CULTIVAR IDENTIFICATION OF OILSEED RAPE, *Brassica napus*, BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AND RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPDs)

A Thesis Submitted to the Faculty of
Graduate Studies, University of Manitoba by

Rodney J. Mailer

In Partial Fulfilment of the Requirements
for the Degree of Doctor of Philosophy

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RODNEY J. MAILER

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

Interest in the methodology for unambiguous identification of plant cultivars has increased in recent years with international recognition of Plant Breeders Rights. Oilseed rape (*Brassica napus* L.) poses a particular difficulty as cultivars of this outcrossing species may be heterogenous populations rather than single genotypes. The methodology for discrimination of oilseed rape cultivars therefore must distinguish characteristics which are more variable among cultivars than within a cultivar.

Two methods were investigated to determine if they were suitable for the identification of oilseed rape cultivars: reversed-phase high-performance liquid chromatography (RP-HPLC) and randomly amplified polymorphic DNA (RAPDs).

The HPLC technique involved analysis of ethanol extractable seed components, some of which were identified as phenolic compounds. A significant influence of environment on seed components was identified in the comparison of samples from different sites. However, sufficient differences were found among cultivars to discriminate among the 29 cultivars in the study. Extraction and chromatographic conditions have been shown to be reproducible over time. Using HPLC together with discriminate analysis, it was possible to identify unknown cultivars correctly, based on the accumulated data set.

RAPD analysis was also successful in discriminating among a selected set of 23 cultivars of *B. napus*. An initial screening of a set of 100 random sequence 10-mer primers identified 22 primers which produced polymorphic markers. A set of six primers were selected which produced a total of 23 clearly discernible polymorphic markers ranging in size from 300 to 2800 base pairs. These markers were sufficient to distinguish among the 23 cultivars. Analysis of seed of five cultivars obtained from field trials at four locations gave evidence of stability of the markers over source of the seed. Repeatability of the test was investigated using four different thermocyclers, each of which produced identical band patterns.

The two methods, RP-HPLC and RAPDs, were shown to have potential as a means of cultivar identification in applications such as Plant Breeders Rights.

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FOREWORD

This thesis was written using manuscript format. The first manuscript, "Optimisation and Evaluation of Reversed-Phase High-Performance Liquid Chromatography For Cultivar Identification of *Brassica napus* L" has been submitted to the Journal of Seed Science and Technology. The second manuscript "Cultivar Identification in *Brassica napus* L. Using RP-HPLC of Ethanol Extracts" has been accepted for publication in The Journal of The American Oilseed Chemists' Society. The third, "Discrimination Among Cultivars of Oilseed Rape (*Brassica napus* L.) Using DNA Polymorphisms Amplified From Arbitrary Primers", has been submitted to Theoretical and Applied Genetics.

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1. INTRODUCTION

Numerous cultivars of rapeseed *(Brassica napus*) 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 Breeders Rights (PBR) legislation in many countries and the need to demonstrate Distinctness, Uniformity and Stability (DUS, Plant Varieties Journal 1991). Rapid, accurate methods of cultivar identification are desirable to allow plant breeders to monitor the use of their genetic material. In addition, these methods could be used to verify seed purity to ensure quality standards are maintained for producers and processors. Plant breeders might apply the methods to select breeding lines with desirable characteristics if desirable quality parameters can be related to markers.

Agronomic and phenotypic characteristics including flower colour, plant height and leaf shape, previously employed for cultivar discrimination, may not show sufficient distinctness to differentiate among cultivars. These characters or traits are often unsatisfactory. They require plants to be grown to full maturity under controlled conditions to avoid environmental influence. The factors may also be dependent on the stage of plant development. However, phenotypic and morphological characteristics such as plant growth period (Halligan et al 1991) and seed shape (Barker et al 1992, Keefe 1992) continue to be used for cultivar identification .

The need for better methods of cultivar discrimination is reflected in the number of methods developed over recent years. Electrophoresis of seed proteins to create cultivar characteristic "fingerprints" has been the most prominent and successful method for cultivar identification of commercial crops such as wheat, *Triticum aestivum* L. (Wrigley et al 1982); cotton *Gossypium* spp. (Rao et al 1990); strawberries, *Fragaria x ananassa* Duch. (Nehra et al 1991) and pasture legumes, *Trifolium* spp. (Gardiner and Forde 1988). High-performance liquid-chromatography (HPLC) of seed proteins has also been used in crops including soybean, *Glycine max* L. (Buehler et al 1989); maize, *Zea mays* L. (Smith 1988); rice, *Oryza sativa* L. (Huebner et al 1990); oats, *Avena sativa* L. (Lookhart 1985); and wheat (Marchylo et al 1988). Applications of chromatography for cultivar identification have been discussed at length by Morgan (1989).

Predominantly autogamous (selfing) species are relatively simple to characterize as the plants within a cultivar are genetically identical. Characterization of predominantly allogamous (outcrossing) species is more difficult as the cultivar consists of a heterogenous population. Despite this, electrophoresis has been shown to be useful for predominantly allogamous species (Gilliland 1989) including the *Brassicas* (Gupta and Robbelen 1986; Chen et al 1989).

The most common rapid tests used by International Seed Testing Association (ISTA) laboratories were shown in a recent survey (Van der Burg and Van Zwol 1991) to be biochemical and electrophoretic tests.

Electrophoresis, restriction fragment length polymorphisms (RFLPs), polymerase chain reaction (PCR), and high-performance liquid-chromatography (HPLC) were described as experimental techniques requiring the development of simple and standardised laboratory protocol. The interest in cultivar discrimination in *Brassicas* has resulted in many specialised tests based on seed composition such as fatty acids in oilseed rape (*B. napus*), (White and Law 1991) or glucosinolates in *Brassica* vegetables (Heaney and Fenwick 1980, Adams et al 1989). Volatile hydrolysis by-products of glucosinolates in swede (*B. napus* L. var *napobrassica* (L.) Peterm.)(Cole and Phelps 1979) have also been used.

In recent years the analysis of plant genomic DNA has become common in characterizing plant and animal species. Restriction fragment length polymorphisms (RFLPs) have been used to investigate genomic relationships (Song et al 1988; Figdore et al 1988). Although RFLP technology is now well established, the technique has several disadvantages including the time and labour requirements and the need for the use of radioactive material.

The relatively recent development of the polymerase chain reaction (PCR) to amplify DNA and subsequently the use of randomly amplified polymorphic DNA (RAPDs, Williams et al 1990) or arbitrarily primed PCR (AP-PCR, Welsh and McClelland 1990) have produced a useful new tool for cultivar discrimination. RAPDs are a method of generating a type of genetic marker based on DNA amplification which requires no knowledge of target DNA sequence. The markers are generated by the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. The primers are generally 10-base oligonucleotides and are used to find polymorphic regions within the genome defined by the primer sequence. The products formed and examined by agarose gel electrophoresis reveal sequence variation in the form of variable numbers of bands of variable length which may be characteristic of the species and/or cultivars within a species. RAPDs may be the best method available for plant cultivar discrimination without many of the limitations of RFLP analysis. RAPD markers are considered to be dominant markers because the presence of a given RAPD band does not distinguish whether its respective locus is homozygous or heterozygous. Although this requires consideration in phylogenetic studies, it does not reduce the usefulness of the technique for fingerprinting and cultivar identification.

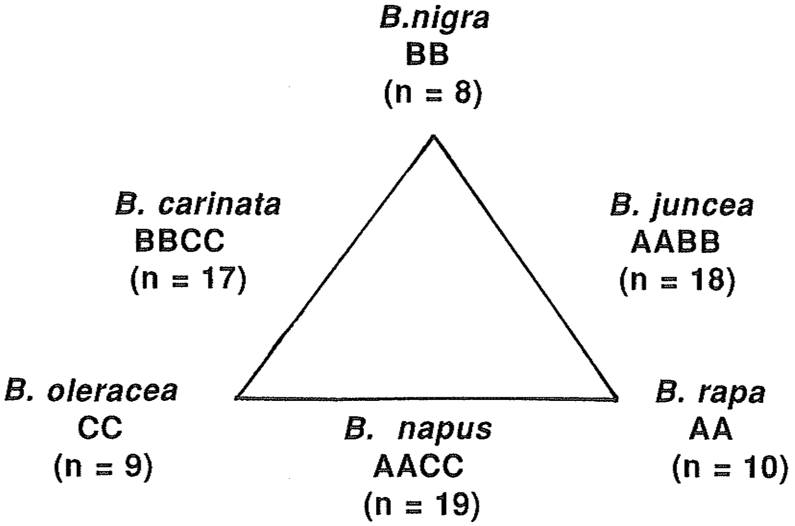
This study investigates the application of RP-HPLC and RAPDs to the discrimination of oilseed rape cultivars. The aims were to determine if there were sufficient differences among *B. napus* cultivars to discriminate between them. Both of the techniques were shown to have potential as a means of successful discrimination, based on the range of cultivars tested. It is also clear that both methods have their own advantages and disadvantages and can be adapted to suit individual needs and facilities.

2. LITERATURE REVIEW

Reproductive Biology of *B. napus*

The genus *Brassica* L. is in the Cruciferae family which includes numerous closely related and important crop plants such as swede and turnip and several oilseed species. Cytological studies have shown that the three species, *Brassica napus* L., *B. juncea* (L.) Czern. & Coss. and *B. carinata* A. Braun, are amphidiploids derived from monogenomic or diploid species *B.nigra* (L.) Koch, *B. rapa* L. and *B. oleracea* L. (Downey and Röbbelen 1989). The relationship among the species is illustrated in Fig. 2.1

Fig. 2.1 Genome relationship of Brassica species. A,B,C are genome symbols and n = number of chromosome pairs (U 1935).



The two rapeseed species *B. napus* and *B. rapa* have both spring and winter types. *Brassica napus* is predominantly self pollinating although under open field conditions, 20-30% outcrossing may result from pollination by wind and insects (Rakow and Woods 1987). *B. rapa* is normally self-incompatible although Downey and Röbbelen (1989) describe bud pollination or the use of high levels of atmospheric CO_2 to be successful procedures to circumvent this natural barrier.

History and Development of Oilseed Rape

Seed oil and meal quality of *B. napus* and *B. rapa* cultivars have been considerably altered from classical oilseed rape cultivars through breeding. The development of these new cultivars has resulted in a new classification of oilseed rape known in Canada as 'canola'. Canola is the seed of *B. napus* and *B. rapa* species, the oil component of which contains less than two percent erucic acid and the solid component of which contains less than 30 μM of any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of oil free solid as measured by the gas liquid chromatographic method of the Canadian Grain Commission (Canola Oil and Meal Standards and Regulations 1990). Low glucosinolate and erucic acid oilseed rape cultivars are often referred to as 'double low' oilseed rape in many countries.

Other breeding objectives include increased seed yield together with increased seed oil and protein concentration (Downey and Robbelen 1989). Further changes to oil quality are also desirable, such as the reduction of the polyunsaturated fatty acid linolenic acid to improve storage characteristics of the oil, as has been achieved in the *B. napus cv* Stellar (Scarth et al 1988). Further improvements of the seed meal are desirable including reduction of fibre content through the production of yellow seed coated cultivars, reduced meal glucosinolates, sinapine and phytic acid concentrations to improve stock feed palatability (Downey and Robbelen 1989).

High losses resulting from frost damage requires that future cultivars have a high frost resistance level (Daun et al 1985). The development of cultivars resistant to disease such as blackleg, caused by *Leptosphaeria maculans* (Desm) Ces. and de Not., and increased tolerance to sclerotinia caused by *Sclerotinia sclerotiorum* are important breeding objectives in Canada (Downey and Robbelen 1989).

Economic Importance of Canola

Fatty acid composition determines the value of seed oil. Current canola oil has a relatively low saturated and high polyunsaturated fatty acid concentration, making it a nutritionally superior oil (Canada's Canola 1988). Canola oil is widely used in margarine, cooking oils and salad dressings in Canada. There also exists a limited market for an oil with high levels of the long chain fatty acid, erucic acid, for industrial purposes (Downey and Robbelen 1989). Canola meal provides a quality animal stockfeed.

Approximate world production of rapeseed is 28.6 million tonnes (protein meal 15.6, and oil 9.4; Fats and Oils in Canada 1991). Canadian oilseed rape production in 1992 was 3.688 million tonnes (Statistics Canada, Fats and Oils Report, 1992). Canada is the world's largest exporter of canola with about 2 million tonnes exported annually (Fats and Oils in Canada 1991).

Plant Breeders Rights

The most pressing needs for simple and unambiguous methods of cultivar identification has been due to the introduction of Plant Breeders Rights. The International Union for the Protection of New Varieties of Plants (UPOV) is concerned with international plant varietal protection. Canada is the 20th member country of UPOV. The Canadian Plant Breeder's Rights Act became law on August 1, 1990 allowing protection of prescribed species for up to 18 years. To meet the criteria for registration under the act (Plant Varieties Journal 1991), varieties must be demonstrated to be new, specifically that the varieties must not have been sold in Canada prior to submitting an application for protection; distinct, in that a variety must be measurably different from all other varieties; uniform, with any variation predictable and commercially acceptable; and the variety must be stable in its characteristics over successive generations. Currently there are six categories covered by Plant Breeders Rights in Canada including wheat, canola/rape, soybean, potato, chrysanthemum and rose (Plant Varieties Journal 1991).

Methods of Cultivar Discrimination

The need for species and varietal markers was realised long before the introduction of Plant Breeders Rights. Numerous tests have been developed to permit taxonomic studies to be undertaken on plant species. Classical plant classification is based on a phenetic comparison of morphological characters. Biochemical tests to study plant constituents such as phenolics and fatty acids (paper chromatography) were developed prior to the instrumental methods which are used more commonly today. In recent years, the development of HPLC, gas-chromatography and electrophoresis techniques have made plant identification less labour intensive and more precise. DNA fingerprinting has been the most recent of these developments and appears to have great potential. DNA techniques are used for identity testing, genome linkage mapping, measuring genetic variation, population and pedigree analysis, localization of quantitative trait loci and epidemiology (Debenham 1992).

Physical Characteristics

Morphology. Morphology refers to the form and structure of the plant. The advantage of morphological descriptors, on the basis of seed characters for marketing and at seed delivery sites, is that the time taken to evaluate seed of the cultivar is minimised. Morphological discriminators of plants are numerous and examples of these are described in detail by Martiniello (1992) for varietal registration of Mediterranean leguminous forage crops. They include: stem characteristics such as height, colour and lateral branches; leaf shape, shape of margin and features of the upper leaf surface; floral characteristics such as number of inflorescences, petal colour, number of flowers per inflorescence; fructiferous organs, shape, pod segmentation, seeds per pod, seed colour, seed shape and hilum colour.

Discrimination on the basis of seed shape is often the only method of routine variety checking of seed bulks at grain intake. In recent years image analysis has been shown to provide relatively accurate discrimination in wheat grains, despite some overlap among cultivars, as most varieties have grains which exhibit a combination of shape and size which is typical or unique to the variety (Keefe 1992, Barker et al 1992). Morphological differences were employed to discriminate among *Cenchrus* pasture species (Nicholson et al 1985) using characteristics of the fascicle including length, shape and colour although shape varied with maturity and was not considered as a reliable character.

Phenology. Phenology is the study of periodic biotic events such as flowering, dry matter and seed production. Individual and species specific characteristics have been studied such as heading date in ryegrass (Halligan et al 1991) in which 18 cultivars were discriminated when grown under specific vernalization and daylength combinations.

Biochemical Methods

Chemical analysis provides an additional number of characters to distinguish among plant cultivars. Although these characters are often more distinct among cultivars than morphological and phenological characters, they are also often subject to the same environmental influences. The compounds which are suitable for discrimination within a species are generally specific characters of the species such as the fatty acid profiles (White and Law 1991) and glucosinolates (Horn and Vaughan 1983) within *Brassica* species.

Glucosinolates: Of particular interest in *Brassica* species is the occurrence and distribution of glucosinolates. Glucosinolates are a class of sulphur containing compounds which when hydrolysed by the myrosinase enzyme present in *Brassica* vegetative and seed tissues, release thiocyanates, isothiocyanates and nitriles which reduce feed palatability and adversely affect iodine uptake of the thyroid gland in non-ruminant animals (Downey and Röbbelen 1989). Horn and Vaughan (1983) showed glucosinolates to be useful as chemotaxonomic markers among 14 *Brassica* species studied. Glucosinolates, however, were less reliable for cultivar discrimination in *B. napus* (Adams et al 1985) in which only two patterns were observed, one exhibited in all cultivars with high levels of glucosinolates in the seed meal and the other common to all cultivars with low levels of glucosinolates. Adams et al (1989) had some success in distinguishing among nine cultivars of swede (*Brassica napus* L. *var. napobrassica* (L.) Peterm.) when they used glucosinolates extracted from plant roots. The volatile products released from cruciferous material upon enzymatic reaction on glucosinolates include isothiocyanates and nitriles. Using gas chromatography-mass spectrometry of isothiocyanates and nitriles and canonical variate analysis, Cole and Phelps (1979), were able to differentiate among 14 cultivars of swede. Although the study involved analyses of samples before and after storage, there was no study carried out to determine the effect of environment which has been shown to alter the levels of glucosinolates (Mailer and Wratten 1985) and presumably also the levels of volatile by-products.

Phenolic Compounds: Other compounds of specific interest for discrimination in *Brassica* species are sinapine (3,5-dimethoxy-hydroxycinnamoylcholine) and related low molecular weight phenolic choline esters. Sinapine is responsible for the disagreeable taste of eggs, a result of the production of trimethylamine, produced by poultry fed with rapeseed meal (Bell 1989). Bouchereau et al

(1991) found qualitative and quantitative distribution patterns of these compounds to be characteristic of the genus and therefore to have potential for *Cruciferae* chemotaxonomy. There are a wide range of phenolic and flavonoid compounds in *Brassica* species as demonstrated in a review listing 120 flavonoids in over 100 species (Fursa et al 1986). These compounds have been used in many studies to generate taxonomic data (Hoshi and Hosoda 1978). Phenolics have also been used in other species including *Pelargonium* (Bauer and Treutter 1990) for cultivar identification.

Oil Composition: Sterols in oils from a selection of seeds of *Brassica* and *Sinapis* species have also been found to be species specific (Appelqvist et al 1981), although no study of cultivar discrimination was reported. No differences were found between spring and winter types of *B. rapa*. Fatty acids in the oil of *B. napus* have also been studied as a chemotaxonomic tool (White and Law 1991) although the effects of temperature on fatty acid profiles have been well documented (Canvin 1965). Despite the success of fatty acid profiles together with canonical variate analysis to distinguish among three cultivars grown in the United Kingdom, it is unlikely that this method would succeed under all growing conditions. Fatty acid profiles have been shown to vary widely across environments in Australia (Mailer 1988).

Electrophoresis

Separation of seed protein by either starch or polyacrylamide gel electrophoresis is a routine procedure for many crops for seed certification, genetic purity testing, to distinguish among cultivars and to study within population genetic variability (Ladizinsky and Hymowitz 1979). The composition of seed proteins is highly stable and only slightly affected by environment (Ladizinsky and Hymowitz 1979) as they are generally storage proteins and not likely to change in dry mature seed. Proteins include prolamins (alcohol soluble), albumins (water soluble), globulins (saline soluble) and glutelins (insoluble in saline solution, alcohol or neutral aqueous solution) based on the protein classification of Osborne (1924). Electrophoresis technology continues to develop as described by McDonald and Drake (1990) using a 'Pharmacia Phast' commercial rapid and automated electrophoresis system for varietal identification of seeds.

Wheat cultivars can be identified by several electrophoretic techniques including gliadin analysis by starch gel electrophoresis or polyacrylamide gel electrophoresis (acid PAGE), and wheat storage proteins (gliadins and glutenins) by sodium dodecyl sulphate gradient polyacrylamide gel electrophoresis (SDSGPAGE) (Marchylo et al 1989). Besides cultivar identification, identification of high molecular weight glutenin subunits is important because of their relationship to breadmaking quality. Similar analysis has been used to identify cultivars of barley (Marchylo 1987), oats [*Avena* *sativa* L.] (Hansen et al 1988) and maize (Smith 1988).

Although electrophoretic analysis of seed storage proteins is widely used as a method for cultivar discrimination in predominantly autogamous species, particularly cereal crops, SDS-PAGE has also been used successfully, in predominantly allogamous species including pasture grasses (Gardiner and Forde 1988). As only partial distinctness is usually achieved among cultivars of allogamous species, electrophoresis was used to test individual plants (Gilliland 1989) and the cultivar description based on the frequency of plants which expressed certain electrophoretic patterns. Alternatively, a bulked sample was used, sufficiently large to represent the genetic variation of the population.

Electrophoresis has been used for cultivar identification in many crops including field beans [*Phaseolus vulgaris* L.] (Hussain et al 1986); watermelon [*Citrullus lanatus* Thund. (Matsum. and Nakai)] (Biles et al 1989); cotton [*Gossypium arboreum* L. and *G. hirsutum* L.] (Rao et al 1990); strawberry [*Fragaria x ananassa* Duch.] (Nehra 1991); sweetpotato [*Ipomoea batatas* (L.) Lam.] (Kennedy and Thompson 1991) and kiwifruit [*Actinidia deliciosa* (A.Chev.) C.F.Liang et A.R. Ferguson] (Messina et al 1991). Ladizinsky and Hymowitz (1979) describe the use of electrophoresis for species identification in 45 different genera from 13 plant families.

Electrophoresis has been used to study relationships among *Brassica* cultivars (Yadava et al 1979; Chen et al 1989; Coulthart and Denford 1982), evolution of cauliflower types (Allen et al 1986) and to identify genetic markers for breeding (Gardiner and Forde 1988). It has become a common technique for cultivar discrimination and seed purity testing in a range of *Brassica* crops including *B. napus* (Gupta and Röbbelen 1986; Chevre et al 1991), *B. oleracea* (Arus et al 1985), and *Raphanus sativus* L. (radish) (Saharan et al 1991).

High Performance Liquid Chromatography (HPLC)

HPLC analysis of proteins for cultivar discrimination has been developed as a method for cultivar identification, particularly for cereal crops (Bietz and Cobb 1985). It is sometimes used in conjunction with electrophoresis to provide positive identification of cultivars (Smith 1988). The application of HPLC to cereal crop analysis permits identification of cultivars as well as the ability to detect seed batch adulteration as each seed in the cultivar should be identical and analysis of single seeds can identify seed variation. HPLC applications to cereal crop identification include wheat (Bietz and Cobb 1985), oats (Lookhart 1985), maize (Smith and Smith 1988), and rice (Huebner et al 1990). Studies have been undertaken to improve HPLC procedures including studies of gradient conditions, elevated temperatures and the addition of internal standards (Bietz and Cobb 1985). They showed that more than 100 analyses per day were possible with faster flow rates and gradients, resulting in improved reproducibility with shorter runs. Improved resolution was also obtained with higher temperatures. Marchylo and Kruger (1985) investigated the use of 3 different columns of 7.5, 10 and 25 cm lengths with C3, C8 and

C18 column support respectively. Advantages and disadvantages of each were described. Extraction solvents and extraction times, and detector wavelength are other variables which have been studied (Kruger and Marchylo 1985). Detector response was shown to be non-linear with injection volume in gliadin and glutenin analysis (Marchylo and Kruger 1988). Effects of environment on the analysis of storage protein for subsequent cultivar discrimination has also been studied in maize (Smith and Smith 1986) and wheat (Marchylo et al 1990)

A major problem with HPLC analysis has been the difficulty in handling the large amount of data generated by the method. Multivariate and cluster analyses has been used to analyze the data (Smith and Smith 1988). Computerized cultivar identification is possible with HPLC (Scanlon et al 1989), allowing automated comparison of unknown cultivars with chromatographic data stored in a data base. A limitation to computerised data analysis is the changes in retention time which occur with column use which can be adjusted manually using commercial chromatography software. Scanlon et al (1989) described computerized analysis using normalized peak retention times.

Analysis of leaf phenols has been used to discriminate among 52 cultivars of pelargonium (*Pelargonium zonale* and *Pelargonium peltatum*) using HPLC (Bauer and Treutter 1990). Discriminant analysis was used to analyze the data and discriminate among cultivars. The method requires that plants be germinated under identical environmental conditions to remove environmental variables.

DNA Fingerprinting

DNA analysis is commonly used in animal pedigree analysis, medical science and anthropology. The results in conjunction with statistical analysis can be used to show identity or relatedness. For plant characterization, genetic markers are ideal as they are not affected by the environmental factors which influence the expression of morphological and agronomic traits. Genetic markers may be produced from isozyme analysis, restriction fragment length polymorphisms (RFLPs) or randomly amplified polymorphic DNA (RAPDs). Isozyme analysis uses polymorphisms of enzymes which differ in electrophoretic mobility as genetic markers. It is rapid and inexpensive but is limited by a lack of polymorphisms and the need for a variety of staining solutions for marker detection (Anderson and Fairbanks 1990).

Restriction Fragment Length Polymorphisms (RFLPs). Substantially more markers are provided by restriction fragment length polymorphisms (RFLPs) than for isozyme analysis. The number of markers is unlimited although costs for material and labour are restrictive. Methods for studying RFLPs to produce DNA markers are well documented (RFLP Training Manual 1989). They are often used in combination with other markers such as isozymes, morphological and phenological characteristics (Landry et al 1987) for varietal identification, genetic analysis of quantitative traits and breeding methodologies. RFLPs have several advantages including the ability to detect Mendelian inheritance (genomic RFLPs) and maternal inheritance (organelle RFLPs). They are detectable in all tissue and at all ages of maturity. Multiple alleles are identified with each probe and an unlimited number of probes are available. In addition, they are not effected by environmental influences (Landry et al 1987).

The majority of published data on RFLPs refers either to the development of linkage maps which are applied to mapping of quantitative trait loci or to phylogenetic analyses. These studies include barley (Heun et al 1991), maize and tomato (Helentjaris et al 1986), lentil (Havey and Muehlbauer 1989), bananas (Gawel et al 1992), lettuce (Landry et al 1987), and pepper (Livneh et al 1990), and several studies on *Brassica* (Chevre et al 1991, Figdore et al 1988, Song et al 1991, Landry et al 1991). The use of RFLPs and the development of linkage maps has made possible selection of breeding lines based on markers linked to genes controlling quantitative traits. These markers have been linked to quality characteristics such as soluble solids in tomato (Osborn et al 1987) or disease resistance, including powdery mildew resistance in barley (Schuller et al 1992) and lettuce (Paran et al 1991).

Polymerase Chain Reaction (PCR) and RAPDs. Since its inception in 1983, application of PCR has had a major impact on molecular biology (Mullis 1990). It allows selective amplification of specific segments of DNA from the total DNA using oligonucleotides as primers. An oligonucleotide is a short chain of specifically ordered nucleotide bases which will bind to a complementary site in single stranded DNA.

The polymerase chain reaction was the basis for the development of a secondary technique referred to as randomly amplified polymorphic DNA (RAPDs, Williams et al 1990) or arbitrarily primed PCR (AP-PCR, Welsh and McClelland 1990). RAPDs involve the amplification of DNA segments using random sequence oligonucleotide primers, generally of 10 bases, to find polymorphic regions within the genome, defined by the primer sequence. The products formed and examined by agarose gel electrophoresis reveal sequence variation in the form of variable numbers of bands of variable length which may be characteristic of the species and/or cultivars within a species. RAPDs require no previous sequence information for fingerprinting of cultivar genomes, none of the probes necessary for RFLPs and no autoradiography. The markers may be visualised and photographed immediately in the gel rather than requiring transfer to a membrane. Polymorphic RAPD markers may also be combined with RFLPs to provide probes, eliminating the need for recombinant DNA cloning of probes in bacteria (Williams et al 1993).

RAPDs may detect a single base change in genomic DNA (Williams et al 1990), although perfect pairing between primer and template may not always occur and there is some evidence of mismatch amplification. Polymorphisms may also result from deletions of a priming site, or insertions that separate priming sites making them too far apart to amplify or insertions that change DNA segment length. The probability of a primer finding binding sites on opposite strands of the template within a distance that can be traversed by the DNA polymerase is simple to calculate (Williams et al 1991 b). It appears that the number of bands is independent of the number of potential sites and appears to be controlled by competition between the substrates rather than by the number of potential sites. Martin et al (1991) developed a formula for calculating the number of random primers necessary in order to find a marker linked to the locus of interest. The probability of obtaining a marker within a specified distance of a target gene in NILs (near isogenic lines) depends on the number of primers screened, the genome size in map units, and the degree of DNA sequence convergence between the NILs in the region surrounding the targeted gene. If the sequences are similar, a primer is less likely to find a polymorphism. The expected minimum distance = $c/2\ (nx + 1)$ where c = genome size in cM, n = number of primers, x = average number of primer products per primer.

DNA Isolation Methods. The first step in DNA fingerprinting by either RFLPs or PCR is the extraction of DNA from plant material. There are many methods published for DNA isolation, the method often being related to the purity of the DNA required for the purpose. Many methods require the use of phenol or chloroform and detergents such as cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS). Even rapid methods for PCR generally require grinding of plant material in pestles that must be cleaned between uses, heating for 60 minutes, and emulsification with chloroform. Often a density gradient centrifugation step in cesium chloride is used to partition the DNA from enzyme inhibiting polysaccharides. This purification step may be replaced with the use of CTAB.

The extraction method used should provide reasonably high quality DNA with a minimum of labour input. Plant material is often harvested onto ice, frozen as quickly as possible at -80°C and stored until required for extraction. The samples are then ground in liquid nitrogen or dry ice, avoiding thawing of plant tissue. It has been shown that it is more convenient to harvest the plant material and lyophilise immediately to avoid using dry ice or liquid nitrogen (Kidwell and Osborn 1992). The lyophilised material can be stored in a dessicator at 20°C.

