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In Vitro Gastrointestinal Digestion Study of Pomegranate Juice Phenolic Compounds, Anthocyanins, and Vitamin C

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Pomegranate is an important source of bioactive compounds, such as anthocyanins, other phenolic compounds, and ascorbic acid. In the present work an in vitro availability method has been used to assay the influence of the physiological conditions in the stomach and small intestine. This method enables the study of the release of anthocyanins, vitamin C, and total phenols from the pomegranate juice and their transformations during gastrointestinal digestion. Results have shown that pomegranate phenolic compounds are available during the digestion in a quite high amount (29%). Nevertheless, due to pH, anthocyanins are largely transformed into non-red forms and/or degraded (97%), and similar results are obtained for vitamin C (>95% degradation).

KEYWORDS: Availability; pomegranate, juice; vitamin C; phenolic compounds; anthocyanins

INTRODUCTION

In the past few years phenolic compounds in general and anthocyanins in particular, as well as vitamin C, have attracted interest once more because of their safety and potential nutritional and therapeutic effects.

In general, epidemiological studies show that consumption of fruits and vegetables with high phenolic content correlates with reduced cardio- and cerebrovascular diseases and cancer mortality (*1*, *2*). Among fruits, pomegranate is an interesting rich source of anthocyanins and other phenolic compounds, with a demonstrated antioxidant activity (*3*). The presence of vitamin C has also been reported in this fruit (*4*). The potent antiatherogenic effects of pomegranate juice have been recently demonstrated in healthy humans and in atherosclerotic mice (*5*−*7*). Also, the antioxidant and antitumoral activity of pomegranate bark tannins (punicacortein) (*7*, *8*) and the antioxidant activity of the fermented pomegranate juice (*9*) have been reported. Pomegranate juice has important clinical implications, and it has even been recommended in the treatment of acquired immune deficiency syndrome (AIDS) (*10*) owing to the fruit rich concentration of diverse bioflavonoids and to their known free radical scavenging activity and inhibition of lipoxygenase. Furthermore, pomegranate is one of nine herbs included in a Japanese-patented formula for treating AIDS (*11*).

Nevertheless, very little is known about the fate of pomegranate juice bioactive compounds (vitamin C and phenolic compounds) during the digestion process, if they are released from the food matrix under the physiological conditions occurring in vivo, and the effect of these conditions on the stability of these compounds. In the present work an in vitro technique (*12*), similar to other methods previously used on phenolic compounds (*13*, *14*), has been chosen as it allows rapid, inexpensive preliminary trials to be conducted on phenols and vitamin C availability without the dangers and difficulties involved with human absorption studies. The methodology here described is used, as far as we are aware, for the first time for the evaluation of the in vitro availability of anthocyanins, total phenols, and vitamin C of pomegranate (cv. Mollar) juice.

MATERIALS AND METHODS

Sample. Sweet pomegranate juice was produced from cv. Mollar pomegranates, harvested in Albatera (Alicante, Spain; Cooperativa "Los Chiguitos") during October 2000. The juice (pH 3.8) was obtained by pressure with a laboratory pilot press (Zumonat C-40). This juice was stored frozen (−20 °C) until analyzed. Prior to analysis, juice was thawed at room temperature.

In Vitro Availability Analysis. A gastrointestinal digestion study was performed with the technique developed by Gil-Izquierdo et al. (*12*), as a modification of Miller's method (*15*). The technique consisted of a first pepsin−HCl digestion for 2 h (to simulate gastric digestion) and a pancreatin digestion with bile salts for 2.5 h, both at 37 °C (to simulate small intestine conditions). For the pepsin−HCl digestion, samples of pomegranate juice (50 mL) were added to 1600 units of pepsin (EC 3.423.1; Sigma, Steinheim, Germany) (1 unit of pepsin will produce a ΔA_{280} of 0.001 per minute at pH 2.0 at 37 °C, measured as TCA-soluble products using hemoglobin as substrate). The pH was adjusted to 2 by the addition of concentrated HCl, and the samples were incubated in a 37 °C shaking water bath (J. P. Selecta S.A., Barcelona, Spain) for 2 h.

