Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 207 (2019) 242–250

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Pre-harvest screening on-vine of spinach quality and safety using NIRS technology

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ARTICLE INFO

Article history:
Received 18 July 2018
Received in revised form 17 September 2018
Accepted 18 September 2018
Available online 19 September 2018

Keywords:
Spinach
Safety
Quality
Portable NIRS
In-situ analysis

ABSTRACT

The study sought to perform a non-destructive and in-situ quality evaluation of spinach plants using near infrared (NIR) spectroscopy in order to establish its suitability for different uses once harvested. Modified partial least square (MPLS) regression models using NIR spectra of intact spinach leaves were developed for nitrate, ascorbic acid and soluble solid contents. The residual predictive deviation (RPD) values were 1.29, 1.21 and 2.54 for nitrate, ascorbic acid and soluble solid contents, respectively. Later, this predictive capacity increased for nitrate content (RPDcv = 1.63) when new models were developed, taking into account the influence on the robustness of the model exercised by the simultaneity between the NIR and laboratory analyses. Subsequently, using partial least squares discriminant analysis (PLS-DA), the ability of NIRS technology to classify spinach as a function of nitrate content was tested. PLS-DA yielded percentages of correctly classified samples ranging from 73.08–76.92% for the class 'spinach able to be used fresh' to 85.71–73.08% for the class 'preserved, deep-frozen or frozen spinach, both for unbalanced and balanced models respectively, based on N—H signal associated with proteins. Overall, the data supports the capability of NIR spectroscopy to establish the final destination of the production of spinach analysed on the plant, as a screening tool for important safety and quality parameters.

1. Introduction

Spinach (*Spinacea oleracea* L.) is a green, leafy vegetable, with a shiny, uniform appearance. When sold, either to industry (for preserving, freezing or deep-freezing) or as a fresh product on the market, it is essential for the leaves to look freshly-picked and tender.

Spinach has an extremely high water content (around 92%) and is very low in carbohydrates and fats. It is also a rich source of vegetable protein, with, like other vegetables, high fibre content [1].

Spinach is also noted for its relatively high content of bioactive substances, including vitamin C (ascorbic acid), which is a powerful antioxidant that participates in the scavenging of the reactive oxygen species, regenerating tocopherols from their radical forms [2,3]. However, one drawback is that they accumulate substances which are harmful for the human organism, such as nitrates [4].

Over recent years, consumers have become increasingly aware of the presence of nitrates in foods, among which are vegetables, since nitrates are a serious threat to human health, due to the conversion of nitrate to nitrite, which may produce methemoglobin due to the oxidation of Fe + 2 in haemoglobin [5]. The impaired capacity of methemoglobin to deliver oxygen to tissues may lead to severe toxic effects and may even prove fatal where methemoglobin accounts for over 70% of total haemoglobin, something which affects infants and very young children almost exclusively [6]. Similarly, a number of studies have highlighted a possible link between nitrate exposure and childhood type 1 insulin-dependent diabetes mellitus [7]. Furthermore, nitrite may react with secondary amines (HNR_2), which occur in many foods, to form nitrosamines. These substances are highly carcinogenic [8].

However, consumers are also well aware that eating vegetables with a high content of antioxidants, such as ascorbic acid, is beneficial for their health. The World Health Organization [9] showed that iron deficiency anemia is one of the most common nutritional disorders which has a major effect on both health and the economy. Main cause of anemia is not only low iron intake but also poor iron absorption [10]. This global health problem can be addressed by improving the dietary iron bioavailability, which can be altered by various components present in the food which can either enhance or inhibit iron absorption. Thus, when iron is present along with ascorbic acid, the absorption of iron has been shown to increase even in the presence of inhibitors [11].

In the case of spinach and in response to this growing public concern about nitrates, the European Union passed Commission Regulation (EC) No 1258/2011 of 2 December 2011 setting maximum levels for nitrates in this leafy vegetable [12]. Thus, the maximum level for nitrates was set

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https://doi.org/10.1016/j.saa.2018.09.035
1386-1425/

for preserved, deep-frozen or frozen spinach at 2000 mg NO_3/kg and for fresh spinach at 3500 mg NO_3/kg.

All this has prompted greater attention to spinach safety and quality concerns. Nitrate accumulation, ascorbic and soluble solid contents in spinach depend not only on genotypic characteristics, but also on a number of other factors, including cultural practices, harvesting date and postharvest handling practices [13,14]. As a result, producers are increasingly anxious to provide consumers with assurances regarding the safety, quality and provenance of this product.

The nitrate content in spinach when harvested is in fact key to determining the final destination of the harvested product. The ascorbic acid content is equally important because of the close relationship between this acid and the bioavailability of iron, and also the soluble solid content, which in turn is linked to the vegetable's quality and shelf-life.

NIR spectroscopy in conjunction with the application of multivariate analysis strategies is an appropriate non-destructive technology for the study of chemical constituents of vegetables at field level. This technology represents a marked change from the conventional analytical methods, because a single spectrum allows the simultaneous characterization of different chemical properties, in a matter of seconds and without sample preparation, thus allowing real-time decision making. In the field, it has become easier to use this technology by the development in recent years of compact, portable hand-held instruments which make it possible to measure the quality and safety parameters of vegetables directly on the plant, thus allowing the product to be instantly analysed.

Several authors have shown the feasibility of using NIRS technology for the non-destructive measurement of nitrate content in various fruits and vegetables, including Japanese radishes [15], the leaf stalk of Qing gin cai [16], pineapple [17] and summer squash [18]. In spinach, Xue and Yang [19] and Itoh et al. [20], are the only ones to have carried out this analysis, although these authors used NIRS instruments with optical performance and wavelength ranges different to those used here.

As regards ascorbic acid content, no references have been found where this parameter has been measured in spinach using NIRS. However, some authors [21–25] have shown how NIRS technology can be used to measure ascorbic acid in apples, zucchini, oranges, potatoes and peppers.

