Journal of Chromatography, 514 (1990) 293–304
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 488

Controlled thermal degradation for the identification and quantification of amine N-oxides in urine

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(First received December 5th, 1989; revised manuscript received April 11th, 1990)

ABSTRACT

Studies of amine N-oxides in urine are important for the evaluation of occupational exposure to amines. These thermolabile compounds are difficult to handle by either gas or liquid chromatography, so a device for controlled thermal degradation has therefore been developed. It consists of a short precolumn with shut-off valves at both ends and an aluminium block for heating, and it was connected to the injection port of a gas chromatograph. After injection of amine N-oxides onto the precolumn and thermal degradation, the degradation products were allowed to enter the analytical column.

Trimethylamine N-oxide (TMAO) and triethylamine N-oxide (TEAO) were investigated. Their thermal degradation patterns could be used for identification and quantification in aqueous solutions and in urine. Linear calibration graphs based on degradation product peaks (trimethylamine and O,N,N-trimethylhydroxylamine from TMAO and diethylamine and triethylamine from TEAO) were obtained for concentrations up to 500 ppm. Detection limits in aqueous solutions were 0.2 ppm (*ca.* 1 ng) for TMAO and 1 ppm for TEAO and the precisions were 6% and 9%, respectively. In urine, similar values were obtained for TEAO. The detection limit for TEAO corresponds to the expected concentration in urine after an 8-h exposure to air containing 0.8 mg/m^3 of triethylamine.

INTRODUCTION

Amine N-oxides are formed in the body as metabolites of tertiary amines from natural sources, such as the enterobacterial metabolism of dietary choline and lecithin[1]. Another source is marine fish which contains N-oxides[2,3].

Occupational exposure to amines is another cause of the formation of amine N-oxides. Amines occur as pollutants in many industrial environments[4], *e.g.*, in polymer production, and various adverse health effects due to this exposure have been

0021-9673/90/$03.50

reported[5,6]. Biological monitoring of industrial pollutants, involving the analysis of urine, blood, etc., is increasingly used to measure individual exposure, in addition to conventional measurements of pollutants in workplace air. For biological monitoring of amine exposure, however, determinations of both the amine N-oxide and the corresponding amine are recommended[7].

The determination of volatile amines both in air and in body fluids can readily be performed using gas chromatography (GC)[8,9]. Amine N-oxides, however, cannot be determined in this way, as they are thermally unstable, non-volatile and very polar. In a recent study[10] the thermal degradation of amine N-oxides in a hot injection port was demonstrated.

Modern liquid chromatography (LC) usually offers good opportunities for the determination of thermolabile compounds in biological samples. However, problems arise when the compounds of interest do not contain functional groups which give a response from the most frequently used LC detectors, *e.g.*, UV, electrochemical and conductimetric detectors. Further, thermolabile substances are not easily identified with LC–mass spectrometric (MS) equipment, as the temperature of the interface is often held relatively high.

With their low UV absorbance, amine N-oxides are difficult to determine with LC at trace levels. For example, a recent LC method with UV detection at 208 and 214 nm gave a detection limit for trimetylamine N-oxide (TMAO) in standard solutions as high as 5 μg[11].

Other direct methods for the determination of amine N-oxides exist, based on IR spectrometry[12], polarography[13] and potentiometry[14]. In all these methods the sensitivity is insufficient for trace analysis and the compound has to be separated from the matrix. Polarograms and cyclic voltammograms obtained at our laboratory for TMAO at pH 1.3, 5.0 and 11.0 showed that high reductive or oxidative voltages (-1.3 and $+1.6$ V *vs.* SCE, respectively) were needed before any reaction occurred. Hence electrochemical LC detectors are expected to give poor selectivity and high background currents.

To avoid the problems mentioned above, amine N-oxides are frequently determined after reduction to the corresponding amines[15–17]. However, in this instance it must be verified that the amines found really emanate from the amine N-oxides.

In this paper we present an alternative method for the identification and quantification of amine N-oxides in urine. It is based on thermal degradation under controlled conditions followed by GC analysis. Additionally, an approach for the simultaneous quantification of volatile tertiary amines and the corresponding amine N-oxides is discussed.

