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Assessment of nitrate levels in greenhouse-grown spinaches by Raman spectroscopy: A tool for sustainable agriculture and food security

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ABSTRACT

Excessive use of nitrogen-based fertilizers in vegetable cultivation has raised significant environmental and health concerns, driving the need for sustainable fertilization practices. Spinach, known for its propensity to accumulate nitrates, demands precise monitoring tools. This study investigates the potential of Raman spectroscopy for quantifying nitrate levels in greenhouse-grown spinach, utilizing multiple linear regression (MLR) and partial least squares regression (PLSR) models. Both models exhibit strong predictive performance, with R^2 exceeding 0.8. Furthermore, integrating Raman spectroscopy with optical sensors like the Dualex can further enhance nitrate prediction accuracy. These findings underscore the feasibility of this non-destructive, scalable approach, offering a promising solution for sustainable agriculture, food security, and environmental protection.

1. Introduction

In recent years, a growing awareness has emerged regarding the significant environmental repercussions stemming from the excessive use of nitrogen-based fertilizers in vegetable crop cultivation. This shift from prioritizing maximum yield to embracing sustainable fertilization strategies has become a central theme in contemporary scientific discourse, driven by concerns over environmental sustainability and resource conservation [1–3]. European regulations, exemplified by the Nitrate Directive implemented since 1991, underscore the critical importance of safeguarding water quality by mitigating nitrate contamination arising from fertilization practices [4,5]. As a response, regulatory measures have been instituted on a national/regional level to limit fertilizer usage and establish legal thresholds for nitrogen application in soil. Nevertheless, persistent challenges, including improper fertilization techniques and temporal misalignments, continue to exacerbate the accumulation of residual nitrogen, leading to issues such as nitrate leaching and increased susceptibility of plants to pests and diseases [6,7].

While recognizing the inherent benefits of nitrates for plant development, it is crucial to acknowledge the potential risks posed by their excessive accumulation not only for crop quality but also for human health [8,9]. Leafy greens, notably spinach, stand out as particularly susceptible to nitrate accumulation, thereby serving as a significant dietary source of nitrates [10]. Despite ongoing concerns regarding the potential health implications of dietary nitrates, accumulating evidence suggests their potential benefits, especially in promoting cardiovascular health and enhancing exercise performances [11]. Nevertheless, effectively managing nitrate levels in crops remains a formidable challenge for farmers, underscoring the necessity for continuous development and adoption of robust assessment methodologies and sustainable agricultural practices to ensure the preservation of both environmental integrity and human well-being [12,13].

Recent advancements in precision agriculture have seen the development and application of non-destructive methods, particularly optical sensors, for monitoring plant nitrogen (N) status. These sensors offer rapid and cost-effective alternatives to traditional destructive analyses, enabling frequent and in situ measurements throughout the growth cycle. Among the prominent optical sensors are the SPAD (Soil-Plant Analysis Development) and Dualex meters, which have gained recognition for their ability to assess leaf chlorophyll content, a key indicator of plant N status [14–17]. The SPAD meter, widely used across various crops including corn, rice, fruit trees, and grapevines, by measuring the transmission of red and infrared light through leaves, provides insights into chlorophyll concentration, which has been shown to correlate with leaf N content [18,19]. This technology has proven valuable in

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predicting the need for additional N fertilizer and optimizing fertilization strategies in agricultural settings [20,21]. Similarly, the Dualex sensor, which combines leaf transmittance and fluorescence measurements, has emerged as a promising tool for monitoring plant N status. By detecting both chlorophyll and epidermal flavonoids, it provides a comprehensive assessment of leaf N content, offering a more robust and sensitive index of plant N status compared to methods focusing solely on chlorophyll measurement. Its linear response to increasing leaf chlorophyll content and efficiency in monitoring crop N status make it a valuable asset in precision agriculture [17,20,22–24]. Recent studies have demonstrated the versatility of the Dualex sensor in various crop species, including grapes and tomatoes, highlighting its capacity to assess N status under diverse environmental conditions, further cementing its role in optimizing nutrient management in field settings [25,26].

Spinaches as well as other green leafy vegetables destined for the ready-to-eat market, were previously investigated by the use of optical methods to assess their leaf nitrogen content [27–30]. However, there were only a few investigations on the possibility of using these methods for the quantification of their nitrate fraction [31–34]. In general, optical sensors may have limitations in determining leaf nitrate levels, as they rely on indirect correlations with chlorophyll or, in the case of Dualex, also flavonoids. Nowadays, destructive assays represent the reference standard procedures for nitrate quantification. However, these procedures require toxic chemicals and imply the production of harmful residuals at risk for individuals as well as are impractical for assessing the nitrate content of a large number of plants.

