JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Near-Infrared Reflectance Models for the Rapid Prediction of Quality of Brewing Raw Materials

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Calibration models for quickly and reliably predicting moisture content and total nitrogen, both "as is" and "dry matter" on malt, as well as moisture content and total lipids, both "as is" and "dry matter", on maize by means of near-infrared (NIR) spectroscopy were developed. The FT-NIR spectra recorded on the finely ground cereals were correlated to the analytical data by means of the multivariate PLS algorithm. In particular, these models were developed on the raw materials, which are used by the main Italian brewing industries. Validation was carried out both by means of cross-validation and test set validation. Regression coefficients (R^2) were higher than 97 for both malt and maize moisture content and higher than 85 and 88 for malt total nitrogen and maize total lipids, respectively. The RMSE values (both RMSECV and RMSEP) were lower than 0.1% m/m for both malt and maize moisture contents, whereas they ranged from 0.024 to 0.042% m/m for malt total nitrogen and from 0.042 to 0.055% m/m for maize total lipids. Repeatability was tested by taking into account more than one sample for each calibration and compared, when possible, to those of the standard methods. Repeatability (r_{95}) ranged from 0.060 to 0.158% m/m and from 0.020 to 0.055% m/m for malt moisture and total nitrogen contents, respectively, and from 0.094 to 0.160% m/m and from 0.076 to 0.208% m/m for maize moisture and total lipids contents, respectively.

KEYWORDS: Near-infrared spectroscopy; calibration; validation; malt; maize; moisture content; total nitrogen; total lipids

INTRODUCTION

Near-infrared spectroscopy is a nondestructive and rapid technique applied increasingly for food quality evaluation in recent years; it is a type of vibrational spectroscopy that employs photon energy ($h\nu$) in the energy range from 2.65×10^{-19} to 7.96×10^{-20} J, which corresponds to the wavelength range of 750−2500 nm (wavenumbers 13300−4000 cm^{-1}) (*1*). Absorption of infrared light by a molecule is due to interaction of the electromagnetic radiation with the vibration of bonds between atoms (*1*). In the infrared spectra, light is absorbed if the frequency of the light is the same as the fundamental frequency of vibration of the molecular bond (*2*). Molecular vibrations are slightly anharmonic, however, and consequently higher frequency light in the near-infrared can also be absorbed if its frequency is the same as that of one of the harmonics of the fundamental. The absorption bands in the near-infrared spectra are thus referred to as either overtone bands, with frequencies of about 2, 3, or more times the fundamental, or combination bands, with frequencies that are the sum of the frequencies of two fundamentals. For combination bands permitted by anharmonicity, it would be necessary that only one of the combining vibrations be active (causing dipole change). This feature may cause some vibrations, which cannot be observed in the mid-infrared, to be displayed by a near-infrared spectrum. For overtone or combination bands to occur in the near-infrared, the frequencies of the fundamental vibrations must be high enough; otherwise, these bands occur at wavelengths in the mid-infrared. This is only true for bonds involving hydrogen, for example, C−H, O−H, N−H, and S−H. The carbonyl (C═O) vibration is an exception, as it is strong enough in the mid-infrared that the second overtone can be seen in the near-infrared (*3*). A near-infrared spectrum typically contains a large number of peaks, broad and overlapped, but now we have computers powerful enough to analyze these complex spectra and extract quantitative and qualitative information about the samples (*3*). These spectra can be collected in reflectance (near-infrared spectroscopy in reflectance, or NIR) or in transmission (near infrared spectroscopy in transmission, or NIT).

When the light is reflected by opaque samples, the diffuse reflectance is the preferred approach.

The analytical methods resulting from the use of the NIR spectroscopic region show significant characteristics: they are fast (≤1 min per sample), nondestructive, and noninvasive, are

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10.1021/jf8022639 CCC: $40.75
Published on Web 12/19/2008

Table 1. Optimal Parameters for NIR Calibrations on Malt (Moisture Content and Total Nitrogen) and Maize (Moisture Content and Total Lipids)

no.	matrix	parameter	regions, cm^{-1}	preprocessing
1	malt	moisture content	9970.4−4246.6	constant offset elimination
2	malt	total nitrogen, as is	9970.4−4246.6	first derivative + vector normalization (SNV)
3	malt	total nitrogen, dry matter	7509.1−4246.6	straight line subtraction
4	maize	moisture content	7501.9−6098, 5453.8−4246.6	vector normalization (SNV)
5	maize	total lipids, as is	7501.9−4246.6	first derivative
6	maize	total lipids, dry matter	7501.9−4246.6	first derivative + vector normalization (SNV)

Table 2. Results of NIR Calibrations for Malt (Moisture Content and Total Nitrogen) and Maize (Moisture Content and Total Lipids) by Means of Cross-Validation (Leave-One-Out)

no.	matrix	parameter	R^2	RMSECV, % m/m	bias, % m/m	rank
1	malt	moisture content	97.83	0.097	0.0004	12
2	malt	total nitrogen, as is	92.86	0.026	−0.0001	9
3	malt	total nitrogen, dry matter	94.08	0.024	−0.0003	15
4	maize	moisture content	98.29	0.074	0.0036	12
5	maize	total lipids, as is	92.96	0.042	−0.00107	9
6	maize	total lipids, dry matter	90.32	0.054	−0.00089	8

characterized by high penetration of the probing radiation beam, are suitable for in-line use, and have nearly universal application (any molecule containing C−H, NH, S−H, or O−H bonds), with minimal sample preparation demands and without the use of hazardous chemicals (*1*).

Most of the quantitative models developed by using NIR spectral information are based on the use of samples for which analyte concentration or property has been determined by a standard, well-accepted analytical procedure designated the reference method. The number of samples employed for calibration has been considered to be of great importance. Recommendations for multicomponent natural samples are in the range of 50−100 samples, depending on the complexity and variability of the matrix accompanying actual samples (*1*).

