FOOD & FUNCTION

Profile of urinary and fecal proanthocyanidin metabolites from common cinnamon (*Cinnamomum zeylanicum* L.) in rats

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Received: October 6, 2011
Revised: November 8, 2011
Accepted: November 25, 2011

Cinnamon (*Cinnamomum zeylanicum* L.) bark is widely used as a spice and in traditional medicine. Its oligomeric and polymeric proanthocyanidins are believed to be partly responsible for the beneficial properties of the plant. We describe here the metabolic fate of cinnamon proanthocyanidins in the urine and feces of rats fed a suspension of the whole bark. The metabolites include ten mono-, di-, and tri- conjugated (epi)catechin phase II metabolites and more than 20 small phenolic acids from intestinal microbial fermentation. Some of these are sulfated conjugates. Feces contain intact (epi)catechin and dimers. This suggests that free radical scavenging species are in contact with the intestinal walls for hours after ingestion of cinnamon. The phenolic metabolite profile of cinnamon bark in urine is consistent with a mixture of proanthocyanidins that are depolymerized into their constitutive (epi)catechin units as well as cleaved into smaller phenolic acids during their transit along the intestinal tract, with subsequent absorption and conjugation into bioavailable metabolites.

Keywords:
Bioavailability / Cinnamon / Mass spectrometry / Polyphenols / Proanthocyanidins

Common cinnamon or Ceylon cinnamon (*Cinnamomum zeylanicum*), an aromatic plant from the family of the Lauraceae, is one of the most commonly consumed spices around the world. The bark, either whole or powdered, as well as being a highly prized spice, has been used in traditional medicine for centuries [1]. During recent years, interest has increased in how cinnamon modulates parameters related with the metabolic syndrome (e.g. blood glucose level, lipid profile), as observed in several animal studies and human clinical trials [1–3].

The biological properties of cinnamon are attributed in part to the phenolic compounds it contains, which mostly belong to the class of proanthocyanidins (PA: oligomers/polymers of flavan-3-ols). In particular, it has been suggested that A-type doubly linked procyanidins (PA constituted of (epi)catechin, EC, units) may have insulin-like biological activity in vitro [4]. Recent studies conducted by our group have revealed that cinnamon bark includes oligomers and polymers that contain (epi)gallocatechin and (epi)catechin gallate units [5], which may contribute to its biological activity [6]. Nevertheless, plant PA are neither directly bioaccessible nor bioavailable since they suffer transformations during transit along the intestinal tract [7]. Therefore, to study the potential biological effects of the PA, it is necessary to define their metabolic fate, i.e. the different transformations that they undergo once ingested and thereby the species that will be in contact with the intestinal tract as well as those that will be absorbed and will circulate in different biological fluids for hours after intake. Once ingested, monomeric and dimeric flavan-3-ols are absorbed in the small intestine and converted into several phase II conjugated metabolites in the liver; these then pass into the bloodstream and are later excreted in urine, or return via the bile to the small intestine. PA that are not absorbed in the small intestine reach the colon intact where, after depolymerization and fermentation by microbiota, they may release small metabolites that are absorbed, transformed in the liver, and have the same fate as the metabolites derived from absorption in the small intestine [7, 8]. The metabolites found in urine indicate the fraction of PA that is bioavailable and that may have an effect on target tissues, while the metabolites excreted in

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Abbreviations: EC, (epi)catechin; **GSH**, glutathione; **Gluc**, glucuronide; **Me**, methyl group; **MRM**, multiple reaction monitoring; **PA**, proanthocyanidins; **Sulf**, sulfate

Table 1. (Epi)catechin and conjugated metabolites in urine and feces from rats fed whole cinnamon bark[a)]