EXPERIMENTAL

Chemicals

Trimethylamine N-oxide (TMAO) dihydrate, trimethylamine (TMA) hydrochloride, ethylamine and dimethylamine (DMA) hydrochloride were obtained from Janssen Chimica (Beerse, Belgium), triethylamine (TEA) from Fluka (Buchs, Switzerland), diethylamine (DEA) from BDH (Poole, U.K.) and triethylamine N-oxide (TEAO) from ICN Pharmaceuticals (Planview, NY, U.S.A.).

Stock solutions (1000 ppm) of amines and amine N-oxides were prepared in 50 m*M* sulphuric acid and 1 m*M* sodium hydroxide solution, respectively. Standard solutions for GC measurements were prepared by dilution with 0.5 *M* sodium hydroxide solution and stored in screw-capped vials sealed with Mininert valves (Alltech, Arlington Heights, IL, U.S.A.).

Equipment

The gas chromatograph (Model 3700; Varian, Walnut Creek, CA, U.S.A.) used for the measurements was equipped with a nitrogen-sensitive detector (TSD; Varian) and connected to a recorder and an integrator (Model 3390A; Hewlett-Packard, Palo Alto, CA, U.S.A.). A thermal decomposition device (see below) was connected to the injection port of the gas chromatograph.

Typical parameter settings were: bias voltage −10 V, bead current 650 scale divisions, detector temperature 200°C, injector temperature 150°C, air flow-rate 170 ml/min and hydrogen flow-rate 3 ml/min. Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min.

A glass column (190 cm × 3 mm I.D.) was used, packed with *ca.* 10 g of 28% Pennwalt 223 with 4% potassium hydroxide on Gas-Chrom R (Alltech)[a]. Further details concerning the chromatographic conditions are given in the figure legends.

A gas chromatograph–mass spectrometer (Model 4021; Finnigan MAT, San José, CA, U.S.A.) was used for the identification of degradation products. The thermal decomposition device was connected to the injection port of the GC–MS system. A glass column of the same type as above was used for the GC stage.

Thermal decomposition device

The thermal decomposition device (Fig. 1) consisted of a precolumn with shut-off valves at both ends. The precolumn was heated by a thermostated aluminium

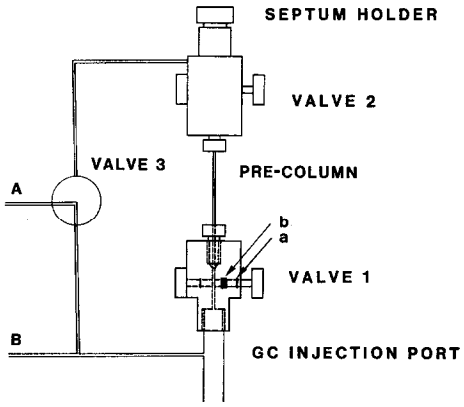

Fig. 1. Thermal degradation apparatus.

[a] Pennwalt 223 is no longer available, but a replacement (Alltech 223) is offered, which is claimed to be identical.

block with a slit, in which the precolumn fitted. The whole device can be easily connected to the injection port of any gas chromatograph with packed columns.

The precolumn was made of steel (7.6 cm × 1 mm I.D.) and packed with *ca.* 24 mg of Chromosorb 103 (Alltech). The rods of the valves were made of PTFE and sealed with O-rings (a) against the surrounding Kel-F blocks. A piece of silicone-rubber tubing (b) ensured complete shut-off in the closed position. In the upper block, a septum holder with a PTFE-lined septum (Microsep F-174; Alltech) was included.

The lower block was directly screwed to the injection port of the gas chromatograph, which was equipped with two independent carrier gas controllers, A and B. One of these (B) was connected to the injector in the normal way. The carrier gas controller A was connected, via a switching valve (3), either to the top of the precolumn or to the injector. In this way, the flow through the precolumn could be adjusted to any value and also switched off while keeping the carrier gas flow-rate through the main column constant. The configuration of the gas streams was the same as described previously[18] in a similar context.