Most of the quantitative applications are targeted to determine major constituents in the sample. In general, the detection limit is about 0.1% (m/m), although, for some specific applications and under favorable characteristics of the sample matrix and analyte, NIR can reach lower values (*1*).

However, NIR relies on a multivariate model to quantify a property or a concentration in complex samples, as demonstrated by the diversity of applications in food and agriculture (*4*).

NIR is indeed used in plant breeding and in the cereal industry for the prediction of a wide range of specific chemical (e.g., water, protein, starch) and physical (e.g., grain hardness) parameters from computerized calibrations using classical statistical and chemometric software (*5*−*7*).

This analytical method is so useful to the brewery industry as a quality assurance and research tool because it can measure organic substances very quickly (5−10 s), without the destruction of a sample or use of hazardous chemicals. It can be used to evaluate raw materials, yeasts, enzymes, nutritional supplements, and production parameters, and it can assist production by monitoring and maintaining control of processes (*3*). One of the main raw materials in the brewery industry is barley malt. However, in many countries the use of amylaceous sources other than barley malt is permitted, because of economic and quantitative reasons. In Italy, the main adjunct used is maize, which can be added as flour in mashing up to 40%, according to Italian legislation (*8*).

Various researchers developed calibration curves using NIR spectroscopy to measure multiple parameters related to malt quality (*9, 10*), for example: moisture (*11*), nitrogen (*11*), amino acids (*12*), malt extract (*13*), starch and total β-glucans (*14*−*16*), and fiber (*17*). All of these studies are carried out on milled and homogenized samples of barley or malt. More recently, researchers have started to develop calibration models for the prediction of malt modification and other grain constituents using NIR on a single grain basis (*18, 19*), but obtaining low correlation coefficients.

One of the most important determinations in malt evaluation is moisture content. This parameter ranges from 3 to 5%. Dry malt is a hygroscopic product; it is necessary to avoid moisture absorption, because the presence of water causes the reactivation of hydrolytic enzymes, resulting in undesired transformations. Moreover, the moisture content of malt is a relevant parameter for brewers, who are interested in the dry matter of the raw material (*20*).

An important determinant of barley quality is the level of protein. For malting barley, a balance between carbohydrates (starch) and protein is important, because an excess of protein decreases the amount of available carbohydrates, giving a negative downstream effect on the brewing process. The protein content of malt must not be high, because it can cause haze and problems of chemical and physical instability in the beer. For these reasons, the total nitrogen must be <1.76% (*20*). Therefore, it is important to have robust techniques to measure protein content in the selection of barley. Near-infrared reflectance spectroscopy (NIRS) has been used routinely to predict barley protein content for many years for grain reception and more recently in barley breeding programs.

Recently, strong correlations for grain protein and NIR wavelengths were found at 1116, 1268, 2040, 2068, 2188, and 2300 nm used for extracted hordein. Multiple linear regression equations provided improved predicting power for barley and malt protein with standard errors of prediction of 0.15 and 0.17%, respectively (*21*).

Another important parameter of malt quality that is possible to determine by NIR is hardness; in barley, malting varieties generally were classified as soft grain, whereas nonmalting or feed varieties were classified as hard. Hardness has also been associated with the level of modification of malt, which would imply that grain components within the endosperm (such as starch granules, starch protein matrix, and cell wall material) directly affect modification (*22*). A particular application of near-infrared spectroscopy in transmission (NIT) is the study of the physiological and physical−chemical basis of barley germination; NIT calibrations can be used to predict vigor in malting grade barley (*23, 24*).

In the evaluation of maize, NIRS can be used to predict dry matter (dm), acid detergent fiber (ADFom), and crude protein (CP) in wet whole maize (WWM) silage samples (*25*).

The use of single-kernel NIRS permits a rapid selection of individual seeds with desired traits. The most accurate models were for predictions of the major components of the kernel including protein, starch, and calorie content as well as seed weight. These data suggest that single-kernel NIR spectra are reporting an absolute amount of each component in the kernel (*26*).

Furthermore, one of the most recent applications of NIRS allows the identification of relevant targets such as the myc-

