RESEARCH ARTICLE

Condensed Tannins in the Diets of Primates: A Matter of Methods?

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To understand the ways in which condensed tannins (CT) affect primate diet selection and nutritional status, correct measurements are essential. In the majority of studies of the CT contents of primate foods, a tannin source such as “quebracho” is used to standardize CT assays, but the CT in quebracho tannin may not be similar to those in the plants of interest. We investigated how the choice of standard to calibrate CT assays affects the estimation of CT in the diets of mountain gorillas (Gorilla beringei). We purified the CT from gorilla foods and compared the actual amounts of CT in the foods with estimates produced by using the quebracho tannin. When quebracho was used, the estimates of CT contents of gorilla foods were, on average, 3.6 times the actual content of CT so that the amounts in frequently eaten gorilla foods were substantially overestimated. The overestimation for a given plant could not be predicted reliably and the ranking of plants by tannin content differed according to the standard used. Our results demonstrate that accurate measurements of CT necessitate the use of tannins purified from the plant species of interest. A reevaluation of primatology studies using interspecific comparisons of tannin content will provide new insights into primate food selection and nutritional ecology. Am. J. Primatol. 71:70–76, 2009. © 2008 Wiley-Liss, Inc.

Key words: proanthocyanidin; nutrition; plant defense compounds; acid butanol assay; food selection

INTRODUCTION

Condensed tannins (CT) are astringent polyphenolic compounds widespread in plants, including potential primate foods. They bind protein and reduce the digestibility and nutritional quality of the diets of herbivores [Hagerman & Klucher, 1986; Robbins et al., 1987a,b], and there is substantial evidence that plants are protected from herbivory using CT as a deterrent [Coley & Barone, 1996; Freeland & Janzen, 1974]. However, CT may also have positive benefits including anti-oxidant properties and potential protection from some types of intestinal pathogens [Min & Hart, 2003; Mueller-Harvey, 2006; Santos-Buelga & Scalbert, 2000]. Given their potential importance in shaping feeding behavior, CT have been considered in studies of food selection by primatologists for decades [Glander, 1982; Milton, 1979; Oates et al., 1980; Wrangham & Waterman, 1981]. Some Primates appear to avoid tannin-rich foods in their natural diets [Leighton, 1993; McKey et al., 1981; Oates et al., 1977], whereas others eat high CT food items [Davies et al., 1988; Kool, 1992], and food selection by some primates does not appear to be influenced by CT [Chapman & Chapman, 2002; Ganzhorn et al., 1985; Milton, 1998; Reynolds et al., 1998]. Many studies point out that the role of tannins in the diets of primates is still uncertain [Kool, 1992; Milton, 1998; Norscia et al., 2006], possibly owing to challenges in interpreting assay results [Yeager et al., 1997]. If we are to understand the ways in which CT affect primates, their correct estimation in primate foods is essential.

Of the many available tannin assays, one of the most commonly used in primatology is the acid butanol assay, a simple spectrophotometric assay that approximates the soluble CT content of a plant extract [Porter et al., 1986]. The assay is based on oxidative depolymerization of the proanthocyanidin (condensed tannin) and spectrophotometric measurement of the red anthocyanidin product. When this reaction occurs, the anthocyanidins produce a red color when the interflavan bond is broken. However, the stability of the bond varies in different

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proanthocyanidins and the color produced by butanol–HCl reactions is a function of the anthocyanidin product released. Consequently, the heterogeneity in chemical structure of CT presents a challenge to this assay, and the use of a single standard to estimate solutions containing CT with unknown structures is unreliable [Schofield et al., 2001]. Giner-Chavez et al. [1997b] demonstrated that the standard curves of six different standards commonly used to estimate the contents of tropical forages all had different slopes, and a study of Spanish shrubs substantiated these results [del Pino et al., 2005]. In a study of purified tannins from foliage of nine species of trees in a coastal forest of northern California, the slopes of the standard curves used to quantify tannin concentrations varied seven-fold among species, with slopes ranging from 1.1 to 7.9 [Kraus et al., 2003].

