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New NIRS Calibrations for Fiber Fractions Reveal Broad Genetic Variation in *Brassica napus* Seed Quality

Benjamin Wittkop, Rod J. Snowdon, and Wolfgang Friedt*

Department of Plant Breeding, IFZ Research Centre for Biosystems, Land Use and Nutrition, Justus Liebig University, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

ABSTRACT: Near-infrared reflectance spectroscopy (NIRS) calibrations were developed for the estimation of neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) in intact seeds of oilseed rape (*Brassica napus*). A set of 338 diverse winter oilseed rape genotypes showing broad variation for seed color was used as a basis for the new calibrations. Different calibrations were generated for 10 or 1 mL seed volumes, respectively. In both seed volumes good coefficients of determination for external validation (R^2) of the calibrations were obtained for ADL, the major antinutritional fiber fraction in oilseed rape meal, and adequate calibrations for NDF and ADF. Evaluation of diverse *B. napus* germplasm with the new calibrations revealed a surprisingly broad variation in contents of ADL in dark-seeded oilseed rape. The ability to use NIRS for efficient selection of low-fiber genotypes, irrespective of seed color, represents an important breakthrough in breeding for improved nutritional quality of seed extraction meals from oilseed rape.

KEYWORDS: *oilseed rape (Brassica napus), near-infrared reflectance spectroscopy (NIRS), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), seed color*

INTRODUCTION

World production of oilseed rape and canola (*Brassica napus*) has risen dramatically during the past two decades because of increasing demands for high-quality vegetable oil and biodiesel. The seed meal residue remaining after oil extraction is an important byproduct used for livestock feeding, having a high protein content of around 40% combined with comparatively high concentrations of essential sulfur amino acids such as methionine and cysteine. On the other hand, however, the use of oilseed rape seed meals in animal nutrition is still limited due to several antinutritive or nonenergetic components, particularly in the feeding of monogastric animals (pigs and poultry). In particular, the small seed size and relatively high proportion of seed coat in the meal lead to high concentrations of the fiber fractions, such as neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL). Animal nutritionists suggest that ADL, consisting largely of lignin-related phenolic compounds, is the most nutritionally relevant fiber fraction. The poor digestibility of ADL in both ruminant and monogastric animals leads to a reduction in energy uptake from high-ADL meals.[1] Whereas dehulling of *B. napus* seeds leads to a significant reduction of fiber compounds, and particularly ADL, in oilseed rape meal,[1,2] a more economically viable option for reduction of antinutritive fiber is breeding for reduced seed coat thickness. Reducing the seed coat contribution to the seed volume is also expected to result in an increased proportion of oil and protein in the seed, leading to an improvement in overall seed value.[3−5]

To date, most efforts to reduce fiber content in oilseed rape seed meal have focused on the use of the yellow seed trait as a selection marker for reduced seed coat thickness. However, seed color is known to be environmentally sensitive, and it can be difficult to develop pure-breeding varieties. On the other hand, we have demonstrated that considerable useful variation for seed fiber content also exists within dark-seeded *B. napus*, which could represent an important resource to breed for improved meal quality.[6] A prerequisite is the establishment of effective selection methods to rapidly measure fiber fractions in breeding materials. Standard wet chemistry analytical techniques are currently used to determine contents of NDF, ADF, and ADL; however, such methods are costly, time-consuming, and destructive, making them unsuitable for early breeding generations for which only small seed quantities are available from breeding lines.

Near-infrared reflectance spectroscopy (NIRS) is a high-throughput analytical technique that is routinely used to estimate numerous quality components for different agricultural crops.[7] In oilseed rape and canola breeding programs NIRS is commonly used for the estimation of important seed quality characteristics such as oil, protein, and glucosinolates contents.[8] Font et al.[9] reported on the possibility to estimate ADF in oilseed rape via NIRS; however, the method has not yet been reported for measurement of other fiber fractions, particularly ADL, in *B. napus*. On the other hand, NIRS has been widely reported to be an efficient analytical method for the screening of different cereal crop species and forage grasses for the rapid prediction of fiber fractions.[10]

In this study we developed new NIRS calibrations for the three major seed fiber fractions for use as a high-throughput, nondestructive selection tool to select oilseed rape and canola breeding lines with improved seed meal quality. Screening of genetically diverse winter oilseed rape populations with the new

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calibrations uncovered a broad range of phenotypic variation for seed fiber in black-seeded progenies.

MATERIALS AND METHODS

Plant Material. A set of 338 genetically diverse winter oilseed rape genotypes from several sources was used for the reference analysis and development of NIRS calibration for seed fiber fractions. The genotypes were divided into subsets for the calibration (289 and 200 seed samples for macro- and microcuvettes, respectively) and the external validation (49 seed samples for macrocuvettes and 40 samples for microcuvettes) in two different seed volumes. The difference in sample numbers for the two seed volumes was because the microcuvette calibration was developed later than the macrocuvette calibration and for 98 samples no remaining seeds were available for generation of microcuvette NIRS spectra. A total of 197 accessions were supplied by the German breeding companies NPZ Lembke (Hohenlieth), KWS Saat (Einbeck), and Deutsche Saatveredelung (Lippstadt) from their commercial breeding programs, whereas the remaining 141 genotypes derived from populations generated by Justus Liebig University, Giessen. Because light seed color is reported to be associated with low fiber contents in *B. napus* seeds,[11] yellow- and brown-seeded genotypes from different genetic backgrounds were also included in the screening panel. To account for environmental variation in seed coat characteristics, the seeds chosen for the screening panel were derived from diverse field locations in different years.

