

Stability of Iron–Quercetin Complexes in Synthetic Wine under *In Vitro* Digestion Conditions

Leticia B. Escudero, Cecilia M. Fusari, Jorgelina C. Altamirano, Alejandra B. Camargo, and Rodolfo G. Wuilloud

Abstract: Wine is a dietary source of polyphenolic compounds with reported health benefits when moderately consumed. Several of these compounds can associate with metals forming complexes. Therefore, this work was conducted to reach a better understanding of the nature and chemical stability of wine-derived Fe^{3+} –quercetin complexes in a digestion model. The stability of the complexes in a synthetic (simulated) wine was studied before and after *in vitro* gastric and intestinal digestions by high-performance liquid chromatography (HPLC) with UV–Vis detection. Metal determination was performed by atomic absorption spectrometry (ETAAS) to evaluate possible dissociation of complexes. During HPLC analysis all peaks eluted from the chromatographic column were collected, acidified, and analyzed by ETAAS. The results showed that complexes remain substantially stable after gastric digestion conditions, with recoveries of 84% to 90%. Although metal complexes were partially degraded during intestinal digestion, 41% to 45% of the Fe^{3+} –quercetin complexes was recovered.

Keywords: iron, gastric and pancreatic digestion, polyphenols, quercetin, wine

Practical Application: This work reveals the chemical stability of Fe^{3+} –quercetin complexes in synthetic wines after an *in vitro* gastrointestinal digestion. The knowledge of this process would be useful to understand the bioavailability of these compounds.

Introduction

Wine is one of the most consumed alcoholic beverages worldwide and it is acknowledged by consumers to contain “healthy” natural compounds. In fact, potential benefits to health due to wine consumption were already reported by Hippocrates of Kos about 2300 years ago, and wine has been used as a tranquilizer, painkiller, and also a diuretic (Böhm and others 2004). Wine contains a high concentration of polyphenols, including flavonoids, flavonols, anthocyanins, oligomeric, and polymeric proanthocyanidins, phenolic acids, stilbenes, and others. Polyphenolic compounds have been widely studied because of their antibacterial, antifungal, and antiviral properties (Chávez and others 2006; Bruno and Sparapano 2007). Moreover, their anti-inflammatory and antimutagenic activities have been demonstrated (Landete 2012). In fact, it has been reported that foods and beverages containing polyphenolic compounds reduce the risk of cancer (Yang and others 2012). Another property of polyphenolic compounds to be mentioned is their ability to protect against free radical damage to cells, which is partially due to their free radical-

scavenging ability and metal–chelating activity. In fact, the current science is moving nowadays to intracellular signaling cascades, as (–)-epigallocatechin–3–gallate has been shown to be useful in modulation of multiple signal transduction pathways in order to control the undesirable proliferation of cells, thus contributing to cancer prevention (Naghma and others 2006).

Polyphenols are able to form complexes with several cations, which has been reported for anthocyanins on metals such as Mg^{2+} and Al^{3+} , quercetin and catechin on Zn^{2+} , as well as quercetin and rutin on Cu^{2+} (Le Nest and others 2004; Estévez and others 2011). In the last years, there has been growing interest in metal complexation by naturally occurring ligands in enological samples. The role of polyphenols on Pb^{2+} and Cu^{2+} complexation in red wines has been studied (Vasconcelos and others 1999, 2000). Furthermore, catechin has been detected as a natural ligand for complexation with Zn^{2+} and Cu^{2+} in the initial stages of grape juice fermentation (Salinas and others 2005).

As part of the diet, polyphenolic compounds are ingested from complex matrices, which then undergo a digestion process. For this reason, it is important to evaluate the chemical stability of these compounds during digestion because changes in their bioavailability can be expected. At present, the information concerning the *in vivo* effects of the digestion process on dietary polyphenolic compounds is still limited. On the other hand, *in vitro* digestion methods have been widely developed for evaluating dietary changes in polyphenols under gastric or intestinal conditions. The bioaccessibility of pistachio polyphenolic compounds during simulated human digestion has been studied by Mandalari and others (2013), while Bouayed and others (2012) have subjected apple polyphenols to an *in vitro* gastrointestinal digestion. Also, Bermúdez-Soto and others (2007) have studied the stability of polyphenols in

