OPTIMISATION OF HPLC CONDITIONS FOR VARIETAL IDENTIFICATION OF BRASSICA NAPUS

R.J. Mailer(1), R. Scarth(1), J.K. Daun(2)

(1) University of Manitoba, Winnipeg, Manitoba, CANADA R3T 2N2
(2) Canadian Grain Commission, 1404-303 Main Street
Winnipeg, CANADA R3C 3G8

INTRODUCTION

Varietal identification of cultivars of Brassica napus has been studied with the use of Reverse Phase - High Performance Liquid Chromatography (RP-HPLC) of ethanol soluble seed components. These components provide a stable extract mixture and subsequent cultivar characteristic profile. The technique appears suitable for verification of seed types and seed purity in addition to possible applications in registration for Plant Breeders' Rights. This paper reports studies undertaken to determine the optimum conditions necessary to achieve the best resolution of chromatographic components and improve distinction between varietal types. The conditions studied include solvent gradients, column temperature, addition of varying amounts of trifluoroacetic acid for protein dissociation, injection volumes, and solvent flow rates. Extraction times to obtain consistent extraction were also studied. Characterization of some of the components has been carried out.

MATERIALS AND METHODS

HPLC-grade water, trifluoroacetic acid (TFA, spectroscopy grade) and acetonitrile (CH_3CN, HPLC grade) were used. All solvents were filtered before use through 0.45 μm filters. Eluent solvents were sparged with helium gas during use. Petroleum ether (bp 40-60^{0}C) and absolute ethanol were AR grade.

The HPLC instrument was a Waters^R system including two 510 pumps, a Wisp autosampler, oven and temperature control unit and a 994 LC UV-Vis Detector. The data system was an IBM AT compatible computer together with Waters "Maxima" integration software which controlled pump gradient rate, peak integration and data handling. A Vydac 218TP54 C_{18}, 5 μm x 300A^0, 0.46 x 25 cm column was used. Eluent solvents were (A) water with 0.1% TFA and (B) 50:50 CH_3CN:water with 0.1% TFA.

Breeder's seed of B. napus cultivars was obtained from Agriculture Canada, Saskatoon; Allelix, Ontario; Garst Seed Company, Winnipeg; Svalof Seed Ltd., Ontario; and University of Manitoba, Winnipeg. Oil was extracted by the F.O.S.F.A. method and the oil-free flour was further ground in a Retsch ZM1 mill (0.5mm sieve). The flour (40mg) was weighed into plastic (1.5 mL) centrifuge tubes, 1 mL of 70% ethanol was added and the tube was shaken in a reciprocal shaker for 60min. The tubes were centrifuged in a high speed centrifuge prior to analysis by RP-HPLC.

RESULTS

Sample Extraction

Several solvent extracts have been examined to study variability of seed components between cultivars (Mailer et al. 1991). The use of aqueous solvents (H_2O, 5% NaCl and 0.2% NaOH) resulted in sample precipitation within 24 hours. Ethanol, (70%) provided a stable extract with cultivar characteristic components as described below. Shaking periods of 15, 30 and 60 minutes showed no significant differences and 60 minute extracts were used for all further analysis. To determine the reproducibility of extraction and the stability of the samples, 10

separate extractions of a sample of Westar were analysed over a period of 5 days and showed excellent reproducibility of those extracts (Fig. 1).

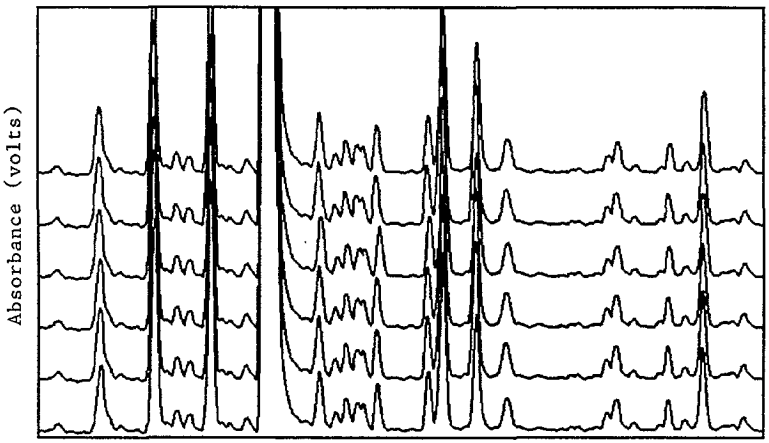

Fig. 1. Chromatograms of six separate extracts of the same Westar meal sampled over 5 days (Absorbance at 210nm, Time 20-40 min)

Solvent gradients

Initial, gradients from 0 to 90% acetonitrile were run over 120 minutes to determine the elution pattern. All components eluted at less than 50% acetonitrile. Subsequent gradients were investigated from 0 to 50% ACN over 30, 45, 60 and 90 minutes to achieve optimum resolution. All chromatograms hence have been acquired using the following conditions:

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 0 -  5 min : 100% A
 5 - 60 min : 100% A to 50% B over 60 minutes
65 - 70 min :  50% B
75 - 80 min :  50% B to 100% A
80 - 85 min : 100% A
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Column temperature, TFA addition and solvent flow rates

Above ambient temperatures were used to maintain day to day reproducibility. Optimum temperature was 40°C, higher temperatures resulting in loss of peak resolution.

