Protein-Precipitating Capacity of Crude Condensed Tannins of Canola and Rapeseed Hulls

M. Naczk[a,*], R. Amarowicz[b], R. Zadernowski[c], and F. Shahidi[d]

[a]Department of Human Nutrition, St. Francis Xavier University, Antigonish, Nova Scotia B2G 2W5, Canada,
[b]Division of Food Science, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland,
[c]Faculty of Food Science, Warminsko-Mazurski University, Olsztyn, Poland,
and [d]Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X9, Canada

ABSTRACT: The protein-precipitating potentials (PPP) of soluble condensed tannins (SCT) were determined in hulls from several samples of canola and rapeseed varieties. The PPP were expressed as slopes of lines (titration curves) reflecting the amount of SCT–protein precipitated vs. the amount of SCT added to the reaction mixture. The slopes (S_p) of titration curves obtained using the protein-precipitation assay ranged from 2.96 to 10.91 (absorbance units at 510 nm per mg SCT), and those of titration curves, obtained using the dye-labeled bovine serum albumin (BSA) assay (S_d), ranged from 28.1 to 267 (% precipitated BSA per mg SCT). For both assays, a statistically significant ($P \leq 0.001$) semilogarithmic linear correlation existed between the slopes and the SCT contents in the canola and rapeseed hulls. Higher amounts of SCT–protein complexes were precipitated at 40°C than at room temperature. Determination of titration curves under standardized conditions (type and concentration of protein, pH and temperature) afforded meaningful differences in the slopes among the range of SCT extracts from canola and rapeseed hulls used in this study.

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KEY WORDS: Canola, condensed tannins, hulls, precipitation of proteins, rapeseed, tannin–protein complexes.

The utilization of rapeseed and canola as sources of food-grade proteins is still thwarted by the presence of glucosinolates, phytates, hulls, and phenolics. Phenolic acids and soluble (SCT) and insoluble (ICT) condensed tannins are the predominant phenolic compounds found in rapeseed and canola products. The phenolic contents of rapeseed and canola products are much higher than those found in other oleaginous products. Therefore, phenolics are important factors when considering canola and rapeseed meals as sources of food-grade proteins because they contribute to the dark color, bitter taste, and astringency of rapeseed protein products (1).

Advances in dehulling may soon bring about the introduction of dehulling to rapeseed and canola processing. The proportion of hulls to entire seeds in canola and rapeseed varieties varies from 10.5 to 20% (1,2). The subsequent use of hulls as a component of feedstuffs or food may be considered by the industry. The hulls may also serve as a source of natural antioxidants (3). Canola hulls contain over 15% protein and 66% dietary fibers (2), and more than 6% phenolic compounds. Canola hulls contain up to 2.8% SCT (2,4), and the combined content of SCT and ICT in canola and rapeseed hulls may be as high as 6% (4). Such concentrations of tannins in *Lotus pedunculatas* are nutritionally detrimental in the diets of ruminants (5).

The biological role of tannins is related to their abilities to form soluble and insoluble complexes with proteins. These interactions are well documented for tannins isolated from sorghum (6) and grape seed (7). Evaluating the protein-precipitating capacity of crude tannin extracts from plants, at selected tannin/protein ratios, is tedious and time consuming because the SCT content of the crude extract is affected by the plant source. Martin and Martin (8) proposed that the slopes of lines, formed by plotting the mg of bovine serum albumin (BSA) precipitated vs. mg (dry weight) of leaf material used, can be used to express the protein-precipitating capacity of plant extracts. These authors reported a lack of correlation between the protein-precipitation capacity of plant extracts (expressed as the slope) and the condensed tannin content in plants.

In this paper, the suitability of using the slope of the line that depicts the relationship between SCT added to the reaction mixture and the amount of insoluble SCT–protein complex formed was examined as a means to evaluate SCT–protein interactions. Better knowledge of the factors affecting SCT–protein interactions would be helpful in developing more efficient methods to produce phenolic-free canola and rapeseed protein products and to remove phenolics from hulls.

MATERIALS AND METHODS

Sample preparation. Seeds of Cyclone, Ebony, PR3113, Westar, and Vanguard canola varieties, grown at several locations in Western Canada in 1991–1996, and seeds of Kamer, Leo, and Ligaret Polish rapeseed varieties, grown in Central Poland in 1995, were dehulled according to the procedure described by Sosulski and Zadernowski (9). Hulls were extracted with hexane for 12 h using a Soxhlet apparatus and then desolventized at room temperature.

