

## AGRICULTURAL MATERIALS

## Derivative Spectrophotometric Determination of Nitrate in Plant Tissue

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**A derivative spectrophotometric method was developed to determine  $\text{NO}_3^-$ -N in plant tissues. The method is based on measurement of the first-derivative spectrum of nitrosalicylic acid in basic solution. The nitrosalicylic acid was obtained by reaction of samples with salicylic acid in concentrated sulfuric acid and was used by Cataldo et al. in nonderivative spectrophotometry. The main strength of this technique is the lack of matrix background interference, typical of plant extracts in traditional spectrophotometric methods. This method is fast, inexpensive, easy-to-apply, and highly selective. The calibration graph was linear in the range of 0.1 and 1.0 mg/L N as  $\text{NO}_3^-$ . Average recovery in real matrixes (lettuce and spinach) was 102.6%; average standard deviation was 3.3. This method has been applied to leaves of 4 types of lettuce.**

The analysis of plant tissue is widely used to determine plant mineral nutrition status. Nitrogen is one of the essential nutrients for plant growth with an adequate content of about 1–2% in dry weight basis. Studies have shown that nitrate content is often a better indicator of plant N requirements than total N concentration (1–6) probably because the former is the main storage form of nitrogen.

For many years, nitrate has indiscriminately been used as a fertilizer. At present, its application is presumably under control because of its deleterious effects on health. A fraction is reduced to nitrites and combines with hemoglobin in blood, causing respiratory problems. It may be involved in the formation of carcinogenic substances such as nitrosamines and nitrosamides (7). For these reasons, the European Commission's Scientific Committee for Food has established the permitted maximum concentration for lettuce and spinach to be put on the market. It is therefore of interest to provide adequate methods to determine plant requirements of nitrate for fertilization.

Nitrate in plant tissue has been quantitatively determined by potentiometric (8, 9), chromatographic (10, 11), and spec-

trophotometric (11–14) methods. The first 2 methods require eliminating interferences, namely, chloride and high salt levels; whereas the principal problem in the classical spectrophotometric method is the presence of pigments and colloidal materials (15). Various methods are available for nitrate determination; Sah (15), in an exhaustive critical review, has attempted to find the strengths and the weaknesses of the reported methods in soil and plant tissue. Because of the difficulties presented by classical spectrophotometry and the advantages of derivative spectrophotometry, a method was designed for nitrate-N determination in plant tissue using first-derivative spectrophotometry.

## Experimental

*Plant Material*

Lettuce and spinach were purchased from a local market. The intact leaves were washed quickly in Tween-20 1% solution followed by washing 3 times in distilled water to remove dust. The samples were dried in a forced-air oven at 65°C for 24 h, ground to pass through a 40-mesh screen, and mixed thoroughly.

*Apparatus*

(a) *Spectrophotometer*.—UNICAM (Cambridge, UK) UV 2 double beam UV Vis with 1 cm quartz cells attached to a printer was used. The spectra were obtained with a spectral bandwidth of 2 nm. The derivative spectra were obtained by instrumental electronic differentiation.

(b) *Water bath*.—Maintain the extract of plant tissue at 45°C.

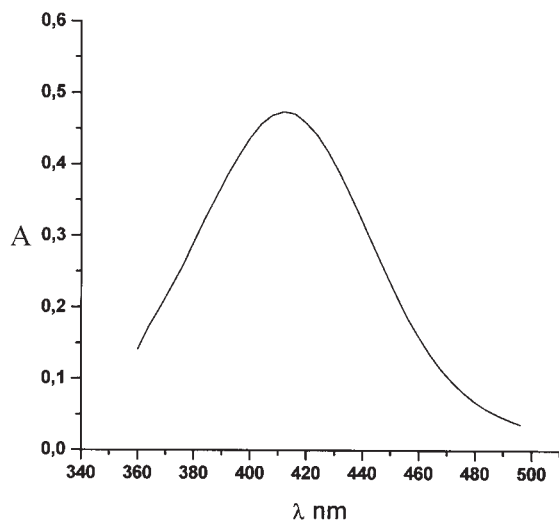
*Reagents*

All reagents were of analytical grade.

