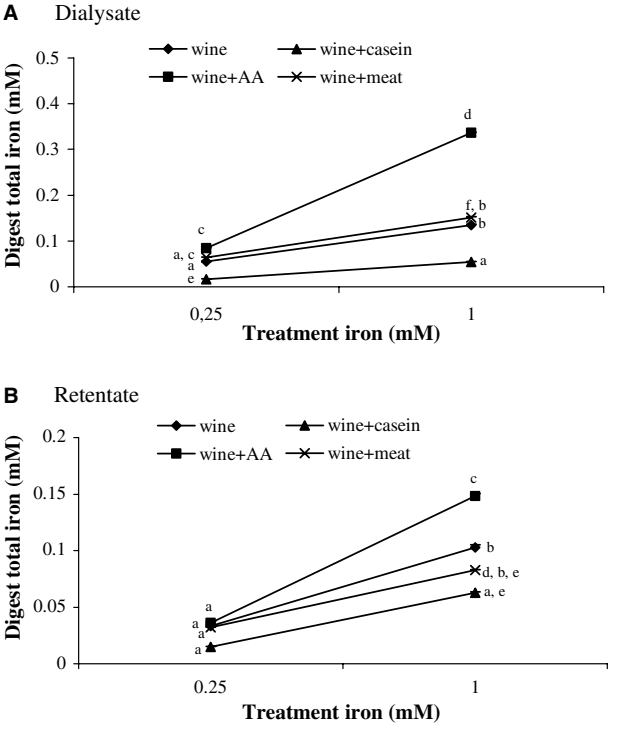

Fig. 4. Total iron content of red wine digested in vitro in the presence of iron and of ascorbic acid, casein or meat. Iron was measured in the dialysate (fraction containing soluble compounds of molecular weight smaller than 6000) and the retentate (fraction containing soluble and insoluble compounds of molecular weight greater than 6000) of the in vitro digests (A and B, respectively) with the ferrozine assay. Means with different letters are significantly different ($P < 0.05$).

4. Discussion

In agreement with the initial hypothesis, the first major finding of this study was that iron may be an

Table 1
Ferrous iron in the dialysate (fraction containing soluble compounds of molecular weight smaller than 6000) and the retentate (fraction containing soluble and insoluble compounds of molecular weight greater than 6000) of the various treatments, estimated as percent of soluble iron in the dialysate and the retentate, respectively. Also, ferrous iron in the dialysate, is estimated as percent of iron added in each treatment. The latter is an index for predicting bioavailability (Kapsokefalou and Miller, 1991)[a,b]

Treatment	Fe^{2+} in dialysate (% of soluble iron in dialysate)		Fe^{2+} in retentate (% of soluble iron in retentate)		Fe^{2+} in dialysate (% of treatment iron) (predicted iron bioavailability)	
	Treatment iron					
	0.25 mM	1 mM	0.25 mM	1 mM	0.25 mM	1 mM
Wine	84.1 ± 3.3a	86.3 ± 7.5b	82.4 ± 5.3a	88.6 ± 18.4b	42.9 ± 0.5d	26.8 ± 0.3c
Wine + AA	91.7 ± 6.4c	91.8 ± 13.6d	92.9 ± 5.7a	103 ± 32.5c	71.4 ± 1.1b	70.9 ± 0.6a
Wine + casein	80.1 ± 4.8e	91.8 ± 3.5ac	112 ± 0.8a	87.0 ± 2.7ab	12.3 ± 0.8a	11.5 ± 0.1d
Wine + meat	97.1 ± 4.2ac	92.8 ± 9.3fb	79.1 ± 2.7a	85.0 ± 9.9ab	57.5 ± 0.7c	32.2 ± 0.4b

[a] Means (±SD) of triplicate analyses.
[b] Means followed by the same letter within a column do not differ significantly ($P < 0.05$).

important modulator of the antioxidant capacity of red wine. The results support the idea that in vitro digests of a mixture of iron and red wine have lower antioxidant capacity than red wine alone. The observed decrease of the antioxidant capacity of wine, when iron was present, depended on the amount of iron added to wine before digestion. Iron produced a statistically significant effect on the modulation of the antioxidant capacity of wine only when it was added at 1 mM before digestion. However, at the lower iron level of 0.25 mM, a trend towards decrease of the antioxidant capacity was also observed.

