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Estimating the fatty acid composition of the oil in intact-seed rapeseed (*Brassica napus* L.) by near-infrared reflectance spectroscopy

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Summary

The objective of this work was to evaluate the potential of near-infrared reflectance spectroscopy (NIRS) as a rapid method to estimate the fatty acid composition of the oil in intact-seed samples of rapeseed. A total of 549 samples (3 g intact seed) from selected mutant and breeding lines were scanned by NIRS, and 220 of them were selected and scanned again by using two different adapters, which reduced the sample size to 300 and 60 mg, respectively. Selected samples were analysed by gas liquid chromatography and calibration equations for individual fatty acids were developed. Calibrations for oleic, linoleic, linolenic, and erucic acid were highly accurate, with values of r^2 in cross validation from 0.95 to 0.98 (samples of 3 g), from 0.93 to 0.97 (300 mg), and from 0.84 to 0.96 (60 mg). Calibrations for palmitic and stearic acid were less accurate, with values of r^2 in cross validation always lower than 0.8, probably because of the narrow range available for these fatty acids. The accuracy of the calibration equations for eicosenoic acid was very low (r^2 = 0.69 in 3 g samples), although improved equations were developed (r^2 from 0.78 to 0.91) when the relationship between erucic and eicosenoic acid was taken into account. We conclude that NIRS is a powerful technique to estimate the fatty acid composition of the oil in rapeseed, provided that samples covering a wide range of fatty acid levels are available, with the advantage that such estimation is possible with few additional costs when NIRS is used for the determination of other seed quality traits.

Introduction

The modification of the fatty acid profile of the seed oil to develop alternative oil types has been one of the most important objectives in the breeding for seed quality in rapeseed. This implies a demand for large number of analyses to determine the fatty acid composition of the oil. The specific chemical method for this determination is the analysis of the methyl esters of fatty acids by gas-liquid chromatography (GLC), which is accurate but also destructive, time-consuming and expensive, and requires the use of toxic and flammable reagents and gases. For plant breeding programmes involving modification of the fatty acid composition of the oil, a non-destructive screening technique with a reduced sample cost and a reduced analysis time is needed.

Near-infrared reflectance spectroscopy (NIRS) is a technique currently used on a large scale for routine analysis of several seed quality traits of intact rapeseed, such as oil, protein, glucosinolates, and chlorophyll content (Daun & Williams, 1995). The NIRS technique is non-destructive, fast, cost effective, environmentally safe, and allows the simultaneous estimation of several traits in a unique measurement, what makes it an ideal technique for plant breeding purposes (Sato et al., 1995). However, the accuracy and reliability of NIRS technique in the estimation of the fatty acid composition of intact-seed samples of rapeseed is not well documented. Koester & Paul (1989) demonstrated that NIRS could be used to estimate accurately the erucic acid content of the seed oil. This fact was later confirmed by Reinhardt & Röbbelen (1991), who found that it was possible to estimate erucic acid content in samples containing significant levels of this fatty acid, and oleic acid content in zero erucic acid samples, but the simultaneous estimation of both fatty acids was not

possible. These authors were unsuccessful in estimating other major fatty acids, such as linoleic or linolenic acid (Reinhardt et al., 1992). Daun et al. (1994) also tried to develop NIRS calibration equations for major fatty acids in rapeseed, but they concluded that the technique was not accurate enough to allow fatty acids to be estimated in routine analysis of rapeseed. Velasco et al. (1995, 1996) reported the successful application of NIRS calibration equations for individual fatty acids within a mutation breeding programme on Ethiopian mustard (*B. carinata* Braun), and the same authors (1997) developed robust and accurate equations for oleic, linoleic, linolenic, and erucic acid in this species, pointing out the critical importance of including samples covering a wide range of values for fatty acid composition.

The objective of this work was to study the potential of NIRS to estimate the fatty acid composition of the oil in intact seed samples of rapeseed. Particular attention was paid to the analysis of small samples.

Materials and methods

Rapeseed samples

A total of 549 intact seed samples of rapeseed were included in this study. Each sample consisted of at least 3 g seeds from a single plant. Most of the samples (469) were selected from the different research programmes in our department. They included several mutant lines with low linolenic acid content (Röbbelen & Nitsch, 1975), or with high oleic acid content (Rücker & Röbbelen, 1995), together with several other breeding lines. These samples corresponded to plants grown in the field or in the greenhouse during the years 1994, 1995, and 1996. A total of 74 samples, showing variability for both oleic and erucic acid content, were provided by Norddeutsche Pflanzenzüchtung Hans-Georg Lembke KG, and six samples with different levels of erucic acid were provided by Dr. W. Lühs, Institute of Crop Science and Plant Breeding, Justus-Liebig-University of Giessen (Germany).