Among the array of optical analysis techniques, Raman spectroscopy stands out due to its exceptional inherent properties, garnering increasing attention in the agri-food area [35–38]. The molecular fingerprints obtained through Raman spectroscopy provide intricate details concerning specific chemical bonds or functional groups, making this technique a powerful and direct tool for qualitative and quantitative analysis of several types of target specimens. Additionally, this spectroscopy boasts several benefits such as rapid data collection, non-invasiveness and non-destructiveness, minimal sample requirement, straightforward or no sample preparation, and the ability to analyze biological and water-containing specimens and biomolecules [39–41]. Despite these advantages, Raman spectroscopy remains underexplored in the quantitative detection of molecules involved in plant biochemistry and metabolism. While it has proven to be an effective tool for characterizing and analyzing plant and food components [42–44], its application in quantifying plant nutrients, particularly in field-grown or commercially cultivated crops, is still in its infancy [45]. Most studies to date have focused on quantifying pigments such as carotenoids [46–50] or flavonoids [51] in fruits and agricultural products, with relatively few efforts directed towards molecules such as leaf nitrate. Furthermore, existing research on nitrate detection has largely been restricted to laboratory-grown species under tightly controlled conditions [52,53].

In this paper, we aimed to test the application of Raman spectroscopy to the quantification of nitrate contents in spinach leaves of greenhouse-grown plants. Raman profiles of spinach leaves were obtained by using a portable and compact Raman system and alternatively subjected to multiple linear regression and partial least squares regression analyses. Finally, we achieved two models for assessing nitrate levels in spinach leaves showing much higher predictivity as compared to approaches based on single Raman band descriptors and overtaking the prediction accuracy of Dualex indexes, taken here as a possible alternative tool to destructive methods.

2. Materials and methods

2.1. Field experiment

The experiment was conducted in a greenhouse field of the farm "Cammelli" (Azienda Agricola Cammelli, Florence, Italy), specialized in managing horticultural crops for commercial distribution. The physiochemical average soil characteristics were: 72 % silt, 15 % sand, 13 % clay, pH 8.23, 3.7 % organic matter, 8.0 % total carbonates, 1.3 % active carbonate, and 1.8 % total N. Baby spinach (*Spinacia Oleracea* L.) was grown under different N fertilization treatments at 0, 200, and 400 kg N ha^{-1} (N0, N200, N400, respectively) to induce variable content of leaf nitrates. The average daily temperature and relative humidity during the growing period were 17 ± 7 °C and 74 ± 15 %, respectively. A sprinkler irrigation system was installed in the greenhouse, providing 12 L/m^2 of water at sowing time and 4 L/m^2 throughout the growing period of the plant. Three plots per treatment, each sized 1.0 × 1.2 m^2, at different sites within the greenhouse were used. Calcium nitrate-based fertilization was applied 42 days after sowing. No other treatment was provided to the analyzed plants. Two measurement sessions 14 days apart were conducted, starting with the first 55 days after the sowing date (November 22, 2023), and the other at the crop harvest. Each collection, consisting of 40 leaves, occurred within the 10:00–11:00 a.m. time interval. The selection of leaves was carefully performed to ensure consistency by collecting those with the same exposure to sunlight while avoiding the youngest leaves. During the initial measurement sessions, conducted during the plant's growth phase, sampled leaves measured approximately 5 cm in length and 3 cm in width. In the second measurement session, fully expanded leaves were collected, which were about 1 cm larger in both length and width compared to those sampled in the first session. A temperature of approximately 15 °C and a relative humidity of about 74 % were measured during sample collection.

2.2. The dualex optical sensor

Each leaf was analyzed with the Dualex (Force-A, France) optical sensor. The leaves were measured indoor at room conditions and within 2 h from collection. Chlorophyll content (CHL) is determined by the ratio between the transmittance at 850 nm (reference signal that is not absorbed by chlorophyll) and at 710 nm. Epidermal flavonoids (FLAV) are calculated by the logarithm of the ratio between chlorophyll fluorescence excited at 650 nm and 375 nm (which is attenuated by the epidermal compounds) [22]. The ratio of CHL over FLAV represents the nitrogen balance index (NBI), which has been shown to account for N changes in the leaf [54]. In our experiment 8 Dualex readings per leaf from the upper to the lower part and on both adaxial sides of the adaxial leaf blade were carried out and averaged and the relevant CHL, FLAV, and NBI values were used to assess their accuracy in predicting the nitrate levels as determined by destructive determination (see 2.4). After optical measurements, each leaf was weighted to determine the fresh weight (FW) and then processed for subsequent destructive analyses.

2.3. Raman measurements

Raman spectra within the 400 - 2500 cm^{-1} range were collected indoor at room conditions (20 °C, 65 % relative humidity) with a portable spectrometer (miniRaman Dual, LightNovo, Denmark) by using a 785 nm laser source that was coupled with a laptop for parameter setting and data visualization under the proprietary software (Fig. 1). The spectrometer was equipped with a f = 15 mm lens (NA = 0.1) producing a 15 mm wide spot and to which it was screwed a custom-made aluminum perforated spacer to allow collecting the maximum signal at the leaf plane surface and maintain a 90° geometry between the irradiation direction and the leaf surface. An acquisition time of 200 ms with 10 accumulations and 70 mW of output power was employed. The leaves, previously measured by Dualex, were subsequently analyzed by Raman spectroscopy under the same environmental conditions. Ten spectra were acquired per leaf (divided into 5 for each half) from the upper leaf blade and lateral to the midrib to minimize possible variability of the leaf signal and averaged.

