Condensed Tannins in Canola Hulls†

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The total content and the biological activity of tannins in canola hulls were determined in several samples of hulls of Westar, Cyclone, Excel, and Delta canola varieties. The contents of condensed tannins in canola hulls ranged from 14 to 1556 mg of catechin equivalents/100 g of hulls as determined by vanillin assay, up to 8 times more condensed tannins than reported previously. Both cultivar differences and the environmental growing conditions may have significant effects on the contents of condensed tannins in canola hulls. Statistically significant linear correlations ($P \leq 0.001$) were found between the tannin content determined by the vanillin and the 4-(dimethylamino)-cinnamaldehyde and the proanthocyanidin assays. Canola tannins had significant biological activity, as determined by the blue BSA assay. The results obtained using the proanthocyanidin and biological assays differed to a lesser extent than those obtained by the vanillin test. This suggests that condensed tannins isolated from low- and high-tannin hulls may differ in molecular weight.

Keywords: *Canola; hulls; condensed tannins; quantification; biological activity*

INTRODUCTION

Tannins are complex phenolic compounds having molecular weights in the range 500–3000. They are widely distributed in foods and feeds of plant origin. Tannins are classified as either condensed or hydrolyzable, on the basis of their structural types and their reactivity toward hydrolytic agents, particularly acids. They may form soluble and insoluble complexes with proteins which may be responsible for the antinutritional effects of tannin-containing ingredients in nonruminant (Martin-Tanguy et al., 1977) and ruminant (Kumar and Singh, 1984) feeds. Tannins are also considered as potent enzyme inhibitors due to their complexation with enzyme proteins. For example, tannins in rapeseed are reported to cause tainting of eggs. Tannins appear to block the metabolism of trimethylamine (TMA) by inhibiting TMA oxidase, an enzyme that converts TMA to odorless, water-soluble TMA oxide (Fenwick et al., 1984).

The presence of condensed tannins in rapeseed hulls was first reported by Bate-Smith and Ribereau-Gayon (1959). This finding was verified by Durkee (1971), who identified cyanidin, pelargonidin, and an artifact, the *n*-butyl derivative of cyanidin, in the hydrolytic products of rapeseed hulls. Later Leung et al. (1979) reported that condensed tannins of rapeseed hulls contained leucocyanidin as their basic units.

Defatted rapeseed cotyledons contain from 0.09% to 0.39% tannins (Blair and Reichert, 1984) as assayed by the modified vanillin method (Price et al., 1978). Recently, Shahidi and Naczk (1989) found that canola varieties contained from 0.68% to 0.77% condensed tannins, as assayed by the modified vanillin method. Rapeseed hulls may account for only 0.02–0.22% of extractable tannins (Mitaru et al., 1982). According to Leung et al. (1979), rapeseed hulls contain up to 0.1% condensed tannins extractable by solvent systems commonly used for the extraction of polyphenols.

The available information on the biological activity of rapeseed tannins is still diverse and fragmentary. Mitaru et al. (1982) reported that condensed tannins isolated from rapeseed hulls did not inhibit α-amylase activity *in vitro*. On the other hand, Leung et al. (1979) found that tannins isolated from rapeseed hulls formed white precipitates after their addition to 1% gelatin solutions. The authors, however, did not attempt to quantify the biological activity of tannins.

Several colorimetric assays have been proposed for the quantification of tannins, but only a few of them are specific toward condensed tannins. These include the modified vanillin method of Price et al. (1978), the proanthocyanidin assay (Mole and Waterman, 1987), and the 4-(dimethylamino)cinnamaldehyde (DAC) method (McMurrough and McDowell, 1978; Thies and Fischer, 1971).

Methods available for the determination of the biological activity of tannins are usually based on their ability to bind and precipitate proteins or to inhibit enzymatic activities. The advances in these methods have been recently reviewed by Makkar (1989). No satisfactory correlations have been found between the tannin concentration and its degree of inhibition of various enzymes (Asquith and Butler, 1985). Accordingly, the estimation of the biological activity of tannins based upon their ability to bind and precipitate protein seems to be the method of choice.

The objective of this study was to determine, comparatively, the total content and the biological activity of tannins in hulls for a range of canola varieties, by utilizing selected chemical and protein precipitation methods commonly used for the quantification of tannins.

