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ARTICLE

Using DRIFT Molecular Spectroscopy with Uni- and Multivariate Spectral Techniques To Detect Protein Molecular Structure Differences among Different Genotypes of Barley

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The objectives of this study were to characterize protein molecular structure using DRIFT spectroscopy with univariate and multivariate molecular spectral analyses and identify the structure differences in both hull and seeds among six spring barley varieties [AC Metcalfe (malting-type), CDC Dolly (feed-type, spring forage type), McLeod (feed-type), CDC Helgason (feed-type), CDC Trey (feed-type), CDC Cowboy (feed-type)]. The molecular structure spectral analyses involved protein amide I and II region ca. 1716−1485 cm^{-1} (attributed to protein amide I C═O and C−N stretching; amide II N−H bending and C−N stretching) together with agglomerative hierarchical cluster (CLA) and principal component analyses (PCA). The results showed that the molecular spectral techniques were able to identify spectral differences associated with the molecular structural differences among the barley varieties. The molecular spectral analyses at the region of ca. 1715−1485 cm^{-1} together with the cluster and principal component analyses were able to show that the molecular structures of the seeds (NOT hull) exhibited distinguished differences among the barley varieties. It was found that CDC Helgason had the distinguished differences from AC Metcalfe, McLeod, and CDC Cowboy in both protein amide I and II. The molecular spectral technique provides a new approach for plant protein molecular structure and biopolymer conformation study.

KEYWORDS: Protein structure; barley variety; molecular spectral analyses

INTRODUCTION

Biopolymer matrix and conformation as well as molecular structure affect barley functionality, quality, rumen degradation kinetics, and biochemical characterization (*1*−*3*). There are two types of barley, malting-type and feed-type. These two types of barley have different degradation kinetics (*4*−*6*) although they have similar chemical composition detected by traditional "wet" chemical analysis. Yu et al. (*5*) showed that Harrington and Valier barley have similar chemical profiling, but Valier barley biodegradation is slower and lower (than Harrington barley), which is more suitable for feed purpose. Harrington barley degradation is faster and higher, which is suitable for beer-making. Modeling nutrient supply results also show there are significant differences in nutrient availability between these two varieties in terms of metabolizable protein (which is total truly digested and absorbed protein in the small intestine).

The biological function differences are expected to be related to molecular structure difference and biopolymer conformation between the varieties of barley. However, conventional "wet" chemical analyses fail to detect the molecular structure difference and fail to detect structural chemical makeup, mainly because the conventional "wet" chemical analyses rely heavily on the use of harsh chemicals and derivatization which can destroy the native physiochemical and molecular structures during the chemical analysis (*7*).

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The use of FTIR spectroscopy to determine malt barley was reported (*8*), but not the use of diffuse reflectance infrared Fourier transform spectroscopy (DRIFT). DRIFT has been developed as a rapid and direct bioanalytical technique. This technique is capable of exploring the molecular chemistry and biopolymer conformation through molecular and functional group spectral analyses. To date there has been very little application of the DRIFT technique plus multivariate molecular spectral analyses (*9*) to the study of barley molecular structure (chemical makeup) in both hull and seeds.

The objective of this study was to use the DRIFT technique with the multivariate analysis technique as a new approach to identify the differences in protein molecular structure (chemical makeup). It was expected that differences in the structural conformation and chemical makeup of the hull and whole seed of barley will help us to understand barley functionality, biodegradation kinetics, nutrient availability and interaction between the structure and biological functions. This study also demonstrated that DRIFT analytical technology combined with the multivariate molecular spectral analyses will provide a new approach and a new opportunity for plant internal structure research.

