Optimisation and Evaluation of Reversed-Phase High-Performance Liquid Chromatography for Cultivar Identification of Brassica napus L.

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Summary

Cultivar identification has become increasingly important with the international recognition of Plant Breeders Rights. The objective of this study was to develop a method to identify cultivars of *Brassica napus* L., an outcrossing species, using reversed-phase high-performance liquid chromatography (RP-HPLC) based on the separation of ethanol extractable seed components. Characteristic profiles were achieved using 70% ethanol extracts of defatted flour. Chromatography was carried out using a Vydac C18 reversed-phase column. A water/acetonitrile gradient, containing 0,1 % trifluoracetic acid, from 0 to 50 % acetonitrile over 60 minutes, provided optimum resolution. Maximum absorbance was obtained at detection wavelengths of 210 nm and 240 nm. Repeated analysis indicated that extraction and chromatographic conditions were reproducible. This study shows that the method has potential for identification of *B. napus* cultivars.

Introduction

Oilseed rape *(Brassica napus)* is a valued commercial crop due to its high quality of oil and meal. New cultivars of rapeseed are constantly being developed from breeding programs around the world. Identification of these cultivars is increasingly important for plant breeders, particularly with the introduction of Plant Breeders Rights (PBR) legislation in many countries. Outcrossing species such as *Brassica* are difficult to characterize as each cultivar consists of a heterogenous population. The ability to identify seeds is important to allow plant breeders to monitor the use of their genetic material. Accurate identification also enables seed purity to be verified by seed testing laboratories and certification authorities to ensure that quality standards are maintained.

Despite numerous investigations for characterization of outcrossing species such as Brassica, unambiguous identification has not been possible. Phenotypic characteristics including flower colour, plant height and leaf shape do not show sufficient distinctness to differentiate between cultivars and are often influenced by environment and stage of plant maturity. Glucosinolate profiles have been used to identify swede (*B. napus* L. var. *napobrassica* (L) Peterm.), a related species (Adams, Vaughan and Fenwick, 1989). Electrophoresis and reversed-phase high-performance liquid chromatography (RP-HPLC) of seed proteins has been used to characterize cultivars of many crops including wheat (Marchylo, Hatcher and Krüger, 1988), oats (Lookhart, 1985), soybean (Buehler et.al., 1989) and maize (Smith, 1988). Although most electrophoresis work has been on autogamous (self-fertile) species, the technique has been shown to be useful for allogamous (outcrossing) species (Gilllliland, 1989). Protein patterns have been utilised for cultivar identification in Brassica species by polyacrylamide gel electrophoresis (PAGE) (Ladizinsky and Hymowitz, 1979 ; Gupta and Röbbelen, 1986). Brassica taxonomy was studied using restriction fragment length polymorphisms (RFLPs) (Song et.al., 1988, Figdore et al. 1988) for the purpose of studying

genome evolution. The wide range of phenolic and flavonoid compounds in *Brassica* species have been used to generate taxonomic data (Hoshi and Hosoda, 1978). The objectives of this study were to develop and optimize a method of RP-HPLC cultivar identification in *B. napus* based on characteristic seed components including proteins and phenolic compounds.

Materials and Methods

Seed samples of five cultivars of *B. napus* cv. Westar, Stellar, Hero, Delta, Regent ; and 1 of *B. rapa* L. var. silvestris (Lam.) Briggs cv. Tobin were obtained from cultivar evaluation trials grown in 1991 at sites in Saskatchewan and Manitoba as detailed in Table 1. Two of the cultivars had similar pedigrees, cv. Stellar and Regent, and the other three cultivars had distinct pedigrees, cv. Delta, Westar and Hero. Two additional seed samples cv. Global and Westar were used to develop the HPLC method.

Analyses were performed using a Water's HPLC system including two 510 pumps, a Wisp autosampler, column oven with temperature control and a 994 LC programmable photodiode array UV-Vis Detector. The data system incorporated an IBM AT compatible computer together with Water's «Maxima» integration software which controlled the solvent gradient, peak integration and data handling. A Vydac 218TP54 C18, 5 µM X300 A°, 0,46 X 25 cm column was used for chromatography. Elution solvents were water (Solvent A) and acetonitrile (ACN, Solvent B), each containing 0,1% trifluoroacetic acid (TFA).

