HPLC Method for the Analysis of the Urease Inhibitor *N*-(*n*-Butyl)thiophosphoric Triamide and Its Metabolites

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A method was developed to analyze the urease inhibitor *N*-(*n*-butyl)thiophosphoric triamide (NBPT) and its metabolites. The method consists of separation of the compounds via reverse-phase liquid chromatography followed by postcolumn hydrolysis using nitric acid and formation of fluorescent products using *o*-phthalaldehyde. The method is extremely sensitive and precise and has been employed to characterize the fate of NBPT and its oxon analogue in soils in the presence of a 20 000-fold molar excess of urea N. Because previous results had suggested that these compounds were unstable under some storage conditions, studies were also conducted to evaluate their stability during storage. Results showed that aqueous solutions containing NBPT could be stored without decomposition for at least 2 weeks at room temperature or when refrigerated. Considerable NBPT decomposition was observed, however, when solutions were stored at −20 °C, especially if they were acidic (pH 5.5). This decomposition at −20 °C was completely eliminated by amending the solutions with methanol to prevent freezing.

INTRODUCTION

The compound *N*-(*n*-butyl)thiophosphoric triamide (NBPT) is the most active compound identified to date for inhibition of soil urease (Bremner and Chai, 1986). Laboratory and field results have shown NBPT to be extremely effective in reducing ammonia losses and increasing plant yields when employed with surface-applied urea and urea–ammonium nitrate solutions (Christianson and Byrnes, 1991; Hendrickson, 1992).

Recent results have demonstrated that NBPT itself is not an active urease inhibitor but instead must be converted to *N*-(*n*-butyl)phosphoric triamide (BNPO), its oxon analogue (McCarty et al., 1990; Byrnes and Christianson, 1988). Little is known at this time concerning the agents responsible for this conversion or the factors controlling the fate of these compounds in soils. Because BNPO is active at concentrations below 10^{-8} M (Creason et al., 1990), proper characterization of the fate of these phosphoramides will require a method that is capable of quantifying them at such low concentrations.

Because both NBPT and BNPO are thermally labile, their analysis is not well suited to GC techniques, but the compounds are easily separated using reverse-phase HPLC. Neither compound is an especially active chromophore, with UV detection limits near 10^{-5} M at 200–210 nm. As such sensitivity is not adequate to characterize these compounds at typical biological concentrations, efforts were initiated to develop a more sensitive method. This paper describes a postcolumn detection method that was adapted from one described by Hill et al. (1984) for analysis of *N*-methylcarbamate pesticides. The method reported here is sufficiently sensitive to enable detection of BNPO in soil extracts at concentrations approaching those at which no inhibitory activity is observed. The method has also been employed to analyze a variety of other phosphoramides and their decomposition products.

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MATERIALS AND METHODS

Apparatus. The high-performance liquid chromatography (HPLC) system used in these studies (Figure 1) consisted of a Varian LC 5060 liquid chromatograph (Walnut Creek, CA) and a Varian 9090 autosampler utilizing a 50-μL loop valve injector. A mobile phase of 40/60 (v/v) methanol and water was pumped at a rate of 0.7 mL min^{-1}. A Du Pont Zorbax-Rx C-8 column (4.6 mm i.d. × 25 cm) was used in conjunction with a Zorbax-Rx Guard column (MAC-MOD Analytical, Chadds Ford, PA). The effluent stream was subsequently reacted, using a 1.59-mm stainless steel low-dead-volume mixing tee, with 0.3 M HNO_3 introduced at a rate of 0.1 mL min^{-1} using an Eldex (San Carlos, CA) positive displacement pump. The combined stream was then heated to 155 °C in a stainless steel coil (0.25 mm i.d. × 9.1 m) contained in a Pierce Reacti-Therm (Rockford, IL) heating module. The stream was then reacted with *o*-phthalaldehyde (OPA) solution (maintained in an ice bath) introduced at a rate of 0.1 mL min^{-1} using a second Eldex pump. The stream then traversed a 7.6-m length of Teflon tubing (0.25 mm i.d.) maintained in a 65 °C water bath prior to entering a RF-535 fluorescence monitor (Shimadzu, Columbia, MD) set at 340-nm excitation and 455-nm emission. The stream terminated at a back-pressure regulator set at 3.4 atm prior to waste collection.

Chemicals. NBPT and BNPO were synthesized by EniChem America, Inc., using proprietary methods. HPLC grade water was prepared using a Milli-Q water system (Millipore, Bedford, MA), and the methanol employed was optima grade from Fisher Scientific (Pittsburgh, PA). The HNO_3 hydrolysis solution was prepared by diluting concentrated nitric acid (Fisher) prior to filtration (0.22 μm). A stock solution of OPA was prepared by dissolving 1 g of *o*-phthalaldehyde (Sigma Chemical, St. Louis, MO) in 100 mL of methanol. This stock solution was stable for several weeks when refrigerated in an opaque bottle. The working OPA solution was prepared daily by adding 5 mL of the OPA stock solution to a final volume of 100 mL of 1.0 M potassium borate adjusted to pH 10.4 (Varian OPA diluent). This solution was filtered (0.22 μm) prior to addition of 200 μL of 2-mercaptoethanol (Sigma) and 500 mL of Brij 35 (30% aqueous solution from Sigma). All solutions, except the OPA, were sparged continuously with helium for purposes of degassing.

Preparation of Soil Extracts. Replicate 2-g samples of a silt loam soil were placed in 20-mL glass vials and were amended with 0.6 mL of a solution providing 0 or 2000 μg g^{-1} of urea N and 0 or 10 μg g^{-1} of NBPT or BNPO. The samples were then incubated at 25 °C for periods of up to 14 days. Upon removal from the incubator, the samples were immediately extracted with 10 mL of deionized water. The samples were then filtered (0.22-

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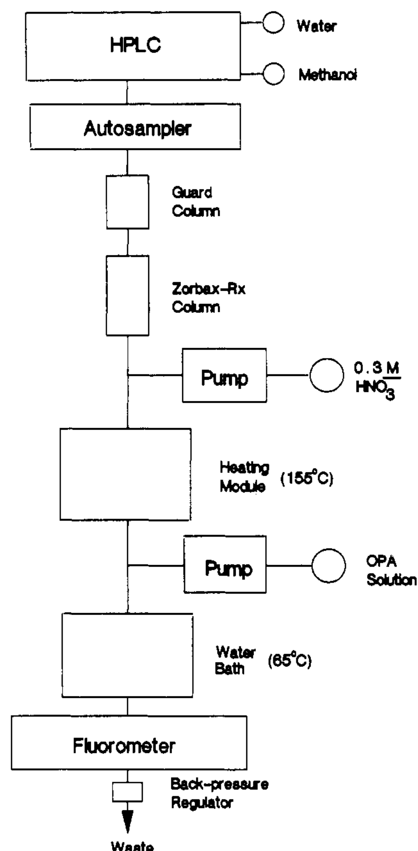


Figure 1. Schematic diagram of HPLC and postcolumn reaction system.

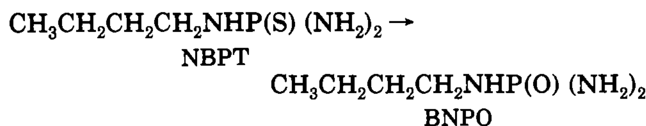
μm), and a 1-mL aliquot of each sample was added to 0.5 mL of methanol prior to analysis.

Sample Preparation and Analysis. Standards containing NBPT and BNPO were prepared daily from recrystallized compounds made up in 40% methanol comparable to the HPLC eluent. All solutions including the standards were filtered (0.22 μm) and placed into disposable vials for automatic injection. Typical automated runs consisted of approximately 100 samples with mixed (NBPT and BNPO) standards (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) at the beginning and end of the run. In addition, 10^{-6} M mixed standards were introduced after each five unknown samples to ensure consistent detection.

Stability during Storage. Solutions containing 10^{-5} M NBPT were prepared in water or in 20% Hoagland's nutrient solution (Hoagland and Arnon, 1950) adjusted to either pH 5.5 or 7.5 with H_2SO_4 or KOH, respectively. Similar solutions were prepared to a final concentration of 40% (v/v) methanol. These solutions were then stored in plastic 20-mL vials at −20, 4, or 25 °C prior to analysis of duplicate samples following 0, 3, 7, and 14 days of storage.

RESULTS AND DISCUSSION

As noted previously, NBPT is converted in the soil to its oxon analogue BNPO:



$$CH_3CH_2CH_2CH_2NHP(S)(NH_2)_2 \rightarrow CH_3CH_2CH_2CH_2NHP(O)(NH_2)_2$$
NBPT → BNPO

Neither parent compound reacts appreciably with known derivatizing agents such as OPA or fluorescamine, but both compounds produce *n*-butylamine and ammonia upon hydrolysis. As such, efforts were initiated to develop a postcolumn system employing hydrolysis coupled with derivatization of the resultant amines. Initial efforts using NaOH to hydrolyze the compounds as described by Hill et al. (1984) were unsuccessful. This was attributed to the presence of significant levels of ammonium or other impurities in the NaOH that gave unacceptable background fluorescence. In addition, since maximal fluorescence with primary amines is obtained over the pH range 9–11 (Svedas et al., 1980), NaOH was not well suited for the system employed as it quickly exceeded the limited capacity of the borate buffer to prevent pH increases.

Table I. Response Obtained with 10^{-5} M NBPT As Affected by Temperature of the Acid Hydrolysis and OPA Reaction Coils

OPA reaction temp, °C	acid hydrolysis temp, °C	area counts × 1000	CV, %	relative response,[a] %
25	25	76	2.3	6.1
25	75	414	1.1	32.9
25	155	650	1.5	51.5
55	25	117	0.8	9.3
55	75	762	1.4	60.4
55	155	1313	1.1	104.1
65	155	1261	0.2	100
75	155	1264	0.4	100.2

[a] As compared to conditions routinely employed.

Because both compounds are quite acid labile, this characteristic instead was exploited for derivatization. The use of HNO_3 was advantageous because the borate buffer was much more effective in resisting pH decreases from its initial pH of 10.5. Several studies were initiated to optimize the hydrolysis and derivatization reactions. Results obtained following variations in the temperature of the acid hydrolysis coil and the OPA reaction coil are shown in Table I. Although the rate of NBPT hydrolysis was relatively slow at both 25 and 75 °C, the compounds hydrolyzed extensively, if not completely, when heated to 155 °C in the acid hydrolysis coil. The reaction of *n*-butylamine and ammonia with OPA solution to form the corresponding isoindole derivatives was also significantly affected by temperature (Table I). While maximum fluorescence was observed over the range 55–75 °C, only 51% of maximum fluorescence was obtained at 25 °C. Although only data from 10^{-5} M NBPT are shown, identical results were obtained for both higher and lower NBPT concentrations.

Consequently, the eventual method utilized the maximum temperature of the heating module for the acid hydrolysis coil (155 °C) and a temperature of 65 °C for the OPA reaction coil. Additional studies showed that increasing or decreasing coil lengths by a factor of 2 had little effect upon the fluorescent signal, again indicating that both reactions were essentially complete under the current concentrations and temperature conditions. The relatively low flow rates (0.1 mL min^{-1}) for the two postcolumn pumps were selected to minimize dilution of the sample stream while maintaining a reliable flow rate. Concerns about potential incompatibility of the hot acid with the introduced OPA solution were unwarranted, as evidenced by the lack of an effect of an additional ice bath between the acid coil and the OPA pump.

Results obtained from a study employing a new column over a 3-day period are shown in Figure 2. Both compounds gave linear responses to concentrations over the range 10^{-8}–10^{-5} M, with r^2 values of 0.996 and 0.994 for NBPT and BNPO, respectively. In addition, essentially identical responses were obtained for both compounds. This would be expected if both compounds were hydrolyzed completely, as both would produce equivalent concentrations of *n*-butylamine and ammonia. Samples as low as 10^{-9} M were effectively integrated in some cases, but contaminants

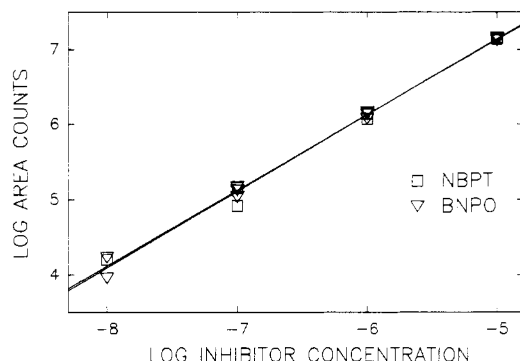


Figure 2. Fluorescence obtained with various concentrations (log M) of NBPT and BNPO over 3 days of analyses. Each data point represents the mean of three injections.

in the reagents often generated appreciable noise, thus making 10^{-8} M a more reasonable useful limit. This sensitivity is similar to the 1 ppb limit given by Hill et al. (1984) for analysis of carbamates, despite their use of a much larger sample loop (700 μL).

NBPT and BNPO in aqueous soil extracts were easily resolved from interfering soil substances and from one another using the C-8 reverse-phase column. Less effective resolution was observed in earlier studies using a C-18 column. Only a slight soil peak was generally observed prior to incubation with urea, but it became progressively larger as the applied urea was hydrolyzed. Although no attempt was made to identify the extracted soil components, they likely consisted of a variety of organic N components that became solubilized as the urea was hydrolyzed and pH increased. Urea was detected to only a limited extent by this system, but extracted ammonium may have contributed to some extent. Even though high concentrations of ammonium were expected in the soil as the applied urea was hydrolyzed, ammonium is not effectively extracted with water. In addition, although ammonia is effectively detected by OPA when buffered near pH 7, it gives much less fluorescence at pH 10.5 (Goyal et al., 1988; Taylor et al., 1974).

The solubilized soil components were essentially not retained by the column (rt = 3.8 min) but gave rise to considerable tailing as their concentrations increased. This occasionally caused considerable overlapping with the BNPO peak (rt = 6.1 min) and, in especially bad samples, tailed into the NBPT peaks (rt = 8.2 min). This problem was largely overcome by diluting the affected samples 10-fold with HPLC eluent prior to analysis, thereby reducing tailing of the soil components. Although this dilution reduced the effective sensitivity of the method to some extent, this was generally not a serious problem. Purification of the soil extract using PVP resins or similar techniques prior to injection was not pursued in these studies but might be employed in future studies to eliminate serious soil interferences. It should be recognized that the soil alone gave few interferences and that the application rate employed in these incubation studies (2000 $\mu g\ g^{-1}$ of urea N) is extremely high under most circumstances. Future studies to evaluate the fate of these compounds in soils without added urea or with lower rates of urea would pose few problems.

Both the NBPT and BNPO peaks were quite symmetrical, but increased tailing was observed following prolonged use of the column for analysis of soil extracts. This was especially evident for NBPT, and this tailing reduced its detection limits to some extent. A tendency for incomplete elution of low concentrations of NBPT was also observed. Retrospectively, some deterioration of the column would not be surprising as many of the soil extracts were quite alkaline (pH 8–9.5), and the recommended range of the column employed was between pH 1.8 and 8. Considerable quantities of solubilized humic materials were introduced over the duration of several hundred samples, and this may have exacerbated the problem.

Table II. Precision of Analyses Conducted on 10^{-6} M Mixed Standards over a 5-Day Period

	log area counts					
	mean		SD		range	
day[a]	BNPO	NBPT	BNPO	NBPT	NBPT	BNPO
1 (5)	6.43	6.01	0.03	0.05	6.39–6.47	5.95–6.08
2 (10)	6.41	5.98	0.01	0.03	6.38–6.43	5.93–6.02
3 (11)	6.38	5.88	0.03	0.08	6.33–6.42	5.76–6.00
4 (15)	6.39	5.72	0.02	0.05	6.35–6.43	5.58–5.76
5 (19)	6.32	5.55	0.06	0.11	6.23–6.39	5.37–5.70

[a] Number in parentheses is the number of separate samples analyzed.

Despite these problems, both NBPT and BNPO were analyzed with considerable precision. Table II summarizes results obtained for 50 10^{-6} M mixed standards that were interspersed throughout several hundred soil extract samples analyzed over a 5-day period. Even though the column had received limited use prior to this study, BNPO gave significantly greater fluorescence than NBPT, and this disparity became more pronounced as the analyses continued. Inasmuch as BNPO gave relatively constant fluorescence over the study, most of this disparity was apparently attributable to incomplete elution of NBPT from the column. Although not shown, both compounds gave very similar fluorescence at 10^{-5} M, but the results were even more disparate at concentrations of 10^{-7} and 10^{-8} M. Nonetheless, both compounds gave extremely linear ($r^2 > 0.96$) standard curves for both BNPO and NBPT each day. The results for both compounds were extremely reproducible each day as indicated by the standard deviation and ranges shown. Some decline in fluorescence was observed during previous prolonged runs (up to 20% over 20 h), presumably due to loss of 2-mercaptoethanol from the OPA reagent. This problem was eliminated by maintaining the OPA solution in an ice bath during the analysis as suggested by May and Brown (1989).

The method was also employed to characterize several additional phosphoramides. The urease inhibitor phenyl phosphorodiamidate (PPDA) was effectively detected with this system, although it provided only 2.3% as much fluorescence as obtained for NBPT. The reduced fluorescence with PPDA is attributable to the fact that it generates only ammonia groups upon hydrolysis. As such, it appears that approximately 98% of the fluorescence observed with NBPT is due to the presence of the *n*-butylamine moiety.

A variety of other alkylphosphoric triamides were also analyzed using this system, giving fluorescence quite similar, although not identical, to NBPT. Such variable fluorescence would be expected because the fluorescence of the isoindoles is known to vary with the structure of the respective alkylamines (Cronin and Hare, 1977). This method was also employed to characterize the appearance of other phosphoramide decomposition products in aqueous solutions. It should be noted that only N-containing products are detected with this method. An alternative method employing acid hydrolysis and phosphomolybdate derivatization (Creason et al., 1990) is capable of detecting all phosphorus-containing decomposition products, albeit with much less sensitivity than this method.

Stability during Storage. In previous soil incubation studies, NBPT and BNPO were shown to be poorly

Table III. Stability of 10^{-5} M NBPT Solutions following Storage for 14 Days at Various Temperatures

methanol, %	solution[a]	% remaining		
		−20 °C	4 °C	25 °C
0	water	96	100	100
0	NS 5.5	58	101	97
0	NS 7.5	94	103	101
40	water	102	96	84
40	NS 5.5	102	98	80
40	NS 7.5	104	103	97

[a] Solutions consisted of deionized water or 20% nutrient solution (NS) adjusted to pH 5.5 or 7.5.

recovered from some acidic soils even when extracted immediately (Douglass and Hendrickson, 1989). Subsequent research instead suggested that the compounds in the soil extracts were decomposing upon storage at −20 °C, even though results of pilot studies had demonstrated that soil extracts containing the compounds were stable for several days when stored at room temperature. Because storage of samples was a necessary component of our research, studies were initiated to evaluate the problem. Since studies to evaluate the uptake of NBPT by plants were in progress, solutions were prepared not only in water but also in 20% nutrient solution that had been adjusted to pH 5.5 or 7.5. Comparable solutions were prepared to a final concentration of 40% methanol, as preliminary studies had shown that solutions containing at least 25% methanol remained unfrozen at −20 °C. These solutions were then stored for intervals of 3, 7, and 14 days prior to analysis.

The results for samples that had been stored for 14 days are summarized in Table III. Surprisingly, the greatest decomposition was observed for samples stored at −20 °C. After 14 days, only 58% of the original NBPT remained in the pH 5.5 solution that had not received methanol. Some minor decomposition was also observed for the other nonacidic samples. However, the samples containing methanol remained intact throughout the study. As such, it appears that decomposition of the solutions is significantly accelerated upon freezing, especially if the solutions are acidic. One might speculate that this acceleration is due to concentration of the compounds and/or the acidity in the initially unfrozen portion of the solutions, thus accelerating the reaction.

The samples stored at 25 °C were much more stable, although significant decomposition was noted in the nonalkaline solutions containing methanol. This suggests that the methanol may be catalyzing NBPT decomposition to some extent. A similar, although much less serious, pattern was observed at 4 °C, again suggesting that the effects of methanol were nonbiological in nature. As such, it appears that NBPT solutions can be stored for at least 2 weeks at either 25 or 4 °C but are less stable at these temperatures when stored with methanol. More importantly, solutions containing these compounds should not be stored at −20 °C unless sufficient methanol is first added to the solutions to prevent freezing. This latter phenomenon should also be considered in the determination of appropriate storage conditions for solutions containing other labile agricultural chemicals.

CONCLUSION

An extremely sensitive, precise method has been developed that enables automated analysis of NBPT and BNPO in over 100 samples daily. The method has been employed to characterize the fate of these compounds in a variety of soils in the presence of up to a 20 000-fold molar excess of urea N. Although problems were encountered due to the solubilization of soil organics following urea hydrolysis, these effects were minimized by dilution of the soil extracts prior to analysis.

Problems were also encountered with incomplete elution and tailing of NBPT that were apparently exacerbated by deterioration of the column following prolonged analysis of soil samples. Further research to evaluate columns that may be more appropriate for alkaline solutions or investigation of solvent modifiers would likely prove fruitful. Solutions containing NBPT and BNPO can be stored for several weeks at room temperature or under refrigeration, but samples should not be stored below 0 °C unless they are first amended with methanol to ensure that they do not freeze.

ABBREVIATIONS USED

NBPT, *N*-(*n*-butyl)thiophosphoric triamide; BNPO, *N*-(*n*-butyl)phosphoric triamide; PPDA, phenyl phosphorodiamidate; OPA, *o*-phthalaldehyde.

LITERATURE CITED

Bremner, J. M.; Chai, H. S. Evaluation of N-(n-butyl)phosphorothioic triamide for retardation of urea hydrolysis in soil. *Commun. Soil Sci. Plant Anal.* **1986**, *17*, 337–351.

Byrnes, B. H.; Christianson, C. B. Development of a urease inhibitor from N-(n-butyl)thiophosphoric triamide. *Agron. Abstr.* **1988**, 212.

Christianson, C. B.; Byrnes, B. H.; Carmona, G. A comparison of the sulfur and oxygen analogs of phosphoric triamide urease inhibitors in reducing urea hydrolysis and ammonia volatilization. *Fert. Res.* **1990**, *26*, 21–27.

Creason, G. L.; Schmidt, M. R.; Douglass, E. A.; Hendrickson, L. L. Urease inhibitory activity associated with N-(n-butyl)-thiophosphoric triamide is due to formation of its oxon analog. *Soil Biol. Biochem.* **1989**, *22*, 209–211.

Cronin, J. R.; Hare, P. E. Chromatographic analysis of amino acids and primary amines with o-phthalaldehyde detection. *Anal. Biochem.* **1977**, *81*, 151–156.

Douglass, E. A.; Hendrickson, L. L. Urease inhibition by N-(n-butyl)thiophosphoric triamide and its oxon analog in diverse soils. *Agron. Abstr.* **1989**, 214.

Goyal, S. S.; Rains, D. W.; Huffaker, R. C. Determination of ammonium ion by fluorometry or spectrophotometry after on-line derivatization with o-phthalaldehyde. *Anal. Chem.* **1988**, *60*, 175–179.

Hendrickson, L. L. Corn yield response to the urease inhibitor N-(n-butyl)thiophosphoric triamide: 5 year summary. *J. Prod. Agric.* **1992**, in press.

Hill, K. M.; Hollowell, R. H.; Dal Cortivo, L. A. Determination of N-methylcarbamate pesticides in well water by liquid chromatography with postcolumn fluorescence derivatization. *Anal. Chem.* **1984**, *56*, 2465–2468.

Hoagland, D. R.; Arnon, D. I. The water culture method for growing plants without soil. California Agriculture Experiment Station Circular 347, 1950.

May, M. E.; Brown, L. L. Instability of orthophthalaldehyde reagent for amino acid analysis. *Anal. Biochem.* **1989**, *181*, 135–139.

McCarty, G. W.; Bremner, J. M.; Chai, H. S. Effect of N-(n-butyl)thiophosphoric triamide on hydrolysis of urea by plant, microbial, and soil ureases. *Biol. Fertil. Soils* **1989**, *8*, 123–127.

Svedas, V-J. K.; Galaev, I. J.; Borisov, I. L.; Berezin, I. L. The interaction of amino acids with o-phthalaldehyde: A kinetic study and spectrophotometric assay of the reaction product. *Anal. Biochem.* **1980**, *101*, 188–195.

Taylor, S.; Ninjoor, V.; Dowd, D. M.; Tappel, A. L. Cathepsin B2 measurement by sensitive fluorometric ammonia analysis. *Anal. Biochem.* **1974**, *60*, 153–162.

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