NIRS scanning

All the samples were scanned on a monochromator NIR Systems model 6500 (NIR Systems, Inc., Silver Springs, MD) equipped with sample autochanger. About 3 g intact seeds were placed in a small ring cup (⌀ 4.7 cm), and reflectance spectra (log 1/R) from 400 to 2500 nm were recorded at 2 nm intervals. A total of 220 selected samples (see underneath) were scanned again by using two different adapters for small samples. The first adapter (⌀ 1.5 cm) required the use of about 300 mg seeds, while the use of the second one (⌀ 0.7 cm) permitted to analyse as few as only 60 mg seeds, i.e., about 15–20 seeds.

Selection of samples

A group of samples were selected according to their spectral variability to make up the calibration set. With this purpose, Select algorithm (ISI vs. 3.10, Infrasoft International, Port Matilda, PA, USA) was applied to reflectance spectra, previously transformed to second derivative (2,5,5,1) and scatter corrected with Detrend and standard normal variate (SNV). A cutoff of H = 0.8 was used, resulting in the selection of 216 samples.

GLC analyses

The fatty acid composition of the selected samples was determined through GLC analyses of fatty acid methyl esters. They were prepared following the procedure developed by Thies (1971), and analysed on a Perkin Elmer gas chromatograph model 8600 (Perkin Elmer Corporation, Norwalk, CT, USA) equipped with a fused silica capillary column FFAP, 25 m × 0.25 mm × 0.25 μm film thickness (Macherey & Nagel GmbH + Co. Kg, Güren, Germany). The oven, detector, and injector temperature were 200, 250, and 250 °C, respectively. The carrier gas was nitrogen, at a pressure of 100 kpa. Two microlitres of sample were injected, at a split rate of 1 : 70. Individual fatty acids were expressed as % of total fatty acids.

NIRS calibration

Reflectance spectra were transformed as described for sample selection, and calibration equations were developed by using the spectral information from 1100 to 2500 nm and modified partial least squares (MPLS) regression. Cross validation was used to prevent overfitting (Shenk & Westerhaus, 1993). The statistic 1 minus variance ratio (1-VR), calculated in cross validation as an estimate of the coefficient of determination, is called r^2 in cross validation throughout the text to facilitate its understanding.

NIRS calibration equations were applied to estimate the fatty acid composition of the whole population. Four samples out of the range of the calibration

Table 1. Fatty acid composition of the oil in the 220 samples of the calibration set

Fatty acid	% of total fatty acids		
	Mean	Range	Standard deviation
Palmitic (C16 : 0)	4.0	2.7– 6.1	0.7
Stearic (C18 : 0)	1.4	0.6– 2.8	0.4
Oleic (C18 : 1)	46.8	9.6–81.5	21.7
Linoleic (C18 : 2)	19.5	5.2–45.5	7.6
Linolenic (C18 : 3)	8.7	1.8–14.8	3.2
Eicosenoic (C20 : 1)	6.2	0.9–18.6	6.0
Erucic (C22 : 1)	11.4	0.0–56.4	15.2

set were detected, analysed by GLC, and added to the calibration set, which included a total of 220 samples. New calibration equations were developed with the expanded calibration set.

Spectral analysis

The most significant wavelength regions for NIRS estimation of individual fatty acids were studied by calculating within the original calibration set the correlation coefficients between the fatty acid values and the spectral values (second derivative, scatter corrected) at each wavelength.

Results and discussion

Variability for fatty acid composition of the oil in the calibration set

Table 1 shows the fatty acid composition of the oil in the 220 samples of the calibration set. The largest variability corresponded to oleic acid content, with a range from 9.6 to 81.5%. This large range of variation was due to the incorporation into the calibration set of samples from several high oleic acid mutants, and high erucic acid samples, with low levels of oleic acid. A large variation was also included for linoleic acid, closely associated to oleic acid, and linolenic acid, with the inclusion of samples from several mutants with reduced levels of this polyunsaturated fatty acid. For erucic acid, all the levels currently available in this species were represented within the calibration set, which also implied a simultaneous variation for eicosenoic acid levels, Unfortunately, the available variation for palmitic and stearic acids was considerably limited.

Table 2. Calibration and cross validation statistics in NIRS equations for percentage of individual fatty acids in the seed oil of intact rapeseed samples scanned with full cap (sample weight about 3 g)

Fatty acid	Calibration		Cross validation	
	r^{2a}	SEC[b]	r^2	SECV[c]
Palmitic (C16 : 0)	0.85	0.26	0.76	0.33
Stearic (C18 : 0)	0.67	0.20	0.62	0.22
Oleic (C18 : 1)	0.99	2.36	0.98	3.21
Linoleic (C18 : 2)	0.97	1.33	0.95	1.69
Linolenic (C18 : 3)	0.98	0.46	0.96	0.66
Eicosenoic (C20 : 1)	0.73	3.13	0.69	3.29
Erucic (C22 : 1)	0.98	1.84	0.98	2.25

[a] r^2 = coefficient of determination. In cross validation, it is estimated through 1 minus variance ratio (1-VR).
[b] SEC = standard error of calibration (% of total fatty acids).
[c] SECV = standard error of cross validation in modified partial least squares regression (% of total fatty acids).

