Effect of tannin source and pH on stability of tannin-protein and fibre complexes

Efeito da fonte de taninos e do pH na estabilidade dos complexos taninos-proteína e taninos-fibra

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Abstract

Quebracho (*Schinopsis quebracho-colorado* Schlecht) is the most extensively tannin extract explored in order to improve the use of protein in ruminant feed. The rock rose (*Cistus ladanifer* L.) and the grape seed (*Vitis vinifera* L.) are alternative sources of condensed tannins (CT). The objective of this study was to evaluate the effect of pH on the stability of complexes formed between the rock rose tannins and grape seed and the protein and fiber of soybean meal and compare the results with those obtained with the quebracho tannin extract. The results obtained show that the rock rose and grape seed tannins, such as quebracho tannins, have the ability to bind to soybean meal protein at pH between 6 and 8, reducing the solubility of nitrogen. These complexes are dissociated at pH 2, releasing the protein. This binding pattern supports the concept of using these tannin sources in ruminant nutrition as additives to protect the protein feed from excessive rumen degradation. However it was observed differences among tannin sources. Rock rose and grape seed tannins seem be more effectives in reducing protein degradation in the rumen than quebracho tannins.

Keywords: *Cistus ladanifer*, grape seed, pH, quebracho, Tannin-protein complexes

Resumo

O extrato de taninos de quebracho (*Schinopsis quebracho-colorado* Schlecht) é o extrato que mais tem sido estudado com o objetivo de melhorar a utilização da proteína dos alimentos para ruminantes. A esteva (*Cistus ladanifer* L.) e a grainha de uva (*Vitis vinifera* L.) são fontes alternativas de taninos condensados (CT). Foi objetivo deste estudo avaliar o efeito do pH na estabilidade dos complexos formados entre os taninos da esteva e da grainha de uva e a proteína e a fibra do bagaço de soja e comparar com a estabilidade dos complexos formados com os taninos de quebracho. Os resultados obtidos mostram que os taninos de esteva e da grainha de uva, tal como os de quebracho, têm a capacidade de se ligar à proteína de soja entre pH 6 e 8, reduzindo a solubilidade do azoto. Estes complexos são dissociados a pH 2, libertando a proteína. Este padrão de ligação suporta o conceito de utilizar estas fontes de tanino como aditivos para proteger a proteína dos alimentos da excessiva degradação ruminal. Existem contudo diferenças entre as fontes de tanino utilizadas parecendo ser os taninos de esteva e de grainha de uva mais eficazes na redução da degradação ruminal da proteína que os taninos de quebracho.

Palavas-chave: *Cistus ladanifer*, complexos tanino-proteína, grainha de uva, pH, quebracho.

INTRODUCTION

Tannins are phenolic compounds functionally defined by their capacity to complex macromolecules (proteins and polysaccharides) and metal ions. Complexation with proteins is the basis of many biological effects of tannins (Min *et al.*, 2003).

Traditionally tannins are grouped into two classes, hydrolysable and condensed tannins (CT) or proanthocyanidins. The CT are the most common type of tannins found in forage legumes, trees and

shrubs (Barry and McNabb, 1999; Mueller-Harvey, 2006) and they are the most commonly responsible for the range of reactions attributed to the presence of tannins in plants (Porter, 1992).

The CT effects on animal nutrition have been extensively studied and are considered to have both, adverse and beneficial effects on the animal depending on concentration and chemical structure (Makkar, 2003b; Min *et al.,* 2003; Mueller-Harvey, 2006).

