Insoluble Condensed Tannins of Canola/Rapeseed

M. Naczk*

Department of Human Nutrition, St. Francis Xavier University, P.O. Box 5000, Antigonish, Nova Scotia B2G 2W5, Canada

R. Amarowicz

Division of Food Science, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland

D. Pink

Food Research Laboratory, St. Francis Xavier University, P.O. Box 5000, Antigonish, Nova Scotia B2G 2W5, Canada

F. Shahidi

Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X9, Canada

The contents of soluble, SDS-extractable, and insoluble condensed tannins were determined in canola/rapeseed hulls from several varieties by utilizing the proanthocyanidin assay. The total amount of tannins in rapeseed/canola hulls ranged from 1913 to 6213 mg per 100 g of oil-free hulls. Insoluble tannins predominated in canola/rapeseed hulls and comprised from 70 to 95.8% of total tannins present. The amounts of SDS-extractable tannins were comparable to those of soluble tannins but constituted only 4.7−14.1% of insoluble tannins present.

Keywords: *Insoluble condensed tannins; hulls; proanthocyanidin assay; canola; rapeseed*

INTRODUCTION

Tannins are complex polyphenolic compounds having molecular masses in the range of 500−3000 Da. They are widely distributed in foods and feeds of plant origin. 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 artifactual *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.

The presence of insoluble condensed tannins in some herbs was first reported by Bate-Smith (1973). Later, Terrill et al. (1992) determined the content of soluble, sodium dodecyl sulfate (SDS)-extractable, and insoluble tannins in a range of forages, cereal grains, and protein concentrates. The procedure proposed by these authors included the extraction of soluble tannins with a mixture of acetone/water/diethyl ether (4.7:2.0:3.3, v/v/v), followed by the extraction of SDS-extractable tannins with a boiling solution of SDS containing 2-mercaptoethanol. The content of insoluble tannins was determined directly on the residue remaining after extraction of SDS-extractable tannins. Recently, Matthews et al. (1996, 1997) reported the presence of insoluble tannins in tree barks. The ground barks were extracted with methanol/water (1:1, v/v). The extract and the residue were then treated with benzyl mercaptan, and the products of acid depolymerization of tannins were analyzed by employing a reverse phase high-performance liquid chromatographic technique.

Advances in the dehulling of rapeseed (Sosulski and Zadernowski, 1981; Greilsamer, 1983; Diosady et al., 1986) may soon bring about the introduction of dehulling to rapeseed/canola processing. The subsequent use of the hulls as a component of feedstuff may be one route for their utilization. Our studies (Naczk et al., 1994) indicated that canola hulls contained up to 2000 mg of soluble condensed tannins per 100 g of oil-free hulls as determined by the vanillin assay, that is, up to 8 times more soluble condensed tannins than reported previously (Leung et al., 1979; Mitaru et al., 1982). Tannins can form soluble and insoluble complexes with proteins, and this may be the reason for the antinutritional effects of tannin-containing ingredients in nonruminant (Martin-Tanguy et al., 1977) and ruminant (Kumar and Singh, 1984) feeds. According to Terrill et al. (1992) both soluble and insoluble tannins can have antinutritional implications.

The objective of this study was to determine the content of insoluble condensed tannins in hulls for a range of canola and rapeseed varieties by utilizing the proanthocyanidin assay.

MATERIALS AND METHODS

Hulls of Cyclone, Ebony, and PR3113 canola and Kamer, Lirajet, Leo, Mar, Marita, and Polo rapeseed varieties were prepared according to the procedure described by Sosulski and

* Author to whom correspondence should be addressed [telephone (902) 867-2205; fax (902) 867-2389; e-mail mnaczk@stfx.ca].

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Table 1. Content of Condensed Tannins in Canola/Rapeseed Hulls As Determined by Using the Vanillin and Proanthocyanidin Assays (Milligrams per 100 g of Hulls)