Calibration equations with large samples

Table 2 shows the results obtained in the calibration equations developed from samples scanned by using the full cup, i.e., about 3 g intact seeds. The equations for oleic, linoleic, linolenic, and erucic acid showed high r^2 values in calibration and cross validation, and low standard errors of calibration and cross validation. This implies that an accurate and reliable estimation of these fatty acid can be obtained by NIRS and demonstrates that, in spite of previous results obtained in this species (Reinhardt & Röbbelen, 1991), the simultaneous estimation of several fatty acids with calibration equations developed from a unique calibration set is possible. Figure 1 shows the relationship of NIRS versus GLC data for oleic, linoleic, linolenic, and erucic acid content in the calibration data set. The good results obtained for these four fatty acids can be explained on the basis of the large variation for them existing in the calibration data set, as it has been pointed out in a previous study on estimation of fatty acid composition in Ethiopian mustard seeds (Velasco et al., 1997). Similarly, the results obtained for fatty acids for which variability in the calibration set was scarce, i.e., palmitic and stearic acid (Table 1), were considerably poorer, with values of r^2 in cross validation of 0.76 and 0.62, respectively, which makes it unsuitable to use these calibration equations in routine analysis. However, future availability of samples covering a wider range for these fatty acids, e.g. from transgenic plants already developed showing high stearic acid content,

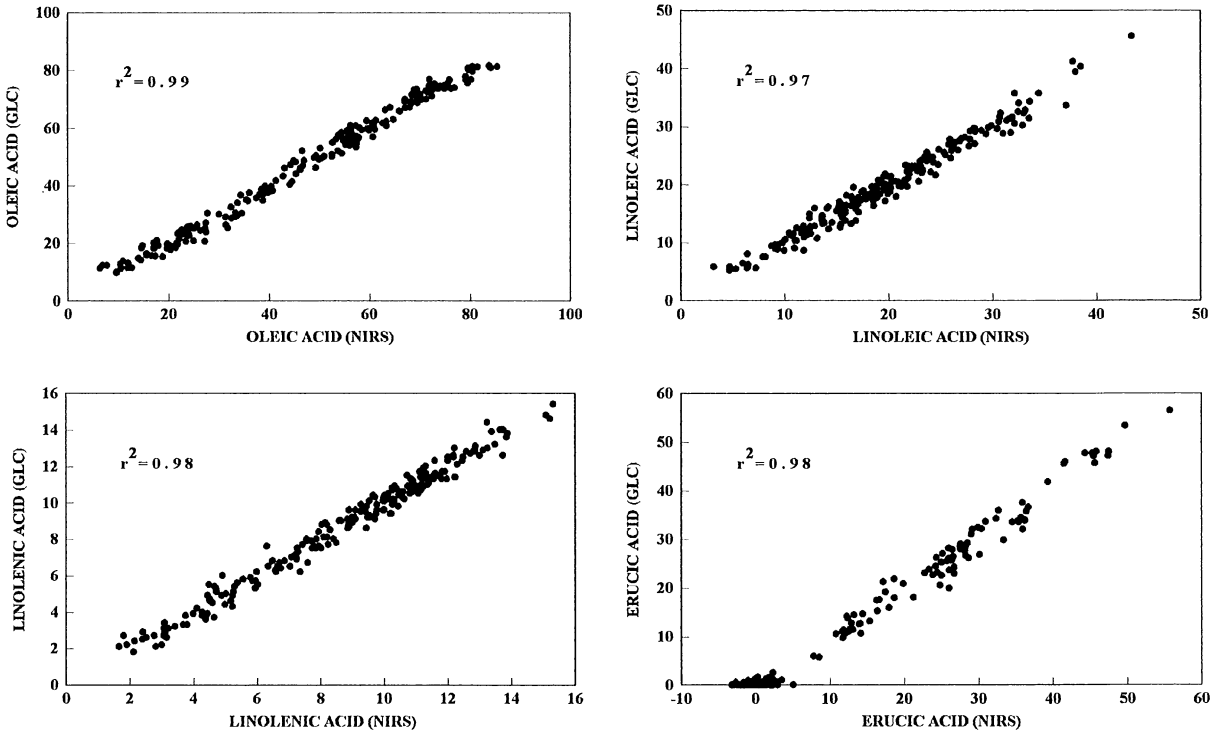

Figure 1. Calibration plots for oleic, linoleic, linolenic, and erucic acid content (NIRS estimated vs. GLC calculated) in 220 samples of about 3 g intact seeds. Individual fatty acids are expressed as % of total fatty acids.

about 25–30% of total fatty acids (Murphy, 1995), will probably result in the attainment of much more accurate calibration equations.