MS 20140629 Submitted 4/14/2014, Accepted 7/12/2014. Authors Escudero, and Wuilloud are with Laboratory of Analytical Chemistry for Research and Development (QUIANID), Facultad de Ciencias Exactas y Naturales, Univ. Nacional de Cuyo, Padre J. Contreras 1300, Mendoza 5500, Argentina. Authors Escudero, Fusari, Altamirano, Camargo, and Wuilloud are with Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. Authors Fusari, and Camargo are with Laboratorio de Cromatografía para Agroalimentos, IBAM-CONICET, Univ. Nacional de Cuyo, Mendoza, Argentina. Author Altamirano is with Inst. Argentino de Nivología, Glaciología y Ciencias Ambientales (IANIGLA), CCT-CONICET-Mendoza, Argentina. Direct inquiries to author Escudero (E-mail: letibelesudero@gmail.com).

chokeberry juice during simulated gastric and pancreatic digestion. These reports have shown different stabilities for several polyphenolic compounds. Although several polyphenolic compounds naturally form complexes with metals in food matrices, the digestibility of metal–polyphenol complexes has not yet been extensively studied.

This study was conducted to reach a better understanding of the nature and chemical stability of metal–polyphenol complexes in a digestion model simulating chemical and biological gastrointestinal conditions. The chemical stability of Fe³⁺–quercetin complexes was assessed before and after *in vitro* gastric and intestinal digestions by high-performance liquid chromatography (HPLC) with UV-Vis detection and electrothermal atomic absorption spectrometry (ETAAS). Element-specific detection of Fe in the chromatographic fractions was performed to evaluate possible dissociation of the Fe³⁺–quercetin complex.

Materials and Methods

Instrumentation

The absorbance of stock solutions was scanned in the 200 to 500 nm spectral range with a Lambda 35 UV/Vis spectrometer (Perkin Elmer, Shelton, Conn., U.S.A.), equipped with 1-cm quartz cuvette. Samples were injected in an HPLC system (200LC series, Perkin Elmer), composed of quaternary pump, column oven, and UV-Vis detector. A Zorbax–SB–Aq column (4.6 × 150 mm, particle size 5 μm), purchased from Agilent Technologies (Santa Clara, Calif., U.S.A.), was used. HPLC analysis was performed with a mobile phase resulting from different ratios of formic acid and water at a flow rate of 1 mL/min for 19 min. The system was equilibrated using the starting conditions for 10 min prior to the injection of the next sample. Before use, mobile phases were filtered through a 0.45-μm filter. Polyphenols were identified by matching their retention times with those of pure standards. Instrumental HPLC conditions were as summarized in Table 1.

Elemental detection was performed using a Perkin Elmer (Überlingen, Germany) Model 5100ZL atomic absorption spectrometer. The spectrometer contained a graphite furnace module, a pyrolytic graphite tube (Perkin Elmer) and a transversely heated graphite atomizer Zeeman-effect background correction system. A Fe (iron) hollow cathode lamp (Perkin Elmer) operated at a current of 30 mA and a wavelength of 248.3 nm with a spectral bandwidth of 0.2 nm was used. The temperature/time program of the ETAAS instrument is fully depicted in Table 2.

A Horiba F-51 pH meter (Kyoto, Japan) was used for pH measurements.

Chemicals and standards

Quercetin dihydrate (99.7%) was purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). A 1000 mg/L standard solution was prepared by dissolving an accurate amount of polyphenol in methanol (Merck, Darmstadt, Germany). Quercetin stock solutions were stored at 4 °C and protected from light. A 1000 mg L⁻¹ stock standard solution of Fe³⁺ was prepared by dissolving FeCl₃·6H₂O (99.9%) (Aldrich, Milwaukee, Wis., U.S.A.) in 1% (v/v) ultrapure nitric acid (Merck). Acetonitrile (99.9%) (J. T. Baker, U.S.A.) and formic acid (98.0%) from Merck were used as mobile phases. Ethanol (Merck) and tartaric acid (99.6%) (Anedra, Buenos Aires, Argentina) were used for the preparation of synthetic wine samples. A 2.0 mol/L acetic acid–acetate solution (Merck) adjusted to pH 3.5 by dissolution of sodium hydroxide (Sigma-Aldrich) was employed as buffer solution.

