Hydrogen bonds in the binding of some polyphenols from Quebracho (*Schinopsis* spp.) to soybean meal protein under *in vitro* ruminal conditions[1]

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ABSTRACT: The extent of binding to soya protein of a commercial tannin (Quebracho), its ethyl acetate extract, the major putative compound (fisetinidol-(4,8)-cathequin-(6,4)-fisetinidol), and the acetylated and methylated derivatives of the major latter was evaluated incubating them with soybean meal under *in vitro* ruminal conditions for 48h. The protein binding activity was estimated measuring changes in ammonia concentration when the tannin was incubated with soybean meal at a proportion of 8% of the dry weight. As compared to soybean meal alone (control), ammonia concentration decreased by 19, 27, and 31% for quebracho, the ethyl acetate extract of quebracho and the major compound, respectively, indicating concentration of the protein binding activity. Acetylation or methylation of the major compound resulted in corresponding decreases in the concentration of ammonia by 21 and 6%. This suggested that as the possibility of hydrogen bond formation decreases (being less with methylation than with acetylation), the binding to the protein is reduced but is not eliminated completely. Therefore, our hypothesis is that the main interactions between fisetinidol-(4,8)-cathequin-(6,4)-fisetinidol and soybean protein in an *in vitro* ruminal system are hydrogen bonds and that only a few hydrophobic interactions are present.

Key words: Fisetinidol-(4,8)-cathequin-(6,4)-fisetinidol, bypass protein, hydrogen bonds, polyphenols, *Schinopsis* spp

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Puentes de hidrógeno en la ligación de algunos polifenoles de Quebracho (*Shinopsis* spp.) a proteína de soya en un sistema ruminal *in vitro*

RESUMEN: Se evaluó la capacidad de ligación a proteína de soya de un tanino comercial (quebracho), su extracto en acetato de etilo, el compuesto causativo mayoritario (fisetinidol-(4,8)-cathequin-(6,4)-fisetinidol), y los derivados acetilado y metilado de éste, incubándolos con torta de soya en un sistema ruminal *in vitro* durante 48 h. Esta capacidad se estimó midiendo los cambios en la concentración de amonio al incubar el tanino con la torta de soya en una relación tanino/proteína de 8% (p/p materia seca). Con relación a la torta de soya sola (control), las concentraciones de amonio disminuyeron en 19, 27 y 31% para el quebracho, el extracto de quebracho en acetato de etilo y el compuesto mayoritario, respectivamente, indicando una concentración de la actividad de ligación a la proteína de soya. La acetilación o la metilación del compuesto mayoritario resultó en disminuciones correspondientes en la concentración de amonio en 21 y 6%. Esto sugiere que a medida que la posibilidad de formación de puentes de hidrógeno disminuye (menor con metilación que con acetilación), la unión a la proteína se debilita, pero no se elimina completamente. Por lo tanto, se postula que las principales interacciones entre el fisetinidol-(4,8)-cathequin-(6,4)-fisetinidol y la proteína de soya en un sistema ruminal *in vitro* son los puentes de hidrógeno y que soló unas pocas interacciones hidrofóbicas estarían presentes.

Palabras clave: Fisetinidol-(4,8)-cathequin-(6,4)-fisetinidol, puentes de hidrógeno, polifenoles, proteína pasante, *Schinopsis* spp

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Introduction

Addition of tannin sources to ruminant diets may improve the efficiency of nitrogen utilisation under certain conditions. Some studies have shown an increase of amino acid flow to the duodenum and amino acid absorption when tannins are included in the diet (Jones and Mangan, 1977; Egan and Utyatt, 1980; Waghorn *et al.,* 1987), whereas other experiments have demonstrated that tannins reduce feed intake, protein and dry matter digestibility and worsen animal performance (Barry and Duncan, 1984; Reed *et al.,* 1990). *In vitro* experiments in our laboratory have shown that a commercial tannin (Quebracho) decreases ammonia, isobutyric and isovaleric acid production from soybean meal, indicating binding of the tannin to soya protein, thus decreasing its *in vitro* availability for fermentation (Gonzalez *et al.,* 1998). Hydrogen bonds and hydrophobic interactions have been reported as the main forces in the binding of tannins to proteins (Siebert *et al.,* 1996). Hydrogen bridges are formed mainly between carbonyl groups of the peptide bond and OH groups of phenols, while hydrophobic interactions occur between the aromatic part of two compounds and involve mainly the pyrrol residue of proline (Oh *et al.,* 1980)

In the present investigation we isolated the major compound responsible for protein binding under *in vitro* ruminal conditions from the ethyl acetate extract of a commercial tannin (Quebracho). To assess the effects of hydrogen bond formation between this compound and soybean meal protein, the former was acetylated and methylated and these derivatives were also tested using the same system. Ammonia concentration was used to estimate the extent of binding of the tannin source and its extract or the major compound and its derivatives to soya protein. Assuming that ruminal micro-organisms can not degrade protein that is bound to tannin, the concentration of ammonia should be inversely proportional to the extent of binding.