MATERIALS AND METHODS

Seeds of Westar, Cyclone, Excel, and Delta canola, grown at several locations in western Canada in 1991–1992 in replicated experimental plots, were bulked, subsampled, and

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Table 1. Chemical Composition of Milled Hull and Cotyledon Fractions from Excel Canola (Percent Dry Basis)

milled fraction	fraction yield	oil	protein N × 6.25	ash	dietary fiber	total analyzed[a]
whole seed	100	42.1 ± 0.7[b]	26.8 ± 0.8	3.8 ± 0.3	17.3 ± 0.4	90.0
cotyledon	74	46.3 ± 0.4	28.3 ± 0.7	3.7 ± 0.1	11.3 ± 0.3	89.6
mixed fines	12	41.7 ± 1.9	26.7 ± 1.4	3.8 ± 0.7	18.0 ± 0.9	90.2
hulls	14	20.1 ± 1.2	19.1 ± 0.4	4.4 ± 0.7	48.0 ± 1.4	91.6
pure hulls[c]	(8.5)	5.5 ± 0.1	15.1 ± 0.1	5.2 ± 0.1	66.3 ± 0.8	92.1

[a] Other constituents include simple sugars and oligosaccharides, polyphenolic compounds, phytate, and residual polar lipids. [b] Standard deviation, $n = 3$. [c] Hand separated from the hull fraction.

dehulled according to the procedure described by Sosulski and Zadernowski (1981). The canola seeds were adjusted to 8% moisture and flaked between smooth rolls and then between corrugated rolls with sieving and air aspiration to separate hulls from the cotyledons. Portions of the embryonic axis and cotyledonary fines were difficult to separate from the hulls, and hand separation was necessary to obtain a pure hull fraction. The pure hull fractions used for the analysis of tannins were finely ground, extracted with hexane for 12 h using a Soxhlet apparatus, and then dried at 50 °C in a forced air oven for 18 h.

Moisture, oil, crude protein, ash, and total dietary fiber in canola and its milled fractions were determined according to methods of the Association of Official Analytical Chemists (AOAC, 1990). Crude protein content was calculated using the nitrogen-to-protein conversion factor of 6.25.

The condensed tannins were isolated as follows. A 1 g sample of hulls was extracted twice with 10 mL of 70% (v/v) aqueous acetone using a Polytron (Brinkman PT 3000) (60 s, 15 000 rpm) at room temperature. After each centrifugation (10 min, 5000 rpm), the supernatants were collected, combined, and evaporated to dryness at 30 °C under vacuum. The extracted phenolics were dissolved in 10 mL of methanol. The methanolic solution of crude tannin extract was used for the quantification of tannins by using chemical and protein precipitation assays.

The condensed tannins were assayed colorimetrically by the modified vanillin method of Price et al. (1978) as follows. Either 5 mL of 0.5% vanillin reagent (sample) or 5 mL of 4% concentrated HCl in methanol (blank) was added to 1 mL of methanolic solution of condensed tannins and mixed well. The absorbance of sample and blank was measured at 500 nm, after they had stood for 20 min in the dark at room temperature. The absorbance of the blank was subtracted from the absorbance of the sample, ΔA_{500}, to account for the potential interference by pre-existing chromophores in the sample. The content of tannins was expressed as ΔA_{500} per gram of hulls. The content of tannins in the hulls was also expressed as catechin equivalents per 100 g of oil-free canola hulls. Catechin (+) containing 3.5 mol of water/mol of catechin (Sigma Chemical Co.) was used as the standard in these experiments. The content of tannins was calculated using the following equation: $C = k\,(1.6835\Delta A_{500} - 0.039)$, with a correlation coefficient $r = 0.999$, where k is a constant and C is the content of tannins in milligrams of catechin equivalents per 100 g of oil-free canola hulls.

The condensed tannins were also assayed by the proanthocyanidin method, as described by Mole and Waterman (1987) as follows. One milliliter of methanolic solution of condensed tannins was added to 10 mL of the 1-butanol–HCl reagent and mixed well. This mixture was heated in sealed ampules for 2 h in a boiling water bath and then allowed to cool. The absorbance of the solution was measured at 550 nm against a reagent-only blank. For $A > 0.75$, a dilution of the reaction mixture was made with 1-butanol. The content of tannins was expressed as ΔA_{550} per gram of hulls. The 1-butanol–HCl reagent was prepared by dissolving 0.7 g of ferrous heptahydrate in 25 mL of concentrated HCl containing a small volume of 1-butanol. This solution was then made up to 1 L with 1-butanol.