MATERIALS AND METHODS

Barley Varieties and Growth Condition. Six varieties of barley [AC Metcalfe (malting-type), CDC Dolly (feed-type), McLeod (feed-type), CDC Helgason (feed-type), CDC Trey (feed-type), CDC Cowboy (feed-type)] were obtained from Professor B. G. Rossnagel, Crop Development Center,

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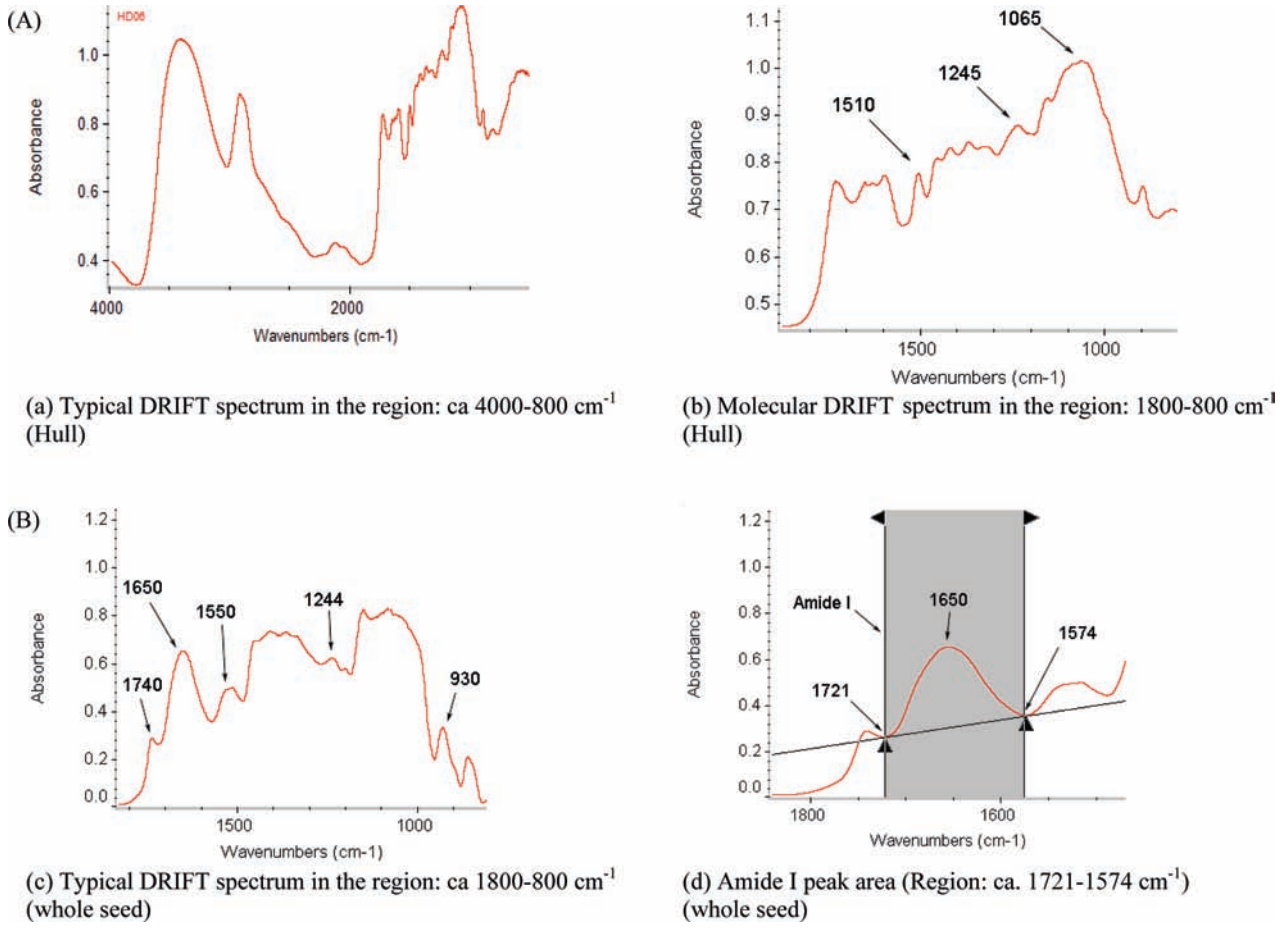


Figure 1. Typical DRIFT spectrum of barley hull (**A**) and whole seeds (**B**). (**a**) Typical DRIFT spectrum in the region ca. 4000−800 cm^{-1} in hull; (**b**) molecular spectrum in the region 1800−800 cm^{-1} in hull; (**c**) typical DRIFT spectrum in the region ca. 1800−800 cm^{-1} in whole seeds; (**d**) amide I peak area: amide I peak center at ca. 1650 cm^{-1}; region ca. 1721−1574 cm^{-1} in whole seeds.