Optimisation of extraction conditions

Samples of two cultivars, Global and Westar, selected because of their distinct pedigrees, were used to develop the HPLC method. Seed was 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 again ground prior to analysis. Extraction of soluble components from the flour was achieved using both water and 70% ethanol. Flour (0.04 g) was weighed into 2 ml plastic microcentrifuge tubes, extraction solvent (1.0 ml) added and the tubes shaken for 1 hour on a reciprocating shaker. The tubes were centrifuged on a high speed centrifuge for 5 minutes and the clear supernatant removed for HPLC-analysis.

Optimisation of elution conditions

1. Evaluation of elution solvent gradients, flow rates and trifluoroacetic acid (TFA) levels. A series of solvent gradients were investigated to determine the optimum gradient for good peak resolution and within a minimum time. Gradients started at 100% water (Solvent A) increasing to 25, 50 or 90% acetonitrile (ACN, Solvent B) over 30, 60, 90 and 120 minutes. Flow rates of 0.5, 1.0 and 2.0 ml min-1 were investigated. Solvents contained TFA ion-pairing reagent, added to decrease non-specific interactions with column matrix. Concentrations of 0, 0,05, 0.10 and 0,20% TFA were added to the elution solvents to determine the ideal amount.

2. Effect of column temperatures. Above ambient column temperatures were necessary to avoid diurnal fluctuations. Temperatures of 30, 40, 50, 60 and 70°C were investigated.

3. Effect of injection volume on resolution. Injection volumes of 5, 10, 20, 50, 100 and 200 l were tested. The relationship of detector response to injection volume was calculated to ensure linearity.

4. Detection wavelength. Chromatograms were acquired and detected at 210 nm and 1.0 absorbance units full scale (AUFS) as is typical for protein analysis in cereals (Marchylo, Hatcher and Krüger, 1988). Using the 994 LC UV-Vis detector, samples were monitored simulaneously at 240 and 280 nm to determine maximum sensitivity.

5. Reproducibility of extraction and injection. Repeated injections (n=10) of a single sample were carried out to test instrumental error. Similarly, 10 independent extracts of a sample of meal were made over a period of two weeks to determine laboratory error.

6. Statistical Analysis. Chromatograms were integrated with Water's Maxima software. Peak areas of the chromatogram components were utilized to study reproducibility of extracts and injection using SAS Proprietary Software Release 6.06.01 (SAS Institute Inc. Cary, NC, USA) and procedure Proc Means.

Results

Optimisation of extraction conditions

Water extracts produced similar chromatograms to those in ethanol. The water extracts produced some additional, poorly resolved, components and precipitated some material (possibly protein) within 16 hours. Higher levels of

B. napus protein are soluble in aqueous solution than in 70% ethanol (Appelqvist, 1972). Electrophoresis of the eluted peaks from the ethanol extracts did not produce evidence of protein, possibly due to insufficient concentrations. As aqueous extracts presented no additional advantage under the conditions described, all subsequent analyses were carried out using 70% v/v ethanol/water.

Optimisation of elution conditions

1. Solvent gradient and TFA concentration. Optimum resolution in minimum time was achieved with the following conditions. Seed components were eluted with a solvent flow rate of 1 ml min-1, with 100% Solvent A for 5 minutes followed by a linear gradient to 50% solvent B in 60 minutes. The final concentration of 50% solvent B was maintained for 10 min. The addition of dissociating agents such as TFA is common in protein analysis to improve peak shape and definition. Despite the fact that most of the peaks appear not to be proteins, based on electrophoresis data, the lack of TFA resulted in some peak broadening and poor resolution. There was no visible difference in the effect of 0.05, 0.10 and 0.20 % v/v TFA. For subsequent analysis, 0.10 % v/v was used to ensure the level was adequate. Using this gradient, flow rate and TFA concentration, a well-resolved chromatogram was obtained (Fig. 1) and the same conditions were used for the remainder of the evaluation.

2. Column temperature. Increased temperatures resulted in reduced retention time and peak resolution (Fig. 2). Lower temperatures produced peak broadening. Optimum resolution was achieved at 40~C using flow rates of 1 ml min-1.

3. Injection volume. Up to 20 1 injections produced sharp well resolved peaks, however, injections of 50 to 200 1 resulted in progressively broader, non-symmetrical peaks. Despite the poor peak shape at higher injection volumes, a linear regession analysis of peak area vs injection volume, determined on 5 independent peaks, indicated that the peak response was linear over the range of injection volumes ($r^2 > 0.95$, Fig. 3).

