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SPECIFIC DETECTION OF NITROGEN IN GAS–LIQUID CHROMATOGRAPHIC EFFLUENTS BY FLUORESCENT DETECTION OF AMMONIA

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SUMMARY

o-Phthalaldehyde (OPA) reacts with ammonia under the same conditions as it does with primary amines and amino acids to yield a similar fluorescent product. The fluorescence is maximal 2 min after the reagents have been mixed and fades rapidly thereafter.

With continuous flow analysis used to keep the time for development of fluorescence constant, ammonia in aqueous solution was measurable at concentrations down to 10^{-3} m*M*. Injection of small volumes of solution into the flowing reagent permitted as little as 10^{-10} g to be detected at the noise level with peaks that were 6 sec wide at half-height. The fluorescent product could also be extracted into ethyl acetate and some other organic solvents, offering increased sensitivity from both enhancement of fluorescence and concentration of the product.

Organic nitrogen in a gas–liquid chromatographic (GLC) effluent can be converted into ammonia by reaction with hydrogen at high temperature in the presence of a catalyst. The ammonia can be "scrubbed" in the OPA reagent, offering the high sensitivity and specificity of this wet-chemical reaction as a detector for GLC.

INTRODUCTION

Organic nitrogen in a stream of gas can be converted into ammonia by reduction with hydrogen over a catalyst such as nickel at high temperatures. Among the gas–liquid chromatographic (GLC) detection systems using this reaction are the Coulson liquid conductivity detector[1], which detects the ammonia by "scrubbing" it in deionized water and measuring the change in the electrical conductivity of the water. Other heteroatoms, such as chlorine and sulfur, interfere and must be removed. On the other hand, the lack of specificity offers the advantage that the detector may be operated in other modes for the detection of these heteroatoms.

In trace analyses of mixtures in which the compounds of interest are present in much lower concentrations than compounds with similar retention times, and particularly when the objective is to identify many of a particular class of compounds in a mixture, increased specificity is desirable. In the work described here we explored the possibility of using a highly specific and sensitive wet-chemical reaction as a detector in GLC to study the possibility of using this general approach.

In 1971, Roth[2] demonstrated that *o*-phthalaldehyde (OPA), in alkaline media and in the presence of a reducing agent, reacts rapidly with amino acids to give fluorescent products with excitation maxima at 340 nm and emission maxima at 455 nm. Ammonia was also detectable. As this highly sensitive reaction has since been widely used to detect amino acids in analysis by liquid and ion-exchange chromatography, it seemed promising for this application also. However, ammonia was detected with a relative fluorescent intensity of only 40 units compared with 25 units for the blank and 1000 units for serine. In a later work, Taylor *et al.*[3] described a more sensitive assay for ammonia using OPA under different conditions in which the excitation and emission spectra of the product were different, and which required an appreciably longer reaction time. We therefore focused our attention first on improving the sensitivity of detecting ammonia by the approach used by Roth and then on a means for coupling it to the GLC effluent.

EXPERIMENTAL

Apparatus

Absorption and emission spectra were measured on an Aminco-Bowman Model J4-8202G spectrophotofluorimeter using 1 × 1 cm cuvettes (American Instrument, Silver Spring, Md., U.S.A.). To follow the time course of the development of fluorescence, the output of the fluorimeter was recorded on a time-base recorder.

When the time between mixing ammonia with reagents and the maximal fluorescence of the product proved critical, we mechanized the assay by using a Technicon AutoAnalyzer II system (Technicon, Tarrytown, N.Y., U.S.A.) including a sampler, proportioning pump and mixing and delay coils (Fig. 1). After the appropriate reaction time, the stream was debubbled and passed through an Aminco Fluoromonitor, Model J4-7461, with a Corning 7-60 primary filter with maximal transmission between 360 and 370 nm and 1% transmission at 310 and 395 nm, and a Wratten 8 emission filter, which transmits more than 85% of the light from 450 to 700 nm and less than 4% at 410 nm.