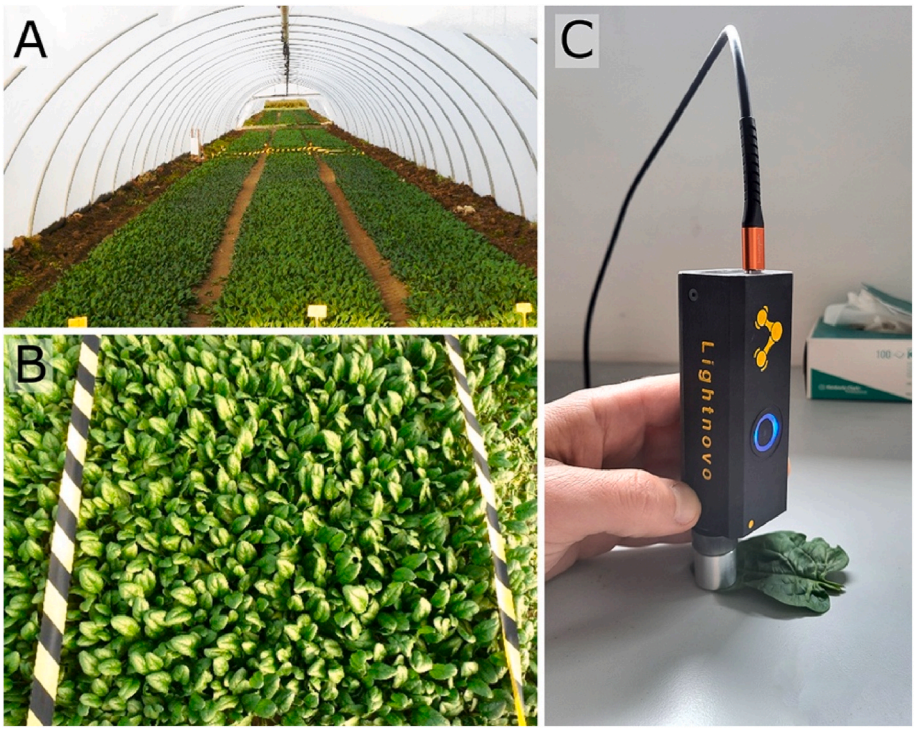


Fig. 1. (A) Greenhouse experimental setup. (B) close-up of a single baby spinach plot. (C) Raman measurement performed in contact with spinach leaf.

2.4. Analytical determination of chlorophyll and nitrates

Chlorophyll determination followed the protocol of Brown et al. [55] with few modifications. Briefly, a 6 mm diameter disk was removed from the topmost part of the leaf and lateral to the midrib using a cork punch. Each disc was placed in a polypropylene tube containing 5 ml of dimethyl-sulphoxide (DMSO) and left overnight in a drying oven at 65 °C. Then a 3 ml sample of the solution was transferred to a 10 mm path-length polystyrene cuvette and the absorbance of each sample was measured at 649 nm and 665 nm with a UV–Vis spectrophotometer (Jasco V-770, Cremella, Italy). Chlorophyll-a and -b concentrations were calculated (in μg ml^{-1}) using Wellburn's [56] equations for DMSO:

$$C_a = 12.19A_{665} - 3.45A_{649}$$

$$C_b = 21.99A_{649} - 5.32A_{665}$$

and finally expressed as mg g^{-1} FW, by considering the extracting volume and the fresh weight of the leaf disk.

The nitrate concentration of dry tissue extract was determined according to the Cataldo et al. [57] colorimetric method with minor modifications as below. The samples were lyophilized inside a polypropylene tube for 39 h at −45 °C with a 0.01 mbar pressure using a Labconco FreeZone 2.5L freeze dryer and powdered in a mortar. 50 mg of dry tissue was suspended in 5 ml water in a polypropylene tube for 1h. After centrifugation, collection of the supernatant and addition of salicylic acid (5 $\%_{w,v}$ in H_2SO_4) followed by quenching with 2N NaOH was carried out. The nitrate content of the leaf was determined spectrophotometrically at 410 nm (using NH_4NO_3 as a standard) and expressed in g kg^{-1} FW.

At the crop harvest (70 days after sowing), a sub-sample of about 200 g of leaves from each plot was collected and sent to a commercial laboratory to analyze its content in total N following the standard method ISO 1871:2009.