4. Detection Wavelengths. The greatest number of peaks were obtained at 210 nm but several individual peaks had higher absorbance at 240 nm (Fig.4). Detail at 280 nm was less informative than 210 or 240 nm and therefore was not used for subsequent analysis. Scanning of individual peaks was possible with the 994 LC UV-Vis detector. Scans of several peaks from 200 to 400 nm indicated maxima at approximately 235 and 320 nm (Fig.5).

5. Reproducibility of extraction and injection. A sample of Westar was extracted and the extract was injected 10 times. In addition, a sample of Westar was extracted 10 times over 2 weeks and the 10 extracts were analyzed. Excellent reproducibility was achieved in both repeated injection of a single extract (retention time cv = 0,04-0.10 %, peak area cv = 1.22-7.50 %, n = 10 peaks) and repeated extraction 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).

The majority of detail eluted in a small area of the chromatogram and only peaks between 20 and 40 minutes appear to be useful. For each of the 6 cultivars, variability between cultivars ranged from small differences in peak area for some cultivars to large differences in others (Fig. 6). Initial evaluation of the chromatograms suggested there was uniformity within chromatograms of the same cultivars and sufficient variation between chromatograms of different cultivars to distinguish between them.

Discussion

Ethanol extracts a wide range of compounds including phenolics, carbohydrates and proteins as indicated by the variable absorbance maxima of individual components. Wavelength scans between 200 and 400 nm produced profiles characteristic of phenolic compounds which indicates that several of the components are phenolics. This is consistent with the findings of Sosulski, Zadernowski and Kozlowska (1980) who identified several phenolic compounds extracted from oilseed rape into 80 % ethanol. Based on a comparison of the U.V. absorption pattern and the retention time of a commercial standard, the large peak at approximately 27 minutes was identified as sinapine, a major component of rapeseed (Fig.5). Several other components with retention times between 20 and 40 minutes were tentatively identified as phenolic compounds, including ferulic acid and coumaric acid, by comparison with commercial standards. Addition of 70% ethanol to the flour in two separate aliquots (0.3 ml water added followed by 0.7 ml ethanol) caused some early eluting peaks, speculated to be glucosinolates hydrolysed by endogenous myrosinase, to disappear. Identification of the majority of chromatographic components was not performed as the profile would serve as a fingerprint of the individual cultivar.

The cultivars selected to evaluate this technique included cultivars with similar pedigrees, Stellar and Regent, and cultivars with distinct pedigrees, Delta, Westar and Hero. It was apparent that there was sufficient differences in profiles between cultivars with similar and distinct pedigrees to discriminate between them (Fig. 6) In previous studies (Mailer, Daun and Scarth, 1993), chromatographic characteristics of the majority of seed components described were shown to be stable over a range of growing sites. These results indicate that environmental influence would not affect the usefulness of characterising cultivars of *B. napus* seeds based on ethanol extractable components.

The usefulness of phenolic compounds in this study is supported by many previous studies of phenolic compounds measured by HPLC analysis in phylogenetic studies such as cultivar identification of *Pelargonium* (Bauer and Teutter, 1990), taxonomy in Brassica (Hoshi and Hosoda, 1978) and eucalyptus (Hillis, 1967) and studies of geographical origins in honey (Ferreres, Tomas-Barberan and Tomas-Lorente, 1991).

This study indicates that RP-HPLC analysis of ethanol extractable components (primarily phenolics) is a useful method for the separation and analysis of ethanol extracts from the defatted flour of seeds of *B. napus* cultivars. Optimum conditions have been described. Characteristic elution profiles for the cultivars studied suggest that this method has potential for the identification of *B. napus* cultivars.

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Table 1 : Sources of seed selected for cultivar comparison studies

Site	Westar	Regent	Delta	Stellar	Hero	Tobin
Dauphin	*		*		*	*
The Pas	*		*			
Roblin	*		*	*	*	
Teulon	*		*	*	*	
Waskada	*		*			*
Canora		*				
North Battleford		*				
Paddockwood		*				
Tisdale		*				
Baggot				*	*	
The Point				*		
Portage				*		
La Salle					*	

Figure 1 : Chromatogram of *B. napus* cv Excel
(flow rate 1 mL min^{-1}, inj. vol. 20 µL, column temperature 40°C, wavelength 200 nm)