Reference Analysis for Fiber Fractions. Reference values for development of NIRS calibrations were generated by wet chemistry analysis of fiber fractions according to the methods of Van Soest[12] and Goering and Van Soest.[13] After defatting of ground seeds with petroleum ether and oven-drying, the samples were boiled in either a neutral or acid detergent solution for extraction of NDF and ADF, respectively. Extraction and boiling were carried out in 600 mL beakers on a six-position serial hot plate (Gerhardt, Germany), with evaporation prevented using 500 mL round-bottomed flasks cooled with circulating cold water. The insoluble fraction after boiling was separated in a 50 mL filter crucible, washed, dried, and weighed, allowing fiber fractions to be determined in percentage of dry weight (% DW). The quantities of remaining dry matter represent the contents of either NDF (comprising mainly hemicellulose, cellulose, and lignin-related compounds) or ADF (cellulose, lignin, and lignin−N bonds). After determination of ADF, this fraction was further treated with 72% sulfuric acid to measure ADL (comprising lignin-related phenolic compounds). Determination of each sample was repeated (two replications) and averaged. On the basis of the NDF, ADF, and ADL fractions, it is possible to determine the approximate contents of each of the three major dietary fiber components hemicellulose (NDF − ADF), cellulose (ADF − ADL), and lignin (ADL).

NIRS Spectra Sampling. Seed samples harvested from the field were cleaned and filled into ring cups with a sample volume of either 10 mL macrocuvettes (approximately 500 seeds) or 1 mL microcuvettes (approximately 50 seeds). All samples were scanned in a NIRSystems 6500 spectrometer (FOSS NIRSystems Inc., Silver Spring, MD) using an autocup sampler. Spectra were acquired in reflectance mode at 2 nm intervals over a wavelength range from 400 to 2500 nm (visible and NIR regions) and reported as $\log(1/R)$ spectra, where a ceramic plate is used as an internal white standard. Contents of seed oil, protein, and glucosinolates were derived using a calibration from VDLUFA NIRS-Qualitätssicherung GmbH (Kassel, Germany).[14] An in-house NIRS calibration for seed color was used.[15] This calibration was developed with a large panel of rapeseed genotypes segregating for seed color, using reference values for visual light absorbance assessed by the digital-optical image analysis system MARVIN (GTA Sensorik, Neubrandenburg, Germany). The calibration describes seed color as a visual light absorbance value on a scale from 1 (light) to 9 (dark).[15] Bright yellow seeds generally give a value of 2−3, whereas black seeds show values of 8−9.

NIRS Calibrations for Fiber Fractions. NIRS calibrations for NDF, ADF, and ADL were performed using WINISI II v.1.50 software (Infrasoft International, LLC, Port Matilda, PA). The modified partial least-squares regression (MPLS) method was used to correlate reference values for NDF, ADF, and ADL against NIR spectra from only the near-infrared spectral region (1100−2500 nm), to avoid spectral interference with seed color. Calibration equations were calculated with transformed raw optical data ($\log 1/R$, where R is reflectance) using the standard normal variate command "SNV and detrend" with different derivatives and smoothing steps as described by Barnes et al.[16] For both seed volumes the following mathematical treatment was used, as described by Shenk and Westerhaus:[17] derivative order = 2; segment of the derivatives (gap) = 5; first smooth = 5; second smooth = 2.

Cross-validation and principal component analyses (PCA) were performed on the calibration set, formed by 289 rapeseed samples for macrocuvettes and 200 seed samples for microcuvettes, to determine the optimal number of terms for the calibration equation and to identify chemical (T) and spectral (H) outliers. The H-outlier samples, identified with PCA before calibration equations were computed, are spectrally different from other samples in the calibration population and have a standardized H value of >3.0 (Mahalanobis distance). The T-outliers are samples with large residuals (T value > 2.5) and represent a higher discrepancy in reference values and spectrum compared with other relationships in the calibration population. A total of seven cross-validation groups and three outlier elimination steps were employed for both seed volumes.

Validation of NIRS Equations. Besides the cross-validation performed during the calibration procedure, an external validation of calibration equations with validation sets was carried out to assess the accuracy and precision of newly developed calibrations for NDF, ADF, and ADL. The validation sets are composed of 49 independent seed samples (every 7th sample from 338) for the 10 mL macrocuvettes and 40 samples (every 6th sample from 240) for the 1 mL microcuvettes. These samples were excluded from the development of the calibration. Calibration quality was evaluated by calculating the coefficient of determination (R^2) and the RPD, defined as the ratio of standard deviation (SD) of validation samples to the standard error of prediction (SEP). Finally, the ratio of SEP to the standard error of laboratory (SEL) was calculated to compare the prediction error of the NIRS calibration in relation to the reference method.[18]

Evaluation of Calibrations by Screening in Breeding Populations. The newly developed NIRS calibrations were used to evaluate seed fiber compounds in comparison to other seed quality traits in two different segregating winter oilseed rape doubled-haploid (DH) populations. The population YE2-DH consists of 166 DH lines derived from a cross between the black-seeded inbred line 'Express 617' and the yellow-seeded inbred line '1012-98'. This population therefore shows a strong segregation for seed color. Both 'Express 617' and '1012-98' have "double-low" seed quality (low erucic acid and glucosinolate contents). The population ExV8-DH consists of 250 DH lines from the cross between 'Express 617' and the black-seeded line 'V8', which has high erucic acid and glucosinolate contents.[19] Seed samples from YE2-DH and ExV8-DH lines were collected from field trials at six different locations throughout Germany between 2003 and 2009. NIRS estimates of seed quality traits were used for calculations of phenotypic and genotypic variances as well as for correlation analyses.

