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In Vitro Gastrointestinal Digestion Study of Broccoli Inflorescence Phenolic Compounds, Glucosinolates, and Vitamin C

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Broccoli (*Brassica oleracea* L. var. *italica* cv. Marathon) inflorescences are a good source of bioactive compounds, such as phenolics (flavonoids and hydroxycinnamoyl derivatives), glucosinolates, and vitamin C. In this work, these health-promoting compounds were submitted to digestion under in vitro gastrointestinal conditions (pH, temperature, enzyme, and chemical conditions). This technique differentiated among the compounds associated with macromolecules in soluble and insoluble form and those that are freely soluble. In addition, it evaluates the chemical stability of the broccoli compounds under simulated physiological conditions. The gastric digestion of broccoli caused high losses in glucosinolates (69% loss), whereas phenolics and vitamin C presented higher stability under these conditions. Thus, there were no losses in flavonoids, a 7% loss of vitamin C, and a variable rate of loss (6−25%) in hydroxycinnamic acid derivatives. The stability of all of the compounds was affected by the in vitro intestinal conditions. Under the in vitro conditions, flavonoids and hydroxycinnamoyl acid derivatives were of low availability, due to their significant losses under these conditions, at the end of the experiment (84 and 80% loss, respectively). Vitamin C was the metabolite that showed the greater decrease after intestinal digestion (91% loss). Regarding the remaining glucosinolates, these compounds presented higher stability under intestinal conditions, rendering an availability similar to that found for phenolics (75% loss). Therefore, broccoli components were affected by gastric and/or intestinal conditions depending on the type of compound. Thus, glucosinolates were mainly degraded by gastric conditions, whereas phenolic compounds and vitamin C were degraded by intestinal conditions.

KEYWORDS: Broccoli (*Brassica oleracea* L.); availability; phenolic compounds; glucosinolates; vitamin C; in vitro gastrointestinal digestion

INTRODUCTION

Fresh broccoli is a major *Brassica* crop consumed as a part of the human diet. Thus, in 1998 the two major consumers of fresh broccoli, the United States and Canada, reached values slightly higher than 3.0 kg/year per capita (*1*), whereas in the European Union the values were almost 2.5 kg/year per capita (*2*). In addition, the fresh-market broccoli average consumption began increasing strongly (1.8%) in the early 1990s and continues to increase today, so that it has reached >3 times the average from the early 1980s (*3*, *4*). Due to clear health benefits, increased consumption of fruits and vegetables is recommended, especially vegetables such as broccoli, which contains indole-3-carbinol, sulforaphane, different flavonoids, hydroxycinnamoyl acid derivatives, vitamin C, and other compounds that play an important role in the maintenance of health and disease prevention (*5*, *6*). In addition, there is clear epidemiological evidence of the protective effect of glucosinolates, and to a lesser extent polyphenols, against important human cancers (*7*). The contribution of dietary flavonols, to provide health benefits, may be related to their high antioxidant activity (*8*). Dietary vitamins C, E, and A are important in an optimal diet due to their free radical scavenging activities and play important roles in human nutrition (*9*, *10*). In addition, vitamin C exerts protective effects against mutagenic disorders, as the prevention of carcinogenic nitrosamine formation in the stomach is another protective mechanism attributed to vitamin C (*11*).

Very little is known about the fate of broccoli bioactive compounds, such as phenolic compounds, glucosinolates, and vitamin C, during the digestion process, and particularly their fate in the food matrix under the gastric and intestinal conditions and the effect of these conditions on the stability of these compounds. In the present study, an in vitro gastrointestinal digestion method was used in order to simulate physiological conditions of the stomach and small intestine (pH, temperature,

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and enzyme conditions) (*12*). Two fractions, dialyzed and nondialyzed, are obtained after digestion. The dialyzed fraction contains free soluble phenolics and glucosinolates available to be absorbed in the small intestine and able to cross the membrane by passive diffusion, as chemical by in vitro gastrointestinal studies with soluble flavonols and caffeic acid derivatives of strawberries, onions, and tea, which were available for absorption, whereas the insoluble fraction of flavonols and hydroxycinnamic acid derivatives remains unavailable (*12*−*15*), although they could be metabolized by the gut microflora (*16*). The nondialyzed fraction includes phenolics and glucosinolates present in the soluble and insoluble fractions associated with macromolecules (proteins, fiber. etc.) that could be metabolized by the gut microflora and, therefore, they would not be available in the small intestine. The methodology here described is used, as far as we are aware, for the first time for the evaluation of the availability of phenolic compounds, glucosinolates, and vitamin C of broccoli (cv. Marathon) inflorescences.