The condensed tannins were also quantified by the 4-(dimethylamino)cinnamaldehyde method (McMurrough and McDowell, 1978; Thies and Fischer, 1971). One milliliter of diluted (up to 1:10) methanolic solution of condensed tannins was added to 5 mL of DAC reagent, and the mixture was shaken immediately. The absorbance of the sample was measured at 635 nm, after it had stood for 15 min at room temperature, against an appropriate blank. The contents of tannins were expressed as ΔA_{635} per gram of hulls. The contents of tannins were also expressed as catechin equivalents using the following equation: $C = K(197.94\Delta A_{635} + 0.77)$, with a correlation coefficient $r = 0.997$, where K is a constant and C is the content of tannins in milligram of catechin equivalents per 100 g of oil-free canola hulls. The DAC reagent was prepared by dissolving 1 g of 4-(dimethylamino)cinnamaldehyde in a precooled solution containing 250 mL of concentrated HCl and 700 mL of methanol. Following this, the solution was made up to 1 L with methanol.

The biological activity of condensed tannins in canola hulls was assayed by the dye-labeled protein assay of Asquith and Butler (1985) as follows: 1 mL of methanolic solution of crude tannin extract was added to 4 mL of blue BSA solution containing 2 mg of protein/mL in 0.2 M phosphate buffer, pH 3.5, as modified by Naczk et al. (1994). The mixture was vigorously mixed at 1000 rpm for 5 min at room temperature. The protein–tannin complex was then separated by centrifugation for 15 min at 5000 rpm. The supernatant was carefully discarded and the pellet dissolved in 3.5 mL of 1% (w/v) sodium dodecyl sulfate solution containing 5% (v/v) triethanolamine and 20% (v/v) 2-propanol. The absorbance was measured at 590 nm against appropriate blank. The biological activity of tannins was expressed as milligrams of BSA precipitated per gram of hulls. The blue-labeled BSA was prepared according to the procedure described by Asquith and Butler (1985). Protein concentration was determined by the Folin phenol method (Peterson, 1983).

All assays were conducted at room temperature (about 22 °C) using appropriate samples and blanks. The results presented in the table are mean values of at least six determinations.

RESULTS AND DISCUSSION

Mechanical methods for separation of hulls from canola seeds are inefficient, and dehulling is not a standard practice in canola oil extraction plants. The present canola samples contained 16–18% hulls but only about 14% was separated by air aspiration, as shown for Excel canola (Table 1), and further hand separation was necessary to obtain a sample of pure hulls for each cultivar. The high oil contents of canola and its milled fractions are associated with the difficulty in separating hulls, and only hand separation gave a fraction with 5.5% oil. The pure hull sample contained over 15% protein and 66% dietary fiber as well as the tannins being quantitated in this study.

The contents of condensed tannins in several samples of hulls of four canola cultivars are summarized in Table 2. The tannin contents are expressed as the difference in the absorbances between sample and appropriate blank per gram of sample, because the tannins isolated from plant materials are a series of polymeric compounds (Salunkhe et al., 1990) that differ in their sensitivity toward the reagents used for their assaying (Butler et al., 1982; Scalbert, 1992; McMurrough and McDowell, 1978). This makes the selection of an appropriate standard for the quantification of tannins difficult.

Table 2. Content of Condensed Tannins in Canola Hulls As Determined by Chemical and Biological Assays[a]

canola variety	vanillin assay: mg/100 g of hulls[b]	vanillin assay: ΔA_{500}/g of hulls	DAC assay: mg/100 g of hulls[b]	DAC assay: ΔA_{635}/g of hulls	proanthocyanidin assay (ΔA_{550}/g of hulls)	dye-labeled BSA assay (mg of BSA/g of hulls)
Westar						
sample 1	1556 ± 54.0	9.27 ± 0.32	797 ± 26	39.9 ± 1.3	22.2 ± 0.8	58.6 ± 1.9
sample 2	173 ± 12.0	1.26 ± 0.09	215 ± 4	10.8 ± 0.2	5.5 ± 0.3	30.7 ± 1.4
sample 3	57 ± 3.8	0.57 ± 0.04	168 ± 4	8.4 ± 0.2	4.0 ± 0.4	23.2 ± 1.3
sample 4	142 ± 2.0	1.07 ± 0.02	192 ± 12	9.6 ± 0.6	4.8 ± 0.2	25.7 ± 1.8
sample 5	98 ± 4.8	0.81 ± 0.04	158 ± 8	7.9 ± 0.4	3.3 ± 0.2	32.2 ± 0.5
Cyclone						
sample 1	1307 ± 85.0	8.00 ± 0.52	671 ± 62	33.5 ± 3.1	192 ± 1.5	52.2 ± 1.0
sample 2	994 ± 45.0	6.14 ± 0.28	655 ± 32	32.7 ± 1.6	17.1 ± 0.6	44.2 ± 1.4
sample 3	1574 ± 68.0	9.58 ± 0.41	792 ± 18	39.6 ± 0.9	22.9 ± 1.4	52.7 ± 1.4
Excel	144 ± 7.0	1.09 ± 0.05	235 ± 8	11.8 ± 0.4	6.0 ± 0.2	33.2 ± 0.5
Delta	14 ± 1.0	0.32 ± 0.02	144 ± 8	7.2 ± 0.4	3.4 ± 0.3	

