Multivariate Model for the Prediction of Soluble Condensed Tannins in Crude Extracts of Polyphenols from Canola and Rapeseed Hulls

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ABSTRACT: The feasibility of using ultraviolet spectrophotometry to develop multivariate models for prediction of soluble condensed tannins (SCT) content in crude polyphenols extracts from canola and rapeseed hulls was investigated. The polyphenols were extracted from hulls using 70% (vol/vol) aqueous acetone. Partial least squares regression was used to correlate the spectral data of the crude polyphenols in methanol between 265–295 nm with the SCT content in hulls. Both the proanthocyanidin (P) and the vanillin (V) assays were used to provide reference data for creating the models. The predictive ability of the models is good, as indicated by the RPD values [the ratio of the standard deviation of data to the standard error of calibration (SEC)] of above 5. Additionally, the SEC values suggest that P is superior to V in predicting the SCT content of hulls using this method.

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KEY WORDS: Canola, hulls, PLS, rapeseed, soluble condensed tannins, UV spectroscopy.

Phenolic compounds of oilseeds are hydroxylated derivatives of benzoic and cinnamic acids, coumarins, flavonoids, and lignins (1). Of these, the major phenolic compounds found in rapeseed and canola products are phenolic acids (2) and soluble and insoluble condensed tannins (3,4).

Condensed tannins are complex polyphenolic compounds having molecular weights in the range 500 to 20,000 daltons. They are widely distributed in foods and feeds of plant origin in soluble (SCT) and insoluble forms (5). SCT can form soluble and insoluble complexes with proteins (6), and this may be the reason for the antinutritional effects of tannin-containing feed ingredients in nonruminants (7) and ruminants (8).

Advances in the dehulling of rapeseed may soon bring about the introduction of dehulling to rapeseed and canola processing. The use of the hulls as a component of feedstuff may be one way for their utilization. According to Amarowicz *et al.* (9), hulls may also serve as source of natural antioxidants. Canola hulls contained up to 2,000 mg SCT per 100 g oil-free sample as determined by the vanillin assay (VA) (3), i.e., up to eight times more SCT than that reported previously (10). The total content of condensed tannins (soluble and insoluble) in canola and rapeseed hulls may be as high as 6% (4).

The analysis of polyphenols is affected by their chemical nature, the extraction procedure employed, sample particle size, storage history (time and conditions), selection of standard, and the assay employed for quantification as well as the presence of interfering substances such as waxes, fats, terpenes, and chlorophylls. Numerous methodologies have been proposed for assaying polyphenols in plant material, and these may be classified as either those that determine the total polyphenol content or those that measure specific groups or classes of phenolic compounds. One group of methods is based on ultraviolet (UV) spectrophotometric assays as each class of phenolic compounds is characterized by one or more UV absorption maxima. The suitability of UV methodology depends on the material to be analyzed (11,12). UV spectroscopic assays have been proposed for the estimation of polyphenols content in tea and beer (13), cereals and legumes (14), as well as oilseeds (15).

The objectives of this study were: (i) to examine whether it is possible to predict SCT in canola and rapeseed hulls from UV spectra of crude polyphenol extracts, using partial least squares (PLS) regression method, in order to simplify future analyses; (ii) to determine which of the commonly used chemical assays for quantification of SCT [the VA and/or the proanthocyanidin assays (PA)] provides better reference data for prediction of SCT .

MATERIALS & METHODS

Hulls of Cyclone, Ebony, PR3113, Vanguard and Westar canola varieties and Kamer, Lirajet, Leo, Mar, Marita, and Polo Polish rapeseed varieties (21 samples in total) were prepared according to the procedure described by Sosulski and Zadernowski (16). The hulls were defatted with hexane for 12 h using a Soxhlet apparatus and then dried at room temperature.

The SCT were isolated from hulls as follows. A sample of hulls (1.0 g) was extracted twice with 10 mL of 70% (vol/vol) aqueous acetone using a Polytron homogenizer (Brinkman PT 3000; Kinematica, AG, Littau, Switzerland) (60 s, 15,000 rpm) at room temperature. The extract was centrifuged for 10 min at maximum speed (1,750 × *g*), 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. This residue was dissolved in 10 mL of methanol and centrifuged again as described above. For the spectral analysis, the methanolic solution of crude SCT was diluted with methanol at a ratio of 1:20 (vol/vol).

