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Application Potential of ATR-FT/IR Molecular Spectroscopy in Animal Nutrition: Revelation of Protein Molecular Structures of Canola Meal and Presscake, As Affected by Heat-Processing Methods, in Relationship with Their Protein Digestive Behavior and Utilization for Dairy Cattle

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Supporting Information

ABSTRACT: Protein quality relies not only on total protein but also on protein inherent structures. The most commonly occurring protein secondary structures (α-helix and β-sheet) may influence protein quality, nutrient utilization, and digestive behavior. The objectives of this study were to reveal the protein molecular structures of canola meal (yellow and brown) and presscake as affected by the heat-processing methods and to investigate the relationship between structure changes and protein rumen degradations kinetics, estimated protein intestinal digestibility, degraded protein balance, and metabolizable protein. Heat-processing conditions resulted in a higher value for α-helix and β-sheet for brown canola presscake compared to brown canola meal. The multivariate molecular spectral analyses (PCA, CLA) showed that there were significant molecular structural differences in the protein amide I and II fingerprint region (ca. 1700−1480 cm^{-1}) between the brown canola meal and presscake. The in situ degradation parameters, amide I and II, and α-helix to β-sheet ratio (R_a_β) were positively correlated with the degradable fraction and the degradation rate. Modeling results showed that α-helix was positively correlated with the truly absorbed rumen synthesized microbial protein in the small intestine when using both the Dutch DVE/OEB system and the NRC-2001 model. Concerning the protein profiles, R_a_β was a better predictor for crude protein (79%) and for neutral detergent insoluble crude protein (68%). In conclusion, ATR-FT/IR molecular spectroscopy may be used to rapidly characterize feed structures at the molecular level and also as a potential predictor of feed functionality, digestive behavior, and nutrient utilization of canola feed.

KEYWORDS: *canola, protein secondary structure, ATR-FT/IR molecular spectroscopy, α-helix, protein nutritive value, heat-processing condition*

INTRODUCTION

Heat treatment of animal feed is currently employed to improve the utilization and availability of its protein[1] and inactivate any antinutrition factors[2] by reducing fermentation and metabolism in the rumen. As a result, increased amounts of protein enter the small intestine for further absorption and digestion.[1] The effectiveness of heat processing as a tool for optimization of the feed protein value has been well documented in the literature, and several mechanisms that alter the degradation and digestive behavior of protein have been proposed for the findings. Generally any temperature change in the environment of the protein that is able to influence the noncovalent interactions involved in the structure may lead to an alteration of the protein structure, including protein secondary structures.[3]

Protein quality relies not only on total protein and amino acid content but also on protein inherent structures such as protein secondary structures and nutrient matrix.[4] The most commonly occurring protein secondary structures include the α-helix and the β-sheet.[5−7] The protein secondary structure profiles may influence protein quality and nutrient utilization, availability, or digestive behavior.[8−11] For the reasons mentioned, studying the secondary structure of proteins is often of interest to understand their digestive behavior, nutritive quality, utilization, and availability in animals and humans.[10]

However, few studies on protein structures and alteration of their inherent structures due to heat processing conditions, in relation to nutritive value and digestive behavior of protein, exist. An approach would be the use of attenuated total reflectance-Fourier-transformed infrared vibration spectroscopy (ATR-FT/IR), which is considered to be a well-established experimental technique for studying the secondary structural composition, stability, and conformational changes (effects of temperature, pH, and pressure).[12−14] Such an approach determines the protein value of canola feed by revealing inherent molecular protein structure and will provide basic and useful information for the animal feed processing and plant breeding as well as human food industries to maintain and improve protein quality.

Therefore, it is hypothesized that heat-processing methods potentially change the protein molecular structure of canola meal

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and presscake, which might be associated with their protein nutrient utilization and availability. The objectives of the present study were (1) to reveal the protein molecular structures of canola meal and presscake by using ATR-FT/IR molecular spectroscopy; (2) to investigate the relationship between protein molecular structures (in terms of protein α-helix, β-sheet intensity, and their ratio and amide I to amide II intensity and their ratio) and (i) protein rumen degradations kinetics (rate and extent), (ii) estimated protein intestinal digestibility, (iii) degraded protein balance, and (iv) total truly absorbed protein in the small intestine (metabolizable protein); and (3) to determine the most important structural features for canola, which could be used as predictors of protein nutrient availability and digestive characteristics.

MATERIALS AND METHODS

Canola Meal and Presscake and Nutrient Analysis. Canola meal (CM) and canola presscake (CPC) were used in this model study as a feed protein source. Two different varieties of canola, yellow-seeded (CM_Y) (*Brassica juncea*) and brown-seeded (CM_B) (*Brassica napus*) solvent-extracted canola meal, were provided by Agriculture and Agri-Food Canada, Lethbridge Research Center, Alberta, Canada, in May 2010, and the second source was provided by Bunge Altona, Manitoba, Canada, in October 2011. The brown-seeded (*B. napus*) canola presscake (CPC_B) was produced and obtained from Milligan Biotech (Foam Lake, Saskatchewan, Canada). In this research study were used two different batches for CM_Y (total = 4 kg), two different batches for CM_B (total = 4 kg), and two different batches for CPC_B (total = 10 kg). The detailed methods and calculations for Cornell Net Carbohydrate and Protein System (CNCPS) protein fractionation nutrient modeling, chemicals and nutrients analyzed, in situ rumen degradation kinetics, and estimated intestinal protein digestion were reported previously by Theodoridou and Yu.[15,16] The following is a brief explanation to understand the concept of these methods and the way the calculations were made.

Crude protein fractions were partitioned according to the Cornell Net Carbohydrate and Protein System (CNCPS). The characterizations of the CP fractions used in this system are as follows: fraction PA is nonprotein nitrogen, fraction PB is true protein, and fraction PC is unavailable protein. Fraction PB is further divided into three fractions (PB1, PB2, and PB3) that are believed to have different rates of degradation in the rumen. Fraction PB2 is fermented in the rumen at a lower rate than buffer-soluble fractions, and some of the PB2 fraction escapes to the lower gut. Fraction PB3 is believed to be more slowly degraded in the rumen than fractions PB1 and PB2 because of its association with the plant cell wall; a large proportion of PB3 is thus believed to escape the rumen. Fraction PC is the acid detergent insoluble N, which is highly resistant to breakdown by microbial and mammalian enzymes, and it is assumed to be unavailable for the animal.