Metabolite	MRM parent	Identification	Urine	Feces
EC	289→245	Standard retention time	X	X
Procyanidin dimer	577→289	MS/MS: 577; 451; 425; 405; 289; 245		X
Mono-conjugated metabolites				
Gluc-EC-1	465→289	MS/MS: 465; 289; 245; 163; 113	X	
Gluc-EC-2	465→289	MS/MS: 465; 289; 245; 113	X	
Gluc-EC-3	465→289	MS/MS: 465; 289; 245; 217; 113	X	
Sulf-EC-1	369→289	MS/MS: 369; 289; 245; 113	X	
Sulf-EC-2	369→289	MRM daughter 289→245	X	
GSH-EC-1	594→289	MRM daughter 289→245	X	
Di-conjugated metabolites				
Me-Gluc-EC-1	479→303	MS/MS: 479; 303; 289; 245; 175; 137; 113	X	
Me-Gluc-EC-2	479→303	MS/MS: 479; 303; 285; 259; 175; 137; 113	X	
di-Gluc-EC-1	641→289	MS/MS: 641; 465; 289	X	
Tri-conjugated metabolites				
di-Me-Sulf-EC-1	397→289	MRM daughter 289→245		X

MRM: multiple reaction monitoring; Gluc: glucuronide; EC: (epi)catechin; Sulf: sulfate; GSH: glutathione; Me: methyl group.
a) Metabolites not detected in the control group or detected from signals at least tenfold stronger.

feces are in contact with the colonic tissue and may influence gut health [8, 9].

The study we report here analyzes the urinary and fecal PA metabolites derived from cinnamon. Female Sprague–Dawley rats ($n = 5$), fed a polyphenol-free diet (TD 94048, Harlan Interfauna Ibérica SL, Barcelona, Spain) were administered an acute dose of powdered common cinnamon (1 g/kg body weight via a solution of 0.6 g/10 mL) by oral gavage after 12 h fasting. PA metabolites, including EC derivatives and microbial-derived phenolic metabolites, were analyzed in urine and feces samples collected over the 24 h after intake, and compared to those of a control group that was only administered tap water. The experimental design, included in the Spanish National Research Project AGL-2009–12374-C03–03/ was approved by the Bioethics-Committee-CSIC.

Urine samples were concentrated by nitrogen stream and then resuspended in 2 mL of acid water (addition of phosphoric acid to pH 3). An Oasis HLB (60 mg) cartridge from Waters Corp. (Milford, MA, USA) was used for the solid phase extraction. The cartridge was activated with 1 mL of methanol and 2 mL of acid water and the samples were loaded onto the cartridge. To remove interfering components, the sample was washed with 9 mL of acid water. The phenolic compounds were then eluted with 1 mL of methanol [9]. Feces samples (0.5 g) were defatted with 10 mL of hexane and the remnant was extracted at room temperature with 10 mL methanol:water:acetic acid (8.0:1.9:0.1, v/v/v) and concentrated by nitrogen stream. Taxifolin (50 mg/L solution) was added to each sample as the internal standard, to obtain a final concentration of 5 mg/L. The temperature of evaporation was kept under 30°C to avoid deterioration of the phenolic compounds. All the samples were filtered through a polytetrafluoroethylene (PTFE) 0.45-μm membrane into amber vials for liquid chromatography (LC)–mass spectrometry (MS)/MS analysis.

A Quattro LC triple quadrupole mass spectrometer with an electrospray source (Waters Corp.) was used in the negative mode to obtain MS and MS/MS data. LC separations were performed on an Alliance 2695 system from Waters Corp. equipped with a Phenomenex (Torrance, CA, USA) Luna C18 (50 × 2.1 mm id) 3.5-μm particle size column and a Phenomenex Security guard C18 (4 × 3 mm id) column. Gradient elution was performed with a binary system consisting of (A) 0.1% aqueous formic acid and (B) 0.1% formic acid in CH_3CN. An increasing linear gradient (v/v) of (B) was used, (*t*(min),%B): 0,8; 10,23; 15,50; 20,50; 21,100; followed by a re-equilibration step. Metabolites were identified by multiple reaction monitoring (MRM) transitions of the putative metabolites using a dwell time of 100 ms and additionally by product ion scan experiments. The cycle time used was 2 s. Cone energy and collision energy in MRM mode were optimized for each group of metabolites.