canola/rapeseed variety	soluble tannins		insoluble tannins		total tannins [I] + [S]
	vanillin assay	proanthocyanidin assay [S]	SDS-extractable	total [I]	
			Canola		
Cyclone sample 1	1073 ± 43	982 ± 21	510 ± 20	5231 ± 239	6213 ± 240
Cyclone sample 2	2131 ± 22	1501 ± 41	627 ± 21	4438 ± 340	5939 ± 343
Ebony	1317 ± 33	1071 ± 16	395 ± 39	4726 ± 236	5797 ± 237
PR3113	534 ± 29	589 ± 9	276 ± 30	3298 ± 189	3887 ± 189
			Rapeseed		
Kamer	57 ± 4	139 ± 17	232 ± 6	2131 ± 116	2270 ± 118
Lirajet	181 ± 3	293 ± 6	311 ± 17	4867 ± 222	5160 ± 222
Leo sample 1	43 ± 2	89 ± 12	198 ± 17	2053 ± 78	2142 ± 79
Leo sample 2	2442 ± 31	1847 ± 23	552 ± 18	4285 ± 284	6132 ± 285
Mar	78 ± 3	159 ± 3	236 ± 11	1754 ± 111	1913 ± 111
Marita	86 ± 9	160 ± 6	144 ± 11	3060 ± 177	3220 ± 177
Polo	1065 ± 44	950 ± 13	348 ± 23	3417 ± 163	4367 ± 164

Zadernowski (1981). Hulls were extracted with hexane for 12 h using a Soxhlet apparatus and then dried at room temperature.

The soluble condensed tannins were isolated from hulls as follows. A (1.0 g) sample of hulls was extracted twice with 10 mL of 70% (v/v) aqueous acetone using a Polytron (Brinkman PT 3000; Littau, Switzerland) (60 s, 15000 rpm) at room temperature. The extract was centrifuged for 10 min at maximum speed, using an IEC clinical centrifuge (International Equipment Co., a Division of Damon, Needham Heights, MA), and the supernatants were collected, combined, and evaporated to near dryness at 40 °C under vacuum. The extracted crude tannins were dissolved in 10 mL of methanol and centrifuged again as described above. The hull residue, after acetone extraction, was dried at room temperature and then finely ground.

The soluble condensed tannins were assayed colorimetrically according to the modified vanillin method of Price et al. (1978) and the proanthocyanidin method of Mole and Waterman (1987) as described by Naczk et al. (1994). The tannin content, C, in milligrams per 100 g of oil-free hulls, was calculated using the equations $C = k(1.70A_{500} - 0.00595)$ [vanillin assay; correlation coefficient $r = 0.997$ and standard error (SE) of estimate = 0.0244] and $C = k(0.516A_{550} - 0.0135)$ (proanthocyanidin assay; correlation coefficient $r = 0.991$ and SE of estimate = 0.0156). Here k is the dilution factor, ranging from 1000 to 2500, and A_{500} and A_{550} are absorbance values at 500 and 550 nm, respectively. A calibration curve was prepared using tannins isolated from Cyclone sample 2 (see Table 1) and purified according to the method described by Strumeyer and Malin (1975).

The insoluble condensed tannins were assayed according to the proanthocyanidin method of Mole and Waterman (1987) as follows. A mixture of 1 mL of methanol and 10 mL of the butanol/HCl reagent was added to 20 mg of finely ground sample and 70% (v/v) acetone-extracted hull and mixed well. The suspension was heated in capped tubes for 1 h in a boiling water bath, and the contents were vortexed for 5 s every 10 min; the mixture was allowed to cool in an ice bath and then centrifuged for 10 min at maximum speed using an IEC clinical centrifuge (International Equipment Co.). This treatment of the hull residue was repeated five more times. The absorbance of each supernatant was measured at 550 nm against a reagent-only blank. For absorbance values of >0.75 a dilution of the reaction mixture was made with 1-butanol. The tannin content, C_n, in milligrams per 100 g of oil-free hulls, in each supernatant, was calculated using the equation $C_n = k(0.516A_{550} - 0.0135)$ (correlation coefficient $r = 0.991$ and SE of estimate = 0.0156). Again, the dilution factor, k, ranges from 1000 to 2500, and A_{550} is the absorbance value at 550 nm. A calibration curve was prepared using tannins isolated from Cyclone sample 2 and purified according to the method described by Strumeyer and Malin (1975). The total content of insoluble tannins, C, was calculated using the equation $C = \Sigma C_n$, where C_n is the tannin content in the supernatant at the nth treatment step (above) with $n = 1-6$.

