A Tutorial on Near Infrared Spectroscopy and Its Calibration

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Near infrared spectroscopy (NIRS) has had rapid usage growth since its first application in the 1960s in the grain industry. Since then, material science, food, environment, medicine, pharmaceutics, agriculture, archeology, and others have reported successful applications of near infrared technologies. Evolution and improvement of instrumentation is expected to continue with increasing users and applications. This review provides a guide for NIR beginners. Theory of NIR measurements, instrumentation, and stages of calibration development are covered in a non-mathematical approach, focusing on critical operating processes to provide new users a starting point for application and study.

Keywords NIR, spectrophotometer, chemometrics, calibration

INTRODUCTION TO NEAR INFRARED SPECTROSCOPY

Light is electromagnetic energy defined by the properties of wavelength, frequency, and energy. The energy content is indirectly proportional to the light wavelength—short wavelengths being more energetic—and is directly proportional to the wave frequency. Light can be arranged by any of those properties to form the electromagnetic spectrum. The infrared region is located in the middle of the electromagnetic spectrum, and has three major regions: far-infrared [300 GHz (1 mm) to 30 THz (10 μm)], mid-infrared (30 to 120 THz or 10 to 2.5 μm), and near-infrared (from 120 to 400 THz or 2,500 to 750 nm). The absorption by a material of any wavelength induces molecular vibrations. Changes in light energy between the three regions lead to varying absorptions from different molecules and bonds and induce different types of vibrations. For instance, the least energetic far infrared region light (FIR) is absorbed by heavy atoms, such as some inorganic and organometallic substances, while the mid infrared region (MIR) is popular for organic chemical analyses.

The near infrared region (NIR) is the most energetic infrared region and is close to the visible region in the electromagnetic spectrum, as discovered by Herschel in 1800. His experiments in measuring the heat produced by filtering the sun light on colors with a thermometer lead him to realize that temperature increased from going blue (450–475 nm) to red (620–750 nm).

Temperature kept rising even after positioning the thermometer further from the visible red, which meant that more energy was present beyond the visible spectrum (1). Further significant research on the NIR region was not done for 150 years. MIR in analytical chemistry became popular, while the NIR region was ignored as it was considered to lack relevant chemical information: NIR spectra from any sample showed broad and overlapped low intensity bands, between 10 and 100 times attenuated compared to the sharper MIR fundamental absorptions (2). NIR broad peaks could not be directly assigned to specific chemical compounds or interpreted in a straight-forward manner as MIR spectra. Term spectrum (or its plural spectra) is commonly used in spectroscopy and it will be often used in this review to refer the light intensity measurements (after being either reflected or transmitted through a sample) as a function of wavelength.

NIR spectra are formed of overtones and combination bands. Overtones are electron excitations to higher energy levels which occur at multiples of the MIR fundamental frequencies. The entire NIR spectra contains up to four overtones (although the fourth overtone is very weak and ignored) from the absorptions of methyl C-H, aromatic C-H, methylene C-H, methoxy C-H, carbonyl associated C-H, N-H from primary and secondary amides, N-H from amides (primary, secondary, and tertiary), N-H of amine salts, O-H (alcohols and water), S-H, and C=O groups (3). Note that all those groups are found in organic molecules and water and absorb in MIR; hence, each NIR overtone repeats the chemical information of MIR but with absorption bands decreasing with overtone level. The combination bands region is located at higher NIR wavelengths (1900

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to 2500 nm), and basically involves a combination of vibrations from the same chemical groups of the overtones, but as a result of interactions between molecular vibrational frequencies, overlapped information from Fermi resonances, and inactive MIR bounds among other phenomena (4). The bottom line is that chemical information in NIR spectra is repeated and highly overlapped through the whole wavelength range, a fact that discouraged researchers for a long time.

The beginning of the 1960s was an inflection point for NIR spectroscopy. Karl Norris, known to be the pioneer on NIR analytical development, and his U.S. Department of Agriculture team could determine moisture content from seed extracts (and later for whole seeds) using NIR bands with a multi-variate calibration approach (5, 6). In the early 1980s, the use of NIR by reflectance became an analytical, recognized method by the American Association of Cereal Chemists (AACC) for measuring protein in wheat, and the list was later expanded with methods for protein and oil determination in ground and whole soybeans, hardness determination of wheat, and protein content in small grains in general (7). The impact of Karl Norris' work was huge not only on the grain sector where NIR would prove to save significant time and money (8), but also in other non-agricultural fields such as pharmaceutics, polymers, material science, medicine, art, textiles, animal feed, and food where NIR is still leading to an extensive variety of applications and emerging NIR-related technologies.

This review intends to provide a summary on the basic principles behind NIR and its analytical use. Fourier transform NIR and NIR chemical imaging are briefly discussed as two of the most popular NIR-related technologies. Since the exponential growth of NIR applications can be mainly attributed to advances in instrumentation and data analysis methods, instrumentation and calibration development sections are emphasized using a practical, user-oriented approach. Critical calibration development stages are listed and common statistical methods discussed.

USING NIR LIGHT IN ANALYTICAL CHEMISTRY

When a sample is irradiated with light, according to energy conservation law, fractions are reflected, transmitted, and absorbed all summing to 1.0. The proportions depend on the light wavelength and sample properties (composition and thickness among others). Beer's law, well-known in molecular spectroscopy, defines the correlation of analyte concentration with its absorbance at specific wavelengths. Beer's law is not directly applicable in NIR spectroscopy because of several restrictive assumptions: no correlation between multiple absorbers, homogeneous samples, negligible light scattering, and constant path length. Notwithstanding this, Beer's law implication is still held by NIR analysis, but some further detailed challenges must be overcome first.

Although absorbed light cannot be directly measured, transmittance and diffuse reflectance can be correlated to light absorption according to Eqs. 1 and 2, respectively.

$$Apparent\ Absorbance = \log(P_o/P) = \log(100\ /\ T(\%)) \qquad [1]$$

$$Absorbance = -\log(R_{relative}) = \log\left(\frac{1}{R_{relative}}\right)$$
$$= \log\left(\frac{R_{s\tan dard}}{R_{sample}}\right) = \log(1/R_{sample})$$
$$+ \log(R_{s\tan dard}) \qquad [2]$$

Transmittance (T) is defined as the ratio of radiation passing a sample per unit area (P) divided by the initial radiation power (P/P_o), expressed as percentage. Log(1/T), also known as optical density, is called apparent absorbance because the effects from light dispersion in the sample are not taken in account. Although it is a close approximation, it is not exactly the same as the absolute absorbance as some of the emitted radiation is reflected before being transmitted (9).