Extraction of DNA as described by Rogers and Bendich (1988) involves breaking, generally by grinding, or digestion of cell walls and disruption of cell membranes, in the presence of detergent including SDS or CTAB. DNA is protected from endogenous nucleases with detergents and ethylenediaminetetra acetic acid (EDTA). EDTA, a chelating agent, binds Mg^{++} ions generally considered a necessary cofactor for most nucleases. The buffer/tissue mixture is emulsified with chloroform or phenol to denature and separate proteins from the DNA. During extraction, shearing of DNA strands must be avoided. The time between thawing frozen, pulverized tissue and exposure to extraction buffer should be minimized to avoid nucleolytic degradation of the DNA.

A miniprep method was developed by Kidwell and Osborn (1992) for RFLP and PCR analysis using CTAB. CTAB forms complexes with polysaccharides but precipitates nucleic acid when NaCl concentrations are lower then 0.5 M. DNA is precipitated in buffer (pH 8.0) in the presence of CTAB at low NaCl concentration, thus removing many polysaccharides which are still soluble at the lower NaCl concentration.

A simple method was described by Oard and Dronavalli (1992), requiring no phenol, chloroform, detergents or mortar and pestles. Small pieces of frozen plant material are ground in 1.5 ml Eppendorf tubes using disposable grinding sticks, TE extraction buffer (trizma base 10 mM (pH 8.0), 1 mM EDTA) with mercaptoethanol is added, the tube is heated in boiling water for 10 min. and centrifuged. DNA is precipitated from the supernatant with ammonium acetate/ethanol, resuspended in TE buffer and incubated with RNase.

Edwards et al (1991) collected plant material using sterile Eppendorf tubes to pinch out leaf disks to prevent the possibility of contamination from handling. The tissue is macerated with a disposable grinder and DNA extracted in 400 µl TE buffer (pH 7.5) containing SDS. The samples are extracted for 1 hour at room temperature, centrifuged and the DNA precipitated from the supernatant with isopropanol. After drying, the sample is resuspended in TE buffer, and stored at 4°C at which temperature it is stable for more than a year. Between 2.5 and 25 µl are used for PCR, depending on tissue maturity.

Applications of Randomly Amplified Polymorphic DNA (RAPDs)

Applications of RAPDs have included the identification of *Brassica* species (Quiros et al 1991) and conifers (Carlson et al 1991), identification of species of *Pyrenophora* Ito and Kuribyashi, (Reeves and Ball 1991) and isolates of *Colletotrichum graminicola* (Ces.) G.W. Wils., (Guthrie et al 1992), parentage determination in maize hybrids (Welsh and McClelland 1990) and cultivar identification of broccoli and cauliflower (Hu and Quiros 1991).

RAPDs, in conjunction with NILs can be used to identify markers linked to genes of interest. To create NIL, the F_1 from an initial cross is backcrossed to a recurrent parent, the parent in which the desirable characteristic will be incorporated. Repeated backcrossing with repeated selection for a desirable character at each generation results in a genotype essentially identical at all loci to the recurrent parent with the exception of the loci surrounding the gene of interest. Polymorphisms detected in NIL therefore have a high probability of being linked to that gene. Using RAPDs to identify polymorphisms in NIL, Martin et al (1991) identified three markers from 144 random primers, tightly linked to a gene conferring resistance to *Pseudomonas syringae* pv. tomato. The identification of RAPD markers took four weeks compared to a predicted two years using RFLPs.

Michelmore et al (1991) used bulked segregant analysis from the homozygous individuals of an F_2 population, in which DNA from individuals segregating for downy mildew resistance was bulked, as was DNA from susceptible plants. The two bulked samples, which were homozygous for alternative alleles at the resistance gene loci, have heterozygous backgrounds due to contribution of other unselected alleles from the individuals in the bulk. Any polymorphisms detected were therefore likely to be linked to resistance genes. In 300 PCR reactions, three markers were identified which were linked to the target locus.

Traits controlled by polygenic systems include yield and quality and are characterized by continuous variation across the population. This variation can be visualised as a normal distribution (Fig. 2.2), with individuals at extremes of the curve being low in the number of individual genotypes present. DNA from the individuals in the tails of the distribution are pooled to form a bulk and screened with RAPDs to identify markers linked to the quantitative trait. The efficiency of this method is improved with the use of recombinant inbred lines (RIL) and in particular, doubled haploid families since dominance related effects are absent and linkage data can be obtained directly. RIL are derived by repeated sibling crosses starting with the second (F_2) generation from a cross resulting in homozygous plants. The family can be extensively replicated assisting in the partitioning of phenotypic variation into heritable and non-heritable components, for example, in *Arabidopsis thaliana* (Reiter et al 1992).

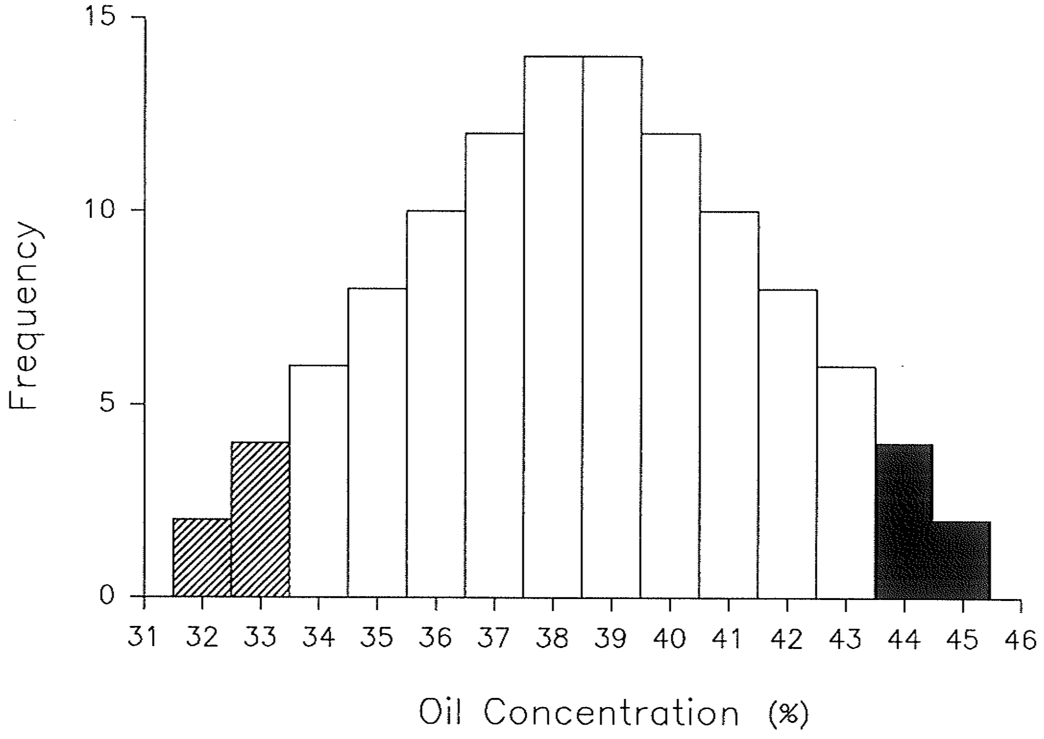


Fig. 2.2. Markers of a polygenic trait (e.g. oil concentration) can be selected by screening samples from the extremes of a continuously varying population (Frequency = number of individual genotypes) (from Waugh and Powell 1992).

Map positions of polymorphisms can be determined using RAPDs and recombinant inbreds. Two polymorphisms closely linked genetically will either both be present or both absent in any RIL. In markers which are more distantly linked, co-segregation diminishes because of recombination between chromosomes from the parents during the breeding process. Well saturated genetic maps can quickly be constructed using RAPD markers as described for

Neurospora crassa (Kubelik 1991) and *Arabidopsis thaliana* (Reiter et al 1992). Paran et al (1991) using NIL and 200 RAPD primers to generate 1880 RAPD bands, identified 10 markers linked to downey mildew resistant genes in lettuce.

RAPD (or AP-PCR) is far easier than other techniques for identifying DNA markers as no RFLP probes have to be identified. The generation of clones and plasmid purification are unnecessary. Polymorphisms can be visualised directly from the gel by ethidium bromide or fluorescent detection.

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 define a method to identify cultivars of *Brassica napus* L., a predominantly self pollinating 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% trifluoroacetic acid (TFA), from 0 to 50% acetonitrile over 60 minutes, provided optimum resolution. Maximum number of peaks were obtained at detection wavelength 210 nm and several peaks had greater absorbance at 240 nm. Repeated analysis indicated that extraction and chromatographic conditions were reproducible. The results of this study show that the method has potential for characterization of *B. napus* cultivars.

Introduction

Oilseed rape *(Brassica napus* L. var. *oleifera* Metzg.) is a valued commercial crop due to the high quality 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. Although *B. napus* is a predominantly self pollinating species, up to 30% outcrossing may occur (Rakow and Woods 1987). It is therefore 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 allows seed purity to be verified by seed testing laboratories and certification authorities and ensure quality standards are maintained.

Despite numerous investigations for characterization of outcrossing species such as *B. napus*, unambiguous identification has not been possible. Phenotypic characteristics, including flower colour, plant height and leaf shape do not show sufficient distinctness to differentiate among 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 taxon (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, *Triticum aestivum* L. (Marchylo, Hatcher and Kruger, 1988); oats, *Avena sativa* L. (Lookhart, 1985); soybean, *Glycine max* L. (Buehler, et al. 1989); and maize *Zea mays* L. (Smith, 1988). Although most electrophoresis work has been on predominantly autogamous (selfing) species, the technique has been shown to be useful for predominantly allogamous (outcrossing) species (Gilliland, 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). The wide range of phenolic and flavonoid compounds in *Brassica* species have been used to generate taxonomic data (Hoshi and Hosoda, 1978).

The objective of this study was to develop and optimize a method of cultivar identification in *B. napus* based on RP-HPLC analysis of seed components including proteins and phenolic compounds.

Materials and Methods

Seed samples of five cultivars of *B. napus, cv* Westar, Stellar, Hero, Delta and Regent; and 1 of *B. rapa* L. *cv* Tobin, were obtained from cultivar evaluation trials grown in 1991 at sites in Saskatchewan and Manitoba as detailed in Table 3.1. Two of the cultivars had similar pedigrees, *cv* Stellar and Regent, and the other three cultivars, *cv* Delta, Westar and Hero had distinct pedigrees. Two additional seed samples, *cv* Global and Westar, were used to develop the HPLC method.

The analyses were carried out using a Waters 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 Waters "Maxima" integration software which controlled the solvent gradient, peak integration and data handling. A Vydac 218TP54 C18, 5 µm x 30 nm, 0.46 x 25 cm column was used for chromatography. Elution solvents were water (Solvent A), purified through a Millipore Super-Q system (Millipore Corp., Bedford, Mass.) and Millipore type HA 0.45-µm filter, and HPLC grade acetonitrile (ACN, Solvent B) obtained from Fisher Scientific (Fair Lawn, N.J.). Trifluoroacetic acid (TFA, Sequanol grade) was obtained from Pierce Chemical Co. (Rockford, Ill.).

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 TFA levels. A series of solvent gradients were investigated to determine the optimum gradient for good peak resolution 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 (Buehler et al 1989), added to decrease non specific interactions with the 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 volume has been shown to be non-linear in the quantitative analysis of gliadins and glutenins (Marchylo and Kruger 1988). To determine if quantitative errors may occur as a result of injection volume, volumes of 5, 10, 20, 50, 100 and 200 µl 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 as is typical for protein analysis in cereals (Marchylo, Hatcher and Kruger 1988). Using the 994 LC UV-Vis detector, samples were monitored simultaneously at 240 and 280 nm to determine the maximum sensitivity.

5. Reproducibility of extraction and injection. Repeated injections (n=10) of a single sample were carried out to test instrumental variability. 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 Waters' 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, Cary, NC, USA) and procedure Proc Means.

Results

Optimisation of extraction conditions

Similar chromatograms were obtained from water or ethanol extracts. The water extracts contained 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 (Buehler et al 1989) 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 (figure 3.1) 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 (figure 3.2). 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 µl injections produced sharp well resolved peaks, however, 50 to 200 µl resulted in progressively broader, leading peaks. Despite the poor peak shape at higher injection volumes, a linear regression analysis of peak area vs injection volume, determined on 5 arbitrary peaks, indicated that the peak response was linear over the range of injection volumes ($r^2 > 0.95$, figure 3.3; Appendix II).

4. Detection Wavelengths. Greatest number of peaks were detected and integrated at 210 nm but several individual peaks had higher absorbance at 240 nm (figure 3.4). 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 (figure 3.5).

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 = 3.45-7.50, 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)(Appendix I a and I b).

Over 100 peaks were initially integrated from each of the chromatograms. For statistical analysis of the data it was necessary to select a reduced number of components. The majority of peaks eluted in a small area of the chromatogram and only peaks between 20 and 43 minutes were selected. For each of the 6 cultivars, variability among cultivars ranged from small differences in peak area for some cultivars to large differences in others (figure 3.6). Initial observation of the chromatograms suggested there was greater uniformity within chromatograms of the same cultivar and sufficient variation among chromatograms of different cultivars to distinguish among them.

Discussion

The conditions established in this study produced well resolved chromatograms. Reproducibility of retention times compares favourably with those of Marchylo and Kruger (1985) using an Aquapore RP-300 column (cv of 0.19-0.47%) although no measure of peak reproducibility was given. Bietz and Cobb (1985) reported retention times of 9 peaks with 27 replicate wheat analyses (cv = 0.00-0.47 %) but again peak area statistics were not reported. For wheat grown under different environmental conditions, chromatograms were divided into 4 segments for statistical analysis (cv=1.5-10.9%, Marchylo et al 1990). Ethanol extracts a wide range of compounds as indicated by the variable absorbance maxima of individual components. To provide some indication of the characteristics of these compounds, absorption ranges of some peaks were scanned between 200 and 400 nm using a photodiode array ultra-violet (U.V.) detector. The wavelength spectrum produced profiles characteristic of phenolic compounds indicating 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 in 80% ethanol. The spectral maxima at 235 and 320 nm correspond to the hydroxyl group of the phenolic compound and the C_3 group respectively (Harbourne 1964). 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 *B. napus* meal (figure 4.1). Several other components with retention times between 20 and 43 minutes appeared to be phenolic compounds based on their spectrum, including ferulic acid (R.T.= 28.24 min) and coumaric acid (R.T.= 26.03 min) identified by comparison with commercial standards. Addition of the 70% ethanol to the flour in two parts (0.3 ml water added followed by 0.7 ml ethanol) caused some early eluting peaks to disappear. These were speculated to be glucosinolates hydrolysed by endogenous myrosinase. Identification of the majority of chromatographic components was not carried out as the objective was to use the profile 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. There were sufficient differences between even the cultivars with similar pedigrees to discriminate between them (figure 3.6). In previous studies (Mailer, Daun and Scarth, 1993), chromatographic differences of the majority of seed components described have been shown to show some variation over a range of growing sites. These results indicate that environmental influence may have some effect on the characterization of cultivars of *B. napus* seeds based on ethanol extractable components. The differences among cultivars however was sufficient to discriminate them despite environmental influence.

The utility of phenolic compounds for diagnostic purposes is supported by many previous studies of phenolic compounds measured by HPLC analysis such as cultivar identification of *Pelargonium* (Bauer and Treutter, 1990), taxonomy of *Brassica* (Hoshi and Hosoda, 1978), and eucalyptus (Hillis, 1967) and studies of geographical origins in honey (Ferreres et al 1991).

These results indicate that RP-HPLC of ethanol extractable components 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. The characteristic profiles for the cultivars studied indicate that this method has potential for the identification of *B. napus* cultivars.

Table 3.1. Sources of seed selected for RP-HPLC 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 3.1. Chromatogram of *B. napus* cv Westar (flow rate 1 ml min^{-1}, inj. vol. 20 µL, column temperature 40°C, detector 210 nm).

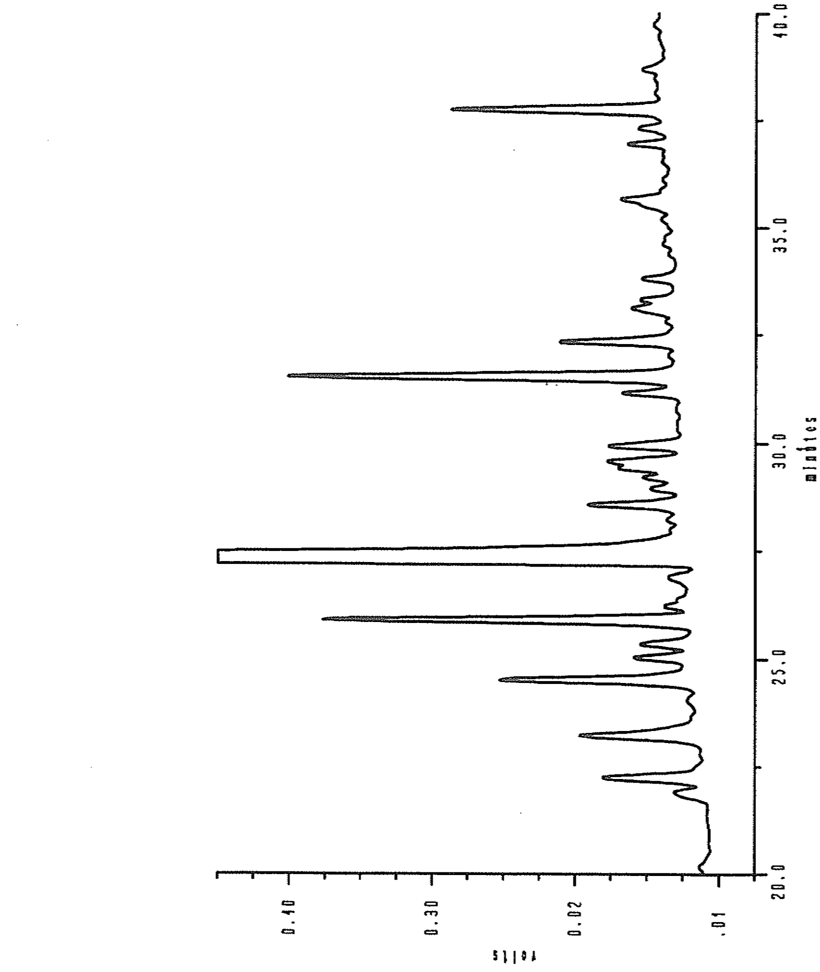

0.40
0.30
0.02
.01
volts
20.0
25.0
30.0
35.0
40.0
minutes

Figure 3.2. The effect of temperature on peak resolution (other operating conditions as for figure 3.1)

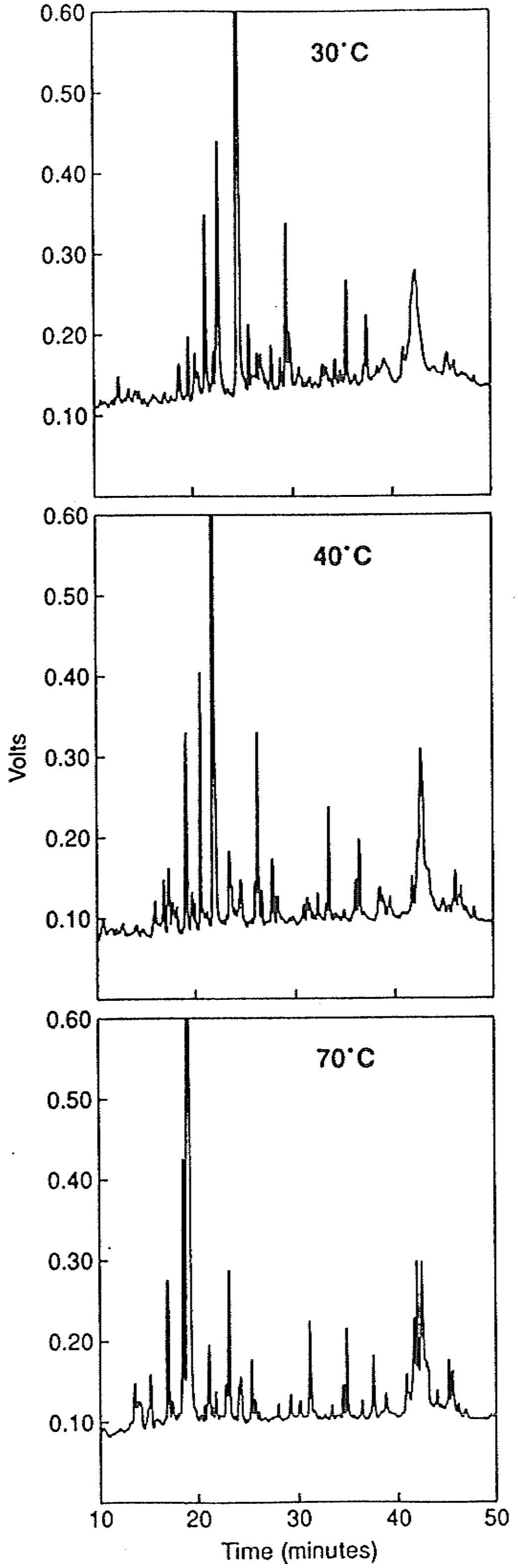

30˚C
40˚C
70˚C
0.60
0.50
0.40
0.30
0.20
0.10
Volts
10
20
30
40
50
Time (minutes)

Figure 3.3. The linear response of increasing injection volume on five chromatogram peaks (other operating conditions as for figure 3.1)(Appendix II).

3.19

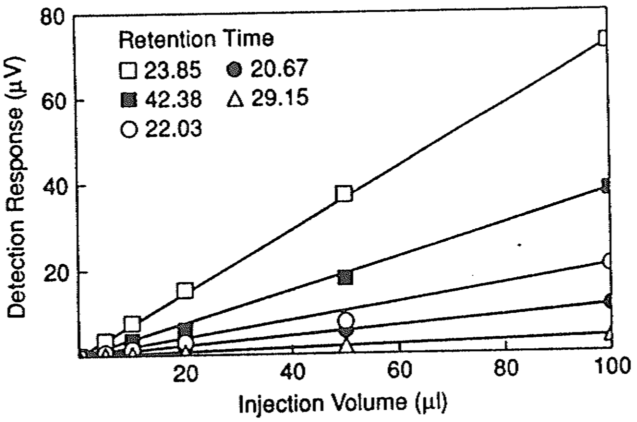

Retention Time
□ 23.85
● 20.67
■ 42.38
△ 29.15
○ 22.03
Detection Response (μV)
80
60
40
20
20
40
60
80
100
Injection Volume (μl)

Figure 3.4. Detector response at three wavelengths; 210 nm, 240 nm, and 280 nm (other operating conditions as for figure 3.1.)

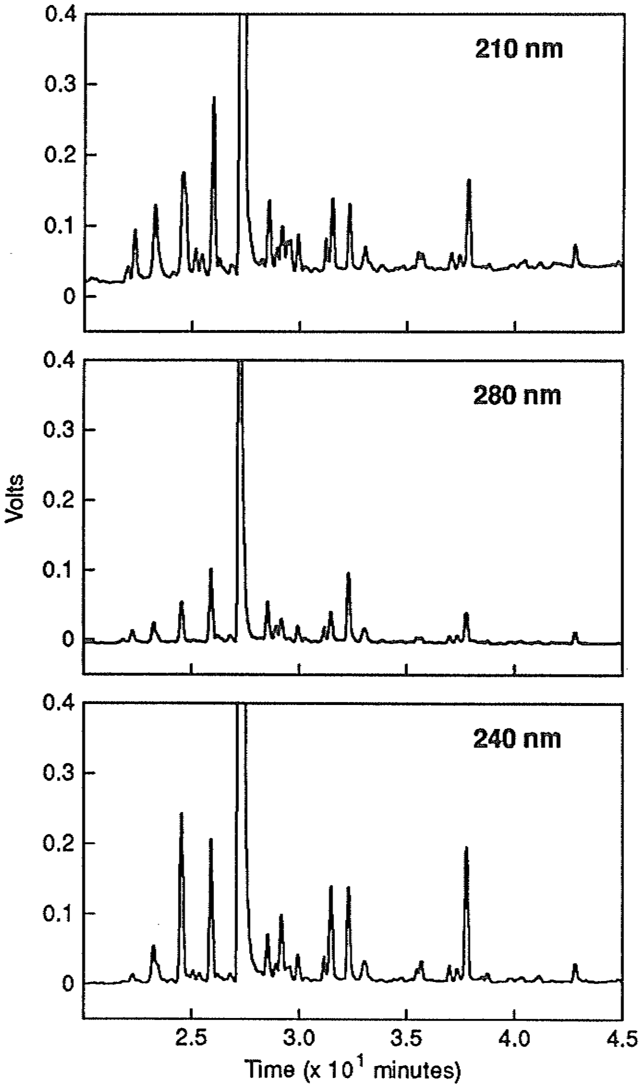

0.4
0.3
0.2
0.1
0
210 nm
280 nm
240 nm
Volts
2.5
3.0
3.5
4.0
4.5
Time (x 10^1 minutes)

Figure 3.5. Scan of chromatogram peak (27 minutes) between 200 and 400 nm (A - *cv* Westar, B - sinapine standard)

3.23

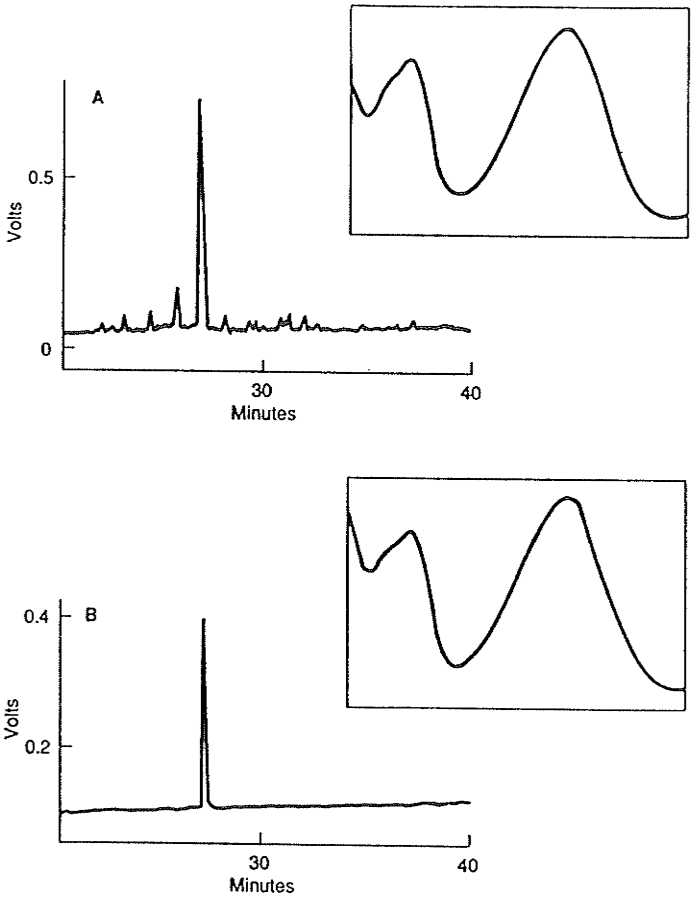
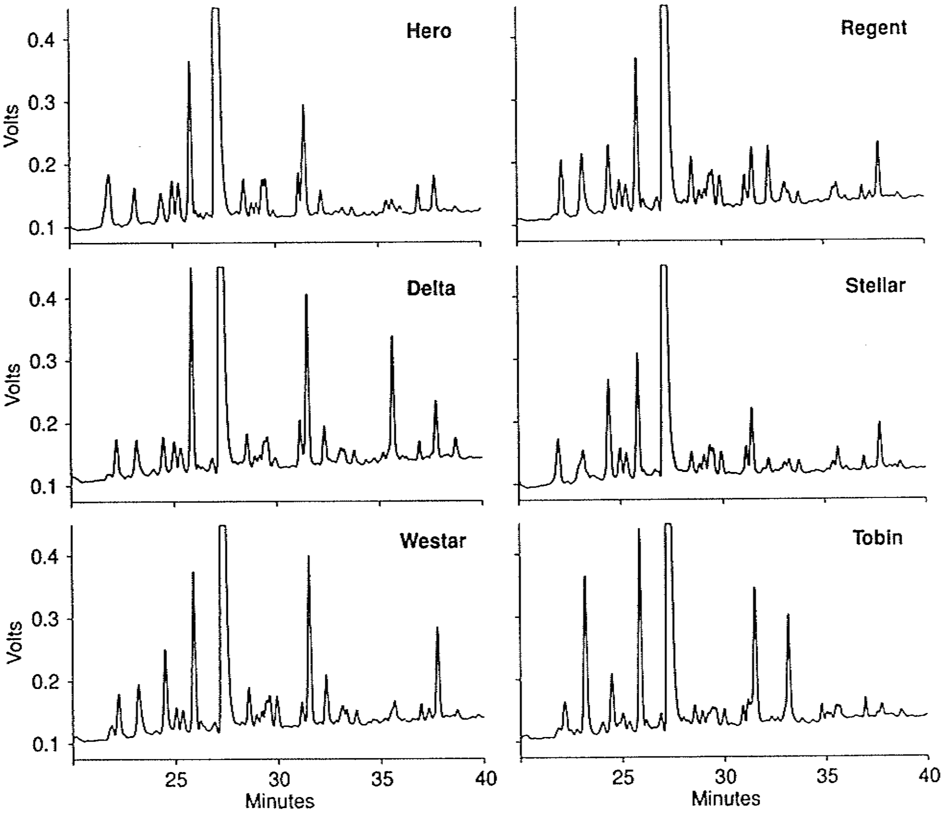


Figure 3.6. Chromatograms of five *B. napus cvs* Stellar, Regent, Delta, Westar, Hero and 1 *B. rapa cv* Tobin (operating conditions as for figure 3.1)


Hero
Regent
Delta
Stellar
Westar
Tobin
Volts
0.1
0.2
0.3
0.4
25
30
35
40
Minutes

Cultivar Identification in *Brassica napus* L.
Using RP-HPLC of Ethanol Extracts

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ABSTRACT

A method has been developed to identify cultivars of oilseed rape (*Brassica napus* L.), an outcrossing species, using reverse-phase high-performance liquid chromatography (RP-HPLC) based on the separation of ethanol extractable seed components. Defatted flour was extracted into 70% ethanol in water and chromatography was carried out using a reverse-phase C18 column. Optimum results were achieved using water solvent with 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 found to be reproducible and significant differences were found in the chromatograms of 29 cultivars studied. The chromatographic differences among these cultivars were found to be stable over a range of growing environments. It was possible to allocate unknown cultivars to their correct classes using the HPLC method together with discriminant analysis . This method has potential for use in characterizing new cultivars for cultivar registration.

