

Cocoa procyanidins are stable during gastric transit in humans¹⁻³

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ABSTRACT

Background: Polyphenolic procyanidins are abundant flavonoid polymers in Western diets. In vitro biological activity has been reported for these compounds, but activity in vivo depends on the amount and chemical nature of the flavonoids reaching the gastrointestinal tract. Degradation of procyanidins under simulated gastric conditions at pH 2.0 has been reported in vitro.

Objective: The objective was to examine whether depolymerization of procyanidins occurs in the stomach of human subjects in vivo.

Design: After an overnight fast, 6 healthy subjects (3 men and 3 women) consumed 500 mL of a cocoa beverage containing 733 mg procyanidin polymers and 351 mg structurally related flavanol monomers. With the use of a nasogastric tube, stomach contents were collected every 10 min after beverage ingestion until the stomach was emptied. Flavanols and procyanidins (up to pentamers) were quantified by normal and reversed-phase HPLC.

Results: In all subjects, gastric transit lasted \approx 50–60 min. No change in the HPLC profile of procyanidins was observed during this period, showing that procyanidins were remarkably stable in the stomach environment.

Conclusion: The results suggest that most ingested procyanidins reach the small intestine intact and are available for absorption or metabolism. *Am J Clin Nutr* 2002;76:1106–10.

KEY WORDS Cocoa, chocolate, flavonoid, procyanidin, flavanol, epicatechin, catechin, stomach, gastric pH, absorption

INTRODUCTION

Procyanidins are a class of polyphenolic polymers composed of flavan-3-ol units (catechin and epicatechin) (**Figure 1**). Red wine, cranberries, apples, tea, and cocoa or chocolate are among the richest food sources of procyanidins (1–6); however, their content is dependent on agricultural postharvest and food processing practices (7). Although the dietary intake of flavanols is still unknown because of their structural complexity, consumption of flavanols is estimated to vary from several tens to several hundreds of milligrams per day depending on the diet (8). Procyanidins are usually combined with flavanol monomers (catechins) in these estimations in food (5). In a detailed study of flavanol consumption in a representative sample of the Dutch population, chocolate contributed \approx 20% of the total flavanol intake, which was estimated at 50 mg/d, and epicatechin was the major compound (9). In chocolate, procyanidins composed largely of epicatechin units are 4–5 times more abundant than are epicatechin monomers and thus may account for a significant fraction of the amount of flavonoids ingested in the Western diet (10–12).

Procyanidins are of great interest in nutrition and medicine because of their potent antioxidant capacity and possible protective effects on human health in reducing the risk of chronic diseases such as cardiovascular diseases and cancers (8). In vitro, cocoa flavanols and procyanidins prevent LDL oxidation (13–15), suppress peroxynitrite-induced nitration of tyrosine (16, 17), and modulate platelet activation and enhance endothelium-dependent relaxation (18–20). To produce a biological effect in vivo, it is essential that sufficient quantities reach target tissues. The bioavailability of these compounds depends on gut absorption, which is determined in turn by the chemical structure and molecular weight of the compounds. Little is known about the absorption and uptake of the oligomeric procyanidins despite their common occurrence in the diet (21). Previous studies showed that epicatechin monomers are well absorbed, reaching a maximum plasma concentration 2–3 h after chocolate consumption (22, 23). Recently, procyanidin oligomers (trimers to hexamers) were shown to be degraded under in vitro conditions that simulated gastric juice (37 °C, pH 2.0, 1–4 h, no digestive enzymes) to mixtures of epicatechin monomers and dimers that could be absorbed once they reached the small intestine (24). To study the fate of procyanidins in the stomach in vivo, we had volunteers ingest a procyanidin-rich cocoa beverage. Procyanidins were analyzed in the gastric contents, which were regularly sampled for 1 h after cocoa ingestion.