Faba bean (*Vicia faba*) seeds were obtained from the Institute of Horticulture of Warminsko–Mazurski University in Olsztyn, Poland. Ground faba beans were extracted with hexane for 12 h using a Soxhlet apparatus, desolventized at

*To whom correspondence should be addressed at Department of Human Nutrition, St. Francis Xavier University, Antigonish, P.O. Box 5000, NS B2G 2W5, Canada. E-mail: mnaczk@stfx.ca

room temperature, and then stored in airtight containers for further analysis.

Extraction of SCT. A 2.0-g sample of hulls was extracted twice with 20 mL of 70% (vol/vol) aqueous acetone using a Polytron (Brinkman PT 3000; Littau, Switzerland) homogenizer (60 s, 15,000 rpm) at room temperature. The extract was centrifuged at 1750 × *g* for 10 min and the supernatants were collected, combined, and evaporated to near dryness at 40°C under vacuum. This residue was dissolved in 25 mL of methanol and centrifuged again as described above.

Quantifying SCT. The solutions of crude SCT in methanol were assayed colorimetrically by the modified vanillin method of Price *et al.* (10) as described by Naczk *et al.* (2). The SCT content, *C*, in mg per 100 g oil-free hulls was calculated using the equations: $C = k(1.70A_{500} - 0.00595)$ (correlation coefficient $r = 0.997$ and standard error of estimate = 0.0244), where *k* is the dilution factor, ranging from 1000 to 2500, and A_{500} is the absorbance value at 500 nm. A standard curve was prepared by using SCT isolated from Cyclone canola hulls as described by Naczk *et al.* (4).

Purifying crude SCT. The purified SCT from Cyclone (sample 3) and PR3113 canola hulls were prepared as described by Naczk *et al.* (4).

Isolating proteins. Globulin and albumin fractions of faba bean were isolated according to the procedure described by Gwiazda *et al.* (11). Meanwhile, globulin and albumin fractions of rapeseed meals were prepared following the method of Raab and Schwenke (12).

Protein-precipitation assays (PPA). The effects of crude and purified SCT concentrations on the formation of SCT–protein complexes were assayed by the PPA of Hagerman and Butler (13) (at 1 mg BSA per mL) and the dye-labeled protein assay (DLPA) of Asquith and Butler (14) (at 2 mg dye-labeled BSA per mL) with pH modifications as described by Naczk *et al.* (15). The DLPA permits direct measurement of the amount of protein precipitated by tannins, while the PPA allows estimation of the amount of precipitated protein-bound polyphenols. A series of SCT solutions containing 0.1–1.7 mg SCT per mL in methanol was prepared. The amount of SCT (measured by the PPA) precipitated as SCT–protein complex was expressed as absorbance units at 510 nm, A_{510}, per assay. The percentage of precipitated dye-labeled BSA (measured by the DLPA) was calculated using the equation $C = 12.5(4.902A_{590} - 0.024)$, correlation coefficient $r^2 = 1.00$, where *C* is the percentage of dye-labeled BSA precipitated per assay and A_{590} is the absorbance at 590 nm. When the addition of ferric chloride reagent to an SCT–protein pellet dissolved in sodium dodecyl sulfate-triethanolamine solution caused turbidity, the solution was clarified by extracting with diethyl ether. This was carried out by adding 2 mL of diethyl ether to each tube, vortexing the contents for 10 s and separating the layers by centrifuging at 1750 × *g* for 5 min. The absorbance of the aqueous layer was measured at 510 nm against a blank.

Data collection and treatments. All assays were conducted at room temperature (about 22°C) using appropriate samples and blanks. The results are mean values of six determinations. The statistical analysis of data (linear regression analysis, *t*-test, standardized residuals, standard errors of slopes, and standard errors of estimates) was carried out using the SigmaStat v.2.03 (SPSS, Chicago, IL) software package.

RESULTS AND DISCUSSION

SCT content. The contents of SCT in 13 samples of rapeseed and canola hulls used in this study ranged from 58 to 1878 mg SCT per 100 g hulls as determined by the modified vanillin assay (Table 1). These values are up to eight times higher than those previously reported (1). The differences in SCT contents within a canola variety may be due to the cultivation area and seed maturity. Quantifying SCT in canola hulls by chemical methods has already been discussed by Naczk *et al.* (2) and is

TABLE 1
Contents of Condensed Tannins in Canola/Rapeseed Hulls and Linear Regression Statistics of Titration Curves[a]