Tannin-protein complexes are established by non-covalent bonds (hydrogen and hydrophobic interactions, van der Waals forces) or covalent bonds (Bourvellec and Renard, 2006). The covalent bonds are usually irreversible but the non-covalent bonds can be reversible depending on many factors such as pH, structure and molecular weight of CT and proteins (Hagerman, 1989; Bourvellec and Renard, 2006). The pH-dependence of tannin-protein complexes is particularly interesting in ruminant nutrition, whereas pH variation along the digestive system defines the nutritional behaviour of CT. In ruminants, one of the most important effects of CT intake is associated with their capacity to increase the digestive utilization of dietary protein. This effect is due to CT ability to bind proteins under rumen pH conditions (pH 5.5-7.0) preventing the excessive microbial degradation of proteins. Tannin-protein complexes are dissociated in the acidic pH of the abomasum (pH 2.5-3.5) and in alkaline conditions of the distal small intestine (pH ≈ 7.5) and they release the protein for digestion and absorption (Barry *et al.*, 1986).

Quebracho is the most extensively extract explored to modulate ruminal fermentation and to improve protein utilization in ruminants (Frutos *et al.*, 2000; Hervás *et al.*, 2003; Beauchemin *et al.*, 2007; Benchaar *et al.*, 2008; Aljobeili *et al.*, 2013). Rock rose (*Cistus ladanifer* L.) and grape seed are plants rich in tannins and can be alternative tannin sources to improve the digestive utilisation of dietary protein. However different tannin sources can result in different nutritional responses and hence comparative studies with different tannin sources are necessary.

The objective of this study was to examine the effect of pH on the stability of complexes formed between protein and fibre from soybean meal and tannins from rock rose (*Cistus ladanifer* L.), grape seed (*Vitis vinifera* L.) and to compare with quebracho (*Schinopsis quebracho-colorado* Schlecht) extract.

MATERIAL AND METHODS

Three tannin extracts were used: grape seed (La Gardonnenque SCA, France), quebracho (Unitán, Argentina) and rock rose (extract obtained in our laboratory, as described by Dentinho *et al*. (2007)).

Preparation of soybean meal products and washing procedure with different pH buffer solutions

Four samples of 150 g soybean meal (solvent extracted with 440 g kg⁻¹ of crude protein (CP)) were weighed into trays. One sample was treated with 180 ml of 70% aqueous acetone (v/v) and used as control. The remaining three samples were treated using a manual sprayer with 180 ml of 70% aqueous acetone (v/v) and tannin extracts. Extract doses were 9.42, 10.95 and 15.20 g for grape seed, quebracho and rock rose, respectively, in order to obtain soybean meal products with 60 g of total phenols (TP) kg⁻¹ of soybean meal. The amount of extract added to soybean meal was computed using TP and not CT content as it presents a significant correlation with protein precipitation capacity, higher than CT (Makkar, 2003a). Soybean meal control and soybean meal treated with grape seed extract (SG), quebracho extract (SQ) and rock rose extract (SR) were oven dried at 40 °C for 24 h and ground to pass through a 1 mm screen. Potassium phosphate buffers (0.1 M) with pH 6 and 8 were prepared mixing appropriate amounts of solutions of potassium phosphate dibasic (1 M) and potassium phosphate monobasic (1 M) according to Green (1933). The pH 2 and pH 4 buffers were prepared from pH 6 buffer using HCl.

Control samples (4 samples) and treated soybean meal (4 samples / extract type) were weighed (4 g) for 50 ml centrifuge tubes. Thereafter, 20 ml of buffer solutions with different pH were added to centrifuge tubes and then shaken on an orbital shaker (KS 260, IKA®-Werke, Germany) for 1 h at 260 rpm and centrifuged at 1400 *g* for 15 min. Supernatants were collected and stored. This washing procedure was repeated three times. Residues were stored at -20 °C and freeze dried.

Chemical analysis

The tannins extracts were analysed for TP and extractable CT. Treated and soybean meal control as well as residues after washing with buffer solutions were analysed for TP, extractable CT, protein

bound CT and fibre bound CT. Total CT were calculated adding extractable CT, protein bound CT and fibre bound CT.

