Determination of Nitric Oxide in Biological Samples Using *N,N,N′,N′*-Tetramethyl-*p*-phenylenediamine by UV VIS Spectrophotometry

[a]Š. MESÁROŠ * and [b]S. GRUNFELD

[a]*Department of Analytical Chemistry, Faculty of Chemical Technology, Slovak Technical University, SK-812 37 Bratislava*

[b]*Department of Chemistry, Oakland University, Rochester MI 48309, USA*

Received 12 December 1997

A highly sensitive, simple, fast, and specific procedure for the determination of nitric oxide and nitrite, its decay product in biological system is described. The organic dye agent *N, N, N′ N′*-tetramethyl-*p*-phenylenediamine was used with nitric oxide and nitrite to form a brightly coloured species which can be detected by UV VIS spectrophotometry. The redox dye formed (Wurster's Blue) has three absorption bands in the ultraviolet and visible region at the wavelengths 328 nm, 564 nm, and 612 nm. All three bands can be used for quantitative determination of nitric oxide and nitrite. A detection limit of 15 nmol dm^{-3} was achieved for nitric oxide. The method was applied to the measurement of nitric oxide release from macrophage and endothelial cells. The experimental results are compared with those obtained using the Griess method and by electrochemical method.

Nitric oxide ($NO^{\bullet}$) is a small reactive radical with a high affinity for ferrous hemoproteins such as soluble guanylate cyclase and hemoglobin. It plays a role, often as a biological messenger, in an astonishing range of physiologic processes in humans and other animals. The expanding range of known functions includes: neurotransmission, blood pressure control, and a role in the immune system ability to kill tumour cells and intracellular parasites [1].

The most widely used methods of $NO^{\bullet}$ detection are based on three methodologies, electron paramagnetic resonance, UV VIS spectrophotometry, and chemiluminescence [2].

The typical and commonly used spectrophotometric method is rooted in the classical Griess reaction [3]. Because nitrite and nitrate are stable metabolic oxidative products of nitric oxide, the Griess reaction had been used to measure nitrite and nitrate by the Stainton method [4]. It has been reported in recent publications [5—8] that nitric oxide was detected by chemiluminescence while nitrite and nitrate were quantified by the Griess reaction in biological media. The detection limit of this method is 5×10^{-8} mol dm^{-3} An automatic system for the Griess method has been developed [9] with a linear response to both nitrite and nitrate in the range of 1 to 300 μmol dm^{-3}

Although the Griess method is sensitive to nitric oxide and nitrite, the detection limits are still not low enough for the determination of the trace concentrations of nitric oxide that occur in biological systems such macrophages and endothelial cells. Our objective in this work was to use another reagent [10] that would react with nitric oxide to produce visible spectra with higher absorbance than those obtained with the Griess reagent. In this paper, we present a highly sensitive and simple analytical method that can be used to measure the very low concentrations of nitric oxide found in biological systems after reaction of $NO^{\bullet}$ with *N, N, N′, N′*-tetramethyl-*p*-phenylenediamine (NTMPD). The method is applied to the analysis of nitric oxide in synthetic solutions and then extended to the determination of the concentration of nitric oxide in macrophages and endothelial cells.

The spectrophotometric methods with NTMPD were applied previously in a wide range of analytical applications in determination of nitrite [11], ozone [12], and such cations as: Ag^{+} Cu^{2+} Hg^{2+} Os^{8+} and others [13]. In this paper we for the first time use NTMPD for spectrophotometric determination of nitric oxide.

EXPERIMENTAL

The *N, N, N′, N′*-tetramethyl-*p*-phenylenediammonium dichloride, *p*-aminobenzenesulfonamide (sul-

*The author to whom the correspondence should be addressed.

fanilamide), and *N*-(1-naphthyl)ethylenediammonium dichloride were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium chloride and hydrochloric acid were obtained from Fisher Scientific Co. Inc. (Fair Lawn, NJ). Hank's Balanced Salt Solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM), Phosphorus Buffer Saline (PBS), and Fetal Bovine Serum (FBS) were obtained from Beaumont Hospital (Royal Oak, MI). Bradykinin, γ-Interferon (500 units/cm^3), LipoPolysaccharide (LPS) (0.1 mg mm^{-3}), and Calcium Ionophore A23187 were obtained from Sigma Chemical Co. (St. Louis, MO).

