Communications in Soil Science and Plant Analysis
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lcss20

A microscale method for colorimetric determination of urea in soil extracts

N.S. Greenan [a] , R.L. Mulvaney [a] & G. K. Sims [b]

[a] Department of Agronomy , University of Illinos , Urbana, IL, 61801

[b] United States Department of Agriculture Agricultural Research Service , 1102 South Goodwin Avenue, Urbana, IL, 61801

Published online: 11 Nov 2008.

To cite this article: N.S. Greenan , R.L. Mulvaney & G. K. Sims (1995) A microscale method for colorimetric determination of urea in soil extracts, Communications in Soil Science and Plant Analysis, 26:15-16, 2519-2529, DOI: 10.1080/00103629509369465

To link to this article: http://dx.doi.org/10.1080/00103629509369465

COMMUN. SOIL SCI. PLANT ANAL., 26(15&16), 2519-2529 (1995)

A MICROSCALE METHOD FOR COLORIMETRIC DETERMINATION OF UREA IN SOIL EXTRACTS

N. S. Greenan and R. L. Mulvaney
Department of Agronomy, University of Illinos, Urbana, IL 61801

G. K. Sims
United States Department of Agriculture Agricultural Research Service, 1102 South Goodwin Avenue, Urbana, IL 61801

ABSTRACT: The diacetyl monoxime colorimetric method of determining urea in soil extracts was modified for microplate format. A 100-μL aliquot of extract was treated with color reagent in a disposable plastic microtiter plate (96 wells/plate), and color was developed by heating the plate in a low-temperature oven at 87°C for 55 min. After cooling for 20 min at ambient temperature, absorbance measurements were simultaneously performed on all 96 wells using a microplate reader. The microscale method was faster and more convenient than the conventional method; moreover, the volume of waste was markedly reduced. Studies to compare the two methods showed very little difference in accuracy, precision, or sensitivity.

INTRODUCTION

The diacetyl monoxime colorimetric method for urea analysis involves measuring the red color formed when urea is heated with diacetyl monoxime and thiosemicarbazide under acidic conditions (1).

A major difficulty with this method has been the instability of the color formed and its sensitivity to light (2,3). In the modified diacetyl monoxime method of Mulvaney and Bremner (3), color development is carried out in the absence of light, and absorbance measurements are completed as rapidly as possible to minimize the variability due to color fading and instability.

With the advent of automated spectrophotometers designed to carry out colorimetric determinations in microtiter plates, large numbers of colorimetric analyses

may be easily accomplished by a batch process in which absorbance measurements are simultaneously performed on all 96 wells in the microplate. Microplate methods have been developed for determination of ammonium (NH_4^+)-, nitrate (NO_3^-)-, and nitrite (NO_2^-)-nitrogen (N) in soil extracts (4).

The microplate approach has obvious advantages for application to the diacetyl method for urea-N analysis. All samples in the plate are analyzed simultaneously, eliminating the problems associated with color instability and fading. Moreover, a much larger number of samples may be processed than with the conventional diacetyl method, and there is a dramatic reduction in the volume of reagents, and consequently also in the waste stream. Multichannel pipettes may be used to dispense reagents, further increasing the efficiency of sample preparation.

The purpose of the work reported here was to adapt the diacetyl monoxime colorimetric method of determining urea-N in soil extracts to microplate format. The microplate method was evaluated for accuracy, precision, and sensitivity relative to the conventional diacetyl method of urea-N analysis.

MATERIALS AND METHODS

Materials

Corning 96-well Assay Plates with Lids: Cat. #25880-96, Corning Glass Works, Corning, NY[1].

Kimble Shell Vials with Stoppers: Cat. #60930L-4 (21 mm od, 70 mm long).

Digital Microliter Pipette: adjustable to 100 µL.

Multichannel Pipette: adjustable to 150 µL.

Microplate Reader: capable of absorbance measurements at 500-550 nm. The Ceres Model 900 Microplate Workstation, available from BioTek Instruments, Winooski, VT, is satisfactory.

Microplate Shaker.

Laboratory Oven: capable of maintaining a temperature of 87±1°C.