Extraction of phenolic compounds for TP and extractable CT determinations was carried out in four replicates, as described by Khazaal *et al.* (1993). Samples (200 mg) were extracted by ultrasonication (Model 200 TH/2l, VWR International, Lisboa, Portugal) at 120 W using 10 ml of 70% aqueous acetone (v/v) in an ice bath for 10 min. The obtained extract was centrifuged at 1400 g at 4ºC for 30 min and the supernatant was used for TP and extractable CT assays.

TP were analysed using Folin-Ciocalteu's reagents according to Julkunen-Tiitto (1985), and concentration was measured as tannic acid equivalent using tannic acid (ref. 100 773, Merck KGaA, Darmstadt, Germany) as standard. Extractable CT were measured using the proanthocyanidin assay (butanol-HCl method) (Porter *et al.*, 1986) and concentration was expressed as quebracho equivalents using quebracho as standard. Remaining residues from the previous extraction were dried for 12 h at 60 ºC and used for protein-bound CT and fibre bound CT determinations, according to Perez-Maldonado and Norton (1996) and the concentration was expressed as quebracho equivalents using quebracho as standard.

Soluble N in supernatants was measured according Dulphy and Demarquilly (1981).

Experimental design and statistical analysis

The experiment was a randomize complete block design with a 4 × 4 factorial treatment design, where the first factor was the type of CT extract (control – no extract, grape seed, quebracho, rock rose and the second factor was the pH of buffer solutions (2, 4, 6 and 8). The experiment was conducted in two days (block) and in each day three replicates for each treatment were performed. For TP, total CT, extractable CT, protein and fibre bound, the control treatment was excluded from the analysis, because only negligible amounts were detectable.

Data were analysed using Proc GLM of SAS (2004) according to the following model:

$$Y_{ijk} = \mu + T_i + P_j + (T*P)_{ij} + B_k + e_{ijkl}$$

where Yij = dependent variable, μ = overall mean, T = effect of CT extract (i = 1 to 4), P = effect of pH (j = 1 to 4), (T*P)$_{ij}$ = interaction Ti and Pj, B = effect of day (k=1 to 2) and e$_{ijkl}$ = residual error.

When F test was significant, multiple mean comparisons were conducted using the Tukey-Kramer test.

RESULTS

Total phenolic contents of quebracho, grape seed and rock rose extracts were 820 g, 955 g, and 594 g of tannic acid equivalent kg^{-1}, respectively. Table 1 shows the composition of TP, CT and nitrogen of soybean meal control and soybean meal treated with the extracts. In treated soybean meal, the recovery of TP was 63%, 38% and 26% of the expected concentration (60 g kg^{-1}) for SQ, SG and SR, respectively.

Figure 1A shows the N solubilised by washing and recovered in supernatants for control and treated soybean meals. Figure 1B plots the differences between solubilised N from each treated soybean meal and soybean meal control, in order to emphasize the effect of tannin extracts. In control, solubilised N recovered in supernatants increased (*P* < 0.001) with pH, particularly for pH 8. The treatment with tannin extracts decreased (*P* < 0.001) solubilised N recovered in supernatants and this effect was more expressive when pH increased.

Figure 2 shows the effect of the washing buffer pH on TP and CT of tested soybean meals. The amount of TP, total CT, extractable and complexed CT (protein and fibre bound CT) was affected by the type of extract used (*P* < 0.001), by the buffer pH (*P* < 0.001) and by their interaction (*P* < 0.001).

Total phenolics were higher for SQ, followed by SG and finally by SR. It decreased with buffer pH but this effect was dependent of the type of extract (Figure 2A). Total CT were higher for SG, followed by SQ and for SR. As buffer pH increase the total CT decrease in SG and SR but not in SQ (Figure 2B).