2.5. Multivariate data analysis

Preprocessing of the spectra included baseline removal by a Rolling-Circle filter (circle radius 1000 cm^{-1}; scale Y 0.10) to eliminate fluorescence effects and enhance spectral quality. The data were then normalized to the 1884 - 2500 cm^{-1} non-analyte region, ensuring the suppression of any residual fluorescence background and improving the performance of the calibration models. To align the spectral data with the destructive nitrate analysis conducted on a single-leaf basis, the spectra collected from a single leaf were averaged. Finally, the data were smoothed using a 2nd polynomial order Savitsky-Golay filter (7 points) to remove high-frequency noise, ensuring clearer signals for robust multivariate data algorithms [58]. For the analysis, we selected Multiple Linear Regression (MLR) and Partial Least Squares Regression (PLSR) for their interpretability, robustness to multicollinearity, and suitability for the dataset size and spectral characteristics.

2.5.1. Multiple linear regression (MLR)

Fifty-five samples were randomly selected and used as the initial calibration dataset. A preliminary selection of Raman bands was performed by calculating the Pearson's correlation coefficient between each spectral band and the measured nitrate concentration. Only bands with an absolute correlation coefficient >0.15 were retained, ensuring that the selected variables exhibited a meaningful linear relationship with the target variable. These bands were used as independent variables in the MLR model. The statistical rationale for this threshold was to focus on bands most strongly associated with nitrate concentration while excluding those contributing minimal or no predictive value. Selection of the final variables included two additional steps to refine the model. First, bands with regression coefficient showing a p-value <0.05 were retained to ensure statistical significance. Second, the variance inflation factor (VIF) was evaluated for each variable using a standard cutoff of 10, thereby reducing the risk of multicollinearity and enhancing model robustness. The model was further refined by assessing outliers using an influence plot, which evaluated both residual variance and leverage. Samples with high residual variance and leverage >0.5 were removed to prevent undue influence on the regression model.

Approximately 24 % of samples were identified as outliers and excluded, resulting in a final calibration set of 42 samples. The model was validated by leave-one-out cross-validation and finally used in a prediction of nitrate levels on the remaining about 1/3 (n = 19) of the initial dataset. The accuracy of the model was determined using the coefficient of determination (R^2) and the root mean square error $RMSE_C$, $RMSE_V$, and $RMSE_P$ for calibration, validation, and prediction, respectively. The Origin Pro 2024 (OriginLab Corporation) and the Unscrambler (v11.0 by Camo Analytics) software programs were used to process the data.

2.5.2. Partial least squares regressions (PLSR)

Initially, the variables were mean-centered to remove any constant offset and noise, if present in the dataset [59]. An initial 400 - 1700 cm^{-1} spectral range was used for training the model. Leave-one-out cross--validation was performed on the calibration dataset using 7 latent variables (LVs). To refine the model, a Marten's uncertainty test was employed to evaluate the statistical significance of each variable within the spectral range. This test identified variables with a significant contribution to the model, reducing the variable pool to 51 spectral frequencies for the final model. Outliers were identified using an influence plot, evaluating both residual variance and leverage. Samples with high residual variance and leverage >0.5 were excluded to avoid undue influence on the model. Approximately 2 % of the samples were removed, resulting in a robust calibration set. The final PLSR model was then used to predict nitrate levels, with R^2 and RMSE values calculated to evaluate its performance, as conducted for the MLR model.

3. Results and discussion

3.1. Dualex evaluation

A popular strategy to obtain an estimate of some leaf main compounds is provided by optical sensors such as the Dualex. This portable sensor offers a practical and time-saving estimation of leaf chlorophyll (CHL) and epidermal flavonoids (FLAV), which showed a characteristic proportional or inverse trend with leaf N levels, respectively, as highlighted by different authors [17,20,23,24]. Similarly, NBI, calculated as the CHL/FLAV ratio, showed a high quality (R^2 = 0.85–0.87) linear regression with leaf N content in *Paspalum vaginatum* turfgrass [60] and *Ligustrum japonicum* [23]. The relationship between NBI and the leaf N content in spinach (Fig. 2a) confirmed a satisfactory (R^2 = 0.77) linear regression, as well as a negative relationship between FLAV *vs* leaf N (R^2 = 0.68). A flatter regression was found between CHL and leaf N, even if the variation of CHL was significant at $P < 0.001$, according to ANOVA.

Here, we aimed to evaluate the relationship of the Dualex indexes with leaf nitrates, which has not been studied or is only sparsely investigated in the literature using another chlorophyll meter as SPAD, as far as we know [31,32]. In our investigation, we found a significant negative correlation between the FLAV index and leaf nitrate (r = −0.75, R^2 = 0.56) (Table 1 and Fig. S1). Additionally, there was a moderate positive correlation (r = 0.57, R^2 = 0.32) for the NBI (Table 1) that doesn't reach the above-mentioned higher estimate levels of leaf nitrogen, while no correlation between CHL and leaf nitrates was observed. A negative correlation between leaf nitrates and total flavonoids in spinach was previously found and explained due to a differential allocation of plant metabolism between higher carbon-containing compounds and lower nitrogen-containing compounds according to nutrient availability [61]. The lower quality of the linear regression between FLAV and leaf nitrates (Fig. S1) compared to that between FLAV and leaf N (Fig. 2a) can be in part explained by a non-linear relationship between leaf nitrates and leaf N. As can be seen in Fig. 2b, the increase in the N rate supplied to the spinach crop increased, as expected, the leaf level of nitrates. On the contrary, leaf N was not significantly increased as a function of the N rate. A non-linear

