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Non-destructive analysis of rapeseed quality by NIRS of small seed samples and single seeds

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Abstract Near infrared-reflectance spectroscopy (NIRS) is widely used as an efficient and non-destructive method for seed quality analysis in oilseed rape (*Brassica napus* L.). Normally, calibration equations are developed for seed samples sizes of 3–4 g. However, during the breeding process much less than 3 g of samples are frequently only available for analysis. The objective of the present study was to analyse whether calibration equations developed for standard sample sizes can be used to predict the oil, protein, moisture, glucosinolate and fatty acid content of sample sizes ranging from about 450 to 5 mg (single seeds). Special adapters were made for the standard ring cups to hold the smaller samples during NIRS scanning. Close Spearman rank correlations (r_S = 0.82–1.00**) showed that NIRS calibration equations developed for standard small ring cups can be used to predict the oil, protein, and glucosinolate content of smaller samples of 450–100 mg seeds. For oleic, linolenic and erucic acid, close correlations were only found for adapter sizes holding 450–250 mg seeds (r_S = 0.79–0.98**). Only moderate to very low correlations were found for the moisture content. The standard NIRS calibration equations could also be used to predict the oil content (R^2 = 0.79**) and the protein content (R^2 = 0.72**) of single seeds (ca. 5 mg), whereas this was not possible for the glucosinolate content (R^2 = 0.54**). Therefore, single seed NIRS calibration equations were developed for alkenyl, indole and total glucosinolate content. The coefficients of determinations in cross validation ranged from 0.83 (alkenyl) to 0.86 (indole and total glucosinolates).

Keywords *Brassica napus* · Canola · Glucosinolate · Oil quality · Single seed NIRS

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In oilseed rape (*Brassica napus* L.), near-infrared reflectance spectroscopy (NIRS) has been shown to be useful to routinely estimate oil, protein and glucosinolate contents as well as fatty acid composition simultaneously in a quick and non-destructive manner using seed samples of about 3 g (Tkachuk 1981; Daun and Williams 1995; Velasco et al. 1997). However, during the breeding process and when assessing germplasm collections, much less than 3 g of samples are frequently only available for analysis. In some cases, special adapters and separate NIRS calibrations have been developed to predict seed quality traits in those samples (Reinhard 1992; Velasco and Becker 1998a, b; Velasco et al. 1998). When the seed

quality trait is determined by the genotype of the embryo it is useful to have NIRS calibrations for single seeds to enable selection among segregating F2-seeds. Such single seed NIRS-calibrations have been developed for determining fatty acids, seed weight, oil, protein and total glucosinolate content (Sato et al. 1998; Velasco et al. 1999a, b; Velasco and Möllers 2002).

The development and maintenance of precise and robust NIRS calibrations is time consuming and expensive, since much effort has to be put into the selection of seed samples which are representative for a larger germplasm collection and which are derived from a larger geographic area and from different years. It is estimated that a calibration needs to be periodically checked and improved for a longer period including several harvest seasons before it can be expected to give stable results. Typically, such calibrations consist of up to several hundred sample spectra and are developed for seed samples of 3–4 g. The question, whether these calibrations can also be used for the estimation of seed quality traits in seed samples of much less than 3 g is of high interest to breeders and researchers working on oilseed rape quality.

The aim of the present study was to systematically analyse the possibility of using NIRS calibration equations developed for small ring cups (approx. 3 g seed filling) to estimate seed quality traits in seed samples of about 450 mg, 350 mg, 250 mg, 100 mg and of single seeds (5 mg). For this purpose, simple adapters for the standard small ring cup have been made, that are suitable to hold the smaller samples during the NIRS scanning. Furthermore, NIRS calibration equations have been developed for the alkenyl, indole, aromatic and the total glucosinolate content as well as for the oil and protein content of single seeds of oilseed rape.