Table 1 lists all the EC conjugates detected by high-performance liquid chromatography coupled to mass spectrometry (HPLC–MS) and HPLC–MS/MS in urine and feces from the rats fed cinnamon. The metabolites were first identified by MRM transitions previously reported in the literature [9] that correspond to the molecular ion and the main fragment, and the final elucidation was confirmed by a second MRM transition and/or by product ion scan experiments.

Free EC (MRM transition 289 → 245) was detected in both urine and feces from the rats fed cinnamon and signals corresponding to EC dimers (577 → 289) were detected in feces.

Nine conjugated EC metabolites were detected in urine: three glucuronidated forms (465 → 289), two sulfated forms (369 → 289), a monoconjugated metabolite with glutathione (GSH) (594 → 289), two methylated and glucuronidated forms (479 → 303), and a di-glucuronidated form (641 → 289). Figure 1A shows the HPLC–ESI–MS/MS (where ESI is

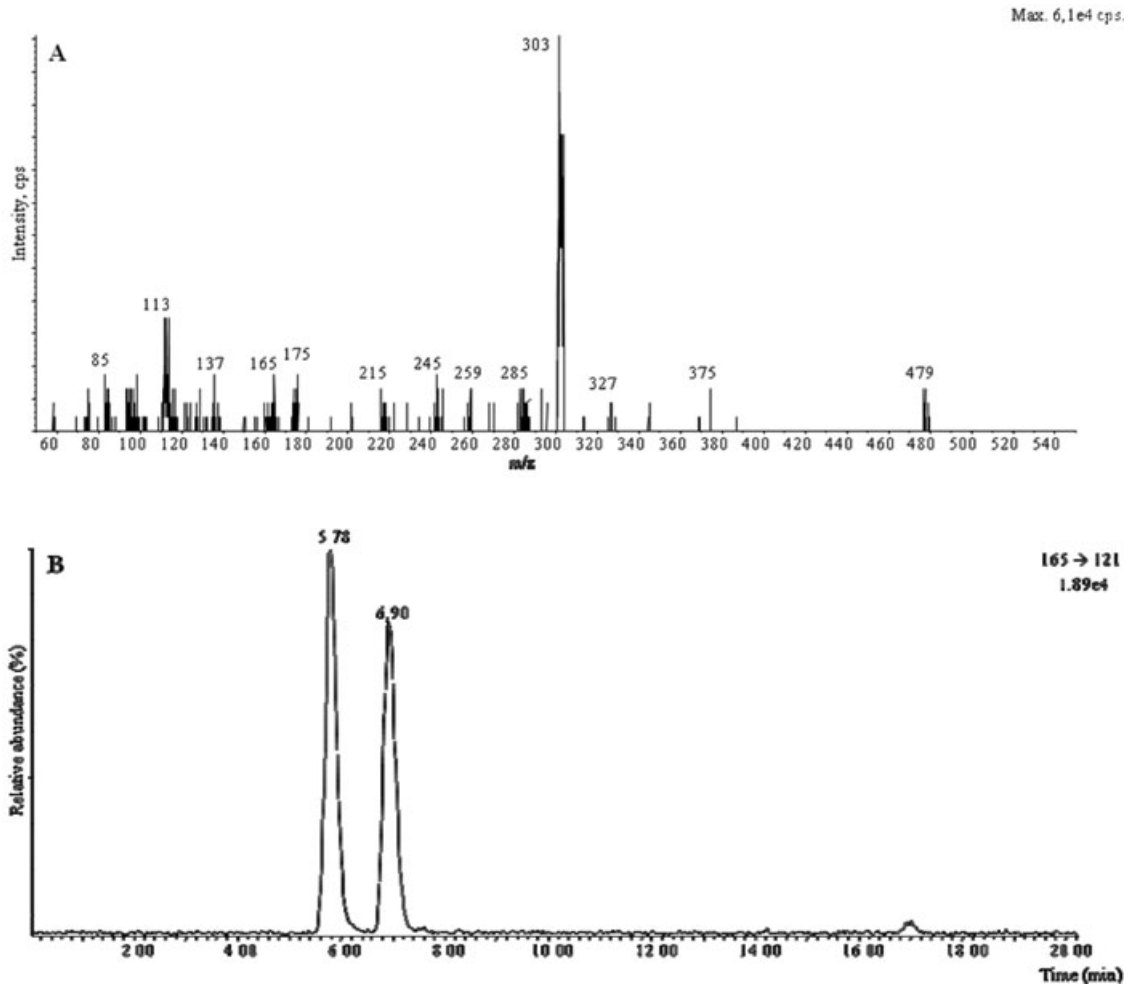

Figure 1. (A) HPLC–ESI–MS/MS product ion scan spectrum of methyl-glucuronide-(epi)catechin (*m/z* 479); and (B) HPLC–ESI–MS profile corresponding to 3- and 4-hydroxyphenylpropionic acid (*m/z* 165) in urine from rats fed whole cinnamon bark.

electrospray ionization) product ion scan spectrum of methyl-glucuronide-EC in urine. The only EC derivative detected in feces from the rats fed cinnamon was dimethylated-sulfated-EC (397 → 289), whose identity was confirmed by a second transition (289 → 245).