1. Mention of a tradename or proprietary product does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

Reagents

Phenylmercuric Acetate (PMA) Solution: Phenylmercuric acetate (50 mg) was dissolved in distilled, deionized (DI) water in a 1-L volumetric flask.

Potassium Chloride-Phenylmercuric Acetate (2M KCl-PMA) Solution: Potassium chloride (1.5 kg) was dissolved in 9 L of DI water, and 1 L of PMA solution was added.

Diacetyl Monoxime (DAM) Solution: Diacetyl monoxime (3.75 g) was dissolved in 100 mL of DI water.

Thiosemicarbazide (TSC) Solution: Thiosemicarbazide (0.375 g) was dissolved in 100 mL DI water.

Acid Reagent: To 150 mL of concentrated (8.7M) phosphoric acid (H_3PO_4), 6 mL of concentrated (18M) sulfuric acid (H_2SO_4) were added, and the mixture was diluted to 200 mL with DI water and mixed thoroughly.

Color Reagent: To 100 mL of the acid reagent were added 5 mL of DAM solution and 3 mL of TSC solution. This reagent was prepared immediately before use.

Standard Urea-N Solution: Pure, dry urea (0.2143 g) was dissolved in 2M KCl-PMA in a 500-mL volumetric flask, diluted to volume with 2M KCl-PMA, and mixed thoroughly. This solution contained 200 mg of urea-N/L and was stored in a refrigerator.

Working Standards for Calibration up to 20 mg of Urea-N: 0-, 1-, 3-, 5-, 10-, 13-, 15-, and 20-mL aliquots of the standard urea-N solution were dispensed into shell vials, brought to 20 mL with KCl-PMA, and mixed thoroughly.

Method

A 100-μL aliquot of each diluted soil extract (see below) and each working standard urea-N solution was pipetted into individual wells of the assay plate. Using a multichannel pipette, 150 μL of color reagent were added to each well. The assay plate was then covered with a matching lid, followed by vortex mixing for 1 min to ensure uniformity. The covered plate was immediately placed in a laboratory oven maintained at 87°C. After 55 min, the covered plate was removed from the oven and allowed to cool for 20 min at room temperature without exposure to light (e.g., by placement in a light-tight drawer). The lid was then removed and the plate was loaded into the microplate reader for absorbance measurements.

A calibration curve was prepared by regression analysis of the data obtained with the working standard urea-N solutions. Absorbance values for soil extracts were converted to urea-N concentrations using the linear regression coefficients from the standards.

Evaluation

Surface (0-15 cm) samples of three soils (Table 1), selected to obtain a wide range in properties, were collected from cultivated or uncultivated sites in California and Illinois. Before use, the samples were air-dried and crushed to pass through a 2-mm screen. The analyses reported in Table 1 were performed as described by Mulvaney and Kurtz (5).

To prepare soil extracts with known concentrations of urea-N, triplicate 5-g samples of soil in 125-mL wide-mouth polyethylene bottles were treated with 1 mL of DI water containing 0.5, 3.75, or 10 mg of urea-N. Samples were immediately treated with 50 mL of 2M KCl-PMA solution. The resulting suspensions were shaken for 1 h, and then filtered through Whatman No. 42 filter paper under vacuum (1). To increase the aliquot volume and thereby improve the accuracy of pipetting for urea analysis by the microplate method, a 1-mL aliquot of each soil extract was pipetted into a shell vial, diluted with 9 mL of KCl-PMA, stoppered, and mixed thoroughly.

RESULTS AND DISCUSSION

Table 2 shows the periods required for maximal color development at different temperatures. As expected, heating markedly increased the rate of color development. The upper limit for heating is 90°C, the melting point of the polystyrene microplate. No warping, fogging, or deformation was observed when heating was carried out at 87°C as recommended. At room temperature, color development was complete after 16 h, but the absorbance was more variable than with heating.

Figure 1 shows the intensity of the color obtained by heating at 87°C for different periods. Maximal color development occurred after 55 min. Color development was incomplete with shorter heating times, whereas heating for longer periods led to a decrease in absorbance. Figure 1 also shows the effect on absorbance when the microplate was allowed to cool at 23°C for various periods. With short periods for heating (20-45 min), color development continued during

TABLE 1. Analyses of Soils.