The results obtained in this work represent a significant improvement as compared with previous calibration equations developed in rapeseed. Thus, the best equation for oleic acid was reported by Reinhardt & Röbbelen (1991), showing an r^2 of 0.80 in validation. For erucic acid, Koester & Paul (1989) obtained an equation with r^2 of 0.94. For the other fatty acids no values of r^2 higher than 0.8 have been reported in this species. In Ethiopian mustard, Velasco et al. (1997) reported values of r^2 in validation from 0.95 to 0.98 for the fatty acids oleic, linoleic, linolenic, and erucic, which are very similar to the ones obtained in this work.

Differences in environmental conditions during cultivation is one important factor that may introduce errors in NIRS analysis of agricultural products (Shenk & Westerhaus, 1993). Velasco et al. (1997) found that calibration equations for fatty acids in Ehtiopian mustard became more robust as new samples from different environments were added to the calibration set. In this study, the calibration set included samples cultivated both in the greenhouse and in the field, in three different locations, and during three different years. It implies that, in addition to their accuracy, the NIRS equations for the major fatty acids developed in this work are supposed to be robust for reasonable environmental variations.

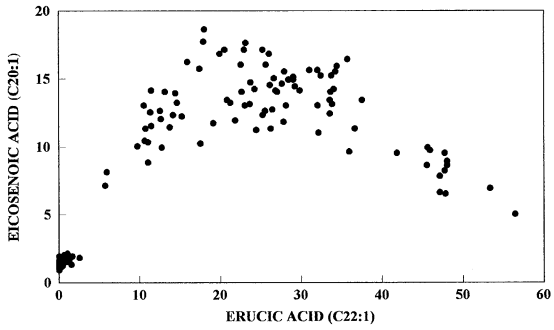

Figure 2. Relationship between eicosenoic and erucic acid (GLC values, expressed as % of total fatty acids) in the 220 samples of the calibration data set.

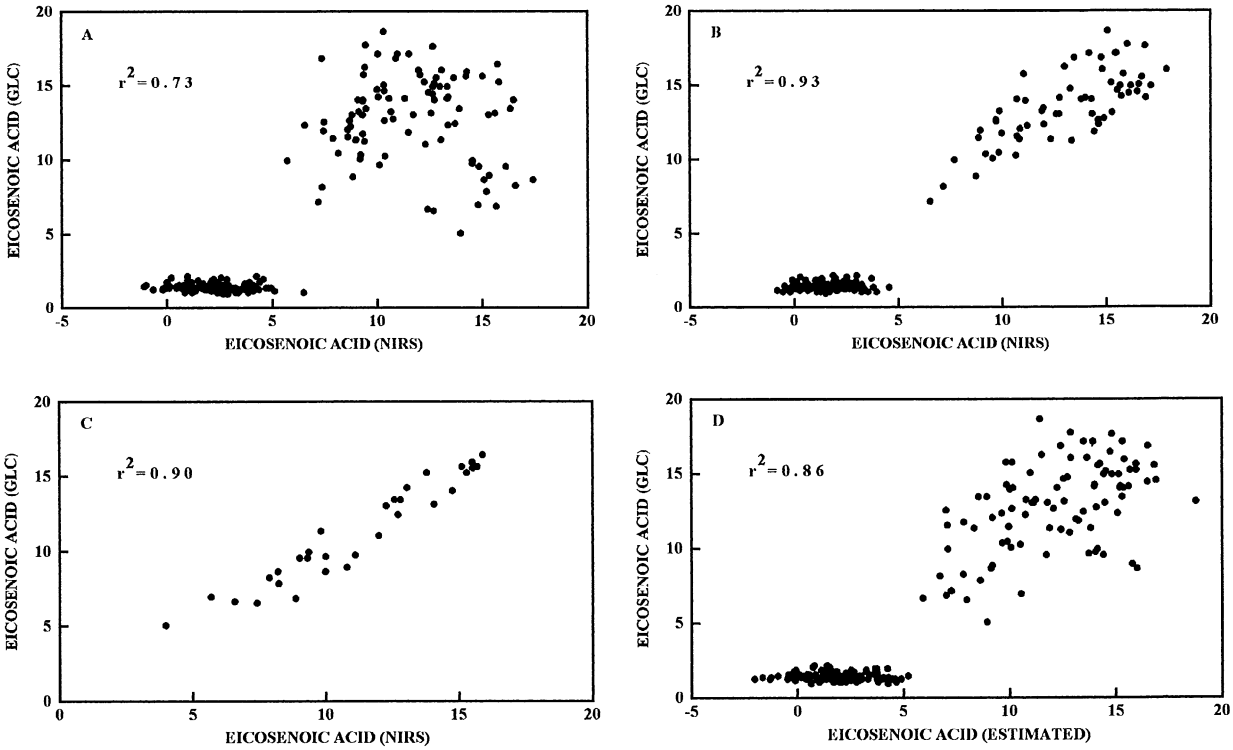

Figure 3. Different strategies to improve NIRS estimation of eicosenoic acid: (A) calibration including all samples from the calibration set; (B) calibration including only samples with erucic acid content lower than 30%; (C) calibration including only samples with erucic acid content higher than 30%; (D) estimation of eicosenoic acid from NIRS equation for erucic acid and for the sum of erucic and eicosenoic acids.