MATERIALS AND METHODS

Material. Broccoli (*Brassica oleracea* L. var. *italica* cv. Marathon) used for the assays were obtained from the Centro de Investigacion y Desarrollo Agroalimentario (CIDA, Murcia, Spain). Sowing date was December 20, 2003. Seedlings were transplanted 30 days after sowing, in one split plot of Finca Torreblanca (southeastern Spain, La Alberca, Murcia). Harvest occurred between 60 and 62 days after planting. Water and pesticides were applied according to standard cultural practices in La Alberca (Murcia). The plot was situated on a clay soil. Before transplanting, the soil was fertilized with 150 kg/ha supplied in the form of ammonium nitrate, 75 kg/ha of P_2O_5, and 200 kg/ha of K_2O; S was applied as calcium sulfate (13% S). Three subplots (size = 15 × 20 m) for each cultivar were used for the statistical design. At commercial maturity, nine uniform size inflorescences comprising three replicates of three inflorescences, free from insect and/or mechanical damage, were randomly harvested and immediately transported to the laboratory for analysis. Subsamples of 20 g from each plant per replicate were combined, weighed, frozen at −70 °C, and freeze-dried. This tissue was ground into a fine powder and stored at −20 °C for further analysis.

In Vitro Gastrointestinal Evaluation of Health-Promoting Compounds. The method described by Gil-Izquierdo et al. (*12*) was used for the determination of the release of broccoli compounds and their behavior and stability under gastrointestinal conditions. With this method it is possible to differentiate between the compounds associated with macromolecules in soluble and insoluble forms and the free soluble compounds, which are likely to be dialyzed. It consisted of a pepsin−HCl digestion for 2 h (simulating gastric conditions) and pancreatin digestion with bile salts for 2.5 h (simulating small intestine conditions). For the pepsin−HCl digestion, samples of broccoli (40 g) were homogenized in 60 mL of Milli-Q purified water (Millipore, Molsheim, France) in an Ultra-Turrax T-25 (Jankel & Kunkel, Staufen, Germany) at 8300*g* for 1 min. For HPLC analysis 30 g of the homogenate was centrifuged at 11400*g* for 10 min at 4 °C (Beckman centrifuge model J2-21) and filtered through a 0.45 μm membrane (type Millex HV13, Millipore), prior to injection. This sample was used as pattern.

An aliquot of the homogenate (40 g) per replicate was added to 31500 units of pepsin (EC 3.423.1; Sigma). The pH was adjusted to 2 by addition of concentrated HCl, and the samples were incubated in a 37 °C shaking water bath (J. P. Selecta, Barcelona, Spain) for 2 h under unbuffered conditions (in order to simulate postconsumption conditions in the stomach). For HPLC analysis, 15 g of the broccoli pepsin digest were centrifuged at 5000*g* for 5 min (centrifuge model Centronic, J. P. Selecta). Supernatant and pellet were separated, extracted, and analyzed by using the technique described below.

After the pepsin digestion (2 h), 20 g of the pancreatin digest, together with segments of cellulose dialysis tubing containing 25 mL of water and $NaHCO_3$ (Sigma), was placed into a 220 mm long polyethylene tube (diameter = 40 mm) to follow the method previously reported (*12*). This digestion generated two phases, a soluble dialyzed and an insoluble nondialyzed or retentate soluble and insoluble part. All of the compounds present in the pancreatin−bile extract were analyzed by HPLC. The dialyzed fraction was directly injected in the HPLC, whereas 10 g of the nondialyzed fraction or retentate was centrifuged at 5000*g* for 5 min in a centrifuge (model Centronic, J. P. Selecta). The flavonoid, glucosinolate, and vitamin C contents of the supernatant and pellet were analyzed by HPLC. All compounds were identified by their chromatographic behavior and UV spectra, HPLC-ESIMS, and chromatographic comparisons with authentic markers.