The microbial-derived PA metabolites detected in urine and/or feces from the rats fed whole cinnamon bark are listed in Table 2. There are 24 metabolites detected in urine and 6 detected in feces. These metabolites included some direct products of microbial fermentation of PA, such as 3- and 4-hydroxyphenylpropionic acid (165 → 121) as well as phenolic acids derived from further transformations in the liver, such as ferulic acid (193 → 134). Other metabolites derived from PA fermentation, such as 3-hydroxyphenylacetic acid (151 → 107) or 3,4-dihydroxyphenylacetic acid (167 → 123) were only detected in urine samples. The HPLC–ESI–MS profile corresponding to the detection of 3- and 4-hydroxyphenylpropionic acid in urine is shown in Fig. 1B.

Some conjugated microbial-derived PA metabolites were found in urine, indicating that microbial metabolites formed in the colon are absorbed, conjugated in the liver, transferred into the bloodstream, and finally excreted in urine. These metabolites were sulfate (sulf)-dihydroxyphenylvaleric acid (289 → 209), sulf-3,4-dihydroxyphenylpropionic acid (261 → 181), sulf-3- or 4-hydroxyphenylpropionic acid (245 → 165) and gluc-3- or 4-hydroxyphenylacetic acid (327 → 151). Finally, hippuric acid (178 → 134), from the conjugation of benzoic acid with glycine, as well as its methylated form (193 → 178) was also detected in urine from the rats fed whole cinnamon bark. It should be noticed that a fraction of the detected hippuric acid may be derived from cinnamaldehyde, also present in common cinnamon [10].

It was commonly thought that only PA dimers and trimers were metabolized in the intestine, while the modification of PA polymers would be negligible [11,12]. More recent studies have suggested that the metabolism of PA is more significant than previously thought since a large number of metabolites

Table 2. Detection of microbial-derived proanthocyanidin metabolites in urine and feces from rats fed with whole cinnamon bark[a)]