The reflected light fraction shows higher complexity. There are two main components of reflected light: specular and diffuse. The specular component angle of reflection is the same as the incident light, is reflected to a single direction, and achieves its maximum intensity when the irradiated light is perpendicular to a smooth sample surface. It lacks NIR relevant information due to its minimum contact with the sample. The NIR diffuse reflectance component refers to the part of the incident beam that achieves a certain degree of sample penetration, it is scattered within the sample, and returned to the surface after within-sample absorption. It can be correlated to absorbance through Eq. 2. Relative reflectance ($R_{relative}$) is measured as the ratio of the sample measured reflectance (R_{sample}) over the measurement from a highly reflective material ($R_{standard}$, with reflectance approximately 100%) such as Teflon or Spectralon.

Transmittance measurements are best taken at lower wavelengths because they are more energetic, have more penetration power, and the absorption is weaker. Instrumentation that works in transmission mode works with a shorter wavelength range, usually not higher than 1800 nm. Measurements by diffuse reflectance are best taken at wavelengths between 1200 and 2500 nm. Above 2500 nm (MIR region), sample absorption becomes very strong. MIR measurements by transmittance cannot be carried out on thick samples; signal to noise ratio is reduced (10).

Summarizing, NIR measurements for analytical purposes can be carried out in two modes: transmission or diffuse reflection. Diffuse reflection mode allows working with thicker and denser samples without inducing as much heating as transmission. While sample path length is pre-determined and must be kept constant for transmittance measurements, the minimum sample required in reflectance mode is highly dependent on the wavelength range used in the analysis and sample characteristics such as density or packing, particle size, and material

absorption (11). Physical characteristics affect reflectance measurements especially at higher wavelengths (combination bands region); hence, any sample changes will create an additional source of variability and noise in the measurements (12).

Overall, reflectance measurements show a shorter dynamic range compared to transmittance (lower sensitivity) because information provided by diffuse reflectance originates from smaller sample portions and has been attenuated (13). Its repeatability is slightly worse which is more noticeable in heterogeneous samples. In specific applications, those limitations may not create significant errors, or may be mitigated by using of a wider range of wavelengths (14). Transmittance measurements exceed the accuracy of reflectance measurements in most pharmaceutical applications, although analytical sensitivity, signal to noise ratio, and limit of detection is highly affected by sample position and changes in geometry (15). Comparison studies in agriculture fields do not lead to a unanimous conclusion regarding superior performance of any of the two measurement modes (16–19). Although there is a general preference towards transmittance measurements when small concentrations need to be measured, differences arise from a combination of factors such as selected wavelength range, instrument and sample characteristics, data processing/analysis, and sampling procedure (14, 15, 20, 21). Due to the reduced flexibility and versatility of measurements by transmittance in sample presentation and characteristics, in-line monitoring, remote sensing, and field applications have been leaded by NIR diffuse reflectance spectroscopy (2, 22).

NIR INSTRUMENTATION: SPECTROPHOTOMETERS

Despite proprietary instrument conformations, any commercial NIR spectrophotometer has five basic sections further detailed: (1) sample compartment, (2) light source, (3) light wave selection system, (4) detector/s, and (5) signal processor or computer. Figure 1 shows the schematic of four of the most common optical bench arrangements in conventional NIR instruments. Note how A, B, and C conformations select the light before it reaches the sample, while conformation D selects it on the reflected/transmitted light after hitting the sample.

Sample Compartment

Instruments working by reflectance do not need sample confinement for in-line measurements, but it is common to use open sample cups or sample cells confined by silica or quartz (materials transparent to NIR light) in laboratory instrumentation. Transmission instruments may work with confined sample cells as well, but with specific pre-set pathlenghts ranging from 0.1 to 10 cm, depending on the product to be analyzed (23). An integrated adjustable sample compartment with automatic flushing is used for whole grain analyzers. One of the advantages of NIR light is its ability to pass through optical glass fibers preserving most of the signal integrity (losses lower than 5% per km of cable), even if the resulting output intensity is low. This is especially useful for measurements to be made far from the phys-
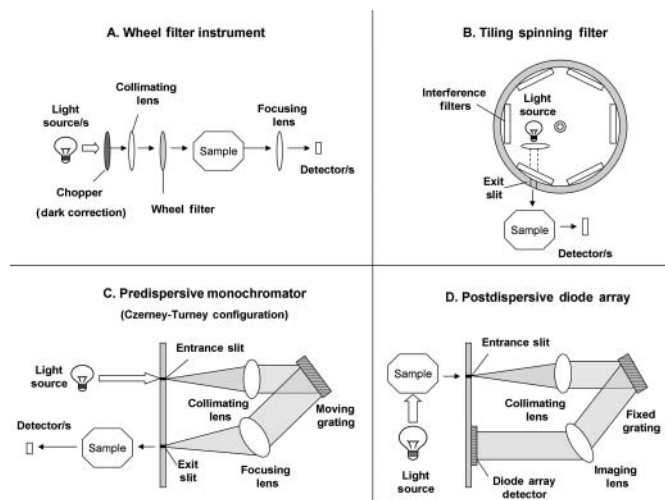

FIG. 1. Four traditional instrument conformations in NIRS. (A) Filter instrument with wheel filter. (B) Tiling spinning interference filter instrument. (C) Pre-dispersive monochromator with grating (Czerney-Turney configuration). (D) Postdispersive diode array.

ical instrument and for multiple sampling/sequential analyses in multi-plexer systems. The use of optic fibers with probes for either transmission or diffuse reflectance measurements allows sampling by immersion in liquids for controlling fermentation or other liquid reaction processes (24–26), contact on small sample areas such as works of art (27), in-vivo medical analysis (28), and development of smaller spectrophotometers (29).

Light Sources

The most popular NIR light source is the tungsten halogen lamp, which has wavelength emission ranges from 320 to 2500 nm. The halogen gas allows recycling of the evaporated tungsten (30), and brings the advantage of a longer lifetime compared to traditional tungsten lamps without halogen.

Light emitting diodes (LED) were used as light source in the first commercial instrument for whole seed analysis in 1985 and in the first portable spectrometers (31). The low power consumption, price, small size, and long lifetime (around 25 years) of LEDs still make them the most suitable light sources for miniaturized instruments and specific screening applications outside the laboratory environment (30, 32). Conventional LEDs emit in short wavelength ranges (30–50 nm) around their center point. Several of them can be mounted in an array with narrowband interference filters if wider wavelength ranges need to be covered, although measuring many wavelengths with this configuration is not an economical approach (33). LED devices have been improved during recent years to overcome some of their limitations. For instance, some commercial instruments allow easy switching of LEDs according to the application.

Finally, the most innovative light sources are tunable diode lasers, also called superluminiscent light-emiting diodes

(SLED). Using the semi-conductor technology of diodes, tunable diode lasers are much smaller than the traditional tunable laser, cheaper, have excellent wavelength resolution, brighter, and have lower noise frequencies than tungsten lamps. SLEDs are suitable for measuring weak absorptions at good signal-to-noise ratio and as light sources in miniature instruments (34). Improvement of tunable diode lasers allows, controlling emitted light at a specific wavelength, combining light source, and wavelength selection features.