SDS-extractable condensed tannins were determined according to the method of Terrill et al. (1992) with the following modification. After the extraction of soluble tannins, 15 mL of SDS reagent (a solution of 10 g/L SDS and 50 g/L 2-mercaptoethanol in 10 mM Tris/chloride, adjusted to pH 8.0 with HCl) was added to the hull residue and shaken well on a vortex mixer. The suspension was heated in a boiling water bath for 45 min, allowed to cool in an ice bath, and centrifuged for 10 min at maximum speed using an IEC clinical centrifuge (International Equipment Co.). The residue was extracted again in the same way. The supernatants were combined, and the volume was adjusted to 100 mL with an SDS solution. A 25 mL sample of this SDS solution of tannins was mixed with 100 mL of methanol and evaporated to near dryness at 40 °C under vacuum. The crude tannin residue was dissolved in 25 mL of methanol and filtered to remove insoluble material. The tannin content of this methanolic solution was determined using the proanthocyanidin assay for soluble tannins, as described above.

Anthocyandins present in a butanol/HCl hydrolysate obtained from hulls or purified canola hulls tannins were separated on a C_{18} Novapak column (5 μm, 3.9 × 150 mm; Waters) with a solvent gradient according to that of Scalbert et al. (1989) and a flow rate of 0.7 mL/min. The separation was performed on a Waters 600E HPLC system equipped with a Waters 996 photodiode array detector and a Waters 715 Ultrawisp sample processor. The wavelength selected for the detection was 530 nm. Identification of compounds was made by cochromatography with a cyanidin standard purchased from Extrasynthese, Genay, France. The retention time of cyanidin was 21.8 min.

The results presented in tables and graphs are mean values of at least six determinations. The data were analyzed using the Table 2D v. 4.0 (SPSS Science, Chicago, IL) and SigmaStat v. 2.03 (SPSS Science) software packages.

RESULTS AND DISCUSSION

The proanthocyanidin assay is commonly used not only for the estimation of soluble tannin content (Porter et al., 1986; Mole and Waterman, 1987; Scalbert et al., 1989) but also for the estimation of insoluble tannin content (Terrill et al., 1992; Makkar et al., 1995). The yield of the reaction depends on the temperature and length of the reaction time, the HCl and water concentrations in the reaction mixture, and the presence of transition metal ions (Scalbert et al., 1989) as well as the length of the proanthocyanidin polymeric chain (Porter et al., 1986).

Figure 1A shows the results of a kinetic study with soluble tannins extracted from Cyclone sample 2 canola hulls. The increase in the absorbance of soluble tannin extract (A_{550}) as a function of reaction time can be described by the equation $A_{550} = 0.599 + 0.209(1 -$

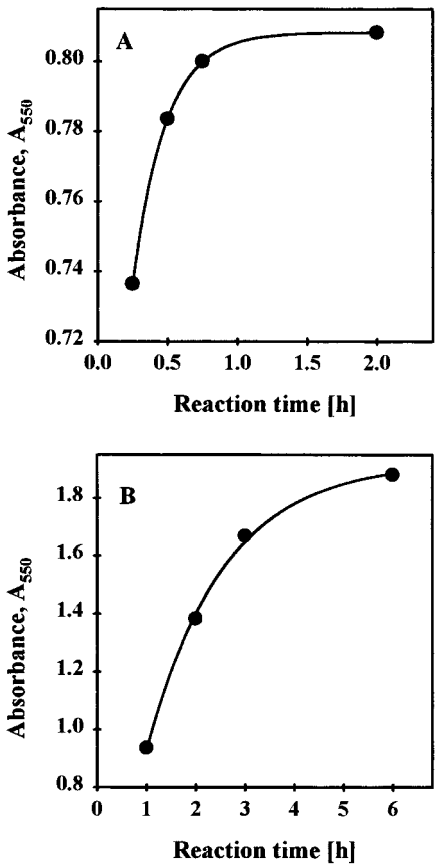

Figure 1. Effect of reaction time on the absorbance, A_{550}, of the reaction mixture of soluble (A) and insoluble (B) Cyclone canola hull tannins and acidified butanol containing ferrous salt.

$e^{-0.0712t}$) (correlation coefficient $r = 0.999$, $P = 0.0022$), where t is the reaction time in minutes. The asymptotic value of absorbance is effectively reached after ~90 min of reaction time. Figure 1B shows a kinetic study with insoluble tannins from 70% (v/v) acetone-extracted Cyclone canola hulls. The increase in the absorbance of the reaction mixture (A_{550}) as a function of reaction time can be described by the equation $A_{550} = 0.0431 + 1.8836(1 - e^{-0.638t})$ (correlation coefficient $r = 0.999$, $P = 0.04$), where t is the reaction time in hours. The asymptotic value of absorbance is effectively reached after ~6 h of reaction time.