In the case of soluble solid content (SSC), no references have been found for measuring this parameter with NIRS in spinach, although several review works indicate that NIRS technology is a viable means of measuring this parameter in fruit and vegetables [26–28].

Taking into account the possibilities of using handheld NIRS sensors to optimize harvesting times and enable the staggered harvesting of spinach for greater quality and safety, thus allowing certain harvested spinach to be used either in the production of baby foods, industrially processed as preserved, deep-frozen or frozen spinach or for fresh consumption, this study sought to assess the feasibility of using NIR spectroscopy to characterize the variations in internal safety and quality—particularly nitrate, ascorbic and soluble solid contents—in intact spinach during on-vine ripening using a low-cost, miniaturized, handheld, near-infrared device based on the MEMS system.

2. Material and Methods

2.1. Sampling

A total of 128 samples of spinach plants (*Spinacia oleracea* L, cv. 'Solomon' (62 samples), 'Novico' (13 samples), 'Meerkat' (10 samples), and 'Gorilla' (43 samples), grown in an open-air plantation in the province of Córdoba (Spain), were harvested between January and March 2017.

2.2. Reference Data

Nitrate content (mg NO_3/kg) was measured using an RQFlex reflectometer (Merck, Darmstadt, Germany) [29]. The reflectometer which measures the colour intensity of Reflectoquant ® test strips (Merck, Darmstadt, Germany) is based on a colorimetric method. For NO_3^- analysis, the spinach leaves were cut into very small pieces and liquified. Next, 5 mL of spinach juice was mixed in a blender with different quantities of deionised water, depending on NO_3^- concentrations. After that, the solution was filtered using a coffee filter and left to settle for 5 min. Subsequently, a test strip was dipped in the supernatant for 2 s, and then the colour was allowed to develop for 1 min. The test strip was then inserted into the reflectometer and the amount of light reflected from the test strip was measured and converted to a concentration by a standard calibration previously introduced into the equipment using a bar-coded plastic strip. The dilution factor was also taken into consideration.

Ascorbic acid content (mg/100 g) was also measured using a reflection photometer (RQflex 10, Merck, Merck, Darmstadt, Germany) [30]. For the ascorbic acid analysis, the analysis procedures were the same as those for nitrate content with the exception that samples containing >450 mg/L of ascorbic acid were diluted with oxalic acid solution 1%. The dilution factor was also taken into consideration.

SSC (°Brix) was measured as the refractometer reading for the spinach juice, using a temperature-compensated digital Abbé-type refractometer (model B, Zeiss, Oberkochen, Würt, Germany).

2.3. Spectral Data Acquisition

The NIR spectra of the spinach leaves were collected in reflectance mode (log 1/R) using a handheld MEMS instrument (Phazir 2400, Polychromix, Inc., Wilmington, MA, USA). The Phazir 2400 is a compact, low-cost near-infrared analyser that incorporates all the essential components to deliver on-vine applications. The spectrophotometer scans at a non-constant interval of approximately 8 nm, across the NIR wavelength range of 1600 to 2400 nm, with a window area of only around 4 mm^2. The sensor integration time was 600 ms. The MEMS device is equipped with quartz protection to prevent the accumulation of dirt. Instrument performance was checked every 10 min, following the diagnostic protocols provided by the manufacturer, and white reference measurement was carried out using Spectralon as reference.

Four spectral measurements were made on each spinach leaf (distal and proximal, on both sides (right and left) of the leaf blade relative to the main vein, on the adaxial side). Because between 4 and 10 leaves were used for the chemical analyses of the parameters of SSC, ascorbic acid and nitrate content when analysing each plant, to obtain a mean spectrum for each spinach sample. A mean spectrum was first obtained from the four spectra for each leaf, and then a mean spectrum was obtained from the four to ten mean spectra for each sample.

2.4. Spectral Repeatability

The spectral repeatability of intact spinach leaves was evaluated using the Root Mean Squared (RMS) statistic. The RMS statistic is defined as the averaged root mean square of differences between the different subsamples scanned at n wavelengths [31,32]. This statistic indicates the similarity between different spectra of a single sample.

For each instrument and sample presentation form, the RMS for an individual subsample (j) of the sample (k) can be calculated using the following expression:

$$RMS_{j,k} = \sqrt{\frac{\sum_{i=1}^{n} D_{ij}^2}{n}}; D_{ij} = y_{ij} - \overline{y}_i$$

where y_{ij} is log (1/R) at wavelength i for subsample j, and $\overline{y_i}$ is log (1/R) at wavelength i for the average spectrum of a sample k; n is the number of data points collected by the instrument (here, 100 data points for the

MEMS instrument). The RMS value obtained in each case was multiplied by 10^6 to facilitate value management and processing.

To determine a cut-off value (RMS_{cutoff}) of each sample presentation form, the mean RMS was calculated along with standard deviation (STD) per sample according to the formulae provided by Martínez et al. [33].

$$STD_k = \sqrt{\sum_{j=1}^{N} (RMS_j)^2/(N-1)}$$

where N are the number of sub-samples.

A STD limit can be calculated for comparing the RMS values of subsamples, following the formula provided by Rosales [34], who demonstrated that the estimated value of the error variance, σ^2 for log (1/R) $y_{i,j}$ is the corresponding to one-way ANOVA:

$$\sigma^2 = \frac{1}{n\,(N-1)} \sum_{i=1}^{n} \sum_{j=1}^{N} \left(y_{i,j} - \overline{y}_i\right)^2$$

This expression corresponds to the STD^2. The sum of squares for error (SSE) can be thus be expressed as:

$$SSE = n\,(N-1)\,STD^2$$

which approximately follows a χ^2 distribution,

$$\frac{n\,(N-1)\left(STD^2\right)}{\sigma_0^2} \sim \chi^2_{[n(N-1)]}$$

with σ_0^2 the parametric value of the error variance. For infinite degrees of freedom (>100), χ^2 tends to a normal distribution. An STD limit can then be calculated for comparing the RMS values of subsamples, following the formula given by Rosales (1993).

$$STD_{limit} = 1.036\sqrt{\sum_{k=1}^{k=m} STD_k^2/m} = 1.036\sqrt{\overline{STD^2}}$$

where STD is the standard deviation per sample and m is the number of samples. The value 1.036 corresponds to a probability level of 85%.