[a] Chemical assays: vanillin, DAC, and proanthocyanidin assays. Biological assays: dye-labeled BSA assay. [b] Expressed as catechin equivalents.

The vanillin method is widely used for quantification of proanthocyanidins due to its specificity for flavanols and dihydrochalcones, both of which possess a single bond at the 2,3-position of the pyran ring and free OH groups at positions 5 and 7 of the benzene ring (Sarkar and Howarth, 1976). Methanol is usually used for carrying out the vanillin reaction because, in methanol, the vanillin reaction is less sensitive to monomer units than it is to the polymeric tannins (Butler et al., 1982). The vanillin assay is commonly standardized with catechin, due to its structural relationship to condensed tannins, but this leads to an overestimation of tannin content (Price et al., 1978). In this study, therefore, the contents of tannins were expressed both as ΔA_{500} per gram and as catechin equivalents per 100 g to facilitate the comparison of the results with those published in the literature.

The total contents of condensed tannins in hulls of four canola cultivars, as determined by the vanillin assay, are summarized in Table 2. The content of condensed tannins in five samples of Westar hulls ranged from 57 to 1556 mg of catechin equivalents/100 g of hulls. The least tannin content was found in the sample of Westar hulls that was considerably contaminated with cotyledons. Seed coats of cereals and legumes are the primary locations of tannins in seeds (Salunkhe et al., 1990). Thus, the low tannin content in this sample may be explained as the effect of hull dilution with cotyledons. The content of tannins in three samples of Cyclone canola hulls ranged from 994 to 1574 mg/100 g, but Delta hulls contained only 14 mg of tannins. These results indicate that canola hulls contained up to 8 times more condensed tannins than reported previously by Mitaru et al. (1982) and Leung et al. (1979). On the other hand, the ΔA_{500} per gram values for high-tannin canola hulls (Table 2) are similar to those reported by Butler (1982) and Price et al. (1978) for high-tannin sorghum varieties. The differences in tannin values (catechin equivalents) within the Westar variety, excluding sample 3, range from 9- to 15-fold. According to Radhakrishnan and Sivaprasad (1980) the local variation in tannin content of sorghum varieties ranged up to 8-fold. The contents of tannins also depend on the stage of seed development. The maximum tannin content in sorghum was found between 25 and 40 days after half-anthesis. The apparent loss of tannin content of mature sorghum seed ranged from 3% to 93% of the maximum content found in immature seed (Price et al., 1979; Butler, 1982). Thus, the differences in tannin content within the canola variety may be due to location, as well as stage of seed development. Accordingly, more detailed studies on the dependence of tannin content upon variety, growing locations, and stage of seed development are necessary.

The DAC reagent is a more specific reagent, in comparison to the vanillin reaction carried out in methanol, since DAC reacts only with the terminal groups of tannins. However, the DAC reagent is sensitive to both monomeric and polymeric units (McMurrough and McDowell, 1978), and this provides an overestimation of the condensed tannin content. To account for the potential interference from chromophores in the samples, 5 mL of 25% HCl in methanol was added to 1 mL samples of methanolic solutions of tannins. The absorbance of the DAC reagent-free sample, measured at 635 nm, against the reagent-only blank was $A_{635} \leq 0.02$ for dilutions of methanolic solutions of condensed tannins less than 1:5. Accordingly, the absorbance of samples containing the DAC reagent was corrected for this contribution. However, for dilutions greater than 1:5 the absorbance of the DAC reagent-free sample was $A_{635} \leq 0.002$ and was negligible.

