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Using EGA-GC to Analyze Nicotine N-oxide in Order to Explain Low Nicotine Concentrations in E-cigarette Liquids

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Using EGA-GC to Analyze Nicotine N-oxide in Order to Explain Low Nicotine Concentrations in E-cigarette Liquids

A Thesis

Presented to the Department of Chemistry and Biochemistry

College of Liberal Arts and Sciences

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In Partial Fulfillment

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Cory Alverson Wuerch

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<u>Abstract</u>

Evolved-gas analysis coupled with gas chromatography (EGA-GC) was used to analyze e-cigarette liquids. Previous analyses of e-cigarette liquids have shown that the determined concentration of nicotine is lower than the advertised concentration. A possible explanation for this phenomenon is that the nicotine in the liquids is being oxidized to nicotine N-oxide by exposure to air and thus reducing the concentration of nicotine. This study focused on analyzing samples of thermally-rearranged nicotine N-oxide. Using EGA-GC, a calibration curve was generated for nicotine N-oxide concentration, which could potentially be used to explain reduced nicotine concentrations in e-cigarette liquids.

Introduction

The use of electronic nicotine delivery systems (ENDS) or electronic cigarettes (e-cigarettes) has become an increasingly popular recreational activity as well as an alternative to tobacco cigarettes. As of 2016, 15.4% of adults in the U.S. had ever used an e-cigarette, and 3.2% currently use e-cigarettes.[1] A recent CDC report showed that a greater percentage of smokers are now using e-cigarettes instead of FDA approved cessation aid such as nicotine patches or nicotine gum.[2] The same report also showed that instead of quitting traditional tobacco cigarettes, many smokers continue using both e-cigarettes and tobacco cigarettes (referred to as "dual use").[2] E-cigarettes seem to be particularly attractive to young people. The percentage of middle and high school students who have ever used e-cigarette products increased from 4.7 to 10.0%.[3] As of 2016, e-cigarettes were the most commonly used tobacco product among high (11.3%) and middle (4.3%) school students.[4] It has also been shown that never-smoking adolescents who try e-cigarettes are at an increased risk of established conventional cigarette smoking.[5] During 2010-2013, e-cigarette awareness among adults also increased from 40.9% to 79.7%.[3] One of the reasons for this dramatic increase in e-cigarette popularity is the perception that e-cigarettes are a "healthier alternative" to traditional tobacco products. There is some truth to this belief, as e-cigarettes do not involve the combustion of tobacco, which generates deadly carcinogens and leads to the numerous adverse health effects associated with tobacco use and second-hand smoking.[6] Proponents of e-cigarettes even claim that these devices have the potential to be cessation aids for traditional tobacco cigarettes; however, the use of e-cigarettes as a cessation aid has not been approved by the FDA.[6,8] Although e-cigarettes have increased in popularity,

there is still little known about the potentially harmful, long-term physiological effects of these products. As of August 8th, 2016, the U.S. Food and Drug Administration has extended its regulation oversight to e-cigarette products.[9] The FDA claims that its goal is "to protect Americans from tobacco-related disease and death" and the new regulation "does not mean [e-cigarettes] are safe to use."[9]

Most e-cigarette liquids ("e-liquid" or simply "juice") contain propylene glycol or vegetable glycerol as the excipient (or humectant carrier), water, nicotine, and flavoring compounds, although other additives and potentially toxic compounds have been detected in e-liquids and the aerosols of e-liquids.[6,9,10-16] When activated, the battery powered heating element (atomizer) vaporizes the e-liquid, delivering a nicotine-containing aerosol to the user and mimicking traditional tobacco cigarettes (see figure 1 for a typical e-cigarette design).[7,12] The health effects from long term exposure to propylene glycol and glycerol have not been investigated.[11] The use of ethylene glycol in place of glycerol or propylene glycol was reported by Hutzler et al.[11] Ethylene glycol is far more toxic than glycerol and propylene glycol and its use as a humectant is prohibited.[11]
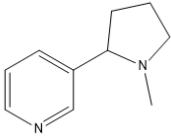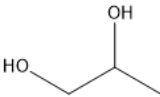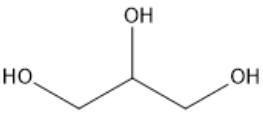
Figure 1: Standard e-cigarette design. The user places his or her mouth on the mouthpiece and then simultaneously activates the battery and inhales through the e-cigarette, delivering a nicotine-containing aerosol to the user. Although this image shows a standard design for e-cigarettes, it should be noted that e-cigarettes come in many different designs.[7]

Table 1 shows the structure, properties, and hazards of nicotine, propylene glycol, glycerol, and ethylene glycol. Lethal dose 50 (LD50) represents the amount of a substance needed to kill half of a test sample and is often used to indicate the toxicity of a substance. The LD50s included in table 1 are the result of oral ingestion by rats. Note the much lower LD50 for nicotine compared to the other three compounds, which means that a smaller dose of nicotine is needed to kill 50% of the test population (i.e., nicotine is more toxic). Due to this higher toxicity of nicotine, precise labeling of nicotine concentrations on e-cigarette liquid packages should be prioritized among manufactures so as to reduce the risk of consumption of a lethal amount of nicotine. Note also that the information reported in table 1 for glycerol and propylene glycol is for the liquid, un-aerosolized forms. When these two compounds (as well as ethylene glycol if it is present in the e-liquid) are vaporized, it is possible for them to decompose into toxic carbonyl compounds (e.g., aldehydes) such as formaldehyde.[11,13-15] In addition to the major

components of e-liquids, other identified toxic substances include tobacco-specific nitrosamines (TSNAs), fine particulate matter, metals, various volatile organic compounds (VOCs), phenolic compounds, polycyclic aromatic hydrocarbons (PAHs), and other potentially toxic flavoring compounds such as diacetyl.[6,10-16]

Table 1: Structures and properties of various compounds found in e-liquids.

Name	Structure	Properties	Hazards
Nicotine		Molecular weight: 162.23 g/mol. Boiling point: 247°C. Colorless, volatile base (pK$_a$ = 8.0)	LD50 (oral-rat): 70 mg/kg. Fatal if swallowed or in contact with skin. No carcinogenicity.
Propylene glycol		Functions as a solvent carrier. Clear, viscous liquid. Molecular weight: 76.09 g/mol. Boiling point: 187°C.	LD50 (Oral-Rat): 20,000 mg/kg. No carcinogenicity.
Glycerol (or Glycerin)		Functions as a solvent carrier. Colorless liquid. Molecular weight: 92.09 g/mol. Boiling point: 182°C.	LD50 (Oral-Rat): 12,600 mg/kg. No carcinogenicity.
Ethylene Glycol		Liquid, clear, sweet-tasting. Molecular weight: 62.07 g/mol, Boiling point: 196 °C.	LD50(Oral-Rat): 4,700 mg/kg. "Do not breathe dust/ fume/ gas/ mist/ vapours/ spray."

