

Multiple Linear Regression Calibrations for Barley and Malt Protein Based on the Spectra of Hordein

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ABSTRACT

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The feasibility of using NIR spectral information from barley and malt hordein was assessed as to the suitability of developing improved NIR calibrations to predict protein in barley and malt. Using extracted hordein it was possible to gain more information on wavelengths relevant to predict protein with reduced errors. Strong correlations for grain protein and NIR wavelengths were found at 1,116, 1,268, 2,040, 2,068, 2,188 and 2,300 nm. Multiple linear regression equations provided improved predicting power for barley and malt protein with a standard error of prediction of 0.15 and 0.17%, respectively, whereas partial least squares regression gave a standard error of prediction of 0.22 and 0.27% for barley and malt, respectively. The use of NIR becomes more pronounced in breeding programs as NIR is a rapid and non-destructive technique allowing the screening of early generation lines with limited grain quantities. Also, the spectral analysis of native components from resting grain components will assist in building calibrations that provide qualitative values rather than just ranking breeding lines.

Key words: Barley, hordein, malt quality, NIR.

INTRODUCTION

The level of protein in barley is an important determinant in considering the final product quality. For malting barley, a balance between carbohydrates (starch) and protein is important, with excess protein reducing the amount of available carbohydrates. Reduced carbohydrates would have negative downstream effects within breweries as well as reduced energy for feeding animals if being used as a feed source. Therefore, it is important to have robust techniques to measure protein content when selecting barley. Near Infrared Reflectance Spectroscopy (NIRS) has been used routinely to predict barley protein content for many years for grain receipt^{8,37} and more recently in barley breeding programs^{14,33,34}.

For the application of NIR in breeding programs, researchers developed calibrations to predict protein using

wavelengths from either filter or monochromator instruments^{13,14,30}. NIR has also been applied to predict amino acid composition in barley. In particular, lysine has received attention due to its importance in feed quality for animals^{11,26,37}. Protein calibrations have always been a measurement of total protein with little consideration given as to the variation in the composition of proteins or how the variation in composition may affect the NIR spectra.

The protein composition of barley varies with the most abundant protein being the storage protein called hordein which comprises between 40 - 50% of total grain protein²⁷. The hordein is stored in protein bodies bound around starch granules in the endosperm²⁷. This protein component is soluble in aqueous alcohol and is comprised of four different fractions labeled D, C, B and A²⁷. Within the hordein matrix, a number of important non-hordein proteins have been identified, including β -amylase¹⁷, limit dextrinase²⁸, endoproteinases and inhibitors¹⁸, and more recently friabilin⁷. The diversity in the hordein family has made it very useful in varietal identification³². This diversity can be explained through the differences within the B and C hordeins that occur between varieties as well as between environments^{20,21}. In addition, research has shown a variation in genetic linkages between the hordein groups⁵.

Many studies have attempted to link individual hordein groups to malting quality^{3,4,17,29,31,32}. However, the role of hordeins in relation to malting quality is yet to be completely defined. Nevertheless, it is well documented hordeins are degraded during the malting process initially by endoproteinases¹⁸ to peptides, which are degraded by exopeptidases to release amino acids. This process impacts significantly on final beer quality. The change in protein composition should be considered if developing total protein calibration. It is pertinent to add that the extraction of hordein from barley and malt demonstrated the quantitative differences between the higher barley and lower malt hordein contents, indicating that the difference in hordein occurs during malting²². To achieve the most efficient results from a quantitative technique such as NIR the use of standards related to the variable of interest would enhance the understanding of spectral information and calibration robustness. Researchers have studied the relationship between chemical standards or purified compounds and NIR wavelengths. Henry¹⁵ used simple sugars in developing multiple linear regression equations for β -glucan. Others studied the mixing properties of wheat flour and associated wavelengths with wheat proteins, gliadin and glutenin^{35,36}.

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In regards to calibration techniques, Partial Least Squares (PLS) is a relatively new technique¹⁹ using the complete NIR spectra to build algorithms. Multiple Linear Regression (MLR) was commonly used when filter instruments used a number of fixed wavelengths. However, for MLR, consideration must be given to the problem of over fitting. Garcia-Olmo et al.¹⁰ and Williams³⁹ recently compared PLS and MLR calibrations for a number of constituents in several grains and concluded that for the prediction of simple constituents there was no difference between PLS and MLR. Wesley et al.³⁵ have applied a curve fitting technique to study specific wavelengths in relation to wheat dough mixing properties.