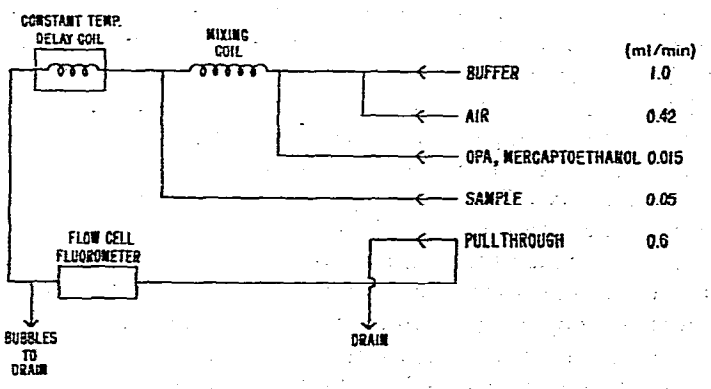

Fig. 1. Flow diagram of system for measuring ammonia in aqueous samples by continuous flow.

Regaents

OPA (1,2-benzyl dicarboxaldehyde) was bought from Aldrich (Milwaukee, Wisc., U.S.A.), boric acid and ammonium sulphate from Mallinckrodt (St. Louis, Mo., U.S.A.), L-amino acids from Calbiochem (Los Angeles, Calif., U.S.A.) and absolute ethanol from U.S. Ind. Chem. (New York, N.Y., U.S.A.). All water used was deionized, with a measured specific conductance of 0.7 $\mu\Omega^{-1}$ or less.

OPA solution was prepared by dissolving 200 mg of OPA in 30 ml of ethanol, adding 0.1 ml of mercaptoethanol and then filtering. This solution is stable for at least 1 week at room temperature. Ammonium sulphate sufficient to form a 2 m*M* solution was dissolved in deionized water and serial dilutions were prepared. The buffers tested included borate (0.4 *M*) at pH 8.0, 8.5, 9.0, 9.5, 9.8 and 10.0, borate (pH 9.0) at 0.05, 0.1, 0.2, 0.3 and 0.4 *M* and phosphate (0.2 *M*) at pH 8.0 and 7.0.

Reaction of ammonia with OPA

A 50-μl volume of the OPA solution containing mercaptoethanol was mixed with 3.0 ml of buffer by inversion in a fluorescence cuvette and the baseline fluorescence was recorded briefly in the Aminco-Bowman spectrophotofluorimeter. A 50-μl volume of ammonia solution was added and the mixing repeated. The change in fluorescence with time was recorded at an excitation wavelength of 350 nm and an emission wavelength of 450 nm.

After the fluorescence had reached a maximum, the excitation and emission spectra were recorded in order to confirm that these were settings that yielded the maximum sensitivity.

In the continuous flow system, OPA solution containing mercaptoethanol was aspirated at 0.015 ml/min and buffer at 1.1 ml/min. To achieve steady-state conditions in the flow cell, samples were aspirated at 0.05 ml/min for 2 min, followed by deionized water for 2 min. The effects of pH and molarity of the buffer were tested using a 0.24 m*M* solution of ammonia. In each experiment different delay coils were chosen so that the fluorescence was maximal when the solution reached the flow cell. Amino acids in 0.02 m*M* concentration were analyzed in similar fashion.

Monitoring ammonia in a gas stream

Organic nitrogen was converted into ammonia in a 20-cm × 5 mm I.D. Vycor (Corning, Corning, N.Y., U.S.A.) tube containing a nickel wire catalyst (Tracor, Austin, Texas, U.S.A.) that was maintained at 800°. Hydrogen was added to the column effluent immediately before it entered the reduction furnace. The effluent from the furnace (*ca.* 50 ml/min) was delivered to a T-connection at the AutoAnalyzer pump where 1 ml/min of the complete OPA reagent solution was pumped into it. The combined stream then entered a six-turn glass helix of diameter 2 cm and with tube I.D. 2 mm in which the ammonia was "scrubbed" from the gas stream by the OPA solution. The stream was then debubbled by allowing it to impinge on the wall of a small plastic funnel constructed by truncating an Eppendorf disposable syringe tip. The solution drained to the bottom of the funnel, and was pumped from the funnel intermittently by the action of the AutoAnalyzer peristaltic pump at approximately twice the rate at which the same pump delivered reagent solution to the T-connection. The result was a stream segmented by bubbles in which diffusion from segment to segment was minimized. For calibrating the response of the system, microliter amounts of ammonia were injected into the stream at the bottom of the funnel.