In situ rumen degradation kinetics of crude protein was determined using the first-order kinetics equation described by Ørskov and McDonald[17] and modified by Robinson et al.[18] and Dhanoa[19] to include lag time: $R(t) = \mathrm{U} + (100 - \mathrm{S} - \mathrm{U}) \times \mathrm{e}^{-K_\mathrm{d} \times (t - \mathrm{T0})}$, where $R(t)$ = residue present at t h of incubation (g kg^{-1}); S = soluble fraction (g kg^{-1}); U = undegradable fraction (g kg^{-1}); D = potentially degradable fraction (g kg^{-1}); $T0$ = lag time (h); and K_d = degradation rate (h^{-1}). On the basis of the nonlinear parameters estimated by the above equation (S, U, and K_d), rumen-degraded feed CP (RDP) and rumen undegraded CP (RUP) were predicted as RDP (g kg^{-1}) = $\mathrm{S} + (\mathrm{D} \times K_\mathrm{d})/(K_\mathrm{p} + K_\mathrm{d})$ and RUP (g kg^{-1}) = $\mathrm{U} + (\mathrm{D} \times K_\mathrm{d})/(K_\mathrm{p} + K_\mathrm{d})$, where D = 100 − S − U (g kg^{-1}) and K_p is the estimated rate of outflow of digesta from the rumen (h^{-1}), which was assumed to be 0.06 h^{-1}.

Predicted nutrient supply to the small intestine was carried out by using the DVE/OEB system[20] and NRC-2001 model.

The DVE/OEB system constitutes a two-part system in which each feed has a DVE and an OEB value. The DVE value comprises digestible feed protein, microbial protein, and an endogenous protein loss correction. The OEB value or degradable protein balance of a feed is the difference between the potential microbial crude protein synthesis based on rumen-degraded feed CP and the potential microbial crude protein synthesis based on energy extracted from anaerobic fermentation.

The NRC-2001 dairy model introduced the concepts of metabolizable protein, defined as true protein that is digested and absorbed by the intestine and contributed by (1) ruminally undegraded feed CP, (2) ruminally synthesized microbial crude protein, and (3) endogenous crude protein from rumen.

Processing Conditions of Feedstuffs. Canola seed was crushed and then was solvent-extracted to separate the oil from the meal. This process (prepress solvent extraction), included the following steps:

(a) Seed cleaning: canola seed was graded according to strict grading standards (i.e., maximum moisture content, seed damage and chlorophyll level) and then was delivered to the crushing plant.

(b) Seed preconditioning and flaking: seed was preheated with grain dryers to approximately 35 °C to prevent shattering, which may occur when cold seed from storage enters the flaking unit. The cleaned seed was first flaked to physically rupture the seed coat.

(c) Seed cooking: later, flakes were cooked/conditioned by passing them through a series of steam-heated drum or stack type cookers. At the start of cooking, the temperature was rapidly increased to 80−90 °C, which served to inactivate the myrosinase enzyme present in canola. The cooking cycle lasted 15−20 min, and the temperatures normally ranged between 80 and 105 °C, with an optimum of about 88 °C.

(d) Pressing the flake to mechanically remove a portion of the oil: cooked canola seed flakes were then pressed in a series of screw presses or expellers. Pressing was performed to remove as much oil as possible, usually 50−60% of the seed oil content, while maximizing the output of the expellers and producing a *canola presscake*.

(e) Solvent extraction of the presscake to remove the remainder of the oil: the presscake was solvent-extracted (hexane) to remove the remaining oil.

(f) Desolventizing and toasting of the meal: solvent was removed from the marc in a desolventizer−toaster. During the desolventization−toasting process the meal was heated to 95−115 °C and moisture increased to 12−18%. The total time spent in the desolventizer−toaster was approximately 30 min. The canola meal was then cooled and dried to approximately 12% moisture by blowing air through it. The *canola meal* was next granulated to a uniform consistency and stored.

ATR-FT/IR Data and Collection Analysis. Molecular spectroscopic experiments were performed at the Department of Animal and Poultry Science, University of Saskatchewan. ATR-FT/IR was performed using a JASCO FT/IR-ATR-4200 (Jasco Inc., Easton, MD, USA) with a ceramic IR light source and a deuterated L-alanine doped triglycine sulfate detector (JASCO Corp., Tokyo, Japan) equipped with a MIRacleTM attenuated total reflectance accessory module and outfitted with a ZnSe crystal and pressure clamp (PIKE Technologies, Madison, WI, USA). Samples were placed in the refrigerator to minimize sticking while grinding. Later, they were fed slowly into the grinder to further prevent sticking during the grinding process and were finely ground using a fit with a 0.5 mm screen. Each sample was analyzed five times. Thirty-two scans per sample were collected in the mid-infrared range from 4.000 to 700 cm^{-1} in transmission mode at a spectral resolution of 4 cm^{-1}. The collected spectra were corrected against air as background.

Univariate Molecular Spectral Analysis of Protein Structure Profiles. The functional spectral bands associated with protein molecular structures were identified with OMNIC 7.2 software (Spectra-Tech Inc., Madison, WI, USA) and assigned according to published studies.[21−26] Unique primary protein features found in peptide bonds (C−O, C−N, and N−H) include amide I (~80% C═O and ~20% C−N stretching vibration; centered at a wavelength of ca. 1655 cm^{-1}) and amide II (~60% N−H bending vibration, ~40% C−N stretching vibration; centered at ca. 1550 cm^{-1}), which are detectable as two absorption peaks within the wavelength region from ca. 1720 to 1485 cm^{-1}.[24,27,28] Absorption peak heights for secondary protein structure α-helices (ca. 1660 cm^{-1}) and β-sheets (ca. 1630 cm^{-1}) are detectable in the amide I area using the second-derivative function of OMNIC 7.2 (Spectra Tech Inc.). The ratios of amide I to amide II and α-helix to β-sheet spectral intensities were calculated.