Seed variety	Tannin content (mg/100 g hulls)	PPA			DLPA		
		S_p	SE	r^2	S_d	SE	r^2
Cyclone canola							
Sample 1	657 ± 12	5.62 ± 0.29	0.193	0.970	78.7 ± 4.9	2.18	0.992
Sample 2	1878 ± 78	2.96 ± 0.06	0.105	0.996	36.5 ± 3.1	3.52	0.989
Sample 3	1472 ± 51	3.68 ± 0.05	0.092	0.998	29.9 ± 1.2	1.36	0.998
Sample 4	1240 ± 34	4.17 ± 0.14	0.087	0.996	55.0 ± 2.9	1.81	0.996
Sample 5	1325 ± 19	3.88 ± 0.08	0.055	0.998	47.9 ± 1.8	1.19	0.996
Ebony canola	1640 ± 74	3.20 ± 0.18	0.080	0.990	27.6 ± 1.7	1.63	0.991
PR3113 canola	790 ± 36	4.46 ± 0.06	0.040	0.999	58.9 ± 3.2	1.27	0.996
Vanguard canola	1535 ± 42	3.67 ± 0.01	0.011	1.00	28.1 ± 2.1	1.88	0.984
Westar canola							
Sample 1	226 ± 12	7.07 ± 0.60	0.073	0.979	213 ± 16.0	1.69	0.983
Sample 2	1345 ± 39	3.73 ± 0.06	0.021	0.999	73 ± 6.5	2.20	0.977
Kamer rapeseed	82 ± 1.7	9.43 ± 0.63	0.054	0.987	266 ± 24.6	2.05	0.975
Leo rapeseed	58 ± 1.7	10.91 ± 1.43	0.043	0.951	267 ± 41.1	2.80	0.934
Lirajet rapeseed	203 ± 6.0	8.25 ± 0.32	0.067	0.997	141 ± 9.2	0.81	0.987

[a]PPA, protein precipitation assay; DLPA, dye-labeled protein assay; S_p and S_d, slopes of titration curves; SE, standard error of estimate; r^2, squared correlation coefficient.

represented by selected data in Table 1 to facilitate discussing the results from the present study.

SCT concentration. Figure 1 shows the curves (here referred to as titration curves) depicting the amount of dye-labeled BSA or SCT precipitated as an SCT–protein complex with increasing quantities of SCT from canola hulls added to a solution containing a known amount of protein (1 mg/mL for the PPA and 2 mg/mL for the DLPA). A statistically significant ($P \leq 0.0001$) linear relationship existed between the amount of SCT added at ≤0.8 mg tannins/mL for SCT extracts from low-tannin hulls (PR3113 canola) and the amount of SCT–protein complex precipitated (Fig. 1), as well as between the amount of SCT added at ≤1.7 mg tannins/mL for SCT extracts from high-tannin hulls (Cyclone canola). The data also indicate that non-SCT phenolics did not contribute to the protein-precipitating capacity of the crude SCT extracts. In previous studies we showed that ethyl acetate–soluble proanthocyanidins did not contribute to the protein-precipitating capacity of the SCT from crude canola hulls (15).

Type of protein. Table 2 shows the S_p values for a set of proteins. In this experiment SCT–protein complexes were precipitated at pH = 4.0 from a solution containing a known amount (1 mg per mL) of protein using the SCT extracted from Cyclone canola hulls, sample 4 (Table 2). The S_p values ranged from 0.95 for faba bean globulins to 4.53 (A_{510} per mg SCT) for gelatin. The rapeseed albumins and globulins exhibited moderate affinities for the SCT of canola hulls. The differences in the S_p values for the proteins we examined may be due to heterogeneity in their binding sites for SCT.

TABLE 2
Linear Regression Statistics of Titration Curves Obtained Using the PPA[a] for a Set of Proteins

	S_p	SE	r^2
BSA	4.23 ± 0.32	0.196	0.984
Fetuin	2.38 ± 0.18	0.157	0.978
Gelatin	4.53 ± 0.19	0.157	0.993
Faba bean albumins	1.06 ± 0.06	0.052	0.992
Faba bean globulins	0.946	—	1.000
Rapeseed albumins	3.60 ± 0.28	0.026	1.000
Rapeseed globulins	3.23 ± 0.03	0.262	0.982

[a]BSA, bovine serum albumin; for other abbreviations see Table 1.

pH. Figure 2 displays the titration curves for BSA and fetuin at two selected pH values, the optimal pH (OPH) for precipitation of SCT–protein complex and at pH = OPH + 1. The precipitation of these two selected proteins by SCT was affected differently by the pH of the medium (13,15). BSA was effectively precipitated by SCT over a wide range of pH (3–5.5), whereas fetuin was precipitated over a narrow pH range (2.5–3.5) (15). At the optimal pH, the S_p value for BSA was somewhat higher than that for fetuin. However, an increase