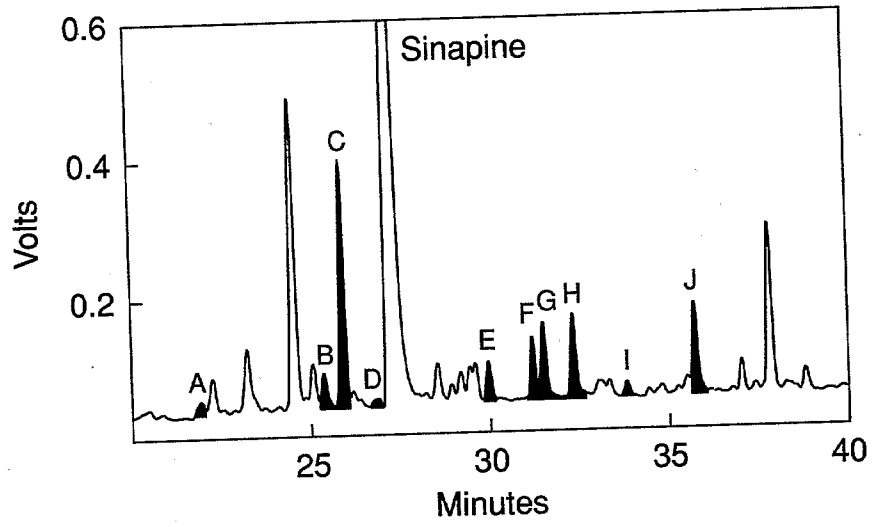


Figure 2 : The effect temperature on peak resolution. Operating conditions as for figure 1

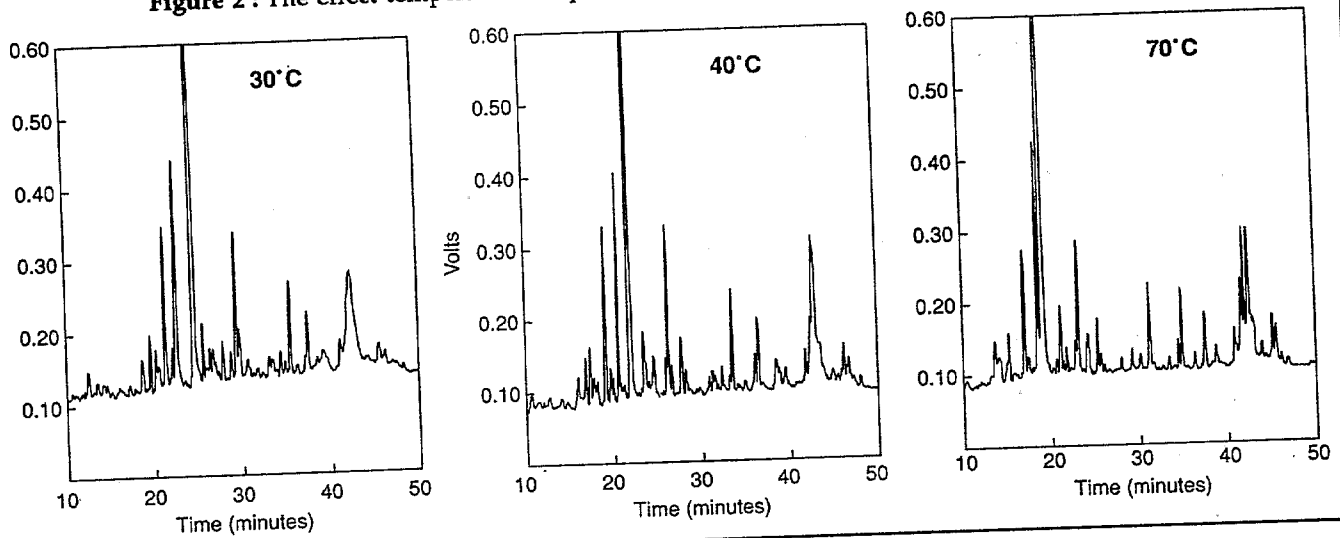


Figure 3 : The linear response of increasing injection volume on 5 chromatogram peaks
(operating conditions as for figure 1)

