Cultivar Identification in *Brassica napus* L. by Reversed-Phase High-Performance Liquid Chromatography of Ethanol Extracts[1]

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A method has been developed to identify cultivars of oilseed rape (*Brassica napus* L.), an outcrossing species, by reversed-phase high-performance liquid chromatography (HPLC) based on the separation of ethanol-extractable seed components. Defatted flour was extracted into 70% ethanol in water, and chromatography was carried out in a reversed-phase C18 column. Optimum results were achieved with water solvent and a gradient to 50% acetonitrile, and peak detection at 210 nm and 240 nm. Trifluoroacetic acid (0.1%) was added to dissociate proteins. Extraction and chromatographic conditions were reproducible, and significant differences were found in the chromatograms of the 29 cultivars studied. The chromatographic differences between these cultivars were stable over a range of growing environments. It was possible to allocate unknown cultivars to their correct classes with the HPLC method together with discriminate analysis. This test has potential as a method of characterization for cultivar registration.

KEY WORDS: *Brassica napus*, canola, chemotaxonomy, cultivar identification, HPLC, phylogeny, rapeseed, taxonomy.

Numerous cultivars of oilseed rape (*Brassica napus* L.) are released annually from breeding programs around the world. The characterization of these cultivars has become increasingly important for plant breeders, particularly with the introduction of Plant Variety Rights legislation in many countries. A rapid, accurate method of cultivar identification would allow plant breeders to monitor the use of their genetic material. Seed purity could be verified to ensure that quality standards are maintained for producers and processors. Plant breeders also could use the method to assist in selecting breeding lines with desirable characteristics.

Many methods have been investigated for identification of canola cultivars. Phenotypic characteristics, including flower color, plant height and leaf shape, may not show sufficient distinctness to differentiate between cultivars. Glucosinolate profiles have been used with some success in swede [*B. napus* L. var. *napobrassica* (L) Peterm.], a related species (1). Protein patterns derived from gel electrophoresis have been utilized for cultivar identification in many species, including *Brassica* (2–4). Although most electrophoresis work is on autogamous (self-fertile) species, the technique is also useful for allogamous (outcrossing) species (5). *Brassica* taxonomy based on restriction fragment length polymorphism has been undertaken by Song *et al.* (6) and Figdore *et al.* (7) for the purpose of studying genome evolution. Reversed-phase high-performance liquid chromatography (RP-HPLC) of seed proteins has been used to characterize cultivars of wheat (8,9), oats (10), soybean (11) and maize (12). Morgan (13) discussed the application of chromatography for cultivar identification at length. This study investigated the application of HPLC to cultivar identification in *B. napus*, based on separation of ethanol-extractable seed components.

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EXPERIMENTAL PROCEDURES

Materials. Samples of *B. napus* cultivars Global and Westar, selected because of their distinct pedigrees, were used to develop the HPLC method. The method was then applied to differentiate between 29 cultivars (*B. napus oleifera annua*, spring types, unless otherwise indicated) with breeder's seed samples obtained from: Agriculture Canada Research Station (Saskatoon, Saskatchewan, Canada): Excel, Tribute, Westar, Tristar, Profit, Bronowski, Oro, Argentine, Midas; Department of Plant Science, University of Manitoba (Manitoba, Canada): Tower, Regent, Reston, Pivot, Stellar, Hero; ICI Seeds, Canada (Winnipeg, Manitoba, Canada): Hyola-40 (hybrid); Pioneer Hybrids (Ontario, Canada): Delta, Winfield *(B. napus oleifera biennis)*, Bounty, Touchdown *(B. napus oleifera biennis)*; and Svalöf Seeds Ltd. (Ontario, Canada): Legend, Vanguard, Celebra, Horizon *(B. rapa oleifera annua)*, Colt *(B. rapa oleifera annua)*, Stallion, Topas, Global, Crystal *(B. napus oleifera biennis)*.