(a) *Nitrate-N stock standard*.—Solution of 500 mg/L was prepared from  $\text{KNO}_3$ . Working standard solutions of 10, 20, 30, 40, 50, 60, 80, and 100 mg/L were prepared by diluting the standard with distilled water and were stored at 4°C.

(b) *Salicylic acid solution 5% (m/v) in concentrated  $\text{H}_2\text{SO}_4$* .—Salicylic acid (5.00 g) was dissolved in concentrated sulfuric acid and diluted to volume (100 mL) with the same acid; prepared at least once each 48 h, and stored in an amber bottle at 4°C.

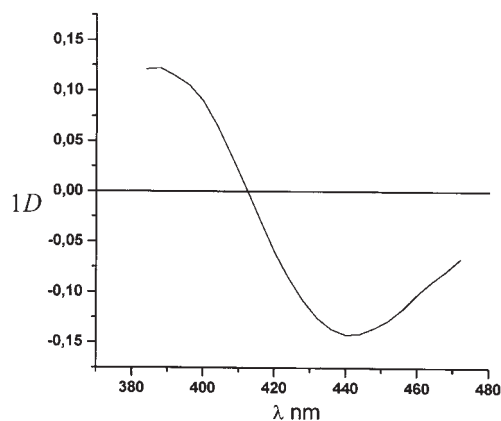
(c) *2N Sodium hydroxide solution*.



**Figure 1.** Zero-order spectrum of nitrosalicylic acid in highly basic solution obtained using standard solution (0.6 mg/L  $\text{NO}_3^-$ -N solution).

#### Calibration

Aliquots (0.1 mL) of working standard solutions (10–100 mg/L  $\text{NO}_3^-$ -N) in a 30 mL tube were mixed thoroughly with 0.4 mL salicylic acid solution. After 20 min at room temperature, 9.5 mL 2N NaOH solution was slowly added to obtain 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 mg/L  $\text{NO}_3^-$ -N solutions. This method is based on the formation of nitrosalicylic acid that shows, in highly basic solution, a maximum absorption at 412 nm in zero-order absorption spectrum,



**Figure 2.** First-derivative spectrum of nitrosalicylic acid in highly basic solution obtained using standard solution  $\Delta\lambda = 4$  nm (0.6 mg/L  $\text{NO}_3^-$ -N solution).

**Table 1.** Regression equation for graph calibration to different  $\Delta\lambda$

$\Delta\lambda$ , nm	Regression equation	Correlation coefficient
1	$1D = 0.1043 Cx - 0.0027$	0.9973
2	$1D = 0.2077 Cx - 0.0053$	0.9977
4	$1D = 0.4166 Cx - 0.0076$	0.9974

and 388 and 440 nm in first-order spectrum ( $1D_{388-440}$ ). The sum of the heights of both peaks (388 and 440 nm) positive and negative (peak-to-peak) of the first derivative was used. The wavelength range selected to obtain the spectrum was 356–500 nm; data interval, 4 nm.

#### Extract Preparation from Plant Tissues

Portions (0.1000 g) of vegetable tissues (lettuce and spinach ground samples) were suspended in 10 mL distilled water, kept at 45°C for 1 h (16), and then filtered through Whatman No. 40 filter paper. Samples were extracted and analyzed immediately or within 24 h after extraction when stored at 4°C.

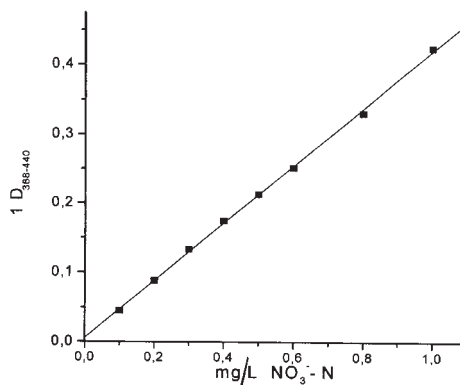
#### Determination

A 0.1 mL volume of the preceding extract was thoroughly mixed in a 30 mL tube with 0.4 mL salicylic acid solution. After 20 min at room temperature, 9.5 mL 2N NaOH solution was slowly added.