The STD_{limit} value was used to establish the RMS_{cutoff} for each product and analysis mode. Hence, the different sources of variation which might cause irregular spectra were controlled, since any spectra in a sample that were above this limit were eliminated, and recalculations were performed until all the values were below the RMS_{cutoff}. Then, the mean spectrum of each leaf was calculated.

To evaluate spectral repeatability, two alternatives were available: first, analysing ten leaves and taking two spectra in each of them, at the same point, and second, analysing twenty leaves and taking four spectra in each, at different points of the leaf.

2.5. Data Analysis: Definition of Calibration and Validation Sets

Principal Component Analysis (PCA) was performed on a set of N = 128 samples in order to decompose and compress the data matrix. After PCA, the center of the spectral population was determined in order to detect outlier samples. The Mahalanobis distance (GH) was calculated between each sample and the center; samples with a GH value >3 were considered outliers [31]. As spectral pre-treatments, the Standard Normal Variate (SNV) plus Detrending (DT) procedure [35] was used to remove the multiplicative interferences of scatter, and first derivative mathematical treatment (Norris derivative) was performed (1,5,5,1), where the first digit is the order of the derivative, the second is the gap over which the derivative is calculated, the third is the number of data points in a running average or smoothing and the fourth is the second smoothing [36,37].

Once spectral outliers had been removed, a set consisting of 124 samples was used to develop the calibration models. The set was divided into two: a training set containing about 75% of the samples (N = 93 samples) and a test set containing the remaining 25% (N = 31 samples). These samples were selected following the method outlined by Shenk and Westerhaus [38] using the CENTER algorithm included in the WinISI software package to calculate the Global Mahalanobis distance (GH). The samples were ordered on the basis of the Mahalanobis distance to the center of the population, and three of every four were selected to be part of the calibration set.

2.6. Chemometric Tools

The data were subjected to chemometric treatment using the WinISI software package version 1.50 [37].

NIR calibration models for the prediction of quality parameters (nitrate content, ascorbic content, and SSC) in intact spinach plants were constructed using modified partial least squares (MPLS) regression [31], with subsequent cross-validation. The calibration set was partitioned into 6 groups; each group was then validated using a calibration derived from the other samples; finally, validation errors were combined to obtain a standard error of cross-validation (SECV).

For each analytical parameter, different mathematical treatments were evaluated. For scatter correction, the standard normal variate (SNV) and detrending (DT) methods were tested [35]. Additionally, a total of four derivative mathematical treatments were tested: 1,5,5,1; 2,5,5,1; 1,10,5,1 and 2,10,5,1.

The statistics used to select the best equations were: the coefficient of determination for calibration (r^2_c), the standard error of calibration (SEC), the coefficient of determination for cross calibration (r^2_{cv}), and the standard error of cross validation (SECV). Furthermore, the Residual Predictive Deviation (RPD) for cross-validation was calculated as the ratio of the standard deviation (SD) of the reference data to the SECV. This statistic enables SECV to be standardized, facilitating the comparison of results obtained with sets of different means [39].

The best-fitting equations obtained for the calibration set, as selected by statistical criteria, were subsequently subjected to external validation following the protocol outlined by Windham et al. [40].

After analysing the results obtained, and in order to test the influence of the simultaneity in time between the NIRS spectrum and the wet-chemistry analysis on the robustness of the model obtained for the prediction of nitrate content in intact spinach, new predictive models were designed for this parameter, dividing the initial total of 128 samples into 2 groups which represent two different analysis strategies.

- Strategy I. Group of samples 1 to 47. On the day the field samples arrived, the corresponding NIR spectra were taken. However, the reference analyses were carried out 24 h later, and the samples were stored in refrigeration conditions during that time (4 °C, RH: 85%).
- Strategy II. Group of samples 48 to 128. The NIRS spectra were taken and the wet analysis performed 24 h after the product arrived in the laboratory, and the samples were stored in refrigeration conditions until both type of analysis (4 °C, RH: 85%).

The same signal pre-treatments and spectral region described earlier were used here for the development of the new quantitative models.

2.7. NIRS Classification Models

The design of models to classify spinach by its nitrate content, in order to evaluate the viability of using NIRS technology to determine the final destination of the harvested spinach (fresh consumption or

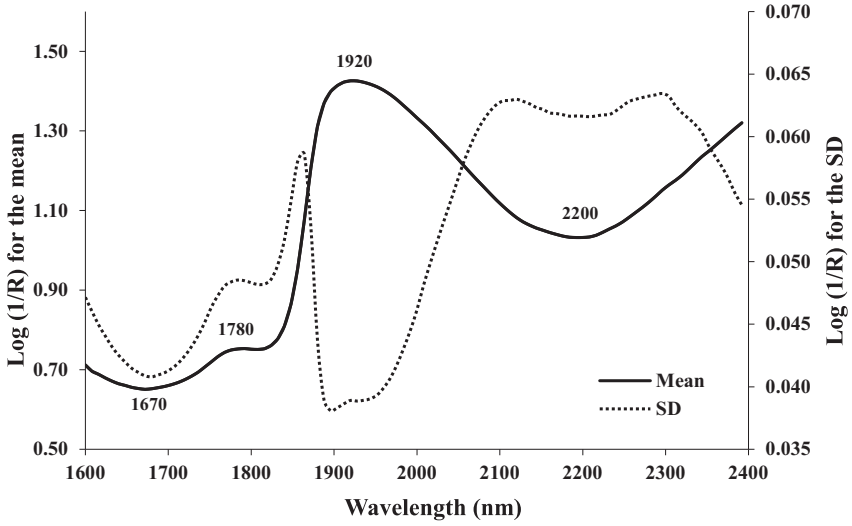

Fig. 1. Mean and standard deviation spectrum for spinach.

preserved, deep-frozen or frozen product), for those samples where the NIR and reference analyses were performed at the same time (Strategy II), comprised two classification groups: 1) spinach which contained 0–2000 mg/kg NO_3^- (N = 45 samples) for preserved, deep-frozen or frozen spinach; 2) spinach with a nitrate content between 2000 and 3500 mg/kg NO_3^- (N = 36 samples) for fresh spinach.