Previous investigations[17-21] have shown that the concentration of nicotine in e-cigarette liquids is often lower than the concentration advertised on the e-liquid containers. E-cigarette liquids that claim to be nicotine free have been shown to contain low concentrations of nicotine, suggesting ingredient "carryover" from the preparation of previous e-liquids.[11,17-18] Other research[11,17,20] has shown that the amount of nicotine delivered to the vapor and the amount of bioavailable nicotine is not strongly related to

the amount of nicotine in the e-liquid. To date, no explanation has been given for the low

concentration of nicotine in e-cigarette liquids. Kim et al.[19] reported a color change in an

e-liquid sample after seven days. This process is colloquially referred to as "steeping"

and e-cigarette users claim that it improves the flavor of their e-liquid; however, the

physiochemical effects of this practice are unknown.[19] The researchers attributed the

color change to the oxidation of nicotine in the e-liquid.[19]

The unreliable labeling of nicotine concentration as well as often potentially fatal

amounts of nicotine in e-cigarette liquids has generated concern over accidental

consumption of e-liquid refill solutions by children.[21] This concern is exacerbated by the

fact that many e-liquids come in flavors (candy, desserts, etc.) and packaging (bright

colors, eye-catching designs) that are attractive to young people (image 1).

Image 1: E-cigarette liquid containers. Notice the bright colors and sweet flavors
(green apple, grape, cake batter, for example), which may be attractive to young people.[22]

Because nicotine is highly addictive, potentially toxic, and is the psychoactive

compound present in e-cigarette liquids, accurate labeling of the amount of nicotine

should be a priority among e-liquid manufactures. Nicotine works by binding to nicotinic

acetylcholine receptors (nAChRs) in the brain and adrenal medulla.[11,23-24] Upon binding to the receptor, the membrane protein channel opens, causing a depolarization of the membrane, which leads to modulation of neuronal activity.[23] Specifically, nicotine binding to nAChRs causes the release of various chemical messengers such as acetylcholine, norepinephrine, epinephrine, dopamine, and serotonin into the synapse or the blood stream, which lead to the various physiochemical effects (sense of euphoria, vasoconstriction, increased heart, etc.) characteristic of nicotine consumption.[23]

Nicotine contains a tertiary amine and is composed of a pyridine and a pyrrolidine ring (Figure 2).[23] Amines are known to be easily oxidized, even by exposure to air.[26] When a tertiary amine is oxidized, it produces the amine oxide.[26] One possible explanation for the low concentration of nicotine in the e-liquids is that the nicotine is reacting with oxygen in the air, producing the zwitterionic nicotine N-oxide (Figure 3). This reaction results in a reduction of the nicotine concentration and an increase in the concentration of nicotine N-oxide. Thus, when the e-liquid is analyzed for nicotine concentration, the amount of nicotine in the e-liquid appears to be less than what is advertised on the packaging. Nicotine N-oxide is a primary metabolite of nicotine and is converted to nicotine by a flavin-containing monooxygenase 3 (FMO3).[25] A relatively small percentage of nicotine (4-7%) is metabolized through this pathway.[25] From here, the nicotine N-oxide can either be processed by the kidneys, eventually being excreted through urine, or it can be reduced back to nicotine, thus serving as a nicotine recycling pathway.[25]
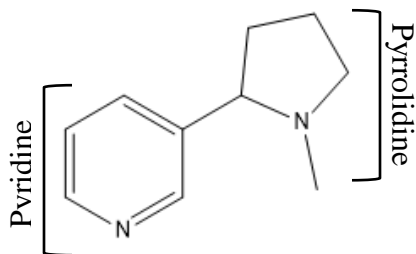
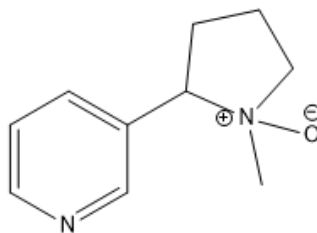**Figure 2: Structure of 3-(1-methylpyrollidin-2-yl)pyridine (nicotine).**

Figure 3: Structure of 1-methyl-2-(pyridine-3-yl)pyrrolidine 1-oxide (nicotine N-oxide). The oxidation of nicotine to nicotine N-oxide can occur simply by exposure to air, possibly explaining the low nicotine concentrations in e-cigarette liquids.

The study presented here utilized evolved-gas analysis coupled with gas chromatography (EGA-GC) to examine the hypothesis that air oxidation of nicotine is responsible for the low concentrations of nicotine in e-liquids. In order to properly test this hypothesis, it is necessary to have a reliable method for the determination of the amount of nicotine N-oxide present in a given e-liquid sample. This study presents a method for the determination of nicotine N-oxide in e-cigarette liquids using EGA-GC, which could have implications for e-cigarette users, e-liquid manufacturers, and regulatory standards.

Experimental

<u>Instrumentation</u>

All nuclear magnetic resonance (NMR) experiments were performed using a

Bruker BioSpin Avance III HD 400 Nanobay System. Deuterated chloroform (CDCl$_3$)

was used as the solvent for each analysis. Chemical shifts are reported versus

tetramethylsilane (TMS).

The microwave reactor used for the conversion of N-oxide to oxazine was a CEM

Discover SP Microwave System w/ActiVent Technology, 12V. Dimethylformamide

(DMF) was used as the solvent for the preparation of microwave reactor samples.

Evolved-gas analysis of nicotine N-oxide was performed using an Agilent

Technologies 6890N Gas Chromatography-Flame Ionization Detector (GC-FID)

modified with an EGA oven inlet by Samide et al.[29] (Figure 4).
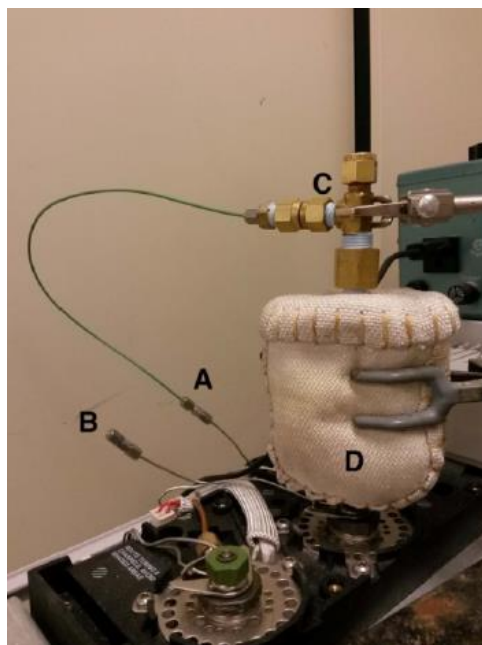

Figure 4: EGA-GC apparatus used for this study. The apparatus was installed on an Agilent 6890 GC/FID. A. Splice in Helium carrier gas line. B. Plug for remaining line welded to inlet. C. Cool zone (ambient). D. Warm zone (180°C). The sample is loaded into a small metal EGA cup and manually injected into C using a sample hook.[29]

Synthesis

3-(1-methylpyrollidin-2-yl)pyridine. HNMR (CDCl$_3$): δ8.55 ppm (1H, s), δ8.5 ppm (1H, d), δ7.7 ppm (1H, d), δ7.3 ppm (1H, t), δ3.25 ppm (1H, t), δ3.1 ppm (1H, t), δ2.3 ppm (1H, quartet), δ2.2 ppm (1H, m), δ2.15 ppm (3H, s), δ1.95 ppm (1H, m), δ1.8 ppm (1H, m), δ1.7 ppm (1H, m) (Figure 8).