Following the pepsin digestion, to simulate small intestine conditions, the pepsin digest (20 mL), together with segments of cellulose dialysis tubing (molecular weight cutoff of 12000 Da; Sigma) containing 25 mL of water and the amount of $NaHCO_3$ (Sigma) equivalent to the titratable acidity measured previously (see below), was placed into a 220 mm long polyethylene tube (diameter = 40 mm). This tube was closed on the top and the bottom ends. In addition, a 150 mm long

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silicone tube was introduced to monitor the pH and add the pancreatin−bile extract when the pH values reached 5.0 (*12*). This tube was closed on the top to avoid contamination and evaporation of the sample during the experiment. Replicates of the filled polyethylene tubes ($n = 3$) were placed into a 500 mL beaker containing distilled water at 37 °C, leaving the top part outside the water to enable pH monitoring and pancreatin−bile extract addition. The beaker was placed in a water bath (J. P. Selecta S.A.) and gently shaken (50 rpm) at 37 °C. Five milliliters of pancreatin mixture [4 g/L; 1.6×10^{-3} U.S. Pharmacopeia (USP) specifications (Sigma)] was added to each tube, and incubation was continued for an additional 2 h. At the end of the incubation period the dialysis tubes were removed and rinsed with distilled water, and the dialysates were weighed and analyzed.

Titrable acidity was determined on a 20 mL aliquot of pepsin digest to which 5 mL of the pancreatin−bile extract mixture was added. Titrable acidity was defined as the number of equivalents of $NaHCO_3$ required to titrate the combined pepsin digest pancreatin−bile extract mixture to pH 7.5 (0.5 N $NaHCO_3$ was used in the titration).

Anthocyanins, phenolic compounds, and vitamin C, present in the fresh pomegranate juice, pepsin digest, and dialyzed and nondialyzed fractions of pancreatin−bile digest, were analyzed as specified below.

Qualitative and Quantitative Analysis of Anthocyanins. The fresh pomegranate juice and the different batches obtained after the in vitro gastrointestinal digestions were filtered through a 0.45 μm filter (type Millex HV13, Millipore Corp., Bedford, MA). Analyses were performed with a Merck-Hitachi L-6200 intelligent pump (Darmstadt, Germany) chromatograph, equipped with a Merck-Hitachi UV−vis detector L-7420 and autoinjector Merck-Hitachi AS-2000 A. Chromatograms were recorded and processed on a D-2500 Chromato-Integrator (Merck-Hitachi). Each sample (20 μL) was analyzed on a Lichrochart 100 RP-18 reversed-phased column (25 × 0.4 cm, particle size = 5 μm) using a mobile phase of 5% formic acid (v/v) (solvent A) and methanol (solvent B). Elution was performed at a flow rate of 1 mL/min using a gradient starting with 15% B, increasing to 35% B at 15 min, to 40% B at 25 min, to 60% B at 30 min, and isocratic elution at 98% B for 5 min. Detection was achieved at 520 nm. All extractions were done in triplicate and results expressed as the mean value. Stardard errors were all within 1% of the mean value.

The different anthocyanins were identified by their UV−vis spectra recorded with a diode array detector, by their mobility on HPLC, and by chromatographic comparison with authentic markers (Polyphenols AS). The different anthocyanins were quantified by their peak areas in the chromatograms by comparison with an external standard of cyanidin 3-rutinoside (Apin Chemicals). Total anthocyanins was calculated by the addition of the amounts of the different anthocyanins detected in each analysis (*16*, *17*).

HPLC Analysis of Vitamin C. Ascorbic acid (AA) and dehydroascorbic acid (DHAA) contents were determined as described by Zapara and Dufour (*18*). The HPLC analysis was achieved after derivatization of DHAA into the fluorophore 3-(1,2-dihydroxyethyl)-furo[3,4-*b*]quinoxaline-1-one (DFQ), with 1,2-phenylenediamine dihydrochloride (OPDA).

Prior to HPLC analyses, samples (10 mL) were acidified to pH 2.2−2.4 with HCl. The solutions were centrifuged at 12000 rpm for 5 min. The supernatant solutions (5 mL) were adsorbed onto a previously activated (with methanol, water, and air) C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA) for solid phase extraction (SPE). The water soluble fraction (750 μL, after the first 3 mL had been rejected) was treated with OPDA (250 μL) as in Zapata and Dufour (*18*).

Detection was achieved 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 (250 × 4 mm; 5 μm particle size, Technokroma, 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. The flow rate was 0.9 mL/min. The detector wavelength was initially set at 348 nm, and after DFQ elution, it was shifted to 261 nm for AA detection. Vitamin C was expressed as the addition of AA and DHAA.

The measures were done in triplicate and results expressed as the mean value. Standard errors were all within 1.5% of the mean value.