For all extracts the highest values for extractable CT, expressed as g kg^{-1} of total CT, were obtained at pH 2 and the lowest values at pH 8. The pattern of decrease of extractable CT with pH was variable among extracts (Figure 2C). Conversely, the values

Table 1 - Composition on total phenolics, condensed tannins and nitrogen of soybean meal control (Control) or treated with extracts of grape seed (SG), quebracho (SQ) and rock rose (SR)

	Control	SG	SQ	SR
Total phenolics[1]	2.02	23.0	37.7	15.6
Total condensed tannins[2]	ND	88.7	54.1	30.3
Extractable condensed tannins [3]	ND	567	795	475
Protein bound condensed tannins[3]	ND	355	172	423
Fibre bound condensed tannins[3]	ND	78	33	102
N total (g kg^{-1} DM)	82.5	78.1	73.8	72.5
N-soluble (g kg^{-1} total N)	130	90.7	96.6	67.4

[1] tannic acid equivalent in g kg^{-1} of DM; [2] quebracho equivalent in g kg^{-1}of DM; [3] quebracho equivalent in g kg^{-1}CT.

for protein and fibre bound CT (as g kg^{-1} of total CT) were lower at pH 2 and increased with pH, tannins remain unchanged in SG and SQ but increased markedly in SR.
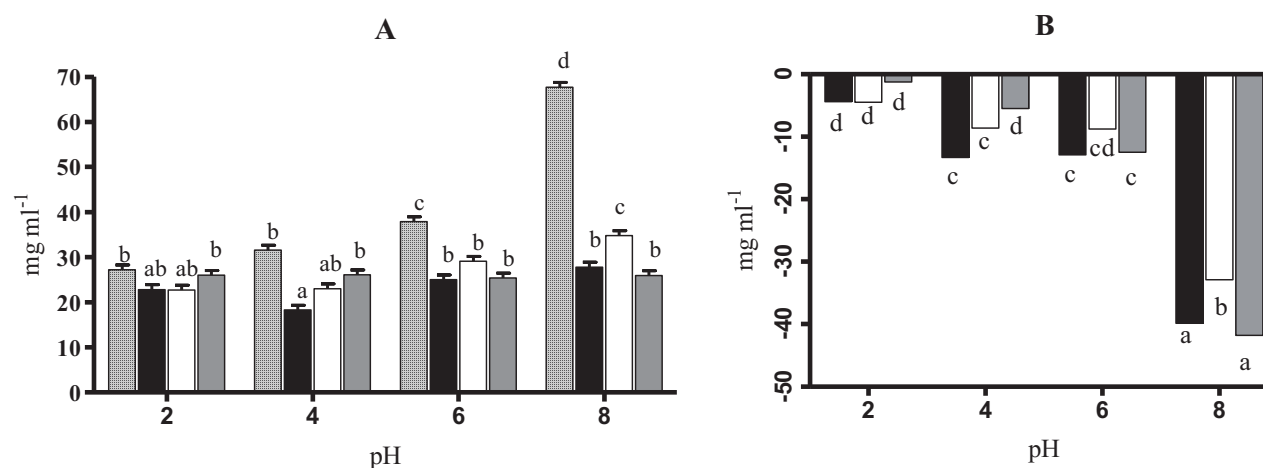

Figure 1 - Effect of washing buffer pH and type of tannins on nitrogen solubility of the soybean meal control (listed column) and treated with tannic extracts of: grape seed (black column), quebracho (white column) and rock rose (grey column). A) Concentration of soluble N in the supernatants and B) Differences between N solubilised of control soybean meal and treated soybean meals.

and the pattern of this increase was also dependent of the type of extracts (Figure 2D and E).

In the SR most of the total CT are protein and fibre bound CT that change across pH, in a more expressive way than in SG or SQ. At pH 2 the protein and fibre bound CT were 368, 247 and 257 g kg^{-1} of total CT for SR, SG and SQ respectively, but at pH 8, the correspondent values were 920, 626 and 408.