Before packing, the precolumn was rinsed with dilute (15%) ammonia followed by a saturated solution of potassium hydroxide in methanol, which was allowed to dry, leaving a layer of potassium hydroxide on the inner wall of the tubing. The channels in the Kel-F blocks were rinsed with dilute ammonia and water.

Injection procedure for amine N-oxides

Samples (usually 5 μl) were injected through the septum onto the precolumn using a 10-μl Hamilton syringe with a fixed needle. On injection the carrier gas was led through the precolumn with valves 1 and 2 (see Fig. 1) open. After 5 s the lower valve (1) and the upper valve (2) were closed and valve 3 was switched, in that order. The precolumn was then heated by applying the aluminium block for typically 5 min at 175°C for TMAO and 3 min at 150°C for TEAO. The degradation products were transferred to the analytical column by switching the carrier gas valve 3 and opening valves 1 and 2, in that order.

To ensure complete transfer of all degradation products, the heating block was kept surrounding the precolumn for 1 min after the opening of the valves. During the entire chromatographic run the valves were kept open and 20 ml/min of carrier gas were passed through the precolumn and 10 ml/min through the lines leading to the injector in order to prevent backflushing.

Injection procedure for a mixture of amines and amine N-oxides

A simultaneous determination of amines and the corresponding amine N-oxides could be performed by keeping the valves open for 30 s after injection at a precolumn temperature of 80°C, permitting the elution of the amines. At this temperature no degradation of the amine N-oxides investigated here was observed. The precolumn was then closed and the amine N-oxides could be determined after thermal degradation at a higher temperature as described above. If needed to complete the amine chromatogram, the degradation can be started later.

Sample pretreatment

Urine samples were stored in acidic solution (pH ≈ 2.0) before further work-up. After centrifugation, the solutions were, if necessary, filtered through 0.45-μm

Millipore membranes. An equal amount of diisopropyl ether was added, the sample was shaken and the organic layer discarded. The aqueous layer was made alkaline (pH 14) by addition of sodium hydroxide. This solution contains both amines and amine N-oxides and was analysed by direct injection followed by thermal degradation as described.

It is also possible to remove the amines by extracting the alkaline solution with diisopropyl ether or diethyl ether. The aqueous phase containing the amine N-oxides was then injected onto the pre-column and treated as above.

Thin-layer chromatographic (TLC) preparation of TEAO

Urine samples collected during exposure to TEA and normal urine samples, with and without addition of TEA and TEAO, were extracted with portions of diethyl ether, in order to remove TEA. The urine samples were lyophilized, extracted with ethanol to remove inorganic salts, dissolved in 50 ml of 0.1 *M* hydrochloric acid and applied to a column of Dowex 50W-X8 (H^+; 200–400 mesh), as described by Baker and Chaykin[12]. The column was washed with water and 0.1% aqueous ammonia. TEAO was eluted with 20 ml of 1% aqueous ammonia. A 10-μl volume of the eluate was transferred to the precoated TLC plate (silica gel 60 F_{254}; Merck, Darmstadt, F.R.G.) and dried by a hot air stream. The plate was developed and the solvent [butanol–acetic acid–water (60:15:25)] was allowed to reach a level of 1 cm from the upper end of the plate. The plate was air-dried overnight. A second elution was made with methanol–acetic acid–water (80:10:10). The plate was sprayed with 0.2% bromocresol green in ethanol from an air-driven atomizer. After a few seconds, bluish spots were seen on the yellow background with R_F values of 0.55 and 0.63 for TEA and TEAO, respectively.

The different spots containing substance and silica were scraped off, transferred to centrifuge tubes, extracted with 500 μl of 0.5 *M* sodium hydroxide solution , shaken

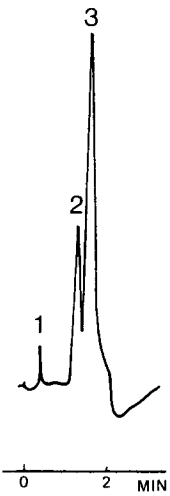

Fig. 2. Chromatogram of 10 ppm of TMAO in 0.5 *M* NaOH, after thermal degradation at 175°C. Attenuation, $8 \cdot 10^{-12}$ a.u.f.s. Temperature programming from 90°C (1 min) at 40°C/min to 155°C. Peaks: 1, sammonia; 2, TMA; 3, Meisenheimer rearrangement product TMHA (see text).

for 40 min and centrifuged. During these steps the test-tubes were capped. Aliquots of the clear solutions were then transferred to capped vials and injected onto the precolumn as above.