Table 1. Structural Characteristics of Protein by Using ATR-FT/IR Molecular Spectroscopy: Comparison among Yellow Canola Meal (CM, *B. juncea*), Brown Canola Meal (CM, *B. napus*), and Canola Presscake Brown (CPC, *B. napus*)

	canola treatment[a]					contrast
item	CM-Yellow *B. juncea* ($n = 2 \times 5$ runs)	CM-Brown *B. napus* ($n = 2 \times 5$ runs)	CPC-Brown *B. napus* ($n = 2 \times 5$ runs)	SEM[b]	*P* value	CM vs CPC *P* value
protein molecular structure spectra profiles (unit: absorbance)						
amide I area	3.21a	2.89a	3.21a	0.157	0.262	0.414
amide II area	1.13ab	0.93b	0.07a	0.074	0.046	0.090
ratio amide I to amide II area	2.90ab	3.19a	2.70b	0.110	0.013	0.014
protein secondary structure profile						
α-helix (height)	0.03ab	0.03b	0.04a	0.002	0.002	0.002
β-sheet (height)	0.04a	0.03b	0.04a	0.004	0.134	0.825
ratio α-helix to β-sheet	0.86b	0.96ab	1.04a	0.033	0.002	0.003

[a]Means with different letters within the same row differ ($P < 0.05$). [b]SEM, standard error of the mean.

Multivariate Molecular Spectral Analysis of Protein Structure Profiles. To compare the spectra of the different feedstuffs used in this study and to determine if there were underlying structural differences, multivariate spectral analyses were applied. CLA results were presented as dendrograms, whereas PCA results were plotted on the basis of the two highest factor scores and illustrated as a function of those scores. In each comparison the eigenvector for factor 1 was plotted against that for factor 2, which accounted for >99% of the variability in the data. These analyses were carried out using Statistica 8 software (StatSoft Inc., Tulsa, OK, USA).

Statistical Analysis. Statistical analysis was performed using the PROC MIXED procedure statistical package of SAS.[29] Significance was declared at $P < 0.5$, and trends were declared at $P \leq 0.10$. Differences among treatments were evaluated using Tukey's test; means with different letters were obtained with "pdmix800 SAS macro".[30] The ATR-FT/IR spectroscopic data were analyzed using a completely randomized design model (CRD)

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

where Y_{ij} was an observation of the dependent variable ij (amide I, amide II, ratio of amide I to amide II, α-helix, β-sheet, or ratio of α-helix to β-sheet), μ was the population mean for the variable; T_i was the effect of feed source (i = 1−3 CM_Y, CM_,B and CPC_B), as a fixed effect, batch as replication, and e_{ij} was the random error associated with the observation ij.

Correlation Analysis. The relationship between the changes in protein structure profiles (in terms of amide I, amide II, ratio of amide I to amide II, α-helix, β-sheet, or ratio of α-helix to β-sheet) and the changes in chemical and nutrient profiles (in terms of chemical composition, protein fractions, in situ rumen degradation kinetics, estimated protein intestinal digestibility, and predicted protein supply to dairy cattle) in the canola meal and presscake samples were analyzed using the PROC CORR procedure of SAS. The normality tests were performed using the UNIVARIATE procedure of SAS with options of NORMAL and PLOT.

Multiple Regression Analysis. To determine which protein molecular structure parameters (amide I, amide II, ratio of amide I to amide II, α-helix, β-sheet, ratio of α-helix to β-sheet) in the samples of canola meal and canola presscake are important in determining protein utilization and availability to cattle, a multiple regression analysis with variable selection analysis was carried out using the PROC REG procedure of SAS with a model as follows:

$$Y = \text{amide I} + \text{amide II} + \text{amide I to amide II ratio (R\_I\_II)} + \alpha\text{-helix} + \beta\text{-sheet} + \alpha\text{-helix to } \beta\text{-sheet ratio (R\_a\_}\beta\text{)}$$

The model used a "STEPWISE" option with variable selection criteria: SLENTRY = 0.05, SLSTAY = 0.05. All variables left in the final prediction models were significant at the $P < 0.05$ level. The residual analysis was carried out to test the regression model assumptions using the UNIVARIATE procedure of SAS with NORMAL and PLOT options.

RESULTS AND DISCUSSION

Quantifying Protein Molecular Structure Profile in Canola Meal and Canola Presscake. *Heat-Induced Changes of Protein Molecular Structure Characteristics.* Goelema[31] summarized that feed heat treatment may result in protein denaturation (disorganization of the overall molecular shape of a protein), unfolding or uncoiling of a coiled or pleated structure, or the separation of the protein into its subunits, which may then unfold or uncoil. In our study, the heat-processing conditions did not significantly change the area of amide I between the two canola meal varieties or between canola meals and presscake (Table 1). However, the amide II area and the amide I to amide II ratio (R_I_II) were different ($P < 0.05$) between the brown-seeded canola meal (CM_B) and presscake (CPC_B). Indeed, R_I_II can be affected by the feed type, heat processing, and even gene transformation, as has been discussed in a recent study.[32]

Depending on the α-helix to β-sheet ratios, tissues of the same protein content may differ in their nutritive value.[33] More specifically, a high percentage of β-sheet structure may partially cause low access to gastrointestinal digestive enzymes, which can result in a low protein value and low protein availability.[8] In the present study a significantly higher β-sheet value was observed for the yellow-seeded canola meal compared to that of the brown one. This result is in accordance with the lower rumen degradability and the higher intestinal digestibility found for the same yellow canola meal in a previous study.[15]

Brown-seeded canola presscake had a significantly higher value for the α-helix and β-sheet compared to brown canola meal. Such a finding can be partly explained by the heat-processing method applied. The canola presscake was not exposed to the high temperature of the desolventizer−toaster, whereas during the process stage of desolventization−toasting the meal was heated to 95−115 °C.[15]