Statistical Analysis of Seed Quality Trait Data. Analysis of variance for seed quality traits estimated by NIRS in seed samples from different years and environments was performed with the PASW Statistics v. 18.0.2 program (IBM Software, SPSS Inc.). On the basis of the trial design the locations were considered as replicates (r), the years as environments (e), and the individual genotypes by year interactions as random; the broad sense heritability (h^2) for each trait was calculated with the formula

$$h^2 = \sigma^2_g/(\sigma^2_e/r + \sigma^2_{ge}/e + \sigma^2_g)$$

Table 1. Cross-Validation Statistics of NIRS Calibrations (Spectral Range 1100−2500 nm; MPLS, SNV, and Detrend 2,5,5,2) for the Seed Fiber Fractions Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), and Acid Detergent Lignin (ADL) Estimated by NIRS in 10 mL Macrocuvettes and 1 mL Microcuvettes, Respectively

fiber fraction	mL	N^a	range (% DW)	SD[b]	SECV[c]	R^2_{cv}[d]	SEL[e]	SD/SECV	SECV/SEL
NDF	10	278	9.25−23.44	2.74	1.67	0.61	0.71	1.64	2.36
ADF	10	280	5.87−17.91	2.49	1.13	0.79	0.64	2.20	1.75
ADL	10	275	0.85−11.56	2.00	0.80	0.82	0.62	2.49	1.29
NDF	1	196	9.68−23.44	2.56	1.66	0.54	0.71	1.54	2.35
ADF	1	194	5.35−17.57	2.51	1.07	0.81	0.64	2.34	1.67
ADL	1	193	0.85−11.56	2.19	0.83	0.85	0.62	2.65	1.33

[a]N = number of seed samples used for calibration. [b]SD = standard deviation for reference values. [c]SECV = standard error of cross-validation. [d]R^2_{cv} = coefficient of determination of cross-validation. [e]SEL = standard error of laboratory (reference analyses).

Table 2. Results of the External Validation of NIRS Calibrations for the Seed Fiber Fractions Neutral Detergent Fiber, Acid Detergent Fiber, and Acid Detergent Lignin Estimated by NIRS in 10 mL Macrocuvettes and 1 mL Microcuvettes, Respectively

fiber fraction	N^a	mL	range (% DW)[b]	mean (% DW)[b]	SD[c]	SEP[d]	R^{2e}	RPD[f]	SEL[g]	SEP/SEL
NDF	49	10	10.44−19.70	15.58	2.52	1.55	0.62	1.62	0.71	2.19
ADF	49	10	5.35−16.10	10.75	2.58	1.42	0.70	1.82	0.64	2.20
ADL	49	10	1.16−9.13	4.64	2.16	0.96	0.81	2.26	0.62	1.54
NDF	40	1	10.67−19.70	15.01	2.62	1.81	0.53	1.44	0.71	2.57
ADF	40	1	6.49−15.89	10.40	2.40	1.19	0.76	2.03	0.64	1.85
ADL	40	1	4.34−8.20	4.34	1.94	0.92	0.78	2.12	0.62	1.47

[a]N = number of seed samples used for calibration. [b]Range and mean from reference values. [c]SD = standard deviation. [d]SEP = standard error of prediction. [e]R^2 = coefficient of determination for external validation. [f]RPD = SD/SEP. [g]SEL = standard error of laboratory (reference analyses).

where σ^2_g is the genetic variance, σ^2_e the mean effective error, r is the number of locations, σ^2_{ge} is the variance in genotype × environment interactions, and e is the number of years. Pearson's pairwise correlation coefficients between different seed quality traits were calculated using the correlation function in SPSS for mean trait values of each genotype from independent environments.

RESULTS

NIRS Calibrations and Cross Validations for Fiber Fractions. A broad range of fiber fractions were captured by both the calibration sets (cf. Table 1) and the validation sets (cf. Table 2). On the basis of the reference values six new NIRS calibrations estimation of NDF, ADF, and ADL in 1 mL microcuvettes and 10 mL macrocuvettes were developed. The results of cross-validation of the calibrations with statistical parameters are shown in Table 1. The lowest SECV values and the highest R^2_{cv} values were obtained for the calibration equations for ADL, followed by the equations for ADF and NDF. Correspondingly, the SECV/SEL ratios, which show the relationship between the errors of the cross-validation and that of the reference wet chemistry, ranged from 1.29 for ADL to 2.36 for NDF in macrocuvettes and from 1.33 for ADL to 2.35 for NDF in microcuvettes. The SD/SECV ratios, which show the relationship between the naturally existing variation in the calibration population and the error of the calibration, ranged from 1.64 for NDF to 2.49 for ADL in the macrocuvettes and from 1.54 for NDF to 2.65 for ADL in microcuvettes.

The variable numbers of samples used for the development of the respective calibrations led to different numbers of T-outlier eliminations for the three fiber fractions. Totals of 11 NDF samples, 9 ADF samples, and 14 ADL samples showed large discrepancies (T values > 2.5) between their reference values and NIRS predictions. Only 4 NDF samples, 6 ADF samples, and 7 ADL samples were identified as T-outliers in the microcuvette estimates.

Modified Partial Least Square Loadings. MPLS loading plots (Figure 1) for the second-derivative (2,5,5,2) transformation illustrate the regression coefficients of each wavelength and indicate which wavelengths predominantly influence the equation model. The three fiber fractions share the same peaks at 1388, 1420, 1868, 1908, and 2052 nm, whereas peaks at 1684, 1708, 1740, 2308, and 2348 nm contribute more to the loadings for ADF and ADL than to NDF. Identification of chemical compounds and groups specific for different wavelengths with the WINISI II v. 1.50 software revealed an association of the MPLS loadings for NDF, ADF, and ADL with several functional groups. Combined vibrations of C−H groups associated with aromatic compounds at 1420 nm and O−H stretch groups at 1908 nm contributed significantly to all three fiber fractions, as did N−H protein groups at 2052 nm. The MPLS spectra for ADF and ADL were particularly strongly influenced by the absorbance of C−H stretching of aromatic compounds at 1684 nm, the S−H stretching groups at 1740 nm, and the C−H and C−O stretching groups related to oil at 2308 and 2348 nm, respectively. The MPLS loadings for microcuvettes (not shown) showed absorbance patterns similar to those of the macrocuvettes, the only visible difference being a slightly lower influence of the spectral region at 1908 nm for all three fiber fractions.