RESULTS AND DISCUSSION

Thermal degradation

The thermal degradation of amine N-oxides results in fragmentation patterns typical of a certain compound. The relative concentration of the degradation products varies with the conditions during the procedure.

TMAO. Fig. 2 shows a chromatogram obtained after thermal degradation of TMAO in 0.5 *M* sodium hydroxide solution. Peaks 1 and 2 were identified by their retention times as ammonia and TMA. The identification of peak 2 was confirmed by MS. The formation of the parent amine by deoxygenation has been observed previously[19]. Peak 3 is probably a result of a Meisenheimer[20] rearrangement of the amine N-oxide to O,N,N-trimethylhydroxylamine (TMHA), which, on further heating, may degrade to DMA and formaldehyde according to Hattori[21]. Thus the following reaction scheme can be written:

$$\text{TMAO} \begin{cases} \rightarrow \text{TMA} \\ \rightarrow \text{TMHA} \; (\rightarrow \text{DMA} + \text{HCHO}) \end{cases}$$

Using MS detection the latter step seems to occur in the hot (*ca.* 250°C) transfer line between the column and the ion source, since peak 3 was identified as DMA, which should have a shorter retention time even than peak 2. This step does not occur to a significant extent in the precolumn as no DMA peak was observed. After peak 3, a negative peak due to the elution of water appears.

Triethylamine N-oxide (TEAO). Fig. 3 shows the fragmentation pattern obtained after thermal degradation of TEAO in 0.5 *M* sodium hydroxide solution. Peaks 1–4 were identified by their retention times as ammonia, ethylamine, DEA and TEA, respectively, and were further confirmed by MS.

The Meisenheimer rearrangement product O,N,N-triethylhydroxylamine (TEHA), formed in a similar way as with TMAO above, seems to decompose to DEA

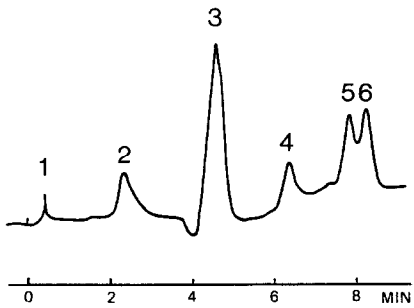

Fig. 3. Chromatogram of 55 ppm of TEAO in 0.5 *M* NaOH after thermal degradation at 150°C. Attenuation, $4 \cdot 10^{-12}$ a.u.f.s. Temperature programming from 75°C (4 min) at 40°C/min to 140°C. Peaks: 1, ammonia; 2, ethylamine; 3, DEA; 4, TEA; 5 and 6, rearrangement products of TEAO.

and acetaldehyde in the precolumn, reflected by a large DEA peak. The acetaldehyde is not present in Fig. 3 as a nitrogen-sensitive detector was used, but at the beginning of the total ion chromatogram obtained by GC–MS a broad peak with $m/z = 44$ is observed.

Peaks 5 and 6 give almost identical mass spectra and obviously result from other rearrangements and degradation reactions in the precolumn. According to the mass spectrum, one of these peaks could be a result of the Cope reaction[22], resulting in the formation of N,N-diethylhydroxylamine (N,N-DEHA) from TEAO after elimination of ethene. The ethene is not observed with the nitrogen-sensitive detector. The other peak could be O,N-diethylhydroxylamine (O,N-DEHA), which should give a very similar mass spectrum and a similar retention time.

Thus, the following reaction scheme is suggested:

$$\text{TEAO} \rightarrow \begin{cases} \text{TEA} \\ \text{TEHA} \rightarrow \text{DEA} + \text{CH}_3\text{CHO} \\ \text{N,N-DEHA} \\ \text{O,N-DEHA} \end{cases}$$

The Cope reaction cannot occur with TMAO owing to the lack of a β-hydrogen.