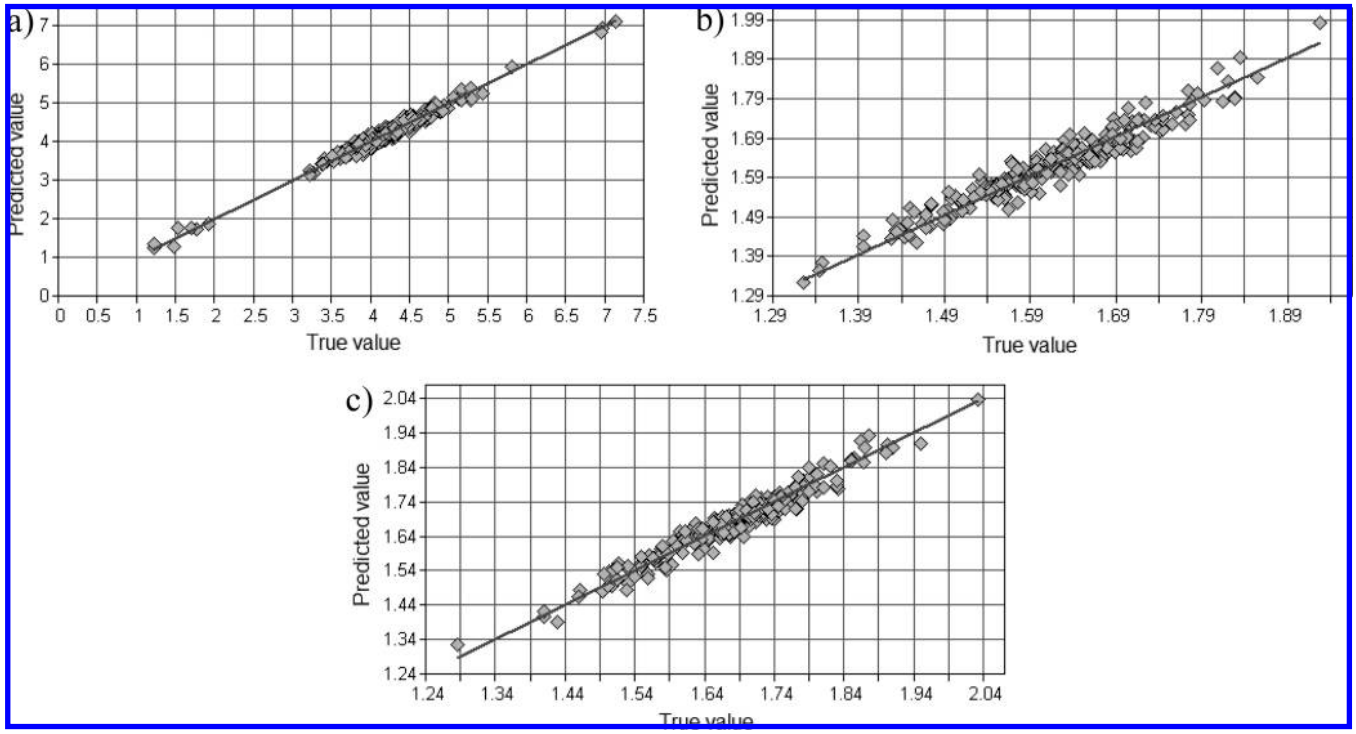

Figure 1. Predicted versus true values for malt calibrations, carried out by means of cross-validation (leave-one-out): (**a**) moisture content; (**b**) total nitrogen, as is; (**c**) total nitrogen, dry matter.

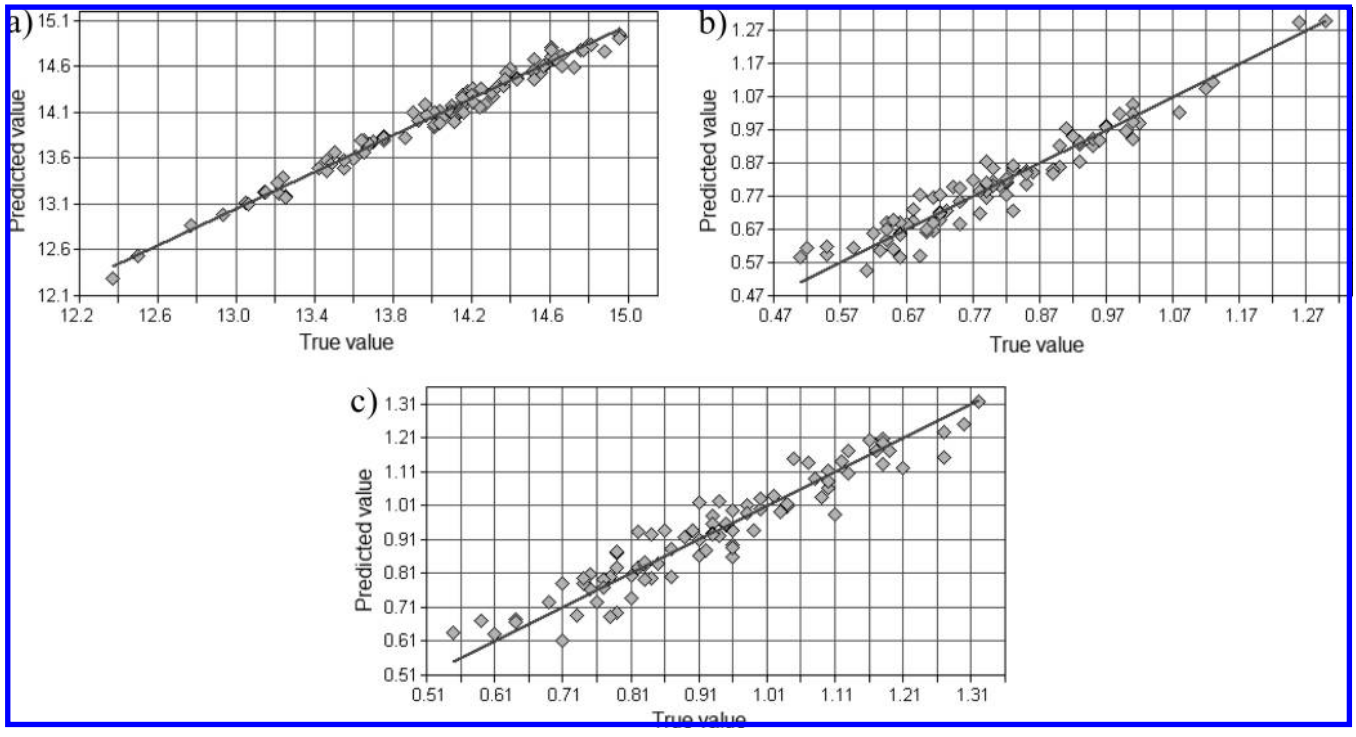

Figure 2. Predicted versus true values for maize calibrations, carried out by means of cross-validation (leave-one-out): (**a**) moisture content; (**b**) total lipids, as is; (**c**) total lipids, dry matter.

otoxigenic fungi and their toxic metabolites produced in naturally and artificially contaminated products (*27*).

Moisture and lipid contents in maize are important parameters for brewers. In particular, the latter has to be kept under control, as a high lipid concentration in the beer can cause problems with the chemical and physical stability, as well as with the keeping of foam. Various researchers have developed calibration curves, using NIRS to measure these parameters, but these models were built using different types of cereals (*28*).