The SCT, dissolved in methanol, were assayed colorimetrically by the modified vanillin method of Price *et al.* (17) and by the proanthocyanidin method of Mole and Waterman (18) as described by Naczk *et al.* (3). The SCT content, *C*, in mg per 100

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g oil-free hulls was calculated using the equations: $C = k(1.70A_{500} - 0.00595)$ (VA assay; correlation coefficient $r = 0.997$ and standard error of estimate = 0.0244), and $C = k(0.516A_{550} - 0.0135)$ (PA; correlation coefficient $r = 0.991$ and standard error of estimate = 0.0156). Here k is the dilution factor, ranging from 1,000 to 2,500, and A_{500} and A_{550} are the absorbance values at 500 and 550 nm, respectively. Standard curves for both the VA and PA were prepared using condensed tannins isolated from Cyclone canola hulls as described by Naczk *et al.* (4).

The spectral analysis of methanolic solutions of crude polyphenols extracts was carried out using a Beckman 7400 diode array UV-visible spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Spectra of the diluted (1:20; vol/vol) methanolic solution of crude polyphenols were recorded from 240 to 400 nm at room temperature (pathlength 1 cm). Pure methanol was used as the blank.

Mathematical analysis of the data was performed using the PLSplus/IQ v.3.0 software (Galactic Industries Corp., Salem, NH). The PLS-1 method was used to develop chemometric calibration models. The statistical analysis of the data (linear regression, *t*-test, standardized residuals, standard errors of estimates) was carried out using the SigmaStat v.2.03 (SPSS, Chicago, IL) software package. The results are mean values of duplicate experiments (with at least three replicates per experiment). No statistically significant difference (*t*-test, $P > 0.05$) was found among the experiments. The bars in each figure represent standard deviations from mean values.

RESULTS AND DISCUSSION

The range of SCT concentration in canola and rapeseed hulls used in this study was found to be 23.4–2719 and 76.3–1539 mg SCT per 100 g sample by the VA and the PA, respectively. These results are in good agreement with those published previously (3). The differences in SCT contents may be explained by the fact that SCT isolated from plant materials are mixtures of polymeric compounds which differ in their sensitivity toward the reagents used for their determination (3,19).

Figure 1 shows UV spectra for samples of high- and low-tannin hulls of the Cyclone canola variety, respectively. These UV spectra show two absorption maxima. Of these, one is related to the presence of SCT and the other to the nontannin fraction of canola hull polyphenols (Fig. 2). The absorption maximum at the longer wavelength may be due to the presence of phenolic acids, notably hydroxycinnamic acid derivatives, and the maximum at the shorter wavelength may be due to the presence of *p*-hydroxybenzoic acid and flavone/flavonol derivatives (1120).

The UV absorption of crude extracts of plant polyphenols is affected by their composition, the nature of the solvent and pH as well as the presence of interfering substances such as proteins, amino acids, and nucleic acids (11). Therefore, the use of traditional UV spectroscopic assays may lead to the overestimation of the content of polyphenols in crude extracts from plant materials. This makes the task of finding a satisfactory traditional UV spectrophotometric assay quite cumbersome; however, problems can be overcome by using a chemometric technique (21). This approach uses information (such as a spectrum) and chemical indices (such as concentration of a component) and establishes a mathematical relationship between the two. It assumes that the chemical index (concentration) is correct and attributes weightings of the spectral information accordingly. The setting up of the model, correlating the information with a chemical index, is known as calibration.

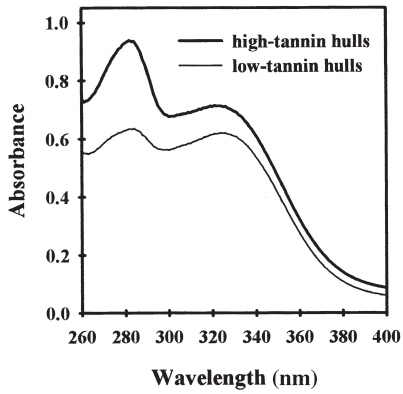

FIG. 1. Ultraviolet (UV) spectra of methanolic solutions of crude condensed tannins isolated from low-(414 mg tannins per 100 g hulls) and high-tannin (1705 mg tannins per 100 g hulls) Cyclone canola hulls.