The aim of this study was to investigate if the use of hordein spectra provided additional spectral information to build improved calibrations to predict barley and malt protein content through the application of PLS and MLR calibrations.

MATERIALS AND METHODS

Barley samples for spectral comparisons

Four commercial malting varieties (Grimmett, Tallon, Lindwall and Schooner) and three advanced breeding lines (CK85, CK123 and B%1302) from the Northern Barley Improvement Program were selected from a Sowing Date x Nitrogen treatment trial, planted at Jondaryan. At each of three sowing dates there were three nitrogen applications (50, 100 and 150 kg of urea/ha). Full details of the field experiment were described in Poulsen et al.²³.

Grain samples for calibration and validation sets

The seven genotypes described above were used for a grain protein calibration. These samples were then micromalted for routine malt quality analysis. Approximately 90 g of whole grain barley and malt samples were scanned in the reflectance mode of a NIRSystems 6500 scanning spectrophotometer. The wavelength range was from 400 to 2500 nm at 2 nm intervals, although the wavelength range 1100 to 2500 nm was used for calibrations. Spectral data was captured in WinISI software. The correlation function was selected to identify wavelengths that had high correlation with grain and total protein. Two calibrations techniques were compared, namely Partial Least Squares Regression (PLSR) and Multiple Linear Regression (MLR). For MLR, step-up regressions were used comparing 3 up to 15 wavelengths. All spectra were pre-treated with Standard Normal Variance and Detrend and Second Derivative was the math treatment applied.

Barley and malt hordein

Barley and malt hordein flours were scanned in the reflectance mode of a NIRSystems 6500 scanning spectrophotometer. The wavelength range was from 400 to 2498 nm at 2 nm intervals, although 1100 to 2498 nm were used for calibrations. Spectral data was captured in WinISI software.

Grain and malt protein content

For grain protein, samples were ground in a Falling Number 8100 hammer mill. Malt protein samples were

milled using a Buhler Miag mill (0.2 mm). Grain and malt protein was determined using a Leco FP428 combustion instrument⁹. The instrument was calibrated using ethylenediaminetetraacetic acid (EDTA (9.56% nitrogen)). Results were initially reported as nitrogen. A factor of 6.25 was used to convert nitrogen values to protein. Moisture was determined by drying 5 g of flour at 105°C for 3 h. The moisture content was used to calculate protein on a dry weight basis.

Hordein extraction

Barley and malt hordein was extracted separately using the process outlined in Osman et al.²². Approximately 5 g of finely ground barley or malt hordein, combined from the above listed cultivars, was used to obtain barley and malt hordein spectra. The nitrogen content of the hordein samples was determined using the Dumas Combustion method outlined above.

Micromalting

Samples for malting were screened to remove the fraction < 2.2 mm. A 100 g aliquot of barley was micromalted (additive free) in a Phoenix automatic micromalting machine. With 6:8:6 steep:air-rest:steep, followed by 96 h germination. All steeping, air-rests and germination stages were carried out at 17°C. The kilning stage was 22 h with temperatures ramping from 17 to 55°C - 4 h, 55-65°C - 6 h, 65-75°C - 6 h and 75-82°C - 6 h. All samples received a 15 min steep at 24 h germination. This steep increased the moisture of all samples to around 45%, although the aim was to obtain 45% moisture in the control sample.

RESULTS AND DISCUSSION

Near Infrared Reflectance (NIR) has been used routinely to predict protein in barley breeding lines by the Northern Barley Improvement Program since 1974. Early filter instrumentation dictated that Multiple Linear Regression (MLR) was used as the calibration technique. Since 1984, a monochromator instrument has been used where initially MLR then Partial Least Squares (PLS) calibrations were developed. Protein is one of the primary grain quality traits used in selecting sites suitable for quality evaluation and subsequently the protein level of barley lines, hence the accuracy of the protein calibration was critical.