Scalbert et al. (1989) observed that for wood extracts the wavelength at the maximum absorption progressively decreased by up to several nanometers as the reaction time of butanol/HCl assay increased. We did not observe such wavelength changes at the maximum absorption for either soluble or insoluble canola tannins at up to 120 min and 6 h of reaction time, respectively (Figure 2). Durkee (1971) identified cyanidin, pelargonidin, and an artifactual *n*-butyl derivative of cyanidin in the hydrolytic products of rapeseed hulls treated with butanol/HCl. However, HPLC analysis of the reaction products obtained from purified canola tannins and acetone-extracted canola hulls showed that cyanidin was the only principal pigment formed.

In a previous study (Naczk et al., 1992), we reported that two-stage extraction of rapeseed meal with 70% (v/v) aqueous acetone was sufficient for the extraction of tannins. In the present study, the effect of the number of extraction stages on the recovery of tannins from Cyclone sample 2 canola hulls was assessed. The

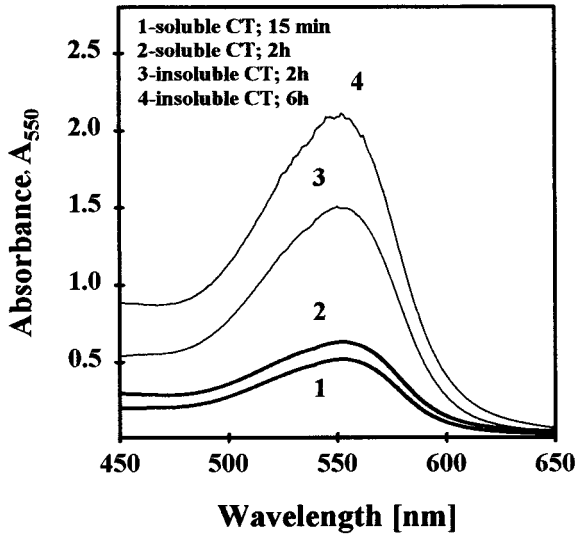

Figure 2. Absorption spectra of soluble and insoluble Cyclone canola hull tannins (CT) as affected by reaction time with acidified butanol containing ferrous salt.

recoveries of tannins (as determined by the vanillin assay) after two- and four-stage extractions of hulls with 70% (v/v) acetone were 2100 ± 15 and 2193 ± 18 mg/100 g of oil-free hulls, respectively, thus indicating that the hull residue after extraction with 70% (v/v) acetone may still contain small quantities of soluble tannins. On the other hand, Terrill et al. (1992) proposed the use of acetone/water/diethyl ether (4.7:2.0:3.3, v/v/v) for the extraction of condensed tannins from forages, cereal grains, and protein concentrates. The results of our studies indicated that the addition of diethyl ether to the acetone/water solvent system lowered the yield of tannin extracted from Cyclone sample 2 canola hulls by 40%. On the basis of the results of these preliminary studies, the two-stage extraction of hulls with 70% (v/v) acetone for the extraction of soluble condensed tannins was selected. The contents of soluble condensed tannins in several samples of hulls of three canola and seven rapeseed varieties, as determined by the vanillin and proanthocyanidin assays, are summarized in Table 1. The differences in the tannin content values between these two assays may be explained as follows. The tannins isolated from plant material are mixtures of polymeric compounds (Salunkhe et al., 1990) that differ in their sensitivity toward the reagents used for their determination (Butler et al., 1982; Scalbert, 1989; Naczk et al., 1994).

The proanthocyanidin assay is the method selected by many researchers (Terrill et al., 1992; Makkar et al., 1995) for the determination of insoluble tannins in plant materials. The above authors subjected plant material, free of soluble tannins, to a one-step treatment with butanol/HCl. In this study we investigated the effect of a multistep treatment of 70% (v/v) acetone-extracted rapeseed/canola hulls with butanol/HCl on the amount of insoluble tannins recovered as anthocyanidin pigments. The content of insoluble tannins in selected canola/rapeseed varieties is summarized in Table 1. It ranges from 1754 mg/100 g of oil-free hulls for the Mar rapeseed variety to 5231 mg/100 g of oil-free hulls for the Cyclone sample 1 canola variety. The differences in the insoluble tannin content, both among and within rapeseed/canola varieties, are much lower than those found for soluble tannin content (Naczk et al., 1994).