Table 3. Calibration and cross validation statistics for eicosenoic acid content in two subsets from the original calibration set, containing the samples with less and more than 30% erucic acid content, respectively

Calibration set	n	Mean eicosenoic	Calibration		Cross validation	
			r^2	SEC	r^2	SECV
Erucic acid < 30%	189	5.2	0.93	1.48	0.91	1.72
Erucic acid > 30%	31	11.3	0.90	1.07	0.78	1.92

The particular case of eicosenoic acid

The precision of NIRS for eicosenoic acid was considerably lower than expected according to the range of variation used which, for example, was wider than the range of linolenic acid (Table 1). The value of r^2 in cross validation was only 0.69, and thus even lower than for palmitic acid in spite of the larger range for eicosenoic acid values. The SECV was extremely high, 3.3% (Table 2), in a calibration set with average eicosenoic acid content of 6.2% (Table 1). To explain these unexpected results, we took into account the specific relationship between eicosenoic acid (C20 : 1) and erucic acid (C22 : 1), two fatty acids with very similar structural characteristics. In fact, all the samples with high erucic acid content showed a large prediction error for eicosenoic acid. Figure 2 shows the relationship between eicosenoic and erucic acid in the 220 samples of the calibration set. Both fatty acids were positively correlated for erucic acid values lower than about 30%, while for higher values the correlation became negative. A similar relationship was reported in rapeseed by Jönsson (1977). As a first hypothesis, we considered that this atypical relationship might produce distortion in NIRS calibration for eicosenoic acid. One important aspect taken into account was that most samples of the

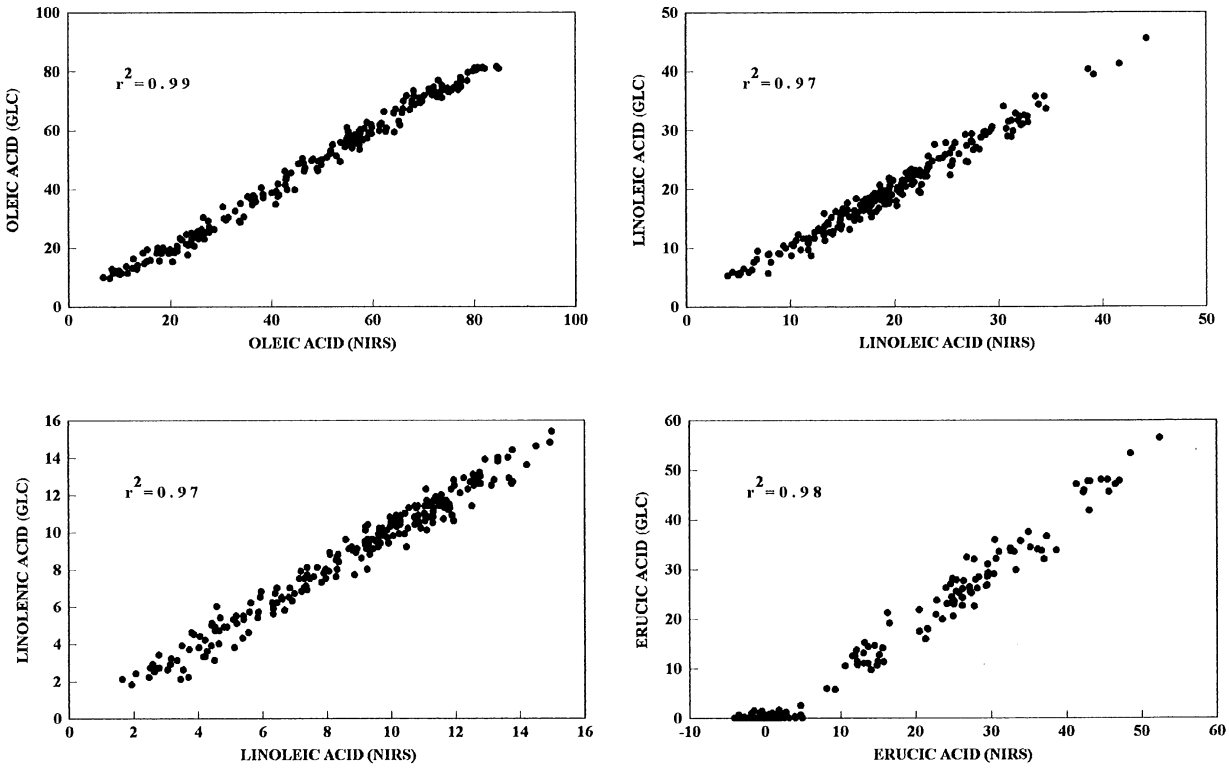

Figure 4. Calibration plots for oleic, linoleic, linolenic, and erucic acid content (NIRS estimated vs. GLC calculated) in 220 samples of rapeseed consisting in about 300 mg intact seeds. Individual fatty acids are expressed as % of total fatty acids.