Wavelength Selection

Most detectors collect light intensity from a relatively wide range of wavelengths. Recording signal values at specific wavelengths is required for analytical purposes. Discrete wavelength values are obtained by filtering the polychromic light beam. Most simple filters work by absorption (absorption filters), which are discrete bandpass filters that absorb all light wavelengths but the one of interest. Narrow bandpass interference filters (Fabry-Perot) achieve better spectral resolution and higher output intensity by selecting wavelengths according the refractive index and thickness of the dielectric material between the two layers of reflective material (9). To select multiple wavelengths, interference filters are mounted in a wheel which can be automatically controlled to rotate and select the suitable filter for the wavelength selected (Fig. 1A and B). This creates spectrometers that provide few spectral measurements. Although filters are an alternative that provides acceptable results, problems of image misalignment and slow operation are common. (35).

Acousto-optic tunable filters (AOTF) and liquid crystal tunable filters (LCTF) allow faster tuning for wavelength selection, and provide better reproducibility without the need for mechanical devices because one filter can create several wavelengths. AOTF filters modulate the light wavelength and intensity through the interaction of sound waves generated in a birefringent TeO_2 crystal. The frequency of the acoustic signal makes the refractive properties of the crystal change allowing wavelength specific transmission. Wavelength discrimination in liquid crystal tunable filters (LCTF) is carried out by applying variable voltage to progressively change the polarity of a liquid crystal (36). Those filters provide a better output quality compared to AOTF filters, but their short wavelength range is limited (below 1800 nm), and give a lower intensity dependent on the selected wavelength (30, 35).

Dispersive-type instruments use a prism or a grating, which diffracts the incident collimated light beam at different degrees while resolving it in discrete wavelengths. Light dispersion can be done before scanning a sample (pre-dispersive instruments) or after radiating the sample with polychromatic light (post-dispersive). Post-dispersive instruments offer advantages such as less environmental interferences with the lamp radiation, analyzing wider sample areas, and holding longer distances between sample and light sources (37, 38). Prisms have been replaced by gratings because of lower cost and better linear wavelength dispersion of the last ones. There are two types: holographic (photosensitive film with fringes) and ruled (concave surface with fringes). Ruled gratings require being complemented with other optical elements such as lens, and show less stray-light rejection than holographic gratings (39, 40).

In the dispersive instruments group, there are monochromators and spectrographs such as diode-array instruments. Monochromators are pre-dispersive instruments that scan a sample with grating mechanical motion. The basic principle is as follows (Fig. 1C): polychromatic NIR light enters through an entrance slit and is then collimated (light rays are made parallel) by a mirror. The light hits the dispersion grating and later hits a focusing mirror, which reflects it to a second exit slit to either hit the sample (transmittance mode) or hit the single-channel detector (reflectance mode). Entrance and exit slits of a monochromator are very carefully designed to have accurate geometry since they are critical for instrument-observed resolution (smallest wavelength difference distinguished by the spectrometer) and effective wavelength bandwidth (full width of a band at half of its maximum value, FWHM). When using grating alone without slits, the resulting resolution is not enough for most chemical measurements in plastic or pharmaceutical applications (41). Small slits (around 0.1 mm) give low band width, more dispersion, and high spectral definition useful in qualitative applications; large slits (around 2 mm) give more intense radiation and are more suitable for quantitative analysis (39).

Diode array spectrographs are post-dispersive instruments that measure all the wavelengths at the same time thanks to a fixed grating and a set of detectors placed in array (multi-channel detectors) (Fig. 1D). There is no need for exit slits. There are fewer optical elements compared to monochromators and resolution depends on the number of elements in the detector array and array characteristics. The latest advances in wavelength selection besides tunable light sources are the micro-electro-mechanical systems (MEMS) created with semi-conductor technologies. MEMS diffraction gratings control light diffraction by electronically controlled movement of diffracting microelements. Their small size and lower cost has lead to a new generation of portable instruments.

Detectors

Detectors transform the incident light energy to electric analog signal. The electrical signal is then amplified and transformed to digital, which may later be further processed by the computer. Detectors and amplifiers are considered the most common sources of non-systematic noise in instruments (random noise). Random noise is reduced in most commercial instrumentation by averaging several spectra from a same sample, improving the signal-to-noise ratio (SNR). SNR achievable values in NIR spectroscopy according to Workman and Weyer range from 25,000:1 to 100,000:1 (3).

An effective detector must have a linear relationship between the energy input and signal output within its dynamic or working range—from the minimum detectable signal to the maximum before reaching saturation Measurement linearity is influenced

by other factors besides detector characteristics; for instance, the number of bits of the analog to digital converter device and slight detector misalignments, which can lead to capturing a small fraction of the reflected specular component (often called stray light) in reflectance mode instruments. Without linearity, more complex and potentially unstable mathematics are needed to calibrate the instrument.

Photo-sensitive detector materials are chosen according to the NIR region to be covered. From 400 to 1100 nm, silicon detectors (Si) are common (30). Si detectors are stable, fast, not too expensive, and sensitive to low light intensity to achieve good performance. Lead sulfide (PbS) or indium gallium arsenide (InGaAs) detectors can cover higher wavelength regions than Si detectors, being usual having both types combined in a same instrument. Photodiode array (PDAs) spectrographs have a set of InGaAs detectors or charged coupled devices (CCDs) in array. While InGaAs PDAs offer high signal precision, high SNR, and less sensitivity to high light intensities when compared to CCD, CCDs have higher signal sensitivity and resolution (42). PDAs take faster measurements (all wavelengths measured at the same time) and can be smaller in size than grating monochromators, in which the optical conformation cannot be easily reduced in size because it would lead to low throughputs and resolution (29).

Selecting Instrumentation: General Aspects

There is currently a wide range of instruments with a wide range of prices in the market: small portable instruments for little over $8,000 and big sophisticated laboratory instruments over $50,000. Instrument price increases with instrument complexity and market position. This need not mean that the most expensive spectrophotometers will lead to better performances; indeed, the opposite may be true if no further considerations are taken before purchasing analytical instrumentation. The important point is to know what the instrument function will be and what it could become in future projections. It should be taken into account that calibration cost increases with instrument cost, and the success of any NIR analytical application is highly dependent on data analysis and calibration development up to the point that instrumentation may become a relative afterthought.

To select a suitable instrument, the user must describe the nature of the materials to be tested (sample physical and chemical properties), identify potential applications or uses (environmental conditions and variability in sampling procedures), and determine the accuracy required for the analysis (i.e., screening or demanding quality purposes). Those points should be written down before looking at instruments. Instrument versatility is a relevant aspect for researchers and for users whose samples show variable composition and physical characteristics. Sampling speed, although usually not a major limiting factor in NIR spectroscopy, must be considered for in-line analysis and process monitoring. For these last applications, grating monochromators would not be recommended as they take longer scanning times and need regular wavelength standardization due to higher number of mechanical moving parts; PDAs would be

more suitable. Instrument robustness is inversely proportional to the number of moving parts and dictates its suitability for rougher environments. An example are miniaturized portable instruments which show high versatility and success in applications such as material sorting for recycling purposes, screening for fraud, narcotic identification, raw material inspection, or paint thickness analysis (43).