The purpose of this study is the optimization of NIR calibration curves to evaluate the quality of brewing raw materials used by the main breweries in Italy, to develop a specific analytical method for breweries and cereal industries. The calibrations were based on the spectra of the finely ground samples: the milling of the cereals is indeed a routine and non-time-consuming operation in all breweries that carry out raw material characterization, and only a small amount of flour, <50 g, is needed to carry out the NIR measurements by means of

Table 3. Results of NIR Calibrations for Malt (Moisture Content and Total Nitrogen) and Maize (Moisture Content and Total Lipids) by Means of Test Set Validation

no.	matrix	parameter	R^2	RMSEP, % m/m	bias, % m/m	rank
1	malt	moisture content	98.30	0.100	0.0080	10
2	malt	total nitrogen, as is	85.41	0.042	0.0051	6
3	malt	total nitrogen, dry matter	90.34	0.029	0.0082	11
4	maize	moisture content	97.68	0.080	−0.0151	9
5	maize	total lipids, as is	90.75	0.044	−0.00355	6
6	maize	total lipids, dry matter	88.19	0.055	−0.0112	5

the integrating sphere sampler. Moreover, the homogeneity of the samples is higher when considering ground cereals rather than whole grains; the corresponding models are hence expected to be more precise. The calibrations were validated, and repeatability, calculated by taking into account more than one sample for each model, was compared with those of the standard analytical methods. Such calibrations were built with the aim of finding a correlation between the NIR spectral data and the moisture content and total nitrogen ("as is" and "dry matter") for malt and moisture and lipids ("as is" and "dry matter") for maize.

MATERIALS AND METHODS

Apparatus included a sample divider (VLB, Berlin, Germany); DLFU type disk mill (Bhüler, Uzwil, Switzerland), set to a gap of 0.2 mm; moisture aluminum dishes (VLB); FD115 electrically heated ventilate oven, capable of holding temperature within ±0.5 °C (Binder, Tuttlingen, Germany); AB204-S balance, accuracy ±0.0005 g (Mettler-Toledo, Greifensee, Switzerland); Elix 3 System (Millipore, Billerica, MA); Foss Tecator Digestor (Foss, Hillerød, Denmark); 2200 Kjeltec Auto Distillation Unit (Foss); Laborota 4600 ECO (Heidolph, Schwabach, Germany); Vector 22/N FT-NIR spectrometer system, equipped with tungsten source, Rocksolid interferometer, fiber optic module equipped with Ge Diode detector, and an integrating sphere module equipped with PsS detector for spectra acquisition in diffuse reflectance (Bruker Optics, Milan, Italy).

Reagents included sulfuric acid (95−97%; Fluka, Milan, Italy); sodium hydroxide >99% (Riedel-de Haën, Milan, Italy); Kjeldhal tablets (Merk, Whitehouse Station, NJ); 35% m/m hydrogen peroxide solution (Riedel-de Haën); 1-octanol >99% (Fluka); boric acid >99% (J. T. Baker, Phillipsburg, NJ); bromocresol green ACS (Carlo Erba Reagents, Milan, Italy); Fixanal 0.05 mol of sulfuric acid (Riedel-de Haën); petroleum ether 45−60 °C (J. T. Baker).

Samples. Malt and maize samples were supplied from industrial malthouses and mills and are representative of the ones available on the Italian market. Samples of malt representative of the different types (i.e., pale, Munich, colored, and caramel) were considered. In particular, the following data sets were used to set the calibrations: 284 malt samples (among which were 13 Munich, 8 colored, 2 caramel, and 1 wheat) for malt moisture content; 275 malt samples (among which were 13 Munich, 7 colored, 2 caramel, and 1 wheat) for malt total nitrogen content, both as is and dry matter; 146 and 95 maize samples for maize moisture content and total lipids (both as is and dry matter), respectively.

Sample Preparation and Spectra Acquisition. Maize grits and malt grain samples (about 1 kg) were homogenized by means of a sample divider and finely ground by means of a DLFU type disk mill set at a distance between the disks of 0.2 mm. The flours were used to record the spectra and to carry out the reference analyses. All spectra were recorded on a quartz-bottom cup (4 cm inner diameter) placed on the integrating sphere optics and, to compensate for the lack of homogeneity, the sample was spun during the measurement (10 rpm). Absorption spectra were collected at room temperature, against a gold-coated background, by means of the software OPUS (version 5.5 or 6.5, Bruker Optics) in the spectral range of 11500−4000 cm^{-1} with a resolution of 8 cm^{-1} using 64 scans/sample (the same number of scans was also used for the background).

Data Processing, Calibration Models, and Validation. Various spectral treatments were employed to avoid baseline shifts arising from scattering: constant offset elimination, first derivate (Savitzky−Golay algorithm with 25 smoothing points), standard normal variate (SNV), and straight line subtraction. Calibration models were constructed using PLS1 (*29*) regression, and both a cross-validation and test set validation were adopted to validate them. In the first case, the internal validation was carried out by means of a leave-one-out procedure, with as many validation subset as the number of samples included in the calibration. The test set validation was carried out instead by selecting 33% of the total samples as a test set from the score plot derived from PCA (component 2 vs component 1), to select a test set representative of all samples. Cross-validation could be too optimistic, because excluding one sample has a lower perturbative effect on the model, whereas test set validation could give a more realistic estimation of the predictivity of the model, the main drawback being the reduction of the number of samples considered. In both cases, the predictivity of the calibrations was quantitatively evaluated as root mean square error, in cross-validation (RMSECV), and in test set validation root mean square error of prediction (RMSEP), as described in eq 1, where M is the number of the samples, which coincides with the total number of samples in cross-validation and with the number of the test set samples in test set validation:

$$\text{RMSECV or RMSEP} = \sqrt{\frac{1}{M}\sum_{i=1}^{M}(X_{i\text{-true}} - X_{i\text{-predicted}})^2} \quad (1)$$