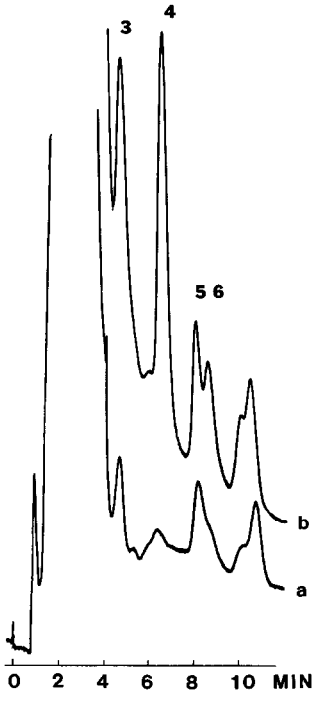

Fig. 4. Chromatograms after thermal degradation at 150°C. Attenuation, $1 \cdot 10^{-11}$ a.u.f.s. (a) Urine; (b) urine spiked with 10 ppm of TEAO. Temperature programme and peak identities as in Fig. 3.

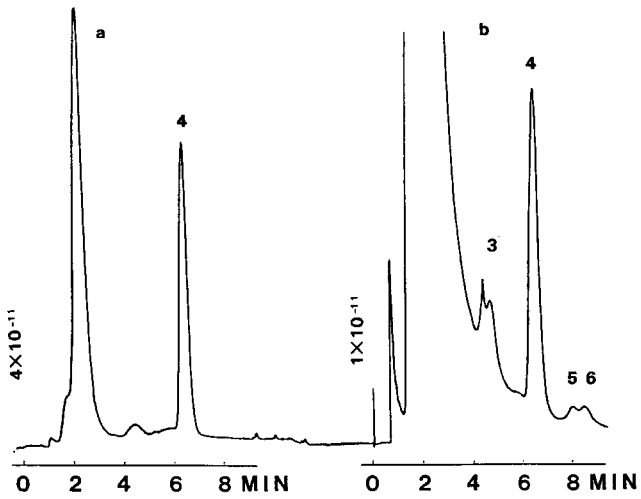

Fig. 5. Chromatograms of urine spiked with 10 ppm of TEAO and 30 ppm of TEA. (a) Direct injection through precolumn at 80°C; attenuation, $4 \cdot 10^{-11}$ a.u.f.s. (b) Chromatogram after heating of precolumn to 150°C; attenuation, $1 \cdot 10^{-11}$ a.u.f.s. Temperature programme and peak identities as in Fig. 3.

Identification of amine N-oxides in urine

These results clearly show that the degradation patterns of TMAO and TEAO can be used as strong evidence for their presence in a sample. It can also be expected that other amine N-oxides can be identified in a similar way.

Urine is an especially interesting matrix in connection with amine N-oxides. The influence of a urine background on the degradation pattern is illustrated in Fig. 4, which shows a direct injection of urine spiked with TEAO, one of the major metabolites after exposure to air containing TEA. It can be seen that the short-chain amines which are abundant in urine do not appreciably influence the degradation pattern. However, in the analysis of urine samples from workers after exposure to TEA in air, the TEA peak will emanate both from TEA and from the degradation of TEAO.

A chromatogram of a mixture of TEAO and TEA in urine is shown in Fig. 5. In this instance the injection procedure for a mixture (described above) was used. The main peak (4) in chromatogram (a) is TEA, which eluted through the precolumn at a temperature of 80°C with the valves open before the thermal degradation was started. Chromatogram (b) shows thermal degradation products of TEAO. The TEA peak is larger here than expected, owing to incomplete elution from the precolumn at 80°C. In spite of this, the first TEA peak is reproducible and could be used for the quantification of TEA.

If only amine N-oxides are of interest, an extraction of an alkalinized sample with *e.g.*, diisopropyl ether minimizes amine interferents. TEA is completely removed from urine samples in this way.