Pepsin (from hog stomach, 600 units/mg), pancreatin (from hog pancreas, P 7545, 8× USP specification activity), the bile salts glycodeoxycholate (≥97.0%) taurodeoxycholate (≥97.0%), taurocholate (≥97.0%), and monobasic potassium phosphate (≥99.0%) were purchased from Sigma. Hydrochloric acid (37.0%) (Ultra pure grade; Figmay, Córdoba, Argentina) and sodium chloride (Merck) were used.

Ultrapure water (18 MΩ cm) was obtained from a Milli-Q Academic A-10 Millipore Water Purification System (Billerica, Mass., U.S.A.). All glassware items used in the experiments were initially cleaned with pure water, then soaked in 0.5 mol/L nitric acid solution at least for 24 h, and finally rinsed 5 times with ultrapure water before use.

Synthetic wine sample preparation

The procedure for preparing the synthetic wine samples was adapted from Garcia-Falcon and others (2007). In this work, 12 mL of ethanol and 260 mg of L-(l)-tartaric acid were added to a flask. The pH was adjusted to 3.5 with 1 mL of 2 mol/L acetate/acetic acid buffer and the volume was made up to 100 mL with ultrapure water.

Subsequently, an aliquot of 375 μL of quercetin standard solution was placed in a 25-mL flask with 25 μL of stock metal solution, and the synthetic wine sample was added to get a final volume of 25 mL. The resulting mixture was homogenized by gentle rotation of the flask and subjected to *in vitro* digestion.

Gastric fluid and intestinal fluid preparations

Initially, gastric fluid was prepared by transferring a mix of 0.5 g of sodium chloride, 0.8 g of pepsin, and 1.75 mL hydrochloric acid to an amber glass bottle, and the volume was made up to 250 mL with ultrapure water. Intestinal fluid was obtained as follows: 1.7 g monobasic potassium phosphate was dissolved in 60 mL pure water. Then, 47.5 mL of 0.2 N sodium hydroxide and 100 mL of pure water were added to this alkaline solution. Finally, 1.5 g bile salts (glycodeoxycholate, taurodeoxycholate, taurocholate) and 2.5 g of pancreatin were added to the solution and the pH was adjusted to 7.5 with diluted sodium hydroxide. Volume was made up to 250 mL in a volumetric flask.

In vitro digestion procedure

The *in vitro* digestion model was adapted from Martínez-Ortega and others (2001). A volume of 25 mL synthetic wine sample was mixed with 25 mL gastric fluid and incubated at 37 °C in a shaking water bath at 100 rpm for 2 h. For intestinal digestion, 25 mL synthetic wine sample or standard solution was mixed with 25 mL intestinal fluid and incubated in a shaking water bath (100 rpm) at 37 °C for 2 h. Both digestion steps were developed in the absence of light.

After gastric and intestinal digestion, each sample was centrifuged at 1200 rpm for 5 min and passed through a 0.45-μm filter prior to HPLC analysis. A schematic diagram of the digestion procedure and chemical analysis is shown in Figure 1.

Results and Discussion

Formation of Fe³⁺–quercetin complex

Spectroscopic studies based on UV-Vis, IR, Raman spectroscopy, and theoretical methods had been developed to evaluate interaction of metal ions with polyphenols, complexation sites, and metal:ligand ratios (Severino and others 2011; Bark and others 2012). In the case of quercetin, several works have focused

Table 1–HPLC instrumental conditions for metal–polyphenol complex determination.

Wavelength	290 nm
LC column	Zorbax–SB–Aq (5 μ m \times 4.6 mm i.d. \times 150 mm)
Guard column	Zorbax Reliance Analytical Cartridge
Flow rate	1 mL/min
Injection volume	20 μ L
Column temperature	20 $^{\circ}$ C
Mobile phases	A: Water (formic acid 0.2% [v/v]) B: Acetonitrile (formic acid 0.2% [v/v])

HPLC gradient program

Step	Initial time (min)	Final time (min)	Final composition of the mobile phase (linear gradient)
0	0	0.5	80% A; 20% B
1	0.5	3	80% A; 20% B
2	3	6	70% A; 30% B
3	6	9	60% A; 40% B
4	9	16	50% A; 50% B
5	16	19	45% A; 55% B

Table 2–ETAAS instrumental conditions for Fe determination.