Another relevant parameter taken into account for the calibrations is the bias. Bias is a quantification of the systematic error of the model. To evaluate the capability of a PLS method necessary to evaluate the two parameters together, RMSEP and bias, the most capable PLS method is the one with the lowest RMSEP and bias value as close as possible to zero. PLS regression gives the dimensionality of the model as an output, that is, the number of factors (rank). Outliers, that is, samples with high error and high leverage, were excluded from the calibration data set. Preprocessing and spectral range were selected for every calibration as a function of the predictivity of the resulting cross-validated models, corresponding to the lower values of RMSECV. Moreover, the spectral regions were selected by taking also into account the NIR position of the absorption bands corresponding to the functional groups, which are characteristic for each calibration, that is, H_2O for the moisture content of both malt and maize, RNH_2 for the total nitrogen content, both as is and dry matter, of malt, and C═O stretching for the total lipids of maize. All operations involving the calibrations (spectral data treatments, construction of PLS regression models, and control of the dependability of the models by RMSECV and RMSEP) were carried out by means of the Quant 2 function, included as part of the OPUS software (versions 5.5 and 6.5).

Reference Analyses. The standard methods from the Analytica European Brewery Convention (A-EBC) were adopted as reference analyses for moisture content in pale and Munich malts (oven-based) (*30*); moisture content in colored malts (oven-based) (*31*); total nitrogen in all malts (Kjeldhal) (*32*); moisture content in maize (oven-based) (*33*); and total lipids in maize (Soxhlet extraction) (*34*).

Validation: Repeatability, Comparison with the Standard Methods, and Uncertainty Determination. Each calibration curve was tested by recording 11 independent spectra under repeatability conditions and evaluating the predicted values. In particular, malt calibrations were tested with three different types of malt, that is, pale, Munich, and colored, whereas maize calibrations were tested with two different maize samples. To do this, samples were finely ground (0.2 mm) and divided in two portions, one for spectra recording and the other for standard analyses. The normality test of the distributions on each data set was performed by means of the Shapiro−Wilk test (*35*, *36*) with a probability level of $p = 95\%$ ($\alpha = 5\%$). Moreover, anomalous data were identified by means of the Huber test, which is based on the

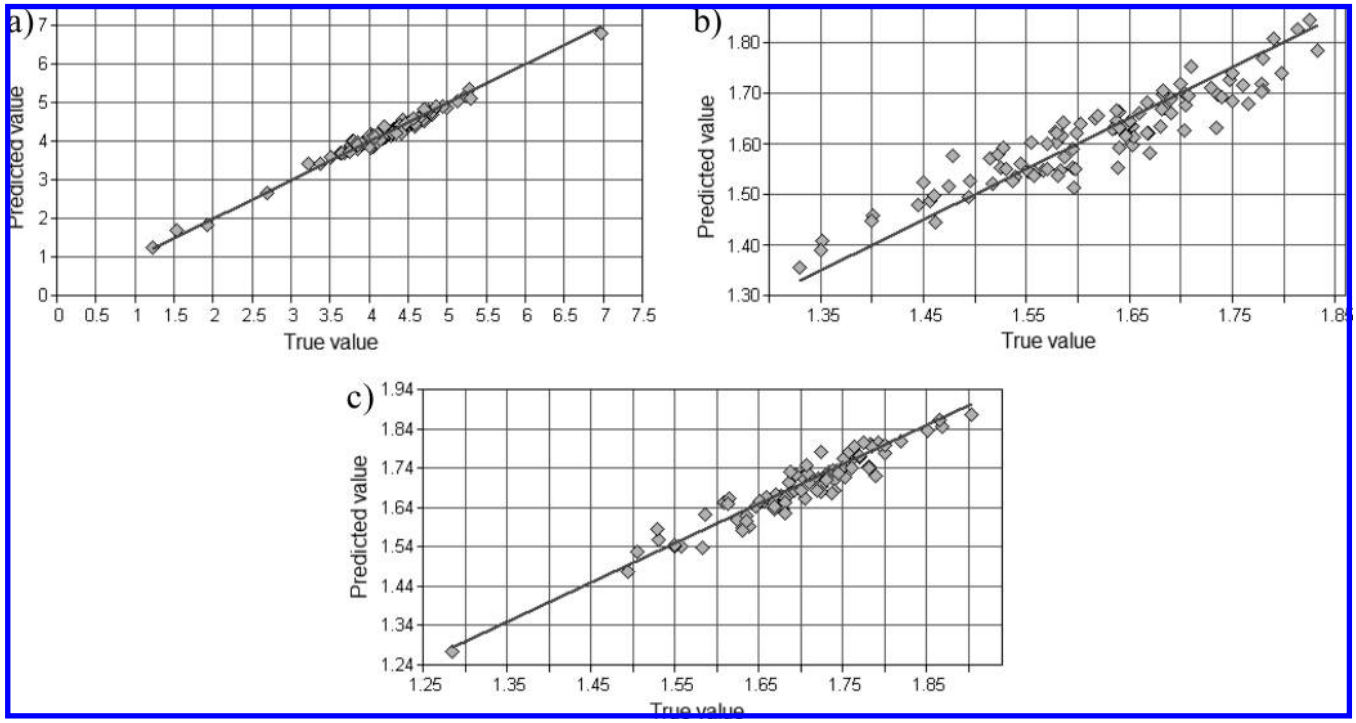

Figure 3. Predicted versus true values for malt calibrations, carried out by means of test set validation: (**a**) moisture content; (**b**) total nitrogen, as is; (**c**) total nitrogen, dry matter.