The iron concentrations of 0.25 and 1 mM refer to mixtures before digestion, corresponding to the iron concentration in a meal before ingestion. For a 250 mL serving, the concentrations of 0.25 and 1 mM are equivalent to 3.5 and 14 mg of iron, respectively. These iron amounts compare well with the recommended daily intake. For example, the Recommended Dietary Allowance for iron varies, according to age and sex group, from 8 to 18 mg/day (Food & Nutrition Board, 2004). This suggests that the iron levels employed herein are within the expected range in a diet.

The observed decrease of antioxidant capacity may be related to the formation of iron–phenol chelates. The decrease in phenolic concentration observed when iron was added to the wine digests is in agreement with the proposed mechanism. This iron–phenol interaction has been observed in various model systems and involves iron in the ferrous or ferric form and adjacent phenolic oxygens (Jovanovic, Simic, Steenken, & Hara, 1998; Kawabata, Schepkin, Haramaki, Phadke, & Packer, 1996; Khokhar & Owusu Apenten, 2003). Phenolics include compounds with different structures, which do not have the same iron-binding characteristics. Khokhar and Owusu Apenten (2003) studied ferric iron binding by phenolic compounds and suggested that the following aromatic hydroxyl groups are important for Fe-binding: (1) *ortho*-dihydroxyl groups, (2) the presence of 5-OH and/or 3-OH in conjunction with a C4 keto group and (3) a large number of OH groups. Red wine contains phenolics with these structural characteristics (Jackson, 1994). The hydroxyl groups are also the functional groups for free radical-scavenging by phenolics. Therefore, their use by iron for the formation of iron–phenol chelates is expected to result in a decrease of antioxidant capacity.

The method employed herein for the measurement of the antioxidant capacity (FRAP assay) is an indirect assay that reflects the total antioxidant power of a sample (Prior & Cao, 1999). However, FRAP is appropriate for testing the mechanism proposed herein because it examines the ability of phenolics to donate a hydrogen atom from an aromatic hydroxyl group to a free radical. Consequently, a decrease in antioxidant capacity depicts a decrease in available hydroxyl groups. It is likely that the decrease in antioxidant capacity is dependent on the formation of iron–phenol chelates. It is possible, however, that other mechanisms operate simultaneously, such as the catalysis of oxidation reactions by iron, not observed herein by the FRAP assay. This catalytic action of iron has been observed in many systems (Kanner & Lapidot, 2001; Kapsokefalou & Miller, 2001). Phenolics may bind iron, thus limiting its potential to participate in Fenton-type reactions, leading to potent reactive oxygen species (Khokhar & Owusu Apenten, 2003; Matsingou, Kapsokefalou, & Salifoglou, 2000; Prior & Cao, 1999).

Despite drawbacks (Prior & Cao, 1999), the FRAP method is a reliable approach for the assessment of antioxidant capacity of phenolics in in vitro systems and in plasma (Pulido, Bravo, & Saura-Calixto, 2000; Serafini et al., 2003). Other methods used for measuring the antioxidant capacity of phenolics, such as ESR (McPhail, Hartley, Gardner, & Duthie, 2003), are also based on the reducing ability of phenolics.

The second finding was that protein may be another important modulator of the antioxidant capacity of red wine. The result that supports this finding is that in vitro

digests of a mixture of red wine and protein, either from meat or from casein, exhibited lower antioxidant capacity than did red wine alone. The decrease was similar for both protein sources. This was not expected, however, because casein and meat interact with iron in different manners. Casein produces mostly high molecular weight ferric iron chelates of low solubility, while meat produces mostly soluble low molecular weight ferrous iron chelates (Kapsokefalou & Miller, 1991). These differences between the interactions of casein and meat with iron have been observed in this study and are depicted in the estimation of predicted iron bioavailability. Data from this study suggest that, at both iron levels, meat and ascorbate are expected to enhance iron bioavailability when added to wine. On the contrary, casein is expected to exert an inhibiting effect on iron bioavailability. These results are in agreement with iron absorption data from human studies (Hurrell, Lynch, Trinidad, Dassenko, & Cook, 1989; Reddy, Hurrell, & Cook, 2000). Clearly, protein is not the only component in meat that may interact with iron. For example, it has been shown that the iron absorption-enhancing properties of meat may involve an interaction between the lean and that fat fraction (Kapsokefalou & Miller, 1993), affecting the solubility, molecular weight and/or valence of iron species in the gastrointestinal lumen (Kapsokefalou & Miller, 1994). Moreover, besides differences in the formation of various iron chelates, meat and casein exert different reducing and antioxidant capacities (Kitts & Weiler, 2003). It appears that this also did not affect the antioxidant capacity of the digests, suggesting that the effect of protein was very strong, prevailing over other properties of the food system that delivers the protein. Others have observed the effect of protein in the past, when milk was the protein source, with variable results (Hollman, Van Het Hof, Tijburg, & Katan, 2001; Serafini et al., 2003). There are no reports on comparable effects of meat.