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

INTRODUCTION

Numerous cultivars of oilseed rape *(Brassica napus*) 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 (PVR) 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 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 unambiguous identification of canola cultivars. Phenotypic characteristics including flower colour, plant height and leaf shape may not show sufficient distinctness to differentiate among cultivars. Glucosinolate profiles have been used with some success in swede (*B. napus* L. var. *napobrassica* (L.) Peterm.), a related taxon (Adams et al. 1989). Protein patterns derived from gel electrophoresis (PAGE) have been utilised for cultivar identification in many commercial crops, including *Brassica* (Ladizinsky and Hymowitz 1979; Coulthart and Denford 1982; Gupta and Röbbelen 1986). Although electrophoresis is generally used for predominantly autogamous (selfing) species, the technique has been shown to be useful for predominantly outcrossing species (Gilliland 1989). Reverse-phase high-performance liquid chromatography (RP-HPLC) of seed proteins has been used to characterize cultivars of wheat, *Triticum aestivum* L. (Bietz and Cobb 1985; Marchylo et al. 1988); oats, *Avena sativa* L. (Lookhart 1985); soybean, *Glycine max* L. (Buehler et al. 1989); and maize, *Zea mays* L. (Smith 1988). The application of chromatography for cultivar identification has been discussed at length by Morgan (1989). This study has investigated the application of RP-HPLC to cultivar identification in *B. napus* based on separation of ethanol extractable seed components.

EXPERIMENTAL PROCEDURES

Materials

Samples of *Brassica 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 among 29 cultivars (*B. napus* var. *oleifera,* summer types "annua", unless otherwise indicated) using breeder's seed samples obtained from:

Agriculture Canada Research Station, Saskatoon,Saskatchewan; Excel, Tribute, Westar, Tristar, Profit , Bronowski, Oro, Argentine, Midas.

Department of Plant Science, University of Manitoba, Manitoba; Tower, Regent, Reston, Pivot, Stellar, Hero.

ICI Seeds, Canada, Winnipeg, Manitoba; Hyola-40 (hybrid).

Pioneer Hybrids, Ontario; Delta, Winfield (*B. napus* var. *oleifera* winter type, "biennis"), Bounty, Touchdown (*B. napus* var. *oleifera* winter type, "biennis"). *Svalöf Seeds Ltd., Ontario*; Legend, Vanguard, Celebra, Horizon (*B. rapa* var. *oleifera,* summer type, "annua"), Colt (*B. rapa* var. *oleifera,* summer type, "annua"), Stallion, Topas, Global, Crystal (*B. napus* var. *oleifera* winter type, "biennis").

Stability of expression over environments was studied using 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 (Table 3.1). Seed samples of turnip rape (*B. rapa* L. 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 using a set of five 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 and were labelled for computer analysis with only their site codes.

Sample preparation and extraction

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 ground again prior to analysis. Extraction of soluble components from the flour was achieved using 70% ethanol. Flour (0.04 g) was weighed into 2 ml plastic microcentrifuge tubes, extraction solvent (1.0 ml,70 % *v/v* ethanol/water) added and the tubes shaken for 1 hour on a reciprocating shaker. The tubes were centrifuged on a high speed centrifuge for 5 min. and the clear supernatant removed for HPLC analysis. Samples were analyzed in duplicate. Electrophoresis of the eluted peaks from the ethanol extracts did not produce evidence of any protein, possibly due to insufficient concentrations.

Chromatography of extracts

The analysis was carried out using a Waters HPLC system including two 510 pumps, a Wisp autosampler, column oven with temperature control and a 994 LC programmable UV-Vis Detector. The data system incorporated an IBM AT compatible computer together with Waters "Maxima" integration software which controlled the solvent gradient, peak integration and data handling. A Vydac 218TP54 C_{18}, 5 μM x 30 nm, 0.46 x 25 cm column was used for chromatography.

Elution solvents were water (Solvent A) and acetonitrile (ACN, Solvent B), containing 0.1% v/v trifluoroacetic acid (TFA), added as a dissociating agent for protein. The solvent flow rate was 1 ml min^{-1} with a gradient of 5 minutes 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, to avoid diurnal fluctuations and achieve optimum peak resolution (Fig. 4.1). Injection volume was 20 µl with larger volumes resulting in progressively broader, leading peaks. Despite the poor peak shape at higher injection volumes, a linear regression analysis of peak area vs injection volume, up to 200 µl, determined on 5 independent peaks, indicated that the peak response was linear over the range of injection volumes ($r^2 > 0.95$). Chromatograms were acquired at 2 wavelengths as most detail was obtained at 210 nm but several individual peaks were more responsive at 240 nm (Fig. 4.2).

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. Excellent reproducibility was achieved in both repeated injection of a single extract (retention time cv = 0.04-0.10, peak area cv = 3.45-7.50, 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) (Appendix I a and I b).

Statistical Analysis

Chromatograms of five cultivars, from several locations, were integrated with Water's Maxima software. Peak area percent data of the chromatogram components acquired at two wavelengths, 210 and 240 nm, were entered into a spreadsheet to form a data matrix. Five samples of cv Westar, identified only by location code numbers were used as unknowns to test the ability of the HPLC method to identify samples. These were also integrated and the data entered into a data matrix (Appendix III a and III b).

Selection of Discriminating Variables - Discriminant Analysis. Sneath and Sokal (1973) described discriminant function as "a linear function 'z' of characters describing OTU's (operational taxonomic units) that weight the characters in such a way that as many as possible of the OTU's in one taxon have high values for 'z' and as many as possible of another have low values such that 'z' serves as a much better discriminant of the two taxa than does any one character of the two taxa taken separately". SAS Discriminant analysis (release 6.07, SAS Institute Inc. Cary, NC USA) was used to select a reduced number of peaks from the initial data matrix which provided the greatest discriminatory power (SAS Proc Stepdisc). Some reduction of the number of peaks was necessary prior to discriminant analysis as the maximum number of discriminating variables that may be used in discriminant analysis is two less than the total number of cases (Klecka 1976). An initial selection was carried out by visual observation of overlaid chromatograms, deleting peaks which were clearly variable within the cultivar group.

Variation Within Cultivar Groups - Canonical Analysis. Canonical discriminant analysis is a multivariate analysis related to principal component analysis. Given a classification variable and several quantitative variables, canonical discriminant analysis summarizes between-class variation and tests the hypothesis that correlations are zero in the population. A plot of the canonical variables can be used to aid visual interpretation of group differences. Data obtained from the five cultivars obtained from separate sites was analysed by canonical discriminant analysis to determine the variation in data within each cultivar group and to provide some information regarding environmental variability. Data from five unidentified samples were included to determine variability within the group and among the five known cultivars. The 1st and 2nd canonical variables were plotted (SAS Proc Plot).

Group Discrimination and Sample Identification. Discriminatory analysis was used to test discrimination between the five cultivar groups (SAS Proc Discrim) to test the ability of the method to identify the unlabelled samples.

Cultivar Similarity - Cluster analysis. Cluster Analysis is a method of phenetic analysis, appropriate for determining the similarity between samples. There are several alternative forms of cluster analysis, including 'average linkage cluster analysis' or unweighted pair group arithmetic average clustering (UPGMA), developed by Sokal and Michener (1958). Average linkage cluster analysis is the average distance between pairs of observations one in each cluster. Average linkage tends to join clusters with small variances and is slightly biased toward producing clusters with the same variance (SAS Users Guide: Statistics. Version 5 edition). Alternative methods are Complete Linkage (distance between clusters is the maximum distance between an observation in one cluster and an observation in another cluster), and Single Linkage (the distance between clusters is the minimum distance between observations in one cluster and observations in another). Average linkage cluster analysis (unweighted pair-group arithmetic average clustering, UPGMA) is the most frequently used clustering strategy (Sneath and Sokal 1973). The data matrix, generally composed of peak areas generated by peak integration with chromatographic software, is converted to a similarity matrix using a simple matching coefficient. Several commercial software packages are available for calculating the similarity matrix such as Phylo software (Baertlein et al 1990) in which relatedness is determined through the calculation of Pearson's product-moment correlations. The two assumptions of this calculation are that a) no characteristic is weighted more heavily than any other, and b) only the relative values of the characteristics for each item are compared. Cluster analysis and construction of similarity trees can be achieved using computer software such as NT-SYS (Rohlf et al 1971) or SAS using the procedure Proc Tree.

A similarity matrix was produced from the HPLC data matrix for the five cultivars from several sites (Appendix VI) and Phylo software. Subsequently, average linkage cluster analysis was then done by the unweighted pair group method (UPGMA, Appendix VII a). Additional analysis by single linkage and complete linkage cluster analysis were also compared (Appendix VII b and c).

Comparison of *Brassica napus* and *Brassica rapa* cultivars. Cluster analysis of 27 cultivars of *B. napus* and two cultivars of *B. rapa* were done to determine the similarity between cultivars. A sample data matrix (Appendix

VIII) and similarity matrix (Appendix IX) were created using integrated peak areas with the same retention times as those described previously for the five cultivars from different locations. Average, single and complete linkage cluster analysis was carried out (Appendix X a, b and c).

RESULTS AND DISCUSSION

Characterization of Chromatogram Components

For the 29 samples of breeder's seed analyzed, variability among cultivars ranged from small differences in peak area for some cultivars to large differences in others, six examples of which are shown in Fig. 4.3. Initial observation of the chromatograms suggested there was sufficient variation in the chromatograms to distinguish among the cultivars. 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 the majority of components over environments as illustrated by the overlaid chromatograms of *cv* Stellar in Fig. 4.4.

Most of the chromatogram peaks eluted between 20 and 43 min. The baseline between 0 to 20 min. and 43 to 60 min. was sometimes variable. For this reason, only peaks between 20 and 43 min. were used in the analysis. Individual peak areas were calculated on peaks for each chromatogram (Appendix III a and III b).

Unlike the studies of wheat protein in which wavelengths of 210 nm (peptide bonds) and 280 nm (aromatic amino acids) are used (Kruger and Marchylo 1985), many of the components in these extracts had higher absorbancies at 240 nm. 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 using a photo diode array ultra-violet (U.V.) detector. 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 (Fig. 4.1) was identified as sinapine, a major component of rapeseed. Several other components with retention times between 20 and 43 minutes were tentatively identified as phenolic compounds including ferulic acid (r. t. = 28.24 min) and coumaric acid (r.t. = 26.03 min), by comparison with commercial standards. Addition of the 70% ethanol to the flour in two parts (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 is not necessary for the profile to serve as a fingerprint of the individual cultivar.

A non-linear response was reported for injection volume in the analysis of quantitative analysis of gliadins and glutenins (Marchylo and Kruger 1988). Investigation showed that absorbance response in this study (Appendix II) was linear for changes in injection volume, providing assurance of accurate estimation of components which vary in concentration among cultivars. In contrast, The use of separate solutions of H_2O + 0.1% TFA and CH_3CN + 0.1% TFA was found to give better reproducibility over extended times than when mixtures of H_2O, CH_3CN + 0.1% TFA were used. This is in agreement with Bietz and Cobb (1985) who found that concentrations of CH_3CN changed over time due to CH_3CN evaporation, particularly as solvents are degassed by sparging with helium over several days.

Statistical Analysis

Data Matrix. Data acquired for two wavelengths, 210 and 240 nm, is presented in Appendix IIIa and IIIb respectively. A total of 10 peaks were selected from the initial set by discriminant analysis for both 210 and 240 nm wavelengths and were entered into a second data matrix (Appendix IIIc and IIId).

Between Class Variation. The canonical analysis plot (Fig. 4.5 and Appendix IV c) of first and second canonical variates indicated that data acquired at 240 nm provided small variation within groups. The groups were clearly separated from each other. The unidentified samples, selections of Westar from separate locations were also grouped closely with other Westar samples. A similar plot for data acquired at 210 nm was less distinct between groups although cultivars were still separated into their correct groups. Again, the unidentified samples were grouped correctly with Westar samples (Appendix IVa and IVb).

Discrimination Between Groups. Discriminant analysis successfully grouped all of the samples of five cultivars into five distinct groups (Appendix V a). Generalized squared distance between groups are given (Appendix V a). Test data for five unidentified samples were identified as misclassified samples from their site code numbers SCG (Saskatchewan Canola Growers samples) and correctly reclassified into the Westar group (Appendix Vb).

Cluster Analysis. Despite the success of discriminant analysis and canonical analysis to separate cultivars into discrete groups, cluster analysis produced some variablity with the cultivar groups (Appendix VII a). The Westar group was separated into two clusters and one Delta sample clustered closer to cultivars other than Delta samples. Analysis of data by alternative cluster methods (i.e. single and complete cluster, Appendix VII b and VII c) at both 210 and 240 nm wavelengths showed similar inconsistencies in clustering.

Samples of 27 cultivars of *B. napus* and 2 cultivars of *B. rapa* were also analysed by cluster analysis. A pairwise correlation matrix was produced (Appendix IX) and average linkage cluster analysis was carried out to determine relatedness among cultivars (Appendix Xa). The analysis clustered the *B. rapa cvs* Colt and Horizon separately from *B. napus*. In several cases cultivars from within the same breeding programs, based on known cultivar pedigrees (Sernyk

1991), clustered together. For example Delta and Bounty (Pioneer Hybrids) were similar, as were the two winter types Winfield and Touchdown (Pioneer Hybrids). Stallion and Crystal (Svalöf Seeds Ltd.) also clustered closely. Argentine, which does not have canola quality, formed a terminal cluster with Canadian cultivars Reston, Regent, Pivot (University of Manitoba), Westar and Oro (Agriculture Canada). Midas, another cultivar which does not have canola quality, also formed a terminal cluster. The clusters were not always representative of breeding programs. This may be expected as there is a basic similarity among the canola cultivars, a result of selection for cultivars with similar quality characteristics e.g. low glucosinolates and low erucic acid concentrations in the seed.

The results demonstrated by discriminant and canonical analysis show that the characterization of the ethanol extractable components, measured by HPLC, can provide a means to differentiate among cultivars. HPLC together with discriminant analysis were useful tools to identify unknown cultivars. Discriminant analysis appears to be the most useful method for analyzing HPLC data for future characterization of cultivars. Canonical analysis is also useful to determine the variability between samples

Some improvement in discrimination could be achieved if the components of interest were extracted from the background of the total ethanol extractable material. This would require that the components be identified. If the majority were free phenolics for example, they could be extracted in methanol followed by extraction into diethyl ether by the method of Sosulski et al (1980). For simplicity, it would appear that the crude extract is sufficient.

A routine method has been described for the analysis of ethanol extracts of rapeseed meal for cultivar identification. The results indicate that RP-HPLC can be used to differentiate and thereby identify cultivars of *B. napus.* This work provides evidence that discriminant analysis may be used in conjunction with RP-HPLC for cultivar identification. Unknown samples can be compared to a previously established data set developed of samples of known cultivars. Due to differences among columns and HPLC instruments, it is necessary for individual laboratories to establish reference standards prior to analysis of unknowns. To identify a particular cultivar, a range of samples which will include the unknown cultivar should be part of the reference set. For the purpose of discriminating a new cultivar from similar existing cultivars, the reference set would include a minimum number of cultivars, such as within a breeding program, and a minimum number of peaks which provide the best discrimination among those cultivars.

FIG. 4.1 HPLC chromatogram of ethanol extract of *B. napus cv* Excel, injection volume 20 µl, column temperature 40°C, flow rate 1 ml min^{-1}, wavelength 210 nm. Peaks A - J have been used for statistical analysis.

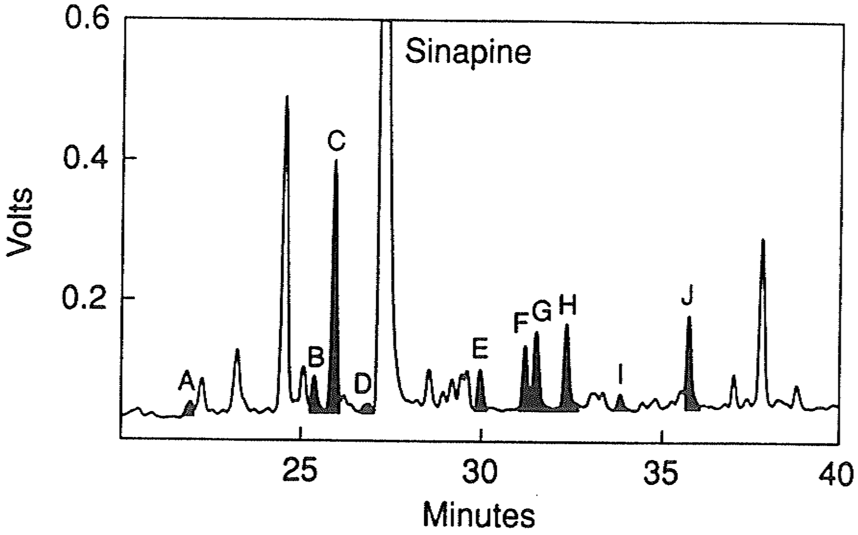

Sinapine
0.6
0.4
0.2
Volts
25
30
35
40
Minutes
A
B
C
D
E
F
G
H
I
J

FIG. 4.2 Detector response at two wavelengths; 210 nm and 240 nm (other operating conditions as for Fig. 4.1)

4.20

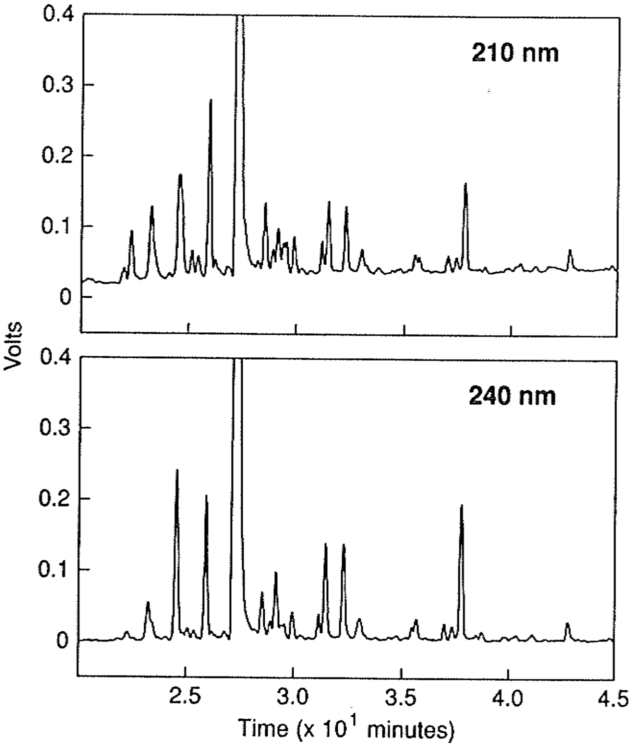


FIG. 4.3 Chromatograms of five cultivars of *B. napus* and one cultivar of *B. rapa*. Chromatograms have been amplified for detail. (operating conditions as for Fig. 4.1)

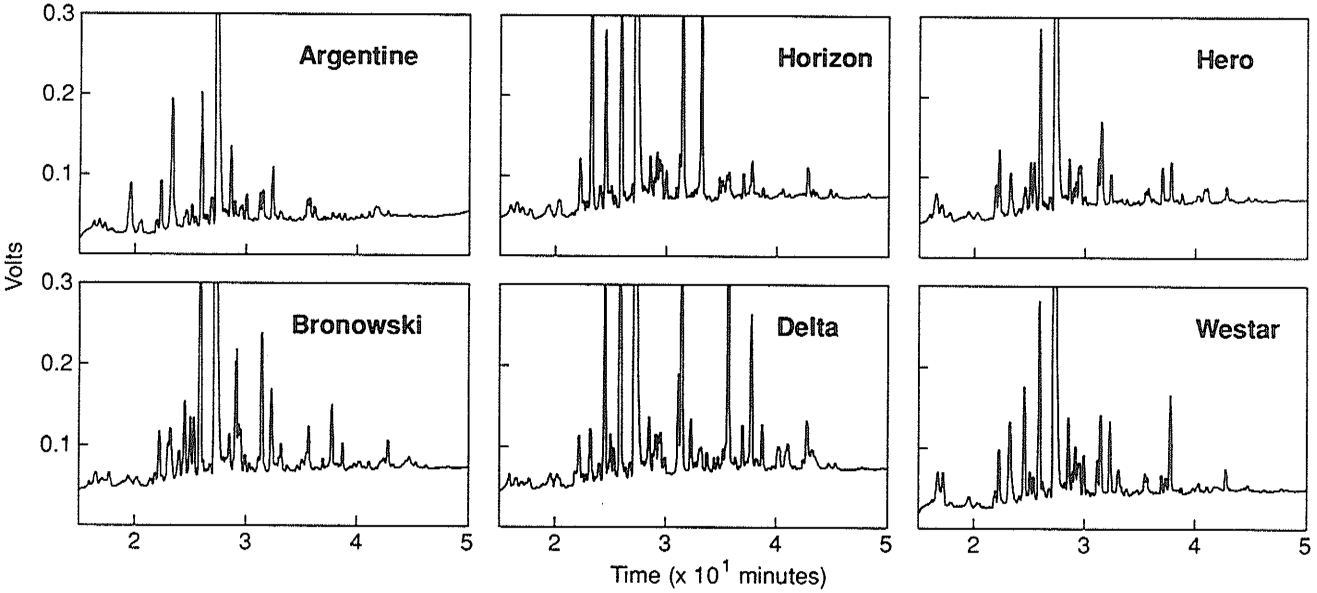

Argentine
Horizon
Hero
Bronowski
Delta
Westar
Volts
0.3
0.2
0.1
0.3
0.2
0.1
2
3
4
5
2
3
4
5
2
3
4
5
Time (x 10^1 minutes)

FIG. 4.4 Five overlaid chromatograms of *B. napus cv* Stellar grown at 5 different sites: A = The Point, B = Baggot, C = Teulon, D = Dauphin and E = Roblin (operating conditions as for Fig. 4.1)

4.24

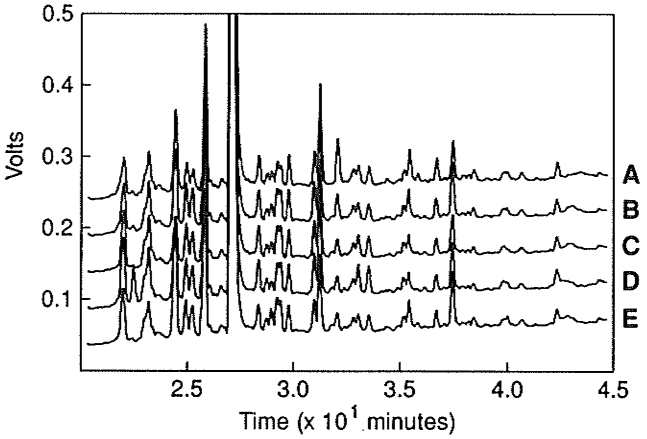


FIG. 4.5 Plot of Canonical Discriminant Analysis (SAS, Appendix IV c.) of five *B. napus* and one *B. rapa* cultivar grown at different sites. D = Delta; H = Hero; S = Stellar; R = Regent; W = Westar based on 10 peaks from Appendix III d acquired at 240 nm. The symbol X indicates the value of unidentified samples (SCGA - Westar) used for testing cultivar identification. Proportion of total variation accounted for by the two axes CAN1 = 37.26%, CAN2 = 28.72%.

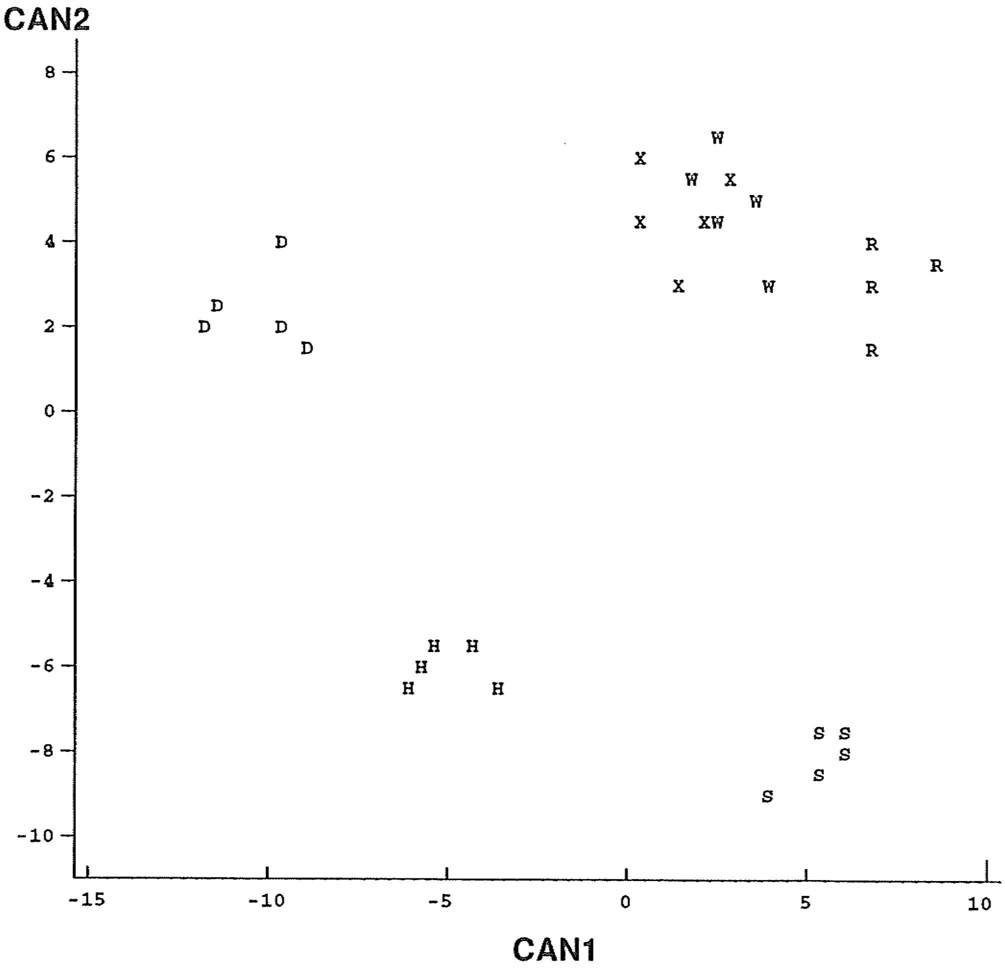
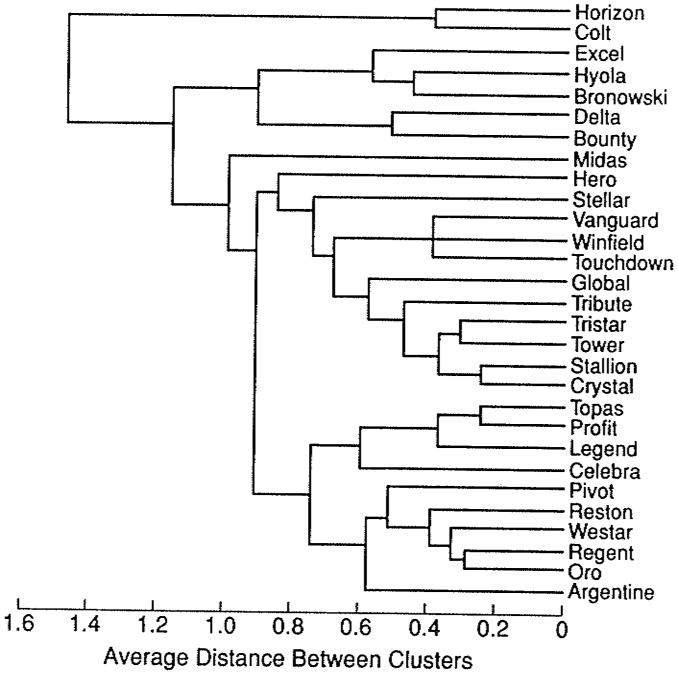

CAN2
8
6
4
2
0
-2
-4
-6
-8
-10
CAN1
-15
-10
-5
0
5
10
D
H
X
W
R
S

FIG. 4.6 Average linkage cluster analysis of 29 cultivars of oilseed rape based on 10 peak areas acquired at 210 nm from Appendix III c.

4.28


Horizon
Colt
Excel
Hyola
Bronowski
Delta
Bounty
Midas
Hero
Stellar
Vanguard
Winfield
Touchdown
Global
Tribute
Tristar
Tower
Stallion
Crystal
Topas
Profit
Legend
Celebra
Pivot
Reston
Westar
Regent
Oro
Argentine
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Average Distance Between Clusters

Discrimination Among Cultivars of Rapeseed (*Brassica napus* L.) Using DNA Polymorphisms Amplified From Arbitrary Primers.

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Summary. RAPDs (Randomly Amplified Polymorphic DNA) were used to discriminate among 23 cultivars of oilseed rape (*Brassica napus*) selected from several breeding programs. A set of 100 random sequence 10-mer primers were tested, of which 70 produced bands and 22 showed evidence of polymorphism. A selection of six primers produced 23 polymorphic bands of between 300 to 2200 base pairs in size, sufficient to distinguish among the cultivars. Analysis of seed of five cultivars obtained from four different sites showed stability of banding pattern over source of seed. The analysis was repeated using four different thermocyclers, each of which produced the same band pattern. Cluster analysis showed that the relationships among cultivars is closer for those from the same breeding program than for those from different programs. The results of this study show that RAPDs can be used as a method of identification of oilseed rape cultivars.