Although advances in estimating the tannin contents of plants have been made by agricultural and ecological chemists [Mueller-Harvey, 2006; Schofield et al., 2001], in many cases these methods have not been adopted by primatologists. Because CT are chemically diverse and difficult to analyze, use of inappropriate methods can lead to invalid results and slowing of advances in primatology. The majority of primate studies examining tannin intake or diet selection rely on use of a single standard to estimate the tannin contents of primate foods: the most common is "quebracho" [e.g., Chapman & Chapman, 2002; Fashing et al., 2007; Lahann, 2007; Voigt et al., 2004], but others have also been used [e.g., leukocyanidin: Hohmann et al., 2006; sorghum: Lucas et al., 2001]. Advances in agriculture and chemical ecology have demonstrated that relating a single CT standard to the CT in a suite of different plant species may pose a problem [reviewed in Rautio et al., 2007].

Used commercially to tan leather, quebracho is the common name for one of three South American trees (Schinopsis lorentzii, S. balansae, Aspidosperma quebracho-blanco), all of which contain high concentrations of tannins [Waterman & Mole, 1994]. Although a crude extract of quebracho is commonly used as a CT standard (as in the studies above), the residue of the tannin extract could contain between 50–80% CT [Asquith & Butler, 1985; Robbins et al., 1991]. As a result, this crude extract contains non-CT impurities. Without purification, the extracted substance cannot be assumed to contain condensed tannin only. Even with the quebracho tannin purification, the reactivity of quebracho in the acid butanol assay may differ from that of the plant of interest. Rautio et al. [2007] obtained tannin values using quebracho as a standard that were 30-fold lower than those obtained using foliage from the trees under study, another indication that use of quebracho standards is inappropriate. Consequently, the use of quebracho tannin as a standard will likely lead to an incorrect estimation of CT content in primate diets; studies using "quebracho equivalents" have suggested that concentrations of tannins in food plant species exceed 100% [e.g., Fashing et al., 2007; Powzyk & Mowry, 2003], a biological impossibility. This problem can be circumvented if tannins are purified from the actual plant species under investigation.

Here, we use our studies of wild mountain gorilla diets to investigate the potential problems of using quebracho tannin as an external standard when estimating the CT contents of primate diets. We explore the use of quebracho as a standard in three ways. First, we examine whether the standard curve created from quebracho tannin differs from the standard curves generated by tannin in the plant species of interest. Second, we compare the estimates of CT content of ten gorilla foods and rank them in order of their CT content using quebracho tannin and CT from the plant species of interest. Third, we estimate the intake of CT in the gorilla diet from five major food items containing tannins [each plant part comprises >5% of the wet weight intake; Rothman et al., 2007] to determine if the use of quebracho tannin results in an overestimation or underestimation of CT intake from these diet items.

METHODS

Field Methods

We studied a group of gorillas in Bwindi Impenetrable National Park (0°53’–1°08’S, 29°35’–29°50’E), southwestern Uganda. Details of plant collections and behavioral methods are available in Rothman et al. [2008]. The gorillas ate a diet that contained mainly the leaves and outer peel of herbaceous and shrub vegetation (67%), but they also ate fruit (15%), tree foliage (7%), pith (6%), decaying wood (4%), tree bark and twigs (0.5%) and herbaceous stems (0.1%) [Rothman et al., 2007]. About 35% of the foods in the Bwindi gorilla diet contained soluble CT [Rothman et al., 2006]. The intake of tannins from the consumption of five major food items that each comprise at least 5% of the wet weight intake of gorillas [Rothman et al., 2007] was estimated using Equation (3) in Rothman et al. [2008].