With all tannins extracts the protein bound CT fraction increased from pH 2 to pH 4 and to pH 6. From pH 6 to pH 8 the protein bound condensed

DISCUSSION

In soybean meals treated with tannin extracts only 63%, 38% and 26% of the total phenols (TP) added (60 g kg^{-1} of soybean meal) were recovered in SQ, SG and SR, respectively. The low solubility of TP in the acetone/water solution used for their extraction might explain the low recover of TP from treated soybean meals. It is well established that a variable but often important fraction of TP particularly tannins may resist to acetone/water extraction (Scalbert, 1992; Cerpa-Calderon and Kennedy, 2008; Hanlin *et al.*, 2010). Interactions with
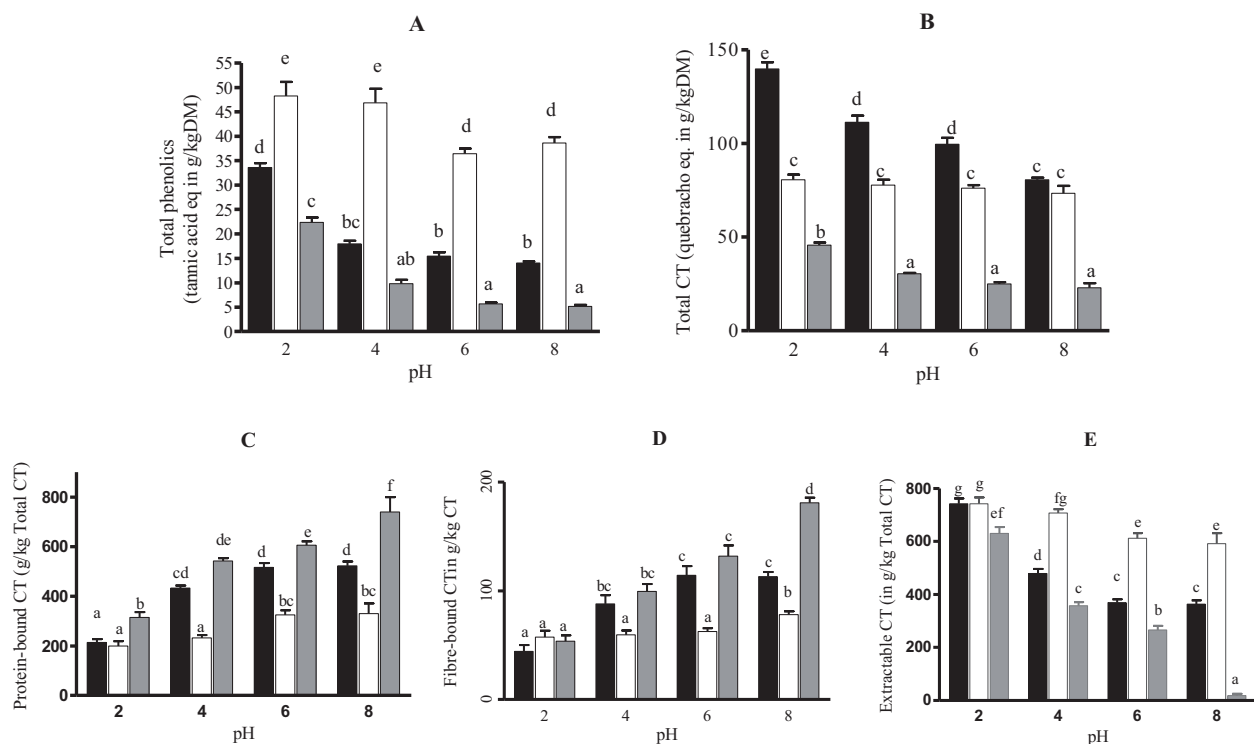
Figure 2 - Effect of pH and tannin extract on the concentration of total phenolics (A) and total condensed tannins (total CT) (B) expressed in g kg^{-1} of DM and extractable (C), protein bound (D) and fibre bound (D) condensed tannins expressed in g kg^{-1} of Total CT of soybean meal treated with a tannic extract of grape seed (black column), quebracho (white column) and rock rose (grey column). Values are least square means with their standard error (n = 6). Differences between treatments are indicated by different letters (P < 0.05).