The standard error (SE) for the reference protein method (Dumas Combustion) was around 0.15% and 0.16% for grain and total protein respectively, for the samples analysed. The SE of Prediction (SEP) would be expected to be slightly larger and for PLS the SEP was 0.22% and 0.27% for grain and total protein respectively, whereas the MLR calibration resulted in a 0.24% and 0.20% SEP for grain and total protein respectively. However, when wavelengths were specifically selected from correlating hordein then the SEP improved to 0.15% and 0.17% for grain and total protein respectively (Table I, Table II). Multiple linear regression calibrations improved when wavelengths were selected on chemical basis. Previous researchers have also compared PLS and MLR with varying results^{12,39}. Williams³⁹ noted that where a Ratio of (Standard Error) Performance to (Standard) Deviation

(RPD) was > 2.5 then the calibration would be suitable for a breeding program. The RPD results from this study suggest that either PLS or MLR calibrations would be acceptable within our breeding program (Table II).

The wavelengths selected for the barley MLR calibration were 1116 nm, 1268 nm, 2040 nm, 2068 nm, 2188 nm and 2300 nm. The latter four wavelengths had been assigned to N-H bonds in the WinISI software. The first two wavelengths had no assignments however it would be expected that at a lower wavelength these would penetrate deeper into the grain and therefore may have some association with protein structure. The wavelength 1116 nm had a high correlation to barley hordein with a correlation coefficient > 0.98. Although the remaining wavelengths were not selected as having a correlation coefficient > 0.9, all correlated positively to hordein. These results agree in part with previous work. Alison^{1,2} used reconstructed spectra and principle components and reported major peaks at 1980 nm, 2070, nm and 2180 with minor peaks 1500 nm, 1700 nm and 2390 nm. The region from 2176 nm to 2186 nm had been assigned to protein. Start³³ and Tragoonrung et al.³⁴ also selected a filter wavelength at 2180 nm for barley protein.

Amino acids and protein spectra have been studied to provide knowledge on the effect of peptide bonding at specific wavelengths⁴⁰ and the relationship with protein structure at wavelengths between 2000nm and 2500 nm²⁵. Yamashita et al.⁴⁰ presented data demonstrating that a strong protein peak at 2170 nm was not due to non-peptide nitrogen such as glutamine. The application of specific standards to understand NIR spectral data has also been applied to wheat proteins and starch³⁶.

TABLE I. Descriptive statistics of calibration and validation samples.

	Barley	Malt
Calibration set		
No of samples	44	44
Range (% protein db)	8.0 – 15.2	8.6 – 14.9
Mean (% protein db)	11.2	11.6
SE (% protein db)	0.10	0.15
Validation set		
No of samples	19	19
Range (% protein db)	8.2 – 14.6	8.7 – 14.4
Mean (% protein db)	11.0	11.7
Standard Deviation	0.065	0.055

TABLE II. Calibration statistics for grain protein and total protein.

	PLS	MLR	MLR ¹
Grain Protein			
r ²	0.989	0.988	0.999
SEP	0.22	0.24	0.15
No of factors ²	10	10	6
RPD ³	3.38	3.69	2.31
Malt Protein			
r ²	0.967	0.998	0.998
SEP	0.27	0.20	0.17
No of factors	4	4	4
RPD ³	4.91	3.64	3.09

¹ Based on selected wavelengths.

² For MLR Factors are wavelengths.

³ Residual Predictive Deviation (Standard Error of Cross Validation or Standard Error of Prediction divided by Standard Deviation of reference values for prediction set)³⁸.

Calibrations for malt protein selected only four wavelengths, namely 1528 nm, 1716 nm, 2048 nm, and 2448 nm. Wavelengths bands 1528 nm and 2048 nm have N-H assignments while the remaining two wavelengths do not have any spectral assignments. Halsey¹³ reported two wavelengths (1690 nm and 2152 nm) for a total nitrogen calibration with 1690 nm having an aromatic assignment.

Protein Spectra

The log 1/r absorbance of the barley and malt samples along with the barley and malt hordein is shown in Fig. 1. The 2nd derivative spectra for these samples is shown in Fig. 2. The spectral difference between barley and malt hordein is shown in Fig. 3.