The effects of processing conditions on protein molecular structure characteristics are equivocal among studies, and this might be due to different heat-processing methods used. Yu et al.[3] found that roasting decreased the percentage of α-helix (from 47.1 to 36.1%), increased the percentage of β-sheet (from 37.2 to 49.8%), and decreased the α-helix to β-sheet ratio (from 1.3 to 0.7) of golden flaxseed tissues. This is in contrast with the results obtained by Doiron et al.,[33] who found that heating flaxseed at 120 °C for 40 and 60 min increased the α-helix to β-sheet ratio. Autoclave and dry heating significantly decreased and increased

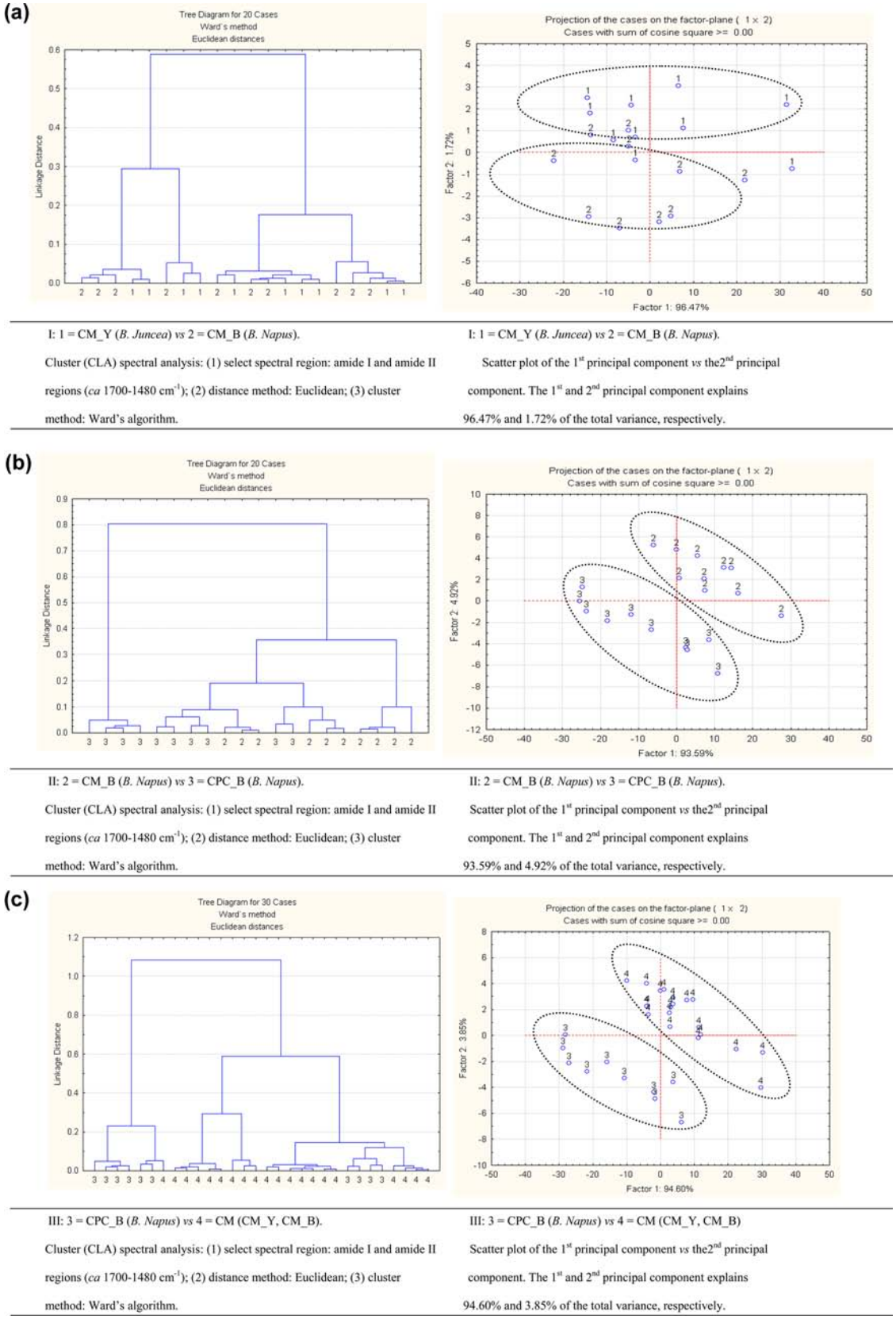

Figure 1. Multivariate molecular spectral analyses of amide I and amide II regions of the feedstuffs used in this study: (a) comparison of yellow canola meal (CM_Y, *B. juncea*) and brown canola meal (CM_B, *B. napus*); (b) comparison of brown canola meal (CM_B, *B. napus*) and canola presscake brown (CPC_B, *B. napus*); (c) comparison of canola meal (CM_Y and CM_B) and canola presscake (CPC_B).

the α-helix to β-sheet ratio, respectively, at the temperature of 120 °C for 1 h as was reported in a study on canola seeds.[34] This alteration in the protein structure ratio was probably caused by denaturation of α-helix and β-sheet during the heating process. In

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Table 2. Correlation between Protein Structures (Amide I, Amide II, and Their Ratio) and Chemical Protein and Nutrient Profiles of Canola Meal and Canola Presscake