Soil			Total	Organic			
Series	Subgroup	pH	N	C	Sand	Silt	Clay
			g/kg				
Flanagan	Aquic Argiudoll	6.3	2.06	24.7	98	624	278
Argonaut	Mollic Haploxeralf	6.1	2.63	29.8	260	540	200
Houghton	Typic Medisaprist	7.6	7.93	126.3	—	—	—

TABLE 2. Periods For Maximal Development of Color at Different Temperatures.

Temperature	Period for color development
°C	min
23	960
45	140
55	100
75	85
87	55

cooling, and the longest cooling time gave the highest absorbance. With longer periods for heating, the color faded during cooling, and the longest cooling time gave the lowest absorbance.

Figure 2 shows the effect of cooling on the precision of absorbance measurements. Cooling for 5 min markedly reduced the variability in absorbance, as compared to analyses when no period was provided for the plate to cool. Cooling for 10 to 20 min led to a further decrease in the coefficient of variation. A 20-min period was chosen as the optimal cooling time because longer periods gave about the same coefficient of variation but a lower absorbance, presumably owing to destruction of the chromophore.

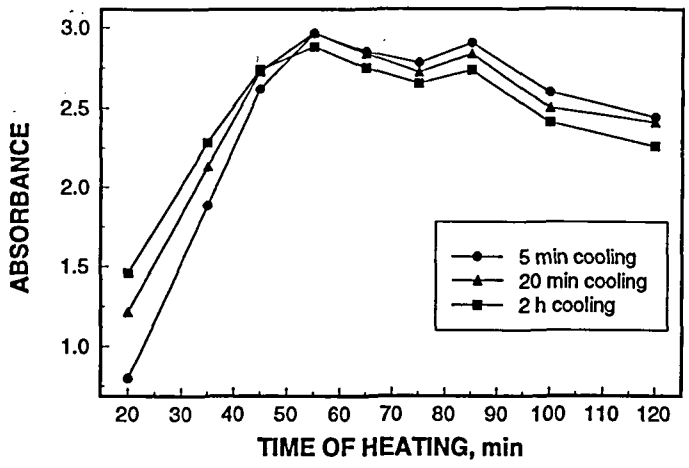

FIGURE 1. Effects of Time of Heating (at 87°C) and Time of Cooling (at 23°C) on Absorbance of Reaction Product.

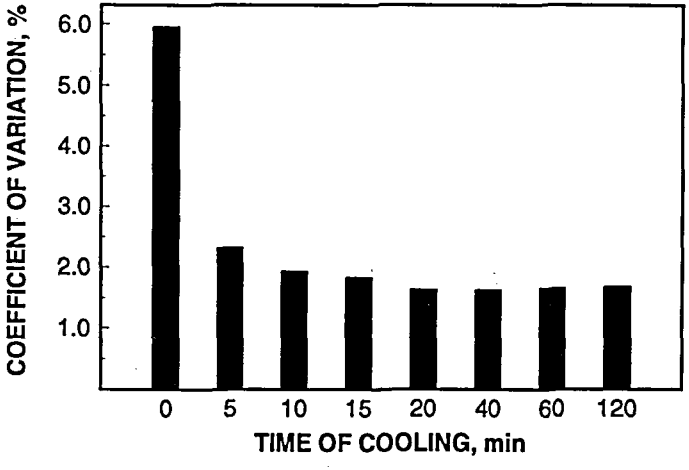

FIGURE 2. Precision of Absorbance Measurements after Various Cooling Times.

Figure 3 plots absorbance measurements by the method described for urea-N concentrations of 0-50 mg/L. Calibration was linear for urea-N concentrations up to 25 mg/L. Figure 4 shows a typical calibration curve obtained with the recommended range of urea-N standards (0-20 mg/L).

As noted in the Introduction, color instability has been a major problem with the conventional diacetyl monoxime method. To minimize this difficulty, Mulvaney and Bremner (3) recommended that heating to develop color be done in the absence of light. In the microplate method described, this was accomplished through the use of a laboratory oven that excluded light. No evidence has been obtained to indicate that the red color formed is sensitive to laboratory lighting during the 20-min period recommended for cooling. However, different batches of H_3PO_4 may cause variable sensitivity to light (3). For this reason, the microplate should be allowed to cool inside a light-tight laboratory drawer.