Key words: *Brassica napus* - cultivar identification - RAPDs - rapeseed - taxonomy

Introduction

Identification of plant cultivars has become increasingly important with the requirement of Plant Breeders Rights (PBR) to demonstrate Distinctness, Uniformity and Stability (DUS) (Plant Varieties Journal 1991) for each new cultivar. Phenology and morphological characteristics may not be significantly distinct and usually require growing plants to full maturity prior to classification. The need for alternative methods of cultivar discrimination is reflected in the number of techniques developed over recent years. In a survey by the International Seed Testing Association (ISTA) (Van den Burg et al. 1991), the most common rapid tests employed by laboratories were biochemical and electrophoretic analyses. Electrophoresis, restriction fragment length polymorphisms (RFLPs), and high-performance liquid-chromatography (HPLC) were described as emerging technologies, requiring the development of simple and standardised laboratory protocol before being adopted as standard tests.

The phenology and morphological characteristics used for cultivar identification include plant growth period (Halligan et al. 1991) and seed shape (Barker et al. 1992). Electrophoresis of seed proteins has been a prominent test in cultivar identification in crops such as wheat, *Triticum aestivum* L. (Wrigley et al 1982); cotton, *Gossypium* spp. (Rao et al. 1990); and pasture legumes, *Trifolium* spp. (Gardiner and Forde 1988). In many cases, high-performance liquid-chromatography (HPLC) has replaced electrophoresis for soybean, *Glycine max* L. (Buehler et al. 1989); maize, *Zea mays* L. (Smith 1988); rice, *Oryza sativa* L. (Huebner et al. 1990); and wheat (Marchylo et al. 1988). Although most electrophoretic work has been on predominantly autogamous (selfing) species the technique has also been shown to be useful for predominantly allogamous (outcrossing) species (Gilliland 1989).

The interest in cultivar discrimination of *Brassica* spp. has resulted in many specialised tests based on seed or plant composition, such as fatty acid in oilseed rape (*B. napus* L.) (White and Law 1991); volatile hydrolysis products in swede (Cole and Phelps 1979); and glucosinolate content of the vegetable forms e.g. brussels sprouts *(B. oleracea* L. var *gemmifera* Zenker.) (Heaney and Fenwick 1980); and swede (*B. napus* L. var *napobrassica* (L.) Peterm.)(Adams et al. 1989).

Restriction fragment length polymorphisms (RFLPs) has been used by Song et al. (1988) and Figdore et al. (1988) for the purpose of studying genome evolution among *Brassica* species. Although the methodology for RFLPs is now well established, the technique has several disadvantages including the time and labour requirements and the need for the use of radioactive material.

The recent development of the polymerase chain reaction (PCR) to amplify DNA and the use of randomly amplified polymorphic DNA (RAPD, Williams et al. 1990) or arbitrarily primed PCR (AP-PCR, Welsh and McClelland 1990) has resulted in a potentially useful tool for cultivar discrimination. RAPD involves the amplification of DNA segments using random sequence primers, generally of 10 bases, to find polymorphic regions within the genome defined by the primer sequence. The products formed and separated by agarose gel electrophoresis reveal sequence variation in the form of variable numbers of bands of variable length which may be characteristic of species and/or cultivars within species. RAPD require no previous sequence information for fingerprinting of cultivar genomes.

This study investigates the application of RAPD to the discrimination of oilseed rape (*B. napus*) cultivars.

Materials and Methods

Plant Material

The *B. napus* cultivars Argentine and Bronowski, selected because of their different backgrounds, were used to optimize the RAPD method and select suitable primers which exhibit polymorphisms between the two cultivars. Bronowski is a Polish cultivar and is the source of the low glucosinolate characteristic in all low glucosinolate oilseed rape cultivars. Argentine, although not a true variety, is a landrace from Argentina which has been used in the early development of many Canadian oilseed rape cultivars.

The method was then used to investigate the possibility of using RAPD to differentiate among 23 cultivars of *B. napus.* Breeders seed was obtained for the study from: Pioneer Hibred International and Svalöf Seeds Ltd., Ontario; Department of Plant Science, University of Manitoba and ICI Seeds Canada, Manitoba; Agriculture Canada Research Station, Saskatoon, Saskatchewan and the New South Wales Department of Agriculture, Australia (Table 5.1). One seed sample of *B. rapa cv.* Horizon was included to observe the differences between the two species.

The possibility of variability in seed samples, due to contamination or outcrossing of seed, interfering with cultivar discrimination was investigated using seed samples of five cultivars harvested from trials at five diverse sites in Saskatchewan and Manitoba in 1991.

Reproducibility of the RAPD analytical procedure was investigated with repeated analyses of samples using a Techne MW-2 thermocycler. To determine if the method was instrument dependent, samples were also tested on a range of thermocyclers, incorporating identical operating conditions but with each instrument's unique method of cooling and/or sample holder. The instruments included: Techne Multiwell 2.01 with a 96 well microtitre tray, circulating refrigerated water cooled, heating block;

Techne PHC-2 1.05 A with 0.5 ml centrifuge tubes, circulating refrigerated water cooled heating block; Thermolyne TEMP.TRONIC refrigerated block with 0.5 Ml centrifuge tube (no water circulation); and Ericomp Ez-Cycler with 0.5 ml centrifuge tube, ambient temperature water cooled heating block. Due to the different methods of cooling, the cycling rate varied considerably among instruments.

Sample preparation

Twenty seeds of each cultivar were grown to two to three leaf stage (2 weeks) in a controlled environment. The true leaves of each cultivar were excised and placed into separate sealed plastic bags on ice, samples of each cultivar bulked together. The bags were placed directly into a freeze drier and lyophilised for 48 hours. After flattening the bags to remove air, they were sealed into air tight containers with desiccant and stored at 4°C.

DNA Isolation

DNA was extracted from the lyophilised leaf tissue (300 mg) using the method of Kidwell and Osborn (1992). Precipitated DNA was hooked out using a hooked glass rod, rinsed several times in ethanol (75%)/ammonium acetate (10 mM) and dried under vacuum. The dried DNA was redissolved in Tris/EDTA buffer (TE) (500 µl and centrifuged (13,000 g, 10 min).

Ribonuclease (1.5 µl 10 µg/ml, Sigma Chemicals R4875 Type 1-A) was added and the solution was incubated at 37°C for 3 hours. DNA was reprecipitated in 3 M sodium acetate (1/10 vol) and isopropanol (1 vol), held at -20°C for 2 hours, centrifuged (5,000 g, 20°C, 30 min.) and washed 3 times with 70% ethanol (-20°C). The DNA was dissolved and stored in TE buffer (1 ml). Concentration was measured using an ultraviolet (UV) spectrophotometer at 260 nm. To check for fragmentation of the DNA, the samples were loaded into a 1.4% agarose gel in 1 x TAE buffer (tris/sodium acetate/EDTA pH 7.8) and separated electrophoretically.

The DNA was of high molecular weight with little fragmentation and free from RNA as indicated by U.V. spectra and gel electrophoresis (data not shown).

RAPD Amplification

Techniques for the generation of RAPD markers are well documented (Rafalski et al 1991). A single primer is used in each reaction. The primer is generally ten nucleotides long (10 mers), short enough to ensure adequate numbers of binding sites on the template DNA which will generate sufficient bands for study. Commercial sets of these 10 mer primers are now available. A set of 100 10 mer primers were obtained from J. Carlson, University of British Columbia. Concentrations of primer, template and TAQ (*Thermus aquaticus* DNA polymerase, Promega M186A) were optimized to give maximum band intensity with minimum quantities of polymerase to conserve costs. Final reaction mixture included 1 x Promega reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (deoxyribonucleoside 5' - phosphates), 0.25 µM primer , 0.75 units of TAQ polymerase, 50 - 100 ng genomic DNA and made to a final volume of 25 µl with sterilized double distilled water. Amplification of the DNA was carried out in microtitre plates (Mandel Scientific) using a Techne MW-2 thermocycler programmed for 1 min at 94°C followed by 45 cycles of 1 min at 94°C (denaturation), 1 min at 37°C (annealing), and 2 min at 72°C (elongation) and a final stage of 10 min at 72°C. Initially only 25 cycles were used but extra cycles produced more intense bands. Although 45 cycles may be excessive, there was no detrimental effect in terms of altered band numbers and as the reaction was carried out overnight, the time factor was not important.

The RAPD products (15 µl) plus blue mix (3 µL, stop buffer/0.5% bromophenol blue/glycerol : 200/400/200), were separated by electrophoresis using 1.4% agarose in 1 x TAE buffer (Thomas C. Osborn, University of Wisconsin, Madison - personal communication). A 1 kb ladder (Bethesda Research Laboratories, BRL) was included as a size marker, and the bands detected with ethidium bromide staining [2.5 µl(10 mg / ml)/100 ml].

Selection of Primers

Initially the entire set of 100 primers were screened using the DNA from the cultivars Bronowski and Argentine. The primers which exhibited polymorphisms among these two cultivars were tested against the remaining cultivars identified in Table 5.1.

Cluster Analysis

Cluster analysis is a method for determining the similarity among samples. The form most frequently used is the unweighted pair group arithmetic average clustering (UPGMA; Sneath and Sokal 1973), developed by Sokal and Michener (1958). Average linkage cluster analysis is the average distance between pairs of observations, one in each cluster. Alternative methods are complete linkage (maximum distance between an observation in one cluster and an observation in another cluster), and single linkage (minimum distance between observations in one cluster and observations in another).

Fragment sizes of RAPD were estimated from the gel by comparison with a 1 kb ladder marker. The bands were recorded as present (1) or absent (0) and assembled into a data matrix table . Pairwise similarity comparisons were calculated from the data matrix according to Nei and Li (1979) and NEIRAPD software. The similarity index was calculated using the equation:

$$S = 2n_{XY} \ / \ (n_X + n_Y)$$

in which n_X and n_Y are the number of fragments in populations X and Y respectively, whereas n_{XY} is the number of fragments shared by the two populations (Nei and Li 1979). Cluster analysis (UPGMA) of the similarity indices was then carried out, using NT-SYS software (Rohlf et al 1971) .

Results

Primer Selection.

Of the 100 primers (oligonucleotides) tested, 70 primers produced some product and of these, 22 primers showed evidence of polymorphisms. Six primers were ultimately selected for further evaluation which produced a total of 43 clear, easily detectable bands, 23 of which were polymorphic bands (Appendix XI). Easily detectable, well resolved bands were those which were reproducible over repeated runs, with sufficient intensity to determine presence or absence in samples with the same relative band intensity. The sequences of the primers are shown in Table 5.2. Other polymorphic primers produced patterns which were either faint and hard to detect or not reproducible. The total number of bands produced per primer varied from 6 to 15 although only one to seven of these were polymorphic and useable. The size of bands ranged from 350 to 2200 base pairs (bp), Appendix XI .

Cultivar Identification.

Examples of polymorphic bands used for discrimination within the sample set are shown in Fig. 5.1. Comparison of band patterns among 23 *B. napus* cultivars using six primers, indicated discrimination of all but the two cultivars, Legend and Vanguard. There was a difference of two bands between the two Australian cultivars, Oscar (cultivar 23 on Table 5.1.) and Yickadee (24), and up to ten band differences among all of the others e.g. Tower (22) and Oscar (23). *B. rapa cv.* Horizon (13) differed by up to 37 of the total 43 bands from the other cultivars.

Effect of Variable Seed Source.

Analysis of five cultivars collected from trials at four separate sites produced similar profiles within each cultivar (Fig. 5.2). All cultivars produced band patterns identical to other samples of the same cultivar when tested with primer #303. The patterns of three cultivars were similar *cv* Stellar, Westar and Regent, whilst the other two cultivars Hero and Delta were distinct.

When analyzed with primer #329 (Fig. 5.2), the same three cultivars were similar and Hero and Delta again had unique bands. With primer #329 one sample of *cv* Stellar had a less intense band at 750 bp than the other three Stellar samples . One sample of *cv* Hero had a slightly more intense band then the other Hero samples, also at 750 bp. This variability in bands may represent genetic heterogeneity of the cultivar, rather than variation in experimental conditions. Despite these differences, there was sufficient information using either primer #329, or a combination of primers, to discriminate among the other cultivars.

Instrumental Variation.

Four cultivars (three *B. napus* and one *B. rapa*) were selected which had distinct polymorphic patterns with primer #329. Identical band patterns were obtained for each of the four cultivars using four different thermocyclers, despite several hours difference in cycling time among the instruments, (Fig. 5.4).

DNA Template Quality

The DNA extracted from 24 cultivars of oilseed rape, and the five samples of cultivars from the separate trial sites, were extracted over several days.

Although concentrations were normalised during dilution, observation of this DNA on agarose gel showed some variation in the fragmentation of the DNA among samples. Despite this, the RAPD bands were consistent in their intensity, indicating that variation in DNA quality was not a critical factor in the discrimination of the cultivars.

DNA templates were frozen and thawed several times during evaluation of the range of primers and were observed to degrade significantly as indicated by streaking when intact DNA was run on agarose gel. Although the band patterns were consistent throughout the study, higher levels of DNA template were required to maintain the intensity of the bands after 1-2 months. The problems of DNA degradation were overcome by separating the bulked DNA into several containers prior to freezing and storing one for current use at 4°C.

Statistical Analysis.

The number of bands and the individual sizes of the bands recorded are presented as a data matrix in Appendix XI. Of 43 bands shown, 23 are polymorphic and 20 are monomorphic excluding sample 13 (*Brassica rapa*). Analysis of similarity indices (Nei and Lei 1979), based on the data in Appendix IX, produced a similarity matrix, Appendix XII.

Average linkage cluster analysis (Fig 5.3) of the similarity indices,

Appendix XII, indicates that distance separating cv Horizon from *B. napus* types is greater than the distance between all other cultivars. The cluster further indicates that there is a basic similarity among the *B. napus* cultivars used in this study, which is to be expected as result of selection for similar quality factors.

Discussion

The use of Argentine and Bronowski to select primers was based on available pedigree information (Sernyk 1991). These two cultivars had dissimilar backgrounds and were expected to display a high number of polymorphic markers. This deliberate selection may have introduced some bias in that every primer used in subsequent analysis would have at least one band different between the two cultivars. This may have contributed to the distance between these cultivars displayed in the cluster analysis (Fig. 5.3) although, this distance was in agreement with the pedigree information.

Despite the possible bias, some observations could be made regarding the clustering of particular cultivars, based on pedigree information (Sernyk 1991). Legend (Andor / SV02215) and Vanguard (Andor // Hermes / Bronowski / Gulle) both Swedish cultivars from *Svalöf Seed Ltd.* had the same band pattern. Oscar and Yickadee, Australian cultivars, clustered together. Crystal and Stallion (Sweden, *Svalöf Seed Ltd.*), Global (Denmark, *Svalöf Seed Ltd.*) and Delta (Sweden, *Pioneer Hibred International*) clustered closely. Argentine and Midas, which are not canola quality cultivars, had similar patterns. Westar, Excel, Profit (Canada, Agriculture Canada) and Regent (Canada, University of Manitoba) also clustered together. Clustering of cultivars of similar seed source adds verification to the RAPD technique.

The RAPD technique discriminated among all of the cultivars tested, with the exception of two, using only 6 primers. There are several hundred primers available commercially which would ensure the ability of the RAPD technique to differentiate between any two distinct cultivars, as long as the seed is pure and representative of the original breeders' seed.

The technique for DNA extraction is relatively simple, allowing for the extraction of at least 12 samples per day. Higher numbers have been processed in a similar period when a high speed paint shaker (Tai and Tanksley, 1990) was used to grind plant material in place of a mortar and pestle. The quality of the DNA extracted in this study was high and produced reproducible RAPD products (Appendix XIII).

The RAPD technique is largely automated requiring little operator input. Most importantly, data generated by the technique is significantly easier to interpret than that of other methods such as HPLC analysis.

Although the intensity of some of the bands varied for seed from the same cultivar grown at different sites, primers can be selected which produce bands that are consistent among samples from different sites or under different cycling conditions. Since there is a large selection of commercially available primers, and each primer produces several bands, it is possible to select only those bands which give consistent results, while variable bands are ignored. The patterns produced by the *B. rapa* cultivar were distinctly different from *B. napus* cultivars.

There was some variation in band intensity among instruments, however there were no differences in the results obtained by the different instruments. Therefore the RAPD methodology used in this study was sufficiently robust to be used as a standard technique among laboratories employing different thermocyclers.

Outcrossing species pose particular problems in cultivar discrimination as they are heterogenous populations, rather than a single genotype. There may be selection for a particular subset of the population under different environmental conditions. This may be the reason for the inconsistency in intensity of bands in two of the 16 samples compared in Fig. 5.3. The use of other primers makes it possible to discriminate among cultivars over environments despite these inconsistencies. Only twenty plants of each cultivar were bulked together in an attempt to obtain a characteristic fingerprint of the cultivar. No single plant analysis has been done to determine the degree of heterogeneity within the populations.

Sampling DNA from a larger number of plants may overcome the variation observed, particularly in the case of synthetic cultivars. Future studies should include single plant analysis of each cultivar to determine the number of plants required to represent the cultivar for backcrossed cultivars, synthetics and hybrids.

The RAPD technique requires time and effort, considering the period required to grow the seedlings, extract the DNA and the subsequent analysis. With prudent selection of primers, it appears to offer a reliable method for cultivar identification and to have advantages over many of the chemotaxonomic methods used for plant identification such as glucosinolate or fatty acid concentration which are susceptible to environmental variation.

Table 5.1. Cultivars and seed suppliers used for method development (all cultivars are *B. napus* var. *oleifera,* summer types, "annua", unless otherwise specified). The sample numbers are used elsewhere in the paper to identify cultivars: I. *Agriculture Canada*; II. *ICI Seeds, Canada*; III. *Department of Plant Science University of Manitoba*; IV. *Pioneer Hibred International*; V. *Svalöf Seed Ltd. , Ontario*; VI. *New South Wales Department of Agriculture, Australia.*

[Y] = *B. rapa* var *oleifera* "annua", [Z] = *B. napus* var *oleifera* "biennis"

Cultivar	**Source**	**Cultivar**	**Source**
1. Stellar	III	13. Horizon [Y]	V
2. Westar	I	14. Crystal [Z]	V
3. Hero	III	15. Legend	V
4. Regent	III	16. Vanguard	V
5. Delta	IV	17. Global	V
6. Excel	I	18. Touchdown [Z]	IV
7. Midas	I	19. Winfield [Z]	IV
8. Tristar	I	20. Bounty	IV
9. Argentine	I	21. Hyola-40 (hybrid)	II
10. Bronowski	Polish	22. Tower	III
11. Profit	I	23. Oscar	VI
12. Stallion	V	24. Yickadee	VI

Table 5.2. Primers obtained from University of British Columbia (UBC) and used for RAPD analysis and discrimination of *B. napus.*

UBC Primer Number	Nucleotide Sequence
302	CGG CCC ACG T
303	GCG GGA GAC C
329	GCG AAC CTC C
341	CTG GGG CCG T
346	TAG GCG AAC G
354	CTA GAG GCC G

Fig. 5.1 RAPD profile of DNA from 23 cultivars (numbered as in Table 5.1) of oilseed rape using primers #329(a) and #341(b) (Table 5.2). Only 23 of the 24 cultivars are shown due to the limitations of the gel box. M = DNA marker

5.12

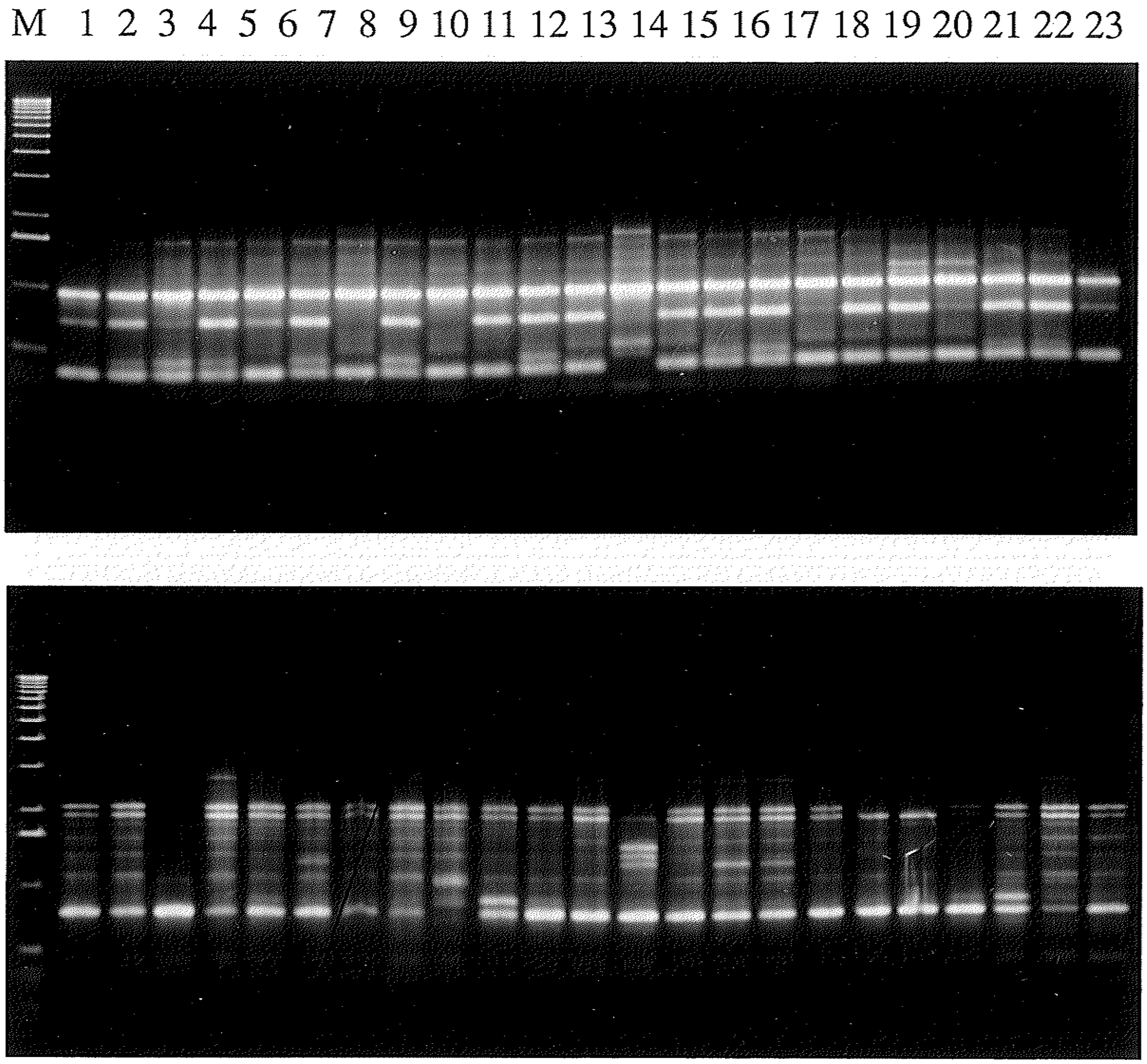


Fig. 5.2 RAPD profiles of four samples of each of 5 cultivars of *B. napus* harvested from separate trials at diverse sites in Manitoba and Saskatchewan

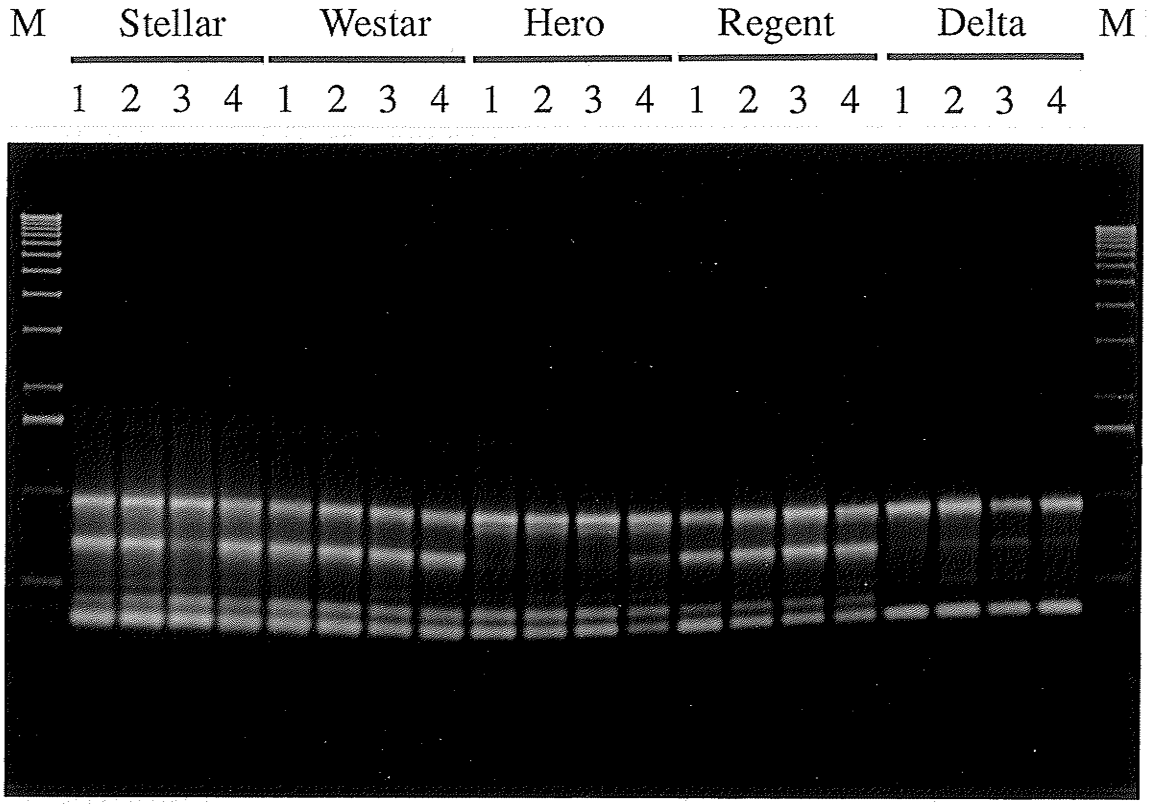

M
Stellar
Westar
Hero
Regent
Delta
M
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

Fig. 5.3 Average linkage cluster analysis of 24 cultivars of *B. napus* using RAPD data from a 24 x 24 matrix of Nei and Li (1979) similarity indices and NT-SYS software (Rohlf et al 1971)

5.26

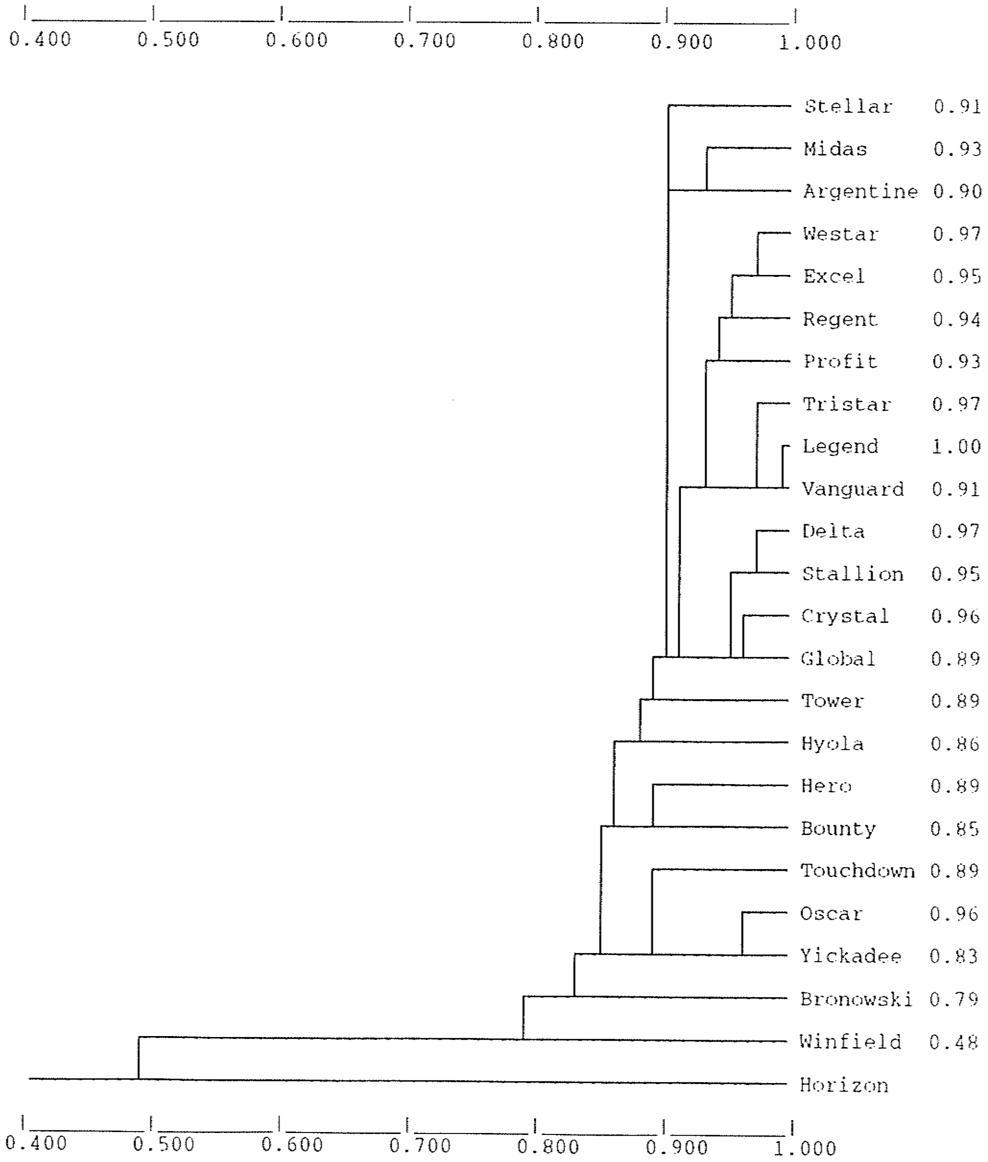
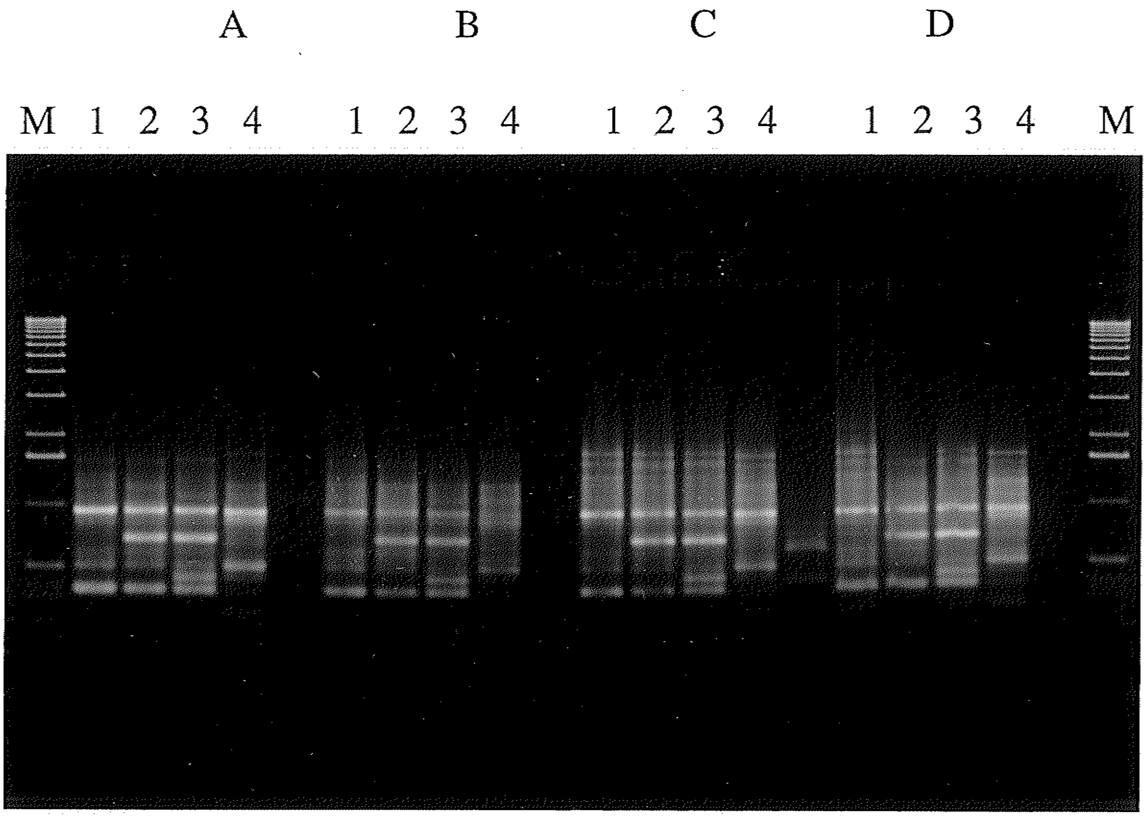

0.400
0.500
0.600
0.700
0.800
0.900
1.000
Stellar 0.91
Midas 0.93
Argentine 0.90
Westar 0.97
Excel 0.95
Regent 0.94
Profit 0.93
Tristar 0.97
Legend 1.00
Vanguard 0.91
Delta 0.97
Stallion 0.95
Crystal 0.96
Global 0.89
Tower 0.89
Hyola 0.86
Hero 0.89
Bounty 0.85
Touchdown 0.89
Oscar 0.96
Yickadee 0.83
Bronowski 0.79
Winfield 0.48
Horizon
0.400
0.500
0.600
0.700
0.800
0.900
1.000

Fig. 5.4 RAPD profiles generated by 4 thermocyclers: A. Techne MW-2; B. Techne PHC-2; C. Thermolyne Temp.Tronic; and D. Ericomp Ez-Cycler, with primer # 329 and 4 cultivars; 1. Midas (*B. napus)*, 2. Bronowski (*B. napus)*, 3. Profit (*B. napus)*, and 4. Horizon (*B. rapa*)

5.28



6. GENERAL DISCUSSION

The aim of this study was to find fingerprints for individual cultivars. For HPLC analysis, the phenotype is displayed as a set of selected ethanol extractable seed components. RAPD analysis provided a set of DNA fragments also useful as phenetic markers and characteristic of cultivars.