Stability of expression over environments was studied with seed samples of five cultivars, cv. Stellar, Westar, Regent, Delta and Hero, grown in 1991 at five different and diverse sites in Saskatchewan and Manitoba. Seed samples of turnip rape [*B. rapa* L. var. *silvestris* (Lam.) Briggs] cv. Tobin, from two sites, Dauphin and Waskada, Manitoba, were included to determine the degree of discrimination between *B. rapa* and *B. napus* species. The ability of the method to identify unknown cultivars was evaluated with a set of five unnamed seed samples of *B. napus* cv. Westar, which were not part of the original data set. These samples were obtained from trials at additional sites in Saskatchewan.

Sample preparation and extraction. Seeds were ground in a Retsch Grinder fitted with a 0.5-mm screen prior to extraction of oil with a Tecator Soxtec apparatus. The flour was ground again prior to analysis. Soluble components were extracted from the flour (0.04 g) into 1.0 mL of ethanol/water (70%, vol/vol). The tubes were shaken for 1 h on a reciprocating shaker and centrifuged for 5 min. The clear supernatant was removed for HPLC analysis. Electrophoretic analysis of the eluted peaks from the ethanol extracts did not produce evidence of any protein contaminants.

Chromatography of extracts. The analysis was carried out with a Waters' HPLC system (Waters Associates, Milford, MA), including two 510 pumps, a Wisp autosampler, column oven with temperature control and a 994 LC programmable UV-Vis detector. The data system

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incorporated an IBM AT-compatible computer and Waters' "Maxima" integration sotware, which controlled the solvent gradient, peak integration and data handling. A Vydac 218TP54 C_{18}, 5 μM × 30 nm, 0.46 × 25 cm column (S.P.E. Ltd., Concord, Ontario, Canada) was used for chromatography.

Elution solvents were water (Solvent A) and acetonitrile (Solvent B), containing 0.1% vol/vol trifluoroacetic acid, added as a dissociating agent for protein. The solvent flow rate was 1 mL min^{-1} with a gradient of 5 min at 100% Solvent A, increasing to 50% Solvent B over 60 min, and held for 10 min. The gradient was then returned to 100% Solvent A in 10 min and held to equilibrate for 10 min. Column temperature was maintained at 40°C. Injection volume was 20 μL. A linear regression analysis of peak area *vs.* injection volume, up to 200 μL, determined on five independent peaks, indicated that the peak response was linear over the range of injection volumes ($r^2 > 0.95$). Chromatograms were acquired at two different wavelengths of 210 nm and 240 nm.

Reproducibility of extraction and injection. Repeated injections (n = 10) of a single sample were carried out to test instrumental error. Similarly, ten independent extracts of a sample of meal were made over a period of two weeks to determine laboratory error. Excellent reproducibility was achieved in both repeated injections of a single extract (retention time cv = 0.04–0.10, peak area cv = 1.22–7.50, n = 10 peaks) and repeated extractions of a single sample over a two-week period (retention time cv = 0.03–0.09, peak area cv = 1.41–7.50, n = 10).

Statistical analysis. Chromatograms were integrated with Waters' Maxima software. Peak areas of the chromatogram components were used to study levels of discrimination between cultivars with SAS Proprietary Software Release 6.06.01 (SAS Institute Inc., Cary, NC). Options utilized included Proc Means (CV), Proc Discrim, Proc Stepdisc, and Proc Cluster analysis. Only statistical analysis of data acquired at 210 nm is shown.