Differences in chromatogram appearance with the addition of TFA at 0.05, 0.10, 0.15 and 0.2% were negligible. However, without the addition of TFA, some additional wide flat peaks were observed which reduced component peak resolution.

Solvent flow rates of 0.5, 1.0, 1.5 and 2.0mL/min have been studied with 1.0mL providing best resolution with minimum elution time.

Samples were finally analysed at 40°C with the addition of 0.1% TFA and with a flow rate of 1mL/min, providing adequate resolution for cultivar comparisons (Fig. 3a-f).

Injection volumes

To determine the linearity of response of variable injection volumes, injections of 5, 10, 20, 50, 100 and 200μL were injected. The peak areas were integrated and linear regressions calculated on several of the major components. From 5 to 100μL injections produced $R^2 = 0.990$ or better. Higher injection volumes resulted in merging of peaks and poor

peak integration with a resulting decline in the linear response. Injection volumes of 10μL have since been used to reduce column contamination whilst providing adequate reproducibility as a result of the accuracy of the Wisp autosampler system and the "maxima" software control.

Wavelengths

Chromatograms were monitored at 210, 240 and 280nm (Fig. 2a-c). Absorbance of the major peaks was highest at 240nm. However the most detail was observed at the more sensitive 210nm. There was little additional detail provided by absorption at 280nm.

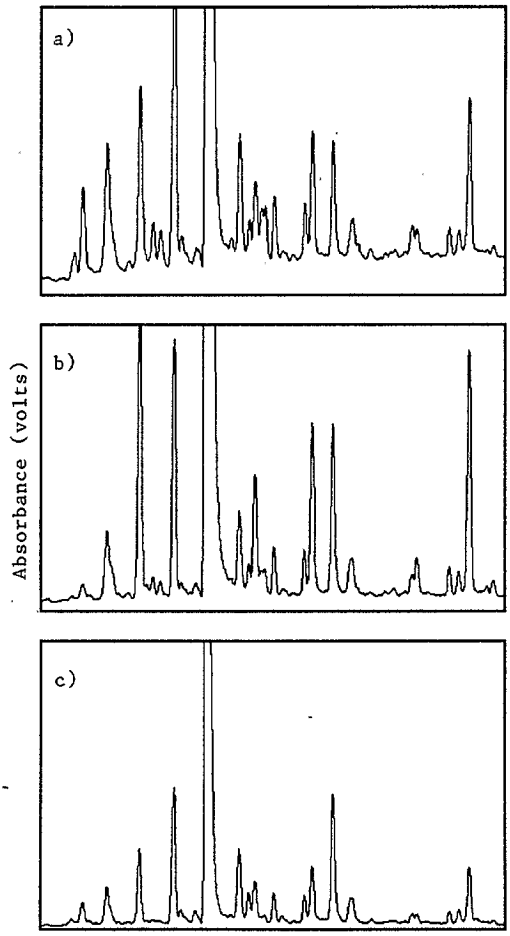

Fig. 2. Comparison of absorbance of Westar meal extract at different wavelengths a) 210nm, b) 240nm, c) 280nm.

DISCUSSION and CONCLUSIONS

The initial technique was based on similar techniques used for gliadin protein comparisons in wheat cultivar identification. Extracts of rapeseed in 70% ethanol contain a range of compounds including proteins, phenolic compounds, carbohydrates, and polar lipids. Although some

characterization of the compounds observed in these extracts has been undertaken, the components at this stage have not been identified. However, these and previous studies indicate that the differences observed in the extractable components are sufficient to provide cultivar identification whilst the components are relatively stable despite differences in environmental conditions.

The need to provide identification of cultivars has stimulated considerable research in various crops. The introduction of Plant Variety Rights (PVR) has increased the requirement to find a measure of uniqueness in cultivars for the purpose of registration. The use of glucosinolates for chemotaxonomy in B. napus has been shown to have limited value (Adams et al. 1985) although some success has been achieved by measuring glucosinolates in the root of swede (Adams et al. 1989), a related species. The differences in protein patterns of Brassica species have also been used to provide cultivar identification using "polyacrylamide porosity gradient gel electrophoresis" (PAGE) (Gupta and Robbelen 1986). However, automated HPLC with data handling abilities has the advantage of reducing operator time, providing accurate component quantification and making sample comparison relatively easy.