Macrophages (RAW 264.7) were obtained from the American Type Culture Collection. Cells were maintained in continuous culture in a T75 tissue-culture flask in DMEM containing 10 % FBS. Cells were harvested by gentle scraping and passaged every 7 days by dilution of a suspension of the cells (12:1) in fresh medium. After discarding the DMEM media, cells were rinsed two times with PBS and eventually replaced with 3.00 cm^3 of PBS containing LPS or 500 units/cm^3 of gamma interferon. Next, the cells were incubated at 37 °C for 1 to 30 h followed by an aliquot of media (20 to 60 mm^3) being transferred to the cuvette and mixed with 1 cm^3 of reagents. The reference used consisted of an identical amount of fresh medium treated in the same manner. After the 5 min elapse for the colour formation reaction to occur, the UV VIS absorption spectrum was recorded.

Endothelial cells from normotensive (WKY) and hypertensive (SHRSP) rats were in 60 mm × 15 mm Petri dishes. After being washed twice with HBSS and then equilibrated with 1 cm^3 of HBSS for 10 min at 37 °C, 15 mm^3 of bradykinin or 30 mm^3 of calcium-ionophore were added to stimulate NO$^\bullet$ release from endothelial cells. After 5 min, an aliquot of 20 to 60 mm^3 of cell culture medium was transferred to the cuvette which contained 1 cm^3 of reagents. The cell culture medium was used as a reference.

All absorbance measurements were performed on a Milton Roy Spectronic 3000 Diode Array UV VIS Spectrophotometer with 1 cm cells. The pH measurements were performed on an Orion Research Model 701A/Digital Ionalyzer with a general purpose glass electrode.

Measurement of Nitric Oxide

0.14 cm^3 of *N*, *N*, *N'*, *N'*-tetramethyl-*p*-phenylenediamine solution was added to a 10 cm^3 flask thus making 0.14 mmol dm^{-3} of the phenylenediamine in final concentration. After the pH was adjusted to 2.00, the solution was purged with nitrogen for about 5 min to deoxygenate. 1.0 cm^3 of this reagent solution was transferred into 1 cm cuvette. For the preliminary investigation of experimental conditions, an aliquot (NO$^\bullet$ concentration 0.5 μmol dm^{-3}) of standard nitric oxide was transferred to this 1 cm cuvette and mixed with the reagents. After 5 min of colour development, absorbances at the wavelengths 328 nm, 564 nm, and 612 nm were measured on spectrophotometer.

Electrochemical Determination of Nitric Oxide

The electrochemical experiments were carried out in cell at 37 °C with the EG&G PAR Potentiostat/Galvanostat M273A (Princeton, NJ) with custom data acquisition and control electrochemical software. The working electrode was a nitric oxide metalloporphyrine biosensor [14]. A saturated calomel reference electrode (SCE) and Pt wire auxiliary electrode were used.

RESULTS AND DISCUSSION

Spectrophotometric determinations of nitric oxide and nitrites with Griess reagent are based on a diazotization reaction followed by a coupling reaction. The azo compound formed from Griess-type reactions has absorptions in the UV and visible region (300 to 700 nm). A pink colour is observed after reaction of nitric oxide and nitrite ions with Griess reagent.

The mechanism of the reaction of NTMPD with NO$^\bullet$ is of the redox type and involves only one step. The radical cation called Wurster's Blue is formed [10]. This step is dependent only on the concentration of NTMPD.

The unreacted NTMPD species shows no absorption in the range from 300 nm to 700 nm. However, after adding 0.5 μmol dm^{-3} of NO$^\bullet$ to the solution, the spectra show three absorption bands at 328 nm, 564 nm, and 612 nm, respectively. The maximum absorption occurs at 328 nm and the bands at 564 nm and 612 nm have almost identical, but lower, absorbance. The absorption spectra of NTMPD alone and with NO$^\bullet$ are shown in Fig. 1. The band at 328 nm was used for NO$^\bullet$ determination.

In order to obtain reproducible results, the NTMPD solution was freshly prepared every time when an experiment was performed. Other parameters that needed to be optimized or studied in the development of the method were pH, reagent concentration, colour development and dye stability, and the effect of interferences.