Next, the structure and spectral variability of the sample population was studied in order to select the samples that would constitute the learning group. To do this, we used the CENTER algorithm, which is included in the WinISI version 1.50 software. This algorithm was applied separately to each of the two training groups. The mathematical treatments SNV (Standard Normal Variate) and DT (Detrend) were applied to correct any scattered radiation phenomena, together with the 1,5,5,1 derivation treatment [31].

After having ordered the sample set by spectral distances (from smallest to greatest distance from the center), a structured selection of the external validation set (10 samples for each classification group), solely on the basis of spectral data was performed [38].

Discriminant models were constructed to classify spinach by its NO_3^- content, using PLS-DA for supervised classification. Specifically, the PLS2 algorithm was applied, using the 'Discriminant Equations' option in the WINISI version 1.50 software package [37].

All the models were constructed using six cross-validation groups. The same signal pre-treatments and spectral regions described earlier for quantitative analysis were used for qualitative model development.

The precision of the models obtained was evaluated using the percentage of correctly-classified samples, both for the global model and for each class.

The difficulty involved in obtaining balanced learning groups in terms of the number of samples per class or classification category led us to assess the influence of this factor on the development of discriminant models. In this way, the results obtained were contrasted with balanced and unbalanced classification models in terms of the number of samples per class.

The samples for the balanced groups were selected using the algorithm SELECT included in the WinISI II software package version 1.50 (Infrasoft International, Port Matilda, PA), which calculates spectral distances (Mahalanobis H), in order to detect samples whose spectrum is very similar to that of others in the population [38]. This algorithm enables spectral selection of a number of samples representative of the population as a whole, by calculating the 'NH' distance (Mahalanobis neighbour distance) between two spectra. An 'NH' of <0.6 implies that two spectra are too similar to each other ('neighbour'). After application of this algorithm, 26 samples of the category 'nitrate content between 0-2,000 mg/kg' were selected, thus making the number of samples of the two classes equal and allowing the classification models to be redesigned.

Next, the best classification models for each of the established types (unbalanced and balanced models) were selected and externally validated. In this case, an external validation procedure was carried out to measure the predictive capacity of the model using a sample group different to that used in the training of the model. In both models (unbalanced and balanced) 20 samples (10 per category) were selected in a structured way [38].

3. Results and Discussion

3.1. Spectral Repeatability

The collection of high quality spectra is crucial for the characterization of spinach plants by quality and safety characteristics and to assess its possible industrial use, as well as to construct discriminant classification models for the product depending on its possible use in the processing industry of fresh or processed vegetables (in this case, preserved, deep-frozen or frozen spinach). The RMS cut-off was calculated for the instrument MEMS used as shown in Section 2.4.

For the first alternative tested (analysing ten leaves and taking two spectra in each of them, at the same point) the mean STD was 48,292 μlog (1/R), representing an RMS cut-off of 64,661 μlog (1/R).

Table 1
Statistical analysis of the calibration and prediction sample sets, i.e. data ranges, means and standard deviations (SD) and coefficients of variation (CV).

Parameter	Item	Calibration set	Validation set
Nitrate content (mg/kg)	Number	93	31
	Range	109.50–5177.00	144.00–3520.00
	Mean	1765.24	1795.48
	SD	1082.34	1088.46
	CV (%)	61.31	60.62
Ascorbic acid content (mg/100 g)	Number	93	31
	Range	156.92–479.23	191.83–453.85
	Mean	298.97	300.96
	SD	66.05	63.52
	CV (%)	22.09	21.10
SSC (°Brix)	Number	93	31
	Range	5.60–14.25	6.25–13.95
	Mean	8.84	8.34
	SD	1.90	1.89
	CV (%)	21.49	22.66

Table 2
Calibration statistics for the best equations obtained for the prediction of quality and safety parameters in intact spinach.

Parameter	Number of samples	Mathematical treatment	Range	Mean	SD	r^2_{cv}	SECV	RPD	SEL
Nitrate (mg/kg)	92	2,5,5,1	109.50–5177.00	1776.16	1083.10	0.41	836.26	1.29	131.61
Ascorbic acid (mg/100 g)	91	2,10,5,1	168.75–467.69	298.55	62.24	0.33	51.46	1.21	10.69
SSC (°Brix)	92	1,10,5,1	5.60–14.25	8.85	1.91	0.85	0.75	2.54	0.07

For the second alternative (analysing twenty leaves and taking four spectra in each, at different points of the leaf) the mean STD and the RMS cut-off were 118,693 μlog (1/R) and 128,437 μlog (1/R), respectively. As can be seen, the result obtained for $STD_{límit}$ in the test in which 10 samples (leaves) were used was lower than that obtained in the test which used 20 samples. This was to be expected, since the former corresponds to the analysis of 10 samples whose two subsamples were taken at the same point, while, in the latter case, 20 samples were analysed by taking the four subsamples at different points in the leaf. The repeatability results obtained therefore indicate that, in the second mode, the analysis reflects the heterogeneity of the leaf, although the reduced window of analysis presented by the spectrophotometer used must also be taken into account.

It was therefore decided to choose the second mode of analysis as the most suitable, that is, to collect 4 spectra per leaf. In this way, a more representative measurement of the analysed product was obtained. Once the RMS value did not exceed the value of the $STD_{límit}$, these spectra were then averaged in order to carry out both the quantitative and qualitative predictive models using the average spectrum of each sample.