Wavelength	248.3 nm
Spectral band width	0.2 nm
Lamp current	30 mA
Injection volume	20 μ L
Matrix modifier	15 μ g Mg [as Mg(NO ₃) ₂]

Graphite furnace temperature program

Step	Temperature ($^{\circ}$ C)	Ramp Time (s)	Hold Time (s)	Argon flow rate (mL/min)
Drying 1	110	15	50	250
Drying 2	300	15	20	250
Pyrolysis	1400	20	20	250
Atomization	2100	0	3	–
Cleaning	2400	1	2	250

on studying its conformational and electronic structure (Chien and others 2006, 2010). Quercetin is a flavonol which has some available sites for metal complexation due to the hydroxyl groups and a carbonyl group present on its C ring. Furthermore, it has been reported to have potential sites for complexation, such as the oxygen atoms of the 3- or 5-hydroxy and the adjacent 4-carbonyl group (Figure 2) (Leopoldini and others 2006). Metal–polyphenol complexes can be affected by pH conditions. It is well known that pKa values for the most acidic phenolic hydrogens are in the range of 7 to 9. However, polyphenols are easily deprotonated at or below physiological pH in the presence of iron to form very stable complexes (Hider and others 2001). Stability constants for Fe³⁺–quercetin are higher than those reported for Fe²⁺–quercetin complexes. It makes sense because deprotonated polyphenolic ligands are hard Lewis bases, forming more stable complexes with hard Lewis acid such as Fe³⁺. Regarding metal:ligand stoichiometries, it has been observed that 1:1 and 1:2 ratios are feasible for Fe³⁺–quercetin complex formation (Dimitrić Marković and others 2011).

In the present work, the formation of the Fe³⁺–quercetin complex was studied by selecting similar conditions to those occurring in real wine samples. It has been observed that significant variations occur in the polyphenolic contents not only between white and red wines, but also young and aged wines. Previous contributions have reported quercetin concentrations in wines in the range of 0.6 to 20 mg/L (Goldberg and others 1996; Šeruga and others 2011; Vergara and others 2011). Wine contains both Fe³⁺ and Fe²⁺ as free species and as part of complexes. The Fe³⁺/Fe²⁺

ratio is influenced by the storage conditions of wine and when wine is subjected to aeration, the Fe³⁺ species is predominant and the beverage is more susceptible to ferric casse (Ribéreau–Gayon and others 2003), a typical alteration of wines caused at high concentrations of iron. Based on these previous findings, the synthetic wine sample was prepared in this work to obtain final concentrations of 15 and 1 mg/L of quercetin and Fe³⁺, respectively. Thus, it was possible to work under similar analyte concentrations as with real wine samples. Moreover, a slight excess of the ligand (1:3 molar ratio) was used in order to force the equilibrium toward total complex formation.

As mentioned above, pH plays an important role in metal–complex formations. In order to maintain similar conditions to those taking place in real wine samples, a pH value of 3.5 was selected for all experiments. An acetic/acetate buffer solution was chosen to keep a constant working pH that allows complex formation and stability. Buffer concentration was studied in the range of 5 \times 10^{–3} to 0.1 mol/L. A concentration of 4 \times 10^{–2} mol/L ensured complex formation, which was confirmed by the highest absorbance obtained at 290 nm. Finally, 4 \times 10^{–2} mol/L concentration of buffer solution was selected for subsequent experiments. The solution was allowed to stand for at least 1 h to ensure complete formation of Fe³⁺–quercetin complexes.

