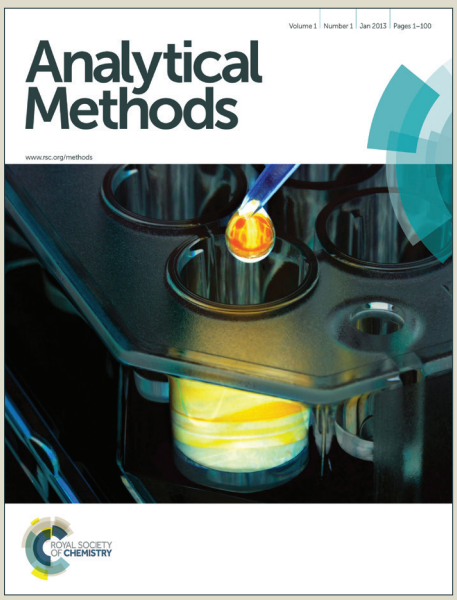
Analytical Methods

Accepted Manuscript



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FEASIBILITY STUDY ON THE USE OF ATTENUATED TOTAL REFLECTANCE MIR SPECTROSCOPY TO MEASURE FRUCTAN CONTENT IN BARLEY

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Abstract

The aim of this study was to evaluate the feasibility of using attenuated total reflectance mid infrared (ATR-MIR) spectroscopy to predict fructan content in both barley and malt flour samples. Samples (n=60) were sourced from commercial and experimental barley grain varieties and their corresponding malts. Fructan content in grain and malt flour was determined using the enzymatic kit from Megazyme (K-FRUC, Megazyme International Ireland). Samples were scanned in a MIR instrument using an ATR single bounce cell (Bruker Optics, Germany). The coefficients of determination in cross validation (R^2) and the standard error of cross validation (SECV) obtained for the prediction of fructan content in the calibration set were 0.76 and 0.20 %, respectively. The residual predictive deviation (RPD=SD/SECV) value obtained was 2.3, indicating that these calibrations can be used for qualitative determination of fructan content (e.g. low, medium and high) in the set of samples analysed. This study showed that ATR-MIR spectroscopy might be used as approximate estimates of the true fructan concentration in barley and malt in order to rank samples (low, medium, high) in the context of a breeding program.

Key words: fructan, mid infrared, partial least squares, attenuated total reflectance, barley

1. Introduction

Fructan is a fructose polymers deriving from sucrose. Like starch, fructan is naturally present in many plants as reserve carbohydrates [1-4]. The synthesis of fructan is induced by high concentrations of sucrose, while breakdown of fructan occurs during the regrowth of plants, for example at initiation of spring growth [5-9]. Fructan is based on sucrose, consisting of a single glucose residue linked to varying numbers of fructose residues [1-4] where the polysaccharide chains may be linear with β-(2, l) linkages between fructose residues (inulin-type), or β-(2, 6) linkages (levan-type) [5-9]. Fructan is the main storage carbohydrates in stems of cereals which accumulate before, during and after anthesis where they might be utilized during grain filling [5-9]. It has been also reported that fructan might enhance the cold and drought tolerance of plants and therefore are considered an important trait for plant breeding [5-9].

In recent years, there is a strong interest in the application and uses of fructan in the food industry due to their potential to improve physicochemical properties of foods as well as their potential health benefits [2-3]. Fructan constitutes part of the dietary fiber and might have an additional positive health effect by the selective stimulation of the beneficial gut bacteria [2-3]. Fructo-oligosaccharides (FOS) including fructan and inulin-type fructan are generally accepted as prebiotics by the food industry since their fermentation induces specific changes in the composition and/or activity of the gastrointestinal microflora that confer several health benefits [2-3]. Grains of both wheat and barley contain a range of so called FOS compounds including fructan [1-4]. In barley, non-starch polysaccharides such as fructan might be important in determining and improving malting and brewing qualities [10-14]. It has been also reported that insufficient degradation of non-starch polysaccharides during malting might have an adverse effect on the subsequent mashing process [13-14].

Recently, the correlation between fructan content and degree of polymerisation in several barley varieties has been reported [14].