RESULTS AND DISCUSSION

Characterization of chromatogram components. Solvent extracts of the seed meal contained a wide range of compounds, including phenolics, carbohydrates and proteins, with variable absorbance maxima of the individual components. To assist in characterizing these compounds, absorption ranges of some peaks were scanned between 200 and 400 nm with a photo diode array ultraviolet (UV) detector. Based on a comparison of the UV absorption pattern and the retention time of a commercial standard, the large peak at approximately 25 min (Fig. 1) was identified as sinapine, a major component of rapeseed. Several other components with retention times between 20 and 40 min were tentatively identified as phenolic compounds, including ferulic acid and coumaric acid, by comparison with commercial standards. Addition of 70% ethanol to the flour (0.3 mL water followed by 0.7 mL ethanol) caused the disappearance of some early-eluting peaks, speculated to be glucosinolates hydrolyzed by endogenous myrosinase. Identification of most chromatographic components is not necessary for the profile to serve as a fingerprint of the individual cultivar. Chromatograms run at 210 nm gave the most detail, but the response of several peaks was intensified at 240 nm (Fig. 2).

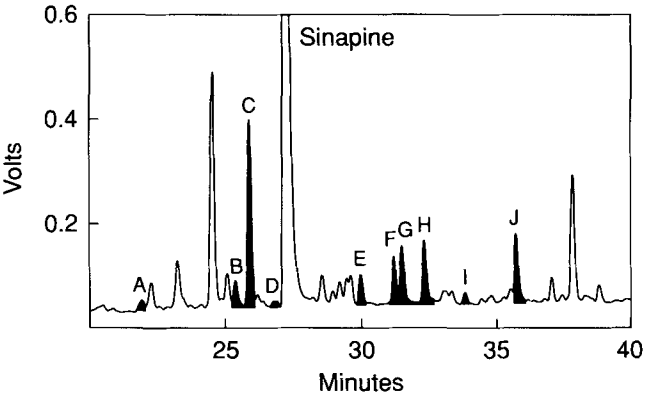

FIG. 1. High-performance liquid chromatography chromatogram of ethanol extract of *Brassica napus* cv. Excel, injection volume 20 μL, column temperature 40°C, flow rate 1 mL min^{-1}, wavelength 210 nm.

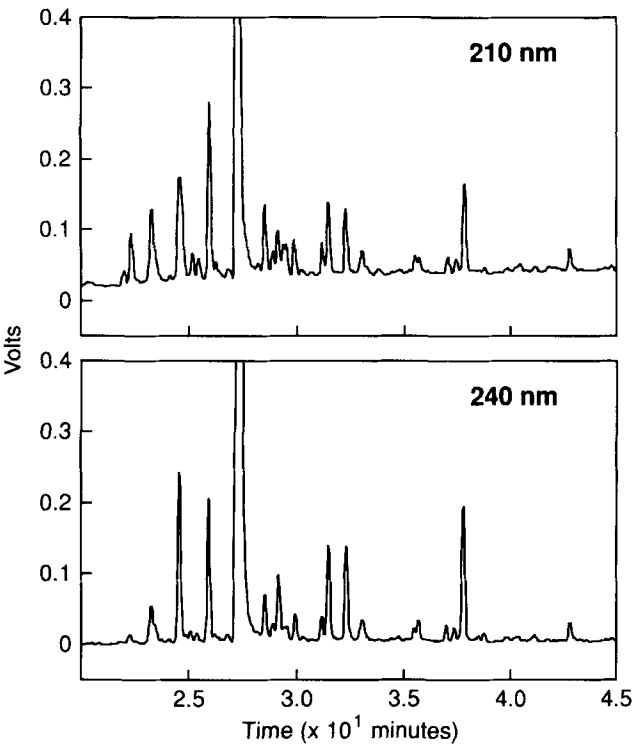

FIG. 2. Detector response at two wavelengths; 210 nm and 240 nm (operating conditions as for Fig. 1).