The calculation of the RMS statistic is extremely important because it aims to ensure high spectral repeatability, which is essential to obtain quality spectral data, and therefore, constitutes an essential step in obtaining robust equations.

No values for this statistic have been found in the scientific literature for spinach analysed on the vine, although the RMS statistic is extremely

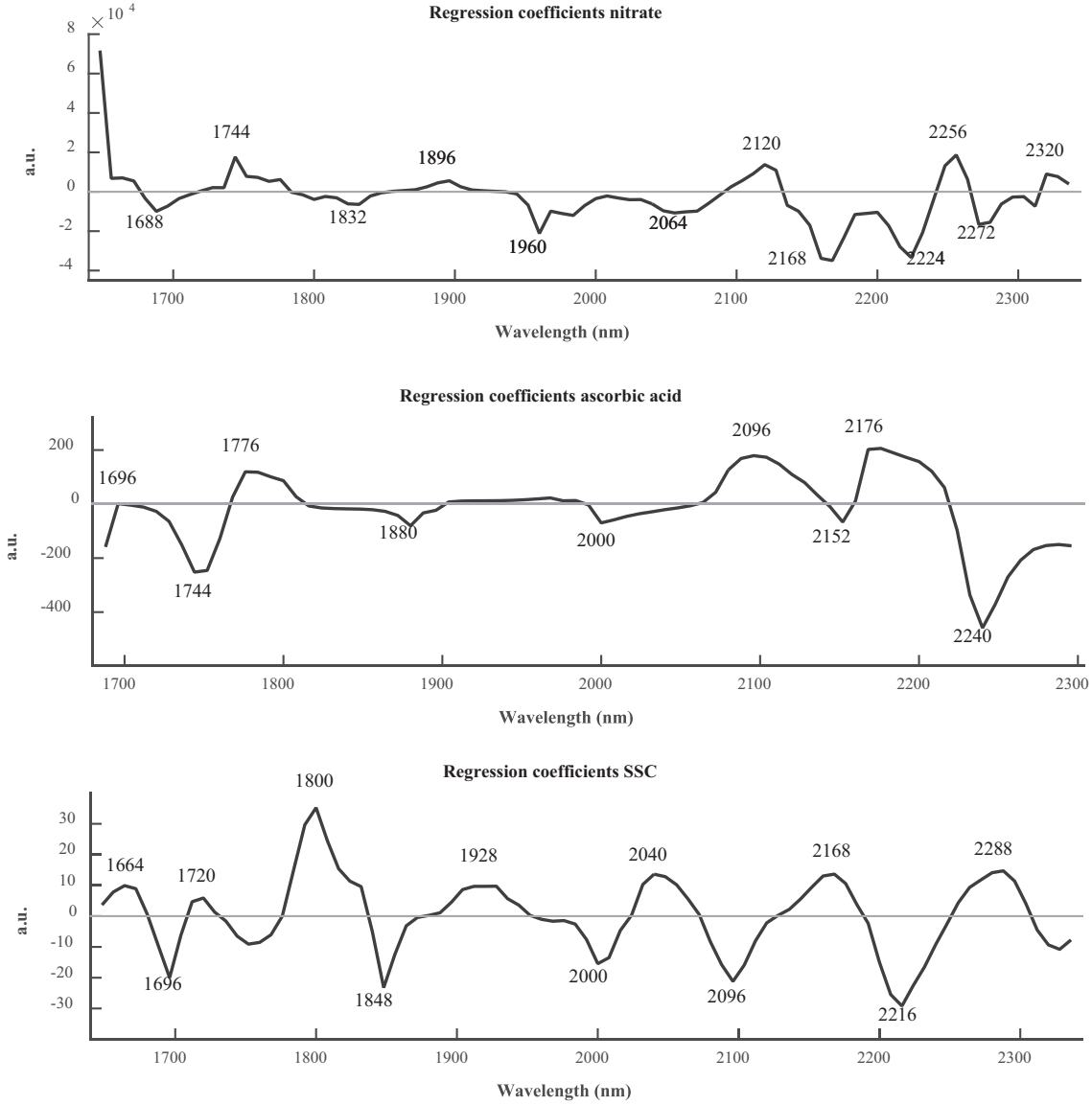

Fig. 2. Regression coefficients for spinach nitrate content, ascorbic acid and SSC during on-vine ripening. * a.u. = arbitrary units.

useful to obtain representative spectral libraries of this vegetable, when analysed on the plant. In fact, this is the first research work to deal with measuring spectral repeatability in leafy vegetables.

3.2. Spectral Features

Mean and standard deviation log (1/R) spectra for intact spinach leaves, captured by the instrument Phazir 2400, together with the most relevant absorption bands, are shown in Fig. 1.

In the NIR region between 1600 and 2400 nm, the highest absorption peak is at the 1920 nm wavelength, which corresponds to water. This was to be expected, since spinach is made up of 90% water [41]. Osborne et al. [42] showed that the peak in the wavelength 1780 nm was directly related to the first overtone of sugars.

3.3. Descriptive Data for NIR Calibration and Validation

Values for number of samples, range, mean, standard deviation (SD) and coefficient of variation (CV) for each of the parameters analysed using the calibration and validation sets after application of the CENTER algorithm and removal of any spectral outliers are shown in Table 1.

The nitrate content, ascorbic acid, and SSC reference values were all fairly normally distributed around the mean values (1765.24 mg/kg, 298.97 mg/100 g and 8.84°Brix, respectively) with standard deviations (SD) of 1082.34 mg/kg, 66.05 mg/100 g and 1.90°Brix for each parameter.