The University of Saskatchewan, SK, Canada. All barley varieties used in this study were grown in 2005, without irrigation, at the Kernen Crop Research Farm with various plots, University of Saskatchewan, Saskatoon, Canada, and were managed using the same and standard agronomic production practices for all barley production. The growth climate conditions were 17.5 °C highest mean temperatures and 455 mm rainfall during the 2005 year. The highest mean temperatures and rainfall were 18.6 °C and 317 mm, respectively. The detailed protein profiles and subfractions as well as physicochemcal features were reported by Hart et al. (*10*) and Du et al. (*11*).

Infrared Spectroscopy−DRIFT Molecular Technique. For DRIFT molecular structural study, all the samples were finely ground in the same way, through 0.25 mm screen two times and then mixed with potassium bromide (KBr, IR grade, P5510, Sigma) in a ratio of 1 part of sample to 4 parts of KBr in a 2 mL centrifuge tube and mixed by vortexing for minutes. DRIFT was performed in a diffuse reflection mode using a Bio-Rad FTS-40 with a Ceramic IR source and MCT detector (Bio-Rad laboratories, Hercules, CA) at the Saskatchewan Structural Sciences Center (SSSC), University of Saskatchewan, Saskatoon, Canada. The data were collected using Win-IR software (Bio-Rad Digilab, Cambridge, MA). Spectra were generated from the 4000−500 cm^{-1} portion of the electromagnetic (EM) spectrum with 256 coadded scans and a spectral resolution of 4 cm^{-1}. Spectral analysis was done with OMNIC 7.3 (Spectra Tech, Madison, WI) software. Typical DRIFT spectra of barley hull and whole seeds in both the mid-IR region (ca. 4000−800 cm^{-1}) and the fingerprint region (ca. 1800−800 cm^{-1}) are present in **Figure 1**. For the details about DRIFT molecular spectroscopy, please see a thesis published by Liu in 2010 (*12*).

Molecular Spectral Data Analyses. *Molecular Spectral Features and Chemical Functional Groups*. Protein molecular spectral features were identified according to published reports (*13*−*21*). The regions of specific interest in the present study included the protein amide I at ca. 1650 cm^{-1} and amide II at ca. 1550 cm^{-1} bands in the IR regions of ca. 1715−1485 cm^{-1} (**Figure 1**)

Univariate and Multivariate Molecular Spectral Data Analyses. Two approaches to analyze molecular spectral data collected under DRIFT usually include univariate and multivariate methods. The univariate methods consisted of various spectral data analysis and included IR absorbed intensities (spectral peak area and height) (*16*, *19*). Spectral peak area or height ratios were obtained by the area or height under one functional group band (such as protein amide I ca. 1650 cm^{-1}) divided by the area or height under another functional group band (such as aromatic lignin ca. 1510 cm^{-1}). The multivariate methods of data analysis created spectral corrections by utilizing the entire spectral information. The multivariate analyses included agglomerative hierarchical cluster analysis (CLA), using Ward's algorithm method without prior parametrization, and principal component analysis (PCA), which were performed using Statistica software 8.0 (StatSoft Inc., Tulsa, OK).

Barley Hull Preparation. Barley hulls were obtained by dehulling the barley grains using a laboratory dehuller (model LH 5095, Codema Inc. Minneapolis, MN) at 100 psi for 30 s. Hulls were screened through a 1.2 mm pore size sieve to remove fines and dusts and continued to be divided into broken kernels and hulls by gently blowing the hulls off using an electric fan. The pure hulls were collected for analysis. Barley samples were ground in a Retsch mill (model ZM-1, Brinkmann Instruments Ltd.,Ontario, Canada) through 0.25 mm pore-size mesh screens twice at 10,000 rpm in preparation for analysis.