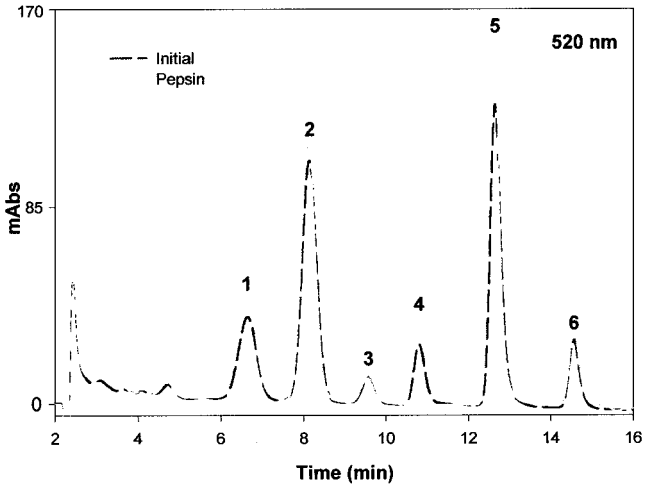

Figure 1. Chromatograms of anthocyanins before and after pepsin digestion: 1, delphinidin 3,5-diglucoside; 2, cyanidin 3,5-diglucoside; 3, pelargonidin 3,5-diglucoside; 4, delphinidin 3-glucoside; 5, cyanidin 3-glucoside; 6, pelargonidin 3-glucoside.

Total Phenolic Compounds Analysis. Total soluble phenolic compounds were determined with Folin−Ciocalteu reagent according to the method of Singleton and Rossi (*19*). Dilutions of 1:10 for fresh juice, pepsin digest, dialyzed and nondialyzed fractions of pancreatin−bile digest were used and carried out per triplicate. Detection was achieved at 765 nm in a UV−vis spectrophotometer (SECOMAN, Anthelie Graphic model). Results were expressed as milligrams of gallic acid equivalents (GAE) per liter. The measures were done in triplicate and results expressed as the mean value. Standard errors were all within 1% of the mean value.

RESULTS AND DISCUSSION

In Vitro Anthocyanin Availability in Pomegranate Juice. HPLC analysis of pomegranate (*Punica granatum*) showed the presence of six anthocyanins, as previously described (*17*, *20*), namely, delphinidin 3-glucoside (5.1%), delphinidin 3,5-diglucoside (18.2%), cyanidin 3-glucoside (27.1%), cyanidin 3,5-diglucoside (41%), pelargonidin 3-glucoside (5%), and pelargonidin 3,5-diglucoside (3.4%). The total amount of anthocyanins was 141 mg/L of fresh juice.

During the in vitro simulation of human digestion and absorption a slight increase in anthocyanin concentration (10%) was observed after stomach digestion (**Figures 1** and **3**), due to a general increase in all of the individual anthocyanins, especially cyanidin and delphinidin glycosides. This would be due to the pH (pH 2) resulting after the pepsin digestion, lower than the fresh original juice (pH 3.8), rendering an increase of the flavylium cation in the solution.

After the pancreatin bile salt digestion (simulation of small intestine digestion), dialyzed and nondialyzed fractions were obtained and analyzed. A significant decrease in anthocyanin concentration was observed, due to a general decrease of all the individual anthocyanins, more marked for delphinidin glycosides (**Figure 2**). Thus, the total dialyzed anthocyanin fraction (**Figure 3**) represented only 2.4% (3.38 mg/L of juice), whereas the nondialyzed fraction was 15.3% (21.60 mg/L of juice). This decrease in the total amount could be partially explained by the transformation of the flavylium cation to the colorless chalcone at the pH of the medium (pH 7.5), as the colorless anthocyanin pseudobases are stable and exist in equilibrium with the colored cationic forms in acidic solutions, but at pH >5 anhydrobases become progressively more stable and increasingly formed until pH 12, when ring fission occurs

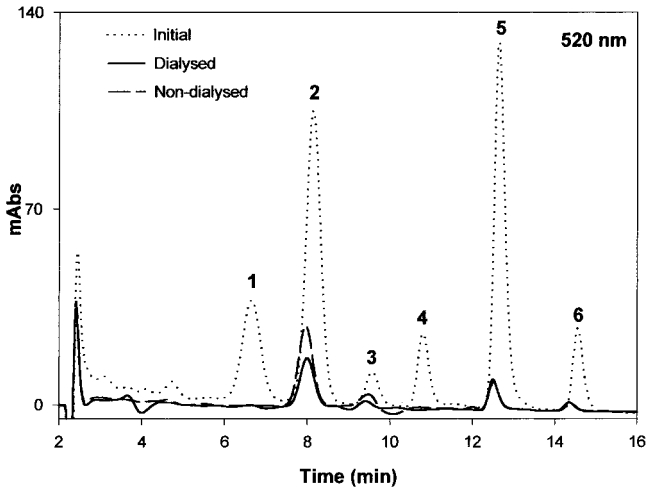

Figure 2. Chromatograms of anthocyanins before and after pancreatin bile salt digestion. Numbers are as in **Figure 1**.