As B. napus naturally outcrosses, the cultivars are heterogeneous populations, thus chemotaxonomic identification was initially considered to be of unlikely value. The variability which exists within a population appears to be overcome by extracting large quantities of seed (e.g. 5g) thus obtaining a "mean" varietal composition. The results obtained on these and several other cultivars of rapeseed over several sites (Mailer - unpublished data) indicated that the ethanol extractable component remains relatively consistent within cultivars.

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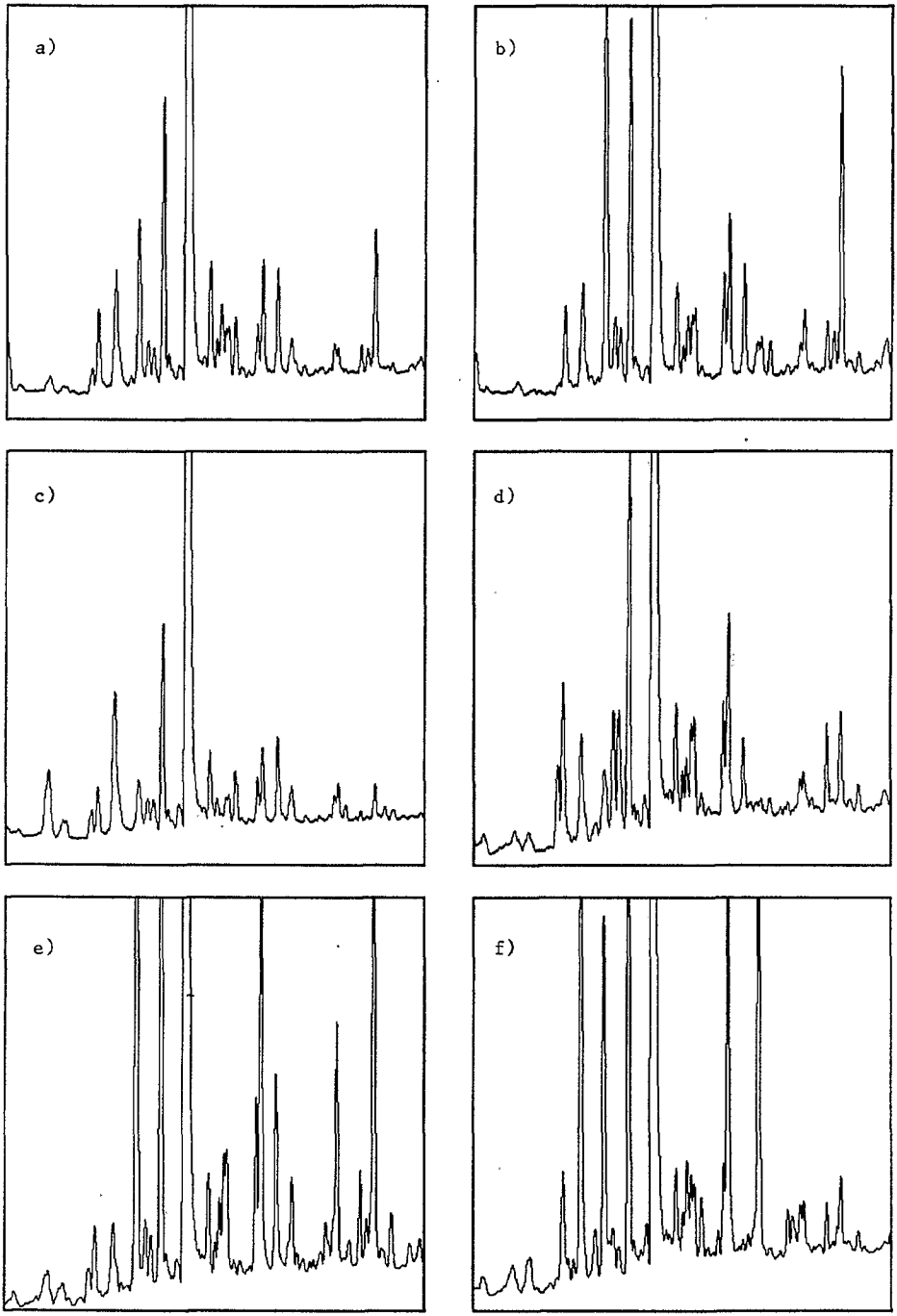

Fig. 3. HPLC profiles from six cultivars of canola/rapeseed a) Westar, b) Tribute, c) Oro, d) Hero, e) OAC Winfield, f) Horizon measured at 210nm.