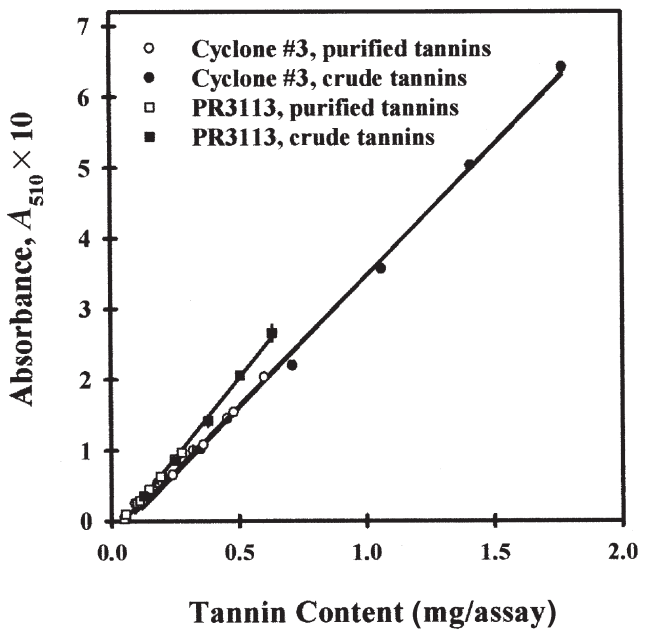

FIG. 1. Titration curves of a known amount of protein with increasing amounts of soluble condensed tannins (SCT) extracted from canola hulls. The protein precipitation assay (PPA) was used for measuring the amount of SCT–protein complex formed. Open symbols indicate SCT purified on a Sephadex LH-20 (Sigma, St. Louis, MO) column; solid symbols indicate crude SCT.

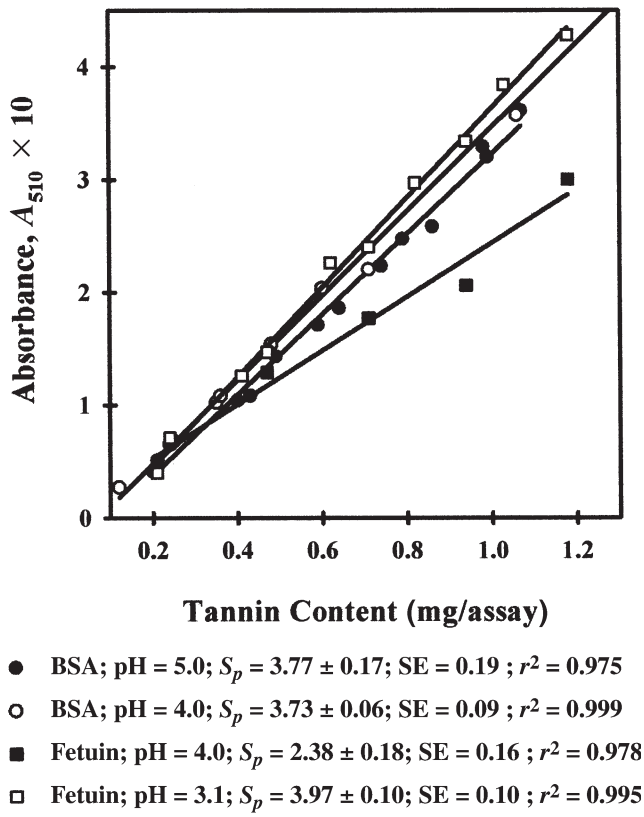

FIG. 2. Titration curves of a known amount of bovine serum albumin (BSA) or fetuin with increasing amounts of Cyclone canola hulls (sample 3) SCT as affected by pH. The PPA was used for measuring the amount of SCT–protein complex formed. S_p, slope values; SE, standard error of estimate; r^2, squared correlation coefficient. For other abbreviations see Figure 1.

in pH from OPH to (OPH + 1) brought about a greater change in the S_p value for fetuin than that for BSA, possibly owing to the apparent differences in their binding sites for SCT. In addition, differences in the overall charge of protein molecules may affect the availability of protein binding sites for SCT.

Protein concentration. Figure 3 shows that the slopes of titration curves are affected by the concentrations of both BSA and dye-labeled BSA in the solutions used for the determination of titration curves. The relationship between the amount of SCT–protein complex precipitated and the quantity of proteins added to a solution containing a known amount of SCT is described by a bell-shaped curve (15–17). The maximal precipitation of tannin–protein complex occurs at the optimal tannin-to-protein ratio referred to as the equivalence point (16). Excess protein leads to the formation of soluble tannin–protein complexes (16), as the precipitation of tannin–protein complex occurs when the number of tannin molecules associated with a protein molecule reaches a critical value (17). Therefore, the changes in the S_p- and S_d-slope values indicate that at protein contents ≤1 mg per mL (for PPA) and ≤4 mg per mL (for DPLA); there was an excess of SCT in the reaction mixture.