	protein molecular structure (amide I, amide II, and their ratio)					
	amide I		amide II		ratio of amide I to amide II	
item	correl coeff R	P	correl coeff R	P	correl coeff R	P
chemical profiles[a] (g kg^{-1} DM)						
DM	−0.44	0.556	−0.45	0.555	−0.94	0.055
EE	−0.97	0.034	−0.97	0.034	−0.16	0.836
NDF	−0.21	0.692	−0.26	0.622	0.24	0.640
ADF	−0.19	0.716	−0.14	0.791	0.03	0.951
ADL	−0.23	0.666	−0.18	0.735	0.059	0.912
protein profiles[b] (g kg^{-1} DM)						
CP	0.54	0.455	0.54	0.457	−0.74	0.263
NPN	−0.50	0.503	−0.50	0.502	−0.92	0.081
SCP	0.07	0.932	0.07	0.930	1.00	0.003
NDICP	0.95	0.051	0.95	0.052	−0.18	0.820
ADICP	0.91	0.272	0.91	0.265	0.88	0.311
protein fractions[c] (g kg^{-1} CP)						
PA	0.02	0.984	0.02	0.983	0.99	0.008
PB1	−0.76	0.244	−0.76	0.243	−0.70	0.301
PB2	0.82	0.188	0.82	0.183	−0.45	0.545
PB3	0.18	0.817	0.18	0.816	0.99	0.009
PC	−0.70	0.297	−0.70	0.297	0.35	0.649
TP	−0.07	0.890	−0.28	0.590	0.41	0.418
digestible nutrients[d] (NRC-2001 summary approach) (g kg^{-1} DM)						
tdCP	0.13	0.799	−0.09	0.8626	0.33	0.5263
TDN_{1X}	0.20	0.698	0.60	0.2086	−0.87	0.0239
in situ protein degradation kinetics[e] (g kg^{-1} CP)						
K_d (h^{-1})	0.95	0.048	0.95	0.049	−0.17	0.829
S	−0.88	0.119	−0.88	0.118	−0.57	0.426
D	0.95	0.049	0.95	0.048	0.38	0.625
EDCP	−0.93	0.073	−0.93	0.073	−0.46	0.537
RUCP	−0.76	0.238	−0.76	0.237	−0.68	0.320
intestinal digestibility of RUCP[f] (g kg^{-1} CP)						
IVCPD	0.89	0.107	0.89	0.108	−0.32	0.679

[a]DM, dry matter; EE, ether extract (crude fat); NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin. [b]CP, crude protein; NPN, non-protein nitrogen; SCP, soluble crude protein; NDICP; neutral detergent insoluble crude protein; ADICP, acid detergent insoluble crude protein. [c]PA, fraction of CP that is instantaneously solubilized at time zero, calculated as NPN; PB1, rapidly degradable protein fraction that is soluble in borate phosphate buffer and precipitated with trichloroacetic acid, calculated as SCP minus NPN; PB2, intermediately degradable protein fraction calculated as total CP minus the sum of fractions PA, PB1, PB3, and PC; PB3, slowly degradable protein fraction, calculated as NDICP minus ADICP; PC, fraction of undegradable protein, calculated as ADICP. TP, true protein = PB1 (g kg^{-1} CP) + PB2 (g kg^{-1} CP) + PB3 (g kg^{-1} CP). [d]tdCP, digestible crude protein; TDN_{1X}, total digestible nutrients at maintenance estimated from NRC dairy model 2001. [e]K_d, degradation rate; S, potential soluble fraction in the in situ ruminal incubation; D, potentially degradable fraction in the in situ ruminal incubation; EDCP, effectively degraded crude protein; RUCP, rumen undegraded crude protein. [f]IVCPD, in vitro crude protein digestibility in small intestine.

contrast, results in a study conducted on canola seeds indicated that both dry and moist heating at 120 °C had no impact on the molecular spectrum in lipid-related functional groups.[35]

Multivariate Analysis of Heat-Induced Changes Protein Structural Spectra. Multivariate spectral analyses (CLA and PCA) are applied when molecular structure differences and functional groups need to be studied. 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. In this study, Ward's algorithm method was used without any prior parametrization of the spectral data in the protein amide IR region. Success in applying multivariate CLA has been reported previously[36] for the three feed inherent structures (structure 1, feed pericarp; structure 2, feed aleurone; structure 3, feed endosperm) and different varieties of canola by Doiran et al.[37] for flaxseed samples with heat treatment and by Liu and Yu[38] for different genotypes of barley. On the other hand, principal component analysis is a statistical data reduction method that 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 a high percentage of variance (>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 as of the information in the original variables as possible, and the results are displayed as scatterplots between the components. Therefore, PCA can be used to allow the feed intrinsic structures to be distinguished and identifies features that differ between feed structures.

Figure 2 in the Supporting Information shows typical spectra, smoothed spectra, and second-derivative spectra of yellow and

Table 3. Correlation between Protein Structures (α-Helix, β-Sheet, and Their Ratio) and Chemical Protein and Nutrient Profiles of Canola Meal and Canola Presscake

	protein molecular structure (amide I, amide II, and their ratio)					
	α-helix		β-sheet		ratio of α-helix to β-sheet	
item	correl coeff *R*	*P*	correl coeff *R*	*P*	correl coeff *R*	*P*
chemical profiles[a] (g kg^{-1} DM)						
DM	−0.21	0.791	−0.99	0.008	0.84	0.165
EE	−0.93	0.071	−0.52	0.476	0.83	0.173
NDF	0.01	0.988	−0.60	0.206	0.80	0.058
ADF	0.11	0.830	−0.52	0.286	0.82	0.044
ADL	0.07	0.900	−0.57	0.238	0.83	0.039
protein profiles[b] (g kg^{-1} DM)						
CP	0.73	0.269	−0.42	0.578	−0.03	0.968
NPN	−0.27	0.731	−0.99	0.008	0.88	0.125
SCP	−0.18	0.819	0.89	0.110	−0.57	0.426
NDICP	−0.35	0.772	−0.51	0.660	0.95	0.194
ADICP	1.00	0.003	0.22	0.780	−0.65	0.354
protein fractions[c] (g kg^{-1} CP)						
PA	−0.23	0.768	0.86	0.136	−0.53	0.472
PB1	−0.58	0.416	−0.90	0.0964	0.94	0.058
PB2	0.94	0.065	−0.07	0.931	−0.40	0.599
PB3	−0.06	0.935	0.93	0.071	−0.65	0.347
PC	−0.79	0.206	0.02	0.9767	0.40	0.603
TP	−0.52	0.294	0.17	0.747	−0.80	0.054
digestible nutrients[d] (NRC-2001 summary approach) (g kg^{-1} DM)						
tdCP	−0.32	0.533	0.38	0.461	−0.88	0.022
TDN_{1X}	0.56	0.250	0.48	0.333	0.02	0.971
in situ protein degradation kinetics[e] (g kg^{-1} CP)						
K_d (h^{-1})	1.00	0.002	0.23	0.771	−0.65	0.346
S	−0.74	0.259	−0.85	0.154	0.98	0.018
D	0.86	0.139	0.70	0.299	−0.93	0.074
EDCP	−0.81	0.185	−0.77	0.233	0.96	0.043
RUCP	−0.59	0.406	−0.89	0.111	0.94	0.065
intestinal digestibility of RUCP[f] (g kg^{-1} CP)						
IVCPD	0.98	0.023	0.08	0.924	−0.53	0.469