A further clean-up of urine samples by TLC may give a more reliable identification and protects the precolumn. It demands, however, considerably more work and leads to lower quantitative accuracy. Fig. 6 shows a chromatogram obtained after precleaning a urine sample spiked with TEAO. The spot giving the chromato-

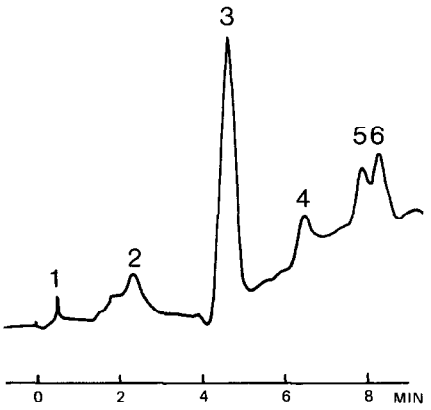

Fig. 6. Chromatogram of urine spiked with 10 ppm of TEAO after precleaning with TLC and thermal degradation. Other conditions and peak identities as in Fig. 3.

gram was identified as TEAO by comparison of the R_F value with that of a standard. The degradation pattern in Fig. 6 is very similar to that in Fig. 3, confirming the identity. Authentic urine samples obtained from exposed workers also gave the same pattern.

For shorter chained amine N-oxides than TEAO, the problem with direct injection of urine increases, as the thermal degradation products will be obscured by the amine background. However, with removal of the amines in an extraction or TLC step and a further optimization of the chromatographic separation, it should be possible to identify even small amounts of the shortest chained amine N-oxide, TMAO, using the peak of TMHA.

Optimization of the system

To perform quantitative determinations of amine N-oxides using the thermal degradation technique, it is necessary to obtain a high and reproducible yield of one or several characteristic degradation products. Several parameters that influence the degradation process were investigated. These include precolumn temperature, heating time and amount of water present in the precolumn. The optimization procedure was performed for both TMAO and TEAO.

TMAO. At a temperature of 125°C significant degradation was observed. However, better conditions from an analytical point of view were obtained using 175°C. At this temperature adsorption of the degradation products in the precolumn was less pronounced, resulting in sharper peaks in the final chromatograms. Furthermore, the degradation was more efficient, giving higher peaks. A temperature of 175°C was chosen, considering the temperature restrictions of the polymeric material (Kel-F) used for the valve blocks.

The thermal degradation procedure was optimized with respect to the amount of water injected and heating time at 175°C. It was found that the most interesting peak, the supposed rearrangement product of TMAO to TMHA (peak 3 in Fig. 2), was largest using a heating time of 5 min and a total injection volume of at least 4 μl.

The injection of large water volumes (up to 17 μl was tested) demands a longer

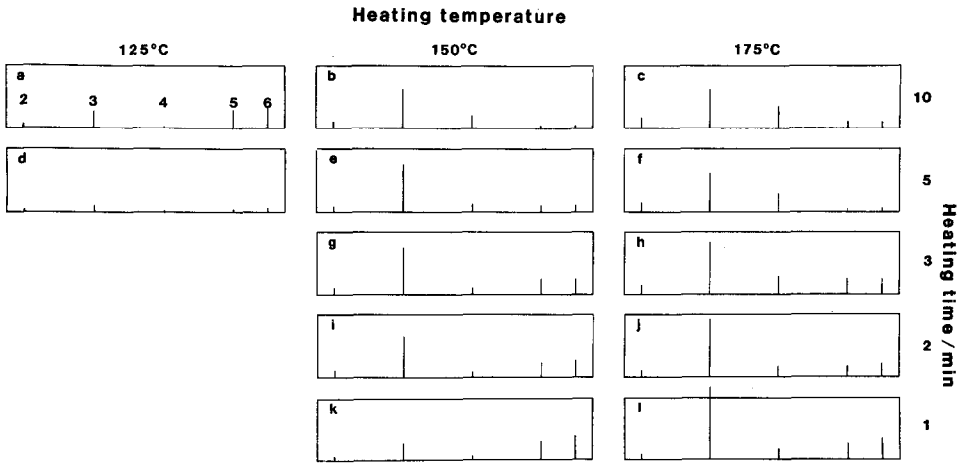

Fig. 7. Schematic chromatograms showing the degradation patterns of TEAO (100 ppm in 0.5 *M* NaOH) after heating in the precolumn for different times and temperatures. Peak identities as in Fig. 3.

heating time. The small inner volume of the precolumn also makes injection of large water volumes difficult. On the other hand, too small water samples are not desirable because of memory effects, *i.e.*, peaks of TMA and TMHA which appear in the following run. This may be due to incomplete degradation of TMAO. As prolongation of the heating time does not decrease the memory effects significantly, access to sufficient amounts of water seems to be necessary for rapid degradation at the chosen temperature.