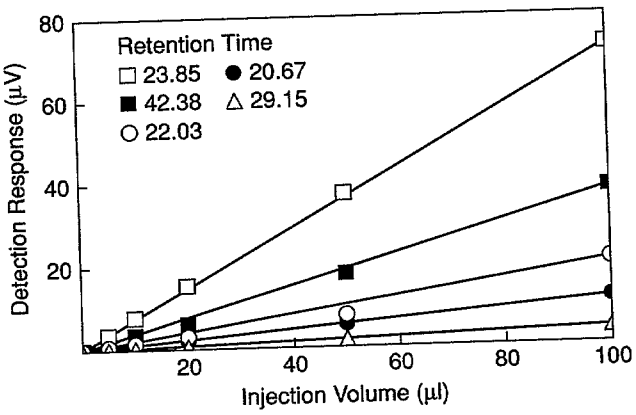


Figure 4 : Detector response at 3 wavelengths ; 210 nm, 240 nm, and 280 nm (operating conditions as for figure 1)

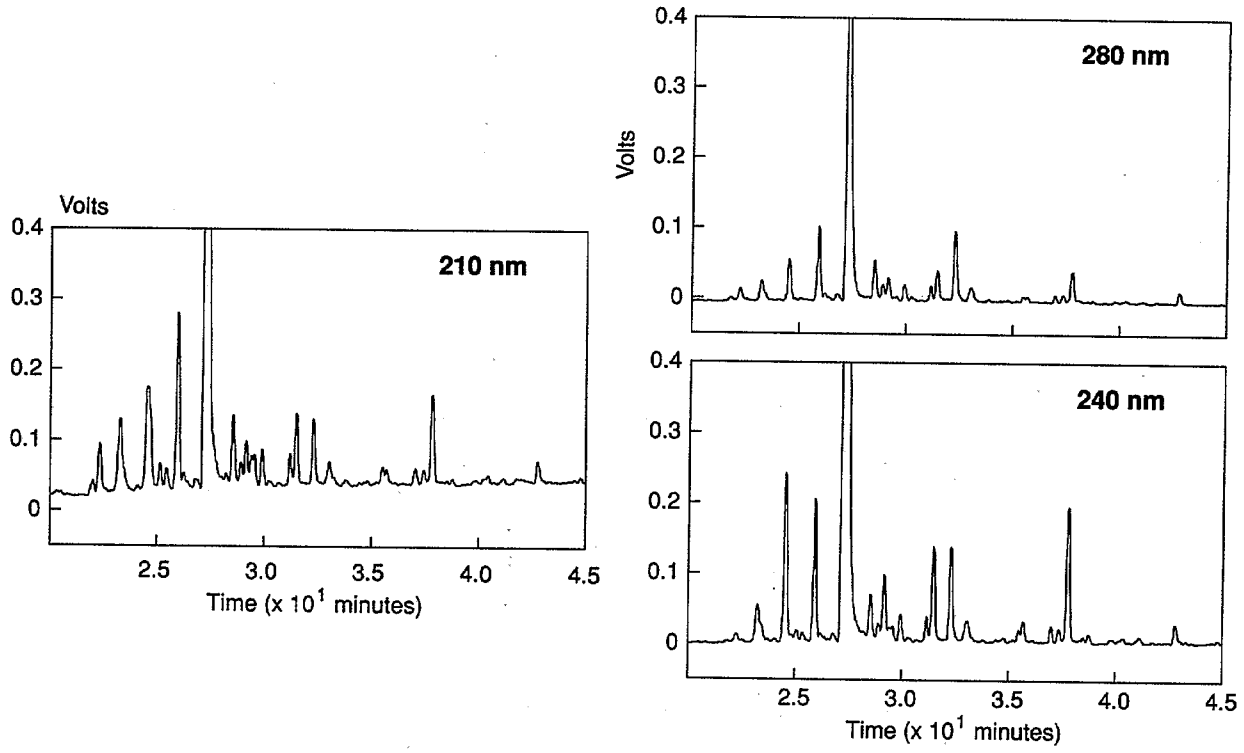


Figure 5 : Ultraviolet spectrum scan of the major peak at 27 min. from 200 to 400 nm from :
(A) a chromatogram of *B. napus* and
(B) a sinapine standard Scans of several other peaks produced similar profiles characteristic of phenolic compounds