Several methods have been described to quantify fructan in food products [4, 10-14], however, they occur at relatively low concentrations (0.7−2.9% on dry basis) in wheat and barley [4, 10-14]. Most of the currently used methods are based on enzymatic hydrolysis of fructan into glucose and fructose followed by detection of the released sugars at specific wavelengths or by analysing the sample after extraction using high performance liquid chromatography (HPLC) [4, 10-14]. However, all these methods are both laborious and time consuming to be used when a large number of samples need to be analysed.

Vibrational spectroscopy, in particular mid infrared (MIR) spectroscopy, has been used to characterize different biochemical and chemical properties in several foods [15-18]. In recent years, the combination of MIR spectroscopy with sampling methods such as attenuated total reflectance (ATR) has been reported as an analytical tool in different food systems [15-18]. This sampling method allows the measurement of solids and paste samples [15-18]. Currently, in conventional and routine chemical analysis of barley, harsh chemical reagents are used that destroy some of the biochemical and biophysical characteristics of the sample that are of importance in order to understand malting quality [19]. Although some reports can be found in the literature on the use of near infrared (NIR) spectroscopy on the measurement of fructan content in stems and leaves of wheat and grasses, no reports on the use of infrared (either NIR or MIR spectroscopy) to measure fructan content in barley grain or malt are available [20-21].

The aim of this study was to evaluate the feasibility of using attenuated total reflectance mid infrared (ATR-MIR) spectroscopy to predict fructan content in both barley and malt flour samples.

2. Materials and Methods

2.1. Grain and malt samples

Commercial barley grain varieties, experimental lines and their corresponding malts (n=60), collected from the 2009, 2010 and 2012 harvests, at two localities in South Australia (Roseworthy and Charlick) were used. Samples were malted using a Phoenix Automatic Micromalting System as reported previously [22-23]. Before analysis flour samples were obtained by milling the grain and malt samples in a laboratory mill UDY Cyclone Mill (Fort Collins, CO, USA) through a 0.8 mm screen.

2.2. Attenuated total reflectance mid infrared spectroscopy

Flour (grain and malt) samples were scanned using a platinum diamond ATR single reflection sampling module cell mounted in a Bruker Alpha instrument (Bruker Optics GmbH, Ettlingen, Germany). The samples were held against the ATR crystal using the pressure applicator or sample clamp mechanism supplied by the instrument manufacturer in order to assure that the same and constant pressure was applied for all samples. Duplicates of each sample were scanned twice (repacking) and the average ATR-MIR spectrum of each sample was used for further analysis. The ATR-MIR spectra were recorded on OPUS software version 7.0 provided by Bruker Optics. The spectrum for each sample was obtained by taking the average of 64 scans (resolution of 8 cm^{-1}, between 4000 and 375 cm^{-1}) with a scanner velocity of 7.5 kHz (background of 64 scans). Air was used as reference background spectra. The ATR diamond surface was cleaned with ethanol (95% v/v) before each sample was scanned [19].

2.3. Fructan reference analysis

Fructan content in grain and malt flour samples was determined using the enzymatic kit from Megazyme (K-FRUC, Megazyme International Ireland) following the method reported and described elsewhere [11-12; AOAC method 999.03, Megazyme Fructan Assay Procedure, Megazyme, Bray, Ireland]. In this method, sucrose, maltose, maltodextrins and starch are hydrolysed to D-glucose and D-fructose. Sucrose is then hydrolysed by a specific sucrase enzyme which has no action on lower degree of polymerisation (DP) FOS such as 1-kestose and 1, 1-kestotetraose [11-12]. Starch and maltodextrins are hydrolysed to maltose and maltotriose by pullulanase and β-amylase, and these oligosaccharides are then hydrolysed to D-glucose by maltase [11-12]. The fructan content in the samples is calculated from the absorbance of all solutions at 410 nm against the reagent blank and expressed as fructan (%) [11-12].