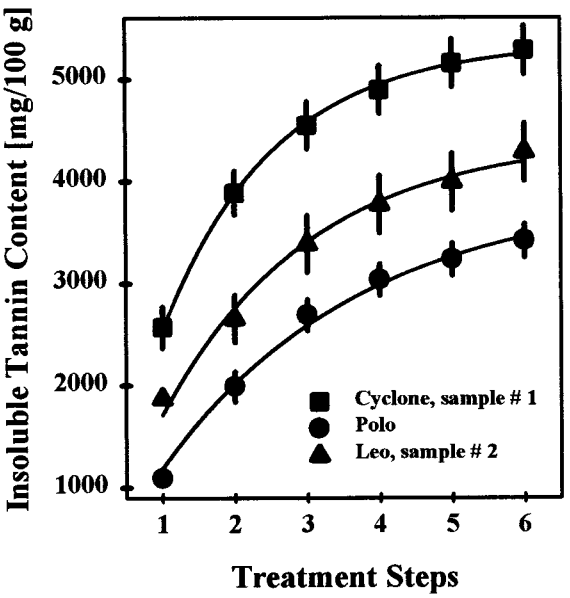

Figure 3. Yield of the extraction of insoluble tannins from hulls as a function of the number of treatment steps with acidified butanol containing ferrous salt.

Figure 3 shows the total amount of insoluble tannins extracted from Cyclone canola sample 1 and Polo and Leo sample 2 rapeseed hulls as a function of the number of treatment steps. This dependence can be described by the nonlinear regression equation $C = a(1 - e^{-bn})$, where C is the total amount of extracted insoluble tannins in milligrams per 100 g of oil-free hulls, n is the number of treatment steps, and a represents the original content of insoluble tannins in hulls in milligrams per 100 g of oil-free hulls (when $n \rightarrow \infty$). The physical meaning of the parameter b can be derived as follows.

Let us assume that at each step, the same fraction, f, of the remaining tannins is extracted as anthocyanidin pigments. Then the amount of tannins removed at the first step is af and $a(1 - f)$ remains. It is then trivial to calculate that after two steps, $a(1 - f)^2$ remains and that after n steps $a(1 - f)^n$ remains. This means that after n treatment steps, the amount of extracted insoluble tannins in milligrams per 100 g of oil-free hulls is $C = a[1 - (1 - f)^n]$. From this we can define $e^{-b} = (1 - f)$ so that $b = |\ln(1 - f)|$. It is then clear that the physical meaning of the parameter b is that it identifies the fraction, f, of tannins removed at each step as anthocyanidin pigments.

Table 2 summarizes the statistics for these nonlinear regression curves describing the amount of insoluble tannins extracted from rapeseed/canola hulls as a function of the number of treatment steps, n. No statistically significant difference was found (t test; $P > 0.05$) between the content of insoluble tannins (Table 1) and corresponding values of a (Table 2). This indicates that six consecutive treatments of hulls with butanol/HCl was sufficient for the extraction of insoluble tannins present in the hulls.

The total amounts of condensed tannins, summarized in Table 1, were calculated as the sum of soluble and insoluble tannin contents as determined by the proanthocyanidin assay. They range from 1913 to 6213 mg/100 g of oil-free hulls for the Mar rapeseed variety and the Cyclone sample 1 canola variety, respectively. In one experiment we also measured the total content of

Table 2. Parameters of the Estimated Nonlinear Regression Curves, $C = A(1 - e^{-bn})$, Depicting the Amount of Insoluble Tannins, C, as a Function of Treatment Steps, n

canola/rapeseed variety	a	b	correl coeff r	probability level P	SE of estimate
		Canola			
Cyclone sample 1	5356 ± 35	0.6132 ± 0.014	0.999	<0.0001	41
Cyclone sample 2	4613 ± 74	0.6492 ± 0.031	0.998	0.0001	66
Ebony	4800 ± 68	0.6236 ± 0.029	0.997	<0.0001	77
PR3113	3268 ± 5	0.8127 ± 0.003	0.999	<0.0001	4
		Rapeseed			
Kamer	2175 ± 31	0.7001 ± 0.031	0.997	0.0001	30
Lirajet	4707 ± 113	0.9918 ± 0.110	0.965	0.0018	186
Leo sample 1	2127 ± 21	0.6292 ± 0.018	0.999	<0.0001	18
Leo sample 2	4430 ± 133	0.4878 ± 0.040	0.994	<0.0001	112
Mar	1841 ± 13	0.6015 ± 0.012	0.999	<0.0001	11
Marita	3208 ± 52	0.4605 ± 0.020	0.998	<0.0001	41
Polo	3890 ± 145	0.3653 ± 0.030	0.996	<0.0001	80

Table 3. Total Content of Condensed Tannins in Canola Hulls (Milligrams per 100 g of Hulls)

canola variety	[S] + [I][a]	hulls
Cyclone sample 1	6213 ± 239	6250 ± 421
Cyclone sample 2	5939 ± 343	6014 ± 242
Ebony	5797 ± 237	5585 ± 450
PR 3113	3887 ± 189	3954 ± 190

[a] [S], soluble tannins content; [I], insoluble tannins content; see Table 1.