Table 1
Pearson's correlation coefficient (r) of chlorophyll$_{a+b}$ levels (Chl_{a+b}), Dualex CHL, FLAV, and NBI indexes, and of selected Raman bands (with a r = ±0.15 cutoff) vs. analytically determined leaf NO_3^- levels. A significant correlation at the level of $p \leq 0.05$ is indicated with *.

Parameter	r
Chl_{a+b} (mg/FW g)	0.15
CHL	−0.20
FLAV	−0.75*
NBI	0.57*
420	−0.43*
450	−0.50*
490	−0.51*
522	0.40*
602	−0.58*
658	0.17
796	−0.28*
824	−0.35*
890	−0.24*
900	−0.26*
936	−0.28*
1042	−0.34*
1086	−0.21
1142	−0.21
1268	−0.30*
1316	−0.28*
1390	−0.33*
1430	−0.45*
1512	−0.18
1548	−0.19
1594	−0.26*
1624	−0.15
1646	−0.18

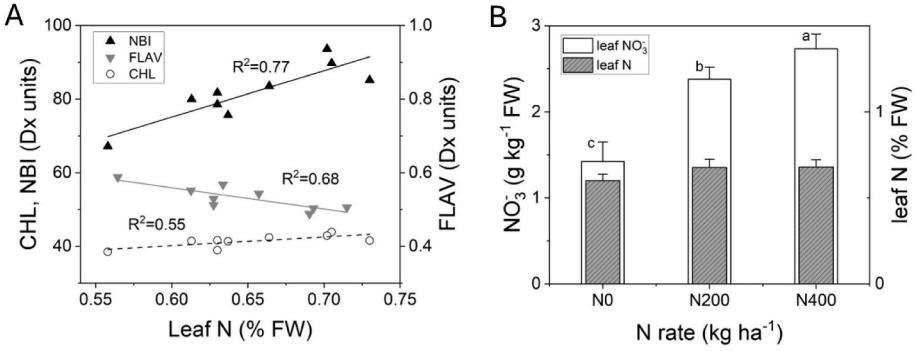


Fig. 2. (A) Relationship between the Dualex CHL, FLAV, and NBI indices and the leaf N content in spinach. (B) Mean (±SD) values of the leaf nitrates and total N as a function of N rates supplied to the spinach crop. For nitrate data, mean values tagged by a different letter are significantly different ($p \leq 0.05$) according to one-way ANOVA with Fisher post hoc test. Mean values of leaf N for the different N rates are not significantly different.

relationship between leaf nitrates and leaf N in spinach (Fig. S2) was also previously observed by Liu et al. [31].

The outcome of using the simple linear regression model to predict leaf nitrates by the FLAV index is reported in Fig. 3. It resulted in a limited accuracy in either calibration ($R^2_c = 0.61$, RMSEC = 0.34 g/kg), validation ($R^2_v = 0.58$, RMSEV = 0.35 g/kg), and prediction ($R^2_p = 0.53$, RMSEP = 0.43 g/kg). However, considering that the safe limit for the leaf nitrate content laid down by the European Union (COMMISSION REGULATION (EU) 2023/915, [62]) is 3.50 g NO_3^- kg^{-1} FW, at this level the nitrate prediction relative error performed by the FLAV model would be still acceptable, being around 12 %.

Overall, the above results underscore the potential of using Dualex for nitrate prediction, although with a moderate accuracy, highlighting the necessity for complementary methods to achieve more precise assessments.

3.2. Raman spectra of spinaches and simple linear regression

The Raman spectral profiles of spinach leaf samples are visualized in Fig. 4. The overall appearance of the spectra denotes a characteristic signature typical of green leafy vegetables under NIR light excitation [43,63], mainly dominated by characteristic carotenoids, chlorophyll, carbohydrates, and polyphenols vibrational modes and showing a moderate variability in intensity and shape throughout the 400 - 1700 cm^{-1} fingerprint region. Carotenoids generate bands at 960, 1006, 1156, 1188, and 1526 cm^{-1} and shoulders at 1117, 1273 and 1494 cm^{-1} [64]. The main bands of chlorophyll (a and b) appear at 747, 917, 1188, 1220, and 1328 cm^{-1}, and shoulders or secondary peaks at 992, 1146, 1486, and 1555 cm^{-1} [65]. Carbohydrates appear as minor peaks in the range 430–490, at 853 cm^{-1} (pectin) and 1097, 1120, 1380 cm^{-1} (cellulose) [66,67]. Polyphenol bands emerge in the ranges 500–596, 625–648, 670–686, 790–860, 1260–1321, 1596 - 1631 cm^{-1} and as shoulders at 1181, 1231, 1353, 1490, 1565 cm^{-1} [68,69].