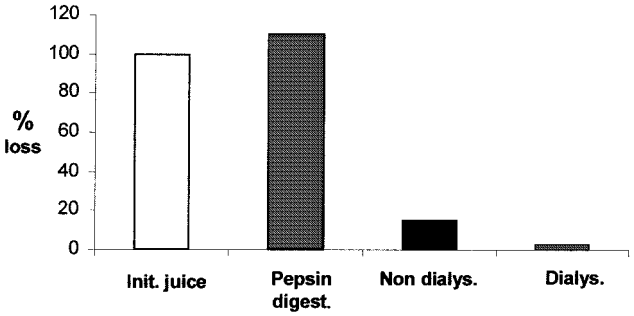

Figure 3. Total anthocyanin concentration of pomegranate fresh juice, after pepsin digestion, and values of the dialyzed and nondialyzed fractions of the pancreatin digest, expressed as percentage.

with formation of ionized chalcones. The initially formed unionized anhydrobases fade rapidly, but those of the 3-glucosides tend to be more persistent than anhydrobases of 3,5-diglucosides (*21*, *22*). This point was confirmed when the resulting fractions after pancreatin bile digestion were acidified (pH 2) and further analyzed by HPLC. Consequently, the dialyzed anthocyanin fraction increased to 22% (31 mg/L of juice), whereas the nondialyzed fraction increased to 48% (67.60 mg/L of juice). Nevertheless, it is important to take into consideration that the flavylium form would not be the predominant form in the human body after pancreatin digestion (due to high pH), but the other anthocyanins forms should be considered when other analyses are developed [e.g., antioxidant activity tested in nonacidic media (*23*)]. Still, the reason for the high loss of anthocyanins remains unknown, although the possibility was considered that part of the anthocyanins is metabolized to some noncolored forms, oxidized, or degraded into other chemicals, which would escape from the detection under the present conditions. It is also remarkable that no aglycons occurred after pancreatin bile digestion, in concordance with previous findings in which anthocyanins are absorbed in glycated forms indicating that no glycoside hydrolysis takes place during digestion (*24*−*26*). The results here found are also in concordance with other studies, in human serum, in which a low bioavailability of the ingested anthocyanins, with a quick degradation, oxidation, or excretion of the compounds, is described (*24*, *25*).

In Vitro Availability of Total Phenolic Compounds. No differences in total phenolics content (**Figure 4**) were evident when the concentrations before and after pepsin digestion (292 mg of GAE/L) were compared. When the concentration after

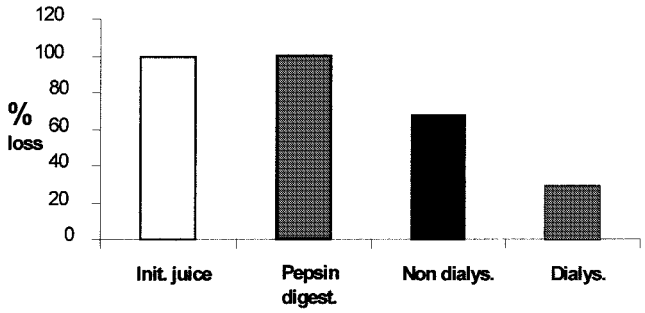

Figure 4. Total phenolic concentration of pomegranate fresh juice, after pepsin digestion, and values of the dialyzed and nondialyzed fractions of the pancreatin digest, expressed as percentage.

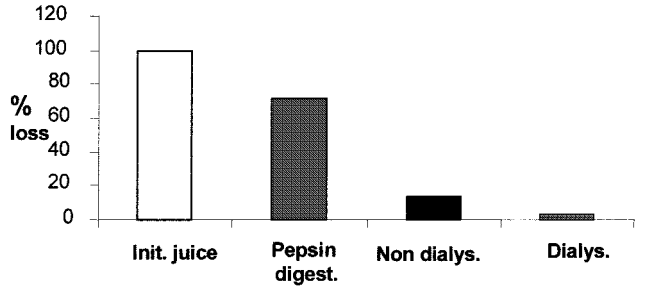

Figure 5. Vitamin C content of pomegranate fresh juice, after pepsin digestion, and values of the dialyzed and nondialyzed fractions of the pancreatin digest, expressed as percentage.

the pancreatin−bile salt digestion was analyzed, 29% of these compounds were present in the dialyzed fraction, whereas the rest remained in the nondialyzed fraction.