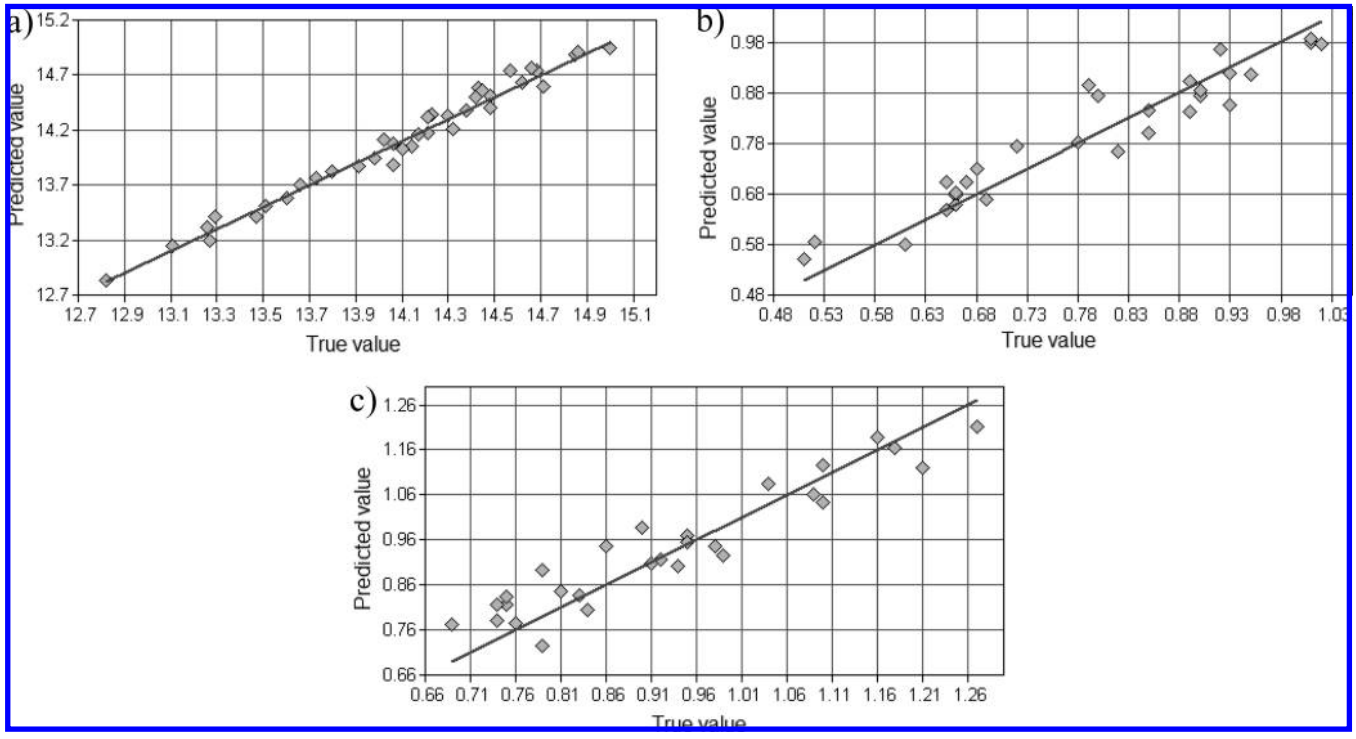

Figure 4. Predicted versus true values for maize calibrations, carried out by means of test set validation: (**a**) moisture content; (**b**) total lipids, as is; (**c**) total lipids, dry matter.

evaluation of the median and is one of the most robust methods (*36*). If both conditions are satisfied, that is, the distribution is normal and there are no anomalous data, the statistical parameters, such as the average value, the standard deviation (s_r), and the repeatability (95% confidence, r_{95}), can be calculated, and a comparison with the repeatability of the standardized methods adopted as primary methods can be carried out.

The average values from each data set were compared with those from standard methods to verify the predictivity of the calibrations. The repeatability of the NIR methods was compared with those of the standard methods through the s_r/σ_r ratio, σ_r being the standard deviations calculated from the collaborative trial determined repeatabilities of the standard A-EBC methods, according to eq 2:

$$\sigma_r = \frac{r_{95}}{t \times \sqrt{2}} \quad (2)$$

As the number of the repetitions is not detailed in the A-EBC methods, a precautionary value of 2 was considered for t in calculating σ_r. The repeatabilities of the NIR methods were considered to be

Table 4. Comparisons between NIR Malt Calibrations and A-EBC Standard Methods and Uncertainties of NIR Methods for Malt[a]

sample	parameter	validation	av	true	diff[b]	r_{95}	s_r	σ_r	s_r/σ_r	R_{95}	U_e
pale	moisture content	internal	4.96	4.92	0.04 (0.8%)	0.060	0.019	0.046[c]	low	0.6[c]	0.4
		test set	4.97	4.92	0.05 (1.0%)	0.061	0.019	0.046[c]	low	0.6[c]	0.4
pale	total nitrogen, as is	internal[d]	1.67	1.63	0.04 (2.5%)	0.044	0.014				
		test set	1.64	1.63	0.01 (0.6%)	0.052	0.016				
pale	total nitrogen, dm	internal	1.72	1.72	0.00	0.036	0.011	0.018[e]	satisfactory	0.13[e]	0.09
		test set	1.72	1.72	0.00	0.049	0.016	0.018[e]	satisfactory	0.13[e]	0.09
Munich	moisture content[f]	internal	2.73	2.54	0.19 (7.5%)	0.083	0.026	0.046[c]	low	0.6[c]	0.4
		test set	2.83	2.54	0.29 (11.4%)	0.102	0.032	0.046[c]	satisfactory	0.6[c]	0.4
Munich	total nitrogen, as is	internal	1.48	1.49	−0.01 (−0.7%)	0.022	0.007				
		test set	1.45	1.49	−0.04 (−2.7%)	0.028	0.009				
Munich	total nitrogen, dm[f]	internal[d]	1.50	1.52	−0.02 (−1.3%)	0.040	0.013	0.018[e]	satisfactory	0.13[e]	0.09
		test set	1.49	1.52	−0.03 (−2.0%)	0.049	0.016	0.018[e]	satisfactory	0.13[e]	0.09
colored	moisture content[f]	internal	2.01	2.07	−0.06 (−2.9%)	0.158	0.050	0.025[g]	high	0.32[g]	
		test set	1.92	2.07	−0.15 (−7.2%)	0.129	0.041	0.025[g]	high	0.32[g]	
colored	total nitrogen, as is	internal	1.85	1.79	0.06 (3.4%)	0.055	0.018				
		test set[d]	1.79	1.79	0.00	0.025	0.008				
colored	total nitrogen, dm	internal[d]	1.83	1.82	0.01 (0.5%)	0.034	0.011	0.018[e]	satisfactory	0.13[e]	0.09
		test set[h]	1.91	1.82	0.09 (4.9%)	0.020	0.006	0.018[e]	low	0.13[e]	0.09