The ranges of nitrate content, ascorbic acid and SSC of the calibration set are 109.50–5177.00 mg/kg, 156.92–479.23 mg/100 g and 5.60–14.25°Brix, respectively; that of the validation set are 144.00–3520.00 mg/kg, 191.83–453.85 mg/100 g and 6.25–13.95°Brix. Since the calibration and validation sets displayed similar values for mean, range and standard deviation for all the parameters studied, a structured selection using only spectral information treatment algorithms such as CENTER proved adequate. Furthermore, the ranges of the validation set lay within those of the calibration set.

All the parameters studied in the calibration set covered a wide range of values. This was truest of nitrate content with a CV of 63.31%, while the CV of ascorbic acid and SSC were practically the same (22.09% and 21.49%, respectively). Pérez-Marín et al. [43] have highlighted the importance of the sample set and of sample distribution within the calibration set, noting that sample sets for calibration should ideally ensure a uniform distribution across the range of the study parameter in question.

For the validation group, the coefficient of variation values were set at 60.62% for nitrates, 21.10% for ascorbic acid and 22.66% for SSC.

3.4. Prediction of Safety and Quality Parameters in Intact Spinach Using MPLS Regression

The best calibration models obtained for predicting one safety parameter (nitrate content) and quality parameters (ascorbic acid and SSC) in spinach using different various mathematical pre-treatments are shown in Table 2. Statistical criteria were used to select the best model for each study parameter.

The models developed for nitrate content have a predictive capacity ($r^2_{cv} = 0.41$; $RDP_{cv} = 1.29$) which allows the samples to be classified under high and low values of this parameter [32,39].

It should be noted that Xue and Yang [19] studied nitrate content in spinach (n = 58 samples), using an ASD Fieldspec FR spectroradiometer and obtained results ($r^2_p = 0.88$) which were higher than those obtained here, although the instrument's optical characteristics and range are significantly different from those of the Phazir 2400.

Itoh et al. [20] also measured the nitrate content in spinach leaves (n = 48 samples), using the FANTEC NIR Gun working on transmittance in a spectral range of 600–1100 nm. The authors reported values of $RPD_p = 2.14$ with the PCR regression and $RPD_p = 2.17$, using PLS regression, which were higher than those obtained in this research study. However, the size and characteristics of the sample group, the means of measurement, the window size (1 cm) and the spectral range of the instrument used, all differed from those used in this study.

As regards ascorbic content, the models designed allow the samples to be classified under high and low values for this parameter ($r^2_{cv} = 0.33$; $RDP_{cv} = 1.21$) [32,39], although the results are limited for routine use. It must be noted that it is especially difficult to measure this parameter in vegetable products - not only with portable instruments, but also with high performance NIRS laboratory instruments.

Kramchote et al. [44] measured ascorbic acid content in cabbage and obtained predictive capacity models ($RPD_P = 1.26$) similar to that obtained here using a spectrophotometer Handy Lamda II (Spectra Co., Ltd., Tokyo, Japan), in reflectance mode in the 310 and 1100 nm spectral range.

When predicting antioxidant content (beta-carotene and ascorbate) in freeze-dried leaves of *Populus* spp., Fernández-Martínez et al. [45]

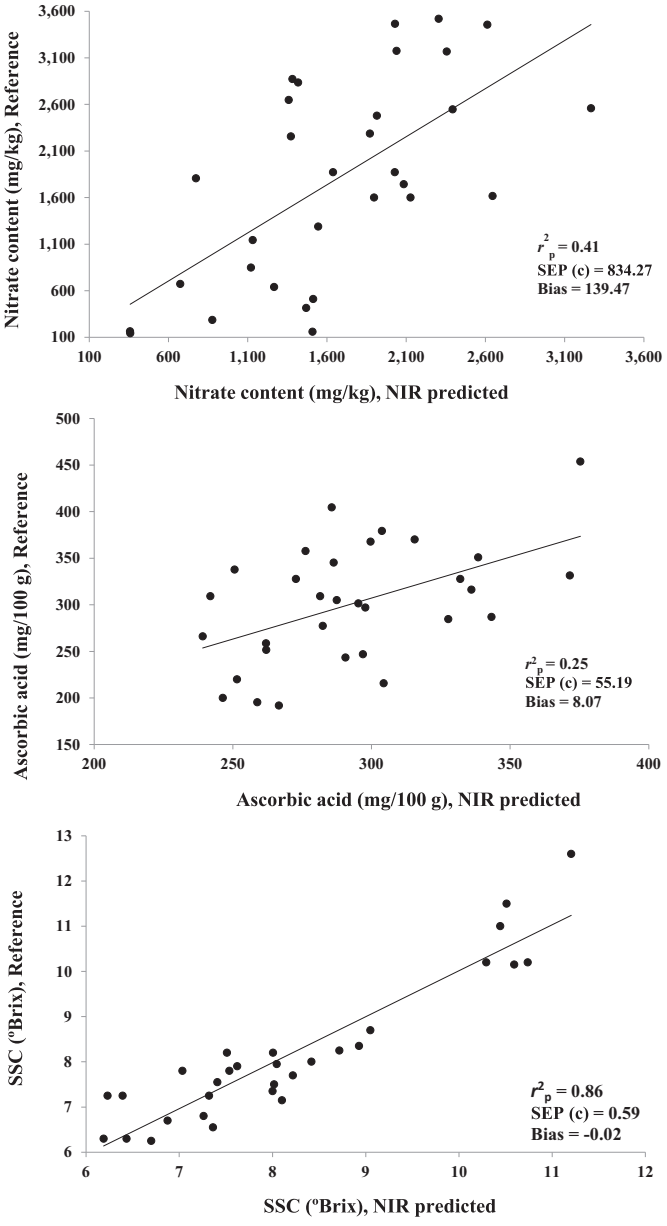

Fig. 3. Reference and NIR predicted values for quality and safety parameters in intact spinach.

Table 3
Calibration statistics for the best equations obtained for the prediction of nitrate content in intact spinach. Strategies I and II.