1-methyl-2-(pyridine-3-yl)pyrrolidine 1-oxide. Hydrogen peroxide (30%, 0.2 mL) was added to a round bottom flask containing 3-(1-methylpyrollidin-2-yl)pyridine (nicotine) (0.5 mL, 0.00305 mol) and water (10 mL). The reaction mixture was left to stir for one week. The water was removed using rotary evaporation, leaving behind an oil. This was washed with absolute ethanol and the ethanol was removed using rotary evaporation. The crude nicotine N-oxide product was analyzed via HNMR (Figure 9). Upon inspection of the HNMR, it was determined that a significant amount of unreacted nicotine was present. In order to convert the remaining nicotine to nicotine N-oxide, the reaction mixture was re-subjected to the reaction conditions. Hydrogen peroxide (30%, 0.3 mL) and water (10 mL) were combined with the reaction mixture and left to stir. After another week, the nicotine N-oxide crude product was again washed with ethanol and the ethanol was removed using rotary evaporation to yield an oil (0.272 g, 0.00152 mol, 49.8%). HNMR (CDCl$_3$): δ8.8 ppm (1H, s), δ8.6 ppm (1H, d), δ8.25 ppm (1H, d), δ7.4 ppm (1H, t), δ3.1 ppm (3H, s) (Figure10).

2-methyl-6-(3-pyridyl)tetrahydro-1,2-oxazine. The 1-methyl-2-(pyridine-3-yl)pyrrolidine 1-oxide (N-oxide) product was dissolved in DMF and subjected to microwave reacting (30 min, 160ºC). The DMF was removed by rotary evaporation to yield the rearrangement product (0.298g, 0.00305mol, 54.8%). The rearrangement product was analyzed by HNMR (Figure 11). Upon inspection of the HNMR, it was

determined that the sample needed to be microwaved again in order to more completely

covert the nicotine N-oxide the rearranged product. Comparison sample: 2-methyl-6-(3-

pyridyl)tetrahydro-1,2-oxazine HNMR (CDCl$_3$): δ8.6 ppm (1H, s), δ8.6 (1H, d), δ7.7

(1H, d), δ7.3 (1H, t), δ4.9 ppm (1H, d), δ3.0 ppm (1H, t), δ2.7 ppm (3H, s), δ2.6 ppm

(1H, t), δ2.0 ppm (1H, m), δ1.9 ppm (1H, m), δ1.7 (1H, m), δ1.6 (1H, q) (Figure 12).

Calibration Curve

N-oxide-rearrangement product (14.6 mg) was combined with acetone (10 mL),

resulting in an $8.19 \times 10^{-3} M$ standard. Serial dilution was performed to generate a

$1.12 \times 10^{-3} M$ standard. Both of these samples were analyzed via EGA-GC. The $8.19 \times$

$10^{-3} M$ and $1.12 \times 10^{-3} M$ standards (10 µL of each) as well as 5 µL of the $8.19 \times$

$10^{-3} M$ standard were analyzed by EGA-GC to determine the relative amount of nicotine

N-oxide in each solution. Analyses were performed in triplicate and peak area was

plotted versus moles of nicotine N-oxide to generate a nicotine N-oxide calibration curve.

The EGA-GC procedure is as follows: once the injector thermometer reaches 180ºC, an

exact volume of the sample is placed in an EGA cup and lowered into the warm zone

(Figure 4). After 2 minutes (the time needed for injection), the sample is raised into the

cool zone. The sample is then analyzed and the retention times and relative amounts

(represented as the area under the curve) of the various volatile compounds in the sample

are collected.

Method Validation

A sample of 2.45% nicotine N-oxide in propylene glycol (a common e-liquid

humectant carrier) was prepared by dissolving nicotine N-oxide (0.074 g, 13.2 mol) in

propylene glycol (3.019 g). The sample was microwaved for 30 minutes at 160ºF in order

to produce the thermal rearrangement product (oxazine). The rearrangement product was analyzed by HNMR (Figure 13). Separate aliquots (~5 mg × 4) were analyzed by EGA-GC in order to verify that the entire sample was analyzed after the first injection and to see if the rearranged product was actually formed. HNMR (CDCl$_3$): δ8.52 ppm (1H, s), δ8.48 ppm (1H, d), δ7.7 ppm (1H, d), δ7.3 (1H, t) (Figures 13-15).

A spike recovery with nicotine was performed. A drop of nicotine was added to a GC vial and the remainder of the vial was filled with acetone so as to dilute the nicotine. A small amount of this solution was then added to the 2-methyl-6-(3-pyridyl)tetrahydro-1,2-oxazine (oxazine) product. This spiked solution was then analyzed by EGA-GC and the retention times and peak areas were recorded.

Results and Discussion

The purpose of this study was to examine the hypothesis that the low

concentration of nicotine in e-cigarette liquids reported by previous investigations is the

result of oxidation of nicotine to nicotine N-oxide by exposure to air. Jacob et al.[27]

developed a method for determining the amount of nicotine N-oxide, where the N-oxide

undergoes a thermal rearrangement to produce 2-methyl-6-(3-pyridyl)tetrahydro-1,2-

oxazine (simply referred to as oxazine) via microwave reaction, which is volatile and can

be analyzed by gas chromatography. The reaction scheme for this process is provided

(Equation 1) as well as a proposed arrow-pushing mechanism (Mechanism 1).

Nicotine   Nicotine N-oxide   Oxazine
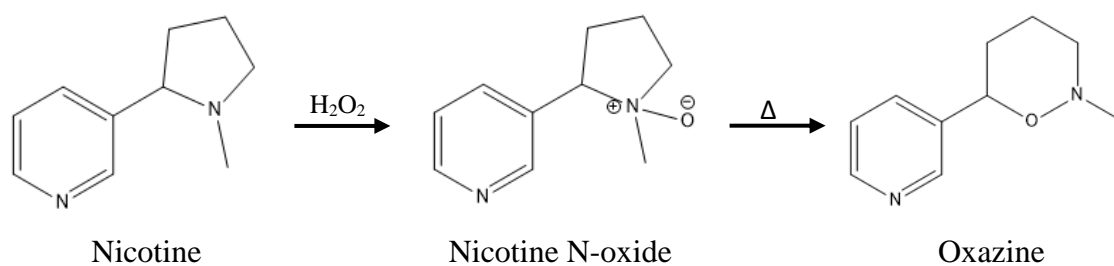
Equation 1: Reaction scheme for the production of nicotine N-oxide and the subsequent thermal conversion to oxazine.
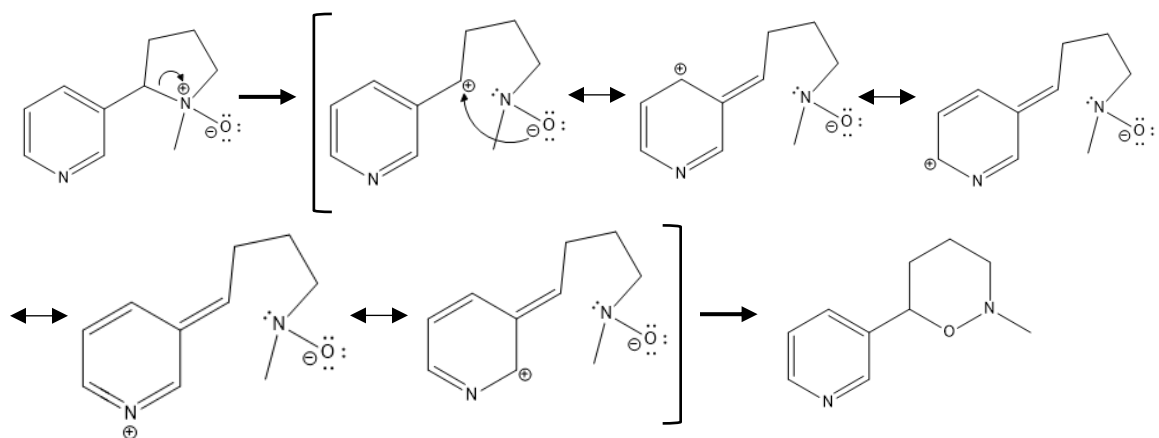
Mechanism 1: Proposed mechanism for the rearrangement of the nicotine N-oxide to the oxazine. The mechanism shown above involves the thermal rearrangement of nicotine N-oxide to generate the oxazine. Note the formation of the resonance-stabilized carbocation. The heat from the microwave reactor is necessary in order to overcome the activation energy barrier associated with the formation of the carbocation.