External Validation of NIRS Ealibrations. External validation of the NIRS calibrations was carried out using 49 seed samples for macrocuvettes and 40 samples for microcuvettes, respectively. The variation captured within the validation samples reflects the same broad distribution for NDF, ADF, and ADL found in the calibration population. Table 2 shows the range, mean, and standard deviation (SD) values for the reference chemistry measurements of the validation set, along with the statistical description of the validation. For both seed volumes the best validation was

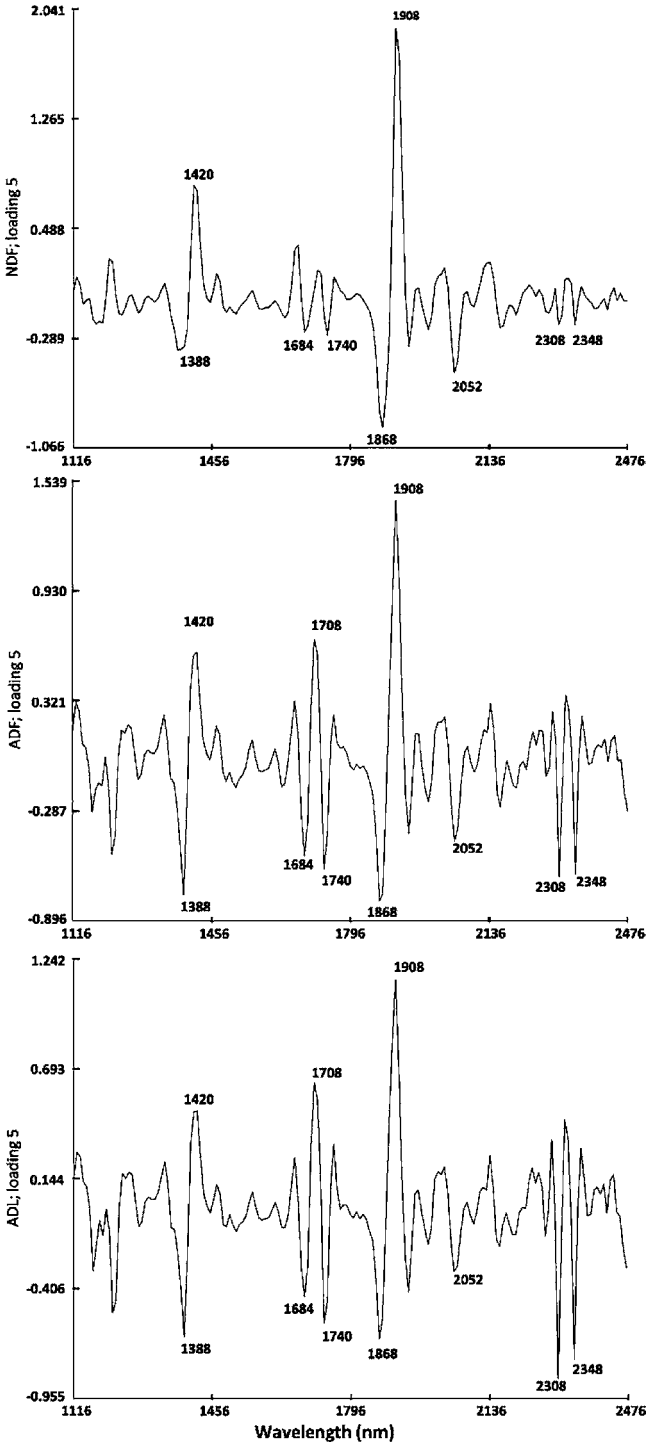

Figure 1. MPLS loading plots showing the highest correlation for neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) in 10 mL macrocuvettes for second-derivative transformation (2,5,5,2).

achieved for the ADL calibrations, with R^2 values of 0.81/0.78 and SEP values of 0.96/0.92 for macrocuvette/microcuvette estimations, respectively. Corresponding to the findings for the calibration and cross-validation statistics, the external validation of ADF contents were better than those for NDF. Figure 2 presents the graphical regressions of NIRS-predicted and wet chemistry reference values, together with the calculated SEP of the external validation for all three fiber fractions and both seed volumes. The distribution of the scattering around the external validation improves from NDF to ADL. These results presumably reflect the different individual chemical compounds that contribute to reflection and absorption in the NIRS analysis. Whereas the calibration equation for ADL takes only the reflection of phenylpropanoid lignin precursors into account, the calibration for NDF additionally incorporates the spectral information of hemicellulose and cellulose components.

The calculated RPD values, which illustrate how the error in prediction compares to the naturally existing trait variation, ranged from 2.26 for ADL to 1.62 for NDF in 10 mL seed volumes and from 2.12 (ADL) to 1.44 (NDF) for 1 mL seed volumes. The ratios observed for SEP/SEL show the relationship between the errors of NIRS prediction and the reference chemistry and reflect again that the developed calibrations are most accurate for ADL followed by ADF and NDF (Table 2).

Evaluation of NIRS Calibration and Phenotypic Trait Anaylsis in Breeding Populations. The application of newly developed NIRS calibrations for fiber fraction in two different winter oilseed rape DH populations uncovered a broad variation for relevant fiber fractions. Table 3 shows all of the observed trait data for the YE2-DH and ExV8-DH populations together with trait values for the different crossing parents. The results clearly demonstrate that the developed NIRS calibrations have the potential to detect strong variation in seed fiber in materials of very different genetic origins (Figure 3).

Interestingly, in the dark-seeded population ExV8-DH several DH lines were identified showing a negative transgressive segregation resulting in lower fiber contents than the crossing parents. The offspring from this cross showed a particularly unexpected segregation for seed ADL content, ranging from 3.2 to 8.4% of seed dry weight, although the population displays only a very limited segregation for seed color in the range from dark brown to black. Hence, the use of seed color as a selection marker for low ADL is not possible, meaning that seed color-independent NIRS calibrations are an extremely useful new selection tool.