The reproducibility of the measurement of fructan content was estimated as the standard deviation of differences (SDD). SDD was calculated on five measurements of the standard samples supplied in the Megazyme kit.

$$SDD = \sqrt{\frac{\sum (d_i - d_m)^2}{(n-1)}}$$

where di = difference in y between five replicate measurements of sample i, dm = mean value of all replicate differences ($\sum di/n$) and n = number of samples.

2.4. Multivariate data analysis

Spectra were exported from the OPUS software in GRAMS format (*.spc) into The Unscrambler X software (version 10.1, CAMO ASA, Oslo, Norway) for pre-processing and

chemometric analysis. Before chemometric analysis (PCA and PLS) the ATR-MIR spectral data was processed using the second derivative Savitzky-Golay (40 smoothing points and second polynomial order) in order to remove and correct for baseline effects [24]. The second derivative is a measure of the change in the slope of the curve ignoring the offset and is very effective in removing both baseline offset and slope from a spectrum [24].

Principal component analysis (PCA) was performed before partial least squares regression (PLS1 algorithm) models were developed to determine any relevant and interpretable structure in the data, as well as to detect sample outliers [25]. The Hotelling T statistics provided in the software was used for this purpose. Hotelling T statistics is a linear function of the leverage that can be compared to a critical limit according to an F-test [25-26]. This statistic is useful for the detection of outliers during modeling or prediction steps. The 95% confidence ellipse was included in the score plot in order to reveal potential outliers, lying outside the ellipse [25-26].

Samples were divided into a calibration and validation set at the ratio of 3:1 as described by other authors [27] in order to guarantee that the range of the actual values in the calibration set covers the values in the validation set [27]. Thus, calibration models (n=40) between reference data (fructan content) and MIR spectra were developed using PLS regression with full cross validation. The optimum number of terms in the PLS calibration models was indicated by the lowest number of factors that gave the minimum value of the prediction residual error sum of squares (PRESS) in cross validation in order to avoid overfitting in the models [25]. Statistics calculated for the calibrations included the coefficient of determination in cross validation (R^2), the standard error of cross validation (SECV), bias and slope. The predictive accuracy of the PLS models developed to measure fructan content was tested using the remaining samples (n=20) as well as by calculating the residual predictive deviation (RPD= SD/SECV) [26].

3. Results and discussion

Figure 1 shows the second derivative of the ATR-MIR mean spectrum of samples having low and high fructan content. Most of the spectroscopic variation in the ATR-MIR was observed between 1100 and 1600 cm^{-1} mainly related to differences in carbohydrate composition between the samples analysed. Intense and characteristic bands in the region between 1500 and 900 cm^{-1} are related to sugars (e.g. glucose and fructose), carbohydrates and cell wall components as reported by other authors [28-31]. These bands are related to the CH-OH and alkyl frequencies for sugars (e.g. glucose and fructose) between 1000 and 1600 cm^{-1} [28-30]. In particular bands between 1500 and 1200 cm^{-1} were assigned to deformations of CH_2 and deformations of C–C–H and H–C–O groups, respectively where peaks between 1200 and 950 cm^{-1} are explained by stretching modes of C–C and C–O [28-30]. Absorption bands in the carbohydrate region between 900 and 1200 cm^{-1} are associated with COC group vibrations in the cyclic structures, and might indicate high content of carbohydrates [28-30]. The absorption bands at 1635 cm^{-1} and 1550 cm^{-1} correspond to the characteristic vibrations of the CONH groups (e.g. Amide I and Amide II), proteins and water [28-31]. Studies by other authors also indicated that inulin in Jerusalem artichoke or chicory roots might have a distinctive and characteristic band at 936 cm^{-1} [31].

Figure 2 shows the score plot of the first two principal components obtained from the ATR-MIR spectra in the calibration set. The first two principal components explain 97% of the variation in the ATR-MIR spectra of barley and malt flour samples analysed. No clear separation was observed between grain and malt flour samples. Only one spectroscopic outlier sample was observed.

The mean, range, standard deviation (SD) and coefficient of variation (CV) for the content of fructan content measured in the set of barley grain and malt flour samples analysed are shown in Table 1. The samples analysed showed a wide range in fructan content as shown by the range (0.89-2.74 %) and the CV (26.1%). The mean values and range observed are in accordance with those reported by other authors [13-14]. The variability in fructan content in the sample set analysed was considered suitable in order to develop ATR-MIR calibrations.