The analytical data in Table 3 show that the microplate method compared very favorably with the conventional method with regard to both accuracy and precision. With the two highest levels of urea-N addition (750 and 2000 mg/kg), recoveries ranged from 96-100%, and the coefficient of variation was usually <1%. Both methods overestimated the recovery when analyses were performed on extracts obtained with the lowest addition of urea-N (100 mg/kg). The overestimation, which was more serious with the conventional method than with the microplate method, can be attributed to the use of a single regression equation for calibration over the entire range of urea-N standards. Overestimation with the microplate method was eliminated when a separate regression was done using absorbance data for standards containing 0-5 mg of urea-N/L, which closely bracketed the data from extracts obtained with the lowest addition of urea-N (Table 3).

The red color formed by reaction of urea with DAM in the presence of TSC exhibits maximal absorbance at 527 nm (2), and the highest sensitivity is achieved when measurements are made at this wavelength. But with most microplate readers, wavelength selection is accomplished through the use of a colored filter rather than a monochromator, and absorbance measurements may not be possible at 527 nm. In such cases, a filter should be used that provides the closest available wavelength, within the range of 500 to 550 nm.

The diacetyl method of urea-N analysis is more specific than other non-enzymatic methods for this analysis, and most of the interferences arise from compounds that are unlikely to occur in soil extracts, such as substituted ureas,

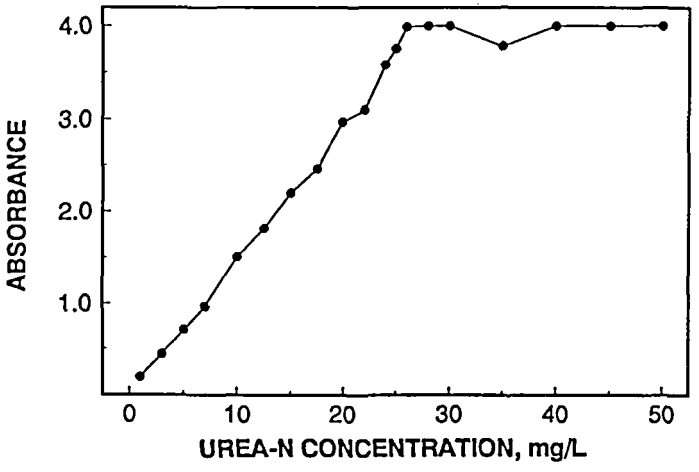

FIGURE 3. Relationship Between Urea-N Concentration and Absorbance of Reaction Product.

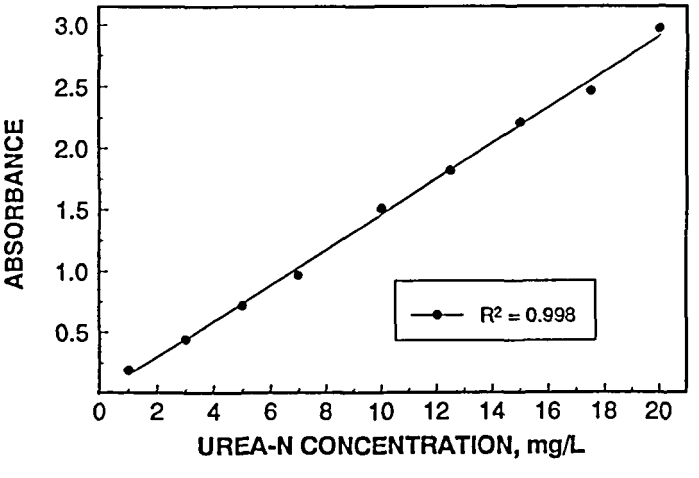

FIGURE 4. Typical Calibration Curve.

TABLE 3. Comparison of Conventional and Microplate Methods for Urea-N Analysis of Soil Extracts.