Statistical Analysis. Statistical analyses were performed using Proc Mixed of SAS (*22*) with a CRD model as follows:

$$Y_{ij} = \mu + v_i + e_{ij}$$

where Y_{ij} is an observation of the dependent variables; μ is the overall mean; v_i is the fixed effect of the ith barley variety ($i = 1-6$); and e_{ij} is the

Table 1. The Structural Characteristics of Protein Amide I and II and Their Ratios in Whole Barley Seed, Revealed Using DRIFT Spectroscopy: Comparison of Six Genotypes of Barley

				molecular characteristics of whole barley seed in terms of IR peak area and their ratios (IR absorbed intensity unit)[a]							
item	peak center (cm^{-1})	region (cm^{-1})	baseline (cm^{-1})	AC Metcalfe[b]	CDC Dolly[c]	McLeod[c]	CDC Helgason[c]	CDC Trey[c]	CDC Cowboy[c]	SEM[d]	*P* value
barley amide I peak area	~1650	1721−1574	1721−1574	24.91 b	23.77 c	23.44 c	23.95 c	24.04 c	27.61 a	0.222	<0.0001
barley amide I peak height	~1650	1721−1574	1721−1574	0.36 a	0.35 b	0.33 c	0.30 d	0.30 d	0.35 ab	0.003	<0.0001
barley amide I and II area		1721−1487	1721−1487	22.73 b	21.08 c	20.46 cd	20.51 cd	19.85 d	23.72 a	0.296	<0.0001

[a] Based on the amide I and II peak area and height. Means with the different letter in the same row are significantly different ($P < 0.05$). [b] Malting type. [c] Feed type. [d] SEM = pooled standard error of means.

error term. The experimental unit was barley sample within each variety. The Fisher's Protected LSD test was used to determine the differences among the treatments. Significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Detecting the Differences in Molecular Structural Makeup in the Seeds among the Barley Varieties. DRIFT spectroscopy is able to identify functional groups. Each biopolymer or biological component or functional group in biological tissues has unique molecular chemical−structural features, which result in unique IR absorption and thus a unique IR spectrum (*18*, *20*). The IR spectrum involving the fundamental vibration from ca. 4000−800 cm^{-1} has been a useful tool for describing the molecular structure of biological compounds. From the IR spectrum, the presence or absence of various organic functional groups is readily observed (*15*, *17*). DRIFT spectroscopy can be used to increase the fundamental understanding of the inherent chemical structures of plant/food/feed tissues. **Table 1** shows the IR absorbed peak area and height of functional groups in the seeds among the six barley varieties at the wavenumbers of ca. 1650 and 1550 cm^{-1} representing protein amide I (peptide C═O bond) and amide II, respectively. The protein spectrum has two primary features, the amide I (ca. 1600−1700 cm^{-1}) and amide II (ca. 1500−1560 cm^{-1}) bands, which arise from specific stretching and bending vibrations of the protein backbone. The amide I band (**Figure 1**) arises predominantly from the C═O stretching vibration (80%) of the amide C═O group plus C−N stretching vibration (*16*, *18*, *20*). The vibrational frequency of the amide I band is particularly sensitive to protein secondary structure (*1*, *13*, *16*, *18*−*20*, *23*) and can be used to predict protein secondary structures in relation to protein values (*2*, *16*, *23*−*25*). The amide II (predominantly an N−H bending vibration (60%) coupled to C−N stretching (40%) is also used to assess protein conformation (*16*). The results show that the area and height absorbed intensities at ca. 1650 cm^{-1} ranged from 23.44 to 27.61 and 0.30 to 0.36, respectively, in the seeds among the barley varieties. There were significant differences ($P < 0.05$) among the barley varieties, indicating that CDC Helgason's protein amide IR absorption intensities were greater than those of the other varieties, which suggested that protein structural conformation (molecular structural makeup) in terms of protein secondary structure profiles (α helix, beta-sheet, random coil and beta-turns) may also be different.