Effect of pH on the Reaction of NO$^\bullet$ with NTMPD

The experiment was performed at pH values of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.4, and 8.5. Hydrochloric acid and sodium hydroxide were used to adjust pH. The effect of pH is shown in Fig. 2. The figure shows that the range of pH that gives maximum absorption is 2.0 to 4.0 whereas a pH above 4.0 produces a rapid decrease in absorbance. Conversely, when pH is higher than 6.0, the absorbance almost decreases to zero. Also the

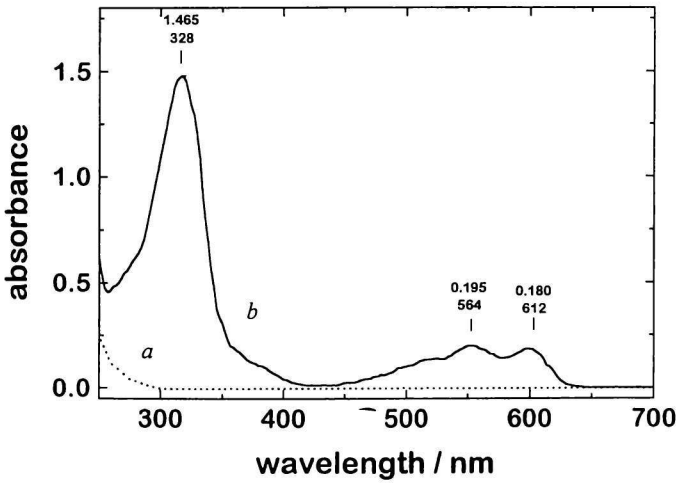

Fig. 1. The absorption spectra of NTMPD reacting with NO$^{\bullet}$ at pH 2.0. *a*) Absorbance of 0.14 mmol dm^{-3} NTMPD *vs.* distilled water – dashed line; *b*) absorbance of 0.14 mmol dm^{-3} NTMPD with 0.5 μmol dm^{-3} NO$^{\bullet}$ *vs.* 0.14 mmol dm^{-3} NTMPD blank – solid line.

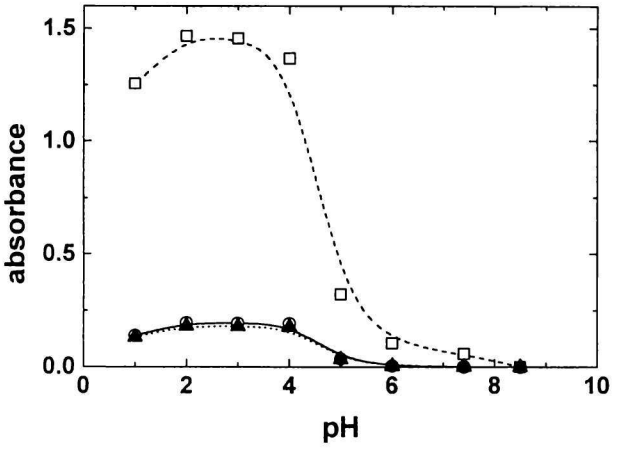

Fig. 2. The effect of media acidity (pH) on the reaction of 0.14 mmol dm^{-3} NTMPD with NO$^{\bullet}$ Responses are absorbances at 328 nm (□, dashed line), 564 nm (○, solid line), and 612 nm (▲, dotted line) *vs.* pH in the presence of 0.5 μmol dm^{-3} NO$^{\bullet}$

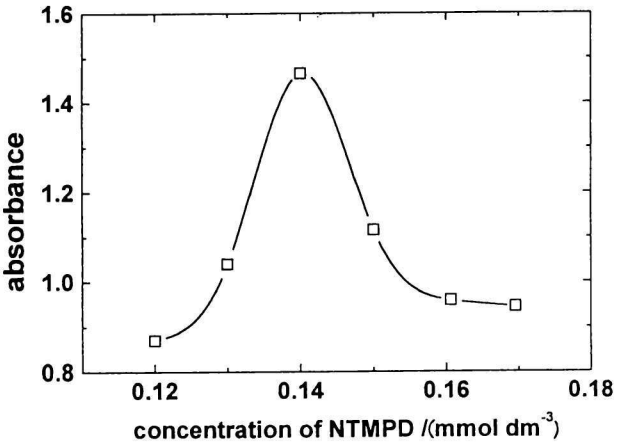

Fig. 3. The dependence of absorbance on concentration of N, N, N', N'-tetramethyl-*p*-phenylenediamine in the presence of 0.5 μmol dm^{-3} NO$^{\bullet}$ in pH 2.0 media.

bluish colour was observed when pH was higher than 4.0 even without addition of nitric oxide and nitrite ion. Therefore, the optimal pH interval for the quantitative analysis is in the range of 2.0 to 4.0.