Taking all these effects into consideration, we chose a sample volume of 5 μl in the subsequent experiments. Under these conditions the memory peaks are of the order of 20% at a concentration of 10 ppm of the TMAO injected and decrease to about 5% in a second blank injection. This must be taken into consideration when quantitative analysis is attempted.

TEAO. With TEAO, more complex influences on the degradation patterns are observed. Fig. 7 shows schematic degradation patterns as a function of temperature and heating time with a 5-μl aqueous sample injection. The conditions for chromatogram (g), heating for 3 min at 150°C, were used in further studies. Chromatogram (h) gives about the same height of the DEA peak, preferably used for quantification of TEAO, but the TEA peak is higher than in chromatogram (g). However, a relative low TEA peak is favourable with respect to possible memory effects in quantitative measurements of TEA in a mixture of TEA and TEAO.

Band broadening

The band broadening process in the precolumn was investigated by direct injection of TMA onto the analytical column. It was found that the peak width was the same, within experimental uncertainty, with and without precolumn. This was also expected as the dead volume in the precolumn (*ca.* 0.05 ml) is small compared with that of the analytical column (*ca.* 9 ml).

Quantification

Alkaline aqueous standard solutions of TMAO and TEAO with concentrations up to 500 ppm gave linear calibration graphs passing close to the origin. For TMA, however, the graph deviated from linearity above 200 ppm. The quantification was based on the peaks from TMA or the supposed rearrangement product THMA in the case of TMAO and on the peaks from either DEA or TEA for TEAO.

The linear correlation coefficients (based on nine points) were 0.996 for TMA, 0.997 for THMA, 0.9997 for DEA and 0.9991 for TEA. In all instances the intercepts on the ordinate did not differ significantly from zero.

The overall precision for TMAO in aqueous solutions was determined using triple injections at a concentration of 10 ppm. Peak heights of TMA and TMHA were measured, giving relative standard deviations of 12% and 6%, respectively. For TEAO at the same concentration, the corresponding figures were 9% based on the peak of DEA and 12% based on the peak of TEA. The detection limits were *ca.* 0.2 ppm (1 ng) for TMAO and *ca.* 1 ppm (5 ng) for TEAO.

The determination of TEAO in a urine matrix is interesting with respect to occupational air exposure. Calibration graphs for TEAO in spiked urine samples were linear in the range 5–100 ppm. The correlation coefficient for DEA was 0.997 and that for TEA was 0.992 and the intercepts on the ordinate did not differ significantly from zero. The precision was 5% for DEA and 8% for TEA (triple injections of urine spiked with 10 ppm of TEAO). The detection limit of TEAO in spiked urine, based on the TEA peak, was *ca.* 1 ppm. Based on the DEA peak it was *ca.* 3 ppm, mostly owing to a higher background.

In urine from workers exposed to TEA, the detection limit for TEAO based on the TEA peak will be higher owing to the contribution from TEA in the urine. Even when TEA is eluted before thermal degradation on the precolumn as in Fig. 5, some memory effects from TEA remain. However, this problem can be eliminated by a preceding basic extraction of TEA from the urine.

With an exposure to 8 mg/m^3 of TEA in air (the Swedish Hygiene Threshold Limit Value from 1990[23]), the concentration in urine of TEAO after 8 h is of the order of 20 ppm[7]. This corresponds to about ten times the detection limit, as the concentration in the sample before the final injection onto the GC column is almost the same as that in the urine.

CONCLUSION

Controlled thermal degradation followed by GC can be used for the identification and quantification of amine N-oxides. As the conditions during the thermal degradation step can easily be varied, *e.g.*, by incorporating different catalysts in the precolumn, it should also be possible to apply this procedure to other thermolabile substances.

ACKNOWLEDGEMENT

This work was supported by a grant from the Swedish Work Environment Fund.

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