Wavelengths in the region from 1460 to 1570 nm had high correlation coefficients for barley and malt hordeins (> 0.98), although there were no wavelengths selected from this region for either barley or malt calibration. Alison¹ suggested that this was a region where a protein with positive influence on hot water extract may exist. In barley and malt grain, the 1540 to 1600 nm spectra was quite flat although for barley and malt hordein there were peaks at 1552 and 1566 nm, a region assigned as CONH. Wave-

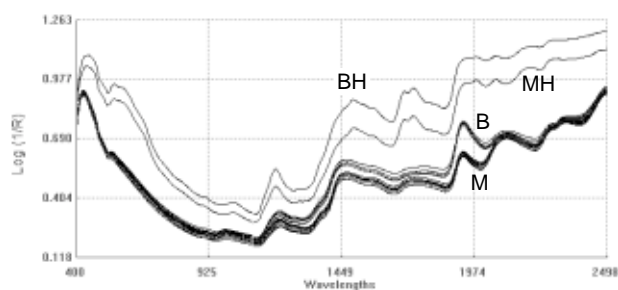


Fig. 1. NIR spectra of barley (BH) and malt hordein (MH) with barley (B), malt (M) samples for cv Grimmatt.

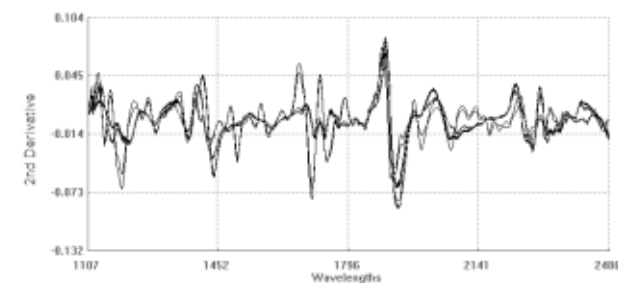


Fig. 2. Second derivative spectra of barley, malt, and hordein samples.

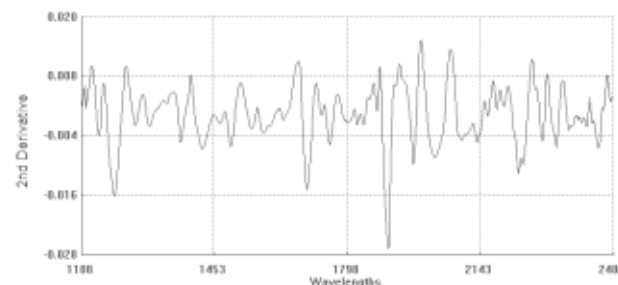


Fig. 3. Spectra differences between barley and malt hordein.

lengths in the region 1550 nm to 1568 nm had high correlation coefficients for barley (>0.99) and malt hordein (> 0.7).

A broad peak was observed from 1600 nm to 1704 nm for barley and malt grain. At 1666 nm, a peak was evident for both barley hordein and malt hordein. The absorbance for malt hordein was greater than barley hordein from 1684 nm to 1710 nm. The region from 1680 nm to 1690 nm was assigned to aromatic structures. A high negative correlation coefficient in both barley and malt hordein was evident (data not shown). This wavelength was also reported by a number of other researchers in both filter and scanning studies including^{6,33} when using for filter instruments. In addition, Williams et al.³⁷ reported 1680 nm in calibrations for aromatic amino acids including phenylalanine and tyrosine in barley.

The region from 1700 nm to 1800 nm revealed little although from 1702 nm to 1704 nm a small peak was evident. Correlation coefficients for barley and malt were 0.67 and 0.82 respectively. Williams et al.³⁷ observed a peak at 1706 nm in proline calibration. Also a trough at 1736 nm had high negative correlation coefficients for barley and malt hordein, -0.62 and -0.82, respectively. This peak has been assigned a -SH combination. This agreed with work of Tragoonrung et al.³⁴ in a filter calibration.

Another area that exhibited a high correlation ($r > 0.99$) to protein was around 2240 nm, although no wavelengths in this region were selected for MLR calibration. In the hordein samples studied in this experiment, there was no apparent decrease in malt hordein absorbance at this wavelength (Fig. 2), suggesting that there was no change in chemical structure which subsequently had an impact of NIR spectra. Alison² reported that 2240 nm may be protein peak but not related specifically to B or C hordein and postulated that this region may be protein of a high molecular weight or of a nature that inhibits endosperm breakdown.

There were a number of regions where there were differences between barley and malt hordein (Fig. 3). For most regions, the barley grain absorbance was greater than that for malt. The largest difference was in the region 1920 – 1940 nm. The large difference could be due to the breakdown in the storage protein matrix as most of the hordein breakdown occurs during malting¹⁸ as well as loss of moisture during the kilning stage.