Temperature. We evaluated the effect of temperature on the formation of insoluble complexes between crude SCT of canola hulls (Cyclone canola hulls, sample 2) and BSA (Fig. 4A) as well as dye-labeled BSA (Fig. 4B). The amounts of SCT and dye-labeled BSA precipitated, as SCT–protein complexes, were higher at 30 and 40°C than at room temperature (22°C). The S_p values increased from 2.96 to 5.44 (A_{510} per mg SCT), and S_d values from 36.5 to 73.2 (% precipitated BSA per mg SCT) as the reaction temperature changed from 22 to 40°C. This temperature sensitivity suggests that hydrophobic interactions (7,18,19) may be involved in canola and rapeseed hulls SCT–protein interactions. Similar effects of temperature on precipitation of BSA by tannins were reported by Hagerman *et al.* (18) and Oh *et al.* (7). Oh *et al.* evaluated the role of hydrophobic groups in the formation and stabilization of grape condensed-tannin–protein complexes. Based on the increased formation of tannin–protein complexes with increasing temperature and ionic strength, they concluded that hydrophobic interactions were involved in the formation and stabilization of tannin–protein complexes. Based on the effect of temperature and organic solvents on the interaction of BSA with pentagalloyl glucose (PGA) and epicatechin$_{16}$ (4 → 8) catechin (EC_{16}-C), Hagerman *et al.* (18) recently demonstrated that the interaction of PGA with proteins was predominantly due to hydrophobic interactions, whereas hydrogen bonding was the dominant mode of interaction of EC_{16}-C with proteins.

Type of hulls. The slopes of titration curves obtained using the PPA (S_p) ranged from 2.96 to 10.91 (A_{510} per mg SCT), and those of the DLPA (S_d), ranged from 28.1 to 267 (% precipitated BSA per mg SCT) (Table 1). A linear regression analysis of the data showed the existence of a statistically significant ($P \leq 0.001$) semilogarithmic linear correlation between slope values and the content of SCT (C) in canola and rapeseed hulls ($S_p = -4.96\log[C] + 19.29$; $r = 0.993$ and $S_d = -166.7\log[C] + 566.6$;