Fig. 5a displays a heatmap that visualizes the relationship between spectral data and nitrate concentration. Higher intensity indicates a higher negative (blue) or positive (red) correlation rate. A preliminary screening allowed us to identify 23 initial most influential Raman bands (within a cutoff of r = ±0.15) in the prediction of nitrate levels with maximum correlation coefficients approaching −0.6 (Table 1),

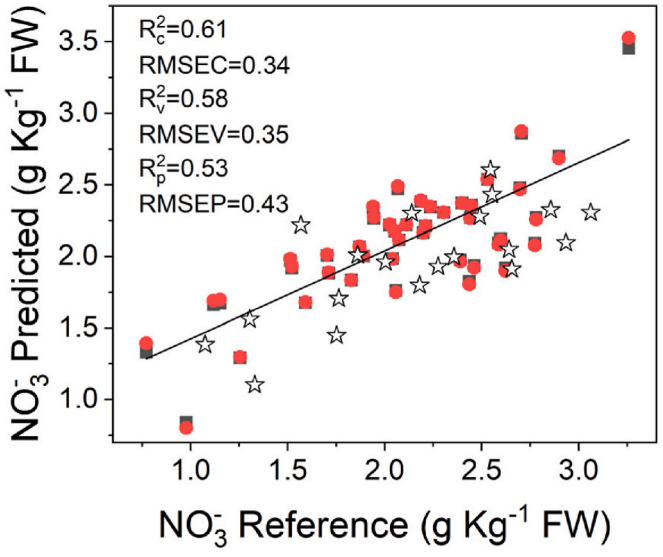


Fig. 3. Results of the SLR model between FLAV and leaf nitrates, including the calibration set (black squares), validation set (red circles), and prediction set (empty stars). A y = 4.58–4.74x dependence between Dx FLAV and leaf nitrates is found. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

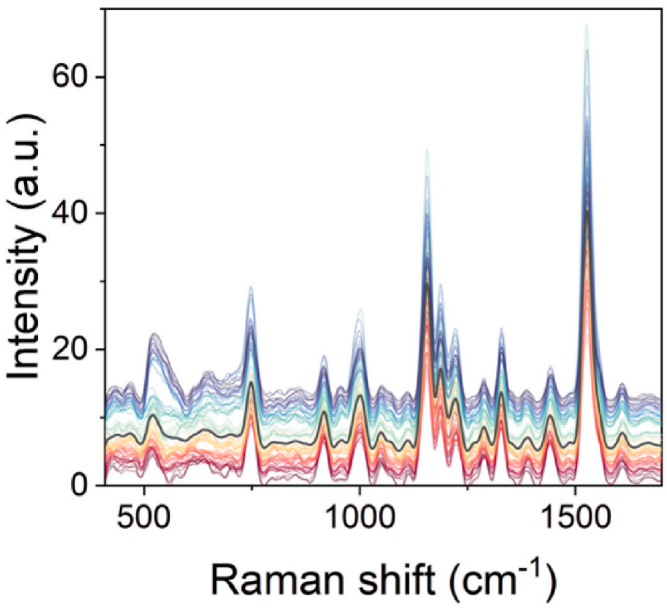


Fig. 4. Stacked Raman spectra of spinach leaves. The black profile represents the average signal of the considered dataset.

indicating a low-moderate dependence from leaf nitrate values. We also noticed a poor linear scaling with nitrate of the 1046 cm^{-1} Raman band previously proposed as a reference parameter in nitrate monitoring [52,53,70] (Fig. S3), likely as a consequence of its colocalization with secondary chlorophyll frequencies [65]. A similar behavior was detected as well when considering the main bands ascribed to chlorophyll (as the 916 and 1328 cm^{-1} bands), which in some recent literature has been shown to vary proportionally with leaf nitrate [31,71], or other main bands (Fig. S3). An inadequate dependence of chlorophyll from nitrate in spinach leaves is also confirmed by a r = 0.15 between extracted $chlorophyll_{a+b}$ and nitrate concentration (Table 1). The above observations point out a scarce accuracy of simple linear regression (*i.e.* a single Raman frequency against analytically determined nitrate) approaches in monitoring nitrate accumulation in leaves and the performance of individual Raman band descriptors appears to be exceeded by those obtained by a widely used optical sensor such as Dualex.