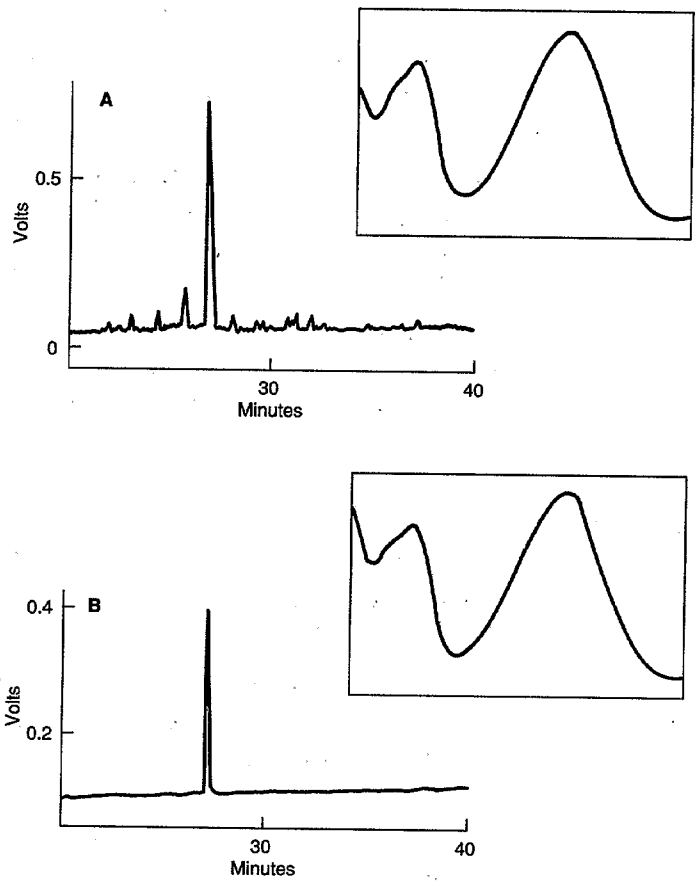
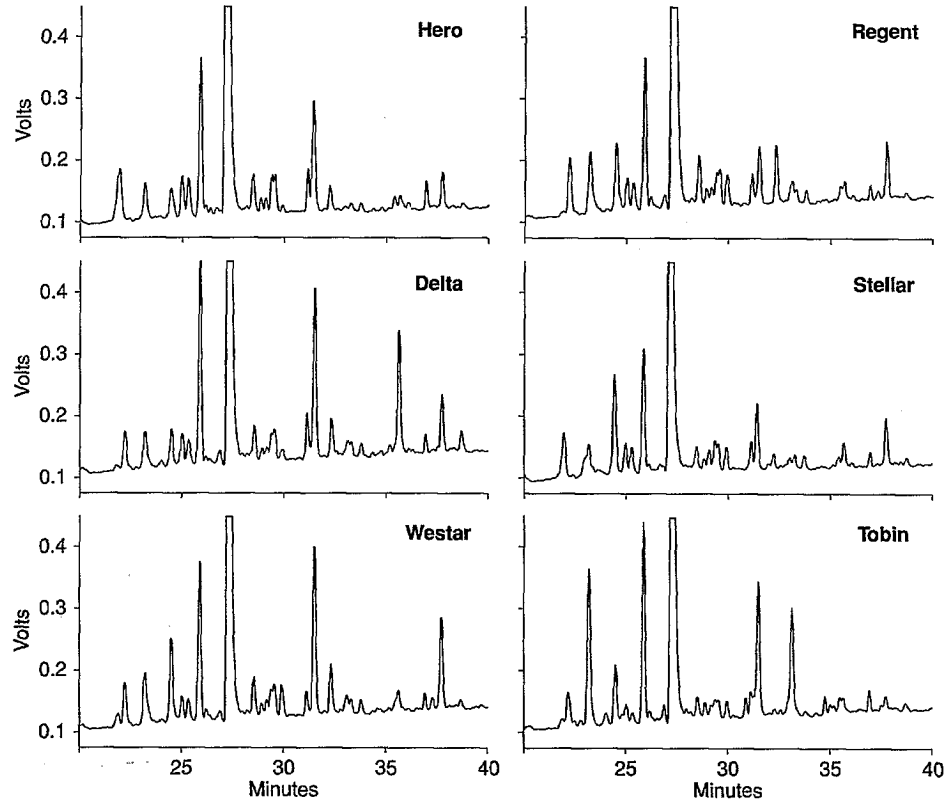


Figure 6 : Chromatograms of 5 cultivars of *B. napus* cv Stellar, Regent, Delta, Westar, Hero and 1 cultivar *B. rapa* cv Tobin (operating conditions as for figure 1)



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