Cultivar identification. For the 29 samples of breeder's seed analyzed, variability between cultivars ranged from small differences in peak area for some cultivars to large differences in others; six examples of which are shown in Figure 3. Initial observation suggested sufficient variation in the chromatograms to distinguish between the cultivars. Peaks with the most detail eluted in a small area of the chromatogram, and only peaks between 20 and 40 min were used in the analysis. Individual peak areas were calculated on 23 peaks for each chromatogram (Fig. 1). Ten peaks, marked as A through J, were used as discriminating variables and provided the greatest discriminatory power (Table 1). They were selected based on a

TABLE 1

Areas and Retention Times of Ten Peaks for 29 Cultivars Used for Statistical Analysis[a]

	Peak number (retention time in parentheses)									
cv.	1 (21.9)	2 (25.4)	3 (25.9)	4 (26.9)	5 (29.9)	6 (31.2)	7 (31.5)	8 (32.3)	9 (33.0)	10 (35.7)
Argentine	1.5	1.8	13.8	4.1	5.0	3.0	5.3	6.9	2.5	2.5
Bounty	1.3	2.5	14.6	0.6	1.6	4.4	8.6	3.5	0.8	9.3
Bronowski	0.6	2.1	17.8	0.9	1.6	5.4	6.2	4.5	0.0	7.5
Celebra	1.4	3.4	10.9	2.0	1.7	3.1	9.1	6.2	1.4	3.3
Colt	0.4	1.4	14.5	1.8	2.0	2.2	13.7	0.3	1.0	2.3
Crystal	1.8	3.8	17.4	0.8	2.2	2.2	8.5	4.9	1.1	3.1
Delta	0.8	1.9	16.2	0.7	1.1	4.2	10.9	2.7	0.9	13.5
Excel	1.1	2.6	13.5	0.6	2.9	3.4	5.2	5.7	2.0	6.0
Global	0.9	4.3	21.0	0.8	1.8	1.5	8.3	6.8	0.9	3.1
Hero	3.7	5.8	17.0	1.9	1.0	3.3	8.5	3.1	0.6	2.2
Horizon	0.4	1.3	14.0	1.8	2.3	2.3	10.9	0.8	1.1	2.4
Hyola	0.8	2.5	16.6	1.0	1.4	3.5	7.3	6.2	0.0	8.7
Legend	1.2	2.8	14.2	1.5	1.3	4.0	9.3	7.5	1.3	3.4
Midas	1.4	3.2	17.9	0.9	0.4	4.2	6.5	7.5	1.3	1.3
Oro	2.1	3.1	14.7	2.7	4.0	3.2	6.0	7.0	4.3	2.7
Pivot	1.4	4.0	13.1	1.0	3.9	2.4	5.8	4.4	3.8	3.4
Profit	0.5	3.0	13.7	0.8	3.0	3.2	8.1	7.0	2.5	3.9
Regent	1.5	3.6	12.8	2.4	3.8	2.7	5.4	5.4	3.3	2.2
Reston	1.2	3.5	12.0	1.0	4.3	2.9	6.7	6.1	2.6	1.5
Stallion	1.2	3.2	18.6	1.1	2.7	2.1	10.1	5.3	0.7	4.0
Stellar	0.8	2.5	16.1	2.4	3.6	3.9	6.1	2.5	1.6	2.9
Topas	1.0	3.0	17.4	0.9	2.1	3.8	9.9	8.8	1.7	5.0
Touchdown	0.6	2.2	17.5	0.7	0.9	3.5	11.0	4.8	0.0	4.5
Tower	0.8	2.9	15.8	1.6	2.8	2.7	7.4	4.8	2.2	2.1
Tribute	0.5	2.8	13.8	0.7	1.0	3.3	6.7	4.7	2.1	2.8
Tristar	1.0	2.6	14.8	0.6	3.2	2.7	8.7	4.7	2.6	2.1
Vanguard	1.1	2.8	17.6	1.0	1.4	4.6	12.3	7.3	2.7	3.6
Westar	1.3	2.4	12.5	1.7	3.2	2.8	6.4	6.1	3.9	2.0
Winfield	1.0	1.4	14.0	0.7	0.7	3.2	10.4	5.2	0.8	5.1

[a]Based on percentage of the total area of the 23 peaks integrated.