Materials and methods

Test set of seed samples and NIRS measurements

A set of $n = 20$ seed samples derived from highly inbred or doubled haploid lines showing a large variation for oil, protein and glucosinolate content as well as for fatty acid composition were analysed. The NIRS calibration raps2001.e-qa (Tillmann 2005) developed for the standard small ring cups (approx. 3 g seed filling) was used to estimate the contents of oil (42–59%), protein (16–27%), moisture (3.8–7.1%), glucosinolates (10–88 μmol/g), oleic acid (35–65%), linolenic acid (8–13%) and erucic acid (0–55%) in these samples. Special adapters made of PVC (4 mm thick, ϕ 38 mm) with a central hole of 14, 12, 10, and 6 mm were used to scan seed samples of about 450 mg, 350 mg, 250 mg and 100 mg, respectively. These adapters were inserted into the standard NIRS small ring cup. The seeds were hold in position from the back with an appropriate size (e.g. ϕ 14 mm) disposable sample cup back which was glued to a normal size disposable sample cup back (ϕ 38 mm). Seed samples were scanned with a NIRS monochromator model 6500 (NIR Systems mod. 6500, NIRSystems, Inc., Silversprings, MD, USA). Spectra were recorded between 400 and 2500 nm, registering the absorbance values log $(1/R)$ at 2 nm intervals for each sample. The samples measured with the adapters 10, 12 and 14 mm were measured twice, the samples measured with the 6 mm adapter were measured three times (ring cup refills from the 3 g seed sample), to avoid sampling errors.

NIRS single seed calibrations

To develop calibration equations for oil, protein, total glucosinolate content and composition (alkenyl, indole and aromatic) for single seeds of oilseed rape, seed samples from different years, locations, genotypes of winter oilseed rape were used, that showed a large range for the different quality traits. Healthy, round and normal weight seeds (about 5.5 to 6.5 mg) were dried at 65°C for 7 h and adjusted to room temperature for about 3 h.

For scanning single seeds, the adapter was made of Teflon (same size as above) but with a 3 mm central hole (Fig. 1). This adapter was inserted into the standard ring cup used for NIRS and the single seeds were put into its central hole. The seed was fixed with a small 5 mm long Teflon rod, which fit into the hole of the adapter from the

Fig. 1 Teflon adapter with a central hole for holding a single seed and a small Teflon rod (left), and the adapter in a standard small ring cup (right)

back. The ring cup was then closed with a disposable sample cup back. NIRS scanning was done as described above, but single seeds were scanned only once.

Analyses of the single seeds with reference methods

After NIRS scanning, each seed was analysed by reference methods. Oil content was measured by a gravimetric method as follows. Glass tubes of 50.0 mm × 6.5 mm diameter were filled with a single seed and weighed. Seeds were finely crushed with a stainless steel rod after adding 100 µl iso-octan:iso-propanol (9:1). Seed meal still sticking to the steel rod was rinsed back into the glass tubes with additional 100 µl of iso-octan:iso-propanol (9:1) Five hundred microliter of petroleum ether (50–70°C boiling point) to extract oil from the seed meal were added and vortexed shortly. After 10 min centrifugation (4000 u/min), the oil containing supernatant was discarded and the oil extraction step with petroleum ether was repeated. The test tubes containing the defatted meal pellet were completely dried at 30°C until no further weight change was detected, which took about 3 h. The total seed oil content was determined from the weight loss after oil extraction and expressed in % seed dry weight. To determine protein content, single seeds were weighed and then individually sealed with aluminium foil. Nitrogen concentration was determined by the Dumas combustion method using an automated CN analyser (varioEL, Elementar GmbH, Hanau, Germany). Nitrogen content was then transformed by the factor 6.25 into protein content (% seed dry weight).

Glucosinolate content and composition were determined by high-performance liquid chromatography (HPLC). Glass tubes of 50.0 mm height and 6.5 mm diameter were filled with a single seed and weighed. Seeds were finely crushed with a stainless steel rod after adding 100 µl iso-octan:iso-propanol (9:1). Seed meal still sticking to the steel rod was rinsed back into the glass tubes with additional 100 µl of iso-octon:iso-propanol (9:1). The seed meal containing glass tubes were completely dried in a desiccator until the weight stayed constant. The glass tubes were then preheated in 70°C water bath for about 5 min. After adding 200 µl of methanol (MeOH) and 10 µl internal standard solution (6 mmol glucotropaeolin l^{-1} water) for GSL extraction, the samples were vortexed shortly and placed back in the water bath for 10 min, during which they were taken out once to vortex. Samples were then added up with 300 µl water, vortexed shortly and centrifuged for 5 min at 4000 rpm. The supernatant of each sample was saved to load 10 mg Sephadex DEAE A25 columns (placed in shortened Pasteur pipettes) and each sample was washed two times with 1.0 ml water, After binding GSL to the columns by this step, 75 µl purified sulfatase (H1, *Helix pomatia* sulfatase, purified to 3.33 mg/m) solution (1:2.5 water) was added to each column. For desulfatation, the columns were incubated overnight in a 39°C oven. By adding two times 500 µl HPLC water/column the desulfoglucosinolates eluted into a 3 ml PS (Polystyrol) tube and vortexed shortly. Finally, the