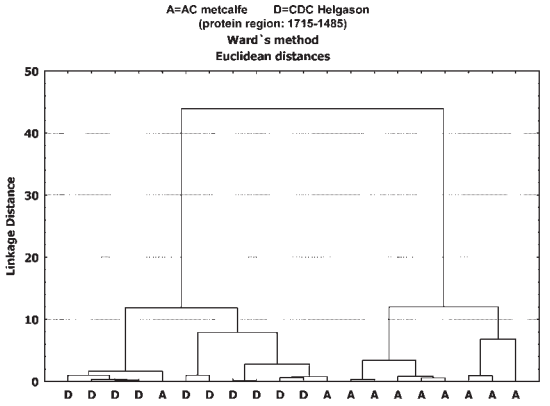
Using Multivariate Molecular Spectral Analyses To Discriminate and Classify Molecular Structure Difference among Barley Varieties. *Multivariate Molecular Spectral Analyses*. In order to study the molecular structure difference, the multivariate molecular spectral analyses (CLA and PCA) were applied. Cluster analysis is a multivariate analysis of which function performs an (agglomerative hierarchical) cluster analysis of an infrared spectra data set and displays the results of cluster analysis as dendrograms (*26*). In this study, the Ward's algorithm method was used without any prior parametrization of the spectral data in the protein amides IR region. This method can give results to show the possibility to discriminate the differences in the structural makeup between the tissues. The principal component analysis is a statistical data reduction method. It transforms the original set of variables to a new set of uncorrelated variables called principal components. The first few principal components will typically account for > 95% variance. The purpose of PCA is to derive a small number of independent linear combinations (principal components) of a set of variables that retain as much of the information in the original variables as possible. This analysis allows studying globally the relationships between *p* quantitative characters (e.g., chemical functional groups such as amide I and II, aromatic lignin, cellulosic compound and carbohydrates) observed in *n* samples (e.g., Fourier transform infrared spectra of the barley hull or seed structures). The outcome of such an analysis can be presented as either 2D (two principal components) or 3D (three principal components) scatter plots (*27*).

Detecting the Molecular Structure Differences in the Seed and Hull among the Barley Varieties. We used both PCA and CLA molecular spectral analyses to compared one barley variety with another in both hull and seeds and found out that there were no differences in the hull among the barley varieties (PCA, CLA figures not shown here), but there were dramatic differences in the seeds, and CDC Helgason was quite different from the other barley varieties. The following are the detailed results (CDC Helgason vs other barley varieties) in protein amide I and II spectral region.

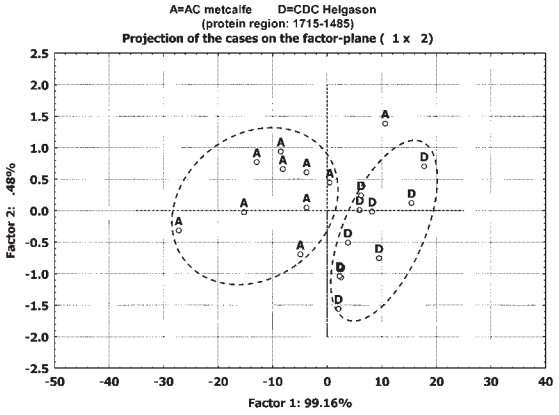
(*a*). *CDC Helgason* (*Feed-Type*) *and AC Metcalfe* (*Malting-Type*) *Differed in Protein Amide I and II Molecular Spectral Region*. Comparing CDC Helgason with AC Metcalfe [**Figure 2** (**1**, left)], two cluster classes can be almost fully distinguished below a linkage distance less than 45, with CDC Helgason and AC Metcalfe groups forming two separate groups. Only two cases of AC Metcalfe's spectra was mixed with CDC Helgason. These cluster analysis results indicated that the spectra from CDC Helgason in the protein amide I and II region of ca. 1715 to 1485 cm^{-1} are almost fully distinguished different from AC Metcalfe. **Figure 2** (**1**, right) shows results from principal component analysis of the spectral data obtained from the CDC Helgason and AC Metcalfe in the seed in the protein amide I and II region of ca. 1715 to 1485 cm^{-1}. First two principal components were plotted, and they explain 99.16% and 0.48% of the variation in the protein molecular spectrum data set, respectively. The results showed that principal component analysis could also distinguish between CDC Helgason and AC Metcalfe. Both the cluster and principal component analyses indicated the different molecular structures (chemical makeup) between CDC Helgason and AC Metcalfe.