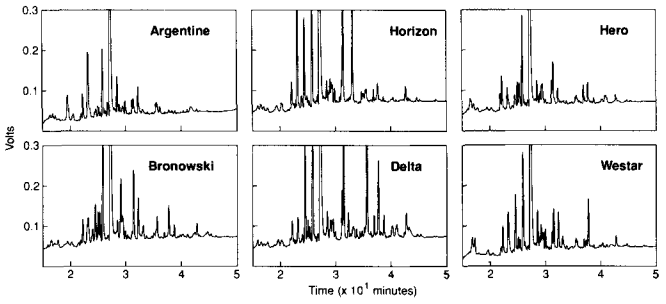

FIG. 3. Chromatograms of five *Brassica napus* and one *B. rapa* cultivar. Chromatograms have been amplified for detail (operating conditions as for Fig. 1).

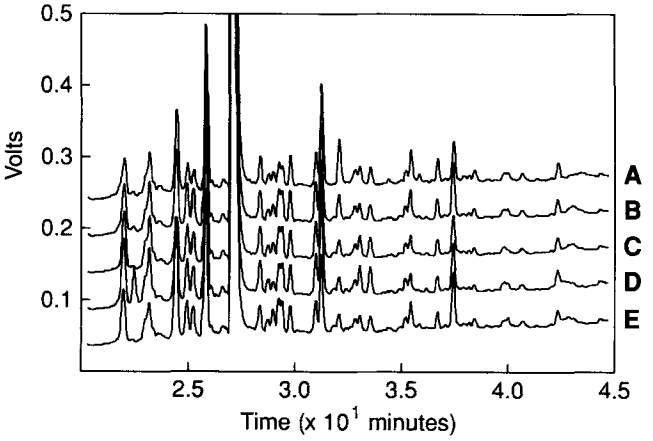

FIG. 4. Five overlaid chromatograms of *Brassica napus* cv. Stellar grown at five different sites. A, The Point; B, Baggot; C, Teulon; D, Dauphin; and E, Roblin (operating conditions as for Fig. 1).

combination of results from SAS stepwise discrimination and visual comparison of the 29 chromatograms. The number of discriminating variables used in SAS discrimination analysis is limited to two less than the total number of cases (14). However, improved discrimination is achieved by reducing the components to the minimum.

Six cultivars selected to test the stability of expression over environments included five cultivars of *B. napus* (two with similar pedigrees, Stellar and Regent, and three with distinct pedigrees, Delta, Westar and Hero) and one cultivar of *B. rapa* cv. Tobin. Analysis of seed samples of these cultivars showed good reproducibility of most components over environments, as illustrated by the overlaid chromatograms of cv. Stellar in Figure 4. These data were analyzed by cluster analysis (Fig. 5), which was generally successful in grouping samples of the same cultivar. The Westar group was separated into two clusters and one Delta sample clustered outside the other Delta samples, indicating environmental influences across the sites selected.

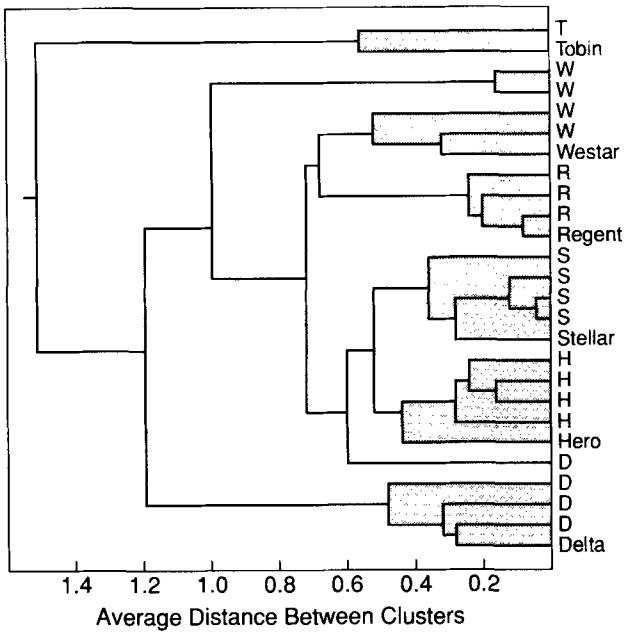

FIG. 5. SAS (SAS Institute Inc., Cary, NC) average linkage cluster analysis of five *Brassica napus* and one *B. rapa* cultivar grown at different sites. T. Tobin; W, Westar; R, Regent; S, Stellar; H, Hero; D, Delta.