[a] Percent m/m. [b] Diff = av − true, values in parentheses are percentages relative to measured values. [c] Reference *28*. [d] $n = 10$. [e] Reference *31*. [f] True value outside the validation range of the A-EBC method, in the same order of magnitude. [g] Reference *30*. [h] $n = 9$.

Table 5. Comparisons between NIR Maize Calibrations and A-EBC Standard Methods and Uncertainties of NIR Methods for Maize[a]

sample	parameter	validation	av	true	diff[b]	r_{95}	s_r	σ_r	s_r/σ_r	R_{95}	U_e
maize 1	moisture	internal[c]	13.57	13.91	−0.34 (−2.4%)	0.137	0.043	0.046[d]	satisfactory	0.60[d]	0.4
		test set[c]	13.65	13.91	−0.26 (−1.9%)	0.124	0.039	0.046[d]	satisfactory	0.60[d]	0.4
maize 1	total lipids, as is[e]	internal[c]	1.02	0.99	0.03 (3.0%)	0.113	0.035	0.028[f]	satisfactory	0.23[f]	0.2
		test set[c]	1.01	0.99	0.02 (2.0%)	0.097	0.030	0.028[f]	satisfactory	0.23[f]	0.2
maize 1	total lipids, dm	internal	1.20	1.15	0.05 (4.3%)	0.208	0.066				
		test set[b]	1.17	1.15	0.02 (1.7%)	0.116	0.036				
maize 2	moisture	internal	12.66	13.04	−0.38 (−2.9%)	0.160	0.051	0.046[d]	satisfactory	0.60[d]	0.4
		test set	12.66	13.04	−0.38 (−2.9%)	0.094	0.030	0.046[d]	satisfactory	0.60[d]	0.4
maize 2	total lipids, as is[e]	internal[c]	1.00	1.02	−0.02 (−2.0%)	0.121	0.038	0.029[f]	satisfactory	0.23[f]	0.2
		test set[g]	0.99	1.02	−0.03 (−2.9%)	0.076	0.023	0.029[f]	satisfactory	0.23[f]	0.2
maize 2	total lipids, dm	internal	1.22	1.17	0.05 (4.3%)	0.130	0.041				
		test set	1.16	1.17	−0.01 (−0.9%)	0.165	0.052				

[a] Percent m/m. [b] Diff = av − true, values in parentheses are percentages relative to measured values. [c] $n = 10$. [d] Reference *32*. [e] True value outside the validation range of the A-EBC method, in the same order of magnitude. [f] Reference *33*. [g] $n = 9$.

comparable to those of the A-EBC methods if the value of the ratio is between a lower (A) and an upper (B) limit, that is, if eq 3 is satisfied:

$$A \leq \frac{s_r}{\sigma_r} \leq B \tag{3}$$

A and B were obtained from Student's test, for $\nu = (n - 1)$ degrees of freedom and $p = 0.95$. When this condition was satisfied, method uncertainties calculated according to the holistic approach allow using the extended uncertainty (U_e) of the reference method, for methods with comparable repeatability. Extended uncertainties were calculated according to eq 4, where k is a coverage factor, conventionally 2:

$$U_e = kU_c \tag{4}$$

U_c is the compose uncertainty, calculated, according to eq 5, from the reproducibility of the standard A-EBC methods with a 95% confidence (R_{95}):

$$U_c = \frac{R_{95}}{t \times \sqrt{2}} \tag{5}$$

Also in this case, t values were considered to be 2.

RESULTS AND DISCUSSION

The NIR calibrations for moisture and total nitrogen content in malt, as well as those for moisture and total lipids contents in maize, were carried out by means of PLS regressions between the spectra of the sample sets and the reference data, with respect to the spectral regions and adopting the spectra preprocessing modes detailed in **Table 1**. These are the selected optimal conditions for each calibration and were adopted for both internal (cross-validation, leave-one-out) and external (test set) validations. Moreover, spectral regions were selected by taking into account the absorption bands characteristic of the analyzed parameters. The region between 9970.4 and 4246.6 cm^{-1} was chosen, for instance, for moisture content of malt, as H_2O absorption bands are in this spectral region.

Table 2 shows the results for the cross-validations, carried out by means of the leave-one-out procedure, and the graphs in **Figures 1** and **2** represent the predicted as a function of the true values, that is, the experimental ones, for malt and maize, respectively. All calibrations can be considered to be satisfactory, considering the low values of the RMSECV parameters. Moisture contents for malt are indeed in the 1−7% m/m range, whereas total nitrogen contents are in the range of 1.3−2% m/m, and in both cases RMSECV values are at least 1 order of magnitude lower. The same can be observed for maize calibrations, as moisture contents for malt are in the 12.3−15% m/m range, and total lipid contents are in the range of 0.5−1.3% m/m.

The bias values, which are the average values of the differences between the values calculated by the model and the those experimentally determined, are lower than 0.01% m/m in all cases, which is at least 2 orders of magnitude smaller

than the measured values. This means that no major systematic errors are present in the calibration results.