Cross validation statistics for the PLS models (n=40) developed for the measurement of fructan in the set of barley grain and malt flour samples analysed are shown in Table 1. The R^2 and SECV for fructan content were 0.76 and 0.20 %, respectively. The R^2 obtained indicated that 76% of the variance in fructan content is accounted by the ATR-MIR spectra. The RPD value obtained for fructan content was 2.3, indicating that these calibrations can be used for qualitative determination of fructan content (e.g. low, medium and high) and might be considered adequate for screening samples for this parameter. In addition, the R^2 is considered adequate for screening and for approximate calibration [24, 26]. It has been also shown that for practical purposes the error derived from a given model to be acceptable should be in the order of SEP $\leq$ 2 x SDD (in this study the SDD = 0.149) [32]. It is important to note that the SDD varies according to the concentration level, mean, range of composition as well as the number of samples included in the calibration set (Table 1). Overall, the SEP values (0.31%) obtained in this feasibility study are only adequate for a large scale screening. A graphical comparison of fructan content determined by the enzymatic method and ATR-MIR predicted data in the validation set is shown in Figure 3. The more accurate the predictive models, the more closely all points cluster near the theoretical 1:1 correspondence shown by the solid line. It was observed that samples that had higher standard error values were more scatter alongside the theoretical regression line.

Examination of the loadings derived from the PLS calibration models might provide insights into the main MIR regions associated with the chemical parameter measured. Loadings are also used to determine which variables (wavenumbers) are important for describing the variation in the data set. They can also be used to determine the inherent dimensionality of the data set as well as to identify unusual variables [24, 32]. In this study, the loadings were used to identify the most important wavenumbers that describe the main variation for the optimal PLS calibration models developed for fructan content in the set of samples analysed. Figure 4 shows the PLS loadings derived from the calibration model developed. The optimal PLS calibration loadings (PLS terms = 4) present strong, sharp and well-defined peaks in the fingerprint region at 979, 1030, 1421 and 1469, 1515 and 1577, and 1706 and 1740 cm^{-1} mostly related to sugars, proteins and water. The loadings around 1030 cm^{-1} might be related to sugars and carbohydrates (e.g. glucose and fructose) as reported by other authors [28-31]. Strong CH-OH loadings at frequencies associated to sugar can be assigned at 1030 cm^{-1}, and with CH_2 around 1420 and 1460 cm^{-1} [28-31]. Loadings around 1515 and 1577, and 1706 and 1740 cm^{-1} might be associated with compounds such as proteins, lipids and carbohydrates [28-31].

Malt quality evaluation is approaching a new age beyond the basic quality analyses [33]. Development and adoption of new technologies that measure aspects of quality not considered or understood in the past will allow a better understanding of malt quality as well as to better facilitate product development and improve efficiencies in the brewing process [33]. Rapid analytical methods based in vibrational spectroscopy are part of these new technologies. They can also offer the possibility to develop relationships between spectra and reference methods in order to measure several parameters simultaneously reducing the time of analysis and requiring minimal sample preparation. Examination of the ATR-MIR regression coefficients or loadings might provide insights into aspects of carbohydrate

chemistry and composition that are related to malt characteristics. These methods also offer the possibility to develop relationships between spectra and reference methods in order to measure several parameters simultaneously reducing the time of analysis, requiring minimal sample preparation.

4. Conclusions

This study showed that the advantages of using ATR-MIR spectroscopy are the potential use of this technology as a tool for high throughput screening in breeding programs. The results from this study also showed that ATR-MIR spectroscopy is capable qualitatively measure fructan content (low, medium and high) in both grain and malt flour samples. However, further studies are needed to include a more diverse set of samples (varieties, harvests) in order to test the capability of ATR-MIR to quantitatively measure fructan content.

Acknowledgments

The authors thank technical staff of the Barley Quality Laboratory and Barley Breeding Program, University of Adelaide. This project (UA00126) is supported by Australia's grain growers through their investment body the Grain Research and Development Corporation (GRDC), with matching funds from the Australian government.