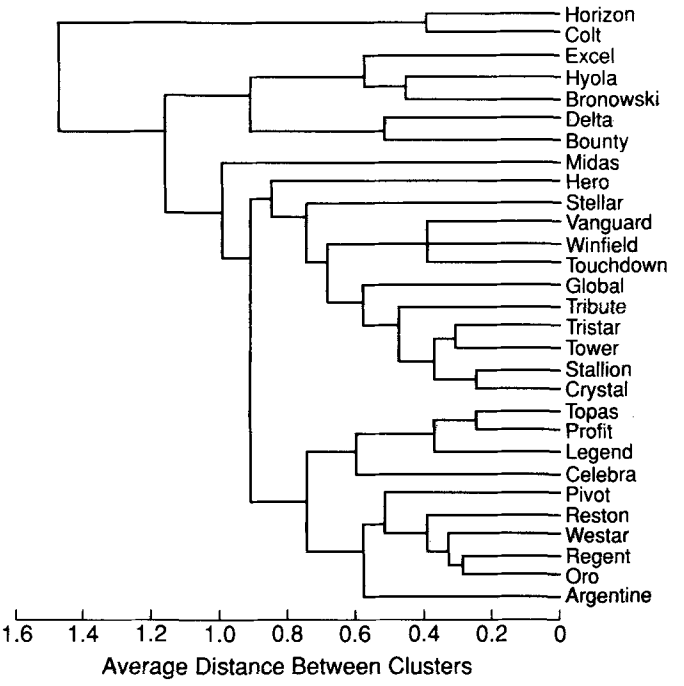

FIG. 6. SAS (SAS Institute, Inc., Cary, NC) average linkage cluster analysis of 29 cultivars of oilseed rape based on ten peak areas from Table 1.

A data set was produced with SAS discriminate analysis by using the data from the five cultivars of *B. napus* at five sites to test environmental stability. The ability of the method to discriminate unknown samples was tested with five *B. napus* cv. Westar seed samples, acquired from Saskatoon Canola Growers Association trials from four additional sites. These samples were labelled with their site code numbers only. From the same 10 peaks identified in Figure 1, all the samples were successfully allocated to the Westar group. These results demonstrated that the characterization of the ethanol-extractable components analyzed by HPLC can provide a means to differentiate between cultivars. Discriminate analysis was a useful tool for testing the ability of HPLC to identify cultivars and appeared to be the most useful method for future characterization of cultivars. Discrimination might be improved if the components of interest were extracted from the background of the total ethanol-extractable material, but when this is not possible, crude extract appears to be sufficient.

The data generated from the analysis of 29 cultivars was used in cluster analysis to investigate the discrimination distances between cultivars (Fig. 6). In addition to successfully discriminating between all 29 cultivars, the analysis tended to cluster cultivars of similar characteristics, such as the *B. rapa* cv. Colt and Horizon and *B. napus oleifera biennis* cv. Winfield and Touchdown.

The results of this study have established a routine method for cultivar identification through the analysis of ethanol extracts of rapeseed meal. The method involves the analysis of a set of reference standards of 3-5 samples of each cultivar by using peak components that are cultivar unique. Due to differences between columns and laboratories, it is necessary for individual laboratories to establish individual reference standards prior to analysis of unknowns. To test for a particular cultivar, samples of that cultivar should be included in the reference set. The set would be further enhanced by a few specific peaks that discriminate between a limited number of cultivars.

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