Materials and Methods

Samples. The soybean meal and a semipurified commercial tannin, Quebracho (*Shinopsis* spp.), produced by Indunor S. A. (Buenos Aires, Argentina), used were analyzed for contents of moisture (AOAC-7.007, 1984) ash (AOAC-7.009, 1984) and neutral detergent fiber (Van Soest *et al.,* 1991). The latter fraction was assayed without sodium sulfite, alpha amylase or residual ash in the case of tannins but with addition of amylase for soybean meal. Soybean meal was analysed for crude protein (AOAC-7.0033-7.037, 1984) and soluble nitrogen according to Van Soest *et al.* (1995). Total phenol and condensed tannins of the semipurified commercial product were determined using the Prussian Blue method (Price and Buttler, 1977) and butanol/HCl method (Porter *et al.,* 1986), respectively. The dried ethyl acetate extract of Quebracho, the major compound, and acetylated and methylated derivatives of the latter were also tested.

Isolation and characterisation of compound C. An aqueous solution of the commercial tannin (500 g/L) was succesively extracted liquid-liquid with petroleum ether, dicloromethane, and finally ethyl acetate. The ethyl acetate phase was concentrated to dryness (39.9 g) and separated by column chromatography on silica gel, eluting with dicloromethane, dicloromethane: ethyl acetate in two proportions (1:1 and 1:4), and ethyl acetate. The ethyl acetate fraction was concentrated to dryness (19 g). A part of this fraction (5 g) was chromatographed on silica gel, eluted with dicloromethane: ethanol (continuous gradient). The fractions eluted with ethanol contained mainly compound C. This fraction was purified by repeated column chromatography on silica gel, obtaining 0.7 g of pure C. This compound was acetylated (Ac_2O/pyridine) and methylated (diazomethane) to yield C1 and C2, respectively. The structure of the isolated compound and its derivatives were elucidated by spectroscopic methods comparing the structure of the methylated derivative with similar compounds (Botha *et al.,* 1982; Viviers *et al.,* 1983).

Soybean and tannin mixtures. Soybean meal (100 mg) was weighed in 100 mL plastic tubes and 8 mg of the commercial tannin, the dried ethyl acetate extract, C, C1 or C2 dissolved in McDougall buffer (5 mL) was added. Triplicate tubes were used for each tannin and for a control (soybean meal alone). Tubes were placed in a thermostat at 39°C for 12h to allow tannin and protein to interact (Figure 1).

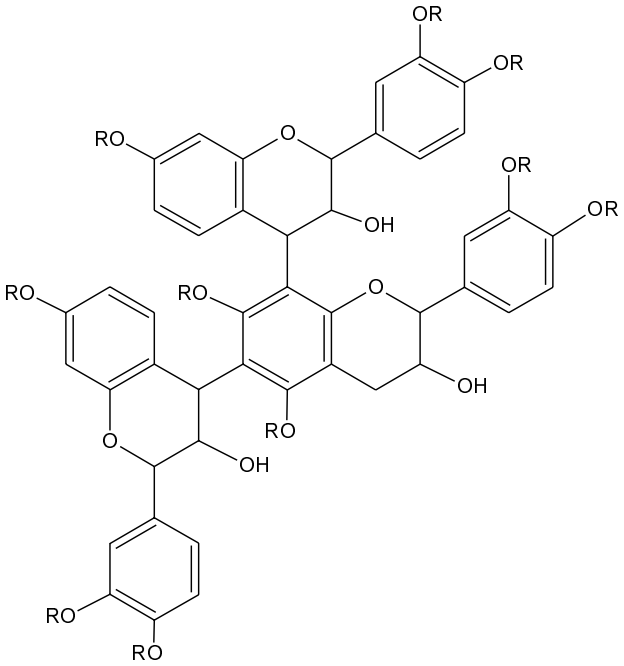
Ruminal *in vitro* incubation. In this work only the first part (degradation by rumen bacteria) of the Tilley and Terry (1963) procedure was used. Ruminal fluid was collected from two steers grazing a pasture of kikuyo grass (*Pennisetum clandestinum*) and white clover (*Trifolium repens*), filtered through cheesecloth and kept at 39°C under CO_2 for 30 minutes. Thereafter, 50 mL of diluted ruminal fluid (ruminal fluid:McDougall buffer-without urea 1:4) were added to tubes that contained soybean meal alone (control) or tannin-treated soybean meal. After gasifying with CO_2, tubes were sealed with rubber stoppers provided with bunsen valves, and incubated at 39°C for 48 h, shaking periodically. Ammonia concentration was determined in the fluid from the incubation tubes.