Laboratory Methods

To identify which of the food items (N=84) eaten during focal samples contained CT, each sample was dried at <22°C in darkness shortly after collection and the following protocol was observed. Each ground food item was immersed in 70% (v/v) aqueous acetone and sonicated in ice water for 20 min. The crude extracts were centrifuged at 2,500 × g for 10 min. The supernatant was collected and stored covered in the dark at 4°C. These extractions were repeated four times and the supernatants
combined to reach a final concentration of 10 mg/ml of crude plant material in 70% acetone [Hagerman, 1988]. The crude extracts were assessed qualitatively for the presence or absence of condensed tannin using a micro version of the butanol–HCl assay [Porter et al., 1986]. Briefly, in 2-ml capped centrifuge tubes, 600 µl of 5% HCl in n-butanol, 100 µl of sample extract, and 20 µl of 2% Fe(NH)2(SO4) in 2N HCl were heated in an oven at 100 °C for 1 hr. After heating, the absorbance of the samples was measured in a spectrophotometer at 550 nm. Samples containing CT turned pink whereas those without CT remained colorless. To control for the interference of plant pigments, the absorbance value determined before heating was subtracted from the final reading [Watterson & Butler, 1983]. The absorbances of the sample solvent and crude quebracho extract were used to standardize assay conditions during these initial qualitative tests.

We purified the tannin from the major dietary items that comprised a substantial portion of the gorilla diet [comprising >5% by weight of gorilla diet; Rothman et al., 2007] that were known to contain CT (n = 5), as well as a few other foods that were occasionally eaten (n = 5). The purification protocol is outlined in Hagerman and Butler [1994] and we slightly modified it. Since both hydrolyzable and CT are purified using this procedure, and we were only interested in CT, we screened the plants for hydrolyzable tannins in addition to CT [Hartzfeld et al., 2002]. This step is important because if a mixture is present, the standard curve might overestimate the amount of condensed tannin in a sample as hydrolyzable tannins are not affected by the acid butanol assay.

Plants were extracted as described above to reach a final concentration of 500 mg/ml of crude plant material in 70% acetone. After evaporating most of the acetone from the sample, leaving about 5 ml of the aqueous solution, the residue was redissolved in 95% ethanol and applied to a slurry of 25 g of equilibrated Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO) in a fritted glass funnel without vacuum. Ninety-five percent ethanol (about 1 ml/min) was applied to the slurry until the absorbance of the eluant at 280 nm approached zero. This eluant containing nontannin phenolics was discarded. Seventy percent (v/v) acetone was slowly applied to remove the brown band of tannins remaining in the Sephadex. After the acetone from the second eluant was evaporated, this portion was extracted three times with equal volumes of ethyl acetate (each time the sample was centrifuged at 2,500 x g and the organic phase was discarded). After removing traces of ethyl acetate, the tannin was frozen at -80°C, lyophilized, weighed, and stored in a desiccator in the dark. During the entire procedure, the apparatus was covered with aluminum foil to minimize light exposure. To construct a six-point standard curve for each plant species (N = 10) using the acid butanol assay, the fluffy tannin powder was redissolved in 70% aqueous acetone (to reach concentrations of 0–1 mg tannin powder/ml). Depending on the tannin source, each dilution represented a 0.1–0.2 mg difference and fell within 0–1.000 OD [Porter et al., 1986]. The standard curve of purified quebracho tannin was obtained from the work of Giner-Chavez et al. [1997b], which was conducted in the same laboratory. The crude extract of quebracho was subjected to the acid butanol assay using the same source of quebracho tannin as the purified CT product from Giner-Chavez et al. [1997b]. The standard curve of crude quebracho was assessed at concentrations of 0–5 mg/ml and each dilution represented a 1 mg/ml difference.

To compare the estimated CT content of foods when using the tannin purified from the plant species of interest, pure quebracho, and crudely extracted quebracho, a within-subjects ANOVA was used. We also used a within-subjects ANOVA to compare the tannin intake when estimated using pure or crudely extracted quebracho, or purified tannin from the food plant of interest. Statistical analysis was performed using SAS (Version 9.1).