3.3. MLR model

Therefore, a MLR model for nitrate prediction was built based on the most influential bands as displayed in Table 1, which were used as independent variables. In the model construction, 11 bands were finally selected as a result of the exclusion of those showing regression coefficients with a p-value >0.05 and/or a VIF >10 in such a way as to rule out a lower statistical significance as well as residual multicollinearity. An overview of the t-values of the selected bands (Fig. 5b) suggests that the model is influenced by polyphenols contributions at 522, 1316, 1594, and 1624 cm^{-1}, carbohydrates at 420 and 490 cm^{-1}, chlorophyll at 1548 cm^{-1} and a more generic 1390 cm^{-1} band ascribed to δCH_2 bending vibration [72]. It is worth noting that N-fertilization can influence plant growth, potentially increasing chlorophyll levels and cell-wall structures while decreasing the polyphenol content [73–75]. The model (Fig. 5c) exhibits enough regression accuracy in either calibration ($R^2_c = 0.94$, $RMSE_C = 0.21$ g/kg), validation ($R^2_v = 0.89$, $RMSE_V = 0.25$ g/kg), and prediction ($R^2_p = 0.80$, $RMSE_P = 0.30$ g/kg). A clear advantage of the MLR model is its effective prediction performance as compared to a simple linear regression approach (Fig. S3). Nonetheless, the model keeps its immediate applicability to a novel dataset thanks to the calculated regression coefficients (listed in Table S1) for the different variables.

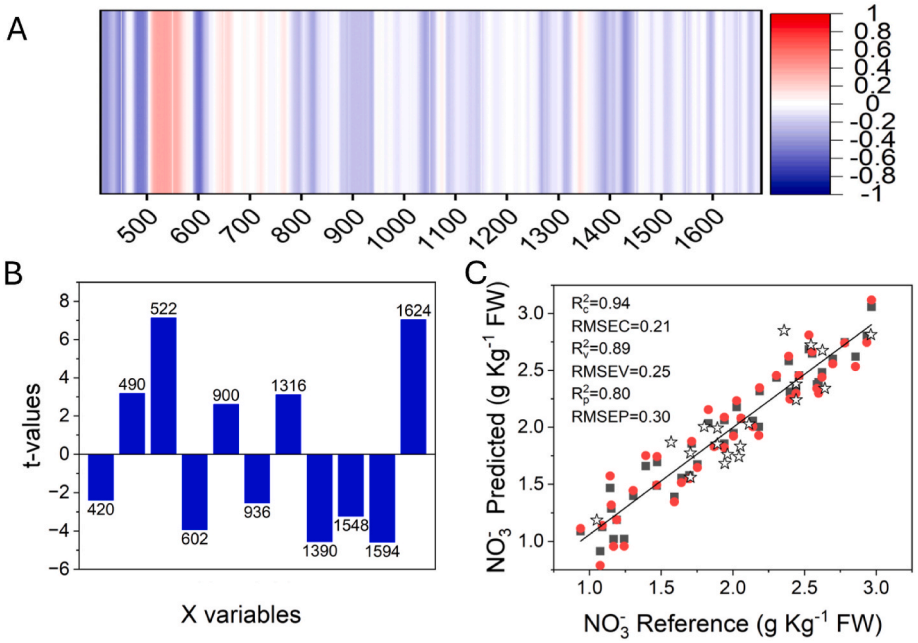


Fig. 5. (A) Heatmap of Pearson's correlation coefficients r between Raman bands of the 400 - 1700 cm^{-1} spectral region and leaf NO_3^- levels; (B) MLR regression t-values of selected independent variables indicating the most influential bands in the model; (C) results of the MLR model including the calibration set (black squares), validation set (red circles), and prediction set (empty stars). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. PLSR model

In recent literature on applications of Raman spectroscopy to food and vegetables, PLSR tools have become a popular strategy in deciphering potential connections between observed parameters and spectral responses [76–82]. Under this picture, we attempted to verify the efficacy of PLSR in the prediction of leaf nitrate as an alternative method to MLR. Fifty-one bands were selected in the final regression model corresponding to the most influential ones as remarked by their high regression t-value (Fig. 6a). Five LVs were required to adequately describe the model with a total variance capturing rate of 94 %. Interestingly, a close agreement with the influential contributions of MLR is found (Fig. 5a and b), endorsing a main role in ruling the regression by polyphenols (514 - 562 cm^{-1}, 798 - 804 cm^{-1}), carbohydrates (424 - 484 cm^{-1}), chlorophyll (976 - 996 cm^{-1}, 1190 - 1244 cm^{-1}, 1534 - 1538 cm^{-1}), and CH vibrations (1384 - 1400 cm^{-1}). The resulting regression accuracy in calibration (R_c^2 = 0.94, $RMSE_C$ = 0.16 g/kg), validation (R_v^2 = 0.90, $RMSE_V$ = 0.20 g/kg), and prediction (R_p^2 = 0.84, $RMSE_P$ = 0.18 g/kg) reaches and exceeds that of MLR (Fig. 6b). Notably, RMSE values appear 1.5-fold reduced on average suggesting a more representative prediction accuracy. Furthermore, in this case, the model covers a larger nitrate level range (~0.7–3.3 g/kg *vs.* ~0.9–3.1 g/kg), possibly as a consequence of the inherently reduced multicollinearity affecting PLS and a significantly greater number of important independent variables governing the prediction that can better describe nitrate variability up to more extreme values. Therefore, from one side the powerful capabilities of PLSR to manage multiple descriptor variables coupled with the multispectral characteristics of Raman spectroscopy succeed in providing an accurate predictive accuracy. However, it's worth remembering a less immediate application of validated PLSR models to