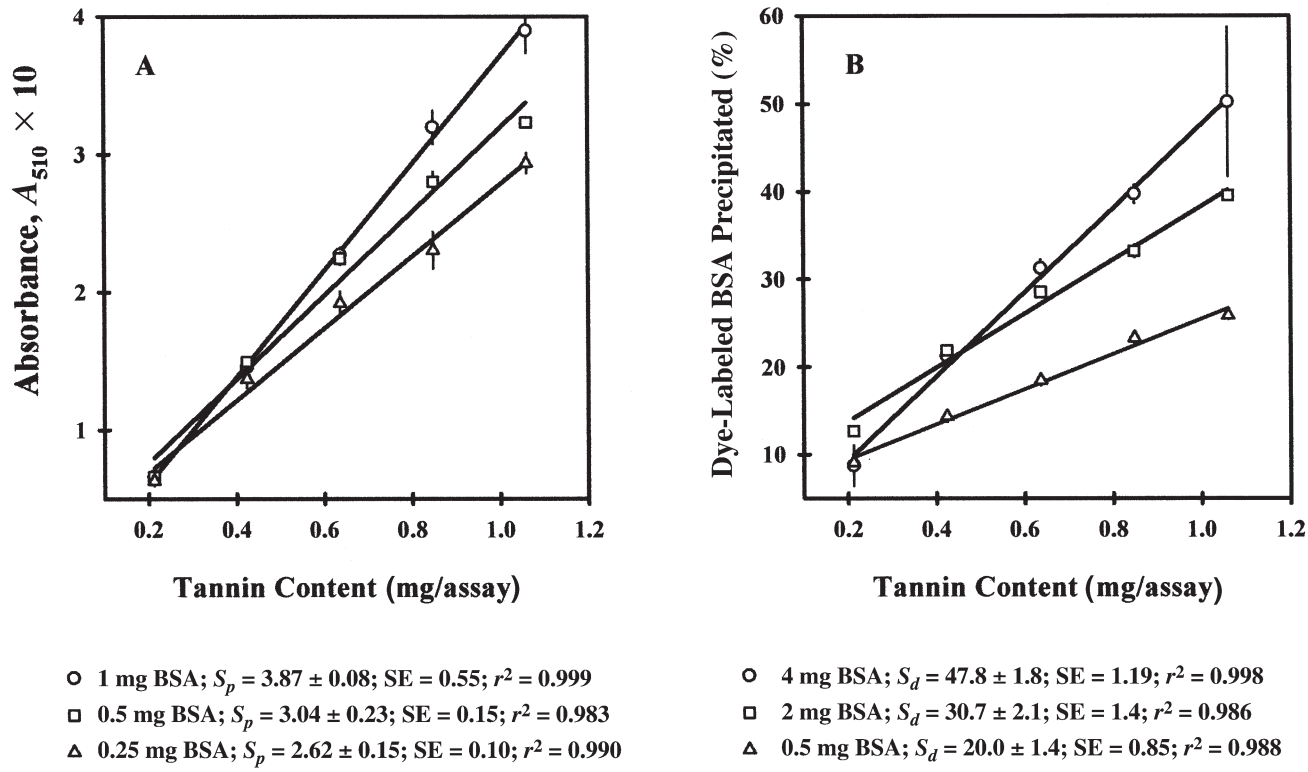

FIG. 3. Effect of protein concentrations on the slope values (S_p and S_d) of titration curves obtained by using (A) PPA and (B) DLPA. SCT were extracted from Cyclone canola hulls, sample 5. For symbols and abbreviations see Figures 1 and 2.

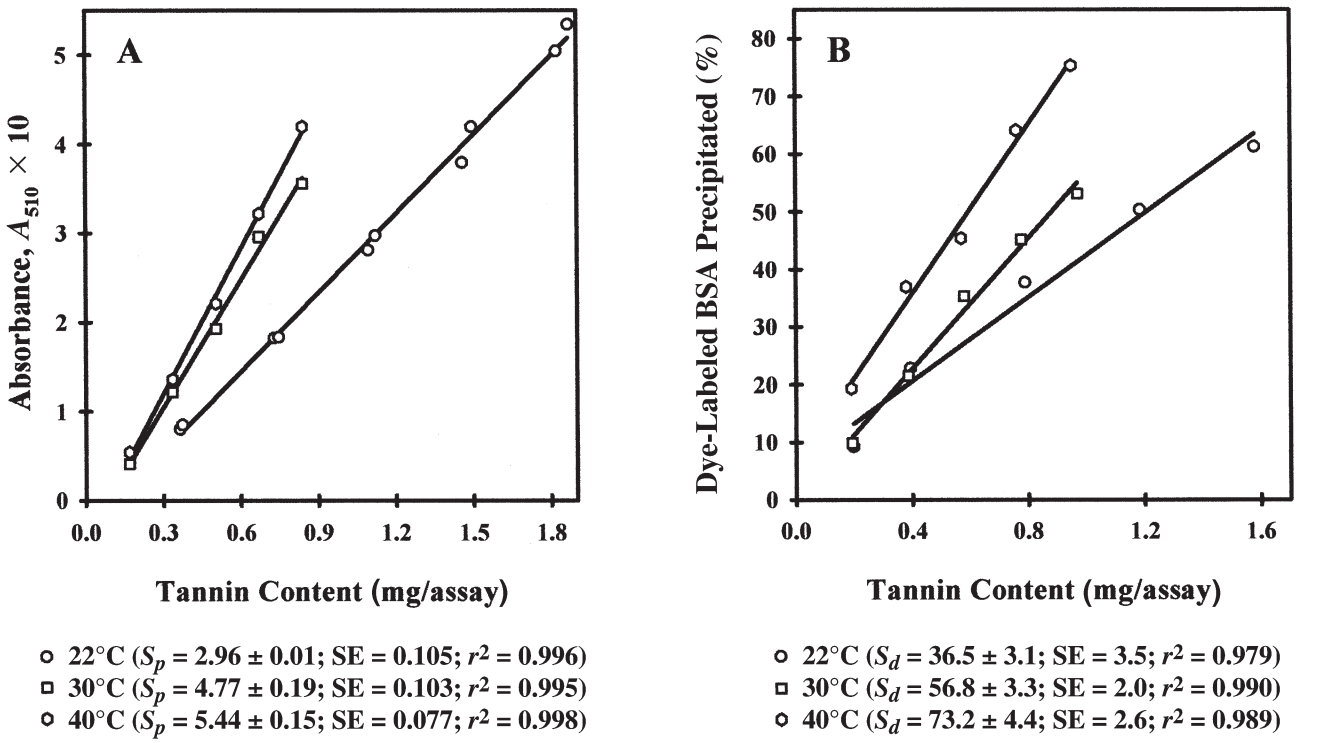

FIG. 4. Effect of temperature on the slopes of the titration curves. SCT used in this study were isolated from Cyclone canola hulls, sample 2. The PPA (A) and the dye-labeled protein assay DLPA (B) were used for measuring the amount of SCT–protein complex formed. For abbreviations see Figures 1 and 2.

r = 0.971). Furthermore, a statistically significant (P < 0.001; r = 0.948) linear correlation existed between the S_p and the S_d values (Fig. 5). The S_p and the S_d values of titration curves for SCT isolated from low-tannin hulls were higher than those isolated from high-tannin hulls. These differences in the slopes among SCT isolated from low- and high-tannin hulls may derive from differences in their affinities for proteins due to their varied degrees of polymerization (2). Porter and Woodruffe (20) demonstrated that the ability of SCT to precipitate proteins depends on the molecular weights of SCT.