Table 4. Calibration and cross validation statistics in NIRS equations for percentage of individual fatty acids in the seed oil of intact rapeseed samples scanned with medium size adapter (sample weight about 300 mg)

Fatty acid	Calibration		Cross validation	
	r^2	SEC	r^2	SECV
Palmitic (C16 : 0)	0.79	0.31	0.72	0.35
Stearic (C18 : 0)	0.74	0.18	0.67	0.20
Oleic (C18 : 1)	0.99	2.35	0.97	3.47
Linoleic (C18 : 2)	0.97	1.33	0.94	1.74
Linolenic (C18 : 3)	0.97	0.58	0.93	0.85
Eicosenoic (C20 : 1)	0.73	3.12	0.70	3.27
Erucic (C22 : 1)	0.98	2.35	0.96	2.95

calibration set had erucic acid values lower than 30% (Figure 2).

Three different strategies were followed to test whether the poor results obtained for eicosenoic acid were due to an influence of the erucic acid levels. Firstly, a new calibration set more homogeneous for erucic acid levels was built and new calibration equations developed. Secondly, the original calibration set was divided into two subsets, containing the samples with erucic acid values higher and lower than 30%, respectively. Thirdly, a calibration equation for the sum of erucic and eicosenoic acid was developed, and the eicosenoic acid content was indirectly estimated by subtracting the estimated erucic acid content from the estimated sum of both fatty acids.

The first strategy led to the development of a new calibration set with only 74 samples, equally distributed over the range of erucic acid content. Although values of r^2 in cross validation of 0.95, 0.87, 0.89, and 0.95 were obtained for oleic, linoleic, linolenic, and erucic acid, respectively, the value of this index in the calibration equation for eicosenoic acid was only 0.21. We concluded that it was not a problem of unbalance in the calibration set.

In the second case, calibration for eicosenoic acid from a subset containing only samples with erucic acid lower than 30% resulted in an equation with r^2 in cross validation equal to 0.91 and SECV equal to 1.72% (Table 3), values that represented a consider-