Figure 3 compares the ADL variation uncovered in both DH populations with regard to seed color. In YE2-DH, which exhibits a broad variation for seed color, a close correlation (r = 0.834) was observed between seed color and ADL content (Table 4). Although ExV8-DH lines reveal only a weak variation for seed color, this population still showed a moderate correlation of (r = 0.621) between seed color and ADL content (Table 4).

The observed trait data for important seed quality components were used to calculate broad sense heritabilities (h^2; Table 3). The h^2 values for ADL of 0.84 in Ye2-DH and 0.91 in ExV8-DH indicate that individual fiber fractions are highly heritable regardless of environmental conditions. This is another very strong advantage of selection for reduced ADL content using NIRS rather than using the environmentally instable seed color as a selection marker.

Further phenotypic correlations were calculated among important seed quality traits on the basis of mean trait values of each genotype from independent environments. Pearson's correlation coefficients (r) among different seed quality traits are presented together in Table 4 for the YE2-DH and ExV8-DH populations. A strong negative correlation was detected between oil content and NDF. In contrast, ADL showed significant but weak positive correlations with oil content for

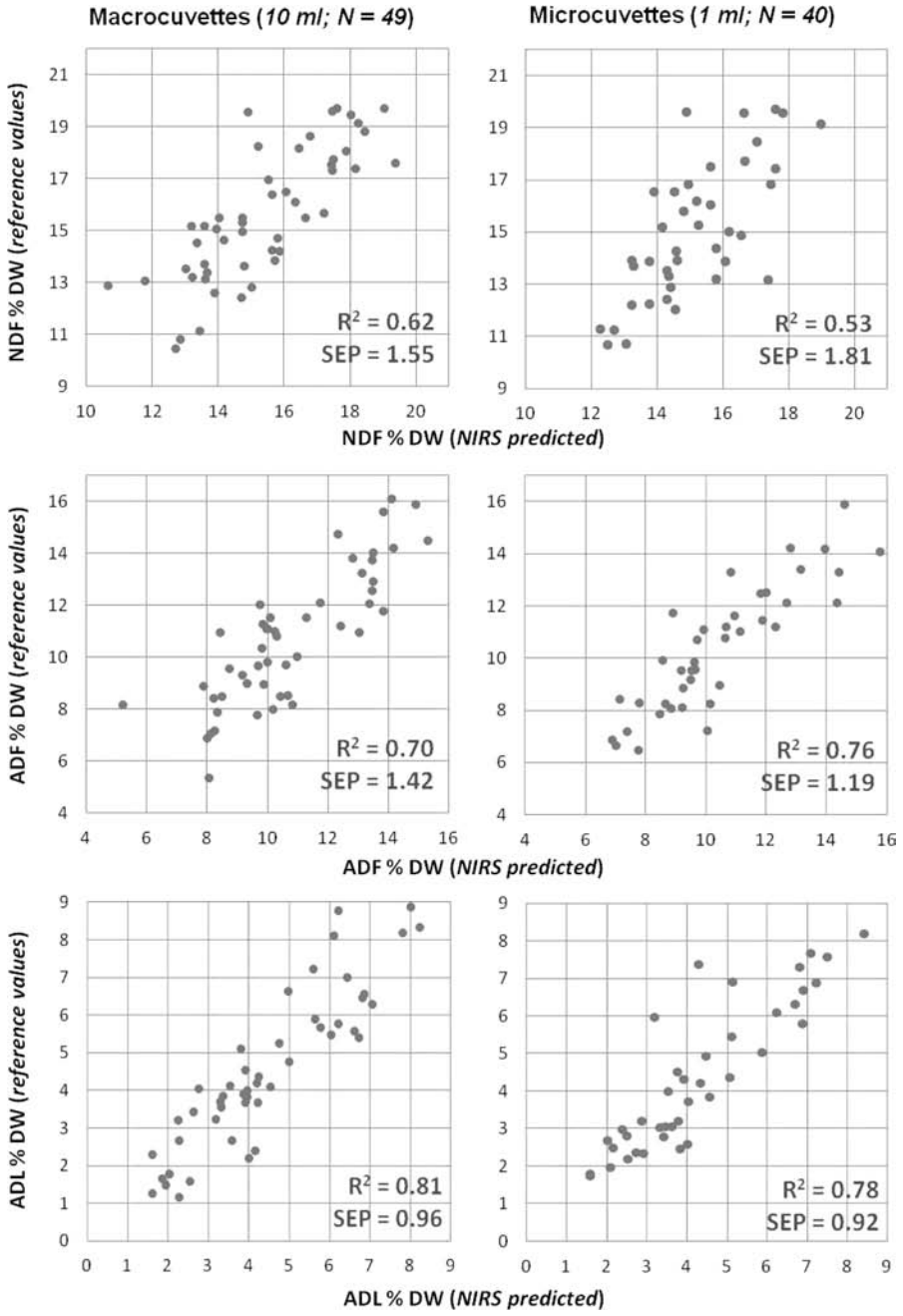

Figure 2. Graphical regression of NIRS-predicted and reference values for NDF, ADF, and ADL in two seed volumes (10 and 1 mL). R^2 = coefficient of determination for independent validation; SEP = standard error of prediction.

both YE2-DH and ExV8-DH, whereas NDF was positively correlated to ADL. These observations reflect the competition between carbon signaling pathways involved in oil biosynthesis, on the one hand, and the synthesis of hemicellulose and cellulose (which are included in the NDF fraction) on the other. In contrast to the widely held view that light-seeded oilseed rape is associated with increased oil content, both YE2-DH and ExV8-DH showed the opposite effect, with seed color and oil content being significantly positively correlated. This indicates that darker-seeded lines in these two populations in fact contained more oil than lighter-seeded lines. Conversely, weak but significant negative correlations were found between seed color and protein content and between ADL and protein content (Table 4). Only a very weak correlation was found between ADL and seed glucosinolate content, demonstrating that antinutritive fiber can be reduced without a negative pleiotropic effect on this other major oilseed rape seed meal quality component.