Spectral resolution provided by manufacturers, known as observed resolution, affects spectral peak location and, hence, may impact measurement accuracies. This term can be often confused with wavelength sampling increment also in nanometers (wavelength increment between two consecutive measurements), which is greater than resolution. Although high resolution (0.1 nm) may look desirable, it is not always required for success. In analyzing biological or materials with complex composition, resolution shows low impact since the NIR absorption happens over broad regions (44, 45). Economical instrumentation with resolution over 4 nm is common and provides acceptable performances in many applications. Resolutions between 1 and 2 nm were required to obtain satisfactory discrimination of compounds with good accuracy when analyzing complex chemical matrices of pharmaceutical and mineral compounds (45, 46). Although both resolution and SNR affect instrument sensitivity and selectivity, enhancing SNR compensates for limitations caused by lower resolutions (42, 47, 48).

Technical support, periodic maintenance, and training by the supplier are important. Instrument maintenance is expensive because most operations beyond replacing the light source need to be performed by supplier personnel. Customer services availability, quality, and training are valuable support, especially during the initial stages of instrument set-up, data collection, and calibration development. Several aspects from the data acquisition software have a direct impact on instrument user-friendliness and efficiency on data managing. Current instrumentation has a wide selection of data file and calibration formats requiring varying user knowledge in data handling. Any facility in managing data is highly desirable. From the time spent since the data is collected and the calibration is developed, 80% can be spent on arranging and organizing the data (i.e., exporting, setting the right formats) and just 20% on the real data analysis (49).

Other NIR-Related Technologies

There are other NIRS technologies and instrumentation use of NIR light under slightly different principles from traditional spectroscopy. Two of the most well-established are Fourier transform NIR (FT-NIR) and NIR chemical imaging. Other emerging technologies, specifically in medical fields, such as NIR fluorescence are not discussed in this review.

Fourier transform (FT) is widely popular in MIR spectroscopy, and it has recently gained high popularity in the NIR range as well. FT technology offers advantages such as high SNR, high light outputs due to the absence of slits, fast measurements, instrumental simplicity, and high resolution and

accuracy (41). Brimmer et al. (50) claim that those advantages are more perceptible when working in the MIR region due to the limitation of higher detector noise relative to the signal when working in the NIR region.

FT-NIR measurements are carried out in time domain and the direct instrument output from sample scanning is an interferogram instead of a spectrum. NIR interferometers split the NIR light beam in two; one of the beams is reflected to a fixed mirror, and the other is reflected to a mirror that moves forward and backward at carefully controlled speed—usually tuned by a HeNe laser. The reflected beams are recombined back in the beam splitter to generate the interferogram signal, which is a result of light interferences. When displacing the moving mirror, the pathlength difference in relation to the fixed mirror changes, leading to different grades of interference between the two reflected beams and which are correlated with different light frequencies. After the interferogram light reaches the sample, the transmitted or reflected signal is read by the detector in time sequence (ms); hence, measurements are fast. Although interferograms contain information from all the frequencies or wavelengths encoded, it has to be first processed with the Fourier transform. The computation takes as an input a time domain wave signal (the interferogram) from which the transform principle states the signal is made from an addition of sinus and cosinus of a set of individual wave frequencies. The processed signal or output looks like the spectra obtained by any traditional spectrometer, but with the expectation of higher throughput and frequency accuracy. One of the drawbacks is the fact that FT-NIR instruments are complex and expensive, and mainly suitable for controlled environments (such as laboratories) due to their sensitivity to external factors such as temperature and vibrations.

Near infrared chemical imaging (NIR-CI), also called NIR hyperspectral imaging, has rapidly become popular, especially in measurements by diffuse reflectance. It combines the advantages of near infrared spectroscopy with digital mapping: the chemical compounds of a sample can be both discriminated and quantified in the sample spatial frame. This is especially useful to analyze compound distribution and sample heterogeneity. Instrument parts and operating principle are very similar to traditional spectrophotometers. The sample scanning procedure can be carried out in two ways: 1) by push-broom or moving imager technique, popular for in-line measurements and sensing, or 2) by fixed staring systems.

Pushbroom instruments measure a spectrum from a whole sample by small consecutive areas or lines while the sample platform is moved and their wavelength selection is usually by dispersion. Staring systems scan on still samples, one wavelength at a time, using either AOTF or CLTF filters. The mapping capability of imaging systems is brought by digital cameras with two dimensional arrays of detectors (pixels) such as CCDs that are effective in lower light intensities. Pixel size or area analyzed per pixel range 49 to 1,600 squared microns in commercial instruments, depending on selected magnification. Higher mag-
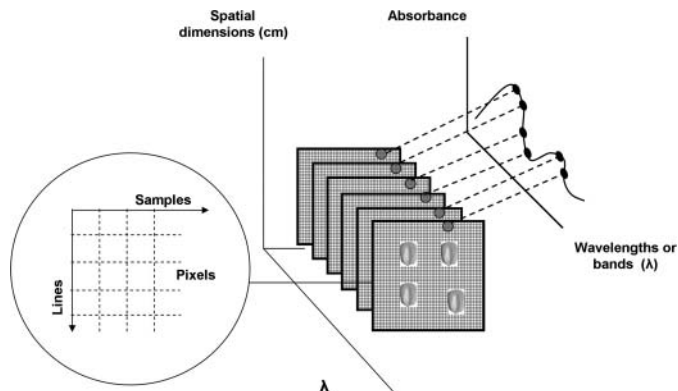

FIG. 2. NIR-CI data configuration, showing the data cube of images at different wavelengths. A single spectrum is obtained from each pixel.

nification (or smaller sample area captured per pixel) will lead to more detailed spatial analysis and a lower dilution effect of the compound of interest within the sample matrix.

NIR-CI data structure can be thought of as a cube or a stack of cards, where two spatial dimensions are combined with a third dimension corresponding to the chemical information or spectra (wavelengths). Depending on the manufacturer, around 320×512 pixels are arranged to capture both sample area and spectra. In that previous example, a total of $320 \times 512 = 163,840$ data points would be generated for a single wavelength and correlated to small sample portions as a chemical map. If the instrument had 200 sampling wavelengths, the final "image" or data cube would have a total of $320 \times 512 \times 200 = 32,768,000$ data points. Although the amount of data generated is large, visual selection of image areas or pattern recognition techniques help in discarding pixels with no relevant information.