SUBJECTS AND METHODS

Chemicals

Procyanidin oligomers (dimer B2 [epicatechin-(4 β →8)-epicatechin], dimer B5 [epicatechin-(4 β →6)-epicatechin], and trimer C1 [epicatechin-(4 β →8)-epicatechin-(4 β →8)-epicatechin]) were purified from cocoa (Cocapro, Mars, Inc, Hackettstown, NJ) as previously reported (2, 21), and their identity

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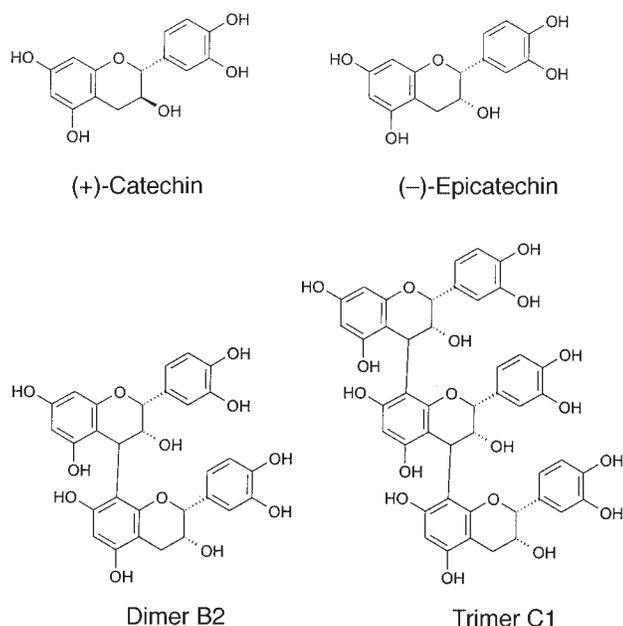


FIGURE 1. Chemical structures of the most common flavanol monomers and procyanidin dimers and trimers in cocoa.

was confirmed by mass spectrometry and nuclear magnetic resonance spectroscopy. Flavanol monomers (–)-epicatechin and (+)-catechin were obtained from Sigma-Aldrich Chemical Co Ltd (Dorset, United Kingdom), and taxifolin was obtained from Extrasynthese (Genay, France). All solvents were HPLC grade.

Subjects

Six healthy, nonsmoking subjects [3 men and 3 women with mean (\pm SD) age, height, and weight of 27 ± 4 y, 174 ± 8 cm, and 68 ± 8 kg, respectively] with no clinical history of gastrointestinal disease participated in this study. They refrained from drinking alcohol for ≥ 48 h before the study and received no medication during the 2 wk before the study. The experimental protocol was approved by the Norwich District Ethics Committee (Ethics Reference LREC 2000/182), and after being informed of the purposes and risks of the study, the subjects provided written consent.

Study design

After the subjects had fasted overnight, a nasogastric tube (outer diameter: 4.67 mm; Sims Portex, Hythe, United Kingdom) was placed in their stomach at the Human Nutrition Unit of the Institute of Food Research. The subjects then drank 500 mL of a cocoa beverage in 3 min. The cocoa beverage was made of cocoa powder (75 g Cocoapro/L), 48 g sucrose/L, 0.75 g carrageenan/L, and 0.032 g ethylvanillin/L in water. All components were food grade (Mars, Inc). Flavanols and procyanidins in cocoa powder were estimated by normal-phase HPLC (25). The test beverage provided 351 mg flavanol monomers and 733 mg procyanidin oligomers. Also, in the test beverage, cocoa powder provided 4 g fat, 74 mg caffeine, and 787 mg theobromine. The pH of the cocoa beverage was 6.5.

At time zero (completion of cocoa beverage ingestion) and every 10 min until the stomach was emptied, a 50-mL gastric sample was taken. A 10-mL volume was divided into 5 aliquots that

were immediately frozen in dry ice and stored at -40°C for further analysis. The remaining 40 mL were promptly reinjected into the subject's stomach via the tube. A sterile air volume of 20 mL was infused to fully drain the gastric sample from the nasogastric tube. Sample collection and mixing took ≈ 1 min. This procedure allowed homogenization of the gastric contents before the collection of each sample.