Our research protocol was reviewed by Cornell University’s Institutional Animal Care and Use Committee and they determined that the protocol did not require approval because our research did not involve more than simple field observations of the gorillas. All research conducted during this study complied with the regulations of the Government of Uganda and was conducted with the permission of the Uganda Wildlife Authority and the Uganda National Council for Science and Technology.

RESULTS

The standard curves generated through the acid butanol assay from purified tannin of different plant species containing CT varied considerably, with slopes ranging from 0.6 to 1.4 (Fig. 1; Table I): Urera sp. herbaceous peel and leaves had the lowest slopes and Myrianthus sp. fruits, leaves, and bark had the steepest slopes. The herbaceous peel of Urera sp. contained hydrolyzable tannin as well as CT. The slopes of all of the standard curves for the internal standards were greater than those of purified and crude quebracho (Fig. 1), indicating that the use of quebracho as a standard to estimate the CT concentrations of gorilla foods will result in an overestimation of CT intake.

The estimated CT content of gorilla food plant species was greater when quebracho was used compared with the values obtained using an internal standard (within-subjects ANOVA, F = 61.786, P < 0.01). The values based on the internal standard were twice as high as those obtained using purified quebracho as the standard, and five times higher.
than the estimates based on a crude quebracho standard (Table I). When the gorilla foods were ranked by descending order of CT content, the rankings differed depending on whether an internal or the quebracho external standard was used and whether the CT from quebracho was purified or if a crude extract of quebracho was used (Table I).

When we estimated the mean daily CT intake resulting from consumption of five food parts that each comprised more than 5% of the wet weight biomass of the gorilla diet, the mean daily tannin intake was 1.1–4.5% on a dry matter basis from these sources, and CT intakes were overestimated using tannin purified from quebracho or the crude extract of quebracho (within-subjects ANOVA, $F = 15.29, P < 0.01$). Intake of CT was overestimated by up to 11% (on a dry matter basis) using CT purified from quebracho and by up to 35% if crude quebracho extract was used (Fig. 2).

### DISCUSSION

We have documented marked differences in estimates of CT content of plant species depending on whether purified standards from the gorilla food plant, purified quebracho tannin, or a crude extract of quebracho was used. These methodological differences resulted in significant differences in estimates of the CT contents of gorilla food items and CT intake. Our results have important implications for studies that use external standards to estimate plant tannin contents and suggest that a reevaluation of results of studies where external standards were used (e.g., quebracho) is needed.

Use of a quebracho tannin standard overestimated the CT contents of gorilla foods, and the magnitude of this overestimation varied among plant species. This has important implications for studies of food selection. If one plant is thought to have more CT than another, then studies aiming to assess food selection or to compare foods for their CT content may lead to incorrect conclusions. For example, *Myrianthus* fruit is one of the most commonly eaten

![Fig. 1. Standard curves of the absorbance vs. tannin content (mg/ml) of tannins generated from purified gorilla food plants, and the purified quebracho, an external standard. As the intercept did not differ from zero for any of the slopes, the regression line was forced through the origin for graphical display. Lowercase letters indicate the standard curve of the following plant species and part: (a) *Myrianthus* bark; (b) *Myrianthus* fruit; (c) *Myrianthus* leaves; (d) *Maesa* leaves; (e) *Triumfetta* leaves; (f) *Ficus* leaves; (g) *Gouiana* leaves; (h) *Smilax* leaves; (i) Urera herb peel; (j) Urera leaves; (k) purified tannin from quebracho; (l) crudely extracted quebracho. Urera herb peel also contains hydrolyzable tannin and therefore, its reaction in the acid butanol assay is based on total tannin (hydrolyzable + condensed tannin).](image)