(*b*). *CDC Helgason* (*Feed-Type*) *and CDC Dolly* (*Feed-Type*) *Did Not Differ in Protein Amide I and II Molecular Spectral Region*. Comparing CDC Helgason with CDC Dolly [**Figure 2** (**2**)], significant molecular structural differences were not found in the amide I and II region (ca. 1715−1485 cm^{-1})

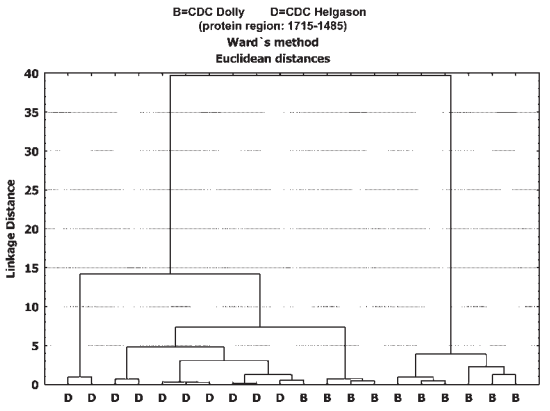
I: Cluster Analysis (CLA): Amides I & II region 1715-1485 cm^{-1} (barley seed) II: Principal component analysis (PCA): Amides I&II region 1715-1485 cm^{-1} (barley seed)



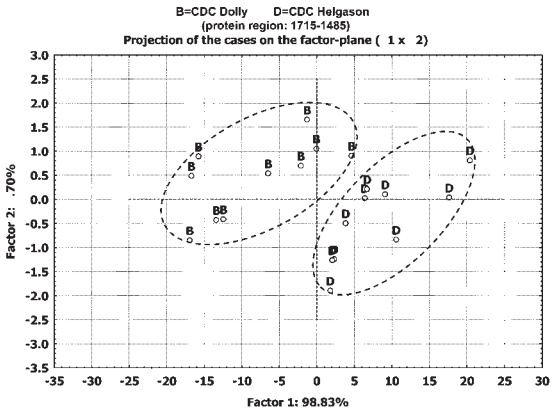
(1) Comparison of CDC Helgason (D) and AC Metcalfe (A)



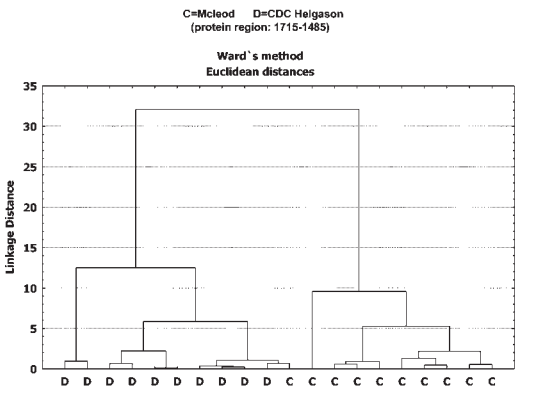
(1) Comparison of CDC Helgason (D) and AC Metcalfe (A): PC1 and PC2 explain 99.16 and 0.48% of the variances, respectively.