Extraction and Determination of Phenolic Compounds. Extraction procedures followed the method previously described for phenolic compounds (*17*). Samples of the homogenated fraction (20 μL) were analyzed on a Merck-Hitachi liquid chromatograph equipped with a pump (model L-6200) and a UV−vis detector (model L-7420). Separations were achieved on a LiChroCART column (Merck, Darmstadt, Germany, ODS-18, 25 × 0.4 cm; 5 μm particle size). The mobile phase was water/formic acid (95:5, v/v) (A) and methanol (B). The flow rate was 1 mL/min, and a linear gradient, starting with 10% B to reach 15% B at 5 min, 30% B at 20 min, 50% B at 35 min, and 90% B at 40 min, was used. Chromatograms were recorded at 320 and 360 nm. Caffeoylquinic acid derivatives were quantified as chlorogenic acid (5-caffeoylquinic acid, Sigma, St. Louis, MO), flavonoids as quercetin 3-rutinoside (Sigma), and sinapic acid derivatives as sinapic acid (Sigma). The results were expressed as milligrams per 100 g of fresh weight broccoli.

Glucosinolate Analysis. For extraction and desulfation of glucosinolates was followed the procedure as described by Vallejo et al. (*19*). Desulfoglucosinolate content was determined according to the method described by Kiddle et al. (*18*). Each sample (20 μL) was analyzed on a Merck-Hitachi HPLC system (Merck-Hitachi Ltd., Tokyo, Japan) consisting on a variable UV detector set at 227 nm and a Lichosphere RP-18 column (Merck) (RP-18, 25 × 0.4 cm; 5 μm particle size). The mobile phase was a mixture of water (A) and acetonitrile (B). Desulfoglucosinolates were eluted off the column in 30 min. The flow rate was 1.5 mL/min in a linear gradient starting with 1% B and reaching 20% B at 28 min. Glucosinolates were identified by their UV spectra, HPLC-ESIMS, and chromatographic comparisons with authentic markers (samples supplied by R. Bennett, IFR, Norwich, U.K.). The results were expressed as milligrams per 100 g of fresh weight broccoli.

Extraction and Determination of Vitamin C. Ascorbic acid (AA) and dehydroascorbic acid (DHAA) contents were determined according to the method of Gil et al. (*20*). HPLC analysis of vitamin C (AA + DHAA) was achieved after derivatization of DHAA into the fluorophore 3-(1,2-dihydroxyethyl)furo[3,4-*b*]quinoxalin-1-one (DFQ), with 1,2-phenylenediamine dihydrochloride (OPDA). Samples (20 μL) were analyzed with a Merck-Hitachi (Tokyo, Japan) liquid chromatograph equipped with an L-4000 UV detector and an L-6000 pump. Separations of DFQ and AA were achieved on a Kromasil 100 C-18 column (25 × 0.4 cm; 5 μm particle size; Tecnokroma, Barcelona, Spain). The mobile phase was methanol/water (5:95, v/v) containing 5 mM cetrimide and 50 mM potassium dihydrogen phosphate at pH 4.5 set at a flow rate of 0.9 mL/min. The detector wavelength was initially set at 348 nm and, after elution of DFQ, was manually shifted to 261 nm for AA detection. Standard solutions, column conditioning, and derivatization procedures have been previously described (*19*). The results were expressed as milligrams per 100 g of fresh weight broccoli.

HPLC-ESIMS was performed according to a previous work (*17*) using an Agilent HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump G1312A, autosampler G1313 A, photodiode array detector G1315B, controlled by Agilent software v. A.08.03, and degasser G1322A, under the same chromatographic conditions as described above for HPLC analyses.