the insoluble matrix, proteins, polysaccharides and other polymers can decrease the solubility of tannins in the extractant, underestimating tannin content of feeds. In fact, the lowest recover of TP was observed in SR, which also present the highest proportion of CT bound to protein and fibre. Nitrogen solubility was used as an indicator of soybean meal protein degradability. Although many factors, other than solubility, affect rumen degradability of proteins, nitrogen solubility is a key factor determining the protein susceptibility to microbial proteases and thus its degradability (Bach *et al.*, 2005). Due to the good correlation observed between nitrogen degradability and solubility, this parameter has been used to predict degradability in protein rationing systems (NRC, 1985). As expected, soluble N of soybean meal was strongly pH dependent, increasing with the increase of pH, which is fully consistent with data reported by Berk (1992). According to this author, close to 80% of soybean meal protein can be extracted at neutral or alkaline conditions, and when acidity increases, solubility rapidly drops and a minimum is observed near the isoelectric region of soybean proteins (i.e. 4.2 to 4.6). In the present study, minimum

N solubility for control soybean was observed at pH 4 and maintained at pH 2.

The soluble N recovered in the supernatant resulting from washing tannin treated soybean meals with different pH buffers expressed as the difference relative to control soybean meal values (Figure 1B) indicates that at pH 6 all tannins extracts clearly reduce the soluble N and that with increasing pH to 8 the quebracho is less effective in reducing the N solubility than grape seed and rock rose. These results support the possibility of using protein treated with grape seed and rock rose tannin to reduce protein degradation in the rumen (pH 5.5-7). At the lowest pH the solubility of soybean meal protein is already low and no major effects are observed with tannin treatments.

As pH increase both, protein and fibre bound CT, tended to increase and thus the extractable CT tended to decrease, although this pattern is much clearer for rock rose and less clear for quebracho. Variation in the binding capacity of different CT sources to proteins has been attributed to the different chemical structure of tannins, namely the

degree of polymerization, stereochemistry and the number of phenolic hydroxyl groups (Xie and Dixon, 2005). Differences in the ability of different tannins to reduce soluble N in silages were observed by Salawu *et al.* (1999) and those to reduce *in vitro* rumen degradability of protein were observed by Aerts *et al.* (1999).

Makkar and Becker (1996) reported that alkaline treatments were effective to inactivate tannins in feedstuffs by diminishing the amount of extractable CT. Our data indicate that, although at mild alkaline conditions (pH 8) the recovering of extractable CT is lowest, notably for rock rose tannins, the fibre and protein tannin complex remain stable and N solubility reduction compared to control is highest. McNabb *et al.* (1998) reported that CT extracted from several forages (*Rumex obtusifolius, Lotus corniculatus, Lotus pedunculatus, Onobrychis viciifolia, and Hedysarum coronarium*) formed complexes with a soluble leaf protein (Rubisco) which also did not dissociate at pH 8.

CONCLUSIONS

The tannins of grape seed, quebracho and rock rose extracts lead to the formation of pH dependent complexes, mainly with proteins and to a lesser extent with fibre. Although the three tannin extracts presented a different "binding behaviour" across pH range, their ability to bind proteins in the pH range 6-8 remains consistent with the reduction of N solubility. Most of these complexes are dissociated at pH 2 and release protein. This binding pattern supports the concept of utilization of these tannin sources in ruminant nutrition as additives to protect feed protein from excessive rumen degradation. However, different efficacy among tannin sources in the protection against rumen degradation must be considered. Our results suggest that rock rose and grape seed tannins might be more effective in reducing rumen protein degradation than quebracho tannins, particularly animals fed diets promoting a higher rumen pH.

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