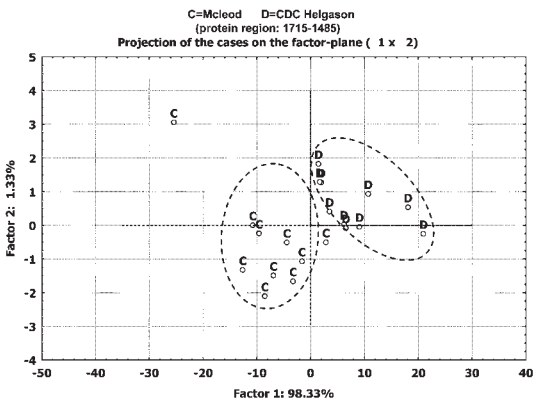
(2) Comparison of CDC Helgason (D) and CDC Dolly (B)



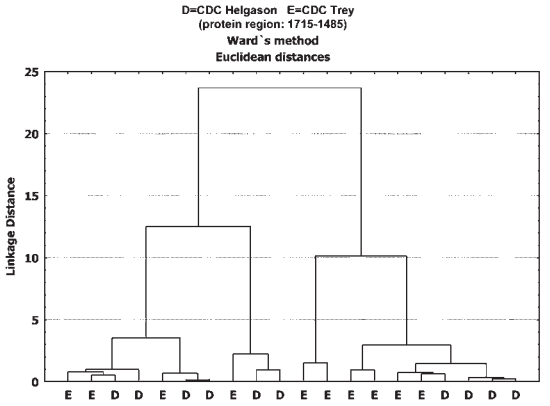
(2) Comparison of CDC Helgason (D) and CDC Dolly (B): PC1 and PC2 explain 98.83 and 0.70% of the variances, respectively.



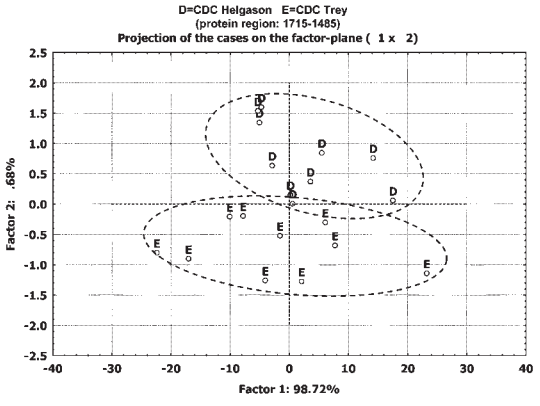
(3) Comparison of CDC Helgason (D) and McLeod (C)



(3) Comparison of CDC Helgason (D) and McLeod (C): PC1 and PC2 explain 98.33 and 1.33% of the variances, respectively.

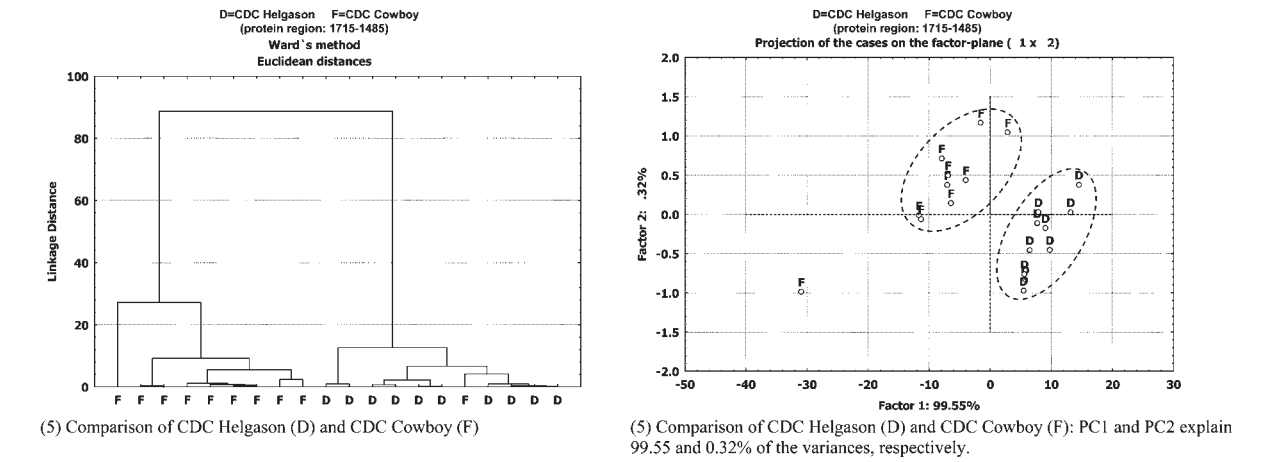


(4) Comparison of CDC Helgason (D) and CDC Trey (E)



(4) Comparison of CDC Helgason (D) and CDC Trey (E): PC1 and PC2 explain 98.72 and 0.68% of the variances, respectively.