The majority of studies of cultivar discrimination have been carried out on self pollinating species, particularly cereals crops, in which plants within the population are genetically similar. Few studies have attempted to discriminate among cultivars (populations) of outcrossing species. This research has investigated two possible methods of discrimination, HPLC and RAPD, for discriminating cultivars of *B. napus*, an outcrossing species.

HPLC generated chromatograms which were visually unique among the cultivars. Discriminant analysis provided evidence that the data obtained from integration of those chromatograms was sufficiently different among cultivars to allow them to be discriminated into distinct groups. Furthermore, plots of canonical discriminate analysis gave additional evidence of the success of HPLC for discrimination by displaying the cultivars as distinct and tightly grouped sets (Fig. 4.5). In addition to discriminating between cultivars, discriminant analysis was capable of identifying unlabelled samples based on this data set. Cluster analysis was not successful in clustering cultivars into discrete groups which would indicate that it is unsuitable for this application.

Both the RAPD and HPLC methods were found to be useful for discriminating among the cultivars tested. The number of markers available by the two methods allowed selection of components which were reliable and provided significant discriminatory power. The characteristic profiles of these cultivars revealed by HPLC and RAPD analysis were sufficiently distinct to allow discrimination among the cultivars tested and subsequent identification of unknown samples.

HPLC is a rapid method and seed can be extracted and analyzed within hours. The HPLC, although an expensive instrument to purchase, is common in most well equipped laboratories. Most HPLC instruments are automated and require minimum labour, input thereby making HPLC an attractive method where large numbers of samples are to be handled. Actual operating costs for the HPLC are relatively low. The major disadvantage of HPLC is the difficulty in handling the type of data generated by the method. Over 100 peaks from each chromatogram were initially integrated a those peaks which provided maximum discriminatory distance among cultivars were selected. The application of multivariate analysis was then necessary to classify the samples. Despite these problems, HPLC provides a large number of components from which to select markers between individual cultivars. Selection and analysis of data would be minimised in a plant breeding program in which a limited number of cultivars are involved. A set of component peaks would be identified from the total chromatogram which are characteristic of a set of cultivars.

The HPLC method and discriminant analysis has the potential to be computerised and automated. The reproducibility of retention times achieved for extractions over two weeks would indicate that application of computerized data handling is possible. Additionally, clear resolution of the major peaks minimises the chance of erroneous peak identification. Automated and computerized HPLC techniques such as used by Scanlon et al. (1989) could be utilised in such a method. The reproducibility shown in overlaid chromatograms of cv Stellar provides further evidence of the feasibility of automation.

The main advantage of RAPD is that it provides discrete data in the form of the presence or absence of a RAPD marker. Although there were instances in this study where a marker was inconsistent in the level of intensity, the large number of markers made it possible to discriminate between samples. The RAPD method generates a relatively small amount of data in comparison to HPLC and a single primer may be sufficient to distinguish one cultivar from another. Although statistical analysis is useful in looking at cultivar relationships, simple observation of the samples run on a gel will generally provide evidence of identity. The RAPD technique is inexpensive to set up but operating costs are high due in large part to the cost of thermally stable polymerase enzyme. A limitation is the time required to grow seedlings for extraction of DNA. Further development of the technique may allow extraction of DNA from seeds which will reduce the time of analysis.

Both of the clusters produced by the HPLC and RAPD data identified the *B. rapa* cultivars as distinct outliers. The grouping of the *B. napus* cultivars was different in the two analyses. For example cv Excel clustered closely to Westar by HPLC but was more distant when analyzed by RAPD. This is not surprising as the markers used in both HPLC and RAPD represent only a small part of the cultivars' phenotype and genotype. There was no reason therefore to expect that the markers from each technique would produce similar clusters. Both the HPLC and RAPD methods have been shown to be capable of discriminating among a wide range of *B. napus* cultivars.

7. SUMMARY AND CONCLUSIONS

The establishment of Plant Breeders Rights requires suitable standardised techniques for a unique description of each new cultivar released. These methods need to meet certain criteria to be useful for breeders. The method must be able to distinguish individual cultivars. The method should not be influenced by differences caused by the environment and should be applicable to seed from a range of sources. The technique must be reproducible in other laboratories; and should be simple, rapid and economical. This study has described two techniques which generally meet these criteria.

Outcrossing species pose particular problems in cultivar discrimination as they are heterogenous populations. The results of the HPLC analysis presented here show that there is sufficient uniformity within the cultivar, and sufficient differences among cultivars, that even closely related cultivars can be discriminated. Although the majority of cultivars investigated were *B. napus*, the success of HPLC in differentiating between *B. rapa* cultivars (*cv* Colt and Horizon) would indicate that the methods may also be suitable for other *Brassica* crops.

Only a small number (100) of the commercially available primers (>700) were investigated for cultivar discrimination by RAPD analysis. This was sufficient to discriminate between all but two of the cultivars studied. This would indicate that by increasing the number of primers used, the method has potential to discriminate between any two different cultivars of *B. napus*. The method is sufficiently robust that it can be repeated on several different instruments and therefore would produce similar results in different laboratories.

It is likely that the major requirements for cultivar discrimination such as described in this study would be used by breeders to discriminate among a restricted selection of cultivars. In particular, it could be applied to the registration of a new cultivar to differentiate it from cultivars of similar pedigree. In this study, a wide spectrum of cultivars were utilised including European, Canadian and Australian cultivars as well as species of *B. napus* and *B. rapa*. It is likely therefore that increased success could be achieved if these methods were more closely adapted to the variability which occurs within breeding programs, or even between the limited number of cultivars grown within a country at a given time.

Both HPLC and RAPD methods rely on the analysis of a bulked sample of seed to provide a 'fingerprint' which reflects the average population of the cultivar. Unless components are identified which are unique to particular cultivars, it is not possible to characterize a blended sample. Variation in seed samples from different sources may occur due to environmental influence, seed contamination and outcrossing. However, the data obtained from both HPLC and RAPD analysis of samples of five cultivars from different sites indicates that the cultivars could be discriminated.

This study has shown that cultivars of *B. napus* can be readily discriminated using either of two methods. The RAPD method has some advantages over HPLC in its simplicity and stability across environments. The HPLC method has the advantage of a large number of components from which to select discriminating variables. These methods have potential as techniques for the characterization of cultivars of *B. napus* and for cultivar registration for Plant Breeders Rights.

8. RECOMMENDATIONS FOR FURTHER STUDY

Two methods, HPLC and RAPDs, have been optimised and evaluated for the discrimination of cultivars of *B. napus* using a wide range of cultivars from Canada, Sweden and Australia and including *B. napus* var *oleifera* summer "annua" and winter "biennis" types and *B. rapa.* To incorporate these methods into a breeding program, the range of cultivars specific to that breeding program should be analyzed and a data set established. It is likely that distinct markers such as individual HPLC peaks or RAPD bands may be identified which are unique to particular cultivars, thereby simplifying the analysis. For the purpose of plant variety registration, these methods are ideal to identify markers for new cultivars to distinguish them from existing cultivars.

RAPD analysis, using discrete data (presence or absence of RAPD bands), is more precise than HPLC analysis. The method is also more stable as there appears to be no environmental influence on the genetic material. To take maximum advantage of this technique, as many markers as possible should be identified using all of the available primers. This should result in an improved ability to discriminate genotypes. Such data could be used in gene banks to determine the variation within the germplasm gene pool.

The major drawback is the time required to analyze a sample because of the need to grow seedlings for DNA extraction. Future study should consider the possibility of extracting DNA from seeds to allow more rapid analysis procedures. Although seeds contain large amounts of carbohydrates and protein which may reduce the DNA quality, it may be possible to extract DNA of sufficient quality and quantity to carry out RAPD analysis.

The results of these studies indicate application of these techniques go beyond cultivar discrimination. HPLC fingerprinting is based on the quantitative variation of several phenolic compounds. Some of these are recognised as antinutritional compounds such as sinapine, responsible for low feed palatability of *B. napus* meal. The HPLC method could be used to screen breeding material for the reduction of these compounds. It is likely that additional markers for RAPD analysis, generated from the several hundred primers available commercially, may provide markers linked to quality traits, suitable for screening and selection of breeding material using techniques described in chapter 2.

For RAPD, no single plant analysis has been done to determine the degree of heterogeneity within the population. Only twenty plants of each cultivar were bulked together to obtain a characteristic fingerprint of the cultivar. The variation observed for seed from different sources may be due to the sample size being too small, particularly in the case of synthetic cultivars. Future studies should include single plant analysis of each cultivar to determine the number of plants required to represent the cultivar for backcrossed cultivars, synthetics and hybrids.

Current methods of plant cultivar description for the purpose of plant breeders rights are many and varied. Two techniques, HPLC and RAPD analysis, have been shown to provide accurate and reliable fingerprints of *B. napus* cultivars. Such techniques could be used to standardise cultivar registration and permit comparisons of cultivars from breeding programs throughout the world. HPLC and RAPD methodologies could be used to create an international data base for comparison of new cultivars.

9. REFERENCES

Adams, H., J.G. Vaughan and G.R. Fenwick, 1985. The limited chemotaxonomic value of glucosinolates for cultivar identification in *Brassica napus* L. var. oleifera (Metzg.). Zeitschrift fur Pflanzenzüchtg. 95:97-102.

Adams, H., J.G. Vaughan and G.R. Fenwick, 1989. The use of glucosinolates for cultivar identification in Swede, *Brassica napus* L var *napobrassica* (L) Peterm. Journal of the Science of Food and Agriculture. 46:319-324.

Allen, B.W., P.W. Goodenough, J.S.Lee and P.P.Rutherford, 1986. Evolution of cauliflower types grown in Great Britian as indicated by the isozyme composition of the cauliflower curds. Euphytica. 35:25-31.

Anderson, W.R. and D.J. Fairbanks, 1990. Molecular markers: important tools for plant genetic resource characterization. Diversity. 6(3&4):51-53.

Appelqvist, L.A., 1972. *In* L. A. Appelqvist and R. Ohlson (eds.), *Rapeseed: cultivation, composition, processing and utilisation,* pp. 123-173. Elsevier, Amsterdam.

Appelqvist, L.D., A.K. Kornfeldt and J.E. Wennerholm, 1981. Sterols and sterol esters in some *brassica* and *sinapis* seeds. Phytochemistry. 20:207-210.

Arus, P., C.R. Shields and T.J. Orton, 1985. Application of isozyme electrophoresis for purity testing and cultivar identification of F_1 hybrids of *Brassica oleracea*. Euphytica. 34:651-657.

Baertlein, D.A. B.A. Baertlein and R.G. McDaniel, 1990. PHYLO: A Cluster analysis program. The Journal of Hereditary 81(1): 87.

Barker, D.A., T.A.Vuori, M.R.Hegedus and D.G.Myers, 1992. The use of ray parameters for the discrimination of Australian wheat varieties. Plant Varieties and Seeds. 5:35-45.

Bauer, H. and D. Treutter, 1990. Identification of *Pelargonium* genotypes by phenolic fingerprints II. Cultivar identification by HPLC analysis of leaf phenols combined with discriminate analysis. Gartenbauwissenschaft. 55(4)S:187-191.

Bell, J.M. 1989. Nutritional characteristics and protein uses of oilseed meals, pp. 192-207. *In* G. Röbbelen, R.K. Downey and A. Ashri (eds.), *Oil Crops of The World.* McGraw-Hill Pub. Co. N.Y.

Bietz, J.A. and L.A. Cobb, 1985. Improved procedures for rapid wheat varietal identification by reversed-phase high-performance liquid chromatography of gliadin. Cereal Chemistry. 62(5):332-339.

Biles, C.L., R.D. Martyn and H.D. Wilson, 1989. Isozymes and general proteins from various watermelon cultivars and tissue types. Hortscience. 24(5):810-812.

Bouchereau, A., J. Hamelin, I. Lamour, M. Renard and F. Larher, 1991. Distribution of sinapine and related compounds in seeds of *Brassica* and allied genera. Phytochemistry. 30(6):1873-1881.

Buehler, R.E., T.T. Van Toai and M.B. McDonald Jr., 1989. The optimisation of elution and extraction conditions for the separation of soybean seed proteins using RP-HPLC. Seed Science and Technology. 17:193-204.

Canada's Canola, 1988. Canola Council of Canada. Winnipeg, Manitoba, Canada.

Canvin, D.T., 1965. The effect of temperature on the oil content and fatty acid composition of the oils from several oilseed crops. Canadian Journal of Botany. 43:63-69.

Carlson, J.F., L.K.Tulsieram, J.C. Glaubitz, V.W. Luk, C. Kauffeldt and R. Rutledge, 1991. Segregation of random amplified DNA markers in F1 progeny of conifers. Theoretical and Applied Genetics. 83:194-200.

Chen, B.Y., W.K. Heneen and V. Simonsen, 1989. Comparative and Genetic studies of isozymes in resynthesized and cultivated *Brassica napus* L., *B. campestris* L. and *B.alboglabra* Bailey. Theoretical and Applied Genetics. 77:673-679.

Chevre, A.M., R. Delourme, F. Eber and P. Arus, 1991. First results of rapeseed variety identification by isozyme electrophoresis. Cruciferae Newsletter Nos. 14/15:70-71.

Cole, R.A. and K. Phelps, 1979. Use of canonical variate analysis in the differentiation of swede cultivars by gas-liquid chromatography of volatile hydrolysis products. Journal of the Science of Food and Agriculture. 30:669-676.

Coulthart, M. and K.E. Denford, 1982. Isozyme studies in *Brassica*. I. Electrophoretic techniques for leaf enzymes and comparison of *B. napus*, *B. campestris* and *B. oleracea* using phosphoglucomutase. Canadian Journal of Plant Science. 62:621-630.

Daun, J.K., K.M. Clear and J.T. Mills, 1985. Effect of frost damage on the quality of Canola (*B. napus*). Journal of the American Oil Chemists Society. 62(4):715-719.

Debenham, P.G., 1992. Probing identity: the changing face of DNA fingerprinting. Trends in Biotechnology. 10:96-102.

Downey, R.K. and G. Röbbelen, 1989. *Brassica* Species, pp. 339-362. *In* G. Röbbelen, R.K. Downey and A. Ashri (eds.), *Oil Crops of The World.* McGraw-Hill Pub. Co. N.Y.

Edwards, K., C. Johnstone and C. Thompson, (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic acids Research. 19(6):1349.

Ferreres, F., F.A. Tomàs-Barberàn, M.I. Gil and F. Tomàs-Lorente, 1991. An HPLC technique for flavonoid analysis in honey. Journal of the Science of Food and Agriculture. 56:49-56.

Figdore, S.S., W.C. Kennard, K.M. Song, M.K. Slocum and T.C. Osborn, 1988. Assessment of the degree of restriction fragment length polymorphisms in *Brassica*. Theoretical and Applied Genetics. 75:833-840.

Fursa, N.S., V.I. Litvinenko and V.E. Avetisyan, 1986. Natural compounds of the family *Brassicaceae* as probable chemotaxonomic characteristics. Report 3. Flavonoids. Rastit. Resur. 22(1):113-130.

Gardiner, S.E., and M.B. Forde, 1988. Identification of cultivars and species of pasture legumes by sodium dodecylsulphate polyacrylamide gel electrophoresis of seed proteins. Plant Varieties and Seeds. 1:13-26.

Gawel, N.J., R.L. Jarret, and A.P. Whittermore, 1992. Restriction fragment length polymorphism (RFLP)-based phylogenic analysis of *Musa*. Theoretical and Applied Genetics. 84:286-290.

Gilliland, T.J., 1989. Electrophoresis of sexually and vegetatively propagated cultivars of allogamous species. Plant Varieties and Seeds. 2:15-25.

Gupta, S.K. and G. Röbbelen, 1986. Identification of rapeseed (*Brassica napus*) cultivars by electrophoresis. Zeitschrift fur Pflanzenzüchtg. 96:363-370.

Guthrie, P.A.I., C.W. Magill, R.A. Frederiksen and G.N. Odvody, 1992 Random amplified polymorphic DNA markers: A system for identifying and differentiating isolates of *Colletotrichum graminicola*. Phytopathology. 82(8):832-835.

Halligan, E.A., M.B. Forde and I.J. Warrington, 1991. Discrimination of ryegrasses by heading date under various combinations of vernalization and daylength: Westerwolds, Italian and hybrid ryegrass varieties. Plant varieties and Seeds. 4:115-123.

Hansen, A.E., A. Nassuth and I. Altosaar, 1988. Rapid electrophoresis of oat (*Avena sativa* L.) prolamins from single seeds for cultivar identification. Cereal Chemistry. 65:153-154.

Harbourne J.B., 1964. In 'Methods in polyphenol chemistry' (ed. J.B. Pridham). Pergammon Press, Oxford.

Havey, M.J. and F.J. Muehlbauer, 1989. Linkages between restriction fragment length, isozyme, and morphological markers in lentil. Theoretical and Applied Genetics. 77:395-401.

Heaney, R.K. and G.R. Fenwick, 1980. The glucosinolate content of *Brassica* vegetables. A chemotaxonomic approach to cultivar identification. Journal of Science of Food and Agriculture. 31: 794-801.

Helentjaris, T., M. Slocum, S.Wright, A.Schaefer and J.Nienhuis, 1986. Construction of gentic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theoretical and Applied Genetics. 72:761-769.

Heun, M., A.E. Kennedy, J.A. Anderson, N.L. Lapitan, M.E. Sorrels and S.D. Tanksley, 1991. Construction of a restriction fragment length polymorphism map for barley (*Hordeum vulgare*). Genome. 34:437-447.

Hillel, J., T. Schaap, A. Haberfeld, A.J. Jeffreys, Y. Plotzky, A. Cahaner and U. Lavi, 1990. DNA fingerprinting applied to gene introgression in breeding programs. Genetics. 124:783-789.

Hillis. W.E., 1967. Polyphenols in the leaves of *eucalyptus*: a chemotaxonomic survey-V. Phytochemistry. 6:845-856.

Horn P.J., and J.G. Vaughan, 1983. Seed glucosinolates of fourteen wild *Brassica* species. Phytochemistry. 22(2):465-470.

Hoshi, T. and T. Hosoda, 1978. Interrelation between the genome and flavonoid composition in the genus *Brassica*. Japanese Journal of Breeding. 28(2):137-142.

Hu, J. and C.E. Quiros, 1991. Identification of broccoli and cauliflower cultivars with RAPD markers. Plant Cell Reports. 10:505-511.

Huebner, F.R., J.A. Bietz, B.D. Webb and B.O. Juliano, 1990. Rice cultivar identification by high-performance liquid chromatography of endosperm proteins. Cereal Chemistry. 67(2):129-135.

Hussain, A., H. Ramirez, W. Bushuk and W. Roca, 1986. Field bean (Phaseolus vulgaris L.) cultivar identification by electrophoregrams of cotyledon storage proteins. Euphytica. 35:729-732.

Keefe, P.D., 1992. A dedicated wheat grain image analyser. Plant Varieties and Seeds. 5:27-33.

Kennedy, L.S. and P.G. Thompson, 1991. Identification of sweetpotato cultivars using isozyme analysis. Hortscience. 26(3):300-302.

Kidwell, K.K. and T.C. Osborn, 1992. Simple plant DNA isolation procedures. *In* Beckman JS, Osborn TC (eds) Plant Genomes: Methods and Physical Mapping. Kluwer Academic Publishers, Netherlands.

Klecka, W.R., 1976. Quantitative Applications in the Social Sciences. pp. 8-11. *In Analysis of Variance*, Sage University Papers, Series: 07-019, Beverly Hills and London, Sage Publications.

Kruger, J.E., and B.A. Marchylo, 1985. Selection of column and operating conditions for reversed-phase high-performance liquid chromatography of proteins in Canadian Wheat. Canadian Jouranl of Plant Science. 65:285-298.

Kubelik, A., 1991. A RAPD map of *Neurospora crassa* from Rafalski J.A., S.V. Tingey and J.G.K. Williams, 1991. Biotech News and Information. 3(4):645-648.

Ladizinsky, G. and T. Hymowitz, 1979. Seed protein electrophoresis in taxonomic and evolutionary studies. Theoretical and Applied Genetics. 54:145-151.

Landry, B.S., R.V. Kesseli, B. Farrara and R.W. Michelmore, 1987. A genetic map of lettuce (*Lactuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance and morphological markers. Genetics. 116:331-337.

Landry, B.S., N. Hubert, T. Etoh, J.J. Harada and S.E. Lincoln., 1991. A genetic map for *Brassica napus* based on restriction fragment length polymorhisms detected with expressed DNA sequences. Genome 34:543-552.

Livneh, O., Y.Nagler, Y.Tal, S.B.Harush, Y.Gafni, J.S.Beckman and I.Sela, 1990. RFLP analysis of a hybrid cultivar of pepper (*Capsicum annuum*) and its use in distinguishing between parental lines and in hybrd identification. Seed Science and Technology. 18:209-214.

Lookhart, G.L., 1985. Identification of oat cultivars by combining polyacrylamide gel electrophoresis and reversed-phase high-performance liquid chromatography. Cereal Chemistry. 62:345-350.

Mailer, R.J., 1988. Environmental effects on rapeseed quality with emphasis on glucosinolate concentration. MSc. Thesis. Australian National University, Canberra, Australia.

Mailer, R.J., J. Daun and R. Scarth, 1993. Cultivar identification in *Brassica napus* L. using RP-HPLC of ethanol extracts. Journal of The American Oil Chemists' Society (in press).

Mailer, R.J. and N. Wratten, 1985. Comparison and estimation of glucosinolate levels in Australian rapeseed cultivars. Australian Journal of Experimental Agriculture. 25:932-938.

Marchylo, B.A., 1987. Barley cultivar identification by SDS gradient page analysis of hordein. Canadian Journal of Plant Science. 67:927-944.

Marchylo, B.A., K.A. Handel and V.J. Mellish, 1989. Fast horizontal sodium dodecyl sulphate gradient polyacrylamide gel electrophoresis for rapid wheat cultivar identification and analysis of high molecular weight glutenin subunits. Cereal Chemistry. 66(3):186-192.

Marchylo, B.A., D.W. Hatcher and J.E. Kruger, 1988. Identification of wheat cultivars by reversed-phase high-performance liquid chromatography of storage proteins. Cereal Chemistry. 65(1):28-40.

Marchylo, B.A. and J.E. Kruger, 1985. Assessment of RP-HPLC columns to separate hordein proteins and identify cultivars of barley and barley malt. Journal of the American Society of Brewing Chemists. 43:29-35.

Marchylo, B.A. and J.E. Kruger, 1988. The effect of injection volume on the quantitative analysis of gliadins and glutenins by RP-HPLC. Cereal Chemistry. 65:192-198.

Marchylo, B.A., J.E. Kruger and D.W. Hatcher, 1990. Effect of environment on wheat storage proteins as determined by quantitative reversed-phase high-performance liquid chromatography. Cereal Chemistry. 67(4):372-376.

Martin, G.B., J.G.K. Williams and S.D. Tanksley, 1991. RAPD identification of markers linked to *Pseudomonas* resistance gene in tomato by using random primers and near isogenic lines. Proceedings of the National Academy of Sciences, USA. 88:2336-2340.

Martiniello, P., 1992. Morphological aspects involved in varietal registration and the production of certified seed of typical Mediterranean leguminous forage crops. Plant Varieties and Seeds. 5:71-81.

McDonald, M.B. and D.M. Drake, 1990. An evaluation of a rapid and automated electrophoresis system for varietal identification of seeds. Seed Science and Technology. 18:89-96.

Messina, R., R. Testolin and M. Morgante, 1991. Isozymes for cultivar identification in kiwifruit. Hortscience. 26(7):899-902.

Michelmore, R.W., I. Paran and R.V. Kesseli, 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proceedings of the National Academy of Science, USA. 88:9828-9832.

Morgan, A.G., 1989. Chromatographic applications in cultivar identification. Plant Varieties and Seeds. 2:35-44.

Mullis, K., 1990. The unusual origin of the polymerase chain reaction. Scientific American, vol 262. April. pp 56-65.

Nehra, N.S., K.K. Kartha and C. Stushnoff, 1991. Isozymes as markers for identification of tissue culture and greenhouse-grown strawberry cultivars. Canadian Journal of Plant Science. 71:1195-1201.

Nei, M., 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Nei, M. and W.H. Li, 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Science. USA. 76:5269-5273.

Nicholson, C.H.L., L.L. Conlan and S.J. Cook, 1985. *Cenchrus* pasture species and cultivar identification using morhological and biochemical techniques. Seed Science and Technology. 13:243-255.

Oard, J.H. and S. Dronavalli, 1992. Rapid isolation of rice and maize DNA for analysis by random-primer PCR. Plant Molecular Biology Reporter. 10(3):236-241.

Osborne, T.B., 1924. The vegetable proteins. Longman, Green Co. Ltd.

Osborn, T.C., D.C. Alexander and J.F. Fobes, 1987. Identification of restriction fragment length polymorhisms linked to genes controlling soluble solids content in tomato fruit. Theoretical and Applied Genetics. 73:350-356.

Paran, I., R. Kesseli and R. Michelmore, 1991. Identification of restriction fragment length polymorphism and random amplified polymorhic DNA markers linked to downey mildew resistance genes in lettuce, using near isogenic lines. Genome. 34:1021-1027.

Paterson, A.H., E.S. Lander, J.D. Hewitt, S. Peterson, S.E. Lincoln and S. Tanksley, 1988. Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. Nature. 335:721-726.

Plant Varieties Journal, (1991). Agriculture Canada. Plant Breeders Rights Office, Ottawa, Ontario.

Quiros, C.F., J. Hu, P. This, A.M. Chevre and M. Delseny, 1991. Development and chromosomal localization of genome-specific markers by polymerase chain reaction in *Brassica*. Theoretical and Applied Genetics. 82:627-632.