Ammonia determinations. Aliquots of 4 mL were taken from each ruminal incubation tube and acidified with 80 mL of H_2SO_4 (80% v/v). Aliquots of 1mL were centrifuged at 9000 x*g* for 10 min. Ammonia concentration was determined in the supernatant using a colorimetric method (McCullough, 1967).

Results

The composition of soybean meal and quebracho are presented in Table 1.

The major compound C, isolated from the ethyl acetate extract of a commercial sample of quebracho (*Schinopsis*



C R=H
C1 R=Ac
C2 R=Me

Fig 1. Chemical structures of compounds C, C1 and C2.

Table 1. Composition of soybean meal and tannin sources.

	Soybean meal	Quebracho
DM (%)	87.8	91.4
CP (%)	50.2	-
Soluble protein (%)[1]	23.0	-
NDF (%)	12.3	-
Total phenols (%)	-	39.3
Condensed tannins ($A_{550} \times mg^{-1}$)	-	0.82
Hydrolysable tannins (%)[2]	-	0

DM = Dry Matter, CP = Crude Protein, NDF = Neutral Detergent Fiber.
1. % of CP.
2. As gallic acid.

Table 2. Ammonia concentration after addition of quebracho, the ethyl acetate extract or the major compound to soybean meal in an *in vitro* ruminal incubation system

Treatment	Ammonia concentration (NH_3 mg × dL^{-1})	Change in ammonia concentration relative to control (%)
Soybean meal (Control)	20.5 ± 0.4	-
Quebracho	16.7 ± 0.3	-19
Ethyl acetate extract	15.0 ± 0.2	-27
Major compound (C)	14.2 ± 0.2	-31
Acetylated compound (C1)	16.2 ± 0.3	-21
Methylated compound (C2)	19.3 ± 0.4	-6

spp.), was characterised as fisetinidol-(4,8)-cathequin-(6,4)-fisetinidol by the spectroscopic methods used.

Upon testing the *in vitro* system, lower levels of ammonia were obtained relative to the control when quebracho, the ethyl acetate extract, or the major compound C were added to soybean meal at the 8% level. Higher levels of ammonia were found with the acetylated (C1) or methylated (C2) derivations than when C itself was added (Table 2).

Discussion

In the present investigation an *in vitro* ruminal system using a 48h incubation time was used. Other researchers have suggested that to estimate ruminal protein degradation shorter incubation times and an inhibitor of both deamination and ammonia uptake should be used (Broderick, 1978; Broderick, 1987). Our system did not take into account the limitations of interpretation associated with microbial synthesis and incorporation of ammonia to the bacteria at longer incubation times. However, the results showed that the system is sensible to the effects of adding different types of compounds. This system may not be accurate to measure the absolute extent of degradation but it could be used to compare the effects of different types of compounds on protein degradability.

The present results agree with those of experiments *in vivo* and *in vitro* showing a decrease in ruminal protein degradation by addition of tannins (Zelter and Leroy, 1966; Driedger and Hatfield, 1972; Barry and Manley, 1984; Asquith and Butler, 1986; Waghorn *et al.,* 1987). In our experiment, effect on ammonia levels decreased in the following order: Quebracho < Ethyl acetate extract < C, indicating concentration of the protein binding capacity. Twice as much protection of soybean meal from ruminal degradation was found with C (fisetinidol-(4,8)-cathequin-(6,4)-fisetinidol) than with the commercial quebracho. According to the molecular structure of compound C interactions with soybean meal protein might be expected through hydrogen bridges or hydrophobic interactions. In order to evaluate the contribution of these two types of bonding, the acetylated and methylated derivatives of compound C were synthesised and tested in the same system. Acetylation or methylation of compound C decreased ammonia levels, relative to the control by 21 and 6%, respectively, indicating that there is still some binding to protein in both compounds. In the acetylated compound the carbonyl group could interact with protein to form hydrogen bonds while this is not so in the methylated derivative. However, hydrophobic interactions could still be present between the aromatic rings of polyphenol and protein, explaining the remaining activity of the methylated compound. Similar conclusions have been reached upon lowering the pH in a system where tannin and protein are present. Hydrogen bonds are susceptible to pH changes and decreasing pH reduces but does not eliminate the binding of tannin and protein, which suggests the pres-

ence of both hydrogen bonds and hydrophobic interactions (Oh *et al.,*1980; Fajardo *et al.,* 1998; Gonzalez *et al.,* 1998).

Conclusion

The main interactions between the major putative compound C obtained from quebracho and soybean meal protein are probably hydrogen bonds but hydrophobic interactions also contribute to this binding.

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