Study of HPLC conditions

HPLC-based methods have been widely applied for the separation of polyphenolic compounds in several wines. Since chromatographic separation of Fe³⁺–quercetin complexes in synthetic wine

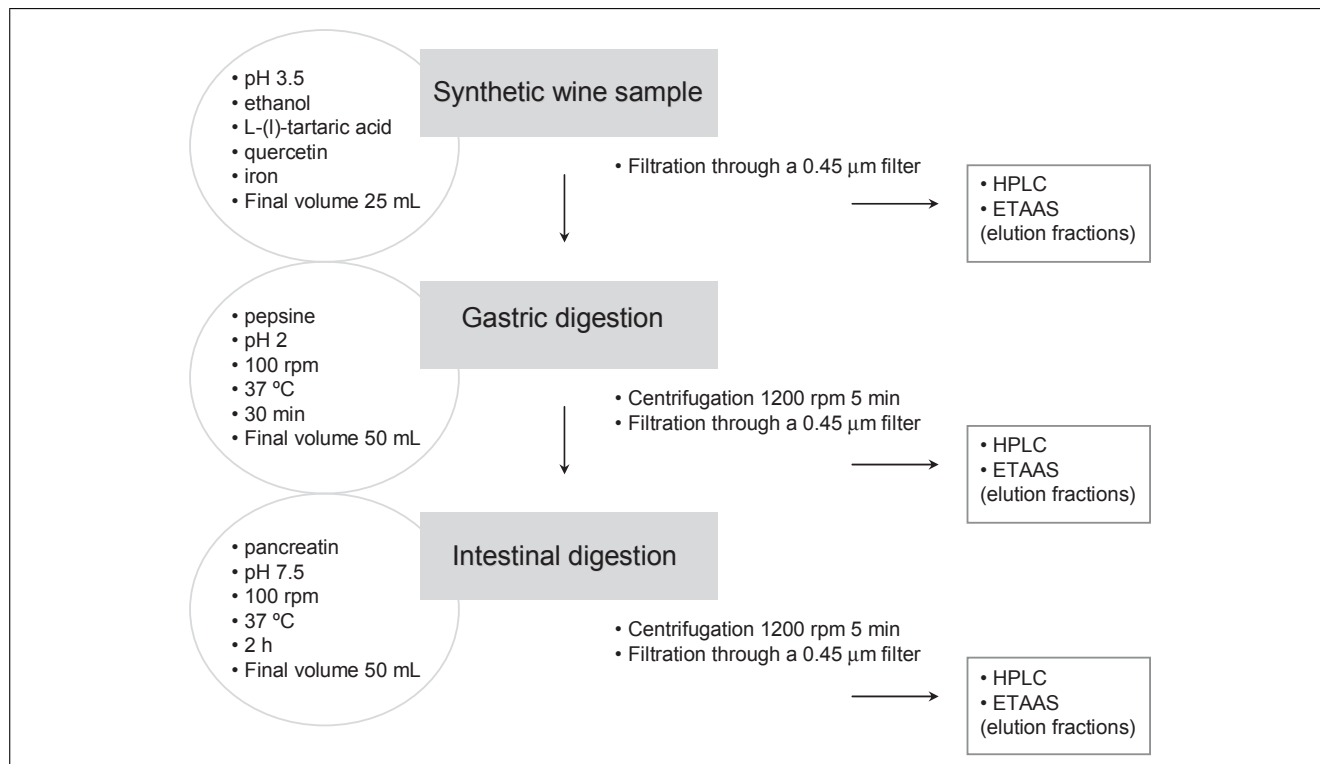


Figure 1–Schematic diagram of the *in vitro* digestion of synthetic wine, including gastric and intestinal phases; and chemical analysis.

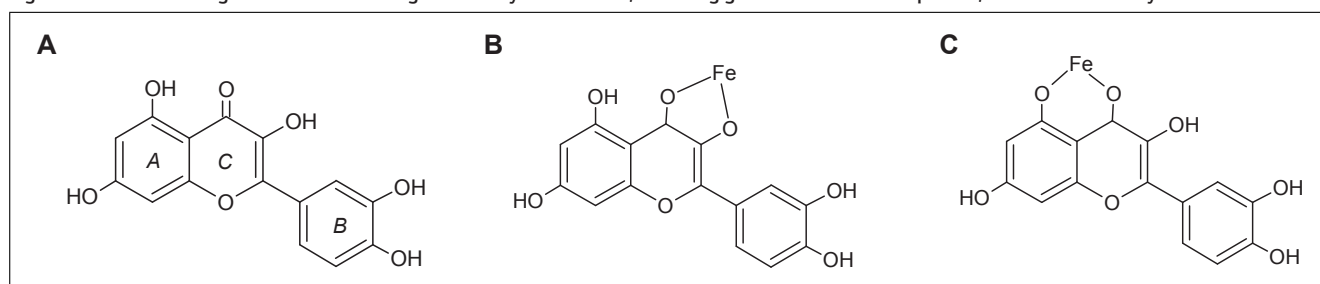


Figure 2–(A) Chemical structure of quercetin; (B) and (C) potential sites for iron–quercetin complexation.