The contents of tannins in canola hulls, as determined by using the DAC reagent, are given in Table 2. The content of tannins ranged from 144 to 797 mg of catechin equivalents/100 g of hulls. On the other hand, absorbance values, ΔA_{635} per gram, ranged from 7.2 for tannins extracted from Delta hulls to about 40 for tannins isolated from high-tannin samples of hulls of the Westar and Cyclone varieties. The absorbance values obtained using the DAC reagent were much higher than those obtained using the vanillin reagent. Thus, due to its greater sensitivity toward tannins, the DAC reagent seemed to be more suitable than the vanillin reagent for the determination of tannins in low-tannin samples of canola hulls.

The proanthocyanidin assay is carried out in butanol and depends on the acid hydrolysis of the interflavan bonds of condensed tannins to produce anthocyanidins. The yield of reaction depends on HCl concentration, temperature and length of the reaction time, proportion of water in the reaction mixture, and presence of transition metal ions (Scalbert, 1992) as well as the length of the proanthocyanidin chain (Porter et al., 1986). In preliminary studies the presence of the pigment in the extract was accounted for by mixing 1.0 mL samples of methanolic solutions of tannins with 10 mL of 15% (v/v) 0.1 N acetic acid, 15% (v/v) methanol, and 70% (v/v) l-butanol (Butler, 1982). The absorbance values of the samples measured at 550 nm against the reagent-only blank were $A_{550} \leq 0.007$ and were negligible.

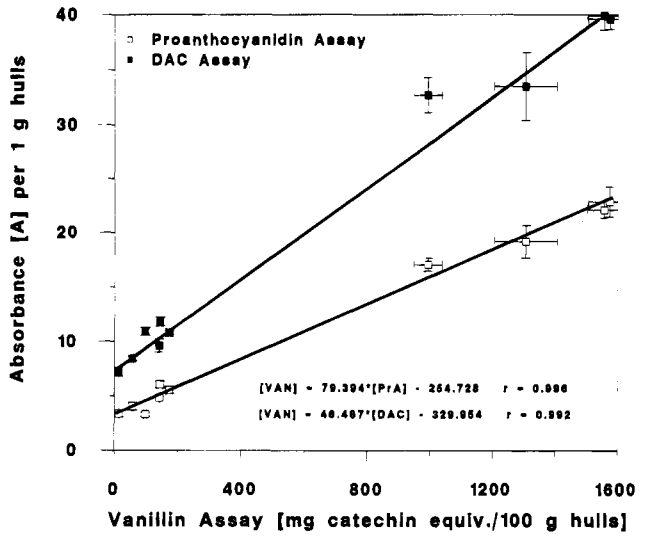

Figure 1. Plot indicating the linear relationships between different chemical assays used for the quantification of canola tannins. [VAN], [PrA], and [DAC] indicate the concentration of tannins as determined by the vanillin, proanthocyanidin, and DAC assays.

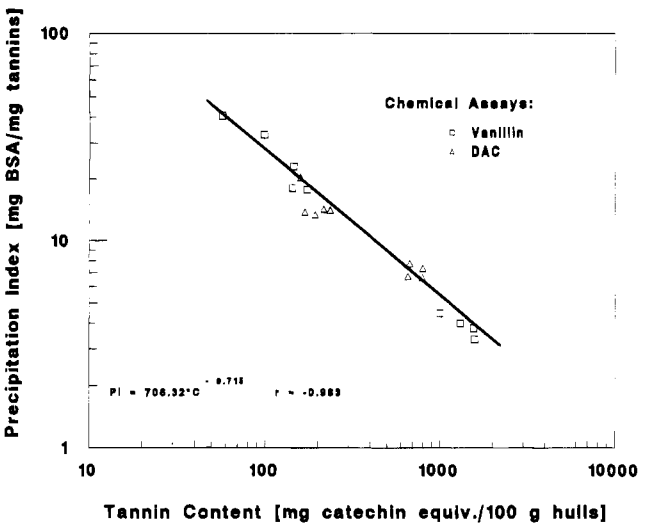

Figure 2. Logarithmic plot indicating the relationship between precipitation index, PI, and tannin content, C, determined by the vanillin and DAC assays.