Rafalski, J.A., S.V. Tingey and J.G.K. Williams, 1991. RAPD markers - a new technology for genetic mapping and plant breeding. Biotech News and Information. 3(4):645-648.

Rakow, G. and D.L. Woods, 1987. Outcrossing in rape and mustard under Saskatchewan praire conditions. Canadian Journal of Plant Science 67: 147-151.

Rao, T.N., Y.S. Nerkar and V.D. Patil, 1990. Identification of cultivars of cotton by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) of soluble seed proteins. Plant Varieties and Seeds. 3:7-13.

Reeves, J.C. and S.F. Ball, 1991. Preliminary results on the identification of *Pyrenophora* species using DNA polymorphisms amplified from arbitrary primers. Plant Varieties and Seeds. 4:185-189.

Reiter, R.S., J.G.K. Williams, K.A. Feldman, J.A. Rafalski, S.V. Tingey and P.A. Scolnik, 1992. Global and local genome mapping in *Arabidopsis thalia* by using recombinant inbred lines and random amplified polymorphic DNAs. Proceedings of National Academy of Science. USA. 89:1477-1481.

RFLP Training Course Laboratory Manual, Training course held at Cornell University and sponsored by the Rockefeller Foundation July, 1989.

Rogers, S.O. and A.J. Bendich, 1988. Extraction of DNA from plant tissue. Plant Molecular Biology Manual A6:1-10.

Rohlf, F.J., J. Kishpaugh, and D. Kirk, 1971. NT-SYS. Numerical Taxonomy System of Multivariate statistical Programs. Tech. Rep. State University of New York at Stony Brook, New York.

Saharan, B.S., C. Samimy and A.S. Malik, 1991. Research note: Identification of varieties of radish (*Raphanus sativus* L.) by isozyme phenotypes. Plant Varieties and Seeds. 4:87-88.

Scanlon, M.G., H.D. Sapirstein and W.Bushuk, 1989. Computerized wheat varietal identification by high-performance liquid chromatography. Cereal Chemistry. 66(5):439-443.

Scarth, R., P.B.E. McVetty, S.R. Rimmer and B.R. Stefansson, 1988. Stellar low linolenic-high linoleic acid summer rape. Canadian Journal of Plant Science. 68:509-511.

Schüller, C., G. Backes, G. Fishbeck and A. Jahoor, 1992. RFLP markers to identify the alleles on the *Mla* locus conferring powdery mildew resistance in barley. Theoretical and Applied Genetics. 84:330-338.

Smith J.S.C., 1988. Identification of pedigrees of hybrid maize (Zea mays L.) cultivars by isozyme electrophoresis and reversed-phase high performance liquid chromatography. Euphytica. 39:199-205.

Smith, J.S.C. and O.S. Smith, 1986. Environmental effects on zein chromatograms of maize inbred lines revealed by reversed-phase high-performance liquid chromatography. Theoretical and Applied Genetics. 71:607-612.

Smith, J.S.C. and O.S. Smith, 1988. Association among inbred lines of maize using electrophoretic, chromatographic, and pediree data. 2. Multivariate and cluster analysis of data from Iowa Stiff Stalk Synthetic derived lines. Theoretical and Applied Genetics. 76:39-44.

Sneath, P.H. and R.R. Sokal, 1973. Numerical Taxonomy. (*ed.* D. Kennedy and R.B. Park). W.H. Freeman and Co., San Francisco.

Sokal, R.R. and C. D. Michener, 1958. "A statistical method for evaluating systematic relationships," University of Kansas Science Bulletin, 38, 1409-1438.

Song, K.M., T.C. Osborn and P.H. Williams, 1988. *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs) 2. Theoretical and Applied Genetics. 76:593-600.

Song, K.M., J.Y. Suzuki, M.K. Slocum, P.H. Williams and T.C. Osborne, 1991. A linkage map of *Brassica rapa* (syn *campestris*) based on restriction fragment length polymorphism loci. Theoretical and Applied Genetics. 82:296-304.

Sosulski, F., R. Zadernowski and H. Kozlowska, 1980. Analytical Chemistry of rapeseed and it's products - a symposium May 5 & 6, Winnipeg, Manitoba.

Tai, T.H. and S.D. Tanksley, 1990. A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. Plant Molecular Biology Report. 8:297-303.

U, N., 1935. Genomic analysis of *Brassica* with special reference to the experimental formation of *B. napus* and its peculiar mode of fertilization. Japanese Journal of Botany. 7:389-452.

Van Der Burg, W.J., and R.A. Van Zwol, 1991. Rapid identification techniques used in laboratories of the International Seed Testing Association: a survey. Seed Science & Technology. 19:687-700.

Waugh, R. and W. Powell, 1992. Using RAPD markers for crop improvement. Trends in Biotechnology. 10:186-191.

Welsh, J., R.J. Honeycutt, M. McClelland and B.W.S. Sobral, 1991. Parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (AP-PCR). Theoretical and Applied Genetics. 82:473-476.

Welsh, J. and M. McClelland, 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic acids Research. 18:7213-7218.

White, J. and J.R. Law, 1991. Differentiation between varieties of oilseed rape (*Brassica napus* L.) on the basis of the fatty acid composition of the oil. Plant Varieties and Seeds. 4:125-132.

Williams, J.G., M.K. Hanafey, J.A. Rafalski and S.V. Tingey, 1993. Genetic analysis using random amplified polymorphic DNA markers. Methods in Enzymology. 218:704-740.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V.Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research. 18:6531-6535.

Williams, J.G., A.R. Kubelik, J.A. Rafalski and S.V. Tingey, 1991. Genetic analysis with RAPD markers. In: *More Gene Manipulations in Fungi.* Orlando, Fl, USA; Academic Press.

Williams, J.G., J.A. Rafalski and S.V. Tingey, 1991. Genetic analysis using RAPD markers. In: *Methods in Enzymology.* Orlando, Fl, USA; Academic Press.

Wrigley C.W. J.C. Autran and W. Bushuk, 1982. Identification of cereal varieties by gel electrophoresis of the grain proteins. Page 211 in: Advances in Cereal Science and Technology. Vol. 5. Y. Pomeranz, ed. Am. Association of Cereal Chemists. St Paul, MN.

Yadava, J.S., J.B. Chowdhury, S.N.Kakar and H.S. Nainawatee, 1979. Comparative electrophoretic studies of proteins and enzymes of some *Brassica* species. Theoretical and Applied Genetics. 54:89-91.

10. APPENDIX

Appendix I a. Repeatibility of HPLC Analysis - Single Extract of *cv* Westar analysed 10 times - Data acquired at 210 nm.

Appendix I b. Repeatibility of HPLC Analysis - Repeated Extraction of *cv* Westar Analysed 10 times Over Two Weeks - Data acquired at 210 nm.

Appendix II. Linearity of absorbance - Peak Area and retention times for HPLC analysis of cv Westar injected at six different volumes.

Appendix III a. HPLC peak area (% of total peaks 210 nm) of five Cultivars and five unidentified samples from a range of sites.

Appendix III b. HPLC peak area (% of total peaks 240 nm) of five Cultivars and five unidentified samples from a range of sites.

Appendix III c. Areas (% of total peaks 210 nm) and retention times (min.) of 10 peaks selected from Appendix III a. by SAS discriminant analysis for five cultivars of *B. napus* and one of *B. rapa* grown at different sites.

Appendix III d. Areas (% of total peaks 240 nm) and retention times (min.) of 10 peaks selected from Appendix III (b) by SAS discriminant analysis for five cultivars of *B. napus* and one of *B. rapa* grown at different sites.

Appendix IV a. Canonical Discriminate Analysis (SAS) of 10 peaks (Appendix III c.) acquired at 210 nm.

Appendix IV b. Plot of Canonical Discriminate Analysis (SAS) of 10 peaks (Appendix III c.) acquired at 210 nm.

Appendix IV c. Canonical Discriminate Analysis (SAS) of 10 peaks (Appendix III d.) acquired at 240 nm.

Appendix V a. Discriminant Analysis (SAS) of 10 HPLC Peaks (Appendix III d.) acquired at 240 nm.

Appendix V b. Discriminant Analysis (SAS) of 10 HPLC Peaks (Appendix III d.) acquired at 240 nm (cont.)

Appendix VI. Correlation Matrix of HPLC data (Appendix III c.) acquired at 210 nm for five Cultivars of *B. napus* from different sites analysed with Phylo software (Pearson's product-moment correlation coefficients).

Appendix VII a. Average Linkage Cluster Analysis of five cultivars of *B. napus* and one of *B. rapa* grown at different sites. Based on 10 peaks (Appendix III c.) acquired at 210 nm.

Appendix VII b. Single Linkage Cluster Analysis of five cultivars of *B. napus* and one of *B. rapa* grown at different sites. Based on 10 peaks (Appendix III c.) acquired at 210 nm.

Appendix VII c. Complete Linkage Cluster Analysisof five cultivars of *B. napus* and one of *B. rapa* grown at different sites. Based on 10 peaks (Appendix III c.) acquired at 210 nm.

Appendix VIII. Areas (% of total peaks) and retention times (min.) of 10 peaks for 27 cultivars of *B. napus* and two of *B. rapa* acquired at 210 nm.

Appendix IX. Correlation matrix of HPLC data (Appendix VIII) acquired at 210 nm on 27 cultivars of *B. napus* and two of *B. rapa* analysed with PHYLO software, utilising Pearson's product moment correlation coefficients.

Appendix X a. Average Linkage Cluster Analysis of 27 cultivars of *B. napus* and two of *B. rapa*. Based on 10 peaks (Appendix VIII) acquired at 210 nm.

Appendix X b. Single Linkage Cluster Analysis of 27 cultivars of *B. napus* and two of *B. rapa*. Based on 10 peaks (Appendix VIII) acquired at 210 nm.

Appendix X c. Complete Linkage Cluster Analysis Analysis of 27 cultivars of *B. napus* and two of *B. rapa*. Based on 10 peaks (Appendix VIII) acquired at 210 nm.

Appendix XI. Data matrix for 24 cultivars of *B. napus*. Primer are identified with UBC (University of British Columbia) code numbers. Marker size is in kilobases.

Appendix XII. Pairwise comparisons of Neis' similarity indices (Nei and Li 1979) determined from data matrix in Appendix XI. Cultivar identification as for Table 5.2.

Appendix XIII. Ultra-violet scan of DNA extracted from *B. napus* as described (5.7).

Appendix I a. Repeatibility of HPLC Analysis

Single Extract of *cv* Westar analysed 10 times
Data acquired at 210 nm.

Peak Area (μvolt sec)

Peak No.	Std Dev.	Minimum	Maximum	Range	Std Error	CV
T1	0.0838738	1.4400000	1.6950000	0.2550000	0.0242123	5.2369287
T2	0.0967707	2.4000000	2.7500000	0.3500000	0.0279353	3.7760491
T3	1.1253001	13.2390000	16.8800000	3.6410000	0.3248462	7.4406974
T4	0.1897366	5.0980000	5.6970000	0.5990000	0.0547722	3.4548860
T5	0.1086781	2.6200000	3.0110000	0.3910000	0.0313727	3.7936333
T6	0.1817221	2.9080000	3.5500000	0.6420000	0.0524587	5.6845899
T7	0.1924253	3.1490000	3.6810000	0.5320000	0.0555484	5.6336103
T8	0.3169570	6.2840000	7.1350000	0.8510000	0.0914976	4.7081557
T9	0.3175709	4.3400000	5.3750000	1.0350000	0.0916748	6.5903176
T10	0.0775955	1.3340000	1.5700000	0.2360000	0.0223999	5.3008421

Retention Times (min)

Peak No.	Mean	Std Dev	Minimum	Maximum	Range	Std Error	CV
T1	21.9249167	0.0218942	21.9000000	21.9580000	0.0580000	0.0063203	0.0998599
T2	25.3805833	0.0095580	25.3670000	25.3920000	0.0250000	0.0027592	0.0376589
T3	25.8999167	0.0101036	25.8830000	25.9170000	0.0340000	0.0029167	0.0390103
T4	28.5194167	0.0203356	28.4830000	28.5500000	0.0670000	0.0058704	0.0713045
T5	29.5798333	0.0157182	29.5580000	29.6170000	0.0590000	0.0045374	0.0531381
T6	29.9159167	0.0209912	29.8830000	29.9670000	0.0840000	0.0060596	0.0701672
T7	31.1470833	0.0157276	31.1250000	31.1750000	0.0500000	0.0045402	0.0504945
T8	31.4875833	0.0131457	31.4670000	31.5170000	0.0500000	0.0037948	0.0417490
T9	32.3105000	0.0184021	32.2750000	32.3330000	0.0580000	0.0053122	0.0569539
T10	35.4757500	0.0167935	35.4500000	35.5080000	0.0580000	0.0048479	0.0473381

Appendix I b. Repeatibility of HPLC Analysis

Repeated Extraction of *cv* Westar Analysed 10 times Over Two Weeks
Data acquired at 210 nm.

Peak Area (μvolt sec)

Peak No.	Mean	Std Dev	Minimum	Maximum	Range	Std Error	CV
T1	0.6964545	0.0406162	0.6360000	0.7580000	0.1220000	0.0122462	5.8318470
T2	4.0868182	0.0576209	3.9980000	4.1930000	0.1950000	0.0173733	1.4099199
T3	11.9320909	0.1859072	11.6120000	12.2530000	0.6410000	0.0560531	1.5580439
T4	1.4503636	0.0226595	1.4120000	1.4810000	0.0690000	0.0068321	1.5623348
T5	0.4560909	0.0196390	0.4230000	0.4830000	0.0600000	0.0059214	4.3059431
T6	2.8844545	0.0515526	2.7900000	2.9560000	0.1660000	0.0155437	1.7872572
T7	8.9516364	0.1100093	8.7850000	9.1350000	0.3500000	0.0331691	1.2289299
T8	4.4943636	0.2205463	4.0430000	4.6630000	0.6200000	0.0664972	4.9071744
T9	1.7677273	0.1317354	1.5180000	1.9140000	0.3960000	0.0397197	7.4522474
T1	2.3747273	0.0458652	2.3160000	2.4820000	0.1660000	0.0138289	1.9313888

Retention Time (min)

Peak No.	Mean	Std Dev	Minimum	Maximum	Range	Std Error	CV
T1	21.9310000	0.0124258	21.9000000	21.9420000	0.0420000	0.0037465	0.0566585
T2	25.3697273	0.0099608	25.3500000	25.3830000	0.0330000	0.0030033	0.0392627
T3	25.8983636	0.0117582	25.8830000	25.9170000	0.0340000	0.0035452	0.0454012
T4	26.7924545	0.0242832	26.7500000	26.8420000	0.0920000	0.0073217	0.0906344
T5	29.9325455	0.0132164	29.9080000	29.9500000	0.0420000	0.0039849	0.0441539
T6	31.1530909	0.0125655	31.1330000	31.1750000	0.0420000	0.0037886	0.0403346
T7	31.4650909	0.0110856	31.4500000	31.4830000	0.0330000	0.0033424	0.0352315
T8	32.2780909	0.0168194	32.2500000	32.3000000	0.0500000	0.0050712	0.0521077
T9	33.0552727	0.0135727	33.0330000	33.0750000	0.0420000	0.0040923	0.0410606
T10	35.6764545	0.0096888	35.6580000	35.6920000	0.0340000	0.0029213	0.0271574

Appendix II. Linearity of absorbance - Peak Area and retention times for HPLC analysis of cv Westar injected at six different volumes (210 nm).

Peak Area (μVolts)

Peak No.	R.T.	5	10	20	50	100	200
1	18.983	162269	296006	571304	1295187	5923226	7466568
2	19.333	144755	289113	570812	1777295	2128578	5774416
3	20.667	493737	1000232	1982232	5611325	11235230	17459449
4	22.033	778524	1614726	3076608	7682273	20600029	16092227
5	23.850	3640817	7656485	15338912	37238942	72603212	77903430
6	25.033	185977	422574	780041	1832401	3202532	6814568
7	25.817	174588	310512	654035	2032430	4968427	16459829
8	27.267	76802	152661	485899	1069804	2831288	9242249
9	28.467	486940	1014615	2149791	5346950	11160081	29234984
10	29.150	158342	335283	724733	1909166	3943430	9816437
11	34.867	283913	582885	1378067	3500646	7084356	15261560
12	37.033	220954	420800	863272	2254144	5612629	12196794
13	42.383	1480581	3546942	6048957	17776103	37910816	91785688
Total Area		8288199	17642834	34624663	89326666	189203834	315508199

Retention Times (min)

Peak No.	5	10	20	50	100	200
1	18.983	18.967	18.967	18.983	19.017	19.100
2	19.333	19.317	19.283	19.300	19.317	19.417
3	20.667	20.650	20.650	20.650	20.683	20.783
4	23.850	23.783	23.717	23.567	23.533	23.583
6	25.033	25.033	25.050	25.017	25.033	25.133
7	25.817	25.833	25.850	25.850	25.833	25.967
8	27.267	27.267	27.300	27.283	27.283	27.383
9	28.467	28.467	28.483	28.467	28.450	28.567
10	29.150	29.167	29.167	29.150	29.133	29.233
11	34.867	34.883	34.900	34.900	34.900	35.017
12	37.033	37.067	37.100	37.083	37.083	37.200
13	42.383	42.383	42.383	42.350	42.350	42.433

Appendix III a. HPLC peak area (210 nm) of five Cultivars and five unidentified samples from a range of sites.

Peak No.		*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*
Retention Time		*21.8*	*22.2*	*22.8*	*23.2*	*23.6*	*24.0*	*24.5*	*24.8*	*25.0*	*25.3*	*25.9*	*26.2*	*26.3*	*26.6*	*26.8*	*27.2*	*27.9*	*28.2*	*28.5*	*28.9*	*29.1*	*29.4*	*29.5*
DELTA	Dauphin	.270	1.607	.100	2.023	.000	.312	.918	.000	1.480	1.229	6.430	.280	.326	0.000	.657	29.504	.061	.388	1.819	.617	.433	.884	1.329
DELTA	The Pas	.680	.782	.076	1.370	.000	.390	1.994	.000	1.340	1.054	5.843	.268	.318	0.000	.517	24.361	.058	.102	1.514	.658	.321	.660	1.015
DELTA	Roblin	.421	1.718	.108	1.976	.000	.370	1.564	.000	1.371	1.132	5.759	.304	.343	0.000	.652	28.646	.058	.435	1.850	.642	.437	.800	1.077
DELTA	Teulon	.340	1.554	.122	1.878	.000	.437	1.489	.000	1.349	1.153	5.586	.287	.367	0.000	.669	27.131	.044	.354	1.564	.548	.512	.798	1.109
DELTA	Waskada	.371	2.189	.129	3.014	.000	.593	1.200	.000	1.373	1.288	4.998	.430	.367	0.000	.845	27.764	.054	.472	1.758	.615	.493	.966	1.258
REGENT	Canora	.246	2.426	.099	3.409	.000	.268	3.030	.000	1.399	1.258	5.089	.808	.000	0.000	.715	34.916	.026	.474	2.114	.788	.737	1.108	1.292
REGENT	Baggot	.245	2.287	.154	3.290	.000	.383	2.567	.000	1.509	1.388	5.242	.478	.311	0.000	.774	32.845	.026	.445	1.968	.704	.637	1.122	1.330
REGENT	P/wood	.272	2.030	.111	2.709	.000	.400	4.665	.000	1.637	1.431	5.539	1.039	.000	0.000	.759	27.032	.022	.480	1.977	.784	.868	1.181	1.459
REGENT	Tisdale	.266	2.170	.119	2.825	.000	.338	4.075	.000	1.333	1.236	4.823	.792	.000	0.000	.655	31.027	.021	.417	1.906	.751	.750	1.081	1.280
WESTAR	Dauphin	.700	1.898	.093	2.931	.000	.418	2.666	.000	1.347	1.172	5.129	.424	.353	0.000	.551	30.596	.031	.360	2.083	.667	.954	1.131	1.375
WESTAR	The Pas	1.209	.880	.065	1.991	.00	.610	5.696	.000	1.270	.986	4.720	.871	.000	0.000	.442	23.931	.023	.100	1.659	.777	.420	.762	1.083
WESTAR	Roblin	1.540	2.143	.244	2.935	.00	1.020	3.586	.000	1.394	1.317	4.643	.617	.504	0.000	.827	24.417	.033	.507	2.103	.810	.573	1.028	1.232
WESTAR	Teulon	.631	1.856	.084	2.772	.000	.386	3.368	.000	1.081	1.038	4.867	.464	.304	0.000	.579	29.097	.026	.428	1.738	.652	.665	.876	1.213
WESTAR	Waskada	.804	2.711	.109	3.873	.00	.454	.894	.000	1.850	1.163	3.989	.933	.000	0.000	.881	27.878	.030	.502	2.041	.716	.386	.911	1.325
HERO	La Salla	0	3.422	.041	1.422	.00	.000	2.371	.000	1.772	1.693	5.256	.362	.375	.357	.167	31.962	.000	.543	1.563	.664	.449	1.193	1.288
HERO	Baggot	0	3.436	.147	1.793	.137	.216	1.603	.000	1.668	1.610	5.147	.344	.299	.413	.154	33.690	.000	.472	1.631	.517	.496	1.167	1.227
HERO	Teulon	0	2.883	.036	1.504	.171	.271	2.153	.000	1.696	1.710	4.438	.393	.308	.442	.239	31.445	.000	.657	1.505	.609	.655	1.084	1.145
HERO	Dauphin	0	3.008	.026	1.571	.157	.000	2.037	.000	1.710	1.701	4.647	.322	.284	.370	.184	32.374	.000	.514	1.499	.515	.521	1.120	1.165
HERO	Roblin	0	3.694	.226	2.552	.631	.000	1.294	.000	1.816	1.990	4.631	.389	.473	1.103	.000	31.179	.000	.092	1.556	.670	.499	1.503	1.738
STELLAR	Point	0	2.559	.126	2.621	.559	.000	4.257	.000	1.346	1.117	4.427	.300	.085	.376	.138	31.475	.027	.084	.786	.282	.646	1.041	.818
STELLAR	Baggot	0	2.833	1.225	3.178	.693	.00	3.100	.000	1.487	1.311	4.695	.460	.248	.609	.238	30.133	.000	.019	1.538	.625	.547	.937	1.092
STELLAR	Teulon	0	2.537	.217	3.315	.763	.000	2.252	.000	1.527	1.300	4.581	.480	.223	.720	.238	30.126	.022	.329	1.600	.578	.649	1.008	1.084
STELLAR	Portage	0	2.259	.256	2.894	.752	.000	2.880	.000	1.657	1.478	4.658	.471	.221	.818	.000	32.392	.000	.305	1.471	.521	.656	.980	1.003
STELLAR	Roblin	0	2.185	.173	2.675	.630	.000	3.064	.000	1.141	.891	4.624	.319	.169	.585	.000	32.366	.037	.191	1.179	.410	.470	.730	.551
TOBIN	Dauphin	.392	1.174	.520	4.952	.000	.601	3.387	.409	.780	.562	6.396	.489	.144	0.000	.593	28.029	.063	.053	.939	.546	.799	.745	.785
TOBIN	Waskada	.414	1.404	.551	5.146	.000	.629	2.021	.342	.763	.528	5.273	.547	.168	0.000	.682	25.912	.095	.063	1.069	.633	.477	.781	.646

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*
RET	*TIME*	*21.8*	*22.2*	*22.8*	*23.2*	*23.6*	*24.0*	*24.5*	*24.8*	*25.0*	*25.3*	*25.9*	*26.2*	*26.3*	*26.6*	*26.8*	*27.2*	*27.9*	*28.2*	*28.5*	*28.9*	*29.1*	*29.4*	*29.5*
SCGA7	M/STONE	.49	1.475	.096	3.222	.02	.327	3.005	0	1.11	1.099	4.326	.675	0	0	.523	33.703	0	.086	1.851	.691	.563	.935	1.123
SCGA1	W/BEND	.57	1.745	0	3.317	.017	.318	2.42	0	1.008	.964	4.964	.522	.405	0	.606	29.402	0	.411	1.958	.689	.854	.9	1.032
SCGA1	R/HURST	.688	1.748	.076	4.209	.035	.124	2.74	0	1.087	1.06	5.269	.388	.233	0	.614	32.659	.264	.353	1.978	.56	.458	1.097	1.187
SCGA4	NIACAM	.843	1.673	.054	2.695	.009	.276	2.335	0	.681	.74	4.351	.664	.883	0	.642	30.527	0	.082	1.647	.598	.629	.624	.840
SCGA7	R/DALE	.47	1.296	.039	2.933	.025	.215	4.346	0	1.037	.96	5.000	.788	0	0	.508	33.403	0	.098	1.707	.655	.788	.767	.901

Appendix III a. HPLC peak area (210 nm) cont.

24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
29.9	*30.4*	*30.8*	*31.1*	*31.5*	*32.0*	*32.3*	*32.6*	*32.7*	*33.1*	*33.3*	*33.8*	*34.3*	*34.7*	*35.2*	*35.6*	*36.1*	*36.9*	*37.3*	*37.7*	*38.3*	*38.7*	*40.2*	*40.8*	*41.0*
.584	.150	.164	1.521	4.903	.029	1.316	.226	.145	.758	.524	.500	.114	.213	.417	4.171	.345	.503	.165	1.315	.390	.762	.583	.139	.207
.481	.300	.194	1.295	3.893	.020	1.034	.344	.000	.660	.411	.387	.118	.268	.532	4.636	.290	.493	.171	1.470	.340	.666	.574	.174	.250
.645	.200	.203	1.386	4.512	.017	1.332	.261	.132	.777	.588	.595	.116	.258	.454	3.920	.331	.457	.196	1.250	.390	.641	.614	.133	.236
.624	.242	.221	1.375	5.439	.028	1.393	.238	.209	.784	.592	.659	.194	.336	.484	4.234	.254	.594	.252	2.030	.480	.891	.667	.214	.252
.636	.336	.301	1.158	3.024	.015	1.190	.255	.195	.740	.589	.550	.115	.300	.329	2.589	.022	.438	.213	.600	.480	.472	.549	.152	.209
1.273	.200	.178	1.051	2.335	.000	2.363	.000	.133	1.070	.463	.402	.069	.105	.158	1.400	.257	.465	.362	2.003	.300	.289	.653	.111	.304
1.296	.340	.227	1.203	2.202	.007	2.420	.000	.200	1.049	.477	.567	.310	.175	.224	1.470	.462	.604	.380	1.860	.424	.390	.631	.112	.262
1.401	.400	.303	1.252	2.684	.160	2.305	.000	.187	.940	.687	.740	.310	.168	.294	1.400	.261	.498	.546	2.323	.530	.348	.867	.154	.327
1.328	.330	.235	.933	2.740	.127	2.218	.000	.189	.934	.606	.621	.260	.132	.195	1.380	.216	.460	.420	2.470	.400	.312	.701	.101	.299
1.251	.220	.123	1.025	5.966	.198	1.970	.000	.168	.880	.500	.452	.230	.107	.189	1.470	.195	.576	.456	2.463	.480	.370	.788	.137	.291
1.182	.420	.229	.842	4.076	.178	1.669	.000	.182	.814	.340	.437	.250	.210	.112	2.030	.197	.531	.627	3.840	.570	.425	.549	.127	.309
1.305	.450	.307	1.162	3.902	.255	1.977	.000	.266	.919	.644	.765	.400	.180	.350	1.360	.311	.512	.520	2.004	.510	.324	.751	.146	.277
1.242	.260	.211	.875	5.595	.238	1.955	.000	.188	.896	.579	.543	.280	.137	.261	1.337	.174	.509	.466	3.070	.460	.371	.677	.111	.328
1.098	.320	.323	.797	3.054	.152	1.855	.000	.229	.846	.541	.468	.270	.109	.227	1.050	.152	.334	.251	.758	.380	.248	.312	.000	.072
.353	.000	.000	1.630	4.827	.181	1.084	.240	.000	.543	.401	.110	.058	.120	.147	1.530	.380	.924	.228	1.765	.289	.341	.694	.000	.278
.222	.000	.000	1.199	4.095	.129	.992	.142	.000	.276	.410	.349	.122	.167	.135	1.314	.337	.923	.259	1.471	.340	.315	.775	.000	.338
.515	.000	.000	1.459	4.178	.192	1.297	.224	.000	.447	.502	.426	.233	.132	.155	1.381	.289	.854	.265	1.780	.249	.329	.643	.000	.274
.247	.000	.000	1.069	3.342	.098	1.014	.186	.000	.292	.279	.266	.130	.094	.130	1.308	.280	.879	.274	1.721	.264	.342	.620	.000	.291
.376	.000	.274	1.273	3.659	.371	1.104	.316	.000	.439	.490	.468	.319	.151	.181	1.430	.638	.831	.203	.804	.325	.469	.421	.000	.351
.790	.000	.000	.979	2.500	.238	.636	.147	.000	.648	.611	.541	.054	.185	.146	1.547	.260	.536	.186	2.051	.468	.419	.678	.072	.359
1.085	.000	.251	1.274	3.110	.306	.660	.298	.000	.733	.958	.874	.138	.290	.202	1.142	.184	.443	.159	1.482	.301	.173	.466	.000	.231
1.082	.000	.287	1.230	3.115	.336	.730	.252	.000	.753	.852	.747	.140	.260	.220	1.243	.201	.463	.171	1.362	.308	.201	.314	.000	.196
1.077	.000	.215	1.220	2.977	.236	.912	.203	.000	.729	.637	.541	.083	.190	.152	1.317	.114	.487	.131	1.641	.307	.247	.586	.000	.292
.865	.000	.000	.934	3.178	.000	1.602	.154	.000	.776	.728	.615	.137	.200	.175	1.654	.371	.686	.214	1.488	.497	.468	.627	.068	.362
.596	.000	.784	.853	5.9	.258	.364	.226	.000	4.362	.000	.214	.189	.509	.634	1.412	.000	.712	.126	.785	.234	.331	.181	.000	.041
.680	.122	.734	.736	4.816	.315	.411	.269	.000	3.964	.000	.292	.176	.544	.611	1.295	.000	.647	.204	.560	.467	.425	.445	.240	.150

24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
29.9	*30.4*	*30.8*	*31.1*	*31.5*	*32.0*	*32.3*	*32.6*	*32.7*	*33.1*	*33.3*	*33.8*	*34.3*	*34.7*	*35.2*	*35.6*	*36.1*	*36.9*	*37.3*	*37.7*	*38.3*	*38.7*	*40.2*	*40.8*	*41.0*
1.207	.11	.162	.819	3.729	.104	2.06	0	.13	.97	.47	.4	.22	.1	.18	1.35	.14	.59	.27	1.7	.32	.32	.62	.11	.46
1.211	.26	.148	1.081	3.034	.11	1.78	0	.13	.89	.32	.25	.18	.08	.17	1.16	.17	.67	.36	1.6	.35	.25	.55	.12	.32
1.219	.151	.094	1.217	2.638	.05	1.86	0	.11	1.21	0	.24	.17	.12	.17	1.37	.23	.71	.26	1.11	.28	.29	.62	.02	.07
.991	.26	.254	1.186	2.034	.836	1.47	0	.1	.93	0	.2	.27	.09	.09	.96	.16	.5	.26	1.88	.02	.12	1.11	.03	.12
1.008	.042	0	.751	3.113	.061	2.11	0	.094	.917	.303	.24	.22	.13	.21	1.63	.21	.78	.49	3.33	.4	.42	1.45	.15	.27

Appendix III b. HPLC peak area (240 nm) of five Cultivars and five unidentified samples from a range of sites.