RESULTS

Addition of ammonia to the basic OPA reagent mixture containing mercaptoethanol described by Roth for amino acid analysis caused the solution to become fluorescent. The product had the same excitation and emission maxima (350 and 450 nm, respectively) as those reported previously with amino acids. The fluorescence increased to a maximum in 1–6 min, depending on pH, and then decreased to half the maximal intensity in 2–17 min. The reaction of alanine with the same reagent was more rapid and the fluorescence persisted appreciably longer (Fig. 2).

There was no difference in the rate of decay of the fluorescence (1) in the presence or absence of the exciting light beam, suggesting that photodecomposition did not play a major role, (2) in reagent from which most of the dissolved oxygen had been removed compared with reagent containing dissolved air or (3) in reactions

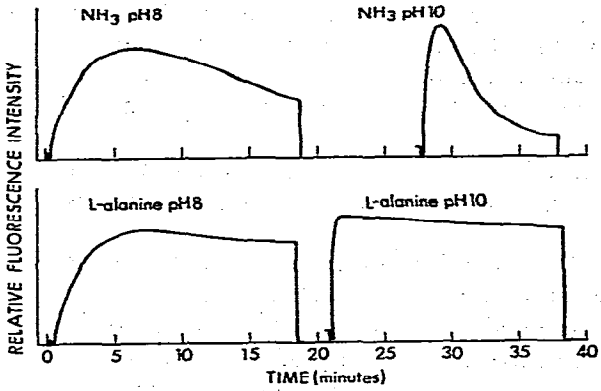

Fig. 2. Effect of pH on time course of fluorescence. The fluorescence of OPA and ammonia develops and decays more rapidly at higher pH. The product with L-alanine is more stable at both pHs.

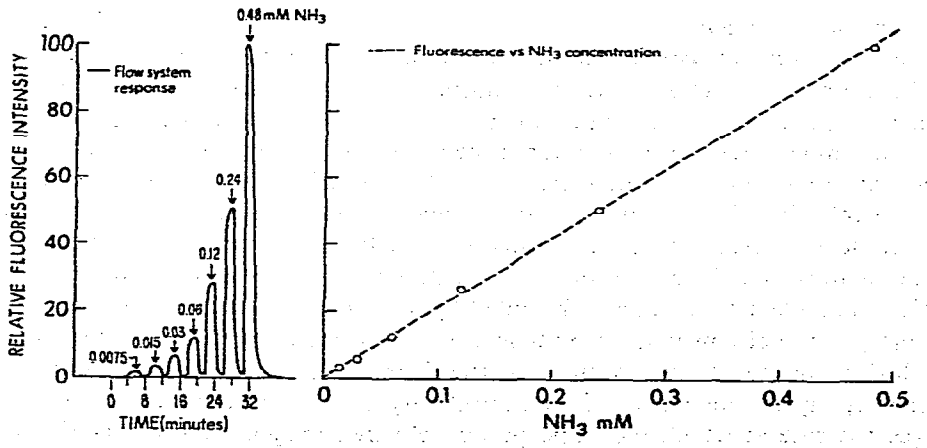

Fig. 3. Proportionality of fluorescence intensity to ammonia concentration. The left-hand graph shows the response of the flow system to graded concentrations. Ammonia solution was aspirated for 2 min, followed by a 2-min water wash. The delay coil was changed for each pH, temperature and molarity of buffer to measure the product at time of maximal fluorescence.

in tightly stoppered cuvettes compared with reactions in open cuvettes, suggesting that loss of ammonia from the basic solution was not responsible.

The fluorescence 2 min after mixing was directly proportional to the concentration of ammonia (Fig. 3). The maximal fluorescence intensity was greatest at pH 9.0–9.5, which is a slightly lower pH than that for most of the amino acids (Table I). The rate at which the maximum was reached and the rate of decay were also greater at higher pH (Fig. 2). The former rate increased with increase in temperature from 20–50°, but the maximal fluorescence decreased (Fig. 4).