The contents of tannins in canola hulls as determined using the proanthocyanidin assay are summarized in Table 2. Absorbance values, ΔA_{550} per gram, ranged from 3.4 for tannins extracted from Delta hulls to 22.9 for tannins isolated from one Cyclone hull sample. The results of the proanthocyanidin assay for low- and high-tannin canola hulls differed to a lesser extent than those obtained from the vanillin test. This may be explained by differences in the molecular weight between condensed tannins isolated from high- and low-tannin samples of canola hulls. The longer the polymer chain, the more anthocyanidin pigment is formed (Scalbert, 1992); on the other hand, less of the red condensation product of vanillin reaction is then formed due to an increase in steric hindrance to the vanillin reaction (Mole and Waterman, 1987). Moreover, it has been suggested that the ratio between A^1_1 of proanthocyanidin (PrA) and A^1_1 of vanillin (V_{me}) assays carried out in methanol is related to the length of condensed tannin chains (Goldstein and Swain, 1963). The (PrA/V_{me}) values for high-tannin canola hulls are about 2.4 and for low-tannin canola hulls between 4 and 8. These data suggest that tannins isolated from low-tannin canola hulls may have longer polymer chains than those found in high-tannin canola hulls. Thus, more detailed analysis to determine the polymer chain lengths of tannin isolated from canola hulls is still needed.

Figure 1 shows the data for the DAC and proanthocyanidin assays plotted against the vanillin assay results. Inspection of the data indicates the existence of linear relationships between these three estimates of tannin content. Statistically significant linear correlations ($P \leq 0.001$) were found between the tannin contents determined by the vanillin and other chemical assays.

The biological and ecological role of tannins is attributed to their abilities to bind/precipitate proteins (Bate-Smith, 1973; Hagerman and Butler, 1978; Salunkhe et al., 1990). Several methods are available for the determination of protein precipitation capacity of condensed tannins. Of these methods, the dye-labeled BSA assay, developed by Asquith and Butler (1985), was selected for quantification of biological activity of canola tannins as this assay allows for the direct measurement of protein precipitated by tannins. The biological activities of canola tannins, as determined by using this assay, are summarized in Table 2. The presence of tannins in the protein precipitates was corroborated by using the assay for protein-precipitable phenols developed by Hagerman and Butler (1978) (Naczk et al., 1994). Condensed tannins, extracted from a canola hull sample, precipitated from 23.2 to 58.6 mg of BSA/g of hulls. The complete precipitation of the blue BSA from the solution corresponds to 80 mg of BSA/g of hulls. The affinities of canola tannins for proteins may be characterized by the precipitation index (PI), calculated using the following equation: $PI = B/C$, where B represents biological activity expressed as milligrams of blue BSA precipitated per gram of hulls and C represents tannin content determined by vanillin assay and expressed as milligrams of catechin equivalents per gram of hulls. The PI values for tannins isolated from the samples of high-tannin canola hulls did not exceed 5.0 mg of BSA/mg of tannins, but, for tannins of low-tannin canola hulls, PI ranged from 17.7 to 40.7 mg of BSA/mg of tannins. Similarly, the PI values, calculated using the tannin contents estimated by the DAC assay, ranged from 6.6 to 7.8 and from 13.4 to 20.4 mg of BSA/mg of tannins for high- and low-tannin hulls, respectively. Thus, the tannins isolated from low-tannin canola hulls show greater affinities for proteins than those from high-tannin canola hulls. This may be explained as a result of the differences in the molecular weight between tannins isolated from high- and low-tannin samples of canola hulls. According to Porter and Woodruffe (1984) the ability of condensed tannins to precipitate proteins depends on their molecular weight. The precipitation indexes correlated well with the tannin contents determined by both the vanillin and the DAC assays and expressed as catechin equivalents. Statistically significant correlation ($P \leq 0.001$) was found between the logarithms of PI values and the logarithms of tannin contents (Figure 2).

CONCLUSIONS

Canola hulls were found to contain up to 8 times more condensed tannins than reported previously by Mitaru et al. (1982) and Leung et al. (1979). The results suggest that both cultivar differences and the environ-

mental growing conditions may have significant effects on the content of condensed tannins in canola hulls. Thus, more detailed studies on the dependence of tannin content upon these variables are necessary. Statistically significant linear correlations ($P \leq 0.001$) were found between the tannin contents determined by the vanillin and other assays. Canola tannins exhibit significant biological activity. The results obtained using the proanthocyanidin and biological assays differed to a lesser extent than those obtained by the vanillin test. This suggests that condensed tannins isolated from low- and high-tannin hulls may differ in molecular weight. Thus, more detailed analyses to determine the polymer chain lengths and molecular weights of tannins isolated from canola hulls are still needed.

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