DISCUSSION

NIRS Calibrations and External Validation. Seed fiber compounds are of high interest in the breeding of new oilseed rape and canola varieties with improved seed meal digestibility. For reliable selection of improved breeding lines an accurate and high-throughput analytical technique for seed fiber fractions is required. Font et al.[9,20] demonstrated the utility of NIRS calibrations for ADF screening in intact *B. napus* seeds. In our study we developed new NIRS calibrations for NDF,

Table 3. Results of the NIRS Analysis of Important Seed Quality Traits for the Parents (Express 617V, V8V, and 1012-98V) and the Two *B. napus* DH Populations ExV8-DHV and YE2-DHV

	Express 617		V8		1012-98		YE2-DH (N = 166)			ExV8-DH (N = 250)		
	mean/range	SD[a]	mean/range	SD[a]	mean/range	SD[a]	mean/range	SD[a]	h^{2b}	mean/range	SD[a]	h^{2b}
seed color[c]	8.43	0.45	8.15	0.31	4.08	0.61	6.24	1.24	0.87	8.48	0.32	0.71
	8.15–8.95		7.90–8.50		3.55–4.75		2.70–8.98			6.95–9.28		
NDF (% DW)	15.92	0.46	15.13	0.71	14.83	0.23	15.87	1.38	0.73	15.99	0.79	0.92
	15.60–16.45		14.50–15.90		14.60–15.05		12.20–19.75			11.90–19.30		
ADF (% DW)	11.35	0.67	11.12	0.60	8.97	0.31	10.65	1.24	0.82	11.57	1.11	0.69
	10.85–11.15		10.55–11.75		8.70–9.30		6.70–14.41			7.35–15.00		
ADL (% DW)	6.17	0.38	5.55	0.23	2.83	0.25	4.03	1.06	0.84	5.83	0.75	0.91
	5.80–6.55		5.35–5.80		2.55–3.00		1.20–7.60			3.20–8.40		
oil (% DW)	48.82	0.63	51.12	2.05	43.48	0.71	44.56	3.99	0.31	49.48	2.79	0.79
	48.25–49.50		48.75–52.40		42.70–44.10		34.20–55.80			39.70–58.70		
protein (% DW)	20.82	0.15	22.10	0.83	22.53	1.02	22.70	3.22	0.14	21.18	1.61	0.41
	20.65–20.95		21.55–23.05		21.85–23.70		13.60–29.70			14.45–22.05		
glucosinolates (μmol/g)	20.63	1.46	48.75	7.49	23.27	7.39	21.23	9.57	0.82	33.26	12.45	0.93
	19.40–22.25		40.10–53.10		17–35–31.55		5.21–67.25			7.50–71.15		

[a]SD = standard deviation. [b]h^2 = broad sense heritability. [c]Seed color values: 1 = light, 9 = dark.

ADF, and also ADL, the most nutritionally relevant fiber compound for use of oilseed rape and canola meals in monogastric animal feeding. During the selection of genotypes with a broad variation for the calibration, the seed color of *B. napus* was taken into account, because it is reported that the yellow seed character is associated with decreasing fiber contents.[11] Font et al.[20] used the total spectral range from the visible and near-infrared region (450−2500 nm) for the development of NIRS calibrations for ADF, reasoning that lower amounts of fiber are always associated with lighter seed color and that the observed absorption in the visible region should thus improve the calibration accuracy. In contrast, we chose not to include the spectral information from the visible region in our calibrations, because the identification of H-outliers during principal component analysis showed a clear clustering of light-seeded and dark-seeded genotypes when the visible region was taken into account. For representative NIRS calibrations such an inhomogeneity of the calibration population is undesirable; hence, it was necessary to remove the visible spectral data from the calibration.

The RPD (SD/SEP) values for the new ADL calibrations were slightly below the cutoff value of RPD = 3 recommended by Williams and Sobering[18] for the use of NIRS equations as a suitable screening technique. On the other hand, some papers described NIRS calibrations as adequate when the SEP was within 2 times the SEL.[10,21,22] The ratios observed in this study fall within this range for both seed volumes. Furthermore, the observed RPD values, at least for ADL, are sufficient for recommendation of the calibrations as a useful selection tool in modern rapeseed breeding programs. A comparable RPD value of 2.5 for ADL was reported by Kong et al.,[10] who used second-derivate mathematical treatments (2,4,4,1 or 2,5,5,1) to evaluate NIRS calibrations for fiber fractions in rice. The ADF calibrations were less accurate than those for ADL; however, the R^2 statistics were still comparable with previously reported results for NIRS calibrations for ADF in different *Brassica* species and ADL in *Oryza sativa*.[9,10,20]

Although the precision of the NDF calibrations was lower compared to those for ADF and ADL, these are still sufficient to differentiate between high and low NDF oilseed rape breeding material. Analogous results were presented by Kong et al.[10] for NIRS calibrations in rice, where the calibrations for NDF are less accurate than those for ADF and ADL.

In both seed volumes (10 and 1 mL) comparable validation statistics were generated with slight differences in the observed R^2 values. A reasonable explanation is the variable amount of seeds, which may influence the strength of essential reflections and absorptions during the NIRS measurement. Nevertheless, the results obtained in this study are precise enough to recommend the use of calibrations for both the macrocuvettes and microcuvettes as selection tools for seed meal quality. The ability to use microcuvettes for NIRS-based selection is a major advantage in early breeding generations when only small quantities of seed are available and sufficient material is to be saved for planting the next breeding generation.