TABLE I

EFFECT OF pH ON MAXIMAL OPA FLUORESCENCE OF AMMONIA COMPARED WITH L-ALANINE (Ala), L-ARGININE (Arg), L-GLUTAMIC ACID (Glu), L-HISTIDINE (His), L-METHIONINE (Met), L-PHENYLALANINE (Phe) AND L-TYROSINE (Tyr)

Results given in relative fluorescence units.

Buffer	*Source of amino group*							
	NH_3 (0.24mM)	*Ala (0.02mM)*	*Arg (0.02mM)*	*Glu (0.02mM)*	*His (0.02mM)*	*Met (0.02mM)*	*Phe (0.02mM)*	*Tyr (0.02mM)*
Borate, pH 10.0	26	28	24	23	24	25	24	14
Borate, pH 9.8	29	16	22	17	23	25	24	16
Borate, pH 9.5	33	16	22	18	22	23	22	17
Borate, pH 9.0	34	23	23	19	22	24	22	21
Borate, pH 8.5	21	22	23	15	22	23	18	19
Borate, pH 8.0	18	20	19	15	19	22	20	20
Phosphate, pH 8.0	2	9	13	8	14	13	10	10
Phosphate, pH 7.0	0.2	0.9	2.3	1.4	2.4	3.1	3.2	3.5

A greater maximal fluorescence was obtained with more concentrated buffer (Table II). The fluorescence in borate buffer was considerably higher than that in phosphate buffer at pH 8.0.

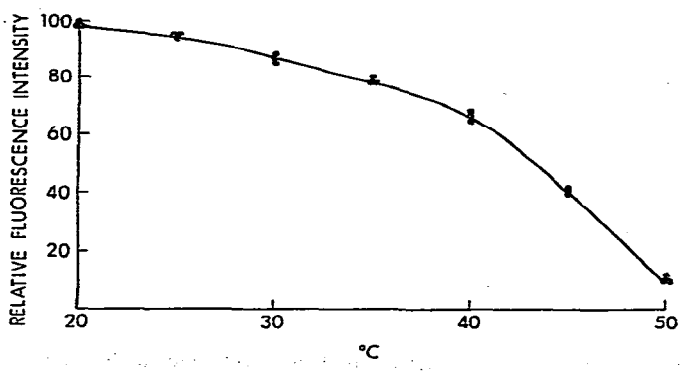

Fig. 4. Effect of temperature on the intensity of fluorescence. The flow system delay coil was changed for each temperature to ensure measurement at time of maximal fluorescence.

TABLE II

EFFECT OF MOLARITY OF BORATE BUFFER AT pH 9 ON FLUORESCENCE WITH OPA WITH AMMONIA, L-ALANINE (Ala), L-ARGININE (Arg), L-GLUTAMIC ACID (Glu), L-HISTIDINE (His), L-METHIONINE (Met), L-PHENYLALANINE (Phe) AND L-TYROSINE (Tyr)

Results given in relative fluorescence units.

Concentration of buffer (M)	*Source of amino group*							
	NH_3 (0.24mM)	*Ala (0.02mM)*	*Arg (0.02mM)*	*Glu (0.02mM)*	*His (0.02mM)*	*Met (0.02mM)*	*Phe (0.02mM)*	*Tyr (0.02mM)*
0.4	34	23	23	19	22	24	22	21
0.3	30	23	22	19	22	22	21	20
0.2	28	22	21	18	21	22	21	20
0.1	27	23	21	18	21	23	20	20
0.05	22	22	20	18	20	23	21	20

Extraction of the fluorescent product into one of several organic solvents stabilized the fluorescence. For example, extraction into an equal volume of ethyl acetate resulted in a decrease in fluorescence of only 13% in 30 min compared with an almost complete loss when the same product remained in aqueous solution. The fluorescence was also enhanced to twice the intensity. The sensitivity of the assay could be increased further by extraction into a smaller volume of the ethyl acetate into which it was preferentially partitioned.