This concept is illustrated in fig. 2, where each squared surface is like a picture taken at one single wavelength and the small squares within represent pixels. In common imaging terminology, "samples" and "lines" specify the number of columns and rows of pixels; "bands" refer to the discrete number of wavelengths, or following the previous analogy, the number of cards in the stack.

DATA ANALYSIS AND CALIBRATION DEVELOPMENT

NIRS data analysis requires chemometrics. Chemometrics, a term widely used in NIRS-related literature, refers to the use of mathematics, statistics, and computational devices in chemical analysis. Without computing capabilities and multivariate methods, NIRS applications would not be possible. Chemometrics made possible the dealing of NIR units in resolving highly overlapped and broad peaks, high sensitivity to sample physical characteristics, and high information redundancy. Workman (3) pointed out that C-H associated vibrational information is repeated eight times in the NIR region (690 to 3000 nm). While information redundancy can be an advantage and can allow
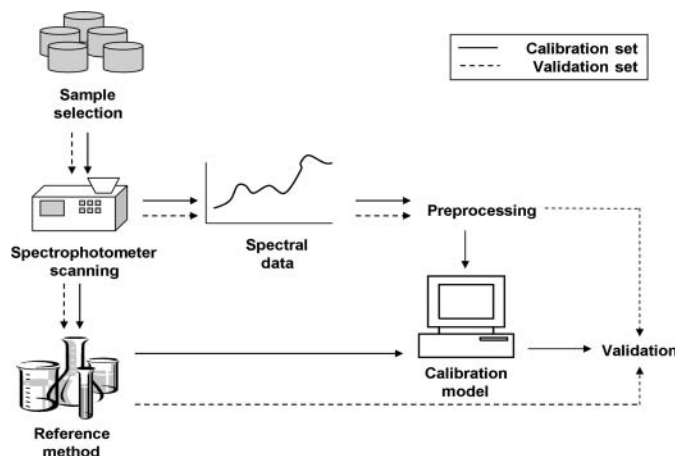
FIG. 3. Block diagram with the basic steps for NIR calibration development.

working with different wavelength ranges, determining which wavelengths hold information of interest without having correlation between them is not a problem which can be efficiently solved by trial-error experimentation.

Figure 3 shows a block diagram with the basic steps for developing a NIRS calibration. In that procedure, the broad absorptions (spectra) from a sample irradiated with NIR light are correlated with the compound concentration or sample characteristic which the user pretends to analyze. The compound to be measured should either be of organic nature (direct measurement) or be correlated with sample physical characteristics or another organic compound (indirect measurement). Some relevant aspects of the calibration procedure can be pointed out from the diagram Fig. 3: 1) there is the need for a fundamental analytical method, called the reference method, in order to obtain the dependent variable to be calibrated; 2) a suitable number of samples uniformly covering a wide enough range of analyte concentration should be part of the calibration set, and 3) the calibration model should be later validated to test the model performance on future samples.

Selecting Samples, Reference Method, and Spectral Data

The importance of choosing an adequate calibration set is often underestimated and not usually covered in the literature. There is no fixed number or rule-of-thumb to determine the number of samples to be included in a calibration. At least between 20 and 30 samples should be taken for feasibility studies and initial calibrations (51), but more robust calibrations may use a few hundred (for instance, instrument built-in calibrations for grain analysis). Calibrations of homogeneous mixtures (i.e., pharmaceutical powders) may require smaller calibration sets than agriculture samples of high compositional complexity and heterogeneity, such as whole grains or forages.

Users work under the constraints of sample availability and reduced budget. Nevertheless, there is not enough emphasis on the ultimate consequence of using calibrations developed with

inadequate calibration sets: calibrations with low predictive ability. An ideal calibration set should cover the chemical, spectral, and physical characteristics of the population to be analyzed and avoid future extrapolations when predicting new samples (52). The distribution of reference values should be uniform. If the distribution is normal (bell shaped distribution), samples belonging to either higher or lower concentrations have the chance to get more relevance in the calibration, which would not be desirable.

Because reference values are not always known and reference analyses of large sample sets may be expensive, there are other methods to select an initial calibration set, using spectra. A method developed by Naes (53) and later illustrated by Naes et al. (54) uses principal component analysis (PCA) on the spectra and cluster analysis of the data.

PCA is a technique that projects the spectral data to a new reduced dimensional space. Wavelengths, the initial set of variables, are projected in the new space defined by axis called principal components (PCs). PCs are consecutively created following the directions of data variability in descending order while holding an orthogonally constraint among them. During this procedure, the initially highly redundant variables (wavelength absorbances) are substituted by a small new set of uncorrelated variables (PCs).

NIRS calibrations can match or virtually achieve better precision and accuracy than traditional wet chemistry methods (55), but paradoxically, NIRS relies on them for calibrations. The quality of the reference data influences NIRS calibrations. Careful search for the suitable method and laboratory should be carried out. In the case of NIRS instrumentation for grain analysis, calibrations are often pre-loaded, e.g., wheat protein. Although this may seem to be an opportunity to save time and resources in developing custom calibrations, the performance of any built-in calibration must be carefully validated to determine its suitability for a particular situation. Calibrations from an instrument brand and model may not perform successfully when loaded to a similar instrument, or used on different samples than the original calibration population.

Outliers from either reference values or spectral data exist and most calibration methods are highly sensitive to them (56, 57). Some tests and statistics such as Dixon test (58) or Grubbs studentized mean deviation (59) can be used as an assessment for potential outliers from the reference data a priori. For instrumental data, visual check of the spectra can identify abnormal and noisy spectra. A visual check is often not enough, and possible outliers may not be detected until data is either pre-processed, or a first attempt of calibration has been carried out.

Detecting multiple potential outliers is not simple; their effect is masked with each other. Traditional approaches to detect single outliers do not perform well (60, 61). The use of influence measures such as leverage or Hotelling's T^2 statistic in combination with checking model residuals are a powerful alternative for outlier detection (61). Influence statistics give an idea of how different a sample is from the rest of the data in a given

dimension. While that does not explicitly make a data point an outlier, high leverage followed with a high residual value (the sample was poorly modeled by the model) give chances that the sample is an influential outlier. Its exclusion from the calibration set could improve the calibration. However, if removed, enough similar samples should remain in the calibration set to avoid significant reduction of representativeness, especially in reduced data sets.

Spectra Pre-treatments

Pre-treatments or spectral pre-processing methods are a set of mathematical procedures on spectra before developing a calibration model. Mathematical pre-treatment of spectra reduces noise or background information (smoothing techniques) and increases signal from the chemical information (differentiation). Any pre-treatment must lead a robust model with good predictive ability. Basically, pre-processing methods can be classified as baseline correction—normalization, signal enhancement, and statistical filtering of signal noise. Mean centering the spectra is a basic pre-treatment that removes the absolute absorbance value (absolute baseline) and thus the need for a model intercept, and enhances the absorbance from each individual wavelength. This pre-treatment is commonly used for PCA-based calibration methods, such as partial least square regression (PLS). Centering the data to the mean value reduces the final model complexity, often reducing the number of variables to be employed by one (62).