PEAK	*No.*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
Retention Time		*21.8*	*22.2*	*22.5*	*23.2*	*24.5*	*25.0*	*25.3*	*25.9*	*26.2*	*26.6*	*27.3*	*28.5*	*28.9*	*29.4*	*29.9*	*30.3*	*31.1*	*31.5*
WESTAR	Dauphin	.229	.359	.097	1.679	4.084	.604	.620	4.470	.430	.470	42.859	1.458	2.020	1.210	.989	.310	.912	9.667
WESTAR	Pas	.660	.293	.145	1.375	10.015	.542	.610	4.171	.554	.370	34.159	1.208	1.130	.960	.985	.370	.686	6.428
WESTAR	Roblin	.186	.314	.107	1.767	6.311	.603	.620	4.649	.510	.420	42.013	1.581	1.330	1.190	1.003	.310	.939	6.847
WESTAR	Teulon	.120	.422	.099	1.614	4.993	.507	.540	4.177	.410	.420	42.234	1.211	1.430	1.020	.941	.290	.731	9.025
WESTAR	Waskada	.000	.472	.000	2.186	1.557	.707	.567	4.008	.370	.560	48.814	1.529	1.110	1.150	.848	.100	.582	5.237
REGENT	Canora	.129	.411	.060	1.704	4.250	.596	.543	4.079	.301	.410	49.182	1.389	1.460	1.100	.901	.180	.814	3.163
REGENT	N.'bat	.148	.427	.063	1.880	4.011	.687	.635	4.698	.280	.410	49.689	1.333	1.250	1.140	.900	.160	.948	3.101
REGENT	P.'wood	.163	.386	.095	1.574	7.811	.795	.800	4.879	.566	.470	40.868	1.435	1.660	1.360	1.096	.330	1.040	3.893
REGENT	Tisdale	.119	.424	.085	1.544	6.296	.657	.650	4.124	.411	.450	45.433	1.348	1.570	1.190	1.036	.390	.775	4.091
DELTA	Dauphin	.132	.358	.000	1.475	1.277	.823	.567	5.842	.350	.420	43.812	1.224	.920	1.070	.333	.050	1.331	7.833
DELTA	Pas	.326	.305	.000	1.050	3.556	.642	.527	5.633	.380	.310	38.345	1.128	.900	.890	.333	.210	1.180	6.438
DELTA	Roblin	.167	.309	.059	1.265	2.388	.802	.553	5.126	.390	.410	41.827	1.284	1.030	.940	.447	.250	1.179	6.995
DELTA	Teulon	.178	.358	.080	1.379	2.203	.635	.570	5.037	.420	.400	40.482	1.019	1.050	.950	.404	.260	1.199	8.611
DELTA	Waskada	0.000	.600	.000	1.764	1.284	.817	.620	5.154	.380	.450	48.327	1.263	1.180	1.130	.327	.150	.972	4.783
HERO	LaSalle	.045	.274	.000	.654	2.230	.611	.484	2.936	.250	.160	29.138	.659	.610	.680	.099	.022	.796	4.668
HERO	Baggot	.044	.273	.000	.746	1.266	.533	.439	2.889	.210	.200	30.690	.729	.660	.710	.110	.020	.699	4.108
HERO	Teulon	.032	.250	.000	.540	1.749	.453	.425	2.283	.170	.160	29.319	.575	.670	.570	.125	.027	.622	3.885
HERO	Dauphin	.101	.312	.000	.577	1.661	.527	.436	2.415	.180	.170	29.112	.668	.730	.650	.126	.030	.584	3.105
HERO	Roblin	.146	.380	.056	.767	.622	.514	.493	2.562	.170	.272	29.386	.578	.550	.840	.067	.000	.616	3.378
STELLAR	Point	.000	.510	.056	.956	5.093	.446	.378	2.811	.148	.220	34.409	.597	1.030	.670	.526	.037	.623	2.910
STELLAR	Baggot	.000	.520	.856	1.297	3.463	.483	.387	3.357	.170	.290	36.621	.694	.690	.640	.548	.000	.745	3.883
STELLAR	Teulon	.000	.190	.022	.959	1.917	.398	.303	2.581	.128	.262	29.970	.571	.690	.570	.456	.056	.556	3.071
STELLAR	Portage	.132	.440	.119	1.138	3.232	.530	.454	3.140	.170	.284	36.706	.669	.800	.640	.581	.041	.723	3.541
STELLAR	Roblin	.173	.290	.020	1.000	3.529	.361	.250	3.137	.180	.200	36.300	.669	.760	.510	.627	.100	.647	3.934
SCGA	M.'stone	.204	.349	.107	1.668	4.500	.528	.539	3.710	.322	.435	46.4	1.273	.493	.841	.867	.152	.699	5.664
SCGA	W.'bend	.179	.442	.093	1.890	4.025	.489	.513	4.657	.544	.594	44.164	1.555	.543	1.463	1.047	.211	1.046	4.848
SCGA	R.'hurst	.100	.601	.154	2.153	4.547	.550	.570	4.800	.483	.447	47.281	1.541	.514	.794	1.083	.177	1.142	4.076
SCGA	Niacam	.179	.466	.019	1.464	3.245	.339	.308	3.490	1.176	.545	43.917	1.229	.460	1.026	.815	.232	1.200	3.237
SCGA	R.'dale	.120	.351	.065	1.562	6.726	.462	.388	4.099	.452	.288	45.382	1.259	.498	1.266	.881	.138	.682	4.735

Appendix III b. HPLC peak area (240 nm) cont.

19 32.3	20 32.8	21 33.8	22 34.4	23 34.6	24 34.8	25 35.2	26 35.7	27 36.1	28 36.9	29 37.3	30 37.8	31 38.1	32 38.7	33 39.7	34 40.3	35 40.6	36 41.1	37 41.7	38 42.8
2.942	1.410	.232	.099	.138	.130	.189	1.417	.222	.595	.407	3.827	.118	.472	.101	.413	.042	.240	.051	.667
2.395	1.160	.241	.135	.171	.201	.169	1.852	.170	.534	.591	6.238	.158	.570	.214	.333	.055	.333	.078	.636
2.985	1.430	.240	.092	.100	.106	.184	1.213	.223	.543	.454	3.637	.134	.366	.156	.499	.071	.264	.051	.615
2.843	1.340	.234	.085	.136	.124	.209	1.356	.187	.537	.430	4.942	.129	.505	.182	.409	.053	.221	.053	.653
2.942	1.080	.090	.020	.093	.040	.119	1.033	.110	.377	.185	1.219	.106	.356	.045	.236	.000	.068	.014	.335
3.362	1.360	.143	.062	.050	.081	.111	1.234	.232	.471	.301	3.155	.084	.368	.126	.348	.028	.223	.055	.721
3.481	1.380	.151	.084	.054	.084	.067	1.268	.328	.596	.295	2.979	.300	.426	.121	.368	.031	.217	.043	.744
3.400	1.500	.304	.107	.092	.120	.210	1.372	.216	.491	.485	3.791	.420	.451	.199	.540	.069	.299	.069	.677
3.318	1.460	.274	.096	.117	.106	.165	1.311	.234	.508	.396	4.074	.390	.458	.186	.450	.051	.278	.062	.685
1.934	1.100	.150	.103	.000	.129	.345	5.916	.247	.490	.082	1.910	.220	.995	.000	.344	.077	.180	.000	.858
1.567	1.040	.106	.123	.000	.171	.503	7.244	.259	.569	.154	2.450	.280	1.019	.014	.317	.106	.278	.021	.826
2.102	1.050	.217	.112	.000	.176	.421	5.482	.290	.509	.166	1.950	.290	.897	.058	.473	.121	.233	.016	.755
2.026	1.220	.210	.165	.000	.263	.423	6.124	.200	.603	.186	3.099	.310	1.150	.070	.410	.142	.248	.029	.923
1.732	.820	.062	.032	.015	.052	.220	3.397	.101	.428	.101	.760	.190	.631	.000	.263	.058	.115	.000	.360
.967	.340	.086	.042	.055	.000	.070	.850	.252	.600	.122	1.753	.090	.273	.047	.280	.000	.166	.029	.352
1.017	.360	.087	.050	.059	.000	.060	.680	.210	.570	.100	1.377	.081	.211	.034	.280	.000	.177	.016	.313
1.140	.350	.069	.031	.048	.000	.064	.810	.132	.501	.112	1.796	.052	.306	.046	.220	.000	.140	.031	.317
.994	.319	.064	.034	.049	.000	.066	.740	.202	.559	.136	1.648	.089	.275	.036	.210	.000	.129	.031	.341
.907	.320	.083	.039	.039	.000	.059	.670	.273	.512	.090	.652	.084	.412	.023	.310	.000	.155	.000	.210
.730	.680	.140	.027	.072	.000	.080	1.100	.118	.348	.082	2.208	.200	.350	.048	.290	.000	.232	.028	.394
.670	.770	.199	.011	.026	.000	.064	.880	.038	.311	.074	1.884	.180	.232	.038	.250	.000	.171	.018	.334
.533	.420	.097	.000	.054	.000	.072	.810	.039	.283	.067	1.338	.130	.208	.000	.180	.000	.127	.000	.284
1.022	.600	.096	.000	.083	0.000	.091	1.240	.104	.393	.090	2.080	.180	.315	.025	.220	.000	.182	.024	.381
2.039	.889	.209	.000	.129	0.000	.107	1.280	.265	.509	.115	1.672	.260	.417	.035	.200	.000	.188	.031	.407
3.163	1.321	.280	.094	.157	.137	.207	1.341	.246	.544	.319	2.738	.135	.502	.199	.402	.062	.350	.075	.527
2.824	1.256	.211	.081	.144	.116	.182	1.228	.212	.565	.372	2.702	.117	.033	.326	.052	.273	.055	.011	.560
3.110	1.278	.224	.073	.091	.115	.142	1.241	.239	.573	.269	1.766	.093	.371	.118	.319	.022	.146	.041	.033
2.252	.925	.215	.106	.265	.115	.148	1.093	.163	.516	.347	2.895	.006	.290	.174	.254	.055	.291	.110	.028
3.209	1.121	.110	.056	.110	.115	.145	1.715	.168	.558	.425	5.096	.113	.573	.148	.322	.033	.289	.062	.032

Appendix III (c). Areas (% of total peaks - 210 nm) and retention times (min.) of 10 peaks selected from Appendix III (a) by SAS discriminant analysis for five cultivars of *B. napus* and one of *B. rapa* grown at different sites.

PEAK IDENTIFICATION

		A	B	C	D	E	F	G	H	I	J
		(Retention Time)									
		21.8	25.3	25.9	26.8	29.9	31.1	31.5	32.3	33.1	35.6
Cultivar	*Site*	*Area %*									
Delta	Dauphin	.270	1.229	6.430	.657	.584	1.521	4.903	1.316	.758	4.171
Delta	The Pas	.680	1.054	5.843	.517	.481	1.295	3.893	1.034	.660	4.636
Delta	Roblin	.421	1.132	5.759	.652	.645	1.386	4.512	1.332	.777	3.920
Delta	Teulon	.340	1.153	5.586	.669	.624	1.375	5.439	1.393	.784	4.234
Delta	Waskada	.371	1.288	4.998	.845	.636	1.158	3.024	1.190	.740	2.589
Regent	Canora	.246	1.258	5.089	.715	1.273	1.051	2.335	2.363	1.070	1.400
Regent	Baggot	.245	1.388	5.242	.774	1.296	1.203	2.202	2.420	1.049	1.470
Regent	P/wood	.272	1.431	5.539	.759	1.401	1.252	2.684	2.305	.940	1.400
Regent	Tisdale	.266	1.236	4.823	.655	1.328	.933	2.740	2.218	.934	1.380
Westar	Dauphin	.700	1.172	5.129	.551	1.251	1.025	5.966	1.970	.880	1.470
Westar	The Pas	1.209	.986	4.720	.442	1.182	.842	4.076	1.669	.814	2.030
Westar	Roblin	1.540	1.317	4.643	.827	1.305	1.162	3.902	1.977	.919	1.360
Westar	Teulon	.631	1.038	4.867	.579	1.242	.875	5.595	1.955	.896	1.337
Westar	Waskada	.804	1.163	3.989	.881	1.098	.797	3.054	1.855	.846	1.050
Hero	La Salle	0	1.693	5.256	.167	.353	1.630	4.827	1.084	.543	1.530
Hero	Baggot	0	1.610	5.147	.154	.222	1.199	4.095	.992	.276	1.314
Hero	Teulon	0	1.710	4.438	.239	.515	1.459	4.178	1.297	.447	1.381
Hero	Dauphin	0	1.701	4.647	.184	.247	1.069	3.342	1.014	.292	1.308
Hero	Roblin	0	1.990	4.631	0	.376	1.273	3.659	1.104	.439	1.430
Stellar	Point	0	1.117	4.427	.138	.790	.979	2.500	.636	.648	1.547
Stellar	Baggot	0	1.311	4.695	.238	1.085	1.274	3.110	.660	.733	1.142
Stellar	Teulon	0	1.300	4.581	.238	1.082	1.230	3.115	.730	.753	1.243
Stellar	Portage	0	1.478	4.658	0	1.077	1.220	2.977	.912	.729	1.317
Stellar	Roblin	0	.891	4.624	0	.865	.934	3.178	1.602	.776	1.654
Tobin	Dauphin	.392	.562	6.396	.593	.596	.853	5.9	.364	4.362	1.412
Tobin	Waskada	.414	.528	5.273	.682	.680	.736	4.816	.411	3.964	1.295
		Test Samples									
SCGA	M/stone	.49	1.099	4.326	.523	1.207	.819	3.729	2.06	.97	1.35
SCGA	W/bend	.57	.964	4.964	.606	1.211	1.081	3.034	1.78	.89	1.16
SCGA	R/hurst	.688	1.06	5.269	.614	1.219	1.217	2.638	1.86	1.21	1.37
SCGA	Niacam	.843	.744	4.351	.642	.991	1.186	2.034	1.47	.93	.96
SCGA	R/dale	.470	.96	5.000	.508	1.008	.751	3.113	2.11	.917	1.63

Appendix III (d). Areas (% of total peaks 240 nm) and retention times (min.) of 10 peaks selected from Appendix III (b) by SAS discriminant analysis for five cultivars of *B. napus* and one of *B. rapa* grown at different sites.

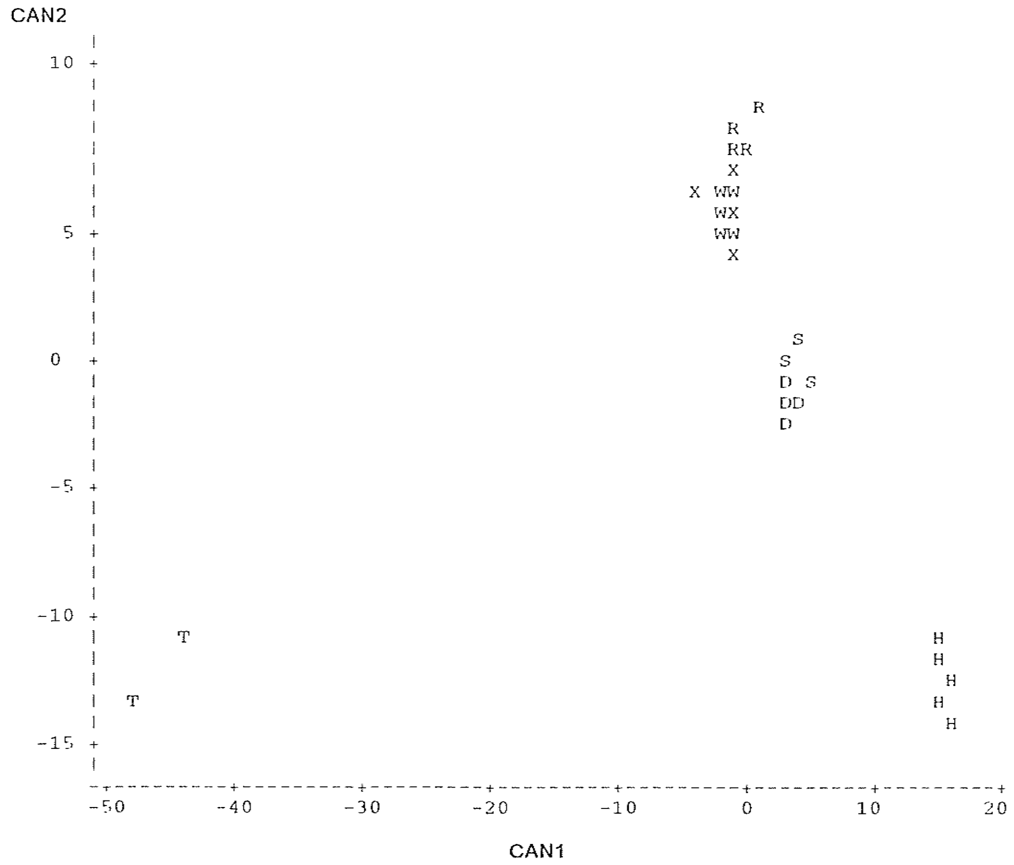
PEAK IDENTIFICATION

		A	B	C	D	E	F	G	H	I	J
		(Retention Time)									
		25.9	28.5	29.9	31.5	32.3	32.8	35.7	37.3	37.8	38.7
Cultivar	*Site*	Area %									
Westar	Dauphin	4.47	1.460	.989	9.67	2.94	1.41	1.42	.407	3.83	.472
Westar	Pas	4.17	1.210	.985	6.43	2.40	1.16	1.85	.591	6.24	.570
Westar	Roblin	4.65	1.580	1.000	6.85	2.98	1.43	1.21	.454	3.64	.366
Westar	Teulon	4.18	1.210	.941	9.03	2.84	1.34	1.36	.430	4.94	.505
Westar	Waskada	4.01	1.530	.848	5.24	2.94	1.08	1.03	.185	1.22	.356
Regent	Canora	4.08	1.390	.901	3.16	3.36	1.36	1.23	.301	3.15	.368
Regent	N/bat	4.70	1.330	.900	3.1	3.48	1.38	1.27	.295	2.98	.426
Regent	P/wood	4.88	1.440	1.100	3.89	3.40	1.50	1.37	.485	3.79	.451
Regent	Tisdale	4.12	1.350	1.040	4.09	3.32	1.46	1.31	.396	4.07	.458
Delta	Dauphin	5.84	1.220	.333	7.83	1.93	1.10	5.92	.082	1.91	.995
Delta	Pas	5.63	1.130	.333	6.44	1.57	1.04	7.24	.154	2.45	1.020
Delta	Roblin	5.13	1.280	.447	7.00	2.10	1.05	5.48	.166	1.95	.897
Delta	Teulon	5.04	1.020	.404	8.61	2.03	1.22	6.12	.186	3.10	1.150
Delta	Waskada	5.15	1.260	.327	4.78	1.73	.82	3.40	.101	.76	.631
Hero	LaSalle	2.94	.659	.099	4.67	.967	.34	.85	.122	1.75	.273
Hero	Baggot	2.89	.729	.110	4.11	1.02	.36	.68	.100	1.38	.211
Hero	Teulon	2.28	.575	.125	3.88	1.14	.35	.81	.112	1.80	.306
Hero	Dauphin	2.42	.668	.126	3.1	.994	.32	.74	.136	1.65	.275
Hero	Roblin	2.56	.578	.067	3.38	.907	.32	.67	.090	.65	.412
Stellar	Point	2.81	.597	.526	2.91	.73	.68	1.10	.082	2.21	.350
Stellar	Baggot	3.36	.694	.548	3.88	.67	.77	.88	.074	1.88	.232
Stellar	Teulon	2.58	.571	.456	3.07	.533	.42	.81	.067	1.34	.208
Stellar	Portage	3.14	.669	.581	3.54	1.02	.60	1.24	.090	2.08	.315
Stellar	Roblin	3.14	.669	.627	3.93	2.04	.889	1.28	.115	1.67	.417
		Test Samples									
SCGA	M/stone	3.71	1.27	.867	5.66	3.16	.28	1.34	.319	2.74	.502
SCGA	W/bend	4.66	1.55	1.05	4.85	2.82	.211	1.23	.372	2.70	.033
SCGA	R/hurst	4.80	1.54	1.08	4.08	3.11	.224	1.24	.269	1.77	.371
SCGA	Niacam	3.49	1.23	.815	3.24	2.25	.215	1.09	.347	2.90	.290
SCGA	R/dale	4.10	1.26	.881	4.74	3.21	.11	1.72	.425	5.10	.573

Appendix IV (a). Canonical Discriminate Analysis (SAS) of 10 peaks (Appendix IV c.) acquired at 210 nm.

```
      29 Observations          28 DF Total
      10 Variables             23 DF Within Classes
       6 Classes                5 DF Between Classes


                    Class Level Information

 CV                Frequency          Weight        Proportion

 D                         5          5.0000          0.161290
 H                         5          5.0000          0.161290
 R                         4          4.0000          0.129032
 S                         5          5.0000          0.161290
 T                         2          2.0000          0.064516
 W                         5          5.0000          0.161290
 X                         5          5.0000          0.161290


Canonical Discriminant Analysis      Pairwise Squared Distances Between Groups

                          2          _   _          -1  _   _
                          D (i|j) = (X - X )' COV    (X - X )
                                      i   j            i   j


Squared Distance to CV

From CV          D          H          R          S        T          W          X

   D             0  432.08327  257.11041  262.91344     2615  244.36699  238.76278
   H     432.08327          0  742.30793  361.54467     3733  656.64256  641.64582
   R     257.11041  742.30793          0  173.76723     2518  137.77187   41.80227
   S     262.91344  361.54467  173.76723          0     2626  244.19785  125.00182
   T          2615       3733       2518       2626        0       2313       2261
   W     244.36699  656.64256  137.77187  244.19785     2313          0   53.90598
   X     238.76278  641.64582   41.80227  125.00182     2261   53.90598          0
```

Appendix IV (b). Plot of Canonical Discriminate Analysis (Can1 * Can2, SAS) of 10 peaks (Appendix III c.) acquired at 210 nm. Proportion of total variation accounted for by the two axes CAN1 = 64.51% CAN2 = 20.58%.



NOTE: 4 obs hidden

Appendix IV (c). Canonical Discriminate Analysis (SAS) of 10 peaks (Appendix III d.) acquired at 240 nm.

```
 29 Observations          28 DF Total
 10 Variables             23 DF Within Classes
  6 Classes                5 DF Between Classes


                  Class Level Information

 CV              Frequency        Weight       Proportion

 D                       5        5.0000         0.172414
 H                       5        5.0000         0.172414
 R                       4        4.0000         0.137931
 S                       5        5.0000         0.172414
 W                       5        5.0000         0.172414
 X                       5        5.0000         0.172414


Canonical Discriminant Analysis     Pairwise Squared Distances Between Groups

                 2        _   _         -1  _   _
                 D (i|j) = (X - X )' COV   (X - X )
                            i   j            i   j


Squared Distance to CV

 From CV         D          H          R          S          W          X
   D             0  171.06623  328.71954  368.07352  208.29978  322.84687
   H     171.06623          0  306.84916  159.99282  212.65443  234.82996
   R     328.71954  306.84916          0  187.24268   66.65750  287.39484
   S     368.07352  159.99282  187.24268          0  207.92248  282.24948
   W     208.29978  212.65443   66.65750  207.92248          0  162.62462
   X     322.84687  234.82996  287.39484  282.24948  162.62462          0
```

Appendix V a. Discriminant Analysis (SAS) of 10 HPLC Peaks acquired at 240 nm.

```
                            Discriminant Analysis

               24 Observations             23 DF Total
               10 Variables                19 DF Within Classes
                5 Classes                   4 DF Between Classes

                           Class Level Information

                                                                    Prior
  CV            Frequency         Weight       Proportion       Probability

   Delta                5          5.0000        0.208333         0.200000
   Hero                 5          5.0000        0.208333         0.200000
   Regent               4          4.0000        0.166667         0.200000
   Stellar              5          5.0000        0.208333         0.200000
   Westar               5          5.0000        0.208333         0.200000


              Pairwise Generalized Squared Distances Between Groups

                  2              _   _            -1   _   _
                 D (i|j) =  (X - X )' COV     (X - X )
                              i   j              i   j


                      Generalized Squared Distance to CV

 From CV     Delta          Hero         Regent        Stellar          Westar

 Delta           0     217.66955      514.44572      849.07825       289.66481
 Hero    217.66955             0      551.09315      615.00516       370.93654
 Regent  514.44572     551.09315              0      251.68730        83.97135
 Stellar 849.07825     615.00516      251.68730              0       377.13766
 Westar  289.66481     370.93654       83.97135      377.13766               0

                  Classification Summary for Calibration Data
           Resubstitution Summary using Linear Discriminant Function
                   Generalized Squared Distance Function:

                     2           _          -1     _
                    D (X)  = (X-X )' COV    (X-X )
                     j           j               j


            Number of Observations and Percent Classified into CV:

  From CV        Delta      Hero     Regent    Stellar     Westar       Total

  Delta              5         0          0          0          0           5
                100.00      0.00       0.00       0.00       0.00      100.00
  Hero               0         5          0          0          0           5
                  0.00    100.00       0.00       0.00       0.00      100.00
  Regent             0         0          4          0          0           4
                  0.00      0.00     100.00       0.00       0.00      100.00
  Stellar            0         0          0          5          0           5
                  0.00      0.00       0.00     100.00       0.00      100.00
  Westar             0         0          0          0          5           5
                  0.00      0.00       0.00       0.00     100.00      100.00

  Total              5         5          4          5          5          24
  Percent        20.83     20.83      16.67      20.83      20.83      100.00
  Priors        0.2000    0.2000     0.2000     0.2000     0.2000
```

Appendix V b. Discriminant Analysis (SAS) of 10 HPLC Peaks acquired at 240 nm.

```
                    Classification Results for Test Data

           Classification Results using Linear Discriminant Function


                     Generalized Squared Distance Function:
                          2         _          -1   _
                         D (X) = (X-X )' COV   (X-X )
                          j          j             j


             Posterior Probability of Membership in each CV:

                               2                      2
             Pr(j|X) = exp(-.5 D (X)) / SUM exp(-.5 D (X))
                               j          k           k


                 Posterior Probability of Membership in CV:

 LOC       From    Classified
             CV      into CV          DEL       HER       REG       STE       WES

M/stone   SCGA      Westar    *    0.0000    0.0000    0.0000    0.0000    1.0000
W/bend    SCGA      Westar    *    0.0000    0.0000    0.0000    0.0000    1.0000
R/hurst   SCGA      Westar    *    0.0000    0.0000    0.0000    0.0000    1.0000
Niacam    SCGA      Westar    *    0.0000    0.0000    0.0000    0.0000    1.0000
R/dale    SCGA      Westar    *    0.0000    0.0000    0.0000    0.0000    1.0000


          Number of Observations and Percent Classified into CV:

 From CV      Delta       Hero      Regent     Stellar    Westar      Total
 SCGA             0          0           0           0         5          5
               0.00       0.00        0.00        0.00    100.00     100.00
 Total            0          0           0           0         5          5
 Percent       0.00       0.00        0.00        0.00    100.00     100.00
```