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Figure 1. Second derivative of barley and malt flour samples having high and low fructan content attenuated total reflectance and mid-infrared spectrum of.

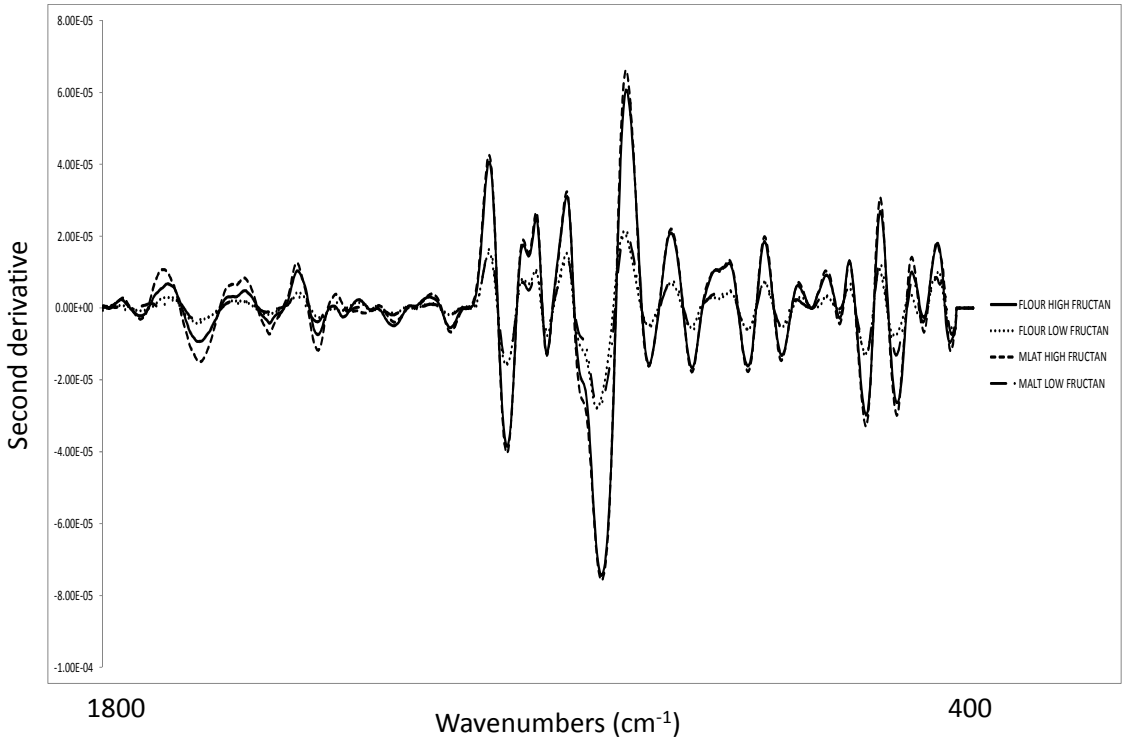

Figure 2. Score plot of the first two principal components of barley and malt flour samples analysed using attenuated total reflectance and mid-infrared spectroscopy. The 95% confidence ellipse is included in the score plot in order to reveal potential outliers.