Scaling spectra involves dividing each wavelength data by its standard deviation, which allows each wavelength to have the same weight or relevance during calibration development. Haaland and Thomas (62) suggest not using scaling when a big part of the spectra do not contain useful information because variables that have more noise than relevant information will get the same importance as the ones with relevant signal.

Multiplicative scatter correction (MSC) (63) and standard normal variate (SNV) (64) are two widely known methods that reduce spectral distortions due to scattering. SNV centers and scales each spectrum individually, so each has a mean equal to 0 and standard deviation equal to 1. MSC is more complex and memory-consuming than SNV and depends on the whole spectra set, while SNV treats each spectrum individually and independently. When applying MSC, the spectra is first averaged and each individual spectrum is regressed by partial least squares to the total average. The regression equation slope and intercept represent the additive and multiplicative effects of light scattering, respectively. Finally, each spectrum is corrected for offset (the offset value is subtracted) and each wavelength of the spectrum is divided over the slope. The regression coefficients should be stored and applied to new data. Generally, both methods provide the same results for most applications (65); this is true with all of the pre-processing methods belonging to the same category. However, MSC and SNV lead to different data geometry in the working space, where SNV shows more curvature in PCA score plots and MSC shows higher tendency to

accentuate outliers (66). Pre-treatments can be very helpful but there is always a tradeoff between information loss and noise reduction: when removing scattering effects, the chemical signal may also be reduced.

The use of derivatives is an alternative for correcting the effect of overlapping peaks (enhancing signal) and removing spectral base line offset (constant drift of the spectra base line intensity across wavelengths) and baseline slope (additive variation of the spectra base line intensity across the wavelengths). The calibrations resulting from applying derivatives usually require fewer variables and models are considered to be more robust (67). Savitzky-Golay derivatives (68) are the most popular. Previously, to carry out derivation to a spectrum, a polynomial function of a selected degree is fit to a window of spectral points by least squares. This step helps smoothing the spectra or carrying out data filtering. Selecting higher polynomial degrees and small window size leads to a high function fit to the data, but the noise is modeled as well, with no smoothing effect. Low polynomial order and wide window size may lead to excessive smoothing and deletion of spectra features containing information. Subsequent derivation of the fitted polynomial differentiates overlapping signal peaks. Although it is possible to work with high-degree derivatives, most of the works in the literature use a maximum of fourth degree for curve sharpening and absorber separation. First and second derivatives are the most common and provide satisfactory results (67). First derivatives removes baseline offset while second derivatives correct the signal terms that vary linearly across the wavelengths (baseline slope) (9).

For most of the cases, the performance obtained by simple processing methods may be better than other more sophisticated methods such as orthogonal signal correction (OSC), developed by Wold et al. (69). OSC creates a model to remove any signal orthogonal (perpendicular in vector terminology) to the information of interest; thus, this method requires having previous reference data and it achieves best performances when most of the irrelevant information is actually orthogonal.

The optimum pre-treatment for a given spectra depends on the type of signal (i.e., transmittance, reflectance), sample characteristics, instrument conformation, and application or final goal (calibration or discrimination). There is no absolute or general rule for choosing the adequate pre-processing method; it usually requires a trial-error process guided by experience. Reflectance measurements often benefit from methods that reduce light scattering effects, such as MSC or SNV. Sometimes, the predictive ability of a calibration model is not improved with further mathematical treatments. Predictive ability may worsen if pre-processing excessively smoothes the signal, affecting the model ability for predicting new samples (generalization capability). Figure 4 shows the effects of several pre-processing methods on absorbance spectra of 100 bulk corn samples obtained from a reflectance mode instrument (Fig. 4, Spectra A). The offset baseline correction can be observed when preprocessing with SNV (Fig. 3, Spectra B), MSC (Fig. 3, Spectra C),

and first and second Savitzky-Golay derivatives (Fig. 3, Spectra D and E, respectively): the 100 spectra are more grouped together after eliminating scatter effects. Second Savitzky-Golay derivatives (Fig. 3, Spectra E) shows flat spectra baseline, similar to linear baseline correction (F), from which the baseline slope has been corrected. Between first and second derivatives, second derivatives lead to more peaks and an enhancement of noise on both spectral extremes. Although pre-processed spectra may look the same in the plots, differences among absorbance intensities across wavelengths still exist and are correlated with the compound to be analyzed during calibration development.

Calibration Models

NIR calibration models correlate either raw or pre-processed spectra with one or more chemical-physical property of a set of samples. As complicated as it may sound, there are several well-developed calibration methods proven to work with most of NIR applications. Those are included in all chemometric software packages. The first assumption when carrying out a calibration is the linear correlation between analyte or property to be measured and its absorbance according to Beer's law. Multiple linear regression (MLR), principal component regression (PCR), and partial least squares (PLS) are three of the best known calibration methods that work under this assumption. Among them, PLS is preferred for several reasons explained later.

MLR was first introduced by Strenberg et al. (70) and was the method that Norris used for his pioneer work in NIR. The method is an extension of bivariate regression for more than two variables. One of the limitations of using MLR with NIR data is the fact that does not account for wavelength multi-collinearity or variable codependency. When variables are correlated—as it is often the case with NIR wavelength—the resulting calibration is unstable and does not hold a unique solution. For this reason, the method is only appropriate if few weakly or non-correlated wavelengths are selected. Both PCR and PLS successfully deal with wavelength correlation. PCR is a direct application of the principal component analysis (PCA) method, and once the spectral data is projected to the new orthogonal non-correlated dimensional axis (PCs) a regression process by least squares is performed between the projected data and the reference values. Wold's introduction of PLS (1975) (71) was an improved alternative to PCR; both methods carry out regression on data projected to a new dimensional space, but the new space coordinates created in a process similar to PCA in PLS regression take into account the information from the reference value matrix, and PLS is thus classified as a supervised regression method; the new variables receive the name of latent variables (LVs) instead of principal components (PCs) as the new variables are not exactly the same as PCs. PCR and PLS calibrations are only based on a relatively small number of PCs/LVs because since they are extracted following the direction of maximum data variability, the last PCs/LVs usually involve noise. If an excessive number of variables are included in the calibration, a

fraction of noise is also modeled and the calibration becomes too specific to the calibration set. This phenomenon is known as overfitting, and as with excessive data smoothing, it leads to a reduction of model accuracy in future predictions.