The third finding was that, in the in vitro digests of wine produced in the experiment reported herein, the effects of iron and of dietary factors on the antioxidant capacity were similar in the low and in the high molecular weight soluble fractions. However, in all in vitro digests, the antioxidant capacity of all samples of high molecular weight was higher, almost twofold, than that of low molecular weight. The high molecular weight fraction also shows a higher concentration of phenolics. Red wine contains anthocyanins and tannins of high molecular weight that may not dialyze and remain in the retentate. The molecular weights of phenolics in red wine may range from 500 to 5000 (Jackson, 1994), but in red wine digests they appear to be higher, particularly when other dietary factors, such as protein, are present. The phenolic composition of wine has been associated with the antioxidant capacity of red wine in a previous study by Serafini, Laranjinha, Almeida, and Maiani (2000). These investigators observed that the ingestion of red wine by humans resulted in a lower and slower increase of the antioxidant capacity of plasma than the ingestion of tea. The authors attributed this finding to the phenolic composition of red wine and to the slow absorption of these polymeric units. Phenolics are a diverse group of compounds. Thus it may be expected that they would respond differently in different food systems and that, in the presence of iron, they may produce different results.

Moreover, results from this study indicate that phenolics may not interact with ferrous iron. This may be observed in the wine + AA digests, where added iron was mostly in the ferrous form. In these samples, the antioxidant capacity decreased but the phenolic concentration of the digests was not affected. We may hypothesize then, that ferrous iron did not form chelates with phenolics during the in vitro digestion, but the observed decrease in antioxidant capacity was related to oxidation of ascorbic acid by iron. Ferric iron chelates are more stable than the ferrous chelates due to low reduction potentials (Kawabata et al., 1996). However, the formation of ferrous chelates with phenolics has been reported (Khokhar & Owusu Apenten, 2003).

Finally, it appears that the use of the in vitro procedure may offer advantages for the study of the effect of dietary factors on the antioxidant properties of phenolics. Albeit removed from real conditions, the in vitro procedure used herein provides a minimal, yet substantive representation of the digestion process. The most important features of the assay are (a) the presence of gastrointestinal enzymes, (b) adjustment of pH and of length of incubation that depict true conditions and (c) the presence of a dialysis membrane that allows separation of soluble digests into low and high molecular weight fractions. This procedure has been developed for the prediction of iron bioavailability and was evaluated as being reliable (Forbes et al., 1989; Kapsokefalou & Miller, 1991; Schricker, Miller, Rasmussen, & Van Campen, 1981). In the past the same procedure has been employed for the measurement of antioxidant behaviour of herbs (Matsingou et al., 2000; Matsingou, Kapsokefalou, & Salifoglou, 2001; Matsingou, Petrakis, Kapsokefalou, & Salifoglou, 2003). It was found that antioxidant activities assessed were in compliance with the results emerging from other systems (Lionis, Faresjo, Skoula, Kapsokefalou, & Faresjo, 1998). Others have reported that events occurring during digestion may affect antioxidant capacity (Friedman & Jurgens, 2000; Halliwell, Zhao, & Whiteman, 2000; Scalbert & Williamson, 2000). A similar system of in vitro digestion has been employed successfully for the study of various foods that contain phenolic compounds (Gil-Izquierdo, Zafrilla, & Tomás-Barberán, 2002; Gil-Izquierdo, Gil, Tomás-Barberán, & Ferreres, 2003; Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002; Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera, 2004). Martínez-Ortega,

García-Parrilla, and Troncoso (2001) have also proposed an in vitro approach for the evaluation of phenolics from wine. Clearly, in vitro procedures, although valuable, present limitations, such as the exclusion of the matrix effects of food or of physiological factors important in the regulation of absorption of nutrients (Ekmekcioglou, 2002).

In conclusion, the in vitro data presented herein suggest that antioxidant capacity of red wine may decrease when iron and/or protein are present. The in vitro procedure employed in this study provides a minimal, yet substantive representation of the digestion process.

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