[a]DM, dry matter; EE, ether extract (crude fat); NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin. [b]CP, crude protein; NPN, non-protein nitrogen; SCP, soluble crude protein; NDICP, neutral detergent insoluble crude protein; ADICP, acid detergent insoluble crude protein. [c]PA, fraction of CP that is instantaneously solubilized at time zero, calculated as NPN; PB1, rapidly degradable protein fraction that is soluble in borate phosphate buffer and precipitated with trichloroacetic acid, calculated as SCP minus NPN; PB2, intermediately degradable protein fraction calculated as total CP minus the sum of fractions PA, PB1, PB3, and PC; PB3, slowly degradable protein fraction, calculated as NDICP minus ADICP; PC, fraction of undegradable protein, calculated as ADICP; TP, true protein = PB1 (g kg^{-1} CP) + PB2 (g kg^{-1} CP) + PB3 (g kg^{-1} CP). [d]tdCP, digestible crude protein; TDN_{1X}, total digestible nutrients at maintenance estimated from NRC dairy model 2001. [e]K_d, degradation rate; S, potential soluble fraction in the in situ ruminal incubation; D, potentially degradable fraction in the in situ ruminal incubation.; EDCP, effectively degraded crude protein; RUCP, rumen undegraded crude protein. [f]IVCPD, in vitro crude protein digestibility in small intestine.

brown canola meal as well as brown canola presscake, revealed by ATR-FT/IR molecular spectroscopy. It is hard to visually detect the spectral difference between the yellow and brown canola meal, in contrast with spectral difference between canola meal and presscake.

The multivariate molecular spectral analyses (PCA and CLA) were applied to reveal the molecular structural difference between the yellow and brown canola meal. CLAs of molecular spectral (amide I and amide II region: ca. 1700−1480 cm^{-1}) obtained from the canola meal samples (Figure 1a) indicated that there were no significant molecular structural differences in the amide I and II regions as they did not form two separate classes and were not grouped in separate ellipses. The scatterplot of the first principal component versus the second principal component of PCA of the spectra obtained from the yellow and brown canola meal samples is shown in Figure 1a. There the first and second PCs explained 96.47 and 1.72% of the total variance, respectively.

The PCA of the spectrum obtained from the brown canola meal and presscake is shown in Figure 1b. The mixed dendrogram of brown canola meal and presscake showed a similarity of spectral data in their amide I and II regions, indicating that these two feedstuffs were not completely different in protein spectroscopic features. PC1 and PC2 explained 93.59 and 4.92% of variation of spectral data, respectively.

Moreover, the molecular spectral comparison between canola meal (both yellow and brown) and canola presscake showed some overlap between them in the dendrogram (Figure 1c). However, this was not the case in the scattered plot of PCA, which showed two clearly distinguishable ellipses in which PC1 and PC2 explained 94.60 and 3.85% of variation of spectral data, respectively.

Correlations between Protein Structure Characteristics and Nutrient and Chemical Profiles. *Heat-Induced Changes in Amide I and Amide II Profiles in Relation to Nutrient and Chemical Profiles.* Amide I and amide II bands are known to have a great sensitivity to protein secondary structural changes.[23,24] Correlations between protein molecular structure and chemical profile, protein profile, protein subfractions, total digestible nutrients, energy value, in situ degradation, and intestinal protein digestibility of the feedstuffs used in this study are presented in Table 2. For chemical profiles, ether extract (EE) was found to be negatively correlated with amide I and II (R = −0.97, P < 0.05) and dry matter tended to be negatively correlated with the amide I to amide II ratio (R = −0.94, P = 0.05). In addition, there was a significant positive correlation between R_I_II and soluble crude protein (SCP) (R = 1.00, P < 0.05). Moreover, a positive correlation was found between neutral acid detergent insoluble crude protein (NDICP) and amide I (R = 0.95, P = 0.051) or amide II (R = 0.95, P = 0.052). However, no correlation (R = −0.97, P > 0.10) was observed between amide I or amide II and the acid detergent insoluble protein (ADICP) (Table 2).

For the CNCPS protein fractionation, the results showed that R_I_II was strongly positively correlated with the non-protein nitrogen fraction (R = 0.99, P < 0.01) and with the slowly degraded protein PB3 fraction (R = 0.99, P < 0.01).

With regard to the in situ degradation parameters, the results indicated that the amide I and amide II bands were positively correlated with the degradable fraction and the degradation rate (R = 0.95, P < 0.05). However, no correlations were noted between the amide I and II bands and the in vitro intestinal digestibility of rumen undegraded protein.

For the prediction of protein supply to dairy cattle, there were no significant correlations between any parameters of the DVE/OEB evaluation system and the protein structure (amide I, amide II, and their ratio) (Table 4). Conversely, amide I and amide II (R

Table 4. Correlation between Protein Structures (Amide I, Amide II, and Their Ratio) and Chemical Protein and Nutrient Profiles of Canola Meal and Canola Presscake

	protein molecular structure (amide I, amide II, and their ratio)					
	amide I		amide II		ratio of amide I to amide II	
item	correl coeff R	P	correl coeff R	P	correl coeff R	P
modeling protein nutrients[a] (DVE/OEB system) (g kg^{-1} DM)						
AMCP^{DVE}	0.90	0.099	0.90	0.0995	−0.30	0.697
ENDP	−0.14	0.862	−0.14	0.863	0.48	0.516
ARUP^{DVE}	0.20	0.709	0.05	0.919	0.13	0.811
DVE	0.83	0.170	0.83	0.171	−0.44	0.564
OEB	0.75	0.252	0.75	0.253	−0.55	0.447
modeling protein nutrients[b] (NRC-2001 model) (g kg^{-1} DM)						
AMCP^{NRC}	1.00	<0.001	1.00	<0.001	0.161	0.839
AECP	−0.04	0.961	−0.04	0.962	0.98	0.025
ARUP^{NRC}	−0.37	0.630	−0.37	0.632	0.84	0.161
MP	0.25	0.754	0.25	0.753	0.99	0.006
DPB	0.20	0.800	0.21	0.798	1.00	0.002