There are different approaches to estimate the appropriate number of PCs/LVs to be kept for the calibration. One of the most employed uses cross-validation, later called as an approximate validation method, and selecting the number of variables that leads to the lowest cross-validation predicted residual error sum of squares (PRESS). The PRESS cumulative function can be plotted versus the number of variables and users may visually select the first minimum from the plot. PLS accuracies may not usually be significantly higher than those of PCR but they are achieved by including fewer latent variables in the final calibration (72–74). Although disadvantages such as overfitting when reference data are noisy and higher model complexity are reported, PLS is preferred because the algorithm is faster, models have higher precision, and it provides more harmonious calibration models (75). There are several PLS-based methods in the literature (modified PLS, hybrid PLS, robust PLS) which may help to improve PLS accuracy in data sets with specific characteristics (i.e., noisy data), although they often require advanced user programming skills, that are not included in commercial chemometric software packages and the resulting models are not compatible with the instrument model files.

There may be cases where the relationship between sample spectra and reference values is not linear. Any of the previously cited calibration methods can handle small non-linearities, but when prediction residuals show certain patterns of positive and negative values or plots of predicted versus reference values show appreciable curvature, non-linearity needs to be addressed (76). Often curvature may not be noticeable but for the fact that calibration statistics are not good. The Durvin-Watson statistic can be used as an assessment tool for detecting non-linearity (77). Once the problem is detected, there are some solutions suggested by Naes et al. (76) such as new preprocessing, deleting wavelengths, adding extra principal components/latent variables to the model, using non-linear calibration models, or splitting the data in subsets using an approach similar to the cluster analysis for sample selection (78).

PCR and PLS when used with locally weighted regression can handle some cases of non-linearity. Locally weighted regression (LWR) is based on the selection of a specified number of data neighbors and applying either PCR or PLS to the local group. When predicting a new sample, all data is retrieved to find the closest data points in the working dimension, a calibration is developed with the selected local points, and the new sample is predicted with the resulting calibration model. The method is very good dealing with highly clustered data but since calibrations are done with small sets of neighbors, extreme care has to be taken to obtain stable local calibrations (79). For this reason the approach requires a higher number of samples than traditional PCR or PLS calibrations. It is recommendable to keep adding samples to the calibration pool (80).
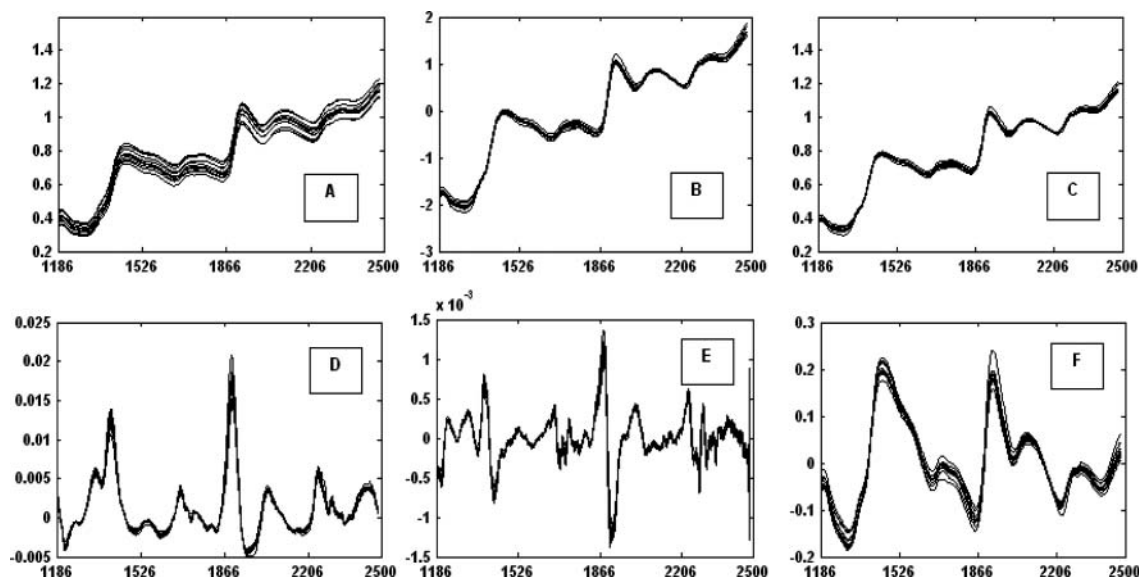
FIG. 4. (A) Raw absorbance spectra. (B) SNV Preprocessed spectra. (C) MSC preprocessed spectra. (D) First derivative spectra, window size 5 and third order polynomial. (E) Second derivative spectra, window size 5 and third order polynomial. (F) Linear baseline correction (slope correction).

Artificial neural networks (ANN) is a computational method that can be applied to NIR data to develop non-linear calibrations. By trying to simulate the human nervous system, ANN uses the calibration set to learn about any relationship that may exist between spectra and references. An artificial neural net is composed by neurons (the basic units) or nodes, layers, and transfer functions. When working with NIR spectra, the input nodes would be either wavelengths or principal components, and the output node would be the predicted value. Other nodes may be created in hidden layers (multi-layer perceptron model), which increase model complexity and the ability to model non-linear relationships. The nodes are linked by transfer functions, which are continuous functions. When the net morphology is defined (i.e., number of input nodes and hidden layer nodes), it is trained usually by backpropagation to start the learning process. In training by backpropagation algorithm, random weights are assigned to each transfer function and are updated according to the prediction error, which is propagated back through the net elements (nodes and transfer functions). This process is done numerous times (epochs or iterations). The learning rate (measurement of the change rate of weights in each epoch) and the number of epochs have to be closely monitored to check for model instability and overfitting (81). Using an additional sample set (early stopping set) besides calibration and validation sets is a common practice to avoid overfitting. Once the training is finalized, the resulting model is a function that depends on several weights coming from transfer functions, which on their turn may depend on other transfer functions and their respective weights. Therefore, the interpretation of weight meanings and the training process is rather complex. Complexity increases as

more nodes and transfer functions are added. For this reason the use of ANN is not as user-friendly as other calibration methods. First, there is a high number of parameters that need to be adjusted in the net morphology and training. Second, large number of samples is needed. Third, there is a high risk of obtaining local minima solutions due to the nature of the error function involved in the training process (79).