matrices had not been previously addressed, a study of those variables involved in HPLC separation was performed. Several HPLC methods have proposed the use of mobile phases consisting of acetonitrile or methanol and water, containing a small amount of acid, for the separation of polyphenolic compounds subjected to *in vitro* digestion models (Cilla and others 2009; Bouayed and others 2012). In our work, water and acetonitrile were evaluated to prepare the mobile phases for separation of compounds. As stated above, the pH of the synthetic wine samples was 3.5; therefore, pH value of water/acetonitrile mobile phases was adjusted to 3.5 with formic acid during HPLC analysis. Also, different concentrations of formic acid (0%, 0.2%, 0.5%, and 1% [v/v]) were evaluated. Under isocratic conditions, it could be observed that 0.2% (v/v) formic acid in water and acetonitrile mobile phases was successful to improve the separation of the Fe³⁺–quercetin peak.

In order to optimize the conditions for complete separation of the analytes, isocratic and gradient elutions were evaluated. Initially, isocratic modes with mobile phases containing water and acetonitrile at 50:50 and 80:20 (v/v) ratios were studied. However, separation of the Fe³⁺–quercetin complex was not satisfactory under these conditions due to the complexity of the matrix. Therefore, HPLC separation of the Fe³⁺–quercetin complex was only achieved with a linear gradient of the above-mentioned mo-

bile phases at a flow rate of 1 mL/min (Table 1). Finally, the application of a Zorbax–SB–Aq column (4.6 × 150 mm, particle size 5 μm) was studied for method development. The column was kept at a constant temperature of 20 °C, and an injection volume of 20 μL was used in all experiments. An acceptable chromatographic separation was obtained using this column, showing satisfactory peak shapes for Fe³⁺–quercetin, even in the presence of the different digestion matrices. The Fe³⁺–quercetin complex was eluted earlier from the column than free quercetin due to the higher polarity of the complex.

Stability of Fe³⁺–quercetin complex under *in vitro* gastric digestion conditions

Our study was designed to evaluate the stability of a metal–polyphenol complex after the simulation of an *in vitro* digestion process applied to synthetic wine samples. Thus, the chemical stability of Fe³⁺–quercetin complex was monitored during gastrointestinal digestion by HPLC and ETAAS. Figure 3 shows the chromatograms obtained for the Fe³⁺–quercetin complex in the synthetic wine sample before and after gastric digestion. The Fe³⁺–quercetin complex was identified by matching chromatographic retention times with those obtained for standard solutions. One

Table 3—Recovery of flavonols after gastric and intestinal simulated digestion.

Sample	Flavonol	Gastric conditions	Recovery (%)	Intestinal conditions	Recovery (%)	Reference
Onions, apples	Quercetin	Pepsin-HCl, pH 2.0, 30 min	100	Pancreatin-bile, pH 6.5, 1 h	25 to 50	(Boyer and others 2005)
Apples	Rutin	Pepsin-HCl, pH 2.0 to 2.5, 1 h	55 to 71	Pancreatin-bile, pH 7.0 to 7.5, 2 h	38 to 41	(Bouayed and others 2012)
Chokeberry juice	Quercetin	Pepsin-HCl, pH 2.0, 2 h	100	Pancreatin-bile, pH 7.5, 2 h	74	(Bermúdez-Soto and others 2007)
Grapes	Quercetin	Pepsin-HCl, pH 2.0, 2 h	97 to 100	Pancreatin-bile, pH 7.5, 2 h	93 to 95	(Tagliazucchi and others 2010)
Raspberries	Rutin	Pepsin-HCl, pH 2.0, 1–2 h	100	Pancreatin-bile, pH 6, 1 h	100	(Gião and others 2012)
Standard mixture of polyphenols	Quercetin	Pepsin-HCl, pH 2.5, 2 h	0	Pancreatin-bile, pH 7.5, 2.5 h	0	(Siracusa and others 2011)
Synthetic wine	Fe ³⁺ -quercetin	Pepsin-HCl, pH 2.0, 30 min	84 to 90	Pancreatin, pH 7.5, 2 h	41 to 45	This work