The use of the PLS method for the development of multivariate calibration models to establish correlations between infrared spectral data and chemical food quality indices has been reviewed by van de Voort (22). Although the PLS method is more commonly used in the infrared (IR) and near-IR spectral range [e.g., Pink *et al.* (23)], it can also be employed in the UV/visible range (24).

The database consisted of 21 samples of hulls of several canola and rapeseed varieties. The modified VA and PA assays were selected to generate reference data, referred to as chemical indices, as these assays are commonly used for quantification of SCT. The optimal number of factors required to develop a satisfactory PLS calibration model was determined on the basis of

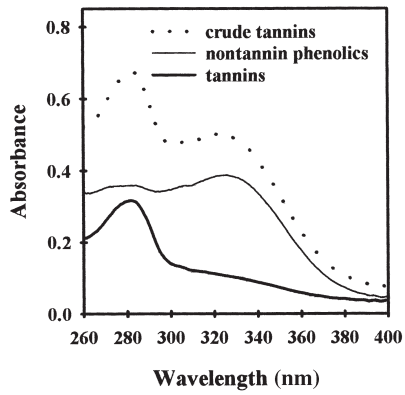

FIG. 2. UV spectra of methanolic solutions of crude condensed tannins isolated from high-tannin Cyclone canola hulls and its nontannin and tannin fractions. The crude condensed tannins were fractionated according to the procedure described by Strumeyer and Malin (31). See Figure 1 for abbreviation.

the prediction residual error sum of squares (PRESS) (21). The data set used in this study was small and therefore employed entirely to develop the PLS calibration model. This model was then tested by the cross-validation procedure involving the rotation of one sample out at each prediction (25). According to Williams and Sobering (26), selection of some samples to serve for validation can, in case of a small database set, lead to artificial improvement in the correlation and statistics. This bias, however, may be avoided by using only the cross-validation procedure.

The PLS-1 method was used because it predicts the analytes (VA and PA data) independently of each other. Of the spectral range 240–400 nm, a narrower region, 265–295 nm, was found to show the best correlation with the chemical data and was therefore selected to develop and then cross-validate the PLS calibration model. This spectral region was also identified in the literature as characteristic of methanolic solutions of SCT (11,20). The optimal number of factors needed to develop a satisfactory calibration model using the reference data from the VA was found to be 5. The results of cross-validation predictions are plotted in Figure 3 in terms of predicted vs. actual tannin contents measured by the VA. On the other hand, only three factors sufficed to develop a satisfactory model using the reference data from the PA. This indicates that the PA is better suited as the reference method. The results of cross-validation predictions are plotted in Figure 4 in terms of predicted vs. actual tannin contents measured by the PA. The *t*-test was used to test the null hypothesis that the intercept and slope values of these linear regressions (equations are given in legends of Figs. 3 and 4) are zero. The results of this analysis indicated that the slopes were different from zero ($P < 0.001$), but the intercepts were not ($P > 0.05$). About 95% of the standardized residuals, used here as a regression diagnostics, were between –2 and +2, indicating that points on the graphs were not far from the regression lines (27). Standard errors of the intercepts (SE_{int}) and slopes (SE_{slope}) are given in legends to the graphs.

The statistics of both cross-validation tests, expressed as the squared coefficient of correlation (r^2) between the spectrally predicted and chemically determined values, were similar (Table 1). The RPD values calculated as the ratio of the standard deviation of the data (SD) to the standard error of calibration (SEC) were 5.57 and 6.17 for the VA and the PA, respectively. According to Williams (28) and Sinnaeve *et al.* (29), this indicates that these PLS models are suitable for quantitative estimation of soluble tannin content in canola/rapeseed hulls. The percentage relative error of calibration calculated as a ratio of SEC to mean tannin content was 15.4 and 22.4% for the PA and VA, respectively. This also suggests that the PA provides a better set of reference data for the development of the PLS calibration model than the VA.