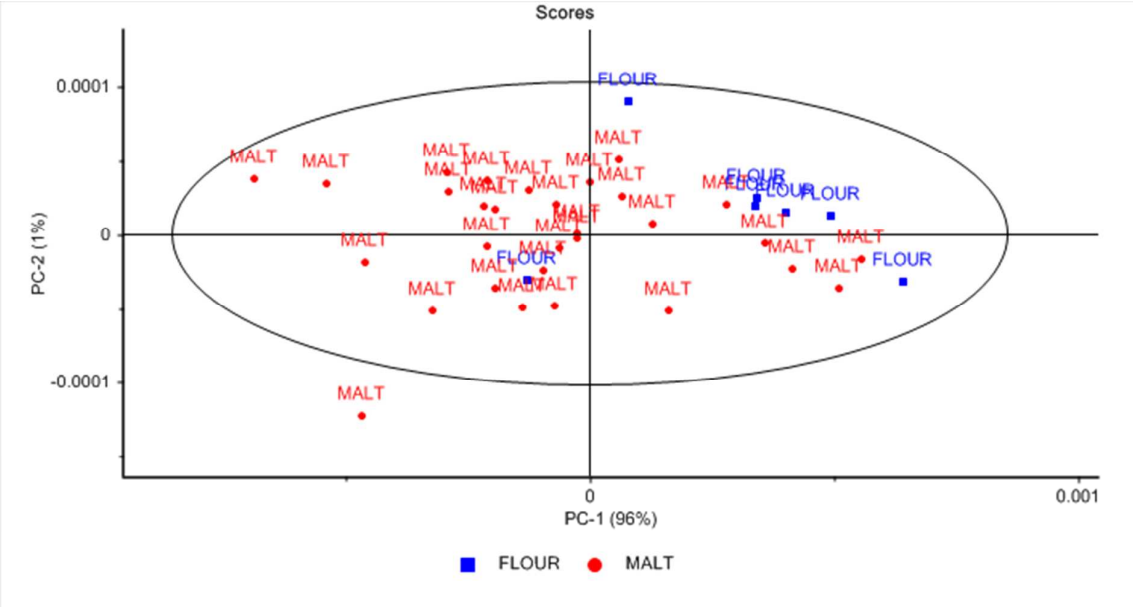

Figure 3. Reference versus predicted fructan content in the set of barley and malt flour samples analysed using attenuated total reflectance mid infrared spectroscopy (whole line 1:1).