CONCLUSIONS

The aim of this experiment was to investigate whether hordein spectra could improve calibrations for barley and malt protein. The results would suggest that it was indeed possible to improve protein calibrations when comparing wavelengths of a chemical basis to those of a raw material.

The results from the analysis of the samples assessed in this experiment demonstrated that NIR again proves to be a reliable tool for breeders and chemists in selecting suitable malting quality traits. A number of options exist to spectroscopists in developing calibration. The use of specific standards assists in identifying spectral regions that have a positive contribution to the improvement in calibrations.

Whole grain NIR analysis provides rapid, non-destructive testing which assists breeders with small amounts of early generation material. The development of calibrations derived from in-grain constituents will improve the selection ability for the breeding program as well as provide an understanding of the grain chemistry and its impact on NIR spectra.

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REFERENCES

1. Alison, M.J., *Journal of the Institute of Brewing*, 1989, **95**, 283.
2. Alison, M.J., Proceedings of 2nd Aviemore Conference on Malting, brewing and distilling, The Institute of Brewing: U.K., 1990, p. 155.
3. van den Berg, R., Muts, G.C.J., Drost, B.W., and Graveland, A., Proceedings of the European Brewery Congress, Copenhagen, IRL Press: Oxford, 1981, p. 461.
4. Brennan, C.S., Harris, N., Smith, D., Cantrell, I., Griggs, D., Tatham, A.S. and Shewery, P.R., Proceedings of the 45th Australian Cereal Chemistry Conference, Y.A. Williams and C.W. Wrigley Eds., Adelaide, Royal Australian Chemical Institute: Melbourne, 1995, p. 438.
5. Brennan, C.S., Smith, D., Harris, N. and Shewery, P.R., *Journal of Cereal Science*, 1998, **28**, 291.
6. Carnielo, M., Grandclerc, J., Muller, P., Moll, M., Grandvoinet, P., Berger, M., Simiand, J.P., Lemaitre, M., Mary, D., L'Hommel, F., Leboeuf, J.P., Rouller, M. and Mabilie, M., *Journal of the Institute of Brewing*, 1985, **91**, 174.
7. Darlington, H.F., Teci, L., Harris, N., Griggs, D.L., Cantrell, I.C. and Shewery, P.R., *Journal of Cereal Science*, 2000, **32**, 21.
8. Downey, G., Proceedings of the 4th International Conference on Near Infrared Spectroscopy, G.D. Batten, P. Flinn, L. Welsh and A.B. Blakeney, Eds., 1995, Royal Australian Chemical Institute: Melbourne p. 136.
9. Fox, G.P., Proceedings of the 44th Royal Australian Chemical Institute Cereal Chemistry Division Conference, J. Panozzo and P. Downie, Eds., Royal Australian Chemical Institute: Melbourne, 1994, p.357.
10. Garcia-Olmo, J., Garrido-Varo, A. and De Pedro, E. In: Near Infrared Spectroscopy: Proceedings of the 9th International Conference on Near Infrared Spectroscopy, A.M.C. Davis and R. Giangiacomo Eds., NIR Publications: Chichester U.K. 2000, p. 253.
11. Gill, A.A., Starr, C. and Smith, D.B., *Journal of Agricultural Science*, 1979, **93**, 727.
12. Goodchild, A.V., El Haramein, F.J., Abd El Moneim, A., Makkar, H.P.S. and Williams, P.C., *Journal of the Near Infrared Spectroscopy*, 1998, **7**, 175.
13. Halsey, S.A., *Journal of the Institute of Brewing*, 1987, **93**, 461.
14. Henry, R.J., *Journal of the Science of Food and Agriculture*, 1985, **36**, 249.
15. Henry, R.J., *Journal of the Institute of Brewing*, 1985, **91**, 393.
16. Howard, K.A., Gayler, K.R., Eagles, H.A. and Halloran, G.M., *Journal of Cereal Science*, 1996, **24**, 47.
17. Janes, P.W. and Skerritt, J.H., *Journal of the Institute of Brewing*, 1993, **99**, 77.
18. Jones, B. L., Proceedings of the 8th International Barley Genetics Symposium, S. Logue, Ed., Vol. I, University of Adelaide: Adelaide, 2000, p. 118.