### TABLE I. Mean Tannin Content (g/kg) and Food Species Ranking by Condensed Tannin Content Estimated From Isolated Plant Condensed Tannin Content, Purified Quebracho Tannin, and Crudely Extracted Residues From Quebracho

<table>
<thead>
<tr>
<th>Food part</th>
<th>Part</th>
<th>Regression equation of standard curve using purified tannin</th>
<th>$R^2$</th>
<th>Internal standard</th>
<th>Rank</th>
<th>Purified quebracho tannin*</th>
<th>Rank</th>
<th>Crude quebracho extract†</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ficus</em> sp.</td>
<td>Leaf</td>
<td>$y = 0.0371 + 1.02 (x)$</td>
<td>99.4</td>
<td>207</td>
<td>1</td>
<td>454</td>
<td>5</td>
<td>1,210</td>
<td>4</td>
</tr>
<tr>
<td><em>Smilax</em> sp</td>
<td>Leaf</td>
<td>$y = -0.0128 + 1.03 (x)$</td>
<td>99.2</td>
<td>203</td>
<td>2</td>
<td>495</td>
<td>2</td>
<td>1,312</td>
<td>2</td>
</tr>
<tr>
<td><em>Myrianthus</em> sp</td>
<td>Bark</td>
<td>$y = 0.107 + 1.22 (x)$</td>
<td>97.0</td>
<td>182</td>
<td>3</td>
<td>635</td>
<td>1</td>
<td>1,662</td>
<td>1</td>
</tr>
<tr>
<td><em>Maesa</em> sp.</td>
<td>Leaf</td>
<td>$y = 0.0233 + 1.25 (x)$</td>
<td>99.5</td>
<td>163</td>
<td>4</td>
<td>461</td>
<td>4</td>
<td>1,172</td>
<td>6</td>
</tr>
<tr>
<td><em>Myrianthus</em> sp</td>
<td>Fruit</td>
<td>$y = 0.0470 + 1.41 (x)$</td>
<td>98.2</td>
<td>129</td>
<td>5</td>
<td>462</td>
<td>3</td>
<td>1,231</td>
<td>3</td>
</tr>
<tr>
<td><em>Gouiana</em> sp.</td>
<td>Leaf</td>
<td>$y = 0.0066 + 1.02 (x)$</td>
<td>99.7</td>
<td>120</td>
<td>6</td>
<td>282</td>
<td>7</td>
<td>780</td>
<td>7</td>
</tr>
<tr>
<td><em>Myrianthus</em> sp</td>
<td>Leaf</td>
<td>$y = 0.0474 + 1.33 (x)$</td>
<td>97.4</td>
<td>112</td>
<td>7</td>
<td>449</td>
<td>6</td>
<td>1,196</td>
<td>5</td>
</tr>
<tr>
<td><em>Triumfetta</em> sp</td>
<td>Leaf</td>
<td>$y = 0.0288 + 1.22 (x)$</td>
<td>98.6</td>
<td>85</td>
<td>8</td>
<td>250</td>
<td>9</td>
<td>644</td>
<td>9</td>
</tr>
<tr>
<td><em>Urera</em> sp.</td>
<td>Peel</td>
<td>$y = 0.0433 + 0.722(x)$</td>
<td>97.7</td>
<td>76</td>
<td>9</td>
<td>266</td>
<td>8</td>
<td>684</td>
<td>8</td>
</tr>
<tr>
<td><em>Urera</em> sp.</td>
<td>Leaf</td>
<td>$y = 0.0411 + 0.608 (x)$</td>
<td>98.2</td>
<td>60</td>
<td>10</td>
<td>117</td>
<td>10</td>
<td>313</td>
<td>10</td>
</tr>
</tbody>
</table>

* $y = -0.01 + 0.44 x$, $r^2 = 0.99$ (Giner-Chavez et al., 1997b).
† $y = 0.6429 + 0.176 x$, $r^2 = 0.99$.
‡ All food plants were collected in May 2003 within the areas where gorillas were feeding.
§ These numbers are biologically invalid.