A relatively new and more robust alternative to ANN, support vector machines (SVM), has been recently introduced for non-linear NIR calibrations, although the original concept of the method was introduced in the 1960s by Vapnik and Lerner (82) was used for linear threshold classifiers. In a later adaptation of the algorithm (83), SVM creates a tube-shaped regression volume with variable diameter. The "kernel trick" originally introduced by Aizerman et al. (84) made the algorithm very popular because it opened the opportunity of applying the linear regression algorithm in higher dimensional data because dimensionality does not matter in the final optimum SVM regression function. This basically says that while a linear correlation may not be possible in the initial dimension, the correlation may be linear in another highly dimensional combination of features. The initial data can be mapped to the higher dimensional space applying a mapping function called kernel or kernel function. There are several kernels with variable complexity [polynomial, Gaussian or radial basis function (RBF) . . .] with which users can experiment, although more complex kernels may be prone to overfitting issues (85). Few parameters such as function regularization and kernel parameters (for instance, width in the case of RBF kernel) need to be chosen for optimum prediction ability. However, the number of parameters to be adjusted is

TABLE 1
Table of Common Validation Statistics for NIR Calibrations

Statistic	Units	Equation
Coefficient of determination (r^2)	Unitless	$r^2 = \dfrac{\left(\sum_{i=1}^{n} \hat{y}_i y - \sum_{i=1}^{n} \hat{y}_i \sum_{i=1}^{n} y_i / n\right)^2}{\left(\sum_{i=1}^{n} \hat{y}_i^2 - \left(\sum_{i=1}^{n} \hat{y}_i\right)^2 / n\right)\left(\sum_{i=1}^{n} y_i^2 - \left(\sum_{i=1}^{n} y_i\right)^2 / n\right)}$
Standard error of prediction (SEP)	Same as reference values	$SEP = \sqrt{\dfrac{\sum_{i=1}^{n} (\hat{y}_i - y_i - bias)^2}{n-1}}$
Root mean square of the error of prediction (RMSEP)	Same as reference values	$RMSEP = \sqrt{\dfrac{\sum_{i=1}^{n} (\hat{y}_i - y_i)^2}{n}}$
Bias (d)	Same as reference values	$d = \dfrac{\sum_{i=1}^{n} (\hat{y}_i - y_i)}{n}$
Ratio of performance of deviation (RPD)	Unitless	$RPD = \dfrac{Sd_y}{SEP}$

$\hat{y}_i = $ ith validation sample predicted value

$y_i = $ ith validation sample reference value

n = number of samples in validation set

$Sd_y = $ standard deviation of reference values from the validation set

much smaller than ANN. Other advantages of SVM over ANN are fewer samples are required and a resistance to local minima since SVM uses a Lagrangian function that has a single general minimum (86). The use of kernels is not reduced to SVM regression; it has been also proposed for ANN transfer functions and even applied in PLS or PCR. Bennet and Embrechts (87) report similar results to SVM with the advantage of simpler optimization of the regression parameters and higher model stability.

Model Validation

An adequate validation of the calibration models is a crucial step to determine the suitability of the model to predict new samples, which is the whole purpose of developing NIR calibrations. Ideally, the best validation should be done with distributed samples which were not previously used for calibrating. Since independent validation may not always be possible, cross-validation can provide a basic assessment regarding calibration performance. The general idea of the method is to keep a single sample (full cross-validation) or a group of samples (k-fold cross-validation) apart and develop a calibration with the remaining samples. The developed calibration is validated with the excluded samples and the prediction values are recorded. This procedure is consecutively done until all the samples have been predicted once. The final calibration model is not tested, but rather several submodels developed with calibration data subsets. Any statistic reported from cross-validation can not be directly compared or interpreted the same way as statistics from a real validation of the final model with new samples. The standard errors from cross-validation are often optimistic and, especially in k-fold validation, highly affected by data artifacts

(88). However, reporting cross-validation statistics are preferred over reporting calibration results alone.

Table 1 shows the most used NIR validation statistics among those suggested and detailed in (51). However, it is not unusual to find literature using other statistics, reporting not so relevant figures of merit, or simply not reporting enough information for a good statistical assessment of the model quality. The coefficient of determination (R^2), which provides an estimation of how much variance between reference and predicted values is explained versus the total variance, seems to be one of the erroneously preferred guides for validation assessment. Its high dependency on the reference value range is often ignored (89). The standard error of prediction (SEP, or SECV when reporting cross-validation results) provides information regarding calibration precision. SEP is corrected for the bias value (or systematic error); thus, when reporting SEP bias must be reported as well. The square root of mean standard error of prediction (RMSEP) is related to SEP and bias according to (Eq. 3). Because RMSEP accounts for bias and provides information regarding calibration accuracy, it can be reported alone, especially when bias is small (then RMSEP $\sim$ SEP) (90).

$$RMSEP^2 = SEP^2 + Bias^2 \qquad [3]$$

The final statistic to be discussed is the ratio of performance of deviation or relative predictive determinant (RPD), which is dimensionless and specific to NIR spectroscopy. It is related with the ability of the model to predict future data in relation to the initial variability of the calibration data. Basically, if a calibration leads to a low SEP but the calibration was carried out with a small range of reference values (standard deviation of reference values almost the same as SEP), the model would only

be predicting the data average. Williams (51) provides ranges of RPD values related to the calibration suitability: values above 8 indicate that the calibration can be used for any purpose, while values below 2.3 indicate a poor calibration performance, with use for predicting new samples not advisable.

FINAL REMARKS

Near infrared technologies offer fast solutions for organic compound discrimination and quantification. With the instrumental market in constant growth and development, cheaper and yet more accurate instruments will probably offer opportunities to explore new applications and fields of work. But choosing a suitable instrument for an application involving the use of NIRS is not even half of the requirements for its success. Sample selection, chemometric methods, and validation are key factors that should not be overlooked. Far from discouraging new NIRS users, this review intended to point out those critical steps and warn for extra care. Although the main focus was on NIRS quantitative analysis, the mentioned steps and methods in this review can be easily adapted for discriminative analysis, and the critical stages remain the same. Although it may have not been especially emphasized in the review, we wish to offer advice about keeping any calibration in constant update, especially when working with samples that may suffer any kind of periodic or seasonal change, and be aware of any change in the reference method as it will negatively impact the calibration. Keeping the sample calibration pool updated by adding new samples when required and using standards for periodic checks is a must to keep a good predictive ability in NIRS calibrations over time. Standardization tasks may be an other periodically unavoidable task since instrument drift and/or environmental changes are not completely under control and impact instrument performance. Readers can refer to several literature sources to learn about keeping the calibration and instrument performance up to date (91, 92).

LIST OF ABBREVIATIONS USED IN THE PAPER

AACC	American Association of Cereal Chemists
ANN	Artificial neural networks
AOTF	Acousto-optic tunable filters
CCD	Charged couple device
FIR	Far infrared
FT	Fourier transform
FWHM	Full width at half maximum
LCTF	Liquid crystal tunable filters
LED	Light emitting diodes
LV	Latent variables
LWR	Locally weighted regression
MEMS	Micro-electro-mechanical systems
MIR	Middle infrared
MLR	Multiple linear regression
MSC	Multiplicative scatter correction
NIR	Near infrared
NIR-CI	Near infrared chemical imaging
NIRS	Near infrared spectroscopy
OSC	Orthogonal signal correction
PCA	Principal component analysis
PCR	Principal component regression
PDA	Photo diode array
PLS	Partial least squares
PRESS	Predicted residual error sum of squares
RBF	Radial basis function
RMSEP	Root mean square of the standard error of prediction
RPD	Relative predictive determinant
SECV	Standard error of cross validation
SEP	Standard error of prediction
SLED	Superluminiscent light emitting diodes
SNR	Signal to noise ratio
SNV	Standard normal variate
SVM	Support vector machines

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