Procyanidin extraction

All samples (gastric content samples and cocoa beverage samples) were prepared for analysis as described by Adamson et al (25) with some modifications. In brief, 2-mL aliquots were thawed and taxifolin (final concentration: $100 \mu\text{mol/L}$) was added as an internal standard. The mixture was defatted by exhaustive extraction with *n*-hexane (3×4 mL). The lipid-free sample (1 mL) was then extracted with 10 mL acetone:water (80:20, by vol). The resulting slurry was centrifuged at $1500 \times g$ for 10 min at 10°C , and the supernatant fluid was retained. The pellet was reextracted once with 3 mL extraction solvent. The 2 supernatants were pooled, dried under nitrogen, and resolubilized in 1 mL methanol. The solution was filtered through $0.2\text{-}\mu\text{m}$ solvent filters.

HPLC analysis of procyanidins

Oligomers up to pentamers were estimated by normal-phase HPLC. Separation of the individual compounds up to trimers [(+)-catechin, (–)-epicatechin, dimer B2, dimer B5, and trimer C1] was achieved by reversed-phase HPLC.

Normal-phase HPLC

Extracted samples were analyzed by using the method described by Adamson et al (25). The HPLC system consisted of an HP 1100 Series (Hewlett-Packard, Palo Alto, CA) equipped with an autoinjector, quaternary HPLC pump, column heater, fluorescence detector, and HP ChemStation (Hewlett-Packard) for data collection and manipulation. Procyanidin oligomers were separated on a Phenomenex Luna silica column (250×4.6 mm, $5 \mu\text{m}$; Cheshire, United Kingdom) at 37°C with a $20\text{-}\mu\text{L}$ injection volume. The ternary mobile phase consisted of dichloromethane (A), methanol (B), and acetic acid:water (1:1, by vol) (C). Separations were effected by a series of linear gradients of B into A with a constant 4% C at a flow rate of 1 mL/min as follows: elution starting with 14% B in A; 14–28.4% B in A, 0–30 min; 28.4–39.2% B in A, 30–45 min; and 39.2–86% B in A, 45–50 min. Fluorescence was recorded at an excitation wavelength of 276 nm and an emission wavelength of 316 nm.

Reversed-phase HPLC

Chromatographic analyses were performed with the same HPLC system used for normal-phase HPLC analyses except with a diode-array detector. Chromatographic separation was performed by using a Phenomenex Luna C_{18} column (250×4.6 mm, $5 \mu\text{m}$; Cheshire, United Kingdom) at 30°C with a $30\text{-}\mu\text{L}$ injection volume. The binary mobile phase consisted of water:tetrahydrofuran:trifluoroacetic acid (98:2:0.1, by vol) (A) and acetonitrile (B). Separations were effected by a series of linear gradients of B into A at a flow rate of 1 mL/min as follows: elution starting with 15% B in A; 15–17% B in A, 0–10 min; 17% B in A, 10–15 min; 17–40% B in A, 15–30 min; 40–50% B in A, 30–35 min; 50–60% B in A, 35–45 min; 60–90% B in A, 45–50 min. Detection was at 220 nm.

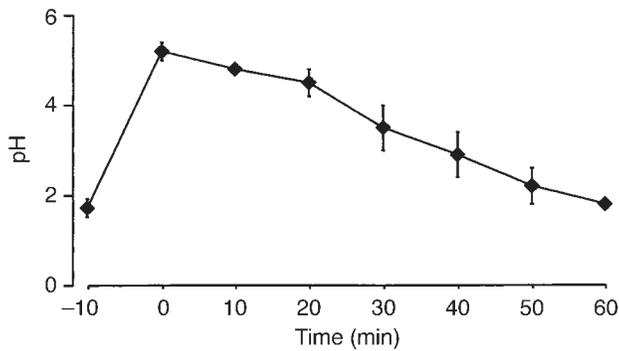


FIGURE 2. Gastric pH after consumption of a cocoa beverage. The subjects ($n = 6$) consumed 500 mL of a cocoa beverage containing 37.5 g cocoa powder in the 3 min preceding time zero.