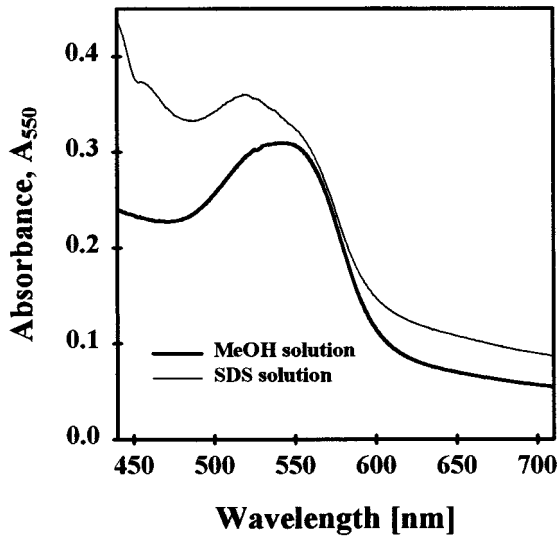

Figure 4. Absorption spectra of the pigments formed as a result of the reaction of SDS-extractable tannins with acidified butanol containing ferrous salt.

condensed tannins in selected unextracted canola hulls by using the multistep butanol/HCl treatment described under Materials and Methods for the determination of insoluble tannin content. The results are shown in Table 3 and are in good agreement with those obtained by the addition of soluble and insoluble tannin contents.

SDS-extractable condensed tannins were extracted from the soluble tannin-free hulls with an SDS solution as described by Terrill et al. (1992). These authors proposed that the tannin content be measured by carrying out the proanthocyanidin assay on this SDS extract. Figure 4 shows the visible spectrum of pigments formed during this reaction (thinner continuous line). The maximum absorption wavelength of the spectrum was shifted from 550 to 520 nm, and a shoulder appeared at 550 nm. However, we found that these

changes in the pigment spectrum can be avoided by evaporating water from the SDS extract in the presence of excess methanol (to prevent excessive foaming), followed by dissolving the residue in methanol and separating the insoluble matter by filtration. The spectrum obtained using the methanolic solution of SDS-soluble tannins has a well-defined maximum at 550 nm. Table 1 summarizes the content of SDS-extractable tannins in rapeseed/canola hulls as determined by using the modified method of Terrill et al. (1992). The content of SDS-extractable tannins ranged from 144 to 627 mg of tannins/100 g of oil-free hulls, and it comprised only 4.7−14.1% of insoluble tannins present in canola/rapeseed hulls. The presence of small quantities of 70% (v/v) acetone-soluble tannins may lead to slight overestimation of the content of SDS-extractable tannins. However, the amounts of SDS-extractable tannins were comparable to that of soluble tannins. The content of SDS-extractable tannins, calculated as the percentage ratio of the content of SDS-extractable tannins to the content of soluble tannins, ranged from 30.6% (Leo sample 1) to 222.5% (Leo sample 2).

CONCLUSIONS

Canola/rapeseed hulls were found to contain up to 6% of condensed tannins. The insoluble tannins may comprise from 70 to 95.8% of the total condensed tannins. The results of this study indicate that the differences in the content of insoluble tannins among and within canola/rapeseed varieties are much lower than those found for soluble tannins. A six-step extraction of hulls with butanol/HCl removed most of the insoluble tannins as anthocyanidin pigments. The insolubility of condensed tannins may be the result of polymerization, as well as the formation of insoluble complexes with the fiber and protein fractions of the canola/rapeseed seed. The amounts of SDS-extractable condensed tannins were comparable with those of soluble tannins, and they comprised from 4.7 to 14.7% of insoluble tannins present in hulls. The determination of SDS-extractable tannins directly on an SDS extract produces an absorption spectrum with the absorption maximum at 520 nm and a shoulder at 550 nm.

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