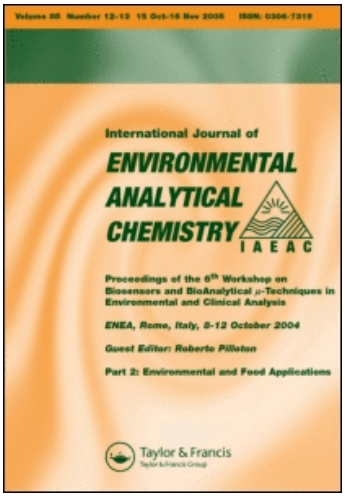


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# Fluorimetric Determination of Alkylating Activity in Particulate Samples

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# Fluorimetric Determination of Alkylating Activity in Particulate Samples

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A sensitive fluorimetric method for the determination of alkylating activity in particulate samples has been developed. The method is based on the alkylation of $NH_3(g)$ and the conversion of the primary amine salts produced to fluorophors using fluorescamine. This method requires no sample treatment prior to reaction with $NH_3(g)$, allows fixation of samples in the field, and is suitable for routine analyses.

KEY WORDS: Alkylating agents, fluorescamine, fluorescence, determination, amines, ammonia, chromatography.

## INTRODUCTION

Alkylating agents generally act as direct mutagens and carcinogens.[1] This is of concern because alkylating agents and direct acting mutagens, some of which are probably alkylating agents, have been found in urban airborne particles,[2,3,4] in particles emitted from coal fired power plants[5,6] and automobiles,[4] in untreated river water,[7] in effluents from paper and pulp mills[8] and sewage treatment plants,[9] and in cigarette smoke condensate.[4,10] Methods for the routine monitoring of alkylating activity in environmental samples are clearly needed.

The most general method used for determination of alkylating agents involves reaction with a nucleophile whose chromophoric or

fluorophoric properties change upon alkylation. The nucleophile, *p*-nitrobenzylpyridine (NBP), has been used extensively.[4,11] However, the chromophore produced in this reaction is unstable. This instability causes major uncertainties in the determination of alkylating agents of unknown structure since the rate of decomposition depends on the alkyl group. A similar nucleophile, 4-nitrothiophenol (NTP), has been shown to work well for the determination of epoxides and alkyl halides,[11,12] however the NTP itself is slowly oxidized in air. The oxidation product interferes with the determination. Also, the Ames tester strain TA100 gives a positive response to NTP and NTP thioethers[13] indicating that these compounds may be hazardous to humans. A fluorimetric method based on the alkylation of nicotinamide and the subsequent conversion of the N-alkylnicotinamide to fluorescent products has been reported.[14–15] This method does not suffer from the reagent instabilities of the above two methods, but it is not well suited for analysis of solid samples since it requires extraction of the alkylating agents into aqueous phosphate buffer prior to determination. Some alkylating agents would be lost by hydrolysis during the extraction.

Other methods, such as GC-MS[5,16,17] and HPLC,[18] have been used to determine specific alkylating agents but are not suitable for determining total alkylating activity.

A nucleophile that has been shown to react quantitatively with the alkylating agent dimethyl sulfate is $NH_3$(g).[6] This reaction produces the monomethylammonium salt of monomethyl sulfate. This reaction has been used for the determination of dimethyl sulfate in environmental samples with no sample treatment prior to reaction with $NH_3$(g). Reaction of $NH_3$(g) with other alkylating agents produces the corresponding primary amine salts. Thus, a determination of the primary amine salts produced by reaction of gaseous ammonia with a sample provides a measure of the alkylating activity in the sample.

Fluorescamine (4-phenylspiro[furan-2(3H), 1′-phthalan]-3, 3′-dione) reacts almost instantaneously with primary amines to produce fluorescent pyrrolinones.[19,21] This reaction has been used extensively for the determination of primary and secondary amines and ammonia.[21–31] In this paper we describe a method for the detection and quantitation of alkylating agents based on the alkylation of $NH_3$(g) and the conversion of the primary amine salts to fluorophors

using fluorescamine. This method avoids the problems associated with reaction and loss of the alkylating agent during sample extraction, allows fixation of the alkylating agents in the field and is suitable for routine analyses.

## EXPERIMENTAL SECTION

*Apparatus* Fluorescence measurements were taken with a Spex Fluorolog or a Perkin Elmer model 204-A spectrofluorimeter with a 1.4 mL quartz cell with a one cm path length.

*Reagents* Fluorescamine (Aldrich), monomethylamine hydrochloride (Eastman Kodak), monoethylamine hydrochloride (ICN Pharmaceuticals), ammonium chloride (Mallinckrodt), $Na_2B_4O_7 . 10H_2O$ (Baker), HCl (Fisher), acetone (MCB Omnisolv) and distilled water were used. The $NH_3$ was purchased from Matheson. The chloroform and ethanol used were reagent grade.

*Solutions* A borate buffer (pH 8.5, 0.01 M) was prepared using $Na_2B_4O_7 . 10H_2O$, HCl and distilled water. Standardized stock solutions (5 mM) of monomethylamine hydrochloride, monoethylamine hydrochloride and ammonium chloride were prepared in distilled water. Dilutions (1:100) of these solutions were also made using distilled water. Standard solutions of the amines were prepared immediately before use by further dilution with borate buffer. The fluorescamine solution was prepared in acetone (25 mg/100 mL).

### Procedure for analysis of samples of particulate matter

To analyze a particulate sample for alkylating activity by this method it was necessary to divide the sample into two equal portions. One portion was exposed to $NH_3(g)$, the other portion not. The portion exposed to $NH_3(g)$ was allowed to stand for at least 20 minutes to insure complete reaction. The same procedure was then followed thereafter for both portions. The sample was purged with argon to remove excess $NH_3(g)$ and to collect any gaseous alkylamines, the gas stream being bubbled through an aliquot of the borate buffer. The same aliquot of buffer was then used to extract the sample in an ultrasonic bath for 20 minutes. The minimum

volume of borate buffer necessary to cover the sample being used. The extract was removed and filtered. One mL of this solution was put in another test tube, stirred on a vortex-type stirrer, 1 mL of fluorescamine in acetone was added and the solution was stirred for another 10 s.

Because ammonia reacts with fluorescamine to produce a fluorescent product,[20] and there is a large excess of ammonia present after exposing a particulate sample to $NH_3(g)$, it was necessary to separate the ammonia-fluorescamine from the alkylamine-fluorescamine reaction product. A TLC method in which the ammonia-fluorescamine product has $R_f = 0.5$ and methyl and ethylamine-fluorescamine have $R_f = 0.8$ to 0.9 proved to be effective. The separation should be equally effective for alkylamines with larger alkyl groups. A 5 $\mu$L aliquot of the solution was spotted on a Whatman $KC_{18}$ thin layer chromatography plate (2.5 × 10 cm). The spot was air dried using no heat and the plate was developed with acetone/chloroform/ethanol (6:4:0.1). The plate was allowed to air dry and the fluorescent spot of interest ($R_f = 0.8$ to 0.9 for methyl and/or ethylamine) was located with a long wave UV lamp. The spot was removed from the glass plate by first cutting around the area and then wetting it with 20 $\mu$L of distilled water. The water causes the thin layer to swell and separate from the glass plate. The thin layer containing the fluorescence was removed with tweezers and put into a test tube. To insure reproducibility the same size area was taken for all of the samples measured. This was conveniently done using a scalpel and a cardboard template. In this work an area 0.8 × 1.2 cm was used. The fluorescent spot was extracted with 1.2 mL of acetone in an ultrasonic bath for five minutes. The suspension was centrifuged, the supernatant was removed, and the fluorescence intensity of the supernatant measured at 475 nm with 390 nm excitation. The difference in fluorescence intensity between the $NH_3(g)$ exposed portion and the unexposed portion is proportional to the alkylating activity present in the sample at the time it was exposed to the $NH_3(g)$.

If necessary the effective detection limit and sensitivity may be increased by an order of magnitude by evaporating the 2 ml of solution obtained after addition of fluorescamine to a volume of 0.2 ml before taking the 5 $\mu$L aliquot for TLC. This is conveniently done in a graduated, tapered test tube in a vacuum oven at 40°C.

### Calibration procedure

Three volumes of primary amine in the buffer (<30 nmol amine/mL) were transferred to a 13 x 100 mm glass test tube. As the test tube was vigorously stirred on a vortex-type stirrer, one volume of the fluorescamine solution was added rapidly. The tube was stirred for an additional 10s. In order to duplicate the procedure used for particulate samples, a TLC plate was spotted, the plate was developed, the spot was removed, and the fluorescence intensity measured as described above.

## RESULTS

Table I gives the calibration data obtained with methyl and ethyl amine. The amount of amine given as "amine taken" in Table I is

TABLE I

A sample set of calibration data for determination of methyl and ethylamine with fluorescamine after separation of the ammonia adduct by TLC.

| Amine taken, picomole | Fluorescence intensity, arbitrary units |
|---|---|
| methylamine | |
| 0 | 5.4, 5.6, 5.4 |
| 57.2 | 8.5 |
| 76.3 | 9.5 |
| 114 | 15.0, 14.5 |
| 181 | 18.4 |
| 305 | 27.0, 27.5, 28.0 |
| 381 | 33.5, 35.5, 34.0, 33.5 |
| 610 | 50.5, 49.0, 51.6 |
| methylamine + ammonium chloride | |
| 305 | 28.6 |
| ethylamine | |
| 120 | 14.0 |
| 480 | 40.0 |

the amount that was spotted on the TLC plate. The detection limit at the 95% confidence level is 5.6 picomole based on the standard deviation of the blank replicates. Obviously, the overall detection limit per sample depends on the volume of borate buffer used to extract the sample. The methylamine data fit a linear calibration curve over the range studied. The linear least square fit of the data in Table I gives Eq. (1)

$$\text{Intensity} = (0.0743 \pm 0.0011)\ (\text{picomoles of amine}) + (5.3 \pm 6.3) \quad (1)$$

where the uncertainties are expressed as the standard deviation. Ethylamine gave the same calibration plot as methylamine. Evaporation of the sample before spotting on the TLC plate increased the scatter in the data by about 2 percent but did not cause any systematic change in the calibration curve.

Table II gives the results of the determination of total alkylating activity in two samples of $<0.5\,\mu$m MMD particulate matter collected in the plume of a coal-fired power plant in Tennessee. No interferences were noted in running these samples.

The same procedure as applied to the particulate matter gave fluorescent products when applied to tubes containing methyl iodide, methyl bromide, ethyl iodide, dimethyl sulfate, and dimethyl sulfite.

TABLE II

Alkylating activity found in $<0.5\,\mu$m MMD particulate matter collected in the plume of a coal-fired power plant.

| Sample[a] | Alkylamine found, picomole | Alkylating activity, picomole/m$^3$ of air |
|---|---|---|
| WC5B17E | 85 | — |
| WC5B17D | 133 | 237 |
| WC3B13E | 137 | — |
| WC3B13D | 278 | 118 |

[a]D samples were exposed to ammonia, E samples were not.

## DISCUSSION

Many alkylating agents, such as epoxides and dimethyl sulfate, are highly reactive and may decompose after collection of the

sample.[6,32] With the method reported here the sample is divided in half, and one half exposed to $NH_3(g)$ in the field upon collection. The sample can then be returned to the lab for analysis. None of the other methods currently available can be used to so conveniently "fix" the alkylating agents.

The method presented here gives an approximation of total alkylating activity since the fluorescent yield is only slightly dependent on the alkyl group unless the alkyl group itself is fluorescent. However, by using some other separation technique, such as HPLC, in place of the TLC separation, identification of the alkyl group added to the $NH_3(g)$ could be accomplished. The TLC method was chosen for this work because it is fast and inexpensive and thus suitable for routine analyses. In any case a separation of some kind is necessary to remove the background fluorescence caused by the reaction of fluorescamine with residual ammonia. Some indication of the nature of the alkyl group is of course obtained from the $R_f$ value of the unknown.

Since each sample is split in half and one half is used as the blank for the sample, interferences are eliminated if the sample is homogeneous. The fluorophore formed is stable for at least one hour at room temperature in light or for days if kept in the dark.[20] We found a loss of fluorescence of approximately $3\%\,h^{-1}$ under normal fluorescent lighting. An acetone solution of fluorescamine is stable for up to twelve weeks at room temperature.[20] In contrast, the chromophore formed with NBP is unstable on a time scale of minutes and in the case of NTP the nucleophile itself is unstable. The method described in this paper also offers a lower detection limit than existing techniques and is applicable to solid samples. Reducing the volume of borate buffer used to extract the sample and using a micro cell in the fluorimeter would further lower the practical limit of detection of alkylating activity by this method.



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