19. Martins, H. and Jensen, S.A., In: Near-Infrared Technology in the Agriculture and Food Industries, P. Williams and K. Norris, Eds., American Society of Cereal Chemists: St. Paul MN, 1987, p. 55.
20. Molina-Cano, J.-L., Ramo, T., Ellis, R.P., Swanston, J.S., Bain, H., Uribe-Echeverria, T. and Perez-Vendrell, A.M., *Journal of the Institute of Brewing*, 1995, **101**, 79.
21. Molina-Cano J.-L., Polo, J.-L., Sozona, A., Voltas, J., Perez-Vendrell, A.-M. and Romagosa, I., *Journal of the Institute of Brewing*, 2000, **106**, 117.
22. Osman, A., Coverdale, S.M., Ferguson, R., Onley-Watson, K., Fox, G.P., Hamilton, S.E. and de Jersey, J., Proceedings of the 10th Australian Barley Technical Symposium, Australia Barley Technical Symposium, 2002.
23. Poulsen, D.M.E., Fox G.P., Butler, D., Sturges, J., Fromm, R., Onley, K., Ferguson, R.A., Johnston, R.P. and Inkerman, P.A. Proceedings of the 7th International Barley Genetics Symposium, University of Adelaide: Adelaide, 2000, p. 183.
24. Ratcliffe, M. and Panozzo, J.F., *Journal of the Institute of Brewing*, 1999, **105**, 85.
25. Robert, P., Renard, D., Mouhous, N. and Dufour, E., In: Near Infrared Spectroscopy: Proceedings of the 9th International Conference on Near Infrared Spectroscopy, A.M.C. Davis and R. Giangiaco, Eds. NIR Publications: Chichester U.K., 2000, p. 63.
26. Rubenthaler, G.L. and Bruinsma, B.L., *Crop Science*, 1978, **18**, 1039.
27. Shewery, P.R. In: Barley Biochemistry and Technology, A.W. MacGregor and R.S. Bhatti, Eds., American Association of Cereal Chemists: St Paul, MN, 1993, p.168.
28. Sissons, M.J., *Journal of American Society of Brewing Chemists*, 1996, **54**, 19.
29. Shewery, P.R., Brennan, C.S., Tatham, A.S., Warburton, T., Fido, R., Smith, D., Griggs, D., Cantrell, I. and Harris, N., Proceedings of the 46th Australian Cereal Chemistry Conference, C.W. Wrigley, Ed., Royal Australian Chemical Institute: Melbourne, 1996, p. 158.
30. Skerritt, J.H., Hayes, G., and Henry, R.J., *Journal of the Institute of Brewing*, 1987, **93**, 413.
31. Skerritt, J.H. and Janes, P.W., *Journal of Cereal Science*, 1989, **93**, 219.
32. Smith, D., *Plant Varieties and Seeds*, 1990, **3**, 63.
33. Starr, C., Morgan, A.G and Smith D.B., *Journal of Agricultural Science*, 1981, **97**, 107.
34. Tragoonrun, S., Hayes, P.M. and Broich, S.L., *Canadian Journal of Plant Science*, 1990, **70**, 71.
35. Wesley, I., Uthayakumaran, S., Anderssen, R.S., Cornish, G.B., Bekes, F., Osborne, B.G. and Skerritt, J.H., *Journal of Near Infrared Spectroscopy*, 1999, **7**, 229.
36. Wesley, I., Larroque, O., Osborne, B.G., Azudin, N., Allen, H. and Skerritt, J., *Journal of Cereal Science*, 1998, **34**, 125.
37. Williams, P.C., Preston, K.R., Norris, K.H. and Starkey, P.M., *Journal of Food Science*, 1984, **49**, 17.
38. Williams, P.C., In: Near Infrared Technology in Agricultural and Food Industries, P.C. Williams and K. Norris, Eds., American Association of Cereal Chemists: St Paul, MN, 1987, p. 147.
39. Williams P.C., In: Near Infrared Spectroscopy, Proceedings of the 9th International Conference on Near Infrared Spectroscopy, A.M.C. Davis and R. Giangiaco Eds. NIR Publications: Chichester U.K., 2000, p. 287.
40. Yamashita, H., Takamura, H. and Matoba, T., *Journal of Near Infrared Spectroscopy*, 1994, **2**, 145.

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