Strategy	Number of samples	Mathematical treatment	Range	Mean	SD	r^2_{cv}	SECV	RPD_{cv}
Strategy I	44	2,5,5,1	109.50–5177.00	1648.82	1037.97	0.53	711.63	1.46
Strategy II	71	1,5,5,1	122.50–3627.00	1821.91	1095.37	0.63	670.81	1.63

obtained models with an average predictive capacity ($RPD_{cv} = 2.10$) for beta-carotene, while for ascorbate, the models were capable of discriminating between high and low values ($RPD_{cv} = 1.56$) [26]. In both studies, reflectance was carried out using high performance Foss NIRS Systems 6500 laboratory equipment, in the 400–2500 nm range.

For SSC – which is a crucial parameter when choosing the optimum time for harvesting and for measuring the shelf-life of spinach, the model developed has a predictive capacity ($r^2_{cv} = 0.85$; $RPD_{cv} = 2.54$) which can be considered as good [32,39].

No reports have been found in the literature regarding the measurement of SSC in spinach using NIR spectroscopy. However, Kramchote et al. [44], in the case of cabbage, obtained models of lower predictive capacity ($RPD_p = 2.05$ and 1.95) using a spectrophotometer (Handy Lamda II, Spectra Co., Ltd., Japan), in interactance and reflectance mode, respectively, in the spectral range 310–1100 nm.

The regression coefficients for the best predictive models for nitrate, ascorbic acid and soluble solids contents are illustrated in Fig. 2. The figure shows the area of the spectra around 1650–1850 nm, which is correspond to the first overtone of C—H stretching bonds [42]. The best models to predict the three parameters tested reflected variations in the wavelengths range 2000–2180 related to N—H and O—H stretching modes besides C═O vibration bands [42]. Absorbance region such as 2200–2280 nm and at 2320 nm could be attributed to C—H stretch and CH_2 deformation [41].

Validations of the best calibration models obtained were performed using a set comprising 31 samples (Fig. 3). Models constructed for predicting SSC in intact spinach using the MEMS instrument met the validation requirements in terms of the coefficient of determination for prediction r^2_p ($r^2_p > 0.6$), while the standard error of prediction corrected for bias or SEP(c), bias and slope were within the confidence limits: the equation thus guarantees an accurate prediction, and can be applied routinely [40].

For nitrate content prediction, the r^2_p value does not comply with the protocol while the values shown by the other statistics lie within the confidence limits, thus complying with this validation protocol [40].

In the case of the ascorbic acid content, the r^2_p values and the slope do not comply with this protocol, while SEP (c) and the bias were below confidence limits.

3.5. Contrasting the Suitability of Performing Reference Analyses Immediately after NIR Measurements

Given the rough predictive capacity of the model designed to measure nitrate content in spinach, and since the non-destructive prediction of this parameter is key in determining the final destination of the harvested product, it was decided to evaluate whether, for spinach leaves, carrying out chemical analyses at times other than when the NIRS spectra are taken significantly affects the predictive capacity of the models. Several authors have pointed out the importance of performing the reference analyses immediately after the NIR analysis, especially in the case of extremely perishable vegetable products [46].

Table 3 shows the best models obtained for predicting nitrates using two analysis strategies: Strategy I: NIRS analysis, and chemical measurement of nitrate content 24 h later; Strategy II: NIRS analysis and wet reference analysis immediately after.

Tables 2 and 3 clearly show the importance of carrying out the NIR and wet process analyses consecutively, and of using the same methodology or systematic analysis for all the samples in the study.

In this way, the joint predictive model (Table 2) shows values of $r^2_{cv} = 0.41$ and SECV = 836.26 mg/kg while the models developed from the two strategies considered (Table 3) had values of $r^2_{cv} = 0.53$ and 0.63, and SECV = 711.63 and 670.81 mg/kg, for strategies I and II, respectively.

It is important to point out that for both strategies, the SECV value was lower. In Strategy II, the SECV value fell by 19.78%, while for Strategy I, it decreased by 14.90%. Likewise, the predictive capacity of the models increased, and the models allowed to distinguish between high, medium and low values of nitrate content for both strategies [32,39].

The results obtained indicate that growers and the agrifood industry could use NIRS technology as a screening technique, permitting a large number of plants to be tested in the field or when they are delivered to the industrial plant, providing results for this parameter and thus enabling growers to take real-time decisions as to the final destination of the harvested product.

These results also confirm the importance of performing these analyses together, in products as perishable as spinach [47] and the suitability of using the same analysis methodology throughout the trial.

3.6. Discriminant Analysis

Results for the best classification models obtained, using PLS2-DA, for predicting the industrial use of spinach depending on its content in nitrates, are shown in Table 4.

The best discriminant models were obtained with D_2 log(1/R) together with SNV + DT for scatter correction (balanced and unbalanced sets).

The unbalanced model correctly classified 80.33% of the samples (85.71% in the 0–2000 mg/kg category and 73.08% in the 2000–3500 mg/kg category), while the balanced model correctly

Table 4
Percentage of spinach plants classified correctly by nitrogen content. PLS2-DA.

Qualitative groups	Unbalanced model		Balanced model	
	Percentage of correctly-classified samples: 80.33% (49/61)		Percentage of correctly-classified samples 75.00% (39/52)	
	Model SECV: 0.46		Model SECV: 0.49	
	Number of synthetic variables: 4		Number of synthetic variables: 5	
	Math treatment: 2,5,5,1		Math treatment: 2,10,5,1	
Industrial destination of spinach according to its content in nitrates	Training set	Validation set	Training set	Validation set
Preserved, deep-frozen or frozen spinach, NO_3^-: 0–2000 mg/kg	85.71% (30/35)	80.00% (8/10)	73.08% (19/26)	90.00% (9/10)
Fresh spinach NO_3^-: 2000–3500 mg/kg	73.08% (19/26)	60.00% (6/10)	76.92% (20/26)	50.00% (5/10)