The regression equations are equations for curves relating the absorbance ($y$) to the mass of tannin standard ($x$).
foods in the gorilla diet and comprises 8.6% of the wet weight intake of the Bwindi gorillas over the year [Rothman et al., 2007]. Maesa leaves were rarely eaten (<1% of the wet weight diet). The Myrianthus fruits contain less tannin than the Maesa leaves, but when estimated using purified or crude quebracho, Myrianthus fruits contained equal or more CT than Maesa (Table I), suggesting that CT does not play a role in food selection when it may. As the majority of primates use an external standard, this result likely does not apply solely to gorillas; a reevaluation of studies using interspecific comparisons of tannin content in primate foods may reveal that primates have different patterns of food selection than what has been previously reported. In addition, because of the complexity of tannin chemistry, quantification of CT content alone does not provide a complete indication of the impact of tannins on food selection, intake, or animal physiology. Biological activity or tannin–protein binding capacity, which is affected by protein structure and animal physiology as well as tannin structure also, must be considered [Giner-Chavez et al., 1997a; Rautio et al., 2007]. Nonetheless, to avoid errors associated with the measurement of CT, internal standards should be used to estimate tannin contents.

When plants contain both hydrolysable and CT, separation of the two chemically distinct structures presents a problem [Hagerman & Butler, 1989]. When methods are not available to determine the precise structures of tannins [Kraus et al., 2003], the total phenolics assay [modified by Graham, 1992; Price & Butler, 1977], which measures all phenolic compounds in a plant, could be used to generate a standard curve for total tannin using purified tannin (purified via Sephadex). However, the biological and chemical properties of hydrolyzable and CT are different. Several assays have been developed to evaluate protein and microbial binding by tannins [Hagerman, 1987; Martin & Martin, 1983; Mueller-Harvey, 2001; Nelson et al., 1997], but similar methodological issues remain [Kraus et al., 2003; Martin & Martin, 1982]. Bioassays also require standards and quebracho frequently is used, but the binding characteristics of quebracho likely differ from those of the tannin of interest, and it is important to note that the protein used in these assays may not react similarly to dietary protein [Giner-Chavez et al., 1997a].

Gorillas in this study ate foods that contained 20% condensed tannin, suggesting that they eat high tannin foods but many staple foods do not contain tannins so actual diet tannin levels are well below 20% owing to dilution effects/cafeteria style feeding [Freeland & Janzen, 1974; Villalba et al., 2004]. Gorillas are thought to be more adapted to lower-quality food sources with higher concentrations of CT in their diets than other apes [Remis et al., 2001], and there is no evidence that gorillas are deterred from eating foods that contain CT [Calvert, 1985; Remis, 2002, 2003; Rogers et al., 1990; Rothman et al., 2006], however we do not have enough data to examine tannin tolerance in gorillas or other primates. Like humans, gorillas and other primates may have proline-rich salivary proteins that bind tannins before they enter the gut; this would likely reduce the effects of CT on protein and diet digestibility [reviewed in Shimada, 2006].

Often primatologists wish to characterize each plant species animals are seen consuming for its nutritional and antinutritional quality, and there is a need for a balance between speed and accuracy in assessing plant chemistry. When relating a single tannin standard to a suite of plants that contain different properties, our results indicate that the accuracy with quicker analyses is compromised too much, and a more rigorous analysis is needed. Our recommendation is to use the acid butanol assay to screen primate foods qualitatively for CT, and depending on the question of interest, tannin-containing plants can then be selected for purification. Mechanisms of secondary compound detoxification by primates are relatively unexplored. A combination of physiological studies and more precise estimations of CT content promise new insights into niche partitioning within primate communities [Ganzhorn, 1998; Wrangham et al., 1998], studies of primate diet, food selection and nutrition [Lambert, 1998], and the evolution of dietary strategies [Milton, 1993, 1999].

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