Data reproducibility. Reproducibility of titration curves was tested for several canola hull samples using freshly prepared SCT solutions on different days. The statistical analysis of data (*t*-test) indicated that the titration curves obtained on different days were not significantly different (P > 0.05). Therefore, determining protein-precipitation capacity of SCT, as a slope value of titration curve, affords reproducible results. Table 3 shows the statistics of the linear regression analysis carried out for the titration curves assayed on different days.

Statistics. The *t*-test was used to examine the null hypothesis that the slopes of regression lines for the titration curves are zero. The results of this analysis indicated that the slopes were different from zero (P < 0.001). About 95% of the standardized residuals, used here as regression diagnostics, were between −2 and +2, indicating that points on the graphs were not far from the regression lines (21).

The slopes of the titration curves obtained using both the PPA (13) and the DLPA (14) are affected by the molecular

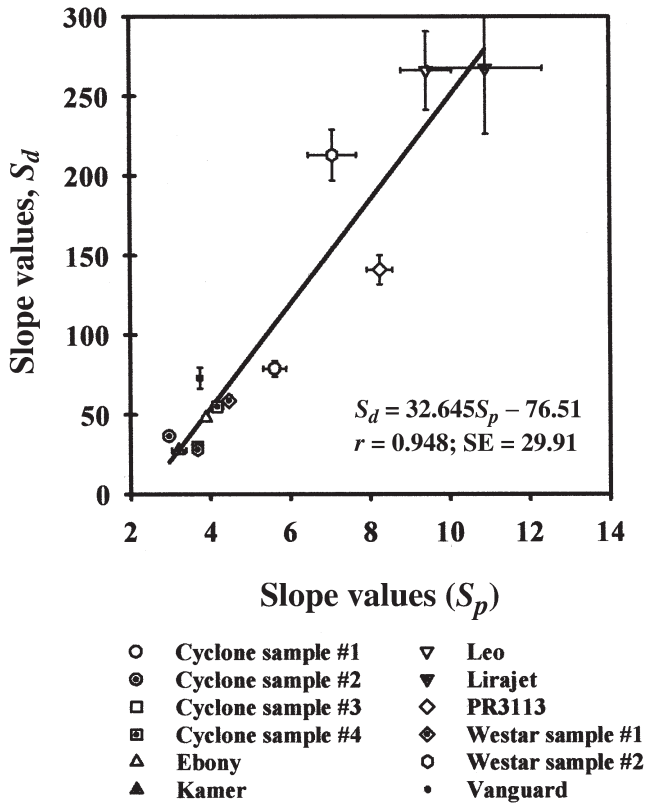

FIG. 5. Plot indicating the linear relationship between the slope values (S_p and S_d). For sample description see text; for abbreviations see Figure 2.

TABLE 3
Linear Regression Statistics of Titration Curves Obtained on Different Assay Days

Canola variety	Day one			Day two		
	S_p[a]	SE	r^2	S_p	SE	r^2
Cyclone						
Sample 1	5.62 ± 0.29	0.193	0.970	5.30 ± 0.26	0.190	0.971
Sample 2	2.96 ± 0.06	0.105	0.996	2.82 ± 0.08	0.090	0.989
Sample 5	3.88 ± 0.08	0.055	0.998	3.84 ± 0.09	0.099	0.999
Ebony	3.20 ± 0.18	0.080	0.990	3.20 ± 0.18	0.152	0.990
PR3113	4.46 ± 0.06	0.040	0.999	4.62 ± 0.12	0.050	0.998

[a]S_p, slope of titration curve obtained using the PPA. For other abbreviations see Table 1.

weights of SCT, the type and concentration of protein, and the pH and temperature of the reaction mixture. Nonetheless, determining titration curves under standardized conditions (type and concentration of protein, pH, and temperature) afforded meaningful differences in the slopes the crude SCT extracts from various canola and rapeseed hulls used in this study. The determination of the slope value is based on statistical analysis (linear regression) of experimental data involving the precipitation of tannin–protein complexes at a minimum of four different tannin levels. Accordingly, the slope is a more meaningful measure of protein-precipitating potential of crude SCT extracts of canola and rapeseed hulls than the measurements carried out at only one nonstandardized tannin/protein ratio (13–15).