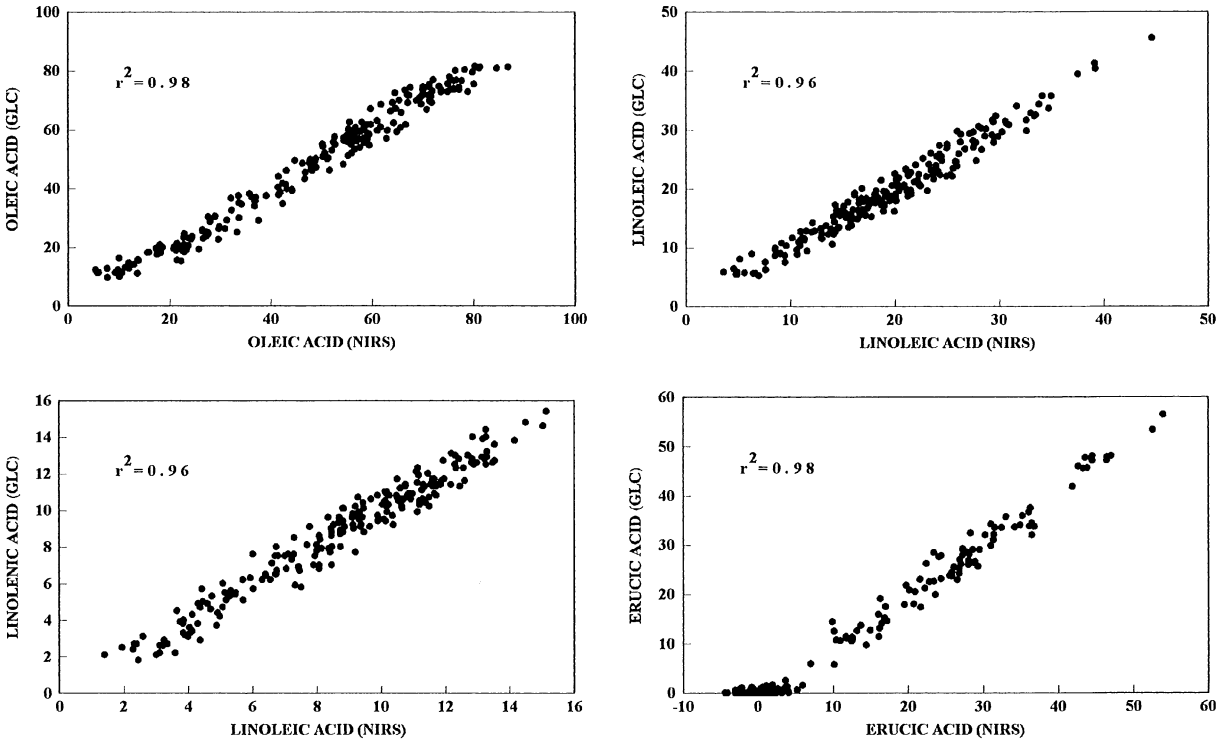

Figure 5. Calibration plots for oleic, linoleic, linolenic, and erucic acid content (NIRS estimated vs. GLC calculated) in 220 samples of rapeseed consisting in about 60 mg intact seeds. Individual fatty acids are expressed as % of total fatty acids.

Table 5. Calibration and cross validation statistics in NIRS equations for percentage of individual fatty acids in the seed oil of intact rapeseed samples scanned with small size adapter (sample weight about 60 mg)

Fatty acid	Calibration		Cross validation	
	r^2	SEC	r^2	SECV
Palmitic (C16 : 0)	0.68	0.37	0.64	0.39
Stearic (C18 : 0)	0.68	0.20	0.60	0.22
Oleic (C18 : 1)	0.98	3.36	0.96	4.44
Linoleic (C18 : 2)	0.96	1.63	0.85	2.98
Linolenic (C18 : 3)	0.96	0.69	0.84	1.31
Eicosenoic (C20 : 1)	0.75	2.99	0.69	3.32
Erucic (C22 : 1)	0.98	2.08	0.96	2.99

able improvement as compared to the use of the whole calibration set (Table 2). Furthermore, the calibration from the subset with samples showing more than 30% erucic acid resulted in an equation with r^2 in cross validation of 0.78, and SECV of 1.92%, also much more accurate than the calibration from the original set.

In the third strategy, calibration for the sum of eicosenoic and erucic acid gave an equation with r^2 in cross validation of 0.98, similar to erucic acid. When eicosenoic acid was estimated by subtracting the estimated erucic acid content from the estimated sum, and compared to GLC values, an r^2 of 0.86 was obtained, and a standard error of the estimation of 2.28%, parameters that improved the results reported in Table 2.

Figure 3 shows the distribution of eicosenoic acid values (GLC vs. NIRS estimated) by using direct calibration, as compared with the two successful strategies previously described.

Calibration equations with small samples

Calibration equations developed by using the same calibration set, but with the samples scanned with a medium-size adapter (sample weight about 300 mg), showed similar accuracy and reliability than the calibration equations developed with the full cup (Table 4). The SECV was slightly higher for most fatty acids, but this was largely compensated by the 10-fold reduction in the sample size. Figure 4 shows the calibration plots for oleic, linoleic, linolenic, and erucic acid. The use of a still smaller adapter, which allowed samples