Effect of Concentration of NTMPD

The concentration of NTMPD was varied from 0.12 mmol dm^{-3} to 0.17 mmol dm^{-3} The absorption of each solution was obtained using the procedure described in Experimental with 0.5 μmol dm^{-3} of NO$^{\bullet}$ at pH 2.0. The results show that the absorbance had a maximum when the concentration of N, N, N', N'-tetramethyl-*p*-phenylenediammonium dichloride was 0.14 mmol dm^{-3} (Fig. 3).

The optimized conditions for the method: pH range 2.0 to 4.0 and 0.14 mmol dm^{-3} of NTMPD were used and a definite amount of nitric oxide (0.5 μmol dm^{-3}) was added to each sample solution for studies of colour development and dye stability and the effect of interferences.

Colour Development and Dye Stability

During the colour development experiments, pH 2.0 and pH 4.0 were used and samples were treated by the procedure described previously.

Colour development for the reaction of NTMPD with NO$^{\bullet}$ solutions subjected to analysis by the method proposed was followed on the Milton Roy Spectronic 3000 Array at 10 s intervals for 450 s in the initial stage and at 60 s intervals for 45 min.

Table 1. Interferences by Selected Species

Species	Final concentration	Absorbance change
	μmol dm^{-3}	%
Calcium	100	0.2
Copper	200	0.4
Sodium	200	1.2
Potassium	200	0.3
Magnesium	100	1.0
Aluminium	100	1.3
Iron(III)	1	> 78[a]
		0.8[b]
Sulfate	250	1.1
Phosphate	250	0.4
Chloride	250	0.3
Glucose	65 000	0.7
Urea	4 540	0.5
Cysteine	794	0.3
Ascorbate	497	0.4
Dopamine	0.03	0.3
Norepinephrine	0.004	0.1
Epinephrine	0.003	0.2

a) Without EDTA; *b*) with EDTA.

At pH 2.0, a rapid increase in absorbance was observed. The absorbance reached a maximum intensity within 6 min and remained constant for at least 45 min. After this time, the colour of the solution started fading. For the pH 4.0 sample, colour development was much slower than for the pH 2.0 sample. Its maximum intensity was reached at 45 min and remained constant for an additional 45 min. Therefore, a solution of pH 2.0 is optimal for determination of NO$^{\cdot}$ because of the short reaction time and higher absorbance. The colour development in this proposed method is faster than that observed in either the Griess method or the Shinn method [3, 15]. The classical Griess method requirement of 10 to 30 min was needed to reach the maximum absorbance [16].

Studies of Interferences

The effect of possible interferences was studied using 0.5 μmol dm^{-3} of nitric oxide with the procedure previously described. Because the proposed method is primarily designed for the analysis of biological samples, the specific interference studies were conducted with ions that commonly occur in such samples: calcium, sodium, potassium, magnesium, aluminium, copper, chloride, sulfate, phosphate, and iron(III).

Initially, potential interference ion was introduced to the reaction mixture. Immediately preceding, the interference ions were successively added in small amount, while the change of absorbance was monitored.

As the results in Table 1 show, calcium, sodium, potassium, magnesium, aluminium, copper, chloride, sulfate, and phosphate do not exhibit changes in absorbances indicating lack of interference. The most serious interferences were observed in the presence of iron(III), one reason may be due to the redox reaction of iron(III) to iron(II). Iron(III) may oxidize NTMPD to a highly coloured product which absorbs at 550 nm. A possible method to eliminate the interference caused by iron(III) is to add a strong complexing agent such as EDTA [17]. Trace amounts of phosphate can inhibit the oxidation of NTMPD by iron(III). Other elements such as copper(II) interfere with most of the Griess-type procedures [18], yet they do not interfere in this procedure. The specificity of the reaction of NTMPD with NO$^{\cdot}$ in biological system was determined for various substrates such as ascorbate, glucose, urea, cysteine, catecholamines, *etc.*, at up to ten times higher concentration than is the maximum physiological concentration. No discernible signal was detected above the background, as can be seen from Table 1.