[a]AMCP^{DVE}, truly absorbed rumen synthesized microbial protein in the small intestine; ENDP, endogenous protein losses in the digestive tract; ARUP^{DVE}, truly absorbed bypass feed protein in the small intestine; DVE, truly absorbed protein in the small intestine contributed by (1) feed protein escaping rumen degradation (RUP^{DVE}), (2) microbial protein synthesized in the rumen (MCP$_{FOM}$), and (3) a correction for endogenous protein losses in the digestive tract (ENDP); OEB, reflects the difference between the potential microbial protein synthesis based on rumen degraded feed crude protein (CP) and that based on energy (rumen fermented OM) available for microbial fermentation in the rumen. [b]AMCP^{NRC}, truly absorbed rumen synthesized microbial protein in the small intestine; AECP, truly absorbed endogenous protein in the small intestine; ARUP^{NRC}, truly absorbed rumen undegraded feed protein in the small intestine; MP, metabolizable protein (true protein that is digested post-ruminally and the component amino acid absorbed by the intestine) contributed by (1) ruminally undegraded feed CP, (2) ruminally synthesized microbial CP, and (3) endogenous CP. DPB^{NRC}, reflects the difference between the potential microbial protein synthesis based on ruminally degraded feed CP and that based on energy-TDN available for microbial fermentation in the rumen.

Table 5. Correlation between Protein Structures (Amide I, Amide II, and Their Ratio) and Chemical Protein and Nutrient Profiles of Canola Meal and Canola Presscake

	protein molecular structure (amide I, amide II, and their ratio)					
	α-helix I		β-sheet		ratio of α-helix to β-sheet	
item	correl coeff R	P	correl coeff R	P	correl coeff R	P
modeling protein nutrients[a] (DVE/OEB system) (g kg^{-1} DM)						
AMCP^{DVE}	0.98	0.020	0.09	0.906	−0.55	0.455
ENDP	−0.26	0.741	0.37	0.634	−0.19	0.809
ARUP^{DVE}	−0.55	0.451	−0.74	0.259	0.79	0.212
DVE	−0.20	0.709	0.51	0.304	−0.90	0.014
OEB	0.94	0.058	−0.051	0.951	−0.42	0.580
modeling protein nutrients[b] (NRC-2001 model) (g kg^{-1} DM)						
AMCP^{NRC}	0.96	0.037	0.54	0.465	−0.87	0.134
AECP	−0.28	0.716	0.83	0.172	−0.49	0.515
ARUP^{NRC}	−0.58	0.418	0.58	0.421	−0.16	0.839
MP	−0.00198	0.998	0.96	0.043	−0.71	0.290
DPB	−0.048	0.952	0.94	0.058	−0.68	0.322

[a]AMCP^{DVE}, truly absorbed rumen synthesized microbial protein in the small intestine; ENDP, endogenous protein losses in the digestive tract; ARUP^{DVE}, truly absorbed bypass feed protein in the small intestine; DVE, truly absorbed protein in the small intestine contributed by (1) feed protein escaping rumen degradation (RUP^{DVE}), (2) microbial protein synthesized in the rumen (MCP$_{FOM}$), and (3) a correction for endogenous protein losses in the digestive tract (ENDP); DPB^{DVE}, reflects the difference between the potential microbial protein synthesis based on rumen degraded feed crude protein (CP) and that based on energy (rumen fermented OM) available for microbial fermentation in the rumen. [b]AMCP^{NRC}, truly absorbed rumen synthesized microbial protein in the small intestine; AECP, truly absorbed endogenous protein in the small intestine; ARUP^{NRC}, truly absorbed rumen undegraded feed protein in the small intestine; MP, metabolizable protein (true protein that is digested post-ruminally and the component amino acid absorbed by the intestine) contributed by (1) ruminally undegraded feed CP, (2) ruminally synthesized microbial CP, and (3) endogenous CP. DPB^{NRC}, reflects the difference between the potential microbial protein synthesis based on ruminally degraded feed CP and that based on energy-TDN available for microbial fermentation in the rumen.

= 1.00, $P < 0.01$) were correlated with the AMCP^{NRC}, whereas AECP^{NRC} was correlated with the amide I to amide II ratio (R = 0.98, $P < 0.05$). In addition, the metabolizable protein and the degraded protein balance based on the NRC-2001 model were significantly correlated with R_I_II.

Heat-Induced Changes in Protein Secondary Profiles in Relation to Nutrient and Chemical Profiles. Significant positive correlations were detected between the acid detergent fiber and lignin with R_a_β (Table 3). However, others found a negative relationship between acid detergent insoluble crude protein and the ruminal and intestinal availability.[39,40] In this study, a positive

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Table 6. Data Obtained from Multiple Regression Analysis Used To Find the Most Important Variables To Predict Protein Nutrient Supply Using Protein Molecular Structural Parameters in Canola Meal and Canola Presscake