Because NIRS methods are based on the reflection and absorption of different chemical molecules in analytical samples, a verification of the trueness of prediction is recommended, particularly for minor seed components such as ADL. In this context rapeseed genotypes segregating for high and low ADL contents were selected for further analytical measurements. ADL represents polymeric phenolic secondary cell wall components. As such, it has its origin in the

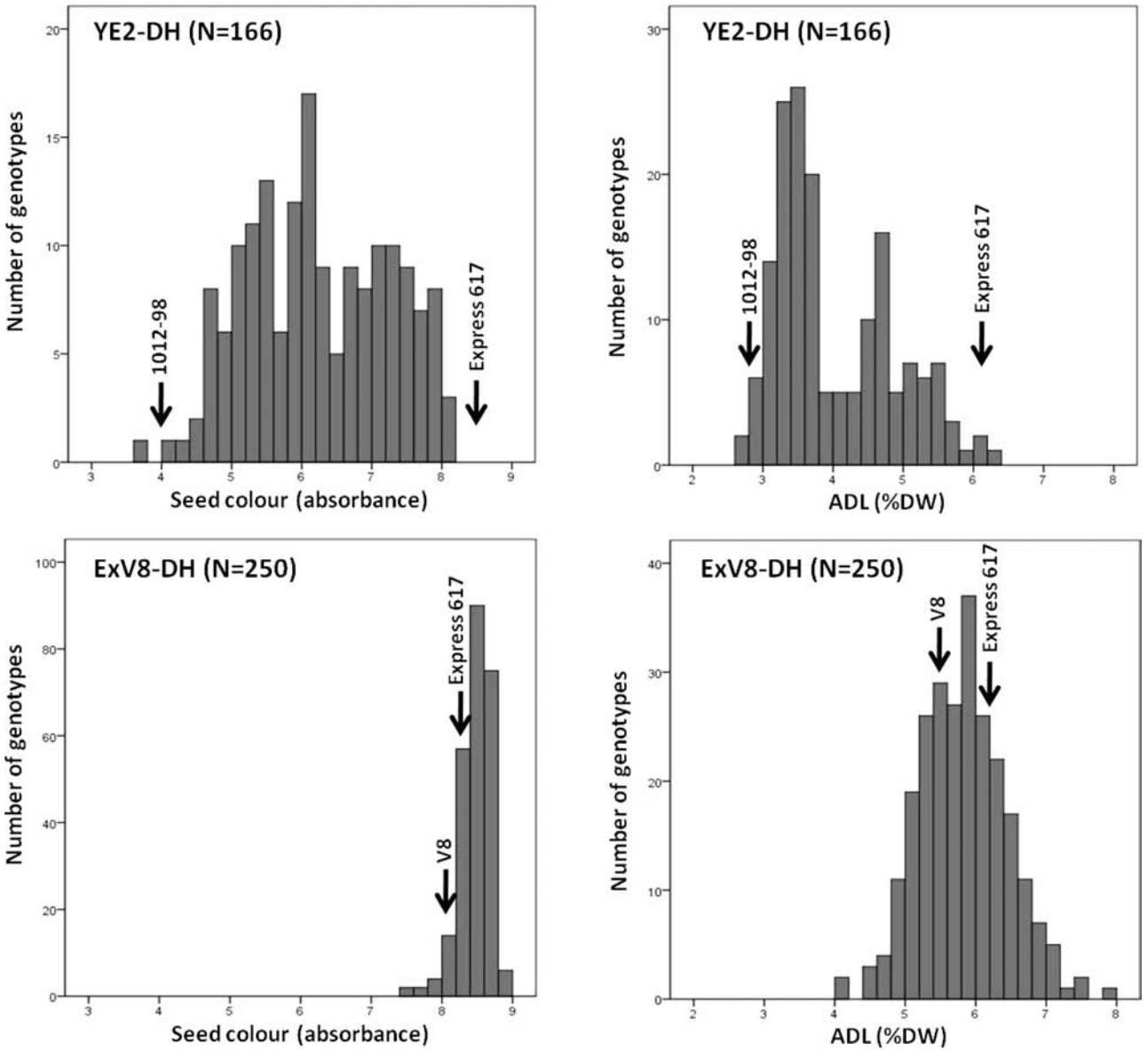

Figure 3. Phenotypic distributions for seed color and acid detergent lignin (ADL) in YE2-DH (N = 166) and ExV8-DH (N = 250) calculated with mean trait values (NIRS estimates) over all environments. Indicated with arrows are the mean values of the parents '1012-98', 'V8', and 'Express 617'.

Table 4. Pearson's Correlation Coefficient (r) among Seed Quality Traits in 166 DH Lines of the Winter Oilseed Rape Population YE2-DH (above Diagonal) and in 250 DH Lines of the Population ExV8-DH (below Diagonal)[a]

	seed color	NDF	ADF	ADL	seed oil	seed protein	glucosinolates
seed color		0.342**	0.575**	0.834**	0.404**	−0.286**	−0.109**
NDF	0.283**		0.836**	0.492**	−0.573**	0.347**	0.111**
ADF	0.578**	0.348**		0.776**	−0.141**	0.023	−0.173**
ADL	0.621**	0.457**	0.830**		0.270**	−0.121**	−0.119**
seed oil	0.227**	−0.537**	0.313**	0.289**		−0.788**	−0.295**
seed protein	−0.129**	0.199**	−0.145**	−0.068**	−0.653**		0.284**
glucosinolates	−0.091**	0.202**	−0.258**	0.070**	−0.105**	0.175**	

[a]*, **: significant at the 0.05 and 0.01 levels, respectively.