Evolved-gas analysis coupled with gas chromatography (EGA-GC) was used in order to indirectly determine the amount of nicotine N-oxide in e-liquids. Evolved-gas analysis (EGA) is a method used to determine the amount of volatile organic compounds (VOCs) present in a sample when the sample is heated into the gas phase.[27-29] When coupled with GC and flame ionization detector (FID), the identity and relative amount of each volatile compound can be determined.

Nicotine N-oxide is not volatile and therefore cannot be analyzed by EGA-GC. As stated earlier, oxazine, produced from a thermal rearrangement of nicotine N-oxide, is volatile and can be analyzed by EGA-GC. Thus, if a sample of nicotine N-oxide undergoes a thermal rearrangement to produce oxazine in a one-to-one ratio, then the amount of oxazine can be determined by EGA-GC, which reflects the amount of nicotine N-oxide that was originally in the sample. The amount of nicotine N-oxide and unreacted nicotine in the sample can be compared to determine whether or not the oxidation of

nicotine is the reason for low nicotine concentrations in e-liquids. A calibration curve was generated using samples with known concentrations of nicotine N-oxide (Table 2, Table 3, Figure 5).

Table 2: Nicotine N-oxide calibration data.

Concentration (M)	Volume (μl)	Moles	Response (Area)
0.00819	10	8.19E-08	5216.38
0.00819	10	8.19E-08	5117.97
0.00819	10	8.19E-08	5110.42
0.00112	10	1.12E-08	558.25
0.00112	10	1.12E-08	518.17
0.00112	10	1.12E-08	540.71
0.00819	5	4.095E-08	2615.06
0.00819	5	4.095E-08	2406.39
0.00819	5	4.095E-08	2611.25

Table 3: Processing of nicotine N-oxide calibration data from table 2.

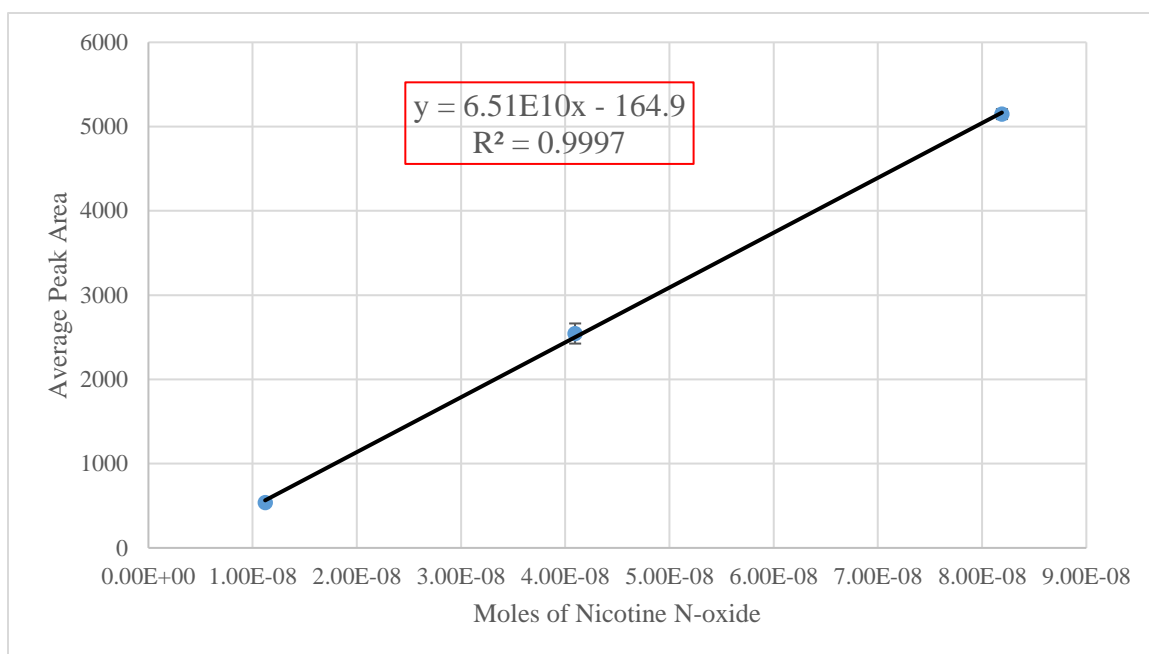| Moles (x)	Average Area (y)	Standard Deviation	%RSD
8.19E-08	5148.26	59.12	1.15
4.10E-08	2544.23	119.39	4.69
1.12E-08	539.04	20.09	3.73

Figure 5: Nicotine N-oxide external calibration curve. Increasing amounts of nicotine N-oxide were analyzed by EGA-GC in triplicate measurements. The average signal response (area) was plotted against the moles of each sample to generate the calibration curve. Standard deviations are shown as error bars. The calibration equation is $y = 6.51 \times 10^{10}x - 164.9$ with an $R^2 = 0.9997$ which indicates a strong linear correlation within the range.

This external nicotine N-oxide calibration curve represents a crucial step towards explaining the origin of reduced nicotine concentration in e-liquids. Previous work with nicotine allowed both nicotine and nicotine N-oxide to be quantified. An e-liquid sample with an unknown concentration of nicotine N-oxide can be analyzed using the EGA-GC method described above. The peak area for nicotine and oxazine can be collected and the moles of nicotine and nicotine N-oxide in the sample can be calculated. If the concentration of nicotine plus the concentration of nicotine N-oxide equals the concentration of nicotine advertised on the e-liquid bottle, then it can be reasonably

concluded (assuming a large enough sample population) that the cause of reduced

nicotine concentration in e-liquids is the air oxidation of nicotine to nicotine N-oxide.

After generating the nicotine N-oxide calibration curve, two important questions

remained regarding the validity of this method: (1) is the microwave reactor used to

convert the nicotine N-oxide to oxazine a viable experimental option for actual e-

cigarette liquids and (2) is the entire nicotine N-oxide sample volatilized after the first

run, or is there some sample still left over in the EGA cup that was not analyzed? The

first question addresses the problem that actual e-cigarette liquids contain a different

chemical composition than that of the nicotine N-oxide standards used to generate the

external calibration. Recall that e-liquids contain either propylene glycol or glycerol as an

excipient (although use of ethylene glycol has been reported), as well as various other

compounds for flavoring. These compounds were not present in the nicotine N-oxide

samples used for the calibration. Subjecting an e-liquid sample to the same reaction

conditions described for the conversion of nicotine N-oxide to oxazine could produce

undesired products due to the presence of these other compounds. In order to address this

problem, a sample with a known concentration of N-oxide was prepared in propylene

glycol, thus more closely mimicking the chemical composition of e-liquids. This sample

was heated in a microwave reactor in order to produce the thermal rearrangement product

(oxazine) and then analyzed using HNMR and EGA-GC (Figures 13-15). The calculated

amount of nicotine N-oxide based on the peak area and the nicotine N-oxide calibration

equation were compared to the known concentration (Table 4).