predicted variable (Y)	variable(s) selection (variables left in the model with $P < 0.05$)	prediction eq (test model: $Y = a + b_1 \times 3x_1 + b_2 \times x_2 + ...$)	R^2	RSD	P
chemical profiles	no variables met the 0.05 significant level for entry in the model				
protein profiles[a] (g kg^{-1} CP)					
CP (g kg^{-1} DM)	ratio of α-helix to β-sheet left in the model	CP = 781.78 − 403.67 × R_a_β	0.792	29.04	0.018
NDICP	ratio of α-helix to β-sheet left in the model	NDICP = −152.82 + 309.90 × R_a_β	0.680	29.83	0.044
PB3	ratio of amide I to amide II left in the model	PB3 = 3.40 + 30.10 × R_I_II	0.738	5.036	0.028
PB2	no variables met the 0.05 significant level for entry in the model				
PB1	no variables met the 0.05 significant level for entry in the model				
PA	no variables met the 0.05 significant level for entry in the model				
PC	no variables met the 0.05 significant level for entry in the model				
TP	no variables met the 0.05 significant level for entry in the model				
digestible nutrients[b] (NRC-2001 summary approach) (g kg^{-1} DM)					
tdCP	ratio of α-helix to β-sheet left in the model	tdCP = 876.08 − 524.53 × R_a_β	0.767	81.10	0.022
TDN_{1X}	ratio of amide I to amide II left in the model	TDN_{1X} = 1335.37 − 213.90 × R_I_II	0.759	67.77	0.024
in situ protein degradation kinetics[c] (g kg^{-1} CP)					
K_d (h^{-1})	ratio of α-helix to β-sheet left in the model	K_d = −6.54 + 14.10 × R_a_β	0.769	1.085	0.022
D	ratio of α-helix to β-sheet left in the model	D = 1365.82 − 511.18 × R_a_β	0.787	37.26	0.018
EDCP	ratio of α-helix to β-sheet left in the model	EDCP = 154.29 + 362.058 × R_a_β	0.785	26.59	0.019
RUP	ratio of α-helix to β-sheet left in the model	RUP = 845.71 − 362.05 × R_a_β	0.785	26.59	0.019
S	no variables met the 0.05 significant level for entry in the model				
intestinal digestibility of RUP (g kg^{-1} CP)					
IVCPD[d]	ratio of α-helix to β-sheet left in the model	IVCPD = 1645.30 − 957.56 × R_a_β	0.759	75.68	0.024

[a]CP, crude protein; NDICP, neutral detergent insoluble crude protein; PB3, slowly degradable protein fraction, calculated as NDICP minus ADICP; PB2, intermediately degradable protein fraction calculated as total CP minus the sum of fractions PA, PB1, PB3, and PC; PB1, rapidly degradable protein fraction that is soluble in borate phosphate buffer and precipitated with trichloroacetic acid, calculated as SCP minus NPN; PA, fraction of CP that is instantaneously solubilized at time zero, calculated as NPN; PC, fraction of undegradable protein, calculated as ADICP. [b]tdCP, digestible crude protein; TDN_{1X}, total digestible nutrients at maintenance estimated from NRC dairy model 2001. [c]K_d, degradation rate; D, potentially degradable fraction in the in situ ruminal incubation; EDCP, effectively degraded crude protein; RUCP, rumen undegraded crude protein; S, potential soluble fraction in the in situ ruminal incubation. [d]IVCPD, in vitro crude protein digestibility in small intestine.

correlation ($R = 1.00$, $P < 0.05$) between the α-helix and ADICP was found, which indicates that a higher α-helix value may result in a lower ruminal and intestinal protein availability of canola meal or presscake.

Our results showed that there was a positive correlation between protein structure, α-helix to β-sheet ratio, and in situ protein degradation in the rumen, which was in agreement with a recently published study by Samadi et al.[34] on heat-treated canola seed. As for the CNCPS protein subfractions, the PB1 fraction and true protein tended to be correlated with R_a_β (Table 3).

Furthermore, modeling results showed that although calculation equations were different between the DVE/OEB system and the NRC-2001 model, α-helix was positively correlated with both $AMCP^{DVE}$ ($R = 0.98$, $P < 0.05$) and $AMCP^{NRC}$ ($R = 0.94$, $P < 0.05$). The α-helix to β-sheet ratio was negatively correlated with the $ARUP^{DVE}$ value and α-helix was not significantly correlated with the DVE value (Table 5). The prediction of protein supply based on the NRC-2001 model showed that β-sheet was significantly positively correlated with the metabolizable protein and the degraded protein balance. The protein secondary structure characteristics were not correlated with the ruminally undegraded crude protein independent of the evaluation system used.

Prediction of Protein Digestive Characteristics from Protein Molecular Structure. Multiple regression equations were successfully established on the basis of the protein profiles and digestive characteristics and protein molecular structure parameters. The tested multiple regression model was Y = amide I + amide II + amide I to amide II ratio (R_I_II) + α-helix + β-sheet + α-helix to β-sheet ratio (R_a_β). This analysis was carried out to explore the most suitable protein structure variable that could be used to predict nutrient supply to dairy cattle.

Using Protein Structure Parameters as Predictors of Protein Profile, in Situ Rumen Degradation Kinetics, Estimated Intestinal Protein Digestibility, and Predicted Nutrient Supply. Concerning protein profiles, the results showed that the α-helix to β-sheet ratio was a better predictor of crude protein and neutral detergent insoluble protein, accounting for 79 and 68% of total variance, respectively (Table 6). As for the CNCPS system, the amide I to amide II ratio could be used as a predictor of the slowly degradable protein fraction (PB3) and accounted for 74% of the variance. These results were in accordance with the study of Liu et al.[32] on different cereal grains (wheat, corn, and triticale) and their dried distillers grains with solubles.

For the in situ degradation kinetics, the α-helix to β-sheet ratio was the only predictor of the degradation rate, degradable fraction, and ruminal degraded and undegradable protein,

whereas the same structure variable could be solely used for the in vitro prediction of intestinal digestibility (Table 6).

According to the DVE/OEB system, the R_a_β sheet was the only variable left in the model for ENDP, $ARUP^{DVE}$, and DVE, accounting for 71, 81, and 81% of the variation, respectively (Table 6). Using the NRC-2001 model, the amide I to II ratio was a better predictor of the $AMCP^{NRC}$ and the degraded protein balance.

In summary, the results of this study indicate that ATR-FT/IR molecular spectroscopy can be used to rapidly characterize feed structures at a molecular level. In addition, it is possible to relate feed functionality, digestive behavior, and nutrient utilization to the specific chemical makeup of the feed intrinsic structures. This ATR-FT/IR molecular spectroscopy technology will provide us with a greater understanding of the plant−animal interface, which is very important for sustainable animal productions. However, a large scale of in vivo research study is required to determine the applicability of the protein molecular structural parameters investigated.

■ ASSOCIATED CONTENT

Supporting Information

Figure 2. Typical spectra (original spectra, smoothed spectra and second-derivative spectra) of yellow canola meal (CM_Y, *B. juncea*), brown canola meal (CM_B, *B. napus*), and canola brown presscake (CPC_B, *B. napus*). This material is available free of charge via the Internet at http://pubs.acs.org.

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