Sensitivity and Detection Limit

The linearity of the change of absorbance with the change of the amounts of nitric oxide present was tested by preparing standard solutions containing nitric oxide and then measuring the absorbance at 328 nm. Lambert—Beer's law is obeyed in this range. Calibration curves were constructed for the nitric oxide concentration range from 0.0 to 1.0 μmol dm^{-3} A calibration curve for nitrite was also constructed using the same method in the concentration range from 0.0 to 10.0 μmol dm^{-3} Equations for the calibration curves were obtained after fitting the experimental data by the least-squares method (Table 2).

The sensitivity of the method expressed as the concentration of nitric oxide corresponding to an absorbance of 0.013 in a 1 cm cell at the wavelength 328 nm is 0.015 μmol dm^{-3} The molar absorptivities are 2.38×10^6 mol^{-1} cm^{-1} dm^3 and 5.27×10^4 mol^{-1} cm^{-1} dm^3 for NO$^{\cdot}$ and nitrite, respectively.

The method using NTMPD reagent has a higher sensitivity than the Griess method: a smaller analyte concentration can produce the same analytical signal as obtained using Griess reagent. The method being proposed has a detection limit about one order of magnitude lower than that reported for the Griess method [3].

The spectrum of the Griess reagent has only one maximum absorbance band at 546 nm; NTMPD has three absorbance peaks with the maximum absorbance at 328 nm. This peak has higher molar absorptivity than the Griess reagent at 546 nm peak. Thus, higher sensitivity can be achieved by using the method described in this paper.

Determination of NO$^{\cdot}$ in Biological Samples

The optimized method was extended to the investigation of two types of cells, macrophages and endothe-

Table 2. The Equations of Calibration Curves for NO$^{\bullet}$ and Nitrite

Nitric oxide	Nitrite
$A = -26.4 \times 10^{-3} + 2.64 \times 10^{-3}$ {[NO$^{\bullet}$]}	$A = -23.4 \times 10^{-3} + 57.36 \times 10^{-2}${[$NO_2^-$]}
conc. range: 0—1000 nmol dm^{-3}	conc. range: 0—10 μmol dm^{-3}
$\varepsilon = 2.38 \times 10^6$ mol^{-1} cm^{-1} dm^3	$\varepsilon = 5.27 \times 10^4$ mol^{-1} cm^{-1} dm^3
$c_{min} = 15$ nmol dm^{-3}	$c_{min} = 110$ nmol dm^{-3}

A – absorbance, ε – molar absorptivity, c_{min} – limit of detection.

Table 3. The Determination of NO$^{\bullet}$ Released from Macrophages (RAW 264.7) Agonized by LPS (0.1 mg mm^{-3})

Incubation time	$c_{NO^{\bullet}}$ A*	$c_{NO^{\bullet}}$ B*	$c_{NO^{\bullet}}$ C*
h	nmol dm^{-3}	nmol dm^{-3}	nmol dm^{-3}
6	44 ± 20	99 ± 45	47 ± 14
9	110 ± 31	179 ± 47	105 ± 24
12	345 ± 42	417 ± 69	362 ± 39
20	993 ± 124	1236 ± 211	976 ± 131

All the values are mean ± interval of confidence ($\alpha = 0.05$) of ten experiments. A – UV VIS method with NTMPD; B Griess method; C – electrochemical determination.

Table 4. The Determination of NO$^{\bullet}$ Released from Endothelial Cells. WKY – Normotensive Rats; SHRSP – Hypertensive Rats

Sample	Agonist	$c_{NO^{\bullet}}$ A*	$c_{NO^{\bullet}}$ B*	$c_{NO^{\bullet}}$ C*
		nmol dm^{-3}	nmol dm^{-3}	nmol dm^{-3}
WKY	Bradykinin	124 ± 15	154 ± 24	127 ± 13
SHRSP	Bradykinin	67 ± 11	83 ± 21	63 ± 14
WKY	Ca-Ionoph	167 ± 18	197 ± 31	159 ± 17
SHRSP	Ca-Ionoph	89 ± 14	105 ± 26	91 ± 16