Soil	Urea-N added	Urea-N recovered†			
		Conventional method		Microplate method‡	
		Mean	SD	Mean	SD
	mg/kg	%			
Flanagan	100	107.2	1.1	101.0 (97.8)	0.9
	750	97.0	1.1	98.3	0.9
	2000	100.1	0.3	100.1	0.8
Argonaut	100	110.6	1.4	103.8 (98.6)	1.2
	750	96.4	0.3	98.1	0.7
	2000	99.7	0.9	99.7	0.2
Houghton	100	109.5	1.9	105.6 (100.0)	1.7
	750	97.3	0.8	99.2	0.6
	2000	99.7	0.5	99.5	0.2

†Three analyses. SD = standard deviation.
‡Values in parentheses were calculated by regression of absorbance data obtained with standards containing 0-5 mg of N/L.

carbamido compounds, or chemical reducing agents (6,7). Nitrite interferes if the concentration of NO_2^--N in the extract is higher than the concentration of urea-N (2,7), but such interference is easily avoided by addition of sulfamic acid to decompose NO_2^- (8).

With the conventional diacetyl monoxime method, up to 96 analyses can be performed in a normal working day. Throughput capacity is substantially higher with the method described here. Up to 88 samples (and eight standards) can be analyzed per microplate, and at least four plates may be prepared and processed during a normal working day. Moreover, the volume of reagents is less than 1% of that required by the conventional method; this reduces the cost of consumables and the amount of analytical waste.

Automated systems utilizing flow analysis have been employed as a means of achieving high throughput capacity in colorimetric determinations of urea-N in soil extracts (7,9,10). The microplate approach has the same advantage and is inherently

simpler, uses less expensive equipment, produces less waste, and poses a lower risk of carryover contamination (4).

CONCLUSIONS

The diacetyl method of urea-N analysis was successfully adapted to microplate format. Errors from color fading and instability are eliminated, because absorbance measurements are simultaneously performed on all 96 wells in the microplate. Throughput capacity is at least four times higher than with the conventional diacetyl method, while requiring less than 1% of the volume of reagents per sample. The microplate method was comparable to the conventional method with respect to accuracy and precision. Because of the very small volumes used with the microplate format, pipetting equipment must be carefully calibrated to achieve high accuracy and precision.

ACKNOWLEDGMENT

We thank D. J. Herman, University of California, Berkeley, for providing the Argonaut soil used in our work.

REFERENCES:

1. Bremner, J. M. 1982. Nitrogen-Urea, pp. 699-709. IN: A. L. Page et al. (eds.) Methods of Soil Analysis, Part 2. Agronomy Monograph 9. 2nd edition. American Society of Agronomy, Madison, WI.

2. Douglas, L. A. and J. M. Bremner. 1970. Colorimetric determination of microgram quantities of urea. Anal. Lett. 3:79-87.

3. Mulvaney, R. L. and J. M. Bremner. 1979. A modified diacetyl monoxime method for colorimetric determination of urea in soil extracts. Commun. Soil Sci. Plant Anal. 10:1163-1170.

4. Sims, G. K, T. R. Ellsworth, and R. L. Mulvaney. 1995. Microscale determination of inorganic nitrogen in water and soil extracts. Commun. Soil Sci. Plant Anal. 26:303-316.

5. Mulvaney, R. L and L. T. Kurtz. 1982. A new method for determination of ^{15}N-labeled nitrous oxide. Soil Sci. Soc. Amer. J. 46:1178-1184.

6. Butler, A. R. and D. Walsh. 1982. Colorimetric non-enzymic methods for the determination of urea. Trends Anal. Chem. 1:120-124.

7. Sullivan, D. M. and J. L. Havlin. 1991. Flow injection analysis of urea nitrogen in soil extracts. Soil Sci. Soc. Amer. J. 55:109-113.

8. Keeney, D. R., and D. W. Nelson. 1982. Nitrogen-Inorganic forms, pp. 643-698. IN: A. L. Page et al. (eds.) Methods of Soil Analysis, Part 2. Agronomy Monograph 9. 2nd edition. American Society of Agronomy, Madison, WI.

9. Douglas, L. A., H. Sochtig, and W. Flaig. 1978. Colorimetric determination of urea in soil extracts using an automated system. Soil Sci. Soc. Amer .J. 42:291-292.

10. Technicon Industrial Systems. 1980. Urea in soil extracts. Industrial Method 600-80A. Technicon Industrial Systems, Tarrytown, NY.