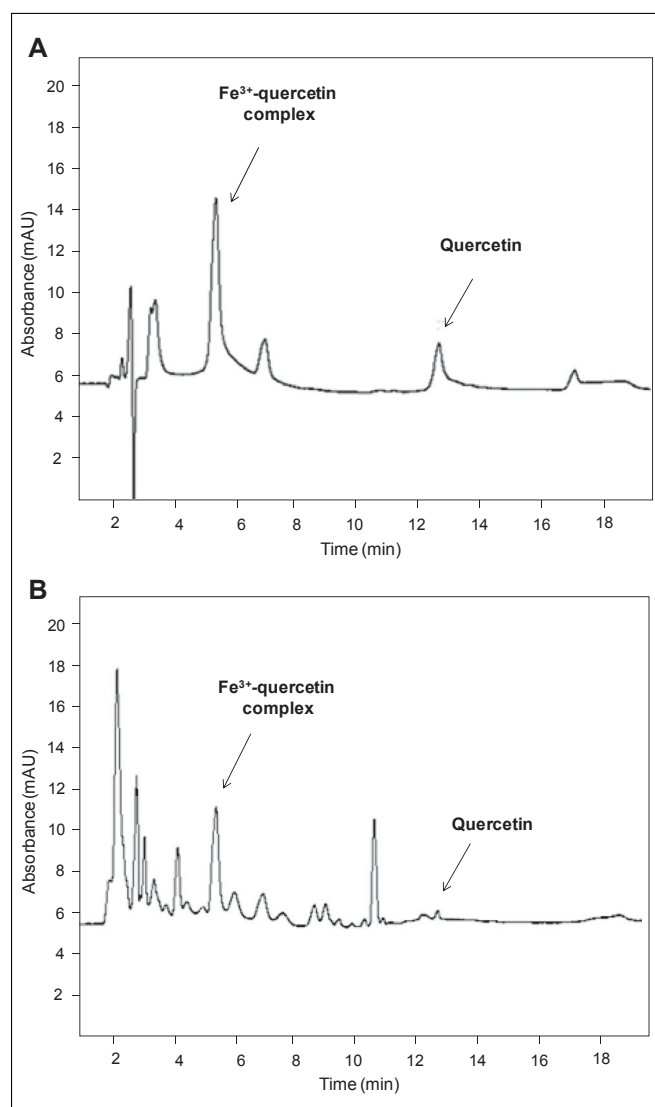


Figure 3—Chromatogram obtained before (A) and after (B) the *in vitro* gastrointestinal digestion processes showing the stability of the Fe³⁺-quercetin complex under these conditions.

peak at 4.8 min for the Fe³⁺-quercetin complex and another at 12.6 min for free quercetin polyphenol were observed (Figure 3A). In accordance with data by others, quercetin was found to be completely degraded after the simulated gastric digestion step (Siracusa and others 2011), as confirmed by the tiny peak observed on the chromatogram (Figure 3B). In this work, the peak at 4.8 min was collected before and after gastric digestion and elemental measurements were performed by ETAAS. No marked difference in metal concentration was found for both stages. Iron concentration after the gastric digestion was barely lower than that of the initial stage. The difference in iron concentration can be directly related to a decrease of Fe³⁺-quercetin complex concentration. Thus, it could be observed that the Fe³⁺-quercetin complex was highly recovered in the gastric phase (84% to 90%), indicating an important degree of stability for the complex under these conditions. This finding shows some relation with results obtained in other works reporting on the stability of flavonols, but also it is indicating a potential effect of Fe³⁺ on quercetin stability under gastric conditions. Table 3 shows a comparison on chemical stability of flavonols and Fe³⁺-quercetin complexes subjected to *in vitro* gastric digestion. Thus, the comparison of chromatograms shown in Figure 3A and B with those mentioned in Table 3, allows us to propose that Fe³⁺ might play a potential role on chemical stability of quercetin when this polyphenol chelates the metal. On the other hand, free quercetin was highly decomposed under gastric conditions (Figure 3B), which is evidenced by significant reduction of peak eluted at 12.6 min.