All the values are mean ± interval of confidence ($\alpha = 0.05$) of ten experiments. A – UV VIS method with NTMPD; B – Griess method; C – electrochemical determination.

lial cells, known to have constitutive NO$^{\bullet}$ synthesis. In biological samples that contain enzyme constitutive nitric oxide synthase (cNOS), nitric oxide will be produced after the addition of an agonist. The half-life of NO$^{\bullet}$ in biological samples is 6 to 30 s. Therefore, most of the NO$^{\bullet}$ produced in biological systems may be partially oxidized to nitrite. However, at pH 2.0 nitrite is converting to NO$^{\bullet}$ Cell treatment was described in Experimental. The nitric oxide concentration was calculated by using the calibration curve.

In order to stimulate protection of NO$^{\bullet}$, macrophage cells were treated with 15 mm^3 of LPS (0.1 mg/mm^3) or 10 mm^3 of γ-interferon (500 units/cm^3). They were incubated at 37°C with 5 % CO_2 for 1 to 30 h. The commutation of nitric oxide and nitrite products was time-dependent and became detectable about six hours after the sample was exposed to LPS. The concentrations of nitric oxide were calculated by using calibration curve equations. The results are listed in Table 3 and compared with electrochemical measurements of NO$^{\bullet}$ by porphyrin microbiosensor.

Endothelial cells were treated with 15 mm^3 of 200 μmol dm^{-3} bradykinin (agonist), with no incubation, and the spectrophotometric response was obtained after 5 min. We also used 30 mm^3 of calcium ionophore as an agonist, the spectra were recorded after 3 min. Results of this experiment are listed in Table 4.

The method which we described in this paper proved to be successful for the determination of nitric oxide in biological samples. The results show more rapid colour development, higher sensitivity, and lower detection limit. These are significant improvements compared to other UV VIS spectrophotometric methods. In addition to these advantages, this method is relatively simple when compared to the electrochemical method (preparation of biosensor) and it can be used with standard UV VIS spectrophotometric equipment available in most chemical and biological laboratories. The method which is presented in this paper can find promising application in physiological, pathological, and pharmacological studies of nitric oxide.

REFERENCES

1. Moncada, S., *Acta Physiol. Scand. 145,* 221 (1992).
2. Archer, S., *FASEB J. 7,* 349 (1993).
3. Griess, J. P., *Ber. Deutsch. Chem. Ges. 12,* 426 (1879).
4. Stainton, M. P., *Anal. Chem. 46,* 1616 (1974).
5. Hibbs, J. B., Jr., Westenfelder, C., Taintor, R. R., Vavrin, Z., Kablitz, C., Baranowski, R. L., Ward, J. H., Menlove, R. L., McMurry, M. P Kushner, J. P and Samlowski, W E., *J. Clin. Invest. 89,* 867 (1992).
6. Myers, P P Guerra, R., Jr., and Harrison, D. G., *Am. J. Physiol. Heart Circ. Phy. 256,* H1030 (1989).
7 Palmer, J. R., Ferrige, A. G., and Moncada, S. *Nature 327,* 524 (1987).
8. Granger, D. L., Hibbs, J. B., Jr. and Broadnax, M., *J. Immunol. 146,* 1294 (1991).
9. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P L., Wishnok, J. S., and Tannenbaum, S. R., *Anal. Biochem. 126,* 131 (1982).
10. Albrecht, A. C. and Simpson, W T., *J. Am. Chem. Soc. 77,* 4454 (1955).

11. Kuemmel, D. F. and Mellon, M. G., *Anal. Chem. 28,* 1674 (1956).
12. Kulberg, L. M. *J. Appl. Chem. 10,* 567 (1937).
13. Galster, H., *Z. Anal. Chem. 186,* 359 (1962).
14. Malinski, T. and Taha, Z., *Nature 358,* 676 (1992).
15. Shinn, M. B., *Anal. Chem. 13,* 33 (1941).
16. Brunauer, S. and Emmett, P H., *J. Am. Chem. Soc. 57,* 1754 (1935).
17. Kershaw, N. F. and Chamberlin, N. S., *Ind. Eng. Chem. Res. 14,* 312 (1942).
18. Foris, A. and Sweet, T. R., *Anal. Chem. 37,* 701 (1965).