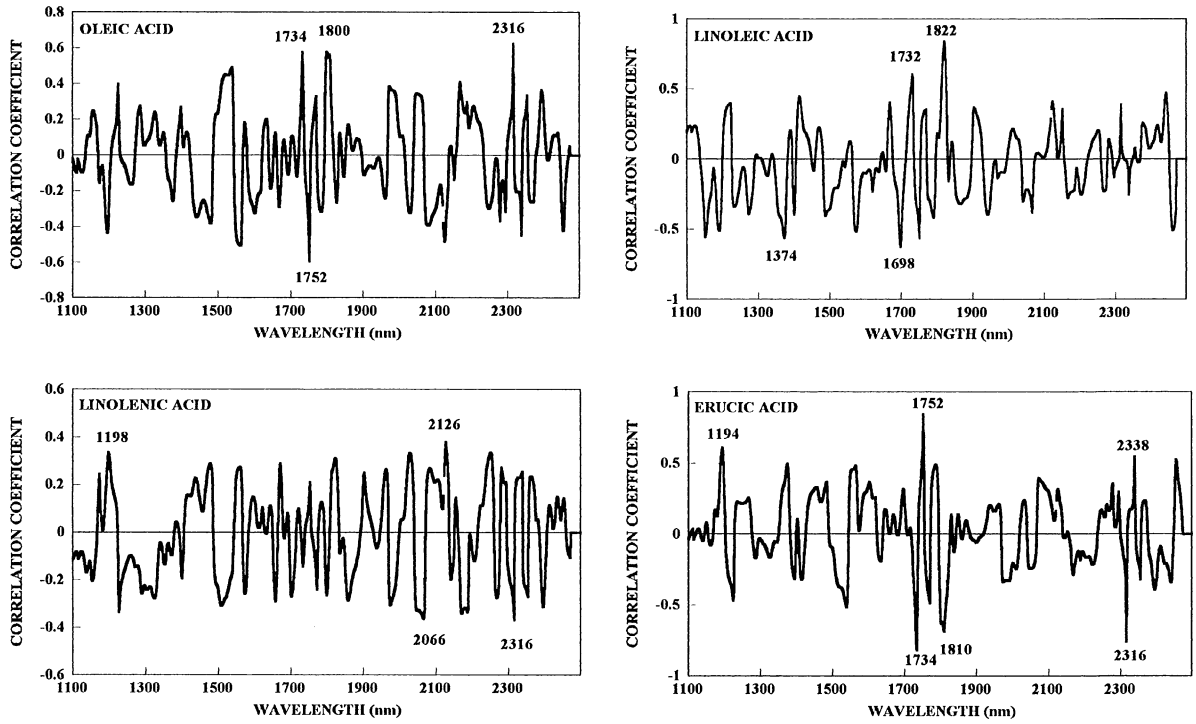

Figure 6. Correlation coefficients between fatty acid values and spectral values (second derivative, scatter corrected spectra) for oleic, linoleic, linolenic, and erucic acid. The wavelengths with the highest correlation coefficients are indicated.

consisting of 60 mg (about 15–20 intact seeds) to be scanned, resulted in a higher reduction of estimation accuracy (Table 5), as it was foreseeable on account of the 50-fold reduction of the sample size. Nevertheless, calibration equations for oleic and erucic acid, the two fatty acids for which the range of variation within the calibration set was larger, were still highly reliable and accurate, with values of r^2 in cross validation of 0.96 in both cases. This fact confirms the critical influence of the range of values in the calibration set to get good calibration equations, which becomes more critical when the sample size is reduced and, consequently, the signal in the detector is weaker. Figure 5 shows the calibration plots for oleic, linoleic, linolenic, and erucic acid by using the small-size adapter.

Spectral analysis

Although NIRS procedure is inherently empirical, its underlying analytical basis can be explored by identifying the spectral regions associated to the analysed trait (Roberts et al., 1997). Figure 6 shows the correlation coefficients at the different wavelengths for the four fatty acids for which highly accurate calibration equations were developed. The highest correlated wavelengths were 2316, 1752, 1800, and 1734 nm for oleic acid, 1822, 1698, 1732, and 1374 nm for linoleic acid, 2126, 2316, 2066, and 1198 nm for linolenic acid, and 1752, 1734, 2316, 1810, 1194, and 2338 nm for erucic acid. Most of them belong to the principal spectral regions associated to fatty acids found by Velasco et al. (1997) in the analysis of Ethiopian mustard intact seeds. The most significant wavelength for erucic acid content was 1752 nm, which fully confirms previous results of Reinhardt & Röbbelen (1991) in rapeseed, and Velasco et al. (1996) in Ethiopian mustard, who found 1750 nm as the most relevant wavelength for erucic acid determination. This wavelength is associated to the first overtones of the C-H stretch in CH_2 groups, and hence to the length of the fatty acid chain (Murray, 1987), which is consistent with the long chain of this fatty acid (C22 : 1). Likewise, the most significant wavelength for linolenic acid, a fatty acid with three double bonds (C18 : 3), was 2126 nm, which is associated to the degree of *cis*-unsaturation (Sato et al., 1991). These results indicate that the estimation of fatty acids through the calibration equations developed in this study, in addition to show a good empirical

adjust with the laboratory values, is mainly based on wavelengths related to fatty acids in previous works, which represent an additional guarantee of robustness for future analyses.

Conclusions

This work demonstrates for the first time that NIRS analysis is useful to estimate the overall fatty acid composition of the oil in intact-seed samples of rapeseed. Although accurate calibration equations were only obtained for oleic, linoleic, linolenic, and erucic acid, it was due to the availability of samples covering a wide range of values for these fatty acids, and it can be speculated that the presence of samples covering a wider range for palmitic or stearic acid will improve considerably the calibration equations for these fatty acids. NIRS estimation of the fatty acid composition of the oil in rapeseed has the main advantage that it may be carried out simultaneously to the estimation of other seed quality traits, such as oil, protein and glucosinolates, which makes NIRS a more powerful technique for the rapid, cheap and non-destructive analysis of rapeseed samples in both research and industrial applications. Furthermore, the possibility of analysing very small samples of about 60 mg in a non destructive way is especially valuable in plant breeding, where handling very small samples is frequently unavoidable. The development of these NIRS equations for individual fatty acids represents only a first step, and they should be tested, expanded, and improved with future samples from different environments and covering a wider range of fatty acid values.

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