Even if these results show that the concentration of phenolic compounds in a soluble form available for absorption, under the conditions of the small intestine, is lower than in the fresh juice, it has to be taken into consideration that pomegranate juice is an important source of phenolic compounds with proven antioxidant activity (*3*), mainly due to the hydrolyzable tannins including punicalagins. These results are especially interesting as complementary to other studies in which it has been proved that the ellagitannins of pomegranate are hydrolyzed extensively in mice, leading to the excretion of ellagic acid in the feces and urine (*27*). Also, in a recent work, using this in vitro method, it has been reported that free ellagic acid of strawberries is released from ellagitannins, leading to an increase of this compound during digestion (*12*). Nevertheless, release of a compound during digestion does not necessarily indicate an antioxidant action in serum, as Caccetta et al. (*28*) have recently demonstrated that the consumption of red wine increased phenolics acids in plasma and serum uric acid concentrations, but this increase was insufficient to influence in vivo lipoprotein oxidation.

In Vitro Availability of Pomegranate Juice Vitamin C. Results showed that pomegranate juice could be a complementary source of vitamin C (50 mg/L), comparable to other fruits or vegetables such as apples, apricots, carrots, cherries, or peaches and better than plums or pears (*4*).

When the availability was analyzed (**Figure 5**), 29% loss was observed after pepsin digestion. Values similar to those found for anthocyanins were present in the dialyzed and nondialyzed fractions after the pancreatin bile salt digestion (loss >80%). These losses could be partly explained if the different pH values of the media and the presence of oxygen are taken into consideration. Ascorbic acid is a moderately strong reducing agent (and it is quite acidic). The molecule shows some unusual

and interesting chemical properties due to the enediol grouping. The dissociation constants are $pK_1 = 4.17$ (at the 3-OH) and $pK_2 = 11.57$ (at the 2-OH).

In aqueous solutions ascorbic acid has a high affinity for oxygen. It is readily oxidized to give DHAA, a reaction that is catalyzed by heavy metal ions. This DHAA can be reduced back to AA. In the presence of oxygen DHAA is irreversibly degraded to diketogulonic acid. In the absence of oxygen a different series of final products have been identified: at lower pH values 2-hydroxyfurfural and carbon dioxide predominate, whereas at a pH near the pK_1 of ascorbic acid, other 5-carbon compounds plus carbon dioxide have been found (*29*).

The results of this study indicate that the AA absorption would be incomplete after pomegranate juice consumption. This concentration would be much lower than what has been proposed (*30*) as the minimal RDA (75−90 mg per day, Dietary Reference Intake, 2000). According to these data, pomegranate juice would, of course, be insufficient itself to make up for this dosage but would contribute as much as many other food sources. It was suggested that AA is absorbed in humans by a specialized transport mechanism (*31*, *32*). Differences among individuals in the values of the parameters associated with this transport process may partially account for the variation noted in the extent of absorption. Furthermore, differences in physiological variables along the gut (e.g., gastric emptying rate) may contribute to this intersubject variation. Compounds transported by specialized processes are generally absorbed only at certain sites along the gastrointestinal tract. This has been illustrated for riboflavin, the absorption of which proceeds from the upper regions of the small intestine (*33*, *34*), and appears to be the case for AA as well (*35*). As a result, factors altering gastric emptying and intestinal transit rates (e.g., food and drugs) may influence the efficiency of absorption from the oral dosage form. Results obtained by Yung et al. (*36*) indicated that AA absorption is incomplete after oral ingestion and that there is considerable intersubject variation in the extent of absorption. The age and habits of the consumers are other influences that factor in absorption (*37*).

We can conclude that these results are quite valuable, due to the potential importance of the here studied compounds in humans health and the relatively high amount present in pomegranate juice. The obtained results indicate that anthocyanins, other phenolic compounds, and vitamin C, all of which demonstrated antioxidant capacity, would be bioavailable after digestion and might contribute to protecting humans from several diseases. Nevertheless, further work has to be carried out to determine the bioavailability of pomegranate juice anthocyanins and other phenolic compounds and their protective effect in humans health, because, as it has been said above, release of a compound during digestion does not necessarily signify an antioxidant action in vivo.

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