Table 4: Results from EGA-GC of thermal rearrangement product.

Sample	Run	Area	Time (min)	Moles	Mass (g)	%Mass
1 (0.0047g)	1	357.94	24.008	--	--	--
	2	436.78	23.963	--	--	--
Total	--	794.72	--	1.47E-08	2.64E-06	0.0562%
2 (0.0048g)	1	803.47	24.019	1.49E-08	2.67E-06	0.0555%
	2	--	--	--	--	--
3 (0.0049g)	1	847.07	24.024	1.55E-08	2.79E-06	0.0569%
	2	--	--	--	--	--
4 (0.0047g)	1	854.15	24.019	1.57E-08	2.81E-06	0.0597%
	2	--	--	--	--	--
Average		824.9		1.52E-08		
Standard Deviation		30.1		4.62E-10		
%RSD		3.65%		3.04%		

The external calibration previously developed using N-oxide rearrangement product and EGA-GC gave a best-fit equation of $y = 6.51 \times 10^{10} x - 164.9$. This equation and the area of each replicate were used to calculate the moles N-oxide rearrangement (which theoretically equals the moles of N-oxide). From the moles of N-oxide-rearrangement and its molecular weight ($179.23 \; ^g/_{mol}$) was calculated the mass of N-oxide-rearrangement. The percent by mass (far right column) was calculated for each sample based on the injection mass of the original sample. These samples were prepared as 2.45% nicotine N-oxide and microwave reacted to produce the N-oxide-rearrangement. The determined mass percentages of N-oxide-rearrangement are much lower than the expected 2.45%, suggesting that the N-oxide was not converted to the rearrangement product in a one-to-one ratio. The retention time used was almost exactly 24 minutes, which is the same retention time used for the calibration. The percent relative standard deviation (%RSD) for the signal response (area) is 3.65%, which indicates good precision.

The results from the EGA-GC of the rearranged product shows that except for the first sample, all of the sample was volatilized. The sum of the areas from the first run is similar to the areas from the subsequent three runs. Due to this similarity, it was inferred that the sum of the two areas from the first run (794.72) is equal to the total amount of nicotine N-oxide in the sample. Thus, this total was factored into the average area.

Additionally, the percent by mass (far right column) of nicotine N-oxide is significantly

lower than the expected 2.45%. The mass percent was calculated by first converting

moles of nicotine N-oxide to grams using the molecular weight. The grams of nicotine N-

oxide from this calculation was converted to a mass percentage by using the total mass of

the sample (in the left column). The low percentages suggests several possible issues: the

retention time of 24 minutes does not correspond to oxazine, unreacted nicotine could be

present in the sample, the microwave reactor did not produce the desired product due to

the presence of propylene glycol, or nicotine N-oxide was not converted to oxazine.

In order to test the possibility that unreacted nicotine was still present in the

sample, the EGA-GC data from oxazine in propylene glycol were reexamined in order to

see if there was a significant peak at the retention time that corresponds to nicotine

(Figure 6). Upon reexamination, there was a significant peak at around 20.2 minutes in

the EGA-GC data; however, it was not known whether or not this peak corresponded to
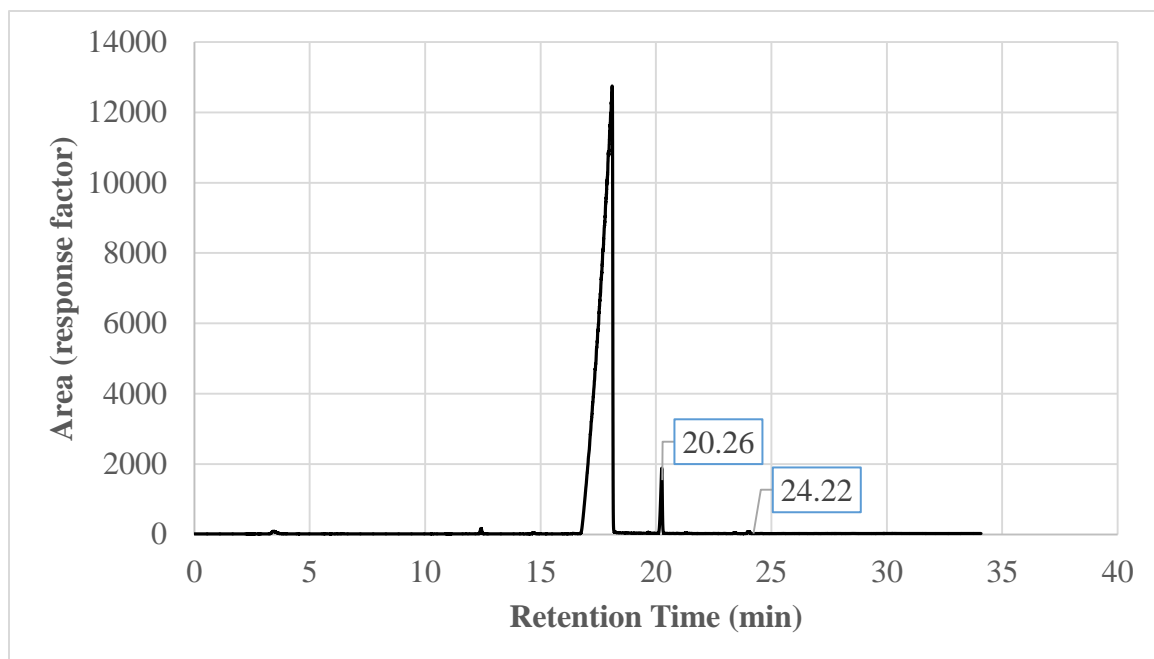
nicotine or to some other compound (Table 5).

Figure 6: EGA-GC chromatogram of oxazine in propylene glycol, sample 1. The peak at around 24 minutes corresponds to oxazine (the thermal rearrangement product). This peak is small compared to the peak at 20.2 minutes, which may correspond to unreacted nicotine.

Table 5: Results of EGA-GC of oxazine in propylene glycol.

Sample	Run	Area	Time (min)
1 (0.0047g)	1	9423.2	20.25
	2	4268.2	20.025
2 (0.0048g)	1	13511.2	20.27
	2	--	--
3 (0.0049g)	1	14573	20.288
	2	--	--
4 (0.0047g)	1	14441.4	20.283
	2	--	--
Average		14175.2	
Stdev		578.79	
%RSD		4.08	

The EGA-GC data was reexamined. A significant peak at ~20.2 minutes was present in all of the EGA-GC results. The identity of this compound is unknown. The second run of the first sample showed a significant peak at 20.025 minutes. Due to its different retention time, sample 1 data was omitted from subsequent calculations.

Notice that the second run of the first sample from table 5 shows a significant peak area at 20.025 minutes instead of 20.2 minutes. Due to this slight difference in retention time, sample 1 was not factored into subsequent calculations. It should be noted, however, that the sum of the first and second run (13691.4) is similar to the areas from the subsequent three samples. A possible explanation for this unique retention time is that the unknown compound whose retention time is normally 20.2 minutes underwent some sort of chemical modification from the heat of the microwave reactor or the EGA oven, resulting in a different compound with a unique retention time.

To verify the retention time of nicotine, a spike recovery of nicotine was performed (Figure 7). The spike recovery showed a significant peak area increase at 20.4 minutes. Additionally, EGA-GC of pure nicotine in acetone resulted in a large peak at 20.4 minutes. These results confirm that 20.4 minutes is the retention time of nicotine.
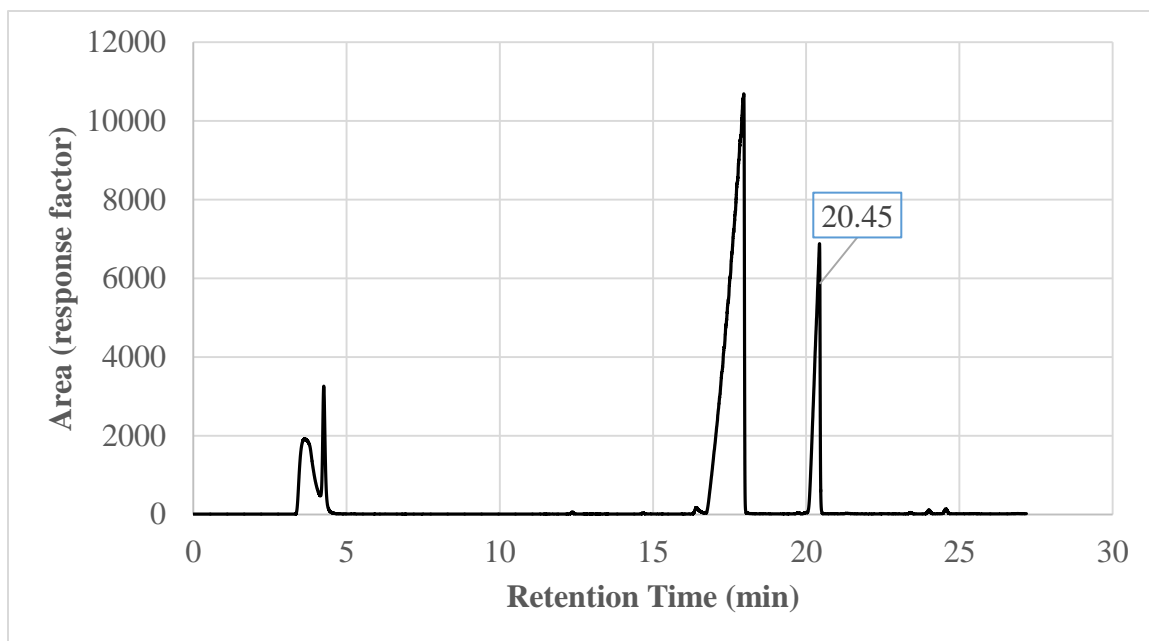

Figure 7: EGA-GC chromatogram of spike recovery of nicotine. Peak area at 20.4 minutes increased significantly, suggesting that this is the retention time for unreacted nicotine.

Considering that 20.4 minutes is close to 20.2 minutes, the identity of the compound whose retention time is 20.2 is likely unreacted nicotine; however, it may also be a side product formed from the thermal rearrangement reaction, thus preventing all of the nicotine N-oxide to be converted to oxazine in a one-to-one ratio. The HNMR of the oxazine in propylene glycol (Figure 13) shows that the NMR mostly detected the propylene glycol and only minimally measured the oxazine due to its low concentration. A closer look at the aromatic region of the HNMR (Figure 14) reveals that the peaks in

the aromatic region line up with the aromatic region of pure oxazine (Figure 12). This, however, is not conclusive evidence for the formation of oxazine due to the fact that unreacted nicotine has the same chemical shift pattern in the aromatic region. The relatively strong peak at δ7.3 ppm is uncharacteristic of both nicotine and oxazine. Notice that the base of this peak is wide and seems to overlap with a shorter peak at the same chemical shift. HNMR of oxazine and nicotine shows a peak at around δ7.3 ppm, but this peak should integrate to the same number of hydrogens (1H) as the other peaks in the aromatic region. Thus, this shorter peak at around δ7.3 ppm may correspond to oxazine or nicotine, but the strong peak at δ7.3 ppm must correspond to a different compound, perhaps the same compound whose EGA-GC retention time is 20.2 minutes. One major difference between the HNMR of oxazine and nicotine is the presence of a peak at δ4.9 ppm in the oxazine HNMR, which corresponds to the HC-O bond. This peak was not present in the oxazine in propylene glycol HNMR (Figure 15), suggesting that either oxazine was not formed (unlikely due to its detection by EGA-GC) or that oxazine was only minimally formed and the high concentration of propylene glycol effectively "hides" this peak. In any case, the presence of propylene glycol in the reaction mixture complicates the conversion of nicotine N-oxide to oxazine and the subsequent analysis of oxazine by EGA-GC and HNMR.

<u>Conclusion</u>

Reliable determination of the amount of nicotine and nicotine N-oxide in an e-cigarette liquid sample represents a crucial step towards explaining the cause of lower than expected nicotine concentration in e-liquids. An explanation for this phenomenon could have implications throughout the e-cigarette community including consumers, manufacturers, and regulators. The chemical composition of e-liquids, which often widely varies between flavors and manufactures, is complicated. Based on the results from the EGA-GC of nicotine oxazine in propylene glycol, significant research is required in order to effectively adapt this method for analysis of e-liquid samples. A major issue with this method appears to be that microwave heating is unable to effectively convert all of the nicotine N-oxide directly to nicotine oxazine in a one-to-one ratio in a chemical environment similar to that of e-liquids. Future directions for this research would be fine tuning this method so as to prevent unwanted reactions. Extraction and purification of nicotine and nicotine N-oxide from an e-liquid sample prior to microwave reaction could circumnavigate the problems brought about by the complex chemical environment of e-liquids.

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Appendix
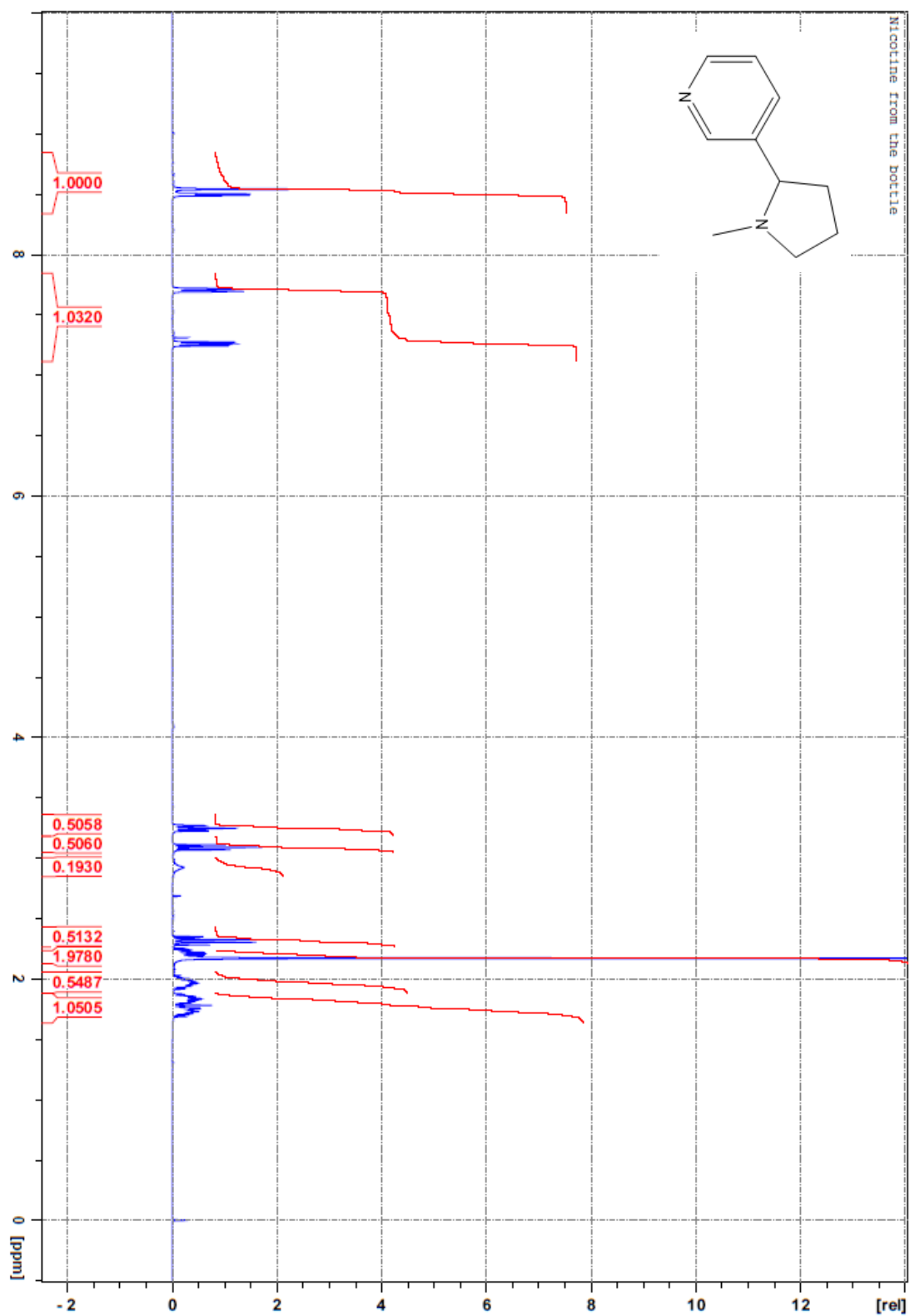
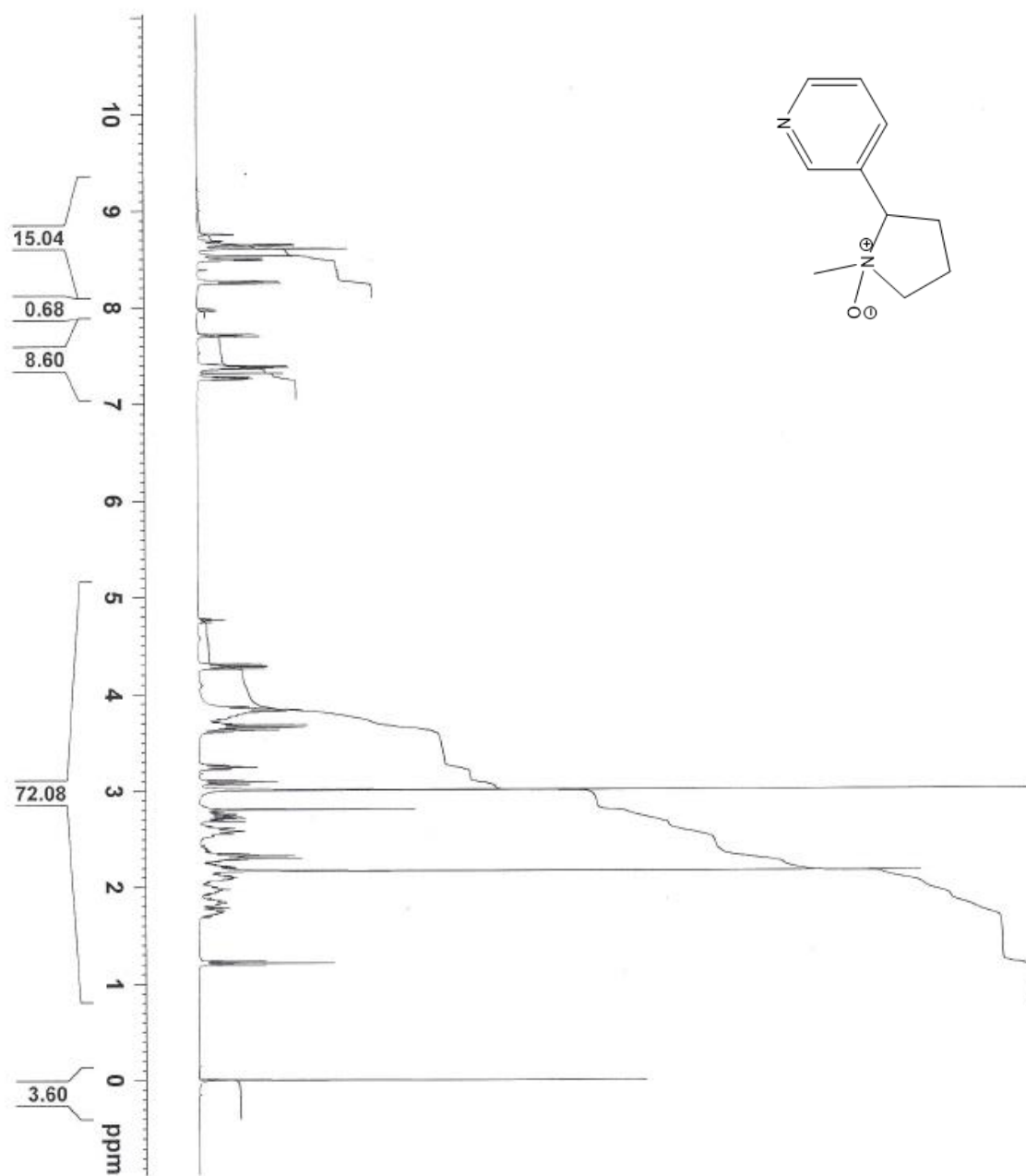**Figure 8: HNMR of 3-(1-methylpyrollidin-2-yl)pyridine (nicotine).**

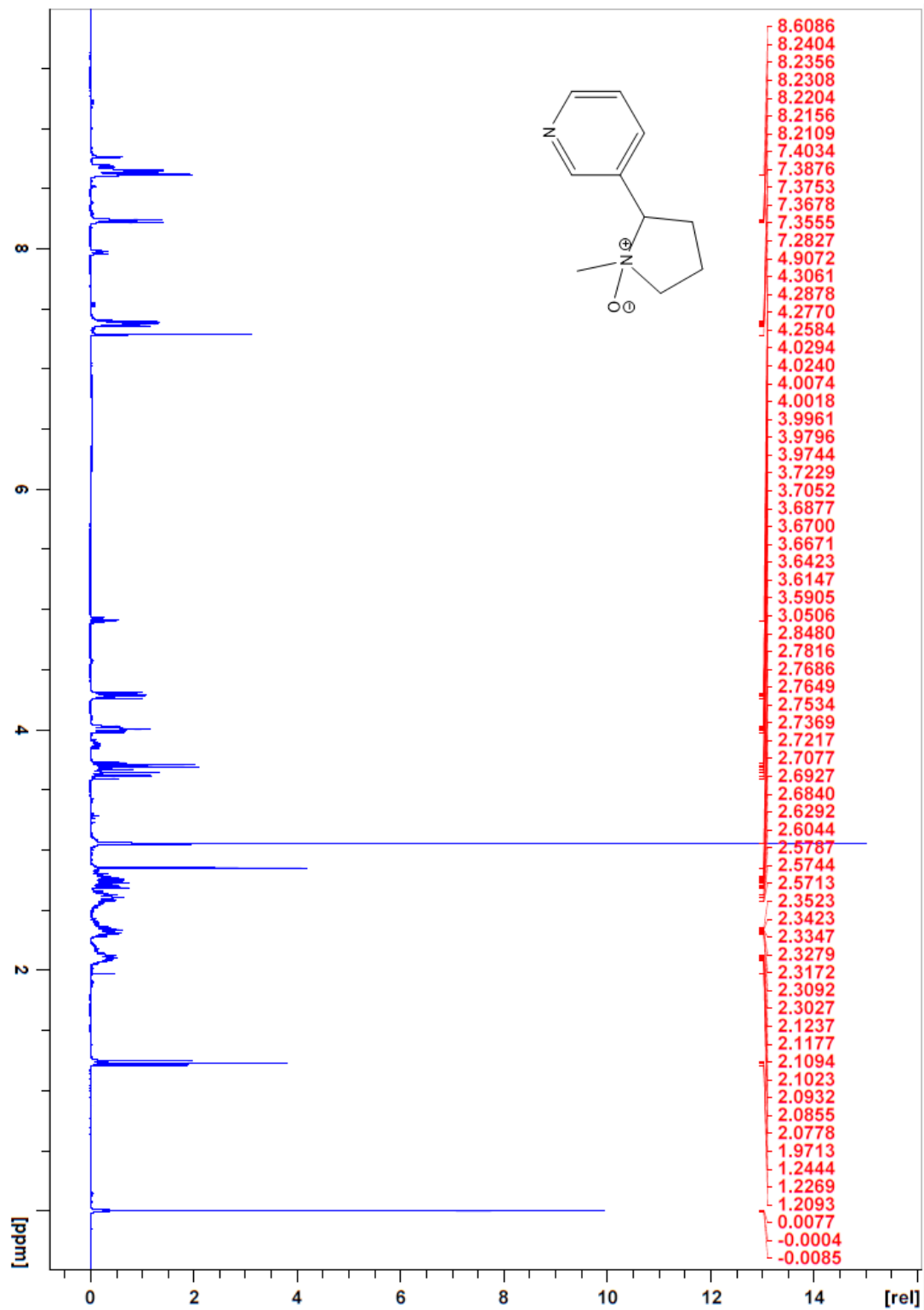**Figure 9: HNMR of crude 1-methyl-2-(pyridine-3-yl)pyrrolidine 1-oxide (N-oxide).**

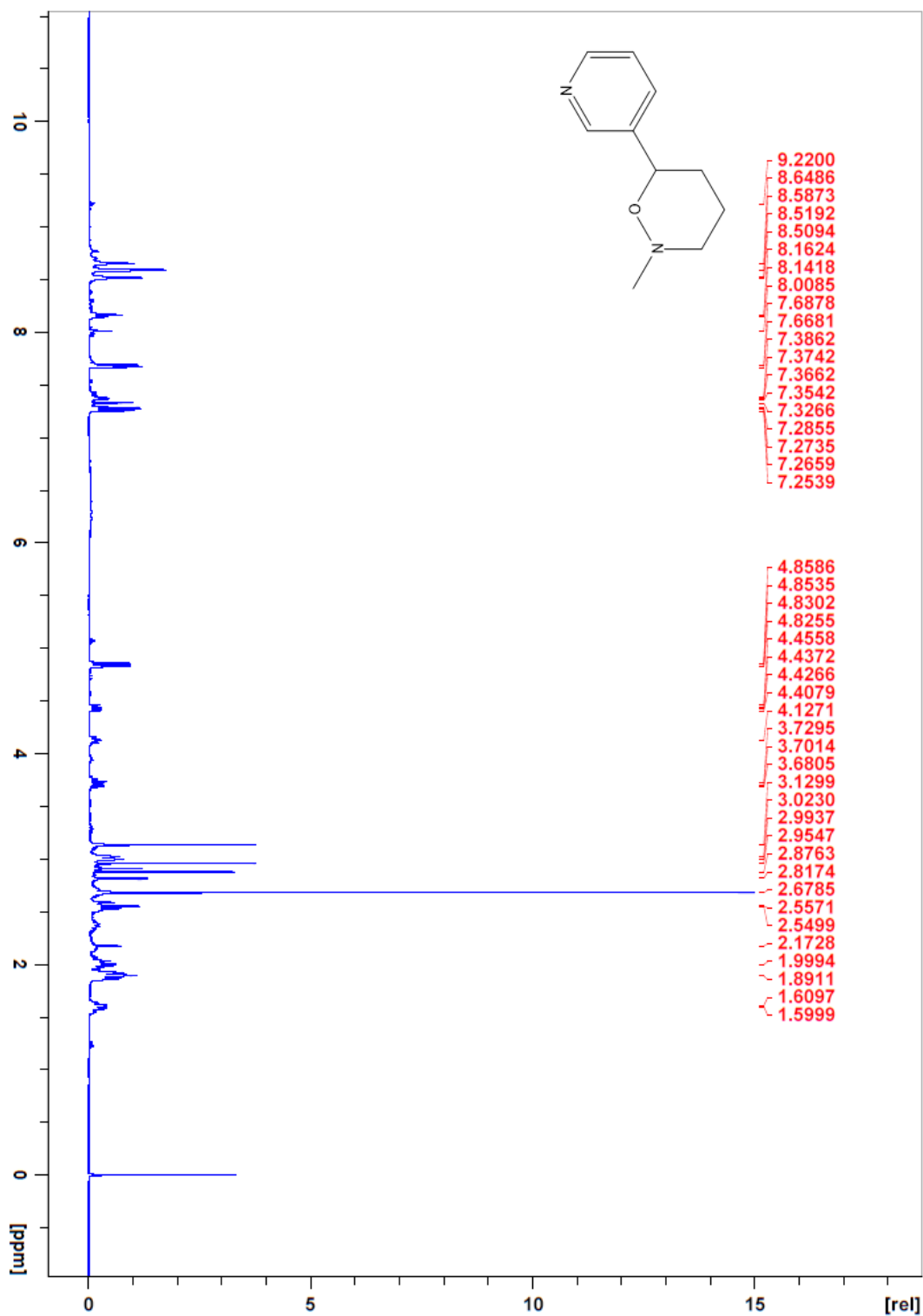**Figure 10: HNMR of 1-methyl-2-(pyridine-3-yl)pyrrolidine 1-oxide (N-oxide).**