Statistics

Results are expressed as means \pm SDs. Analysis of variance was used to test for any significant differences between the 5 time points (0, 10, 20, 30, and 40 min). If the result of the analysis of variance was found to be significant ($P < 0.05$), Dunnett's multiple comparisons test was used to determine the specific differences between the time points when compared with the control time (0 min). Statistical analysis was carried out with INSTAT version 3.00 (Graph Pad Software, San Diego).

RESULTS

Gastric pH

pH was measured in the various samples. At baseline, the pH of the gastric contents was 1.9 ± 0.2 . pH increased to 5.4 ± 0.2 just after cocoa beverage consumption and progressively returned to the baseline value as the stomach emptied within 50–60 min (Figure 2).

Cocoa procyanidins in gastric contents

Epicatechin and catechin monomers, as well as B2 and B5 dimers and the C1 trimer, were clearly seen on reversed-phase HPLC chromatograms of the gastric contents (Figure 3, bottom), and their profile was similar to that found in the cocoa beverage (Figure 3, top). Oligomers with a higher degree of polymerization (up to pentamers) were estimated by normal-phase HPLC, which separates procyanidins according to their molecular weight (25). Here again, the same profiles were observed in the cocoa beverage and gastric content extracts (Figure 4).

Any changes in the concentrations of the different procyanidins in the stomach were monitored by both HPLC methods. The proportions of the different oligomers and monomers, as determined by normal-phase HPLC, did not significantly change over the stomach transit period (Figure 5). Similar results were obtained by reversed-phase HPLC. No change in the ratios of trimer to dimer, trimer to monomer, or dimer to monomer were observed (Figure 6).

Depolymerization of cocoa procyanidins, which are composed primarily of epicatechin units, would have resulted in an increase in epicatechin and a change in the ratio of oligomers to the monomeric epicatechin. In addition, a decrease in the catechin to epicatechin ratio would have been observed on degradation of the

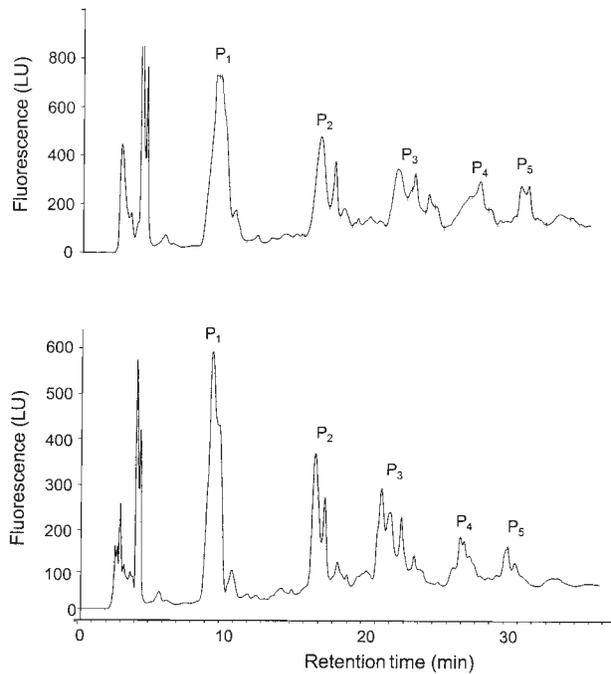


FIGURE 3. Flavanol and procyanidin analysis by normal-phase HPLC of the cocoa beverage (top) and of the gastric contents sampled 20 min after consumption of the beverage (bottom). P₁, monomers; P₂, dimers; P₃, trimers; P₄, tetramers; P₅, pentamers; LU, luminescence units.

procyanidin units and liberation of free epicatechin. No such decrease was seen during digestion (Figure 6). Because epimerization of catechin into epicatechin or of epicatechin into catechin have been reported under acidic conditions (26, 27), we also checked to ensure that the stability of the catechin to epicatechin ratio could not be explained by the conversion of some epicatechin into catechin in the stomach. However, no epimerization was observed at 37 °C (34.2 mmol NaCl/L; pH was adjusted to 2 or 4