#### Calculation

Nitrate-N in plant tissue expressed as  $\mu\text{g NO}_3^-$ -N/g dry weight:

$$C_x = \frac{(1D_{388-440} - a) \times 1000}{b \times w}$$



**Figure 3.** Graph calibration of nitrate determination using first-order derivative spectrophotometry.

**Table 2. Statistical results for calibration graph**

Parameter	Derivative method
Concentration range, mg/L	0.1–1.0
Slope <i>b</i>	0.4138
Standard deviation <i>s<sub>b</sub></i>	0.0048
Intercept <i>a</i>	0.0056
Standard deviation <i>s<sub>a</sub></i>	0.0027
Correlation coefficient	0.9996
<i>n</i>	8

where  $1D_{388-440}$  corresponds to the spectrophotometric measurement of the sample (UD derivative unity); *b* and *a* correspond to the slope and the intercept of the calibration curve  $1D_{388-440}$  (UD) vs  $\text{NO}_3^-$ -N concentration (mg/L), respectively; *w* = tissue weight (g).

**Results and Discussion**

Zero- and first-order spectra for nitrosalicylic acid obtained with standard solutions are shown in Figures 1 and 2, respectively. The conditions of derivative spectrophotometry such as wavelength range of spectrum, derivative order, differential wavelength, and noise attenuation, were studied. The optimum conditions were first-order derivative, wavelength range of 356–500 nm, and  $\Delta\lambda$  4 nm. The first-order derivative spectrum shows more definite signals for analytical purposes; in addition, the spectral range selected avoids the noise at  $\lambda < 356$  nm. In relation to the differential wavelength using 4 nm—the highest option in the UNICAM UV 2 software—a greater sensitivity is reached as shown in Table 1.

Under the above experimental conditions, a linear correlation was obtained from  $1D$  and nitrate-N concentration. In this derivative method, the measurement selected to prepare the analytical calibration graphs was peak-to-peak (sum of the heights of 388 and 440 nm peaks), which exhibits good linearity to nitrate-N concentration. The intercept is near zero. The calibration graph and statistical results are given in Figure 3

and Table 2, respectively. Detection and quantitation limits are 0.028 and 0.094 mg/L, respectively (17).

*Calculation of the Detection Limit*

$$S_{y/x} = \frac{\sum_i (y_i - \hat{y}_i)^2}{n - 2}$$

where  $\hat{y}_i = y$  individual recalculated in calibration curve;  $n - 2$  = degrees of freedom:

$$y \text{ in detection limit} = y_{DL}$$

$$y_{DL} = y_B + 3s_B$$

$$y_B = a$$

$$s_B = s_{x/y}$$

Calibration curve where  $x = \text{NO}_3^-$  -N concentration (mg/L);  $y = 1D_{388-440}$  (UD).

$$y_{DL} = b \text{ conc}_{DL} + a$$

$$\text{Detection limit} = \text{conc}_{DL} = \frac{y_{DL} - a}{b}$$

*Calculation of the Quantitation Limit*

$$y_{QL} = y_B + 10 s_B$$

In calibration curve

$$y_{QL} = b \text{ conc}_{QL} + a$$

$$\text{Quantitation limit} = \text{conc}_{QL} = \frac{y_{QL} - a}{b}$$

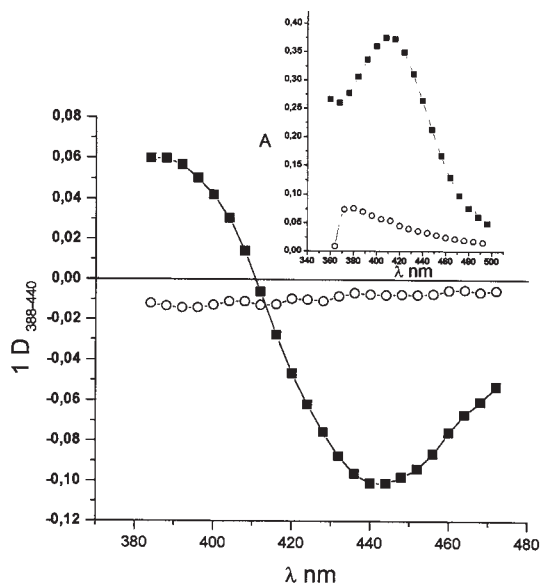
Accuracy and precision were studied in vegetable samples from lettuce and spinach foliage. To this end, recovery assays were performed by spiking with 1, 1.5, 2, and 3  $\mu\text{g}$  N as ( $\text{NO}_3^-$ ) to aliquots of one aqueous extract of lettuce, and with 1 and 2  $\mu\text{g}$  N as ( $\text{NO}_3^-$ ) to aliquots of one aqueous extract of spinach. Each spiking was repeated 4 times. The results are summarized in Table 3.

The accuracy of the method was very good; most recoveries were nearly 100%. However, at low concentrations, mean recovery was 112% as reflected by the spinach determination.

**Table 3. Recovery results of lettuce and spinach samples containing added amount of N as  $\text{NO}_3^-$**

Sample	Sample, $\mu\text{g}$	Added, $\mu\text{g}$	Found, $\mu\text{g}$	Recovery, % <sup>a</sup>
Spinach	0.33	1.0	1.49 ± 0.05	111.6 ± 3.7
	0.33	2.0	2.37 ± 0.08	101.7 ± 3.3
Lettuce	1.75	1.0	2.78 ± 0.08	100.9 ± 3.0
	1.75	2.0	3.83 ± 0.15	102.2 ± 4.2
	3.49	1.5	5.13 ± 0.17	102.7 ± 3.4
	3.49	3.0	6.83 ± 0.14	105.1 ± 2.2

<sup>a</sup> Mean of 4 determinations ± standard deviation.



**Figure 4.** Zero- and first-order absorption spectra of (■) nitrosalicylic acid in highly basic solution obtained using lettuce extract and (O) blank lettuce sample.

Without this value, the mean recovery was  $102.6 \pm 3.3\%$ . In the same preparations, the absorbance at 412 nm was measured as with the method of Cataldo et al. (16), with recovery ranging from 115 to 132%; values higher than 100% are ascribed to the background absorption of the foliar extract (mostly pigments), as can be seen in the zero-order absorption spectrum of the blank sample. Figure 3 shows the zero- and first-order spectra obtained using plant tissue extract with their respective spectra of the sample blank. The sample blank contained 0.1 mL extract of sample, 0.4 mL concentrated sulfuric acid, and, after 20 min, 9.5 mL 2N NaOH. Instead, the first-order derivative spectrum of the blank sample exhibited negative values near zero in the wavelength range under study, originating a displacement of zero in the first-order derivative spectrum of nitrosalicylic acid obtained by foliar extract without affecting the signal used to determine nitrate-N when the sum of the bands at 388 and 440 nm was considered. The described method is adequate for foliar samples containing  $\text{NO}_3^- \text{-N} \geq 1000 \mu\text{g/g}$  dry weight. For samples whose content is  $\leq 1000 \mu\text{g/g}$  dry weight, a greater aliquot of the extract (0.2–0.3 mL) or a modification of tissue weight: water ratio is recommended for the extraction procedure.

Linearity between percent recovery and amount of analyte added of standard solutions was studied in solutions containing 1, 2, 4, 5, 6, and 8  $\mu\text{g NO}_3^- \text{-N}$ . Table 4 shows the data obtained. For different levels, the recoveries were nearly 100%, with a global percent recovery of  $102.7 \pm 5.4$ . The slope and the intercept of the regression linear equation  $\mu\text{g added (x)}$  vs

**Table 4.** Recovery results of standard solutions in the derivative method

Added, $\mu\text{g NO}_3^- \text{-N}$	Found, $\mu\text{g NO}_3^- \text{-N}$	Recovery, % <sup>a</sup>
1	$1.10 \pm 0.06$	$110.2 \pm 5.4$
2	$2.11 \pm 0.06$	$105.5 \pm 2.5$
4	$4.02 \pm 0.22$	$100.5 \pm 4.4$
5	$4.91 \pm 0.23$	$98.1 \pm 4.1$
6	$6.17 \pm 0.09$	$102.9 \pm 1.3$
8	$7.92 \pm 0.30$	$99.0 \pm 3.1$

<sup>a</sup> Mean of 4 determinations  $\pm$  standard deviation.

$\mu\text{g found (y)}$  were 1.0023 and 0.0999, respectively, with a correlation coefficient of 0.9976 for  $n = 24$ . Both parameters, slope, and intercept do not differ significantly from 1 and 0, respectively, at the 95% confidence interval, which points out that systematic errors were not detected (18). In the lowest concentrations, the recovery was  $>100\%$ , as in the recovery assay using plant tissue matrixes.

#### Comparison of Proposed Method with Modified Griess–Illosvay Method (19)

Nitrate-N concentration was determined in one sample of lettuce and one sample of spinach by both methods (Table 5). Each value is the mean value of 4 determinations (4 extracts). The agreement between the 2 methods was acceptable. In the modified Griess–Illosvay method, nitrate was determined by the colorimetric method preceded by the reduction of nitrate to nitrite. The measured signal corresponds to the absorbance at 540 nm, the zone less affected by pigments of the foliar extract.

#### Application

Upon detection of differences in nitrate-N contents in lettuce leaves (*Lactuca sativa* L.) of different ages, the proposed derivative method was used for nitrate-N determination in external, central, and internal leaves of 4 types of lettuces: “costina” (group Cos), “escarola” (group Iceberg), “española,” and “milanesa” (group Butterhead). This approach had a prospective character: only one lettuce of each

**Table 5.** Comparison of derivative method with modified Griess–Illosvay method

Sample	Proposed method	Modified. Griess–Illosvay method <sup>a</sup>
Lettuce	$3350 \pm 149$	$3391 \pm 184$
Spinach	$830 \pm 19$	$1141 \pm 66$

<sup>a</sup> Mean of 4 determinations  $\pm$  standard deviation.

**Table 6. Nitrate content in *Lactuca sativa* L. in leaves of different ages and types,  $\mu\text{g NO}_3^-$ –N/g dry weight**

Type of lettuce	External leaves <sup>a</sup>	Central leaves <sup>a</sup>	Internal leaves <sup>a</sup>
Milanesa	11580 ± 578	4957 ± 89	1535 ± 80
Costina	10056 ± 378	2988 ± 152	—
Española	4118 ± 8	2941 ± 27	—
Escarola	5758 ± 306	2401 ± 123	—

<sup>a</sup> Mean of 4 determinations ± standard deviation.

type was sampled. Each value corresponds to the mean value of 4 determinations (4 extracts from the same sample). Table 6 shows nitrate–N concentrations in different leaves of lettuce. In internal leaves, the younger tissue, nitrate concentration was very low or not detected, as occurs in 3 types, costina, española, and escarola. However, as in most plants, this ion accumulates in old leaves, becoming 2 or 3 times higher than in central leaves. The different nitrate distribution in leaves will allow selection of tissue that is more sensitive for evaluating the application of fertilizers. Other factors, such as light and drought, may also affect nitrate concentration in leaves, but they are less controllable by humans.

### Conclusions

A simple and adequate analytical quality method based on first-order derivative spectrophotometry was developed to determine nitrate in plants. The method allows the assessment of the nitrate status in crops, which is fundamental to the application of fertilizers. Good yields must conciliate with minimum risks to health.

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