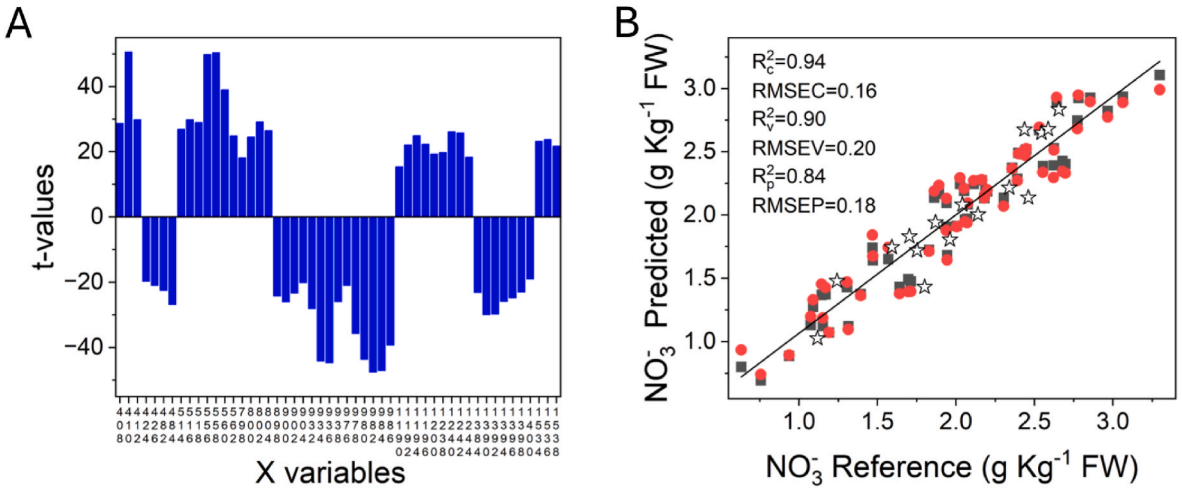


Fig. 6. (A) PLS regression t-values of selected independent variables indicating the most influential bands in the model; (B) results of the PLSR model including the calibration set (black squares), validation set (red circles), and prediction set (empty stars). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

new datasets as in classical linear regression modeling, which is not limited to the knowledge of basic parameters but requires proper handling and adaptation of the model to new data [83].

4. Conclusion

In conclusion, this study underscores the potential of Raman spectroscopy as a non-destructive tool for quantifying nitrate levels in spinach leaves, offering an innovative alternative to traditional destructive methods. The comparative analysis between MLR and PLSR models revealed that both approaches could effectively predict nitrate concentrations, with PLSR demonstrating slightly superior performance due to its ability to capture complex interactions among the spectral data.

The successful application of a portable Raman spectrometer in this study highlights its feasibility for on-field assessments, providing rapid and reliable nitrate measurements that can aid in real-time decision-making for nitrogen management and operational nutrient-management programs for the plant. This advancement aligns with the broader goal of precision agriculture, where accurate and timely information on crop nutrient status can lead to more sustainable fertilization practices, minimizing environmental impacts while optimizing crop yields. However, the scalability of this method for large-scale agricultural operations requires further investigation, particularly regarding its integration with existing farming technologies, operational costs, and the economic feasibility compared to traditional methods or sensors.

Moreover, the integration of Raman spectroscopy with other optical sensors, such as the Dualex, presents a synergistic approach to monitoring plant health and nutrient status. While the Dualex sensor efficiently estimated nitrogen balance indices through chlorophyll and flavonoid measurements, Raman spectroscopy offered a more detailed chemical profile, enhancing the accuracy of nitrate predictions.

The limitations observed in single-band descriptors for nitrate quantification highlight the need for multivariate techniques to fully exploit spectral data. Identifying specific Raman bands correlated with nitrate will be key to refining predictive models and improving accuracy. Future research should expand the dataset to include diverse plant varieties and growing conditions to enhance model generalizability.

Adopting non-destructive nitrate assessment methods offers environmental benefits. By reducing reliance on chemical assays, which generate toxic residues, and enabling more precise fertilizer application, farmers can mitigate nitrate leaching and groundwater contamination, improving food quality. In summary, this study demonstrates the feasibility of using Raman spectroscopy for nitrate quantification in spinach leaves, with promising results that warrant further development, particularly regarding scalability, cost-effectiveness, and potential contributions to sustainable agricultural practices and food security.

CRediT authorship contribution statement

Paolo Matteini: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **Carmelo Distefano:** Writing – review & editing, Methodology, Investigation. **Marella de Angelis:** Writing – review & editing. **Giovanni Agati:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2025.101839.

Data availability

Data will be made available on request.

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