solution from each sample tube was transferred into 1 ml sample vials and analysed by HPLC. Glucosinolates were determined by the HPLC gradient method for desulfated glucosinolates as described by Kräling et al. (1990) and converted to μmol g^{-1} dry weight. The alkenyl glucosinolate content is the sum of progoitrin (PRO), glucoerucin (ERU), sinigrin (SIN), glucobrassicanapin (GBN), gluconapin (GNA), and gluconapoleiferin (GNL). The components of 4-hydroxyindol-3-ylmethyl GSL (4OH), glucobrassicin (GBC), neoglucobrassicin (NEO) and 4-methoxyglucobrassicin (4ME) were combined to express the indole glucosinolate content. Gluconasturtiin (NAS) was the only aromatic glucosinolate. The sum of all three glucosinolate types is reported as the total glucosinolate (GSL) content.

Development of the calibration equations

Calibration equations were developed under WinISI II Project Manager v. 1.02a, with modified partial least squares (MPLS) regression and cross validation technique using spectral information from 400 to 2500 nm. The mathematical treatment was 1.4.4.1 for oil, 2.5.5.1 for protein and 3.6.6.1 for total and subgroup glucosinolate content. The first number is the derivative, the second number the gap, and third and fourth numbers are the smooth.

Statistics

Spearman rank correlations were calculated using Plabstat (Utz 1997). Multiple mean comparisons were made with StatGraphics Plus for Windows 3.0 (Statistical Graphics Corp., Rockville, USA).

Results and discussion

Prediction of seed quality traits using adapter ring cups (6–14 mm)

The mean results of the test seed sample set (n = 20) measured with the standard small ring cup and with the different adapters and as predicted with the equations of the raps2001.eqa are shown in Table 1. As expected for the smaller sample size, the absolute predicted seed quality values showed some deviation from the results when using the standard small ring cup (systematic bias). Interestingly, these deviations were different in size and in direction for the different quality traits. With decreasing seed sample size, the predicted oil content decreased from 50.4% (standard cup) to 38.1% (6 mm adapter cup). The protein content increased only slightly with decreasing sample size. The glucosinolate content did not show a clear trend and mean values were not significantly different. The predicted moisture and oleic acid content increased drastically with decreasing sample size. The opposite was observed for the erucic acid content. The linolenic acid content first increased and then decreased. These deviations may be explained by the fact that the PVC material used for making the 6–14 mm adapters affects the near infrared reflection at specific wavelengths in different directions (Reinhard 1992). The correlations between the predicted values using the standard ring cup and all the different adapter sizes were very close and highly significant for oil, protein and glucosinolate content (Table 2). Only moderate to very low correlations were found for the moisture content. Therefore, particular care should be taken to dry the seed samples to a

Table 1 Prediction of seed quality traits in a set of seed samples (n = 20) using the standard ring cup and different adapter cups and the raps2001.eqa (Means ± SD)

Ring cup	Oil %	Protein %	GSL μmol/g	Moisture %	18:1 %	18:3 %	22:1 %
Standard	50.4 ± 5.8	21.7 ± 2.9	45.1 ± 29.5	5.9 ± 0.9	53.0 ± 10.9	10.4 ± 1.5	19.1 ± 23.1
14 mm	48.2 ± 5.2	22.7 ± 2.7	42.4 ± 28.6	9.6* ± 0.7	64.5* ± 9.1	12.4* ± 1.4	3.7* ± 20.4
12 mm	47.5 ± 4.7	22.6 ± 2.6	42.1 ± 28.2	10.8* ± 0.7	72.0* ± 8.2	12.1* ± 1.2	–5.9* ± 19.3
10 mm	46.7* ± 4.7	22.8 ± 2.3	44.6 ± 27.6	12.6* ± 0.8	81.2* ± 7.6	9.9 ± 1.1	–23.2* ± 15.9
6 mm	38.1* ± 3.1	23.7* ± 1.9	47.1 ± 22.3	18.0* ± 0.8	142.8* ± 5.7	-1.2* ± 1.6	–115.4* ± 10.3

* Significantly different from the standard ring cup mean at 95% LSD (Multiple Range Test)

Table 2 Spearman rank correlations between the values obtained with the standard ring cup and those obtained with the different adapter cups (raps2001.eqa)

Ring cup adapter	Oil	Protein	GSL μmol/g	Moisture	18:1	18:3	22:1
14 mm	0.99**	0.98**	0.99**	0.76**	0.94**	0.95**	0.90**
12 mm	0.99**	0.97**	1.00**	0.54*	0.94**	0.98**	0.80**
10 mm	0.98**	0.99**	0.99**	0.38	0.92**	0.95**	0.79**
6 mm	0.95**	0.82**	0.99**	-0.16	–0.03	0.44*	0.18

*, ** Significant at $P < 0.05$ and $P < 0.01$, respectively

low and equal moisture content, since contents higher than 8% may affect the prediction of other seed quality traits (own unpublished results). For the different fatty acids, close correlations were only found for adapter sizes of 10–14 mm. For quality traits and adapter cup sizes highly correlated with the standard calibration, the systematic bias can be corrected by regression analysis using a test set, as shown as an example in Fig. 2 for the oil content and the 6 mm adapter cup.