Figure 2. Continued



(5) Comparison of CDC Helgason (D) and CDC Cowboy (F)

(5) Comparison of CDC Helgason (D) and CDC Cowboy (F): PC1 and PC2 explain 99.55 and 0.32% of the variances, respectively.

Figure 2. Multivariate spectral analyses of barley structures in the whole seed: comparison of CDC Helgason (D) with other five barley varieties [AC Metcalfe (A), CDC Dolly (B), McLeod (C), CDC Trey (E) and CDC Cowboy (F)]. (**I**) Cluster analysis. Select spectral region: amide I and II region, ~1715 to 1485 cm^{-1}. Distance method: Euclidean. Cluster method: Ward's algorithm. (**II**) Principal component analysis. Scatter plots of the first principal components (PC1) vs the second principal components (PC2).

[**Figure 2** (**2**)], because they did not form two separate classes. This indicated similarity of their protein structure.

(*c*). *CDC Helgason* (*Feed-Type*) *and McLeod* (*Feed-Type*) *Differed in Amide I and II Molecular Spectral Region*. Comparing CDC Helgason with McLeod [**Figure 2** (**3**)], significant molecular structural differences were found in the amide I and II region (ca. 1715−1485 cm^{-1}), because they formed two separate classes in **Figure 2** (**3**, left: cluster analysis) and they could be grouped in separate ellipses in **Figure 2** (**3**, right: principal component analysis).

(*d*). *CDC Helgason* (*Feed-Type*) *and CDC Trey* (*Feed-Type*) *Did Not Differ in Protein Amide I and II Molecular Spectral Region*. Comparing CDC Helgason with CDC Trey [**Figure 2** (**4**)], no significant molecular structural differences were found in the amide I and II region because they could not form two separate classes in **Figure 2** (**4**, left: cluster analysis) and they could be grouped in separate ellipses in **Figure 2** (4, right: principal component analysis).

(*e*). *CDC Helgason* (*Feed-Type*) *and CDC Cowboy* (*Feed-Type*) *Differed in Protein Amide I and II Molecular Spectral Region*. Comparing CDC Helgason with CDC Cowboy [**Figure 2** (**5**)], significant molecular structural differences were found in the amide I and II region (ca. 1715−1485 cm^{-1}) [**Figure 2** (**5**)] because they formed two separate classes and they could be grouped in separate ellipses.

No published results have been found for discrimination of internal structures within barley varieties in both hull and seeds. No comparison could be made with published results. This study indicated that DRIFT spectroscopy with multivariate molecular spectral analyses can be used to study protein molecular structure or bipolymer conformation in plant seeds.

In conclusion, the DRIFT technique was able to identify spectral features associated with molecular structural differences in both hull and seeds among the barley varieties. The molecular spectral analyses at the amide I and II region of ca. 1715−1485 cm^{-1} (attributed to protein amide I and II) together with the agglomerative hierarchical cluster and principal component analyses were able to show that the molecular structures (or structural makeup) of the barley seeds (but not hull) exhibited distinguishing differences among the barley varieties. Multivariate analyses show that CDC Helgason (feed-type) had the distinguishing structural differences from AC Metcalfe (malting-type), McLeod (feed-type), and CDC Cowboy (feed-type) in protein amides I and II, but exhibited no distinguishing differences and CDC Dolly (feed-type) in molecular structural makeup. The DRIFT bioanalytical technique provides a new approach and future potential for plant/seed/feed/food structural molecular study and biopolymer conformation study in relation to functionality, biodegradability, nutrient availability and interaction between plant structure and biological functions.

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