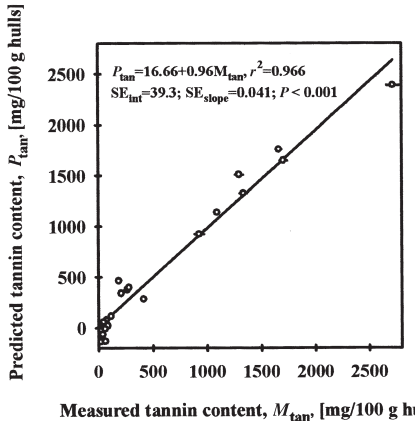

FIG. 3. Plot of condensed tannin content predicted (P_{tan}) by the partial least squares (PLS) model vs. condensed tannin content measured (M_{tan}) by the vanillin assay as shown by the cross-validation results. SE_{int}, standard error of intercept; SE_{slope}, standard error of slope.

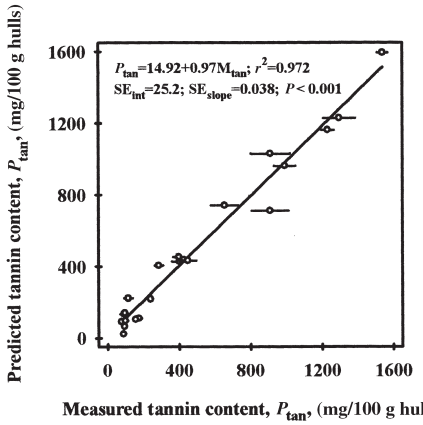

FIG. 4. Plot of condensed tannin content predicted by the PLS model vs. condensed tannin content measured by the proanthocyanidin assay as shown by the cross-validation results. See Figure 3 for abbreviations.

In this study, the PLS method was used to develop multivariate calibration models correlating UV spectral data of methanolic solutions of crude polyphenol extracts with the SCT contents in hulls as determined by the VA and the PA. Results indicated that UV spectroscopy can be used in combination with the PLS method for quantitative prediction of SCT content in crude extracts of polyphenols from either canola or rapeseed hulls, within the concentration range used for the calibration. Similar calibrations can be developed for other plant materials. The best correlation was obtained in the 265–295-nm region which includes the absorption maximum specific for canola and rapeseed tannins in methanol. This study further suggests that if the calibration set can be increased to represent a greater variety of hulls, then, in the future, more accurate prediction of SCT content will be possible by simply recording the UV spectra of crude extracts of polyphenols in methanol and by applying our

TABLE 1
Statistics of the Cross-Validation Predictions of the PLS Model

Assay	Tannin range[a]	Mean tannin content[a]	SD[b]	r^2[c]	SEC[d]	RPD[e]
VA[f]	23.4–2719	600.9	756.1	0.966	135.7	5.57
PA[g]	76.3–1539	490.2	466.9	0.972	75.7	6.17

[a]mg tannins per 100 g hulls; [b]SD, standard deviation; [c]r^2, squared correlation coefficient; [d]SEC, standard error of calibration; [e] RPD = SD/SEC; [f]VA, vanillin; [g]PA, proanthocyanidin; PLS, partial least squares.

calibration. A possible use of this procedure is for screening crude extracts from plant materials as a potential source of natural antioxidants. Hagerman *et al.* (30) recently reported that tannins were 15–30 times more effective in quenching peroxyl radicals than simple phenolics. The research should be expanded to determine whether the absorption maximum at the longer wavelength could be correlated with the total content of phenolic acids in canola and rapeseed hulls.

Prediction of SCT content in crude extracts of canola and rapeseed hulls by the UV-based chemometric technique is ecologically more sound than methods commonly used for quantification of tannins as it reduces the usage of chemicals. The VA requires concentrated HCl and vanillin, and PA employs concentrated HCl, *n*-butanol, and ferrous sulfate heptahydrate as reagents. Furthermore, time required for extraction and preparation of methanolic solutions of crude polyphenols is similar for this chemometric method, and for those of the VA and PA. However, the time required to determine the SCT content in this methanolic solution by the VA is 30–40 min, while that for the PA may be up to 2.5 h (3), but only up to about 10 min using the chemometric procedure described here.

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