Prediction of seed quality traits using a single seed adapter and development of single seed NIRS calibrations

To further test whether the standard small ring cup calibration equations of raps2001.eqa would be also suitable to predict the oil, protein and glucosinolate content of single seeds, another adapter was constructed specifically for scanning single seeds (Fig. 1). The prediction of the oil content of single seeds using the raps2001.eqa developed for standard ring cups showed a high coefficient of determination ($R^2 = 0.79$**) between the values of the reference method and of the NIRS prediction (Fig. 3). Still acceptable correlations between the predicted values and the reference values were also found for protein ($R^2 = 0.72$**), whereas for glucosinolate content the coefficients of determination were only of a much lower size ($R^2 = 0.29$) (data not shown). Based on the available spectra of single seeds and the pertinent reference values, single seed NIRS calibrations were developed for oil, protein and glucosinolate content, to see whether those would give equal or even better results. The coefficient of determinations (RSQ) were 0.98 for oil, 0.99 for protein and 0.97 for total glucosinolate content for the reference and the NIRS values (Table 3). The standard errors of cross validations (SECV) were low for oil and protein content (2.5 and 3.5% of the mean) and high for total glucosinolates (32% of the mean). Compared to the correlations obtained with the raps2001.eqa

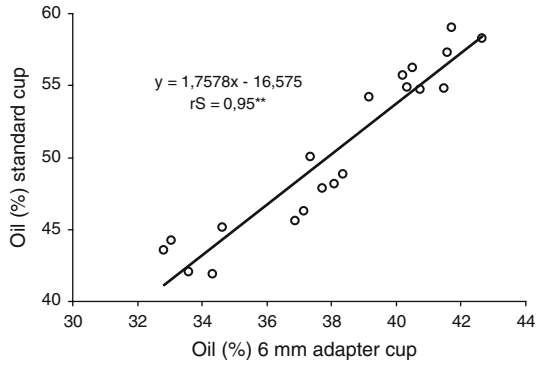

Fig. 2 Regression and Spearman rank correlations between the oil content of a test seed sample set ($n = 20$) as determined by using a 6 mm adapter cup and by using a standard ring cup. Contents were predicted using the raps2001.eqa calibration, developed for the standard ring cup

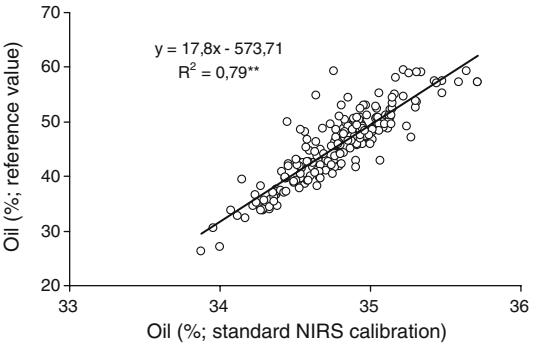

Fig. 3 Coefficient of determination between the gravimetric reference values for oil content of single seeds and the predicted values using the raps2001.eqa calibration, developed for the standard ring cup ($n = 208$)

Table 3 NIRS statistics of the calibrations for the contents of oil, protein and glucosinolates (GSL) in single seeds

Trait	Calibration						Cross validation	
	n	Mean	Range	SD	SEC	RSQ	SECV	1-VR
Oil	206	45.7	26.2–61.1	6.5	0.98	0.98	1.14	0.97
Protein	157	20.9	14.7–32.1	3.6	0.38	0.99	0.74	0.96
Total GSL	111	32.6	0.6–118.9	27.4	5.01	0.97	10.3	0.86
Alkenyl	109	25.7	0.6–86.0	22.2	4.42	0.96	9.29	0.83
Indole	107	3.3	0.2–12.2	3.6	1.05	0.91	1.35	0.86
Aromatic	109	0.9	0.3–2.1	0.44	0.27	0.63	0.34	0.36