Stability of Fe³⁺-quercetin complex under *in vitro* intestinal digestion conditions

Since the Fe³⁺-quercetin complex showed to be resistant to acid hydrolysis of the stomach, this compound was submitted to the subsequent step. In order to determine the stability under simulated intestinal conditions, the synthetic wine sample was subjected to an *in vitro* process and analyzed before and after the intestinal step. Although the complexes under study were partially degraded during the intestinal digestion, 41% to 45% of the Fe³⁺-quercetin complexes were recovered after this mild alkaline process.

The aforementioned result agrees with the fact that most flavonols are degraded during incubation with pancreatin-bile salts at neutral or slightly alkaline pH. In Table 3, a comparison between the stability of some flavonols submitted to *in vitro* intestinal digestion is shown. Thus, it can be observed that there is an

important loss of flavonols for most samples regardless of simulated intestinal conditions.

Up till now, no previous studies on Fe³⁺–quercetin chemical stability under simulated *in vitro* gastrointestinal conditions has been reported. Our results are suggesting that the Fe³⁺–quercetin complex is stable under acidic conditions in the stomach but is partially degraded under alkaline intestinal conditions. However, as information available in the literature shows discrepancies between *in vitro* and *in vivo* results, the *in vitro* methods need to be further validated with *in vivo* studies using animals or humans.

Dissociation studies with elemental-specific detection

As stated above, the concentration of the Fe³⁺–quercetin complex was evaluated before and after *in vitro* gastric and intestinal digestions by HPLC–UV–Vis and ETAAS techniques. ETAAS analysis was performed in order to evaluate possible dissociation of the Fe³⁺–quercetin complex. Basically, during HPLC analysis all peaks eluted from the chromatographic column were collected, the fractions acidified, and finally injected into the graphite furnace of an ETAAS instrument. In agreement with previous results in this work, the presence of Fe was not observed in the collected fractions, with the exception of peak corresponding to the Fe³⁺–quercetin complex. This approach confirmed the presence of the metal–polyphenol complex at 4.8 min retention time. Furthermore, additional studies were developed to reject the possibility that the column could permanently retain the metal during separation. Thus, 1 mg/L Fe³⁺ standard solutions were injected in the HPLC system and the elution fraction was collected and analyzed by ETAAS. It was observed that the metal was not retained in the chromatographic column, since it was completely eluted (100% recovery achieved) at its proper retention time.

Besides the aforementioned study, Fe³⁺ determination was evaluated in the presence of the digestion solutions to check for possible matrix interference during ETAAS analysis. Thus, a recovery study was developed for method validation. Synthetic wine solutions submitted to gastric or intestinal digestion were spiked at 0.5 mg/L Fe³⁺ and measured by ETAAS. The results showed recoveries in the range of 97.5 to 101.8% for Fe³⁺, meaning that our method is reliable for the analysis of Fe³⁺ before and after the *in vitro* gastrointestinal digestion in synthetic wines. Therefore, Fe³⁺ determination by ETAAS was feasible even in the presence of a complex matrix and turned out to be a useful analytical tool to complement HPLC–UV–Vis studies.

Conclusions

This study provides insight into the stability of the Fe³⁺–quercetin complex in wine under simulated gastrointestinal conditions. The results demonstrated that the complex is substantially stable, even when it was subjected to conditions mimicking those that occur in the stomach. Although the complex was partially degraded during the intestinal digestion process, a recovery of 41% to 45% was obtained after this stage. One relevant factor affecting the stability of this complex in the intestinal digestion could be the mild alkaline environment. However, additional studies are needed to better understand the factors influencing this process. Finally, this work presents further analytical pathways to explore the stability of the Fe³⁺–quercetin complex under *in vitro* gastrointestinal digestion with real wine samples.

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