Validations were carried out by means of test set validation, as well. Leave-one-out cross-validation could be indeed too optimistic to estimate the predictivity, in terms of RMSECV, of the models. The calibrations were hence validated by means of an external validation, treating part of the complete sample set as a test set (33%). Such samples were chosen by operating a PCA on the complete sample set, equally dispersed on the score plot built by taking into account the first two components, to select a test set representative of all samples. Test set validation is hence carried out on a lower number of samples, but is supposed to be more realistic in evaluating the predictivity of the models. **Table 3** shows the results for the test set validations, and the graphs in **Figures 3** and **4** represent the predicted as a function of the true values, that is, the experimental ones, for malt and maize, respectively. In this case, the rank values are lower than those reported in **Table 2**, as the number of samples in the training set was decreased and cross-validation methods tipically overfit more than external validation ones. RMSEP values are a little bit higher than RMSECV ones, as expected, but remain lower than the values of the parameters. Hence, the test set validations confirm that the models are satisfactory to predict the values of the considered parameters for malt and maize.

Both internally and externally validated models were tested for their repeatability, considering more than one validation sample for each calibration. In particular, the calibrations for malt were tested by means of three different malts: a pale, a Munich, and a colored malt, representing the three main classes of malt adopted to build the calibrations. Two different maize samples, owing to the two main types of maize adopted to build the calibrations, were used to test the repeatability of maize calibrations. For each sample, 11 independent spectra were recorded under repeatability conditions, that is, in the same laboratory, by means of the same instrument and operator, in a short time interval, on finely ground samples, and used to calculate the values of moisture content, total nitrogen (both as is and dry matter), and total lipids (both as is and dry matter). At the same time, the same values were determined on the same samples by means of the reference methods, that is, the A-EBC. A comparison between NIR and experimentally determined (true) values was carried out to test the predictivity of the calibrations and to verify if it depends on the kind of malt. Multiple measurements were used to calculate the repeatability of the NIR methods with a 95% confidence level (r_{95}) and to compare it with those of the standardized methods, as described under Materials and Methods. As the malt calibrations were built with different kinds of malt, three different kinds of malt were considered to test them: pale, Munich, and colored. Two different maizes were used instead to test the maize calibrations. All data sets satisfied the Shapiro−Wilk normality test, and validation parameters are listed in **Tables 4** and **5** for malt and maize, respectively.

Data relative to malt calibrations are discussed first. No relevant difference was observed between cross-validated and test set validated calibrations. The average values measured by means of NIR calibrations seem to be closer to the true ones for pale rather than for Munich and colored malts. This result was expected, as most of the training set samples are pale malts. The moisture content values for both Munich and colored malt lay outside the ranges considered in the validation of the A-EBC reference methods, which are 3.8−7.3% m/m (*30*) for Munich malts and 4.1−7.7% m/m (*31*) for colored malts. The same was observed for the total nitrogen content, dry matter, of the Munich malt, for which the validation range is 1.56−1.87% m/m (*32*). The repeatability of the standard A-EBC methods was compared with those of the NIR calibrations in all cases, because the values laying outside are in the same order of magnitude of the validation ranges. Repeatability was compared only for those parameters validated by the EBC, that is, moisture content and total nitrogen, dry matter, as no validation parameter for total nitrogen, as is, is reported in the standard A-EBC methods. The comparison was not satisfactory only for moisture content of colored malt, the s_r/σ_r ratio being higher than the upper acceptability limit. When this ratio was instead lower than the inferior acceptability limit, the repeatability of the two methods was considered to be comparable, as the NIR method is supposed to be characterized by fewer experimental variables than the oven-based one. This means that, apart from the NIR method for moisture content determination on colored malt, all calibrations match the repeatability of the standard methods for pale, Munich, and colored malts. Under these conditions, the reproducibility of the standard method can be considered applicable to the NIR method, and their extended uncertainties (U_e) can be calculated according to the holistic method for uncertainty determination, as described in eqs 4 and 5 (Materials and Methods). When extended uncertainty was calculated, all differences between NIR-determined and true values were lower than the uncertainty of the NIR method, the determination of total nitrogen, dry matter, being at the limit.

Data for the maize sample are shown in **Table 5**. Also in this case, no major difference was observed between cross-validated and test set validated models. For both samples, there was good agreement between NIR-determined and true measured values, for both internal and test set validated calibrations. Moreover, the repeatabilities of NIR methods match those of the standard A-EBC methods, for both moisture content and total lipids, as is (total lipids, dry matter, was not validated by the A-EBC collaborative trials), and extended uncertainties were calculated from the reproducibilities of the standard A-EBC methods. All differences between NIR-determined and true values were smaller than the uncertainties of NIR methods.

Hence, the NIR technique can be used to predict the quality of brewing raw materials in a rapid, reliable, and nondestructive way. Due to optimized calibrations, it can be indeed much more advantageous than other analytical techniques, as NIR measurements are very fast and no sample preparation or use of hazardous solvents is required. The results obtained in this work point out the precision of the method for cereals such as malt and maize, to keep under control their moisture, nitrogen, and lipid contents. The calibrations were obtained by considering more than 250 malt samples and 100 maize samples. The optimization of the models gave calibrations characterized by very low values of the RMSEP parameter.

In the end, we gave a positive answer to requests from large, medium, and small brewing industries asking for a rapid and highly repetitive analytical method comparable to the official ones to control malt moisture and nitrogen as well as maize moisture and lipid contents.

ACKNOWLEDGMENT

We gratefully acknowledge Prof. Paolo Fantozzi, Director of the Italian Brewing Research Centre (CERB).

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Received for review July 23, 2008. Revised manuscript received November 11, 2008. Accepted November 11, 2008. We gratefully acknowledge the financial support of the Italian Ministry of University and Research (MUR), by means of the Prin-COFIN (Grant 2006078598) titled "Implementation of the monitoring systems (safety, quality) in the malt and beer production chain, by the use of innovative control methods".

JF8022639