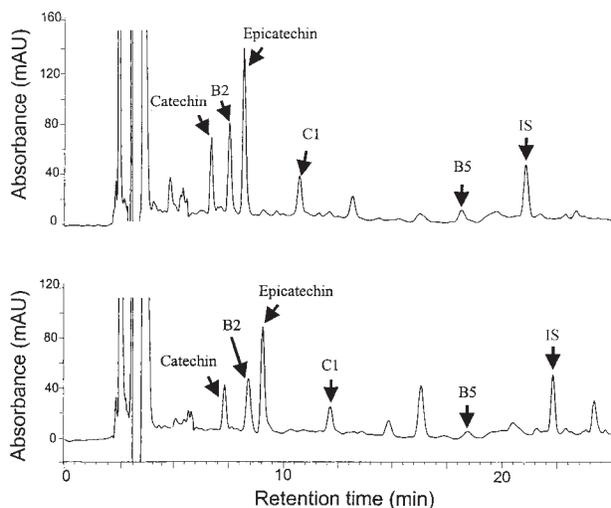


FIGURE 4. Flavanol and procyanidin analysis by reversed-phase HPLC of the cocoa beverage (top) and of the gastric contents sampled 20 min after consumption of the beverage (bottom). IS, internal standard (taxifolin); mAU, milliabsorbance units.

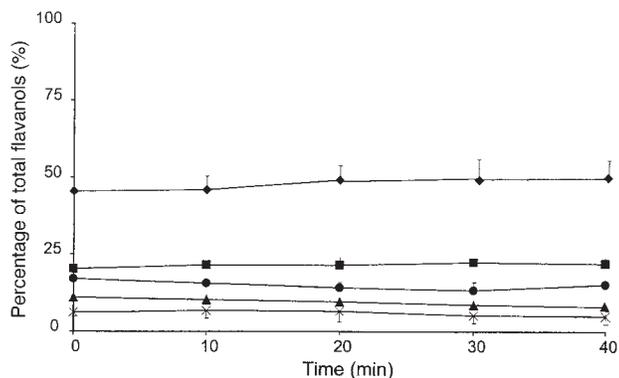


FIGURE 5. Mean (\pm SD) relative concentrations of procyanidins and flavanol monomers in the stomachs of 6 subjects after consumption of a cocoa beverage as established by normal-phase HPLC. Values are percentages of the total flavonols [monomers and oligomers (dimers to pentamers)]. \times , pentamers; \blacktriangle , tetramers; \bullet , trimers; \blacksquare , dimers; \blacklozenge , monomers. ANOVA was used to test for any significant differences between the 5 time points (0, 10, 20, 30, and 40 min); no significant differences were observed.

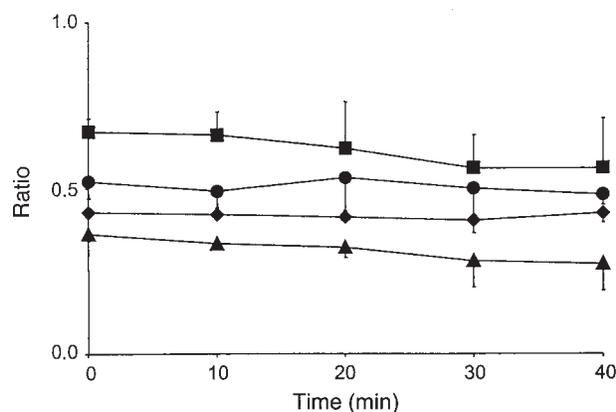


FIGURE 6. Mean (\pm SD) flavanol and procyanidin concentration ratios in the stomachs of 6 subjects after consumption of a cocoa beverage as established by reversed-phase HPLC. \blacksquare , ratio of trimer C1 to dimer B2; \blacktriangle , ratio of trimer C1 to epicatechin; \bullet , ratio of dimer B2 to epicatechin; \blacklozenge , ratio of catechin to epicatechin. ANOVA was used to test for any significant differences between the 5 time points (0, 10, 20, 30, and 40 min); no significant differences were observed.

with hydrochloric acid) within 3 h (data not shown). The results showed that oligomers up to pentamers were stable during stomach transit.