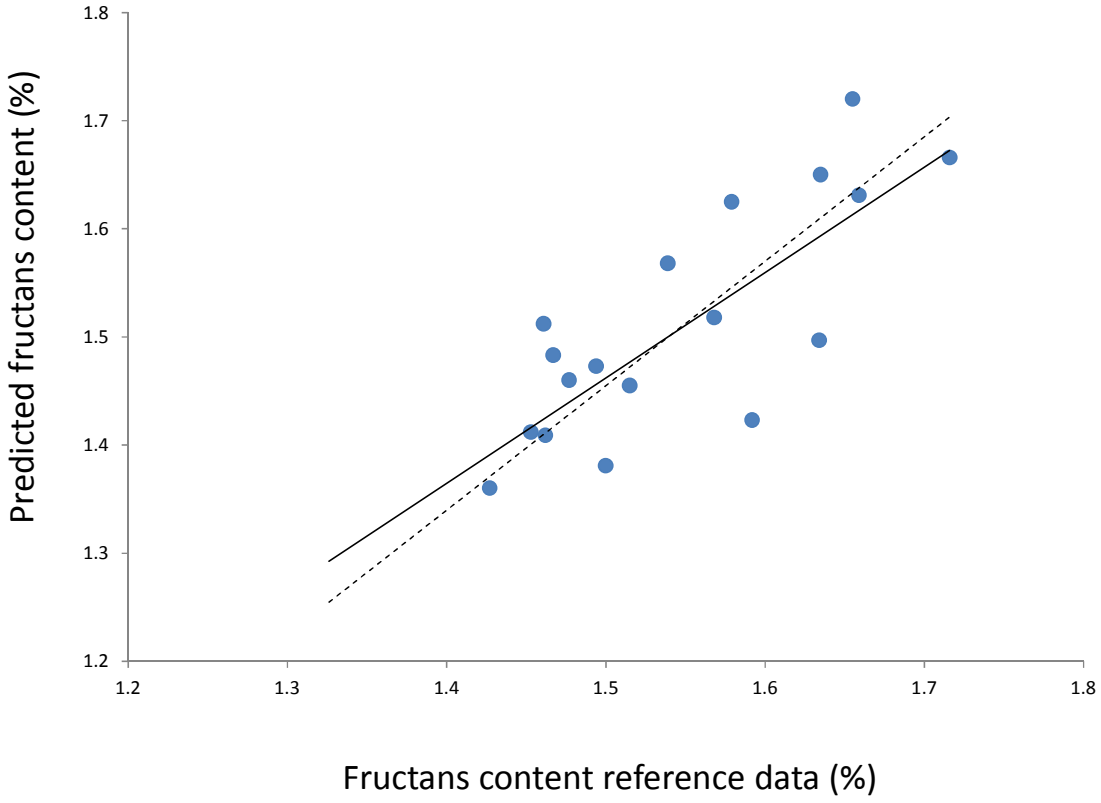

Figure 4. Optimal loadings derived from the partial least squares regression for the prediction of fructan content in barley and malt flour samples analysed using attenuated total reflectance mid infrared spectroscopy.

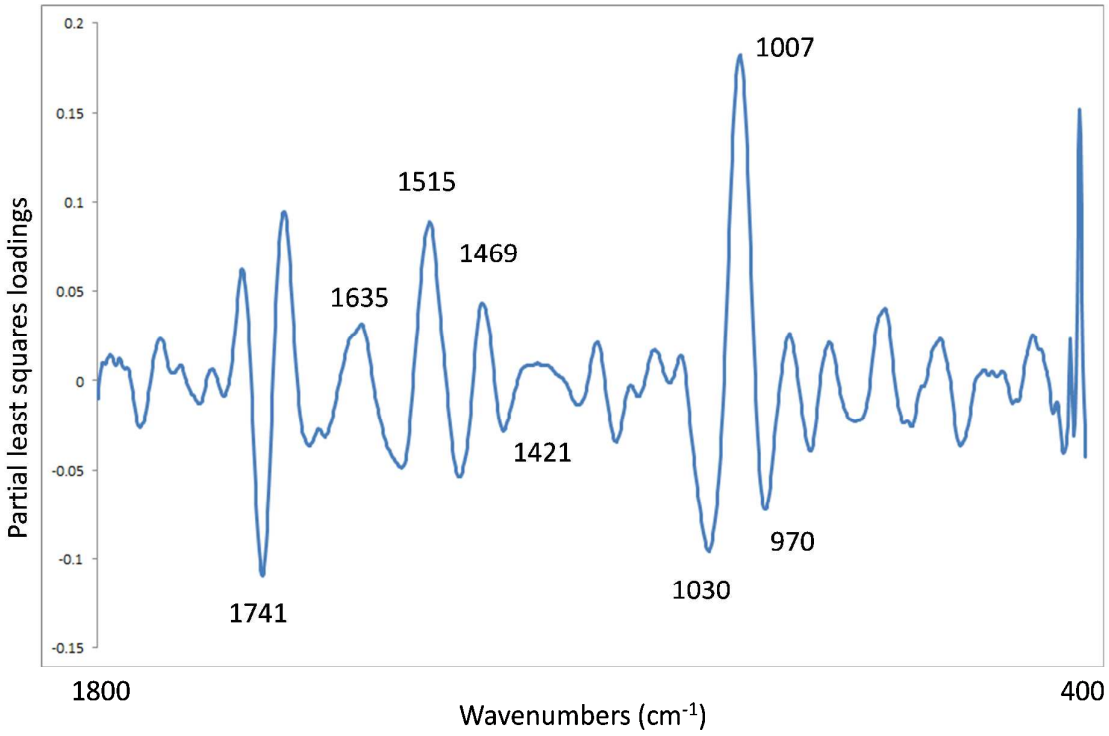

Table 1. Descriptive and calibration statistics for the measurement of fructan content (%) in barley and malt flour samples using attenuated total reflectance mid infrared spectroscopy.

	Descriptive statistics	Calibration (n=40)	Validation (n=20)
Mean	1.76		
Range	0.89-2.74		
SD	0.46		
CV (%)	26.1		
R^2		0.76	0.65[#]
SECV		0.20	0.31[*]
RPD		2.3	1.5
bias		0.008	
Slope		0.71	
PLS terms		4	

SD: standard deviation, CV: coefficient of variation; R^2: coefficient of determination in cross validation, SECV: standard error of cross validation; RPD: SD/SECV, [#] coefficient of correlation, [*] SEP = standard error of prediction.