Metabolite	MRM parent	Identification	Urine	Feces
Valerolactones				
Dihydroxyphenylvalerolactone	207→163	MRM daughter 163→119	X	X
Phenylvaleric acids				
Sulf-dihydroxyphenylvaleric acid	289→209	MRM daughter 209→165	X	
Phenylpropionic acids				
3-Hydroxyphenylpropionic acid	165→121	MS/MS: 165; 121; 93	X	X
4-Hydroxyphenylpropionic acid	165→121	MS/MS: 165; 121; 93	X	X
3,4-Dihydroxyphenylpropionic acid	181→137	Standard retention time	X	
Sulf-3,4-dihydroxyphenylpropionic acid	261→181	MRM daughter 181→137	X	
Suf-3 or 4-hydroxyphenylpropionic acid	245→165	MS/MS: 245; 165; 121	X	
Phenylacetic acids				
3-Hydroxyphenylacetic acid	151→107	Standard retention time	X	
4-Hydroxyphenylacetic acid	151→107	Standard retention time	X	
Gluc-3- or 4-hydroxyphenylacetic acid	327→151	MS/MS: 327; 151; 107	X	
3,4-Dihydroxyphenylacetic acid	167→123	MS/MS: 167; 123; 105; 95	X	
Benzoic acids				
Benzoic acid	121→77	Standard retention time	X	
4-Hydroxybenzoic acid	137→93	Standard retention time	X	X
Sulf-3 or 4-hydroxybenzoic acid	217→137	MS/MS: 217; 137; 121	X	
3,4-Dihidroxybenzoic acid	153→109	Standard retention time	X	
Hippuric acid	178→134	Standard retention time	X	
Me-hippuric acid	193→178	MS/MS: 193; 178; 134	X	X
Cinnamic acids				
Caffeic acid	179→135	Standard retention time	X	
m-coumaric acid	163→119	Standard retention time	X	
p-Coumaric acid	163→119	Standard retention time	X	
Sulf-coumaric acid	243→163	MRM daughter 163→119	X	
Ferulic acid	193→134	Standard retention time	X	X
Lignans				
Enterodiol	301→107	Standard retention time	X	
Sulf-enterolactone	377→297	MRM daughter 297→253	X	

MRM: multiple reaction monitoring; Sulf: sulfate; Gluc: glucuronide.
a) Metabolites not detected in the control group or detected from signals at least tenfold stronger.

have been detected in plasma and/or urine after ingestion of foodstuffs rich in polymers [8, 9, 13]. Many of these metabolites are conjugates of monomeric EC that cannot come exclusively from the small amounts of this monomer contained in the food. Moreover, the observation that some small phenolics are detected in plasma several hours after ingestion confirms the hypothesis that polymers are transformed by the intestinal microbiota [9, 14]. The results obtained in the present study with whole cinnamon are in agreement with those obtained for other polymer-rich sources such as grape pomace and almond skin [8, 9, 13], and are in keeping with the observation that cinnamon is a rich source of PA [5, 15]. Common cinnamon contains more than 3000 mg/100 g of PA (from dimers to polymers; more than 80% of which have a degree of polymerization equal to or higher than 10), while its monomeric EC content is only 24 mg/100 g [15]. We report here the detection of a large number of EC metabolites in the urine of rats fed whole cinnamon bark. The strong MS signals cannot come only from the transformation of monomeric EC, which is a very minor component of the bark. Moreover, we detected 3,4-dihydroxyphenylacetic acid, the major phenolic acid from procyanidin dimer fermentation, which has not been identified in studies performed with pure monomeric flavan-3-ols [16].

In the study reported here, we fed the animals a water suspension of whole cinnamon bark instead of the extracts commonly used in other studies. Therefore, both the extractable and the nonextractable PA fractions were administered. This is important since nonextractable PA are mostly polymeric and are present in higher amounts than extractable PA in many foodstuffs [17]. It may be expected that the PA metabolites detected in this study derive not only from extractable PA, but also from nonextractable PA which have been shown to be bioavailable as phase I and II metabolites [18]. The present results also confirm that polymeric PA are depolymerized into EC units in the intestine and not only directly cleaved into smaller phenolic acids [8, 9]. Furthermore, it should be noted that intact EC and an EC dimer were present in feces. This means that intact free radical scavenging catechol groups are in contact with the intestinal walls for hours after ingestion.

In summary, the bioavailability of cinnamon PA after ingestion of whole bark powder has been evaluated for the first time. A wide variety of PA metabolites, including phase II

EC metabolites and microbial-derived metabolites, were detected in the urine and feces of rats fed cinnamon. They would appear to be derived from the degradation of PA polymers. These circulating metabolites may contribute to the reported beneficial health effects of cinnamon.

This work was supported by the Spanish Ministry of Education and Science (AGL2009–12374-C03–03/ALI). J.P.-J. thanks the Spanish Ministry of Science and Innovation and the ISCIII for her Sara Borrell postdoctoral contract (CD09/00068). Language revision by Christopher Evans is appreciated.

The authors have declared no conflict of interest.

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