RESULTS AND DISCUSSION

Flavonoid Stability of Broccoli after in Vitro Gastrointestinal Digestion. Five to 10 individual flavonoids (depending on the samples) were detected, mainly quercetin and kaempferol

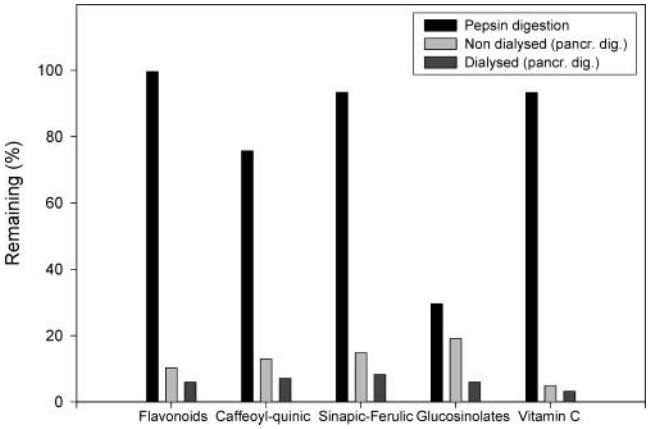

Figure 1. Remaining flavonoids, caffeoylquinic, sinapic, and ferulic acid derivatives, glucosinolates and vitamin C concentration, after pepsin digestion, and the dialyzed and nondialyzed fractions after intestinal digestion, expressed as percentage of broccoli homogenized fraction.

glycosides, which is in agreement with previous studies on broccoli florets (*21*). Other acylated derivatives were also present.

Flavonoid contents of the different samples before and after digestion are shown in **Figure 1**. After in vitro gastric digestion, flavonoid levels showed no losses in comparison to initial fraction (18 mg/100 g) after broccoli homogenization, indicating that pepsin digestion had no effect on flavonoid stability. This was in agreement with previous reports on orange juice phenolics, in which the flavanones, the major flavonoids in this product, were not affected after pepsin digestion (*12*).

On the other hand, after the pancreatin−bile salt digestion (simulation of small intestine digestion) of either dialyzed or nondialyzed fractions was observed, significant decreases in flavonoid concentration of 94.1 and 89.7%, respectively, with regard to the initial concentration were found (**Figure 1**). Thus, the general trend after gastrointestinal digestion is a large decrease of total flavonoid level, as previously reported for other food products (*12*, *22*). It is possible that the pancreatin digestion liberates compounds (macromolecules as proteins and fiber) able to associate with flavonoids. These flavonoids could not cross the dialysis membrane remaining in the retentate (nondialyzed fraction).

Hydroxycinnamoyl Acid Derivative Stability. As previously described (*17*, *23*), HPLC analysis of broccoli (*B. oleracea* L.) showed the presence of two caffeoylquinic derivatives, neochlorogenic acid (86%) and chlorogenic acid (14%), and seven sinapic and ferulic derivatives, as follows: 1,2-disinapoylgentiobiose (10.7%), 1-sinapoyl-2-feruloylgentiobiose (18%), 1,2-diferuloylgentiobiose (23.8%), 1,2,2′-trisinapoylgentiobiose (18%), 1,2′-disinapoyl-2-feruloylgentiobiose (19.7%), 1-sinapoyl-2,2′-diferuloylgentiobiose (4.1%), and 1,2,2′-trisinapoylgentiobiose (5.7%). The total amounts of caffeoylquinic derivatives and sinapic and ferulic derivatives, after broccoli homogenization, were 3.5 and 6.1 mg/100 g, respectively.

The hydroxycinnamoyl derivative content of the different fractions is shown in **Figure 1**. In this case, after in vitro gastric digestion, total and individual hydroxycinnamic acid derivatives levels showed decreases that represented losses between 24.3% for total caffeoylquinic derivatives and 6.6% for total sinapic and ferulic derivatives. With regard to individual compounds, similar loss rates were found for total sinapic and ferulic derivatives. Therefore, pepsin digestion had a significant effect on the composition of the broccoli caffeoylquinic acid derivatives, decreasing their concentration, whereas this effect was small for sinapic acid derivatives.

When the concentrations were analyzed after the pancreatin−bile salt digestion, the total dialyzed caffeoylquinic derivative fraction represented only 7.1%, and the nondialyzed 12.9%, of the initial concentration (**Figure 1**), whereas the total dialyzed sinapic and ferulic derivative fraction represented only 1.8% and the nondialyzed 8.2% of the initial concentration, indicating that a high loss of these compounds was produced after intestinal digestion, following the same trend as that showm for flavonoids. This high loss could be associated with the instability of the caffeoylquinic acids in aqueous solution (*24*). In addition, the pH value of 7.5 and the bile salts could contribute to increase this loss.