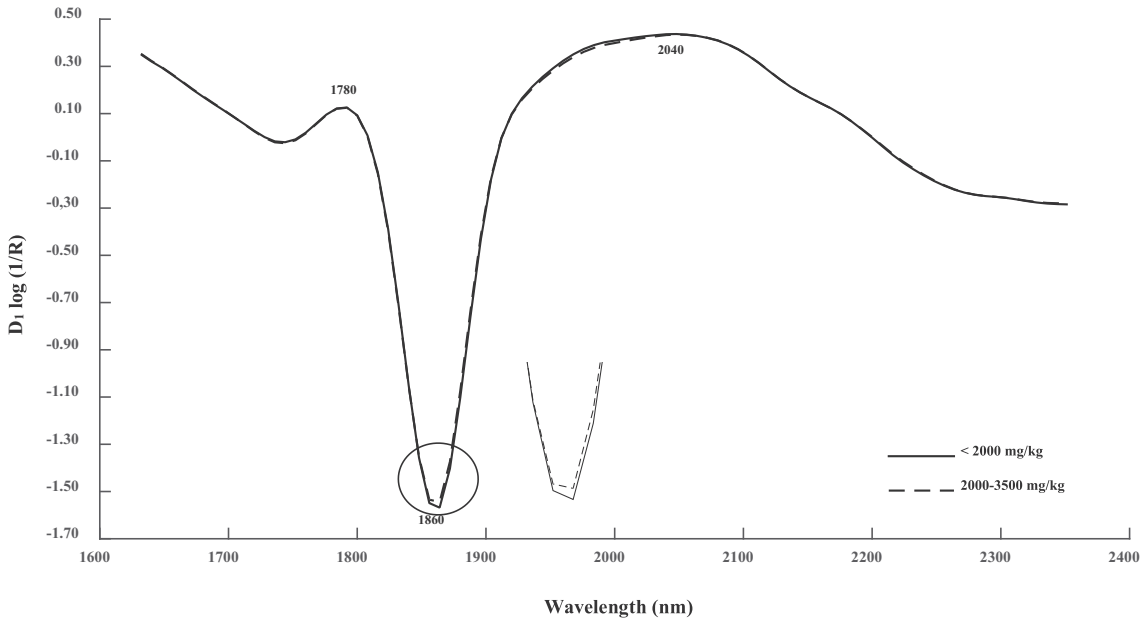

Fig. 4. D_1 log(1/R) of the mean spectra for intact spinach plants with different nitrate content.

classified 75.00% (73.08% in the 0–2000 mg/kg category and 76.92% in the 2000–3500 mg/kg category). For validation, the percentage of correctly classified samples obtained using the unbalanced model was 70% (80% in the 0–2000 mg/kg category and 60% in the 2000–3500 mg/kg category) and for the balanced model, the percentage was 70.00% (90% in the 0–2000 mg/kg category and 50% in the 2000–3500 mg/kg category).

As can be seen in Table 4, the percentage of samples correctly classified by the unbalanced model was slightly higher than by the balanced model, which reflects the low sensitivity of the PLS2 algorithm to the difference between the number of samples of the classes to be discriminated [48].

A detailed study of the unbalanced model reveals that, of the 12 poorly classified samples, 5 had a nitrate content (mg/kg) of between 2000 ± 2 * SEL (SEL = 131.6 mg/kg), a range that can be considered difficult to discriminate as it would fall within the error of the reference method, so that the error of classification may be put down to a typical laboratory error value. The remaining 7 samples did not show nitrate content within this range, and therefore their incorrect classification can be attributed to errors in the model or to their poor representation in the training set, given the limited number of samples available, which only allowed to perform a feasibility study of the potential of technology in this area. It is also important to mention that this constituent is found in ppm in spinach leaves, which makes it difficult to measure by NIRS in whole plants. However, the results obtained are promising and allow us to continue consolidating the application of NIRS technology as a screening technique in the spinach handling and processing industry, permitting, in a non-destructive way and in a matter of seconds, to assess the possible industrial destination of the spinach leaves.

Of the 13 poorly classified samples in the balanced model, 3 had a nitrate content (mg/kg) within the range of 2000 ± 2 * SEL (SEL = 131.6 mg/kg), and any classification errors can again be attributed to the SEL value obtained.

Wavelenghts at 1780 nm, 1860 nm and 2040 nm appeared to have more weight in the classification of intact spinach on-vine by nitrate content (Fig. 4). This indicates that the discrimination of spinach by nitrate content in the NIR region of the spectrum is related to lipids, proteins and N—H combinations [41].

As regards the external validation of the classification models, a percentage of correctly classified samples of 70% was obtained for both the unbalanced and balanced models. For the first, 10 samples from the 0–2000 mg/kg category were used, of which 8 were correctly classified, and 10 from the 2000–3500 mg/kg category, of which 6 were correctly classified, while of the 4 poorly classified samples, 1 had a nitrate content (mg/kg) within the range 2000–2 * SEL. Similarly, in the balanced model, 9 out of 10 samples in the 0–2000 mg/kg category were correctly classified, and 5 from the 2000–3500 mg/kg category.

4. Conclusions

Near infrared spectroscopy is clearly an advantageous technique for the rapid screening of quality and safety according to the SSC and nitrate levels, although further research is needed to make it robust for predicting these parameters. It has also been demonstrated that the NIRS and the laboratory analysis should be performed together.

The results obtained from the classification models of spinach leaves according to their nitrate content, which determines their possible industrial destination, also confirm the feasibility of using NIRS technology both in the field and in the stages of selection and classification of spinach carried out during industrial processing for classification according to the quality and safety characteristics of this vegetable.

Acknowledgements

This research was under the Research Project 'Quality determination of spinach grown in Santaella (Córdoba)', funded by Gelagri Ibérica, S.L. The authors are grateful to Mrs. María Carmen Fernández of the Animal Production Department for her technical assistance. Furthermore, the authors wish to express their gratitude to the Spanish Ministry of Education, Culture and Sports for the support offered to Irina Torres in the form of the Training Programme for Academic Staff (FPU).

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