Appendix VI. Correlation Matrix of HPLC data (210 nm) for 5 Cultivars of *B. napus* from 5 sites analysed with Phylo software (Pearson's product-moment correlation coefficients).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1																										
2	.982																									
3	.999	.984																								
4	.988	.965	.991																							
5	.980	.957	.975	.942																						
6	.817	.760	.809	.753	.893																					
7	.806	.753	.796	.734	.888	.998																				
8	.834	.771	.824	.771	.907	.996	.994																			
9	.851	.784	.844	.803	.909	.992	.983	.994																		
10	.858	.762	.857	.879	.838	.792	.761	.818	.861																	
11	.928	.877	.929	.924	.921	.858	.835	.876	.907	.959																
12	.854	.779	.852	.840	.874	.874	.852	.896	.918	.959	.977															
13	.851	.753	.850	.871	.834	.800	.768	.824	.867	.999	.958	.960														
14	.849	.764	.844	.825	.888	.932	.913	.945	.965	.944	.955	.981	.949													
15	.917	.836	.910	.910	.918	.833	.818	.865	.880	.948	.933	.929	.940	.921												
16	.922	.847	.913	.902	.940	.871	.859	.901	.909	.935	.940	.941	.929	.941	.994											
17	.905	.819	.897	.900	.907	.840	.825	.871	.888	.948	.926	.925	.941	.924	.997	.991										
18	.920	.852	.910	.890	.948	.889	.881	.916	.920	.907	.925	.926	.900	.934	.983	.996	.983									
19	.909	.837	.900	.887	.928	.864	.855	.892	.900	.908	.915	.912	.900	.915	.987	.993	.989	.996								
20	.938	.894	.929	.890	.973	.917	.912	.938	.934	.855	.919	.893	.851	.907	.938	.957	.928	.965	.953							
21	.896	.825	.885	.856	.932	.899	.890	.928	.926	.893	.915	.914	.889	.923	.960	.970	.952	.969	.963	.985						
22	.907	.837	.897	.870	.938	.901	.892	.929	.930	.900	.923	.916	.896	.926	.964	.973	.957	.972	.966	.987	.999					
23	.902	.837	.892	.861	.938	.916	.909	.940	.940	.884	.917	.910	.880	.923	.956	.969	.953	.974	.971	.989	.995	.996				
24	.934	.875	.930	.907	.952	.944	.931	.954	.970	.918	.959	.937	.918	.955	.946	.956	.946	.956	.949	.968	.958	.965	.970			
25	.758	.683	.753	.753	.757	.698	.668	.707	.734	.822	.805	.785	.825	.785	.813	.797	.780	.771	.767	.800	.821	.824	.796	.805		
26	.732	.658	.727	.727	.731	.678	.647	.683	.711	.794	.777	.755	.798	.758	.781	.765	.747	.738	.734	.775	.795	.797	.769	.780	.998	

Appendix VII (a). Average Linkage Cluster Analysis of five cultivars of *B. napus* and one of *B. rapa* grown at different sites. Based on 10 peaks (210 nm) shown in Appendix I (a).

```
      1.6 +
          |
          |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
          |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
A         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
v     1.4 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
e         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
r         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
a         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
g         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
e     1.2 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
          |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
D         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
i         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
s         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
t       1 +XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
a         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXX
n         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXX
c         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXX
e         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXX
      0.8 +XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXX
B         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXX
e         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXX
t         |XXXXXXX XXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXX XXX XXX
w         |XXXXXXX XXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXX XXX XXX
e     0.6 +XXXXXXX XXXXXXXXXXXXXXXXXXXXX XXXXXXX XXXXX XXX XXX
e         |XXXXXXX . XXXXXXXXXXXXXXXXXXX XXXXXXX XXXXX XXX XXX
n         |XXXXXXX . XXXXXXXXX XXXXXXXXX XXXXXXX XXXXX XXX XXX
          |XXXXXXX . XXXXXXXXX XXXXXXXXX XXXXXXX XXX . XXX . .
C         |XXXXX . . XXXXXXXXX XXXXXXXXX XXXXXXX XXX . XXX . .
l     0.4 +XXXXX . . . XXXXXXX XXXXXXXXX XXXXXXX XXX . XXX . .
u         |XXXXX . . . XXXXXXX XXXXXXX . XXXXXXX XXX . XXX . .
s         |XXXXX . . . XXXXXXX XXXXXXX . XXXXXXX . . . XXX . .
t         |XXX . . . . XXXXXXX XXXXXXX . XXXXXXX . . . XXX . .
e         |. . . . . . . XXX . XXXXX . . XXXXXXX . . . XXX . .
r     0.2 +. . . . . . . XXX . XXXXX . . XXXXX . . . . XXX . .
s         |. . . . . . . . . . XXXXX . . XXX . . . . . XXX . .
          |. . . . . . . . . . XXXXX . . XXX . . . . . . . . .
          |. . . . . . . . . . XXX . . . . . . . . . . . . . .
          |. . . . . . . . . . XXX . . . . . . . . . . . . . .
        0 +. . . . . . . . . . . . . . . . . . . . . . . . . .
           D D D D D H H H H H S S S S S R R R R W W W W W T T
           E E E E E E E E E E T T T T T E E E E E E E E E O O
CULTIVAR   L L L L L R R R R R E E E E E G G G G S S S S S B B
           T T T T T O O O O O L L L L L E E E E T T T T T I I
           A A A A A           L L L L L N N N N A A A A A N N
                               A A A A A E E E E R R R R R
                               R R R R R T T T T

           D R T P W L B D R T B T P P R B C P T P R W D T D W
SITE       A O E A A A A E O E A E O O O A A A I A O A A E A A
           U B U S S S G U B U G U R I B X N D S S B S U U U S
```

Appendix VII (b). Single Linkage Cluster Analysis of five cultivars of *B. napus* and one of *B. rapa* grown at different sites. Based on 10 peaks (210 nm) shown in Appendix III (a).

```
      1.4 +
M         |
i         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
n         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
i         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
m     1.2 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
u         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
m         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
          |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
D         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
i       1 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
s         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
t         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
a         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
n         |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
c     0.8 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
e         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
          |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
B         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
e         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
t     0.6 +XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXX
w         |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX . .
e         |XXXXXXX . XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX . .
e         |XXXXXXX . XXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXX . .
n         |XXXXXXX . XXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXX . .
      0.4 +XXXXXXX . XXXXXXXXXXXXXXXXXXX XXXXXXX . XXX XXX . .
C         |XXXXX . . XXXXXXXXX XXXXXXXXX XXXXXXX . XXX XXX . .
l         |XXX . . . XXXXXXXXX XXXXXXX . XXXXXXX . . . XXX . .
u         |. . . . . . . XXXXX XXXXX . . XXXXXXX . . . XXX . .
s         |. . . . . . . . XXX XXXXX . . XXXXXXX . . . XXX . .
t     0.2 +. . . . . . . . XXX XXXXX . . XXXXXXX . . . XXX . .
e         |. . . . . . . . . . XXXXX . . XXX . . . . . . . . .
r         |. . . . . . . . . . XXX . . . XXX . . . . . . . . .
s         |. . . . . . . . . . XXX . . . . . . . . . . . . . .
          |. . . . . . . . . . . . . . . . . . . . . . . . . .
        0 +. . . . . . . . . . . . . . . . . . . . . . . . . .
           D D D D D H H H H H S S S S S R R R R W W W W W T T
           E E E E E E E E E E T T T T T E E E E E E E E E O O
CULTIVAR   L L L L L R R R R R E E E E E G G G G S S S S S B B
           T T T T T O O O O O L L L L L E E E E T T T T T I I
           A A A A A           L L L L L N N N N A A A A A N N
                               A A A A A E E E E R R R R R
                               R R R R R T T T T

           D R T P W L B D R T B T P P R B C P T P R W D T D W
SITE       A O E A A A A A O E A E O O O A A A I A O A A E A A
           U B U S S S G U B U G U R I B X N D S S B S U U U S
```

Appendix VII (c). Complete Linkage Cluster Analysisof five cultivars of *B. napus* and one of *B. rapa* grown at different sites. Based on 10 peaks (210 nm) shown in Appendix III (a).

```
              2.25 +
                   |
            M      |
            a      |
            x    2 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
            i      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
            m      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
            u      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
            m 1.75 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
                   |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
            D      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
            i      |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
            s  1.5 +XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
            t      |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX
            a      |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXX XXX
            n      |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXX XXX
            n      |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXX XXX
            c 1.25 +XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXX XXX
            e      |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXX XXX
                   |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXX XXX XXX
            B      |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXX XXX XXX
            e    1 +XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXX XXX XXX
            t      |XXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXX XXX XXX
            w      |XXXXXXX XXXXXXXXXXXXXXXXXXX XXXXX XXXXXXXXX XXX XXX
            e      |XXXXXXX XXXXXXXXXXXXXXXXXXX XXXXX XXXXXXXXX XXX XXX
            e 0.75 +XXXXXXX XXXXXXXXXXX XXXXXXX XXXXX XXXXXXXXX XXX XXX
            n      |XXXXXXX XXXXXXXXXXX XXXXXXX XXXXX XXXXXXXXX XXX XXX
                   |XXXXXXX XXXXXXXXXXX XXXXXXX XXXXX XXXXXXXXX XXX XXX
            C      |XXXXX . . XXXXXXXXX XXXXXXX XXX . . XXXXXXX XXX XXX
            l  0.5 +XXXXX . . XXXXXXXXX XXXXXXX XXX . . XXXXXXX XXX . .
            u      |XXXXX . . XXXXXXXXX XXXXXXX XXX . . XXXXXXX XXX . .
            s      |XXXXX . . XXXXXXX . XXXXXXX XXX . . XXXXXXX XXX . .
            t      |XXX . . . XXXXXXX . XXXXXXX . . . . XXX XXX XXX . .
            e 0.25 +. . . . . XXXXX . . XXXXX . . . . . . . XXX XXX . .
            r      |. . . . . XXXXX . . XXX . . . . . . . . XXX XXX . .
            s      |. . . . . XXX . . . XXX . . . . . . . . . . . . . .
                   |. . . . . XXX . . . . . . . . . . . . . . . . . . .
                 0 +. . . . . . . . . . . . . . . . . . . . . . . . . .
                    D D D D D H H H H H S S S S S R R R R W W W W W T T
                    E E E E E E E E E E T T T T T E E E E E E E E E O O
CULTIVAR            L L L L L R R R R R E E E E E G G G G S S S S S B B
                    T T T T T O O O O O L L L L L E E E E T T T T T I I
                    A A A A A           L L L L L N N N N A A A A A N N
                                        A A A A A E E E E R R R R R
                                        R R R R R T T T T

                    D R T P W L B D R T B T P P R B C P T P R W D T D W
SITE                A O E A A A A A O E A E O O O A A A I A O A A E A A
                    U B U S S S G U B U G U R I B X N D S S B S U U U S
```

Appendix VIII. Areas (% of total peaks) and retention times (min.) of 10 peaks for 27 cultivars of *B. napus* and two of *B. rapa* (210 nm).

PEAK IDENTIFICATION

	A	B	C	D	E	F	G	H	I	J
					(Retention Time)					
	21.94	25.36	25.90	26.87	29.92	31.15	31.46	32.28	33.03	35.66
Cultivar					Area (%)					
Argentine	3.279	3.864	29.783	8.891	10.765	6.603	11.363	14.808	5.347	5.297
Bounty	2.728	5.189	31.002	1.328	3.312	9.399	18.246	7.413	1.620	19.762
Bronowski	1.233	4.443	38.284	1.845	3.479	11.632	13.361	9.680	0.000	16.043
Celebra	3.379	7.889	25.682	4.755	4.077	7.268	21.327	14.549	3.354	7.720
Colt	.929	3.510	36.794	4.641	4.998	5.545	34.574	.661	2.475	5.874
Crystal	3.919	8.296	37.989	1.845	4.800	4.765	18.545	10.770	2.302	6.768
Delta	1.575	3.621	30.607	1.415	2.138	7.893	20.557	5.018	1.611	25.564
Excel	2.549	6.099	31.348	1.351	6.840	8.016	12.024	13.188	4.634	13.950
Global	1.810	8.653	42.710	1.559	3.551	3.101	16.868	13.729	1.800	6.220
Hero	7.816	12.305	36.204	3.975	2.202	7.013	18.143	6.522	1.217	4.602
Horizon	1.021	3.525	37.617	4.788	6.099	6.169	29.150	2.145	3.054	6.432
Hyola	1.699	5.121	34.712	2.014	2.827	7.352	15.154	12.889	.000	18.232
Legend	2.642	6.108	30.605	3.275	2.698	8.699	19.912	16.089	2.726	7.244
Midas	3.091	7.277	40.022	1.977	0.863	9.483	14.602	16.814	2.958	2.914
Oro	4.287	6.214	29.541	5.340	8.066	6.437	12.059	14.028	8.622	5.408
Pivot	3.297	9.171	30.270	2.363	9.037	5.557	13.413	10.273	8.738	7.881
Profit	1.188	6.479	30.102	1.712	6.507	7.078	17.757	15.315	5.405	8.457
Regent	3.523	8.333	29.699	5.630	8.793	6.243	12.495	12.458	7.704	5.121
Reston	2.783	8.280	28.680	2.471	10.277	6.983	16.114	14.683	6.149	3.581
Stallion	2.527	6.539	37.847	2.293	5.544	4.293	20.661	10.712	1.489	8.095
Stellar	1.888	5.814	38.002	5.580	8.581	9.184	14.487	6.008	3.692	6.763
Topas	1.859	5.620	32.472	1.756	3.828	7.047	18.436	16.461	3.199	9.322
Touchdown	1.300	4.872	38.291	1.580	1.948	7.650	23.972	10.528	.000	9.858
Tower	1.788	6.809	36.658	3.754	6.542	6.347	17.105	11.102	5.024	4.871
Tribute	1.286	7.247	36.095	1.848	2.500	8.669	17.450	12.227	5.394	7.284
Tristar	2.293	6.011	34.484	1.417	7.391	6.293	20.175	10.976	6.051	4.909
Vanguard	1.977	5.114	32.321	1.904	2.599	8.444	22.585	13.451	4.951	6.653
Westar	3.094	5.787	29.457	3.983	7.647	6.510	15.216	14.309	9.328	4.669
Winfield	2.392	3.396	32.869	1.619	1.584	7.633	24.385	12.276	1.896	11.950

Appendix IX. Correlation matrix of HPLC data (210 nm) on 27 cultivars of *B. napus* and two of *B. rapa* analysed with PHYLO software (Pearson's product moment correlation coefficients).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	.704														
3	.834	.958													
4	.817	.839	.838												
5	.715	.817	.770	.883											
6	.892	.870	.916	.933	.875										
7	.595	.981	.894	.763	.778	.779									
8	.873	.924	.972	.854	.733	.929	.853								
9	.917	.847	.914	.921	.822	.993	.749	.940							
10	.796	.815	.863	.877	.864	.965	.717	.834	.946						
11	.784	.849	.830	.894	.992	.917	.799	.798	.873	.896					
12	.814	.969	.979	.872	.761	.911	.923	.975	.913	.835	.811				
13	.870	.856	.892	.965	.846	.952	.766	.906	.949	.891	.876	.907			
14	.910	.790	.894	.905	.757	.956	.668	.911	.970	.919	.813	.873	.957		
15	.965	.745	.855	.857	.748	.941	.633	.911	.962	.858	.813	.839	.909	.959	
16	.894	.819	.882	.851	.803	.962	.728	.933	.966	.893	.860	.867	.885	.919	.960
17	.900	.862	.901	.958	.831	.959	.775	.947	.962	.865	.871	.916	.978	.945	.945
18	.957	.753	.859	.863	.778	.957	.639	.906	.974	.891	.840	.836	.904	.954	.991
19	.923	.734	.816	.908	.798	.942	.615	.879	.947	.862	.843	.808	.930	.936	.959
20	.894	.892	.920	.948	.903	.995	.813	.931	.983	.946	.939	.923	.958	.936	.929
21	.912	.847	.928	.833	.854	.953	.757	.908	.944	.922	.912	.874	.876	.913	.924
22	.891	.884	.920	.968	.824	.961	.799	.950	.965	.879	.864	.940	.990	.956	.932
23	.845	.929	.934	.965	.920	.972	.860	.921	.952	.930	.946	.937	.972	.923	.884
24	.934	.832	.903	.917	.868	.987	.732	.921	.986	.937	.916	.882	.946	.962	.971
25	.887	.879	.933	.937	.851	.979	.786	.943	.979	.932	.896	.919	.970	.975	.949
26	.899	.835	.876	.936	.902	.979	.743	.904	.966	.919	.938	.864	.950	.937	.950
27	.850	.865	.882	.980	.902	.956	.784	.894	.942	.899	.924	.888	.988	.938	.907
28	.939	.754	.836	.899	.801	.942	.648	.896	.953	.854	.851	.828	.934	.950	.988
29	.802	.934	.911	.969	.908	.937	.882	.905	.915	.876	.925	.934	.969	.888	.846

	16	17	18	19	20	21	22	23	24	25	26	27	28	29
17	.945													
18	.976	.943												
19	.948	.964	.968											
20	.951	.966	.944	.938										
21	.943	.899	.946	.897	.950									
22	.920	.992	.924	.939	.968	.891								
23	.905	.959	.893	.899	.985	.926	.971							
24	.972	.964	.984	.964	.983	.971	.955	.956						
25	.955	.974	.954	.938	.974	.945	.976	.972	.983					
26	.965	.972	.960	.970	.981	.945	.956	.962	.988	.974				
27	.904	.975	.905	.930	.965	.892	.978	.981	.955	.975	.973			
28	.958	.966	.979	.975	.938	.908	.947	.905	.973	.958	.973	.944		
29	.863	.951	.843	.865	.958	.869	.967	.989	.916	.946	.936	.978	.881	

Appendix X a. Average Linkage Cluster Analysis of 27 cultivars of *B. napus* and 2 of *B. rapa* based on peak areas of 10 peaks shown in Appendix II (a).

```
           1.4 +
        |
        |
        |
        |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
A       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXX
v   1.2 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXX
e       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXX
r       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXXXX
a       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXXXX
g       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXXXX
e       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXXXX
      1 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXXXX
D       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXX XXX XXXXXXX
i       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXX XXX XXXXXXX
s       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXX XXX XXXXX .
t       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXX XXX XXXXX .
a       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXX . XXX XXXXX .
n   0.8 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXX . XXX XXXXX .
c       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXX . . XXX XXXXX .
e       |XXXXXXXXXXXXXXXXXXXXX XXXXXXX XXXXXXXXXXX . . XXX XXXXX .
        |XXXXXXXXXXXXXXXXXXXXX XXXXXXX XXXXXXXXXXX . . XXX XXXXX .
B       |XXXXXXXXXXXXXXXXXXX . XXXXXXX XXXXXXX XXX . . XXX XXXXX .
e       |XXXXXXXXXXX XXXXXXX . XXXXXXX XXXXX . XXX . . XXX XXXXX .
t   0.6 +XXXXXXXXXXX XXXXXXX . XXXXXXX XXXXX . XXX . . XXX XXXXX .
w       |XXXXXXXXXXX XXXXXXX . XXXXX . XXXXX . XXX . . XXX . XXX .
e       |XXXXXXXXXXX . XXXXX . XXXXX . XXXXX . XXX . . XXX . XXX .
e       |XXX XXXXXXX . XXXXX . . XXX . XXXXX . XXX . . XXX . XXX .
n       |XXX XXXXXXX . XXXXX . . XXX . XXX . . XXX . . XXX . XXX .
        |XXX XXXXXXX . XXXXX . . XXX . XXX . . . . . . XXX . XXX .
C   0.4 +. . XXXXXXX . XXX . . . XXX . XXX . . . . . . XXX . . . .
l       |. . . XXXXX . XXX . . . . . . XXX . . . . . . . . . . . .
u       |. . . XXXXX . XXX . . . . . . XXX . . . . . . . . . . . .
s       |. . . XXX . . XXX . . . . . . . . . . . . . . . . . . . .
t       |. . . XXX . . XXX . . . . . . . . . . . . . . . . . . . .
e       |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r   0.2 +. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
s       |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
        |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
        |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
        |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
        |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
      0 +. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
         A O P R W R S T T T E C L P W C S T G T V M H C H B B H D
         r r i e e e t o r r x e e r i r t o l o a i e o o o r y e
         g o v g s s e w i i c l g o n y a u o p n d r l r u o o l
         e   o e t t l e s b e e e f f s l c b a g a o t i n n l t
         n   t n a o l r t u l b n i i t l h a s u s     z t o a a
         t     t r n a   a t   r d t e a i d l   a       o y w
         i           r   r e   a     l l o o     r       n   s
         n                           d   n w     d           k
         e                                 n                 i
```

Appendix X b. Single Linkage Cluster Analysis of 27 cultivars of *B. napus* and 2 of *B. rapa* based on peak areas of 10 peaks shown in Appendix VII (a).

```
   0.8 +
       |
       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXXXX
M      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXX .
i  0.7 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXX .
n      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXX .
i      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX . XXX XXXXX .
m      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX . XXX XXXXX .
u      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX . XXX XXXXX .
m  0.6 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX . XXX XXXXX .
       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX . XXX XXXXX .
D      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX . XXX . XXX XXXXX .
i      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX . . . XXX . XXX . XXX .
s      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX . . . . XXX . XXX . XXX .
t  0.5 +XXXXXXXXXXX XXXXXXXXXXXXXXXXX . . . . . XXX . XXX . XXX .
a      |XXXXXXXXXXX XXXXXXXXXXXXXXXXX . . . . . . . . XXX . XXX .
n      |XXXXXXXXXXX XXXXXXXXXXXXXXXXX . . . . . . . . XXX . . . .
c      |XXXXXXXXXXX XXXXXXXXXXX . XXX . . . . . . . . XXX . . . .
e      |. XXXXXXXXX XXX XXX . . . XXX . . . . . . . . XXX . . . .
   0.4 +. . XXXXXXX XXX XXX . . . . . . . . . . . . . . . . . . .
B      |. . XXXXXXX XXX XXX . . . . . . . . . . . . . . . . . . .
e      |. . XXXXXXX XXX XXX . . . . . . . . . . . . . . . . . . .
t      |. . XXXXXXX . . XXX . . . . . . . . . . . . . . . . . . .
w      |. . XXXXXXX . . XXX . . . . . . . . . . . . . . . . . . .
e  0.3 +. . . XXX . . . XXX . . . . . . . . . . . . . . . . . . .
e      |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
n      |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
       |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
C      |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
l  0.2 +. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
u      |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
s      |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
t      |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
e      |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r  0.1 +. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
s      |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
       |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
       |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
       |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
     0 +. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
        A O P R W R S T T T E C L P W C S T G T V M H C H B B H D
        r r i e e e t o r r x e e r i r t o l o a i e o o o r y e
        g o v g s s e w i i c l g o n y a u o p n d r l r u o o l
        e   o e t t l e s b e e e f f s l c b a g a o t i n n l t
        n   t n a o l r t u l b n i i t l h a s u s     z t o a a
        t     t r n a   a t   r d t e a i d l   a       o y w
        i           r   r e   a     l l o o     r       n   s
        n                           d   n w     d           k
        e                                 n                 i
```

Appendix X c. Complete Linkage Cluster Analysis Analysis of 27 cultivars of *B. napus* and 2 of *B. rapa* based on peak areas of 10 peaks shown in Appendix VII (a)

```
   2.5 +
       |
       |
       |
M      |
a      |
x      |
i      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
m    2 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXX
u      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXX
m      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXX
       |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXX
D      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXX
i      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXX
s      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXXXX
t      |XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXXXX
a  1.5 +XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXX XXXXXXX
n      |XXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXX XXX XXXXXXX
c      |XXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXX XXX XXXXXXX
e      |XXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXX XXX XXXXXXX
       |XXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXX XXX XXXXXXX
B      |XXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXX XXXXXXX XXX XXXXXXX
e      |XXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXX XXXXXXX XXX XXXXX .
t      |XXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXX XXXXXXX XXX XXXXX .
w    1 +XXXXXXXXXXXXXXXXXXXXX XXXXX XXXXXXXXX XXXXXXX XXX XXXXX .
e      |XXXXXXXXXXXXXXXXXXXXX XXXXX XXXXXXX . XXXXXXX XXX XXXXX .
e      |XXXXXXXXXXXXXXXXXXXXX XXXXX XXXXXXX . XXXXXXX XXX XXXXX .
n      |XXXXXXXXXXX XXXXXXXXX XXXXX XXXXX . . XXXXXXX XXX XXXXX .
       |XXXXXXXXXXX . XXXXXXX XXXXX XXXXX . . XXX XXX XXX XXXXX .
C      |XXX XXXXXXX . XXXXXXX XXXXX XXXXX . . XXX XXX XXX XXXXX .
l      |XXX XXXXXXX . XXXXXXX XXXXX XXXXX . . XXX XXX XXX . XXX .
u      |XXX XXXXXXX . . XXXXX XXXXX XXX . . . XXX XXX XXX . XXX .
s  0.5 +XXX XXXXXXX . . XXXXX . XXX XXX . . . XXX . . XXX . XXX .
t      |XXX . XXXXX . . XXX . . XXX XXX . . . . . . . XXX . . . .
e      |. . . XXXXX . . XXX . . . . XXX . . . . . . . . . . . . .
r      |. . . XXX . . . XXX . . . . . . . . . . . . . . . . . . .
s      |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
       |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
       |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
       |. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
     0 +. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
        A O P R W R S T T T E C L P W C S T G T V M H C H B B H D
        r r i e e e t o r r x e e r i r t o l o a i e o o o r y e
        g o v g s s e w i i c l g o n y a u o p n d r l r u o o l
        e   o e t t l e s b e e e f f s l c b a g a o t i n n l t
        n   t n a o l r t u l b n i i t l h a s u s     z t o a a
        t     t r n a   a t   r d t e a i d l   a       o y w
        i           r   r e   a     l l o o     r       n   s
        n                           d   n w     d           k
        e                                 n                 i
```

Appendix XI. Data matrix of RAPD data for 24 cultivars of *B. napus*. Primer are identified with UBC (University of British Columbia) code numbers. Marker size is in kilobases.

CULTIVAR

Cultivars		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Primer (UBC)	Marker Size																								
302	2.2	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
	2.0	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0
	1.3	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
	1.0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
	0.7	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	0	0	0	1	1	1	1	1
303	1.75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1
	1.7	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1
	1.6	0	0	1	0	1	0	0	0	0	1	0	1	1	1	0	0	1	0	0	1	0	0	1	1
	1.5	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	1	1
	1.1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1.0	1	1	0	0	1	1	1	0	1	0	1	1	0	0	1	1	1	0	0	1	1	1	1	1
	0.7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
	0.6	1	1	0	1	0	1	1	0	1	1	1	1	0	0	0	0	0	1	0	0	1	1	1	1
329	1.6	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	1.5	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
	1.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
	1.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	0.7	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
	0.4	0	1	1	1	0	1	0	1	0	0	1	0	1	0	1	1	0	0	0	0	1	1	0	0
	0.3	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
	0.2	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	0	0
341	2.1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	1
	1.9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1.5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	1.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1.3	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0
	1.1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	0.7	0	0	0	0	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	1	1	0	0	0
	0.6	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
346	2.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1.7	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	0	1	1
	1.5	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	1	0	0
	1.0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
	0.8	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	0
	0.6	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
354	1.6	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
	1.2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	0.9	1	0	0	1	1	0	0	1	1	0	0	1	0	1	1	1	1	0	0	0	0	1	0	0
	0.8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	0.7	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	0.6	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
	0.4	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1

Cultivars:
Stellar 1, Westar 2, Hero 3, Regent 4, Delta 5, Excel 6, Midas 7, Tristar 8, Argentine 9, Bronowski 10, Profit 11, Stallion 12, Horizon 13, Crystal 14, Legend 15, Vanguard 16, Global 17, Touchdown 18, Winfield 19, Bounty 20, Hyola 21, Tower 22, Oscar 23, Yickadee 24.

Appendix XII. Pairwise comparisons of Neis' similarity indices (Nei and Li 1979) determined from data matrix in Table 2. Cultivar identification as for Table 2.

CULTIVAR

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	0.000																							
2	0.929	0.000																						
3	0.852	0.893	0.000																					
4	0.912	0.949	0.877	0.000																				
5	0.897	0.900	0.862	0.918	0.000																			
6	0.897	0.967	0.862	0.951	0.903	0.000																		
7	0.909	0.947	0.873	0.897	0.881	0.915	0.000																	
8	0.893	0.931	0.893	0.949	0.933	0.900	0.877	0.000																
9	0.909	0.912	0.800	0.897	0.915	0.881	0.929	0.912	0.000															
10	0.846	0.815	0.846	0.836	0.857	0.821	0.792	0.815	0.792	0.000														
11	0.912	0.949	0.877	0.933	0.918	0.951	0.897	0.915	0.897	0.873	0.000													
12	0.931	0.933	0.862	0.951	0.968	0.935	0.915	0.900	0.915	0.857	0.918	0.000												
13	0.383	0.490	0.511	0.520	0.510	0.549	0.458	0.490	0.417	0.489	0.480	0.510	0.000											
14	0.909	0.912	0.909	0.931	0.949	0.881	0.893	0.947	0.893	0.830	0.862	0.949	0.500	0.000										
15	0.897	0.933	0.862	0.918	0.935	0.935	0.881	0.967	0.915	0.786	0.918	0.903	0.510	0.915	0.000									
16	0.897	0.933	0.862	0.918	0.935	0.935	0.881	0.967	0.915	0.786	0.918	0.903	0.510	0.915	1.000	0.000								
17	0.909	0.912	0.873	0.897	0.949	0.881	0.893	0.912	0.893	0.830	0.862	0.949	0.500	0.964	0.915	0.915	0.000							
18	0.863	0.868	0.824	0.852	0.800	0.836	0.885	0.830	0.808	0.857	0.815	0.836	0.455	0.846	0.800	0.800	0.846	0.000						
19	0.769	0.815	0.769	0.800	0.786	0.786	0.792	0.815	0.755	0.760	0.764	0.786	0.489	0.830	0.786	0.786	0.830	0.857	0.000					
20	0.857	0.862	0.893	0.814	0.900	0.833	0.877	0.862	0.842	0.815	0.881	0.867	0.449	0.877	0.867	0.867	0.877	0.792	0.778	0.000				
21	0.852	0.921	0.852	0.906	0.892	0.923	0.903	0.889	0.871	0.814	0.938	0.892	0.481	0.839	0.892	0.892	0.839	0.828	0.814	0.857	0.000			
22	0.909	0.912	0.873	0.931	0.881	0.915	0.857	0.877	0.857	0.830	0.931	0.915	0.458	0.857	0.881	0.881	0.857	0.769	0.717	0.807	0.871	0.000		
23	0.909	0.912	0.873	0.862	0.881	0.881	0.929	0.842	0.857	0.830	0.862	0.915	0.458	0.893	0.847	0.847	0.893	0.885	0.792	0.877	0.871	0.821	0.000	
24	0.873	0.877	0.836	0.828	0.847	0.847	0.893	0.807	0.821	0.830	0.828	0.881	0.458	0.857	0.814	0.814	0.857	0.885	0.830	0.842	0.871	0.786	0.964	0.00

Appendix XIII. Ultra-violet scan of DNA extracted from *B. napus* as described (5.7).