DISCUSSION

The present results showed the absence of any significant depolymerization of cocoa procyanidins in the stomach. These results do not support the hypothesis put forward previously after observing the *in vitro* depolymerization of procyanidins at pH 2 (simulated gastric juice) (24). Two main reasons may explain this discrepancy. First, the duration of exposure to acidic conditions was shorter in the present study (< 50 min) than in the *in vitro* study (1–3.5 h). In the *in vitro* study, only partial depolymerization was observed after 1 h (5–60% of the procyanidins). Second, the pH in the stomach in the present study was higher than the pH in the *in vitro* experiment. In the stomach, the acid secretion (pH 2 under basal conditions with an empty stomach) is buffered by the food bolus so that the contents are exposed to much less acidic conditions. When the cocoa beverage was ingested on its own, the pH increased to 5.4 and progressively decreased to the basal value as the stomach emptied. Thus, there was an overall difference in proton concentration between the *in vivo* conditions and the *in vitro* test of 2–3 orders of magnitude. Also, note that the *in vitro* experiment was performed with the use of purified compounds, whereas our *in vivo* experiment was performed with cocoa powder. The simulated gastric juice thus appears to be an inappropriate model to study the fate of procyanidins *in vivo*.

Thus, it is clear that most of the procyanidins ingested with the cocoa beverage reached the small intestine unchanged. This is consistent with a comparison of catechin recovery in the urine of rats fed either pure catechin or a grape-seed extract containing procyanidins together with low amounts of catechin (28). In this experiment, about the same proportion of catechin (\approx 40%) was recovered in the urine after consumption of each diet. Furthermore, no catechin was detected in the urine or plasma of rats fed a diet supplemented with pure procyanidin dimer B3 (28). These

results strongly suggest that procyanidins are not depolymerized into monomers in the gastrointestinal tract of rats.

Some procyanidin dimers and trimers were initially shown to be absorbed through a Caco-2 cell monolayer used as a model of the intestinal epithelium (21). However, 2 recent *in vivo* studies in rats showed that procyanidin dimers fed to rats are not detected in the plasma and thus are not absorbed through the gut barrier in their intact form (28, 29). Only minute amounts of the structurally related theaflavin dimers are absorbed in humans (30). Thus, we conclude that the mucosa of the small intestine is exposed to significant concentrations of procyanidins.

The absence of degradation of procyanidins in the stomach and their very limited absorption in the small intestine suggest that they may influence digestion or the physiology of the gut through direct interactions with the gut mucosa and gut lumen solutes. As reducing agents (31), procyanidins may limit oxidative stress in the gut mucosa and participate in the prevention of various cancers of the gastrointestinal tract (8, 32, 33). Procyanidin oligomers were shown to inhibit the release of cytokines by human blood mononuclear cells and thus have potential immunoregulatory functions in the gut (34). Procyanidins form stable complexes with metal ions and influence the gut absorption and bioavailability of several minerals (35).

Procyanidins also decrease the apparent digestibility of proteins in animals. Consumption of procyanidin-rich faba beans by Egyptian boys also reduced net protein utilization (36). This result was assumed to be explained by procyanidins forming complexes with proteins. However, experiments with ^{15}N -labeled proteins clearly showed that these antinutritional properties were instead explained by an increase in the endogenous secretion of proteins such as proline-rich proteins or digestive proteases and lipases (37). The nutritional significance of these effects in humans is not clear. The average intake of procyanidins in humans is probably too low to significantly affect digestion (8).

Finally, some health effects of procyanidins may also be associated with the formation of low-molecular-weight metabolites by the microflora once the procyanidins reach the colon (38). Recent results have emphasized the quantitative importance of these

metabolites (39), and more work is needed to evaluate their biological properties. 

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