In relation to individual compounds great differences were shown, chlorogenic acid and 1,2-diferuloylgentiobiose being the metabolites that presented lower losses (only 10% of the initial concentrations), in the dialyzed fraction, whereas neochlorogenic acid was the most unstable compound, reaching a loss of >94%. The compounds found in smaller amounts, identified as 1-sinapoyl-2,2′-diiferuloylgentiobiose and 1,2,2′-trisinapoylgentiobiose, were not detected after intestinal digestion.

Glucosinolate Stability. In agreement with previous reports (*25*−*27*), seven glucosinolates were found in broccoli inflorescences, namely, progoitrin (11.5%), glucoraphanin (25.2%), glucoalyssin (19.7%), gluconapin (13.1%), 4-hydroxyglucobrassicin (2.8%), glucobrassicin (17%), and 4-methoxyglucobrassicin (10.7%), representing a total amount of 47.7 mg/100 g of fresh weight. In contrast to what has been described above for phenolic compounds, and following a different trend, after in vitro gastric digestion, total glucosinolate values showed a significant decrease (69% loss) (**Figure 1**). Aliphatic glucosinolates were much more stable than indolic derivatives; only 27% was lost on the gastric digestion in contrast to indolic, which suffered losses of 73%. Therefore, pepsin digestion had a significant effect on the stability of broccoli glucosinolates.

When the effect of the pancreatin−bile salts was analyzed, an additional decrease in total glucosinolate concentration was observed. Thus, after whole gastrointestinal digestion, the total dialyzed glucosinolate fraction represented only 6% of the initial concentration and the nondialyzed, 19%. Hence, we can conclude that, even if the remaining fractions after gastrointestinal digestion were similar to those found for phenolic compounds, glucosinolates were less stable under gastric conditions than the phenolics.

With regard to the individual glucosinolates, 4-methoxyglucobrassicin was the compound that reached the highest dialyzed concentration (12.3%). After intestinal digestion, no 4-hydroxyglucobrassicin was found.

A possible explanation for the high percentage of loss during digestion could be the high degradation of these compounds to nitriles at gastric pH (≅3) (*28*). Also, the low stability of glucosinolates at intestinal pH (≅7) led to a decomposition into their secondary reaction products (isothiocyanates) (*28*).

Vitamin C Stability. When the availability of this vitamin was analyzed (**Figure 1**), a slight loss (6.7%) was observed after pepsin digestion, in relation to initial concentration (63.8 mg/100 g), following the same trend as flavonoids and sinapic and ferulic acid derivatives. These results are in agreement with previous findings (*22*), which proved that in vitro gastric conditions (pH 2 or 3) had very little effect on vitamin C stability.

Nevertheless, after in vitro intestinal digestion, there were significant decreases in vitamin C concentration in both dialyzed

and nondialyzed fractions of 96.8 and 95.1%, respectively, which is in agreement with previous results for orange and pomegranate juice (*12*, *22*), due to the low stability of this compound at high pH. Thus, the performance of vitamin C was similar to that of phenolic compounds and in contrast to that of glucosinolates.

Conclusions. In general, we can conclude that these three classes of health-promoting compounds present in broccoli inflorescences were clearly affected by in vitro gastrointestinal conditions, suffering a severe reduction dependent on the type of compound. Flavonoids and vitamin C were less affected during HCl−pepsin digestion (0 and 7% losses, respectively), whereas glucosinolates were highly affected by these gastric conditions (69% loss). On the contrary, the remaining glucosinolates were only slightly affected by the intestinal digestion conditions (12% additional loss), whereas the remaining phenolics and vitamin C were highly degraded (>70% additional loss).

Finally, it is remarkable that quantitatively the availability of total glucosinolates (2.8 mg/100 g), under in vitro gastrointestinal conditions, was almost 3-fold higher than the availability of total flavonoids (1 mg/100 g) and close to 4-fold higher than the availability of hydroxycinnamoyl derivatives (0.8 mg/100 g) in broccoli, whereas the availability of vitamin C was similar to that of glucosinolates (2.2 mg/100 g).

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