phenylpropanoid pathway where phenolic acids and phenolic mono- and polymers are produced[23] as precursors to lignin polymer formation. In collaboration with colleagues from the Leibniz Institute for Plant Biochemistry (IPB, Halle, Germany), we performed analyses of several phenolic compounds (monomers, polymers, acids) using liquid chromatography–mass spectrometry (LC-MS) and were able to observe strong correlations between ADL contents estimated by NIRS and LC-MS measurements for specific lignin precursors (A. Frolov and B. Wittkop, unpublished data). In particular, a strong correlation (r = 0.914) was identified with sinapic acid, known as the major phenolic acid in rapeseed and a major precursor

for monolignol subunits. Other notable correlations were obtained with 5-hydroxyferulic acid ($r = 0.825$) and *p*-coumaric acid, which are also known as important monolignol precursors.[23] These observations appear to verify that our NIRS calibrations for ADL are indeed very useful for prediction of phenolic fiber contents in oilseed rape seed samples. The presence of lignin, polyphenols (tannins), and phenolic acids as well as their degradation products in different *Brassica* species seed hulls was reported by Durkee[24] and Theander et al.[25] In addition, Theander et al.[25] observed differences of phenolic compounds in light- and dark-seeded turnip rape (*B. rapa*), particularly for contents of total lignin and condensed polyphenols.

MPLS Loading Plots. MPLS loading plots allow an observation of wavelengths with high variation in the calibration set that may be associated with spectral regions of known chemical origin.[24] Examination of loadings for NDF, ADF, and ADL calibration equations suggested that absorbance effects related to C−H, O−H, and N−H groups in aromatic and protein regions are important. Moreover, in all loadings a major influence at 1908 nm was observed, which is reported as a water-specific spectral region.[20] An influence of water-specific O−H groups has been reported previously in NIRS calibrations for fiber in cereal products[26] and is expected in our study because nondried seeds were used. Interestingly, only the ADF and ADL loading plots reveal a significant contribution to the model of the C−H and C−O groups of lipids (2308 and 2348, respectively). Wavelengths for specific absorbance of oil functional groups are known as major contributors to NIRS calibrations for ADF in *Brassica* species[20] and for dietary fiber in high-fat cereal products.[26] In contrast, the contribution of 2308 and 2348 nm to the NDF loading plot was relatively small.

Phenotypic Trait Analysis and NIRS as a Selection Tool. The phenotypic results we obtained with our new calibrations for fiber fractions showed that a huge natural variation exists in oilseed rape breeding material for different fiber fractions, especially ADL. The ADL fraction was found to have a positive correlation with seed color not only in the yellow-seeded population YE2-DH but also in the dark-seeded population ExV8-DH. This is presumably because the flavonoid precursors of seed coat pigments have a common origin to the phenolic lignin precursors represented as ADL in this study.[27] The large variation in seed ADL observed in the dark-seeded ExV8-DH population demonstrates clearly that seed fiber, particularly ADL, needs to be analyzed independently from seed color to uncover the complete existing variation of this trait in diverse *B. napus* breeding material.

Correlations of ADL to seed coat phenolic compounds suggest that low ADL content is associated with reduced seed coat thickness. The reduction of fiber in the seed meal correlated to a simultaneous increase in protein content, presumably due to common amino acid precursors in the phenylpropanoid and storage protein biosynthesis pathways. This correlation means that selection of low-ADL germplasm will have a double-positive effect on the overall seed meal quality.[28] In some low-fiber winter oilseed rape genotypes we were able to decrease fiber and increase protein contents without reducing the oil content.[6] A combined NIRS-based selection for low ADL, high protein, and high oil content without a compromise for increased glucosinolate content represents a great advance in our ability to breed oilseed rape for simultaneous improvement of both oil content and meal quality. Because NIRS selection is already a standard tool in oilseed rape and canola breeding programs, the new fiber calibrations can be integrated into existing NIRS selection programs after further improvements of the calibrations with a larger panel of genotypes in the calibration and validation sets.

The high heritability we estimated for seed ADL content indicates that reliable selection for this trait can already be carried out in early breeding generations. Because seed samples from single plants must be selected in early generations, it is important to use nondestructive selection methods that are suitable for small quantities of seed. Our NIRS calibrations for microcuvettes require only around 50 seeds per sample, enabling a nondestructive screening for low-ADL lines at a very early stage in the breeding process. NIRS selection for seed fiber fractions in young breeding generations can potentially reform and accelerate the breeding of varieties that have higher oil yields combined with a higher meal quality.

In this study we describe new NIRS calibrations for major seed fiber fractions in intact *B. napus* seeds in two different seed volumes. We demonstrate that the calibrations for ADF, NDF, and particularly the most relevant antinutritive fiber component, ADL, can be effectively used to select these fractions by NIRS in both young and advanced practical breeding material as well as in experimental seed lots and commodities. Phenotypic analysis of different winter oilseed rape populations revealed that genetic variation for reduced ADL content is widespread not only in yellow-seeded genotypes but also in dark-seeded materials. We therefore recommend the use of high-throughput, nondestructive NIRS selection for all important seed quality traits (oil, protein, glucosinolate, and fiber) to obtain an overall improved *B. napus* seed quality. Furthermore, these NIRS calibrations can also be efficiently used to determine major quality traits of rape seed commodities.

AUTHOR INFORMATION

Corresponding Author

*Phone: +49-641-9937421. Fax: +49-641-9937429. E-mail: wolfgang.friedt@agrar.uni-giessen.de.

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Notes

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ABBREVATIONS USED

ADF, acid detergent fiber; ADL, acid detergent lignin; NDF, neutral detergent fiber; NIRS, near-infrared reflectance spectroscopy; PCA, principal component analysis; MPLS, modified partial least-squares; R^2, coefficient of determination; R^2_{cv}, coefficient of determination for cross-validation; SD, standard deviation; SEC, standard error of calibration; SECV, standard error of cross-validation; SEP, standard error of prediction; SEL, standard error of laboratory; RPD, ratio between SD and

SEP; h^2, broad sense heritability; r, Pearson's correlation coefficient; DH, doubled haploid.

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