SD = standard deviation, SEC = standard error of calibration, RSQ = coefficient of determination, SECV = standard error of cross validation, 1-VR = RSQ after cross validation

calibration equations, the coefficients of determination in cross validation of the single seed calibrations developed for oil and protein content were higher (1-VR 0.97 and 0.96; see Table 3). It can be anticipated that these values would drop to lower levels in an independent external validation using single seeds not represented in the calibration set, which, however was not performed in the present study. The calibration equations for oil and protein content of the raps2001.eqa are based on spectra of $n = 795$ and $n = 307$ seed samples. These are considerably more samples than used in the present study for the development of the single seed calibrations and hence should give more robust results in independent validation. The goodness of the single seed calibrations for oil and protein content are comparable to calibration equations previously developed for seed oil content (1-VR = 0.90) by Velasco et al. (1999a), and for protein content (1-VR = 0.96) by Velasco and Möllers (2002). A SD/SECV ratio of 5.7 and 4.8 demonstrates the usefulness of these calibration equations for selection (Fontaine et al. 2001). If NIRS is used to select the 5% or 10% seed genotypes with the highest oil content, than 8 out of 10 or 18 out 20 seeds with highest oil content are identified according to the results of the reference method.

The calibration equation of the raps2001.eqa for glucosinolate content did not allow an accurate estimation of the glucosinolate content of single seeds ($R^2 = 0.29$). Hence, the correct estimation of the glucosinolate content of single seeds requires the development of specific calibration equations. The coefficient of determination in cross validation of the single seed glucosinolate calibration developed in the present study was excellent ($R^2 = 0.96$) and compares well to the single seed results ($R^2 = 0.98$) of Velasco et al. (1999b), but also to calibrations for 300 mg seed samples with $R^2 = 0.99$ reported by Velasco and Becker (1998a). The relatively large SECV of 10.04 and a SD/SECV ratio of 2.7 indicates that this calibration equation can, however, only be applied successfully when there is a large variation among single seeds.

The coefficients of determination for alkenyl, indole and aromatic glucosinolates were 0.96, 0.91 and 0.63, respectively (Table 3). High coefficients of determination in cross-validation were found for indole glucosinolate (0.86) and for the alkenyl glucosinolate contents (0.83). The standard errors of cross validations (SECV) were high for the individual glucosinolate groups (36–41% of the mean). The development of single seed calibrations for individual glucosinolates has not yet been reported. In the present study, it was possible to develop separate single seed calibrations for the group of alkenyl, indole and aromatic glucosinolates. Such calibrations can be useful if genotypes with a low alkenyl glucosinolate content shall be combined with genotypes having a low indole glucosinolate content to achieve a further reduction in the total glucosinolate content. For the alkenyl and the indole glucosinolates, the calibration statistics was similar to the one for total glucosinolate content (see Table 2; SD/SECV ratio = 2.4 and 2.7). For the aromatic glucosinolate gluconasturtiin the calibration statistic was not convincing. This can be explained by the limited range found in the present sample collection and the generally very

low contents which may have caused an increased analytical error. The much reduced coefficients of determination in cross validation for the aromatic glucosinolate indicate that this calibration could be improved by including more samples. For standard seed sample sizes of 3–4 g the development of NIRS calibrations for individual glucosinolates has been reported by Velasco and Becker (1998a) and by Font et al. (1999).

In summary, the results of the present work show that NIRS calibrations equations developed for standard seed sample sizes of 3–4 g can be used to reliably predict the oil, protein, and glucosinolate content of smaller seed sample ranging from 450 to 100 mg. With some limitations the oil and protein content of single seeds can also be estimated. As a consequence of this, geneticists and breeders working on seed quality in oilseed rape do not need to develop and maintain separate calibration equations for smaller seed sample sizes, but rather can use standard calibration equations carefully developed and maintained over years by NIRS specialists. The results indicate that for minor constituents like fatty acids (Velaco and Becker 1998b), glucosinolates and probably also sinapic acid esters (Velasco et al. 1998) it is necessary to develop separate calibrations for smaller sample sizes and single seeds. Nevertheless, compared to any other method, the prediction of seed quality by NIRS is rapid, inexpensive, non-destructive, and sufficiently accurate to select in segregating populations. The selection among segregating F_2 seed populations is meaningful if the seed quality trait is completely or to some extent determined by the genotype of the embryo and if the trait has a sufficiently high heritability. There is evidence that seed quality traits like oil, indole and alkenyl glucosinolate content may be significantly influenced by the genotype of the embryo (Hom 2004). Similar effects have been found in transgenic plants which express gene constructs that affect seed quality traits, allowing a selection among segregating single seeds by NIRS (e.g. Kohno-Murase et al. 1995; Zou et al. 1997).

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