HPLC–Ion Chromatographic Analysis of Nitrate in Plant Tissues and Its Application to Sweetclover (*Melilotus* spp.) Seedlings

Alister D. Muir* and Juliana J. Soroka

Agriculture Canada Research Station, 107 Science Place, Saskatoon, Saskatchewan S7N 0X2, Canada

Nitrate ions were extracted from plant samples using a borate–gluconate buffer and quantified using a single-column anion-exchange HPLC–ion chromatography column eluted with the borate–gluconate buffer. Nitrate concentrations detected by ion chromatography were correlated with those obtained by reaction with salicylic acid in a colorimetric assay of borate–gluconate extracts of cotyledons from field-grown seedlings. The salicylic acid method was determined to be subject to nitrite interference. HPLC–ion chromatography was used to determine nitrate levels in 7- and 21-day-old annual sweetclover (*Melilotus infesta* Guss.) and yellow sweetclover (*Melilotus officinalis* (L.) cv. Norgold) seedlings grown in the greenhouse. Cotyledons from 7-day-old Norgold seedlings contained significantly more nitrate (2.83 mg/g) than those from *M. infesta* seedlings (1.70 mg/g). By 21 days the nitrate levels in all parts of the *M. infesta* seedlings were significantly higher than those of the corresponding Norgold seedlings.

INTRODUCTION

Previous work and our own preliminary experiments have shown that analytical techniques applied to the study of nitrate levels in plant samples are subject to interference from other anions and require special precautions to eliminate that interference (Singh, 1988) or require separate analyses to distinguish between nitrate and nitrite (Lowe and Hamilton, 1967). Other techniques including the use of a nitrate electrode require more leaf material than is normally available in an individual seedling (Paul and Carlson, 1968; Singh, 1988) or are not suitable for screening the large number of samples required in a breeding program (Cataldo et al., 1975; Elton-Bott, 1977; Tanaka et al., 1982).

Single-column anion-exchange ion chromatographic techniques developed for analysis of ions in drinking water and soil samples (Nieto and Frankenberger, 1985; Barak and Chen, 1987) have yet to find wide application in the analysis of ions in plant samples. Previous applications of ion chromatography technology to determine plant nitrate levels have significant limitations for a plant breeding program. Dedicated ion chromatographic procedures with single or multiple columns have been applied to plant samples (Kalbasi and Tabatabai, 1985; Grunau and Swiader, 1986); however, the sample requirement is large and the equipment specialized and expensive. The method of Bradfield and Cooke (1985) requires extensive sample purification which limits the number of samples that can be analyzed in a day. Analysis of nitrate by HPLC–ion chromatography utilizing UV detection is free of interference from other ions present in the plant sample and can be used to measure accurately the nitrate levels of 70–100 samples per day.

The sweetclover weevil, *Sitona cylindricollis* Fahraeus, is the principal insect pest of sweetclover (*Melilotus* spp.) in North America (Beirne, 1971; Craig, 1978), and is thought to be the main contributor to the decline in importance of the crop (Craig, 1978). Manglitz and Gorz (1964) found that the annual wild species *M. infesta* Guss. was a poor host for the sweetclover weevil. This lack of weevil damage on *M. infesta* was observed to be associated with significantly higher concentrations of nitrate in the foliage than were found in *M. officinalis* (L.) and *M. alba* (Desr.) (Akeson et al., 1969a, 1969b; Beland et al., 1970). The present studies were initiated to develop methods capable of determining nitrate concentrations in sweetclover seedlings, a stage at which the plant is particularly vulnerable to feeding damage by weevils emerging from their winter diapause (Monroe et al., 1949).

The suitability of the single-column anion-exchange HPLC–ion chromatography method for detecting nitrate levels in cotyledons was evaluated in a greenhouse study of nitrate levels in *M. infesta* and *M. officinalis* seedlings. The effectiveness and reproducibility of the results obtained by ion chromatography were also compared to results obtained using the salicylic acid spectroscopic method using field-grown sweetclover seedlings.