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REFERENCES

1. Naczk, M., R. Amarowicz, A. Sullivan, and F. Shahidi, Current Research Development on Polyphenolics of Rapeseed/Canola: A Review, *Food Chem. 62*:1702–1705 (1998).
2. Naczk, M., T. Nichols, D. Pink, and F. Sosulski, Condensed Tannins in Canola Hulls, *J. Agric. Food Chem. 42*:2196–2200 (1994).
3. Amarowicz, R., M. Naczk, R. Zadernowski, and F. Shahidi, Antioxidant Activity of Condensed Tannins of Beach Pea, Canola Hulls, Evening Primrose, and Faba Beans, *J. Food Lipids 7*:195–206 (2000).
4. Naczk, M., R. Amarowicz, D. Pink, and F. Shahidi, Insoluble Condensed Tannins of Canola/Rapeseed, *J. Agric. Food Chem. 48*:1758–1762 (2000).
5. Barry, T.N., The Role of Condensed Tannins in Nutritional Value of *Lotus pedunculatus* for Sheep. 3. Rates of Body and Wool Growth, *Br. J. Nutr. 54:*211–217 (1985).
6. Hagerman, A.E., and L.G. Butler, The Specificity of Proanthocyanidin–Protein Interaction, *J. Biol. Chem. 256:*4494–4497 (1981).
7. Oh, H.I., J.E. Hoff, G.S. Armstrong, and L.A. Haff, Hydrophobic Interaction in Tannin–Protein Complexes, *J. Agric. Food Chem. 28*:394–398 (1980).
8. Martin, J.S., and M.M. Martin, Tannin Assay in Ecological Studies: Lack of Correlations Between Phenolics, Proanthocyanidins and Protein-Precipitating Constituents in Mature Foliage of Six Oak Species, *Oecologia 54*:205–211 (1982).
9. Sosulski, F., and R. Zadernowski, Fractionation of Rapeseed Meal into Flour and Hull Components, *J. Am. Oil Chem. Soc. 58*:96–98 (1981).
10. Price, M.L., S. Van Scoyoc, and L.G. Butler, A Critical Evaluation of the Vanillin Reaction as an Assay for Tannin in Sorghum Grain, *J. Agric. Food Chem. 26*:1214–1218 (1978).
11. Gwiazda, S., K.D. Schwenke, and A. Rutkowski, Isolation and Partial Characterization of Proteins, *Nahrung 24*:939–950 (1980).
12. Raab, B., and K.D. Schwenke, Simplified Isolation Procedure for 12S Globulin Fraction from Rapeseed (*Brassica napus* L.), *Nahrung 28*:863–866 (1984).
13. Hagerman, A.E., and L.G. Butler, Protein Precipitation Method for the Quantitative Determination of Tannins, *J. Agric. Food Chem. 26*:809–812 (1978).
14. Asquith, T.N., and L.G. Butler, Use of Dye-Labeled Protein as Spectrophotometric Assay for Protein Precipitants Such as Tannins, *J. Chem. Ecol. 11*:1535–1544 (1985).
15. Naczk, M., D. Oickle, D. Pink, and F. Shahidi, Protein-Precipitating Capacity of Crude Canola Tannins: Effect of pH, Tannin and Protein Concentration, *J. Agric. Food Chem. 44*:2144–2148 (1996).
16. Hagerman, A.E., and C.T. Robbins, Implications of Soluble Tannin–Protein Complexes for Tannin Analysis and Plant Defense Mechanisms, *J. Chem. Ecol. 13*:1243–1259 (1987).
17. Silber, M.L., B.B. Davitt, R.F. Khairutdinov, and J.K. Hurst, A Mathematical Model Describing Tannin–Protein Association, *Anal. Biochem. 263*:46–50 (1998).
18. Hagerman, A.E., M.E. Rice, and N.T. Ritchard, Mechanisms of Protein Precipitation of Two Tannins, Pentagalloyl Glucose and Epicatechin$_{16}$ (4→8) Catechin (Procyanidin), *J. Agric. Food Chem. 46*:2590–2595 (1998).
19. Kennedy, R.M., Hydrophobic Chromatography, *Methods Enzymol. 182*:339–343 (1990).
20. Porter, L.J., and J. Woodruffe, Haemanalysis: The Relative Astringency of Proanthocyanidin Polymers, *Phytochemistry 25*:1255–1256 (1984).
21. Montgomery, D.C., *Design and Analysis of Experiments*, John Wiley & Sons, 4th edn., New York, 1977, pp. 63–120.

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