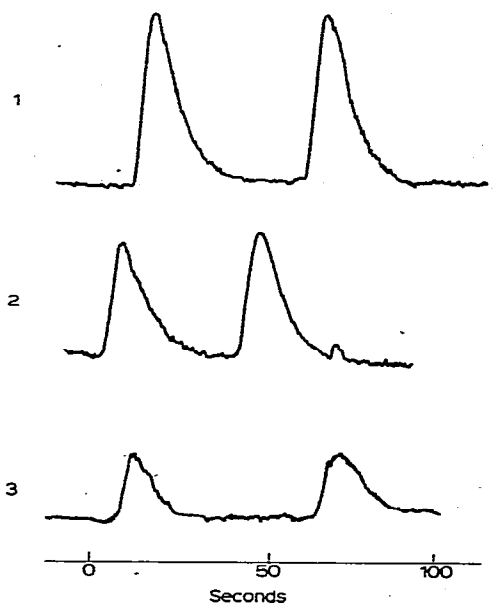

Fig. 5. Results of introduction of ammonia into two liquid segments. Peaks 6 sec wide at half-height were obtained. The records labeled 1, 2, 3 show detection in duplicate of 13.2, 6.6 and 3.3 ng of ammonium sulphate, respectively, equivalent to 2.8, 1.4 and 0.7 ng of ammonia-nitrogen, respectively.

Sensitivity and precision

In twenty consecutive assays of 0.24 m*M* ammonium sulphate solutions by the continuous-flow method the coefficient of variation was 0.8%. The precision was less at lower concentrations. At a concentration of 0.0018 m*M* the signal-to-noise ratio decreased to 4:1. Injection of ammonium sulphate solution into two segments of the flowing reagent stream, after the scrubber, resulted in peaks of fluorescence that were 6 sec wide at half-height. The need to allow 2 min for the development of maximal fluorescence was the primary cause for this degree of peak broadening. Injection of progressively greater dilutions demonstrated a detection of *ca.* 10^{-10} g of ammonia at the noise level (Fig. 5).

These results were obtained by reading the fluorescence of the aqueous solution without either extraction into an organic solvent or concentration.

Recovery

Ammonia could be removed from a gas stream flowing at 50 ml/min into 1 ml/min of flowing reagent with better than 90% efficiency. The efficiency of conversion of nitrobenzene-nitrogen into ammonia in the reduction furnace was estimated to be 40%.

DISCUSSION AND CONCLUSIONS

The proposed method is a sensitive technique for detecting ammonia and for continuously monitoring its concentration in either aqueous solution or gases. In assays of aqueous solutions, a means of distinguishing amines and amino acids, which also react with OPA, must be used when these compounds may be present. Use of the differential solubility of their fluorescent products in organic solvents or their different rates of passage through semi-permeable membranes are two possible means of accomplishing this. In monitoring flowing gas streams, volatile amines could present similar problems amenable to similar approaches.

We have also planned to "scrub" the GLC effluent with OPA reagent without prior reduction. This should permit the selective detection of compounds with primary amino groups that are either free or that can be released by hydrolysis. In this instance interference would be expected from compounds that are themselves fluorescent or that precipitate in the reagent and scatter light.

The method of converting organic nitrogen into ammonia by exposure to hydrogen at high temperatures in the presence of a catalyst has been widely used and its characteristics are well known. It would be interesting to compare the different methods of monitoring the ammonia concentration both for sensitivity and specificity on the same analytical problems. We have not yet had the opportunity of doing so.

The method of detecting ammonia described by Taylor *et al.*[2] also involved mixing ammonia with OPA, but at pH 7. The fluorescent product obtained had different excitation and emission spectra. Amino acids and amines did not react and the product reached maximal fluorescence after 60 min that was stable until 90 min. We chose the method described primarily because the reaction was faster. A reaction that required so long for completion might require off-line monitoring.

Continuous-flow analysis was used here primarily so that each sample was allowed the same time for the development of fluorescence. In assays of solutions

the precision was much lower when more conventional manual techniques were used. Continuous-flow apparatus was also very useful for sampling and monitoring concentrations continuously and for "scrubbing" the ammonia from the gas stream. Its use made the entire sequence of manipulations convenient and practical.

We hope that this work has demonstrated the feasibility of extending to GLC one of the great advantages of high-performance liquid chromatography, namely the possibility of using highly specific detection methods.

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