MATERIALS AND METHODS

Plant Material. Greenhouse trials were conducted with *M. infesta* and *M. officinalis* cv. Norgold grown in flats in soilless potting mix under sodium vapor lamps. In the field, the cv. Norgold was seeded in mid summer in plots containing 8 rows 0.3 m apart and 6.1 m in length and harvested 7 days post-seeding.

Liquid Chromatography. A Millipore-Waters liquid chromatograph equipped with a M600E gradient module, M700 autosampler, and 991 photo diode-array detector (PDA) was used (Millipore (Canada) Ltd., Mississauga). The PDA software was used to integrate the signal. Nitrate and nitrite levels were determined by ion chromatography on a IC-Pak Anion HC column (4.6 × 150 mm) (10 μm) (Millipore (Canada) Ltd.) with a IC-Pak anion precolumn cartridge (Millipore (Canada) Ltd.). Ions were eluted isocratically with a borate–gluconate buffer (Heckenberg et al., 1989) at 2.0 mL/min with UV detection at 214 nm.

Reagents. The borate–gluconate eluent (pH 8.5) was prepared by mixing 20 mL of a borate–gluconate concentrate, 20 mL of 1-butanol, and 120 mL of HPLC-grade acetonitrile in HPLC-grade water and diluting to 1 L. The eluate was filtered prior to use. The borate–gluconate concentrate was prepared by dissolving 16 g of sodium gluconate, 18 g of boric acid, 25 g of sodium tetraborate decahydrate, and 250 mL of glycerol in HPLC-grade water, mixing thoroughly, and diluting to 1 L. All solvents used were HPLC grade or better, all chemicals were Analar grade or better except for sodium nitrite which was reagent grade. All reagents were supplied by BDH Chemicals Inc., Toronto, Canada.

Sample Preparation. Nitrate concentrations were determined in individual cotyledons, unifoliolate leaves, and individual

* Author to whom correspondence should be addressed.

0021-8561/92/1440-1602$03.00/0 Published 1992 by the American Chemical Society

leaflets from the first trifoliolate leaf which were excised and immediately placed in nitrate extraction solution (2 mL of deionized water or borate–gluconate HPLC eluent buffer) in a 16-mL polypropylene centrifuge tube. The excised cotyledons and leaflets were crushed with a Teflon tissue grinder, allowed to extract for 1 h, and then centrifuged at 25000*g* for 15 min, and the supernatant recovered for nitrate analysis. When fresh weights (FW) were determined, the cotyledons were placed in numbered tubes and immediately transported to the lab for weighing. Extraction solution (2 mL) was then added to each tube after the cotyledon weight was determined.

Salicylic Acid Method for Nitrate Determination. Nitrate concentrations were determined by a modification of the method of Cataldo et al. (1975). The salicylic acid reagent was prepared fresh daily by dissolving 2.5 g of salicylic acid in 50 mL of concentrated H_2SO_4. Calibration standards of 0, 4, 20, 40, and 80 ppm nitrate were prepared for each analysis. Aliquots (0.2 mL) of each extract and standard were pipetted into three 50-mL beakers. The salicylic acid reagent (0.8 mL) was added to two of the beakers. Sulfuric acid (0.8 mL) was added to the third beaker (Blank). After 20 min, 19 mL of 2 N NaOH was added slowly to all the beakers and mixed. After the mixtures were cooled to room temperature (15 min), the absorbance at 410 nm was read using the 0 ppm standard blank to zero the spectrophotometer.

Nitrate and Nitrite Working Standards. A 4000 ppm nitrate standard was prepared by dissolving 0.548 g of $NaNO_3$ in water and diluting to 100 mL. A 4000 ppm nitrite standard was prepared by dissolving 0.600 g of $NaNO_2$ in water and diluting to 100 mL. Working standards were prepared by diluting 100 μL of the 4000 ppm solution to 100 mL (4 ppm).

Data Treatment. ANOVA and Pearson correlation coefficients were applied using the Statistical Analysis System (SAS, 1989).

RESULTS AND DISCUSSION

Preliminary experiments comparing nitrate concentrations in cotyledon extracts prepared in either water or borate–gluconate buffer produced similar values for samples analyzed immediately after collection by both the HPLC ion chromatography and salicylic acid methods (data not shown). However, when samples were allowed to sit in autosampler vials for 24 h, variable decreases in nitrate levels were observed in samples extracted in water. Nitrate concentrations in borate–gluconate-extracted samples were unchanged when reanalyzed 1 week later by ion chromatography. No loss of sensitivity in the salicylic acid method was observed when the extracts prepared in the borate–gluconate buffer were compared to water extracts allowing the same extract to be analyzed by either method. All subsequent extractions were made using the borate–gluconate buffer.

Cotyledons collected from field-grown cv. Norgold seedlings were used to evaluate the ion chromatography and salicylic acid methods. The mean nitrate concentration of the same extract determined by the salicylic acid method was greater than that determined by the ion chromatography method, but the difference was not statistically significant ($P = 0.08$). Both methods revealed similar trends in nitrate levels and a significant correlation ($R = 0.84$, $P < 0.01$) was observed between them. There was a greater range of values in the salicylic acid method (SE = ±2.02) compared to the ion chromatography method (SE = ±1.50).

The salicylic acid method also appeared to be more sensitive to interference from nitrite than previous research (Cataldo et al., 1975) had indicated. Nitrite concentrations of 40–80 ppm produced a measurable colored chromophore (Figure 1) when reacted with salicylic acid in sulfuric acid. Ion chromatographic analysis of these nitrite standards indicated that no nitrate was present to account for the

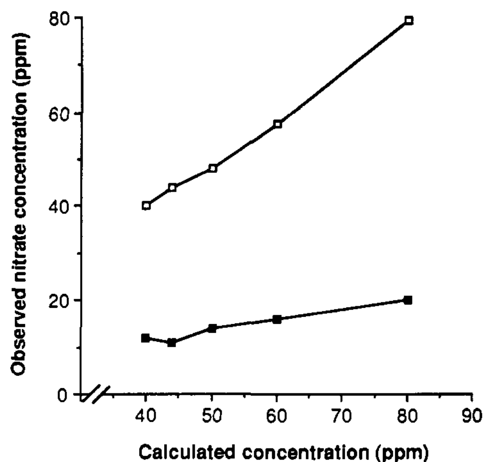

Figure 1. Colorimetric estimation of nitrate (□) and nitrite (■) (40, 44, 50, 60, and 80 ppm) measured as nitrate by reaction with salicylic acid in concentrated sulfuric acid.

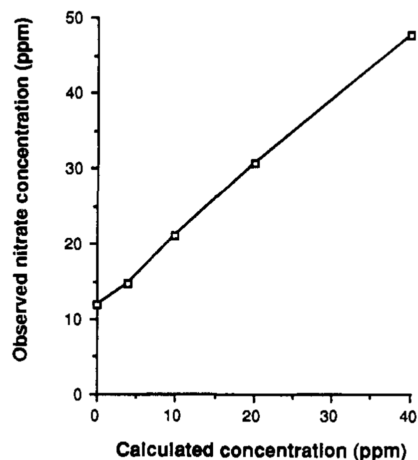

Figure 2. Effect on colorimetric estimation of nitrate of adding 40 ppm nitrite to 0, 4, 10, 20, and 40 ppm nitrate (□) measured as nitrate by reaction with salicylic acid in concentrated sulfuric acid.

color development. When 40 ppm nitrite was added to a range of concentrations of nitrate, the nitrite resulted in an overestimation of nitrate concentrations of up to 15 ppm (Figure 2).

Ion chromatographic determination of nitrate levels in cotyledon samples to which known quantities of nitrate were added indicated 94.8% (SE = ±1.8, $n = 10$) recovery of the added nitrate. No interfering compounds were observed to elute close to either the nitrate or nitrite peaks in any of the plant samples analyzed (Figure 3). In this method, the whole cotyledon was used in each extraction; therefore, duplicate extractions were not possible. When both cotyledons from a single seedling were extracted separately, the variability in nitrate levels between cotyledons was low and the correlation between pairs highly significant (Table I).

One advantage of the ion chromatography solvent system described above is that nitrate and nitrite are easily resolved and the only other ions usually detected by UV are bromide and organic acids such as malate, which elute before nitrite and are easily distinguished from nitrate and nitrite. Nitrate (25 ppm) and nitrite (25 ppm) standards exhibit similar peak areas, with the nitrate peak broader due to its greater retention time (Figure 3). Another advantage is the reagents used are inexpensive and widely available compared to those used in an alternative ion chromatographic system (Bradfield and Cooke, 1985).

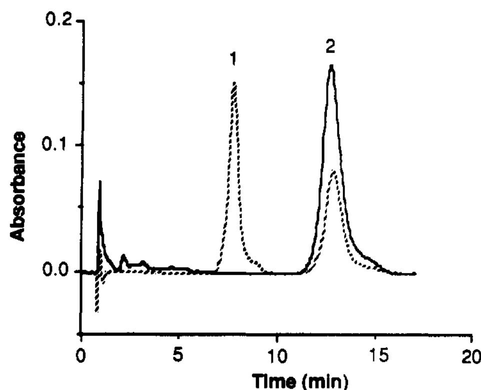

Figure 3. HPLC analysis of nitrate (25 ppm) and nitrite (25 ppm) standards (···) and *M. infesta* cotyledon extracted in borate gluconate buffer (—): 1 = nitrite, 2 = nitrate. Chromatography as per text, 50-μL injection.

Table I. Mean (±SE) Nitrate Levels in Pairs of Cotyledons of Greenhouse-Grown cv. Norgold and *M. infesta* Seedlings at 7 Days Postseeding (n = 20)

	fresh wt, mg	nitrate, μg/cotyledon	nitrate, mg/g of cotyledon FW
cotyledon A	11.80 (±3.00)	24.83 (±5.55)	2.21 (±0.49)
cotyledon B	11.90 (±3.00)	25.81 (±5.77)	2.38 (±0.52)
r	0.97[a]	0.92[a]	0.94[a]

[a] Pearson correlation coefficients significant at $P < 0.01$.

Table II. Mean (±SE) Nitrate Levels in Cotyledons and First Trifoliolate Leaves of *M. officinalis* cv. Norgold and *M. infesta* Seedlings Grown for 7 or 21 Days in a Greenhouse

	M. officinalis cv. Norgold	*M. infesta*
7 Days Postseeding		
cotyledon (n = 20)		
fresh wt, mg	9.0 (±0.6)	14.8 (±0.8)[b]
nitrate, μg/cotyledon	25.63 (±2.17)	25.01 (±2.04) ns
nitrate, mg/g cotyledon	2.83 (±0.10)	1.70 (±0.10)[b]
21 Days Postseeding		
cotyledon (n = 10)		
fresh wt, mg	14.7 (±0.6)	25.3 (±1.5)[b]
nitrate, μg/cotyledon	32.44 (±2.86)	90.6 (±8.87)[b]
nitrate, mg/g cotyledon	2.21 (±0.16)	3.50 (±0.18)[b]
leaf (n = 10)		
fresh wt, mg	39.0 (±3.0)	60.0 (±4.0)[b]
nitrate, μg/leaf	69.49 (±5.11)	227.43 (±22.87)[b]
nitrate, mg/g leaf	2.06 (±0.41)	3.72 (±0.20)[b]
first trifoliolate leaf (n = 20)		
fresh wt, mg	10.0 (±0.8)	10.2 (±0.6) ns
nitrate, μg/leaf	18.34 (±2.09)	24.70 (±2.14)[a]
nitrate, mg/g leaf	1.76 (±0.15)	2.36 (±0.12)[b]

[a,b] Means within rows significantly different at $P < 0.05$, $P < 0.01$, respectively. ns, not significant.

A comparison of nitrate levels in *M. infesta* and *M. officinalis* cv. Norgold cotyledons grown in the greenhouse demonstrated significant differences between the two species at 7 days postseeding. Although nitrate/cotyledon levels were not significantly different between species, cv. Norgold cotyledons contained significantly more nitrate/mg FW than did *M. infesta* cotyledons (Table II). By 21 days postseeding, the nitrate levels were reversed, with nitrate concentrations in the cotyledons, the unfoliolate leaves, and first trifoliolate leaves of *M. infesta* significantly higher than those of cv. Norgold. These results are in contrast with observations made by Kulm (1973) who examined 31-day-old seedlings of each species and found no differences. However, the differences at 21 days are not large, and the methods employed by Kulm (1973) may not have permitted differentiation between them. Akeson et al. (1969a) examined nitrate levels in more mature plants (12–20 cm tall) and found large differences in nitrate levels in mature leaves. In a subsequent experiment (Akeson et al., 1969b), *M. infesta* plants 42–56 days old were found to have higher nitrate levels than fully expanded *M. officinalis* leaves. However, in the very young "pinched" leaves, nitrate levels were lower than in fully expanded *M. officinalis* leaves. At this stage of plant development (42–56 days postseeding), feeding damage by the sweetclover weevil is a less significant factor in plant survival than at the seedling stage. Our results suggest that during the critical seedling emergence period (5–20 days postseeding), nitrate levels in *M. infesta* are not sufficiently elevated over those observed in cv. Norgold to protect *M. infesta* seedlings from weevil attack.

Table III. Pearson Correlation Coefficients[a] between Nitrate Levels in Cotyledons, Unifoliolate, and First Trifoliolate Leaves of Greenhouse-Grown Sweetclover Seedlings (Nitrate/Cotyledon) (n = 20, 50% Plants cv. Norgold, 50% *M. infesta*)

	cotyledon	unifoliolate leaf
unifoliolate leaf	0.955	
first trifoliolate leaf	0.546	0.583

[a] All Pearson correlation coefficients significant at $P < 0.05$. All correlations for nitrate concentrations (ppm/mg fresh weight) were also significant at $P < 0.05$.

In experiments conducted to determine the distribution of extractable nitrate in greenhouse-grown seedlings, all correlations observed between cotyledon, unifoliolate, and first trifoliolate leaf nitrate concentrations were significant (Table III). These results demonstrate that nitrate levels in samples as small as a single trifoliolate leaf can be analyzed by ion chromatography. Therefore, it is possible to estimate the nitrate concentrations in leaf tissue consumed by weevils.

Sufficient plant samples can be collected in a time period short enough to minimize diurnal variations in nitrate concentrations such as those found by Hageman et al. (1961) in maize. This technique can also be used to identify plants with increased cotyledon or first leaf nitrate levels in a breeding program. High nitrate levels present in the mature leaves of *M. infesta* may contribute to the resistance of these tissues to weevil feeding damage; however, the lack of significant elevations in nitrate levels in young *M. infesta* seedlings appears to preclude nitrate from being a significant factor in early seedling resistance to sweetclover weevil feeding. Our greenhouse studies have shown that *M. infesta* seedlings do not contain elevated levels of nitrate at emergence, and consequently any reduction in feeding observed at this stage of plant development must result from factors other than nitrate. These observations support the studies of Akeson et al. (1969c) who found another unidentified deterrent and a stimulant to weevil feeding. Further studies of the chemical attractants and deterrents of *Melilotus* spp. to the sweetclover weevil are needed.

In conclusion, ion chromatography using the IC-Pak anion-exchange column offers a practical method for the study of nitrate levels in small plant samples such as individual cotyledons. This analysis could be implemented with a single-pump HPLC system equipped with a UV detector capable of measuring absorbances at 210 nm, equipment which can now be found in most research laboratories. Ion chromatographic procedures using dedicated ion chromatographs require substantially greater capital investments.

ACKNOWLEDGMENT

We thank K. A. Bassendowski and J. L. Nerland for technical assistance and D. T. Spurr for statistical analysis.

LITERATURE CITED

Akeson, W. R.; Beland, G. L.; Manglitz, G. R. Nitrate as a deterrent to feeding by the sweetclover weevil. *J. Econ. Entomol.* **1969a**, *62*, 1169–1172.

Akeson, W. R.; Beland, G. L.; Haskins, F. A.; Gorz, H. J. Influence of development stage of *Melilotus infesta* leaves on resistance to feeding by the sweetclover weevil. *Crop Sci.* **1969b**, *9*, 667–669.

Akeson, W. R.; Haskins, F. A.; Gorz, H. J. Sweetclover weevil feeding deterrent B. Isolation and identification. *Science* **1969c**, *163*, 293–294.

Barak, P.; Chen, Y. Three-minute analysis of chloride, nitrate, and sulphate by single column anion chromatography. *Soil Sci. Soc. Am. J.* **1987**, *51*, 257–258.

Beirne, B. P. *Pest insects of annual crop plants in Canada. I. Lepidoptera II. Diptera III. Coleoptera*; Memoirs of the Entomol. Soc. Canada; Entomol. Soc. Canada: Ottawa, 1971; No. 78, 124 p.

Beland, G. L.; Akeson, W. R.; Manglitz, G. R. Influence of plant maturity and plant part on nitrate content of the sweetclover weevil-resistant species *Melilotus infesta*. *J. Econ. Entomol.* **1970**, *63*, 1037–9.

Bradfield, E. G.; Cooke, D. T. Determination of inorganic anions in water extracts of plants and soils by ion chromatography. *Analyst* **1985**, *110*, 1409–10.

Cataldo, D. A.; Haroon, H.; Schrader, L. E.; Youngs, V. L. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* **1975**, *6*, 71–80.

Craig, C. H. Damage potential of the sweetclover weevil, *Sitona cylindricollis*, (Coleoptera: Curculionidae), in the Canadian Prairies. *Can. Entomol.* **1978**, *110*, 883–889.

Elton-Bott, R. R. A modified spectrophotometric method for nitrate in plants, soils and water by nitration of 3,4-dimethylphenol. *Anal. Chim. Acta* **1977**, *90*, 215–221.

Grunau, J. A.; Swiader, J. M. Application of ion chromatography to anion analysis in vegetable leaf extracts. *Commun. Soil Sci. Plant Anal.* **1986**, *17*, 321–335.

Hageman, R. H.; Flesher, D.; Gitter, A. Diurnal variation and other light effects influencing the activity of nitrate reductase and nitrogen metabolism in corn. *Crop Sci.* **1961**, *1*, 201–204.

Heckenberg, A. L.; Alden, P. G.; Wildman, B. J.; Krol, J.; Romano, J. P.; Jackson, P. E.; Jandik, P.; Jones, W. R. *Waters innovative methods for ion analysis*; Millipore Corp.: Milford, MA, 1989; pp 1–4.

Kalbasi, M.; Tabatabai, M. A. Simultaneous determination of nitrate, chloride, sulfate, and phosphate in plant materials by ion chromatography. *Commun. Soil Sci. Plant Anal.* **1985**, *16*, 787–800.

Kulm, R. D. Nitrate content and nitrate reductase activity in *Melilotus*. M.Sc. Thesis, University of Nebraska, Lincoln, NE, 1973.

Lowe, R. H.; Hamilton, J. L. Rapid method for determination of nitrate in plant and soil extracts. *J. Agric. Food Chem.* **1967**, *15*, 359–61.

Manglitz, G. R.; Gorz, H. J. Host-range studies with the sweetclover weevil and the sweetclover aphid. *J. Econ. Entomol.* **1964**, *57*, 683–687.

Monroe, J. A.; Leraas, M. A.; Nostdahl, W. D. Biology and control of the sweetclover weevil. *J. Econ. Entomol.* **1949**, *42*, 318–321.

Nieto, K. F.; Frankenberger, W. T. Single column ion chromatography: I. Analysis of inorganic anions in soils. *Soil Sci. Soc. Am. J.* **1985**, *49*, 587–592.

Paul, J. L.; Carlson, R. M. Nitrate determination in plant extracts by the nitrate electrode. *J. Agric. Food Chem.* **1968**, *16*, 766–768.

SAS Institute. *SAS User's Guide*; SAS Institute: Cary, NC, 1989; 596 pp.

Singh, J. P. A rapid method for determination of nitrate in soil and plant extracts. *Plant Soil* **1988**, *110*, 137–139.

Tanaka, A.; Nose, N.; Iwasaki, H. Spectrophotometric determination of nitrate in vegetable products using 2-sec-butylphenol. *Analyst* **1982**, *107*, 190–4.

Received for review March 12, 1992. Revised manuscript received May 26, 1992. Accepted June 9, 1992.

Registry No. Nitrate, 14797-55-8.