ARCHIVOS LATINOAMERICANOS DE NUTRICION
Organo Oficial de la Sociedad Latinoamericana de Nutrición

Vol. 44 N° 4-S, 1994

Tannins: thermostable pigments which complex dietary proteins and inhibit digestive enzymes

Andrés Carmona

Profesor titular, Escuela de Biología, Universidad Central de Venezuela.

SUMMARY. The presence of antinutritional factors in legume seeds and other vegetables has been considered as an expression of the chemical warfare of plants against their predators. As a consequence, the nutritional utilization of these foods has only been possible through the use of a variety of treatments (cooking, fermentation, germination) which increase nutrient bioavailability. Nonetheless, some factors are not destroyed by effect of seed processing, among which stand a family of polymeric polyphenols called tannins. These pigments have the ability to complex and precipitate proteins and inhibit digestive enzymes. This paper describes what has been accomplished in regards to the selection of an appropriate solvent to extract bean polyphenols, the assessment of the most commonly used assay procedures, the purification of bean tannins and the evaluation of their interaction with proteins and digestive enzymes, responsible for their antinutritional effect.

RESUMEN. Taninos: Pigmentos termoestables que se acomplejan con proteínas e inhibiben enzimas digestivas. La presencia de factores antinutricionales en las semillas de leguminosas y otros vegetales se ha interpretado como la expresión de mecanismos de defensa de las plantas contra sus depredadores. Por ello, el aprovechamiento nutricional de dichas semillas sólo ha sido posible a través de la aplicación de diversos tratamientos de cocción, fermentación, germinación, etc., los cuales producen, en general, un aumento en la biodisponibilidad de los nutrientes. No obstante, algunos factores resisten a los tratamientos mencionados. Entre estos se encuentra una familia de polifenoles poliméricos, llamados taninos, que se concentratan en la cáscaras de semillas coloreadas. Los taninos tienen la capacidad de acomplejarse con proteínas, inducir su precipitación e inhibir una diversidad de enzimas digestivas. El presente trabajo describe los logros alcanzados en la búsqueda del solvente de extracción más apropiado para solubilizar los polifenoles de semillas de leguminosas, la evaluación de diferentes procedimientos de cuantificación, la purificación de los taninos de las semillas y el estudio de su interacción con proteínas y enzimas responsables de su efecto antinutricional.

INTRODUCTION

Some vegetable foods contain antinutritional and toxic factors which conform an effective arsenal in the chemical warfare of plants against their predators. Legume seeds are an example of a heavy armored group [1]. Nonetheless, the bean's content of proteins, carbohydrates, dietary fiber, minerals and vitamins confers them a high nutritive potential whose extensive exploitation have only been possible through the development of a variety of processing techniques (ordinary cooking, autoclaving, roasting, germination, fermentation, etc.).

Although these treatments had made possible the consumption of pulses, the digestibility of processed seeds is usually lower than that of animal foods. A variety of reasons have been put forward to explain this finding. Besides the intrinsic nature of some seed proteins (*i.e.* globulins) and starches, other determinants contribute to the relatively low digestibility of cooked beans: The incomplete inactivation of heat-labile antinutritional factors (hydrolase inhibitors and lectins), the presence of heat-stable agents (tannins, alkaloyds and non-protein amino acids), and the formation of complexes among various seed components (proteins, starches, lipids, fiber, minerals) which are resistant to hydrolase attack [2,3,4].

Several reports have pointed to the fact that colored seeds had lower digestibilities than the white varieties [5,6]. In addition, rats or chicks fed diets containing colored *Vicia faba* or sorghum grains showed decreased body weight gain. In these animals, the true digestibility of proteins and, to a lesser extent, that of carbohydrates and fats, was sensibly reduced [7,8]. These effects have been attributed to polymeric

polyphenols (tannins) which concentrate in the coats of most colored seeds.

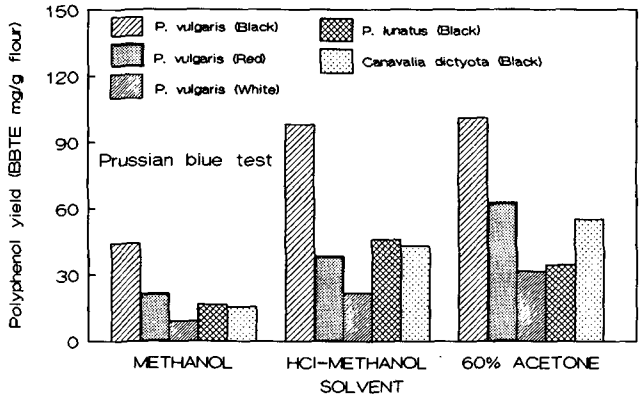
Tannins, an ill defined and chemically diverse class, are able to complex and precipitate proteins [9]. Two major tannin categories have been identified: hydrolyzable and condensed tannins. The latter, abundant in vegetable foods [10], are polymers of flavan-3ols (catechins) or flavan-3,4-diols (leucoantocyanidins). The first group, represented by tannic acid, is composed of esters of phenolic acids with sugars [10,11].

The lack of commercial condensed tannins has prompted the use of tannic acid as a model compound in various **in vivo** and **in vitro** studies depicted to evaluate the antinutritional effects of the whole tannin group. Although some of the changes brought about by the latter are similar to those of condensed tannins, there are also important differences. For instance, diets based on high tannin sorghum do not cause depression of food intake as has been reported for those containing tannic acid [7,12,13]. In addition, condensed tannins seem to be more effective than tannic acid in regards to the reduction of body weight in chicks [14].

To ascertain the antinutritional influence of common bean tannins it was necessary to extract, quantify and extensively purify them, in order to test their effects free of other interferences. These aspects were addressed in the present study.

The ability of methanol, 1% HCl in methanol and 60% acetone to extract legume seed coat polyphenols is depicted in Figure 1. As compared to pure methanol, acidic methanol or the aqueous acetone solution increased two to three times the extraction yield. Water and 60% ethanol were only slightly more effective than methanol (results not shown). Extraction yield was always higher for the colored seeds as compared to white common beans.

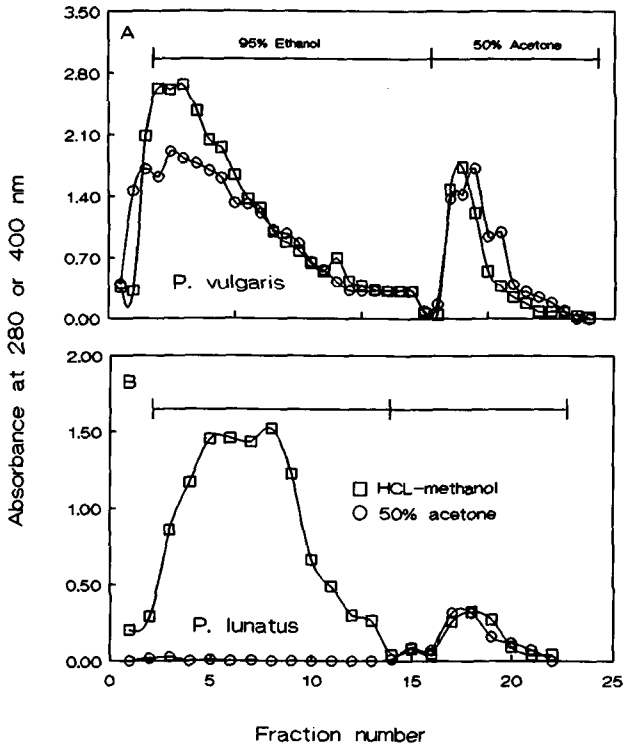
FIGURE 1
Effectivity of various solvents to extract bean polyphenols



Seeds were ground to an average particle size of 820 μm and subjected to four consecutive extractions with the indicated solvents using a flour/solvent ratio of 1:10. Extracts were pooled and their total polyphenol content assessed with the Prussian blue reagent [16]. Results were expressed as Black Bean Tannin Equivalents (BBTE).

The selectivity of extraction solvents to solubilize polyphenols is a frequent concern of researchers in this field. This question can be approached analyzing polyphenol extracts by adsorption chromatography on Sephadex LH-20 [15,16]. In short (4.5 x 2 cm) columns, HCL-methanol and acetone extracts from the coats of *black Phaseolus vulgaris* and *P. lunatus* seeds were resolved into two major fractions: non-tannins eluted with ethanol and condensed tannins eluted with 50% acetone (Figure 2). The chromatographic profiles were very similar for both *P. vulgaris* extracts. For *P. lunatus*, the acidic methanol extract yielded a profile similar to that from *P. vulgaris*, while the acetone one contained almost exclusively condensed tannins. These results indicate that there could be differences in the selectivity of solvents to extract some polyphenols depending upon its source. The uncertainties associated with polyphenol extraction could be solved by direct assessment in the dry vegetable material. Although some attempts have been made using Near Infrared Reflectance Spectroscopy [17] or seed color determination in a chromameter [18], these procedures have not been properly validated.

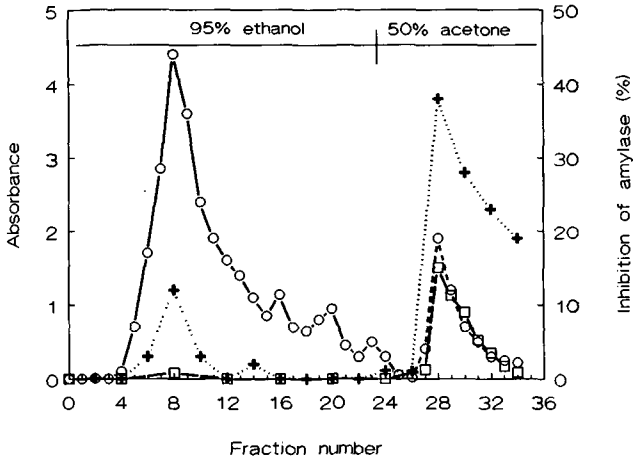
FIGURE 2
Fractionation of *P. vulgaris* and *P. lunatus* polyphenols by adsorption chromatography



HCl-methanol and 60% acetone extracts were seeded on top of a Sephadex LH-20 column (4.5 x 2 cm) [16]. Non- tannin polyphenols were washed with 95% ethanol and their elution followed at 280 nm. Afterwards, tannins were desorbed with 50% acetone and their elution monitored at 400 nm.

The results of a more extensive characterization of common bean polyphenols is presented in Figure 3. Fractions eluted with 50% acetone reacted strongly with the vanillin reagent and inhibited pancreatic α-amylase. These fractions did not react with anthrone (results not shown) indicating the condensed nature of these tannins.

FIGURE 3
Characterization of polyphenols from black beans

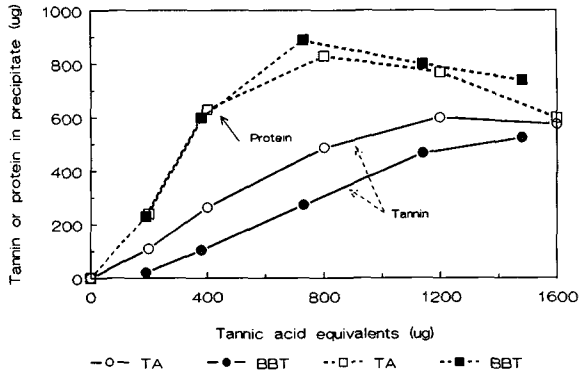


A HCl-methanol extract of black beans was cromatographed in a 20 x 2 cm Sephadex LH-20 column and resolved as indicated in figure 2. Fractions were analyzed for condensed tannins with the vanillin reagent (□) and their ability to inhibit pancreatic α–amylase (+) [16,24].

Tannin-protein interactions are a major issue regarding the nutritional significance of tannins. Using the protein precipitation test designed by Hagerman and Butler [19] it was found that tannic acid and black bean tannins precipitated serum albumin in a similar fashion (Figure 4). Nonetheless, the amount of tannins recovered in the precipitates was usually lower for the condensed ones, particularly at low tannin levels. In spite of the complexities of protein-tannin interactions, these results suggest that bean tannins are more effective promoting albumin precipitation than tannic acid.

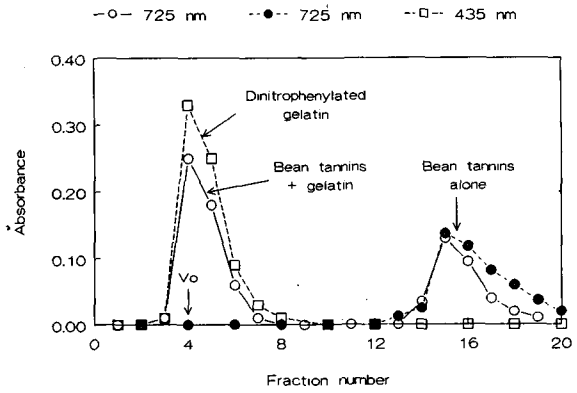
Figure 5 demonstrates the formation of soluble black bean tannins-gelatin complexes. When the supernatant from incubations of gelatin and tannins were chromatographed in Bio-Gel P60, a fraction of the tannins appeared along with the protein in the void volume, while the uncomplexed polyphenols were considerably delayed. These soluble tannin-protein adducts are of utmost importance in regards to enzyme inhibition which may occur at tannin concentration which do not induce protein precipitation.

FIGURE 4
Formation of insoluble tannin-protein complexes



Bovine serum albumin (1 mg/ml) was incubated with the indicated amounts of either black bean tannins or tannic acid [19]. Precipitates were collected, rinsed and analyzed for their tannin content. The amount of protein precipitated was estimated by difference measuring that remaining in the supernatant.

FIGURE 5
Demonstration of soluble tannin-protein complexes



Samples of black bean tannins and of the supernatant from incubations of dinitrophenylated gelatin and black bean tannins were chromatographed on a (0.6 x 29 cm) Bio-Gel P60 column, using 0.15 M NaCl as solvent. The elution of the protein was followed as 435 nm while that of tannins by their reaction with the Folin-Ciocalteus reagent measuring the resulting absorbance at 725 nm. Vo.: Void volume.

Considering that condensed tannins are not absorbed from the gut, the evaluation of their antinutritional potential points to establish their effects on various digestive enzymes: pancreatic proteases (trypsin and chymotrypsin), α-amylase, brush border disaccharidases (maltase, sucrase and lactase) and the intestinal glucose uptake system (Table 1). Black bean tannins inhibited the enzymes concerned with carbohydrate digestion, the proteolytic activity of pancreatin and, to a lesser extent, intestinal glucose uptake. Nonetheless, caution should

be taken in interpreting these figures due to the differences in the assay mixtures employed to measure these enzymes where complex tannin-protein and or tannin-substrate interactions could be established.

TABLE 1
INHIBITION OF DIGESTIVE ENZYMES BY BLACK BEAN TANNINS

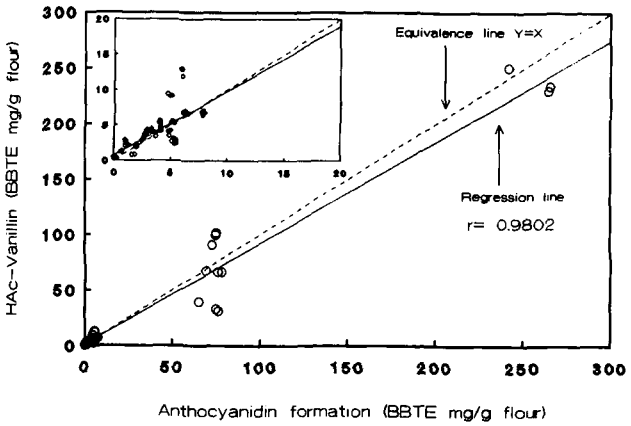
Enzyme[1]	Source	Substrate	Tannin requiered to to inhibit activity by 50% (µg)
Proteases	Bovine pancreatin	Casein	180-200[2]
α-amylase	Bovine pancreatin	Starch	21[3]
α-amylase	Hog pancras (purified)	Starch	50[4]
Maltase	Rat brush border membranes	Maltose	73[5]
Sucrase	Rat brush border membranes	Sucrose	57[5]
Lactase	Rat brush border membranes	Lactose	97[5]
Glucose uptake	Rat *intestinal slevees*	Glucose	> 2000[4]

1. Enzymes were measured using standard assays (21-25).
2. Rojas, M. (unpublished observations).
3. Carmona, A. (unpublished observations).
4. Borges, G. (unpublished observations).
5. Borgudd, L. (unpublished observations).

The concentration dependent nature of the fore mentioned effects of bean tannins emphasize the need to quantify the seed content of polymeric polyphenols able to elicit an antinutritional response. In our laboratory we have critically evaluated a variety of assay procedures, from those based on the general reductive character of polyphenols (*i.e.* Prussian blue test) to those highly specific for condensed tannins such as the vanillin reaction (Figure 2) or the quntitative anthocyanidin formation test [20]. To overcome some of the limitations of the traditional vanillin reaction performed in methanol [16,21], the modified procedure of Butler et al. [22], in glacial acetic acid, proved to be more reliable and led to results which closely paralleled those obtained measuring anthocyanidin release under conditions which minimize phlobaphene formation (Figure 6).

Although the nutritional relevance of the data presented in this study is still uncertain, due in part to the lack of results from *in vivo* experiments, we are opening a way to test the effect of condensed tannins under conditions closer to those prevailing *in vivo*. Considering the consequences of dietary protein complexing and enzyme inhibition, it is likely that condensed tannins may significantly contribute to reduce the digestibility of colored legume seeds.

FIGURE 6
Correlation between the tannin determinations performed with the glacial acetic acid-vanillin and anthocyanidin formation tests



HCl-methanol extracts from the seeds of 8 species and varieties of legumes were analyzed for their tannin content using the acetic acid-vanillin [22] and anthocyanidin formation [20] tests. Results were expressed as Black Bean Tannin Equivalents (BBTE). The equivalence line represents the Y=X theoretical line. The inset expands the scale to show the points around the origin of the plot.

ACKNOWLEDGEMENTS

This manuscript is dedicated to Dr. Werner G. Jaffé who introduced me to this field in 1978. The author expresses his gratitude to the graduate and undergraduate students who have acompained him in the study of legume seed tannins. Supported in part by Grants 03.65.87 fron Consejo de Desarrollo Científico y Humanístico, Central University of Venezuela, and SI 2438, Conicit, Venezuela.

REFERENCES

1. Carmona A, A Gómez-Sotillo & DS Seidl. Uso de pruebas bioquímicas para el estudio de problemas nutricionales en *Canavalia ensiformis*. In: Canavalia ensiformis (L.) DC. Producción, procesamiento y utilización en alimentación animal. R Vargas, A León & A Escobar (Ed.). Editorial Futuro, San Cristobal, Venezuela. 1993, p. 151-162.
2. Seidl DS, M Jaffé & WG Jaffé. Digestibility and proteinase inhibitory action of a kidney bean globulin. J. Agric. Food Chem. 17: 1318-1321, 1969.
3. Tovar J, A de Francisco, I. Bjorck & N-G Asp. Relationship between microstructure and *in vitro* digestibility in precooked leguminous seed flours. Food structure 10: 19-26, 1991.
4. Gómez-Sotillo A, A Carmona & DS Seidl. Fractionation of *canavalia ensiformis* proteins. Distribution of the proteic antinutritional factors. En: Advances of Research on Antinutritional Factors in Legume Seeds. ABF van del Poel, J Huisman & HS. y Saini (Eds.) Wageningen Pers, Wageningen, The Netherlands. 1993. p. 235-239.

5. Jaffé, W.G. Toxic factors in beans. Their practical importance. In: Nutritional aspects of common beans and other legume seeds as animal and human feed. WG Jaffé (Ed.). Archivos Latinoamericanos de Nutrición, Caracas. 1973. p. 199-209.
6. Bressani, R., Hernández, E. and Brahan, J.E. (1988) Relationship between content and intake of bean polyphenols and protein digestibility in humans. Plant Foods Hum. Nutr. 48: 1000-1001.
7. Featherston WR & JC Rogler. Influence of tannin on the utilization of sorghum grain by rats and chicks. Nutr. Rep. Int. 11: 491-497, 1975.
8. Moseley G & DW Griffiths. . Varietal variation in antinutritive effect of field beans when fed to rats. J. Sci. Food Agric. 30: 772-778, 1979.
9. Butler LG & KD Bos. Analysis and characterization of tannins in faba beans, cereals and other seeds. A literature review. In: Recent advances of research in antinutritional factors in legume seeds. ABF van der Poel, J Huisman & HS Saini (Eds.). Wageningen Pers, Wageningen, The Netherlands, 1993, p. 81-89.
10. Salunkhe DK, JK Chavan & SS Kadan. Dietary tannins: Consecuences and remedies. CRC Press, Boca Raton, 1990, p. 1-28.
11. Porter LJ. Tannins. En: Methods in Plant Research, JB Harborne (Ed.), Vol. I, Academic Press, Londres, 1989, p. 389-419.
12. Glick Z & MA Joslin. Food intake depression and other metabolic effects of tannic acid in the rat. J. Nutr. 100: 509-515, 1970.
13. Janbunathan R & ET Mertz. Relationship between tannin levels, rat growth, and distribution of protein in sorghum. J. Agric. Food Chem. 21: 692-696, 1973.
14. Marquardt RR, AT Ward, LD Campbell & PE Cansfield. Purification, identification and characterization of a growth inhibitor in faba beans (Vicia faba L. var, minor). J. Nutr. 107: 1313-1324, 1977.
15. Strumayer, D.H. and Malin, M.J. 1975. Condensed tannins in grain sorghum: isolation, fractionation, and characterization. J. Agric. Food Chem. 23: 909-914.
16. Carmona, A, DS Seidl & WG Jaffé. Comparison of extraction methods and assay procedures for the determination of the apparent tannin content of common beans. J. Sci. Food Agric. 56: 291-301, 1991.
17. Sinnaeve, G, P Dardenne & R Biston. Prediction of antinutritional factors in pea by Near Infrared Reflectance spectroscopy (NIRS). In: Advances of Research on Antinutritional Factors in Legume Seeds, ABF van del Poel, J Huisman & HS Saini (Eds.), Wageningen Pers, Wageningen, The Netherlands, 1993, p. 1.01-105.
18. Black, RG. Use of objective colour measurement of pea seeds to classify low or high tannin varieties. In: Advances of Research on Antinutritional Factors in Legume Seeds, ABF van del Poel, J Huisman & HS Saini (Eds.), Wageningen Pers, Wageningen, The Netherlands, 1993, p. 113-115.
19 Hagerman AE & LG Butler. Protein precipitation method for the quntitative determination of tannins. J. Agric. Food Chem. 26: 809-812, 1978.
20. Porter LJ, LN Hrstich & BG Cahn. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 25: 223-230, 1986.
21. Carmona, A, AI Laurentín & G Borges. Extraction and assay of legume seed tannins. Effect of temperature on polyphenol yield. In: Advances of Research on Antinutritional Factors in Legume Seeds, ABF van del Poel, J Huisman & HS Saini (Eds.), Wageningen Pers, Wageningen, The Netherlands, 1993, p. 107-111.
22. Butler LG, ML Price & JE Brotherton. Vanillin assay for proanthocyanidin (condensed tannin): Modification of the solvent for estimation of the degree of polymerization. J. Agric. Food Chem. 30: 1087-1089, 1982.
23. Kakade ML, DH Swenson & IE Liener. Note on the determination of trypsin and chymotrypsin inhibitor activity using casein. Anal Biochem. 22: 550-555, 1970.
24. Bergmeyer HU. Alpha-amylase. In: Methods of Enzymatic Analysis, HU Bergmeyer and K Gawhen (Eds.), Academic Press, New York, 1974, p. 432.
25. Dahlqvist A. . Method for assay of intestinal disaccharidases. Anal. Biochem. 7: 18-25, 1964.
26. Asp, NG, A Dahlqvist Human small intestine beta-galactosidades: Specific assay of three different enzymes. Anal. Biochem. 47: 527-538, 1972.
27. Karasov WH & JM Diamond. A simple method for measuring intestinal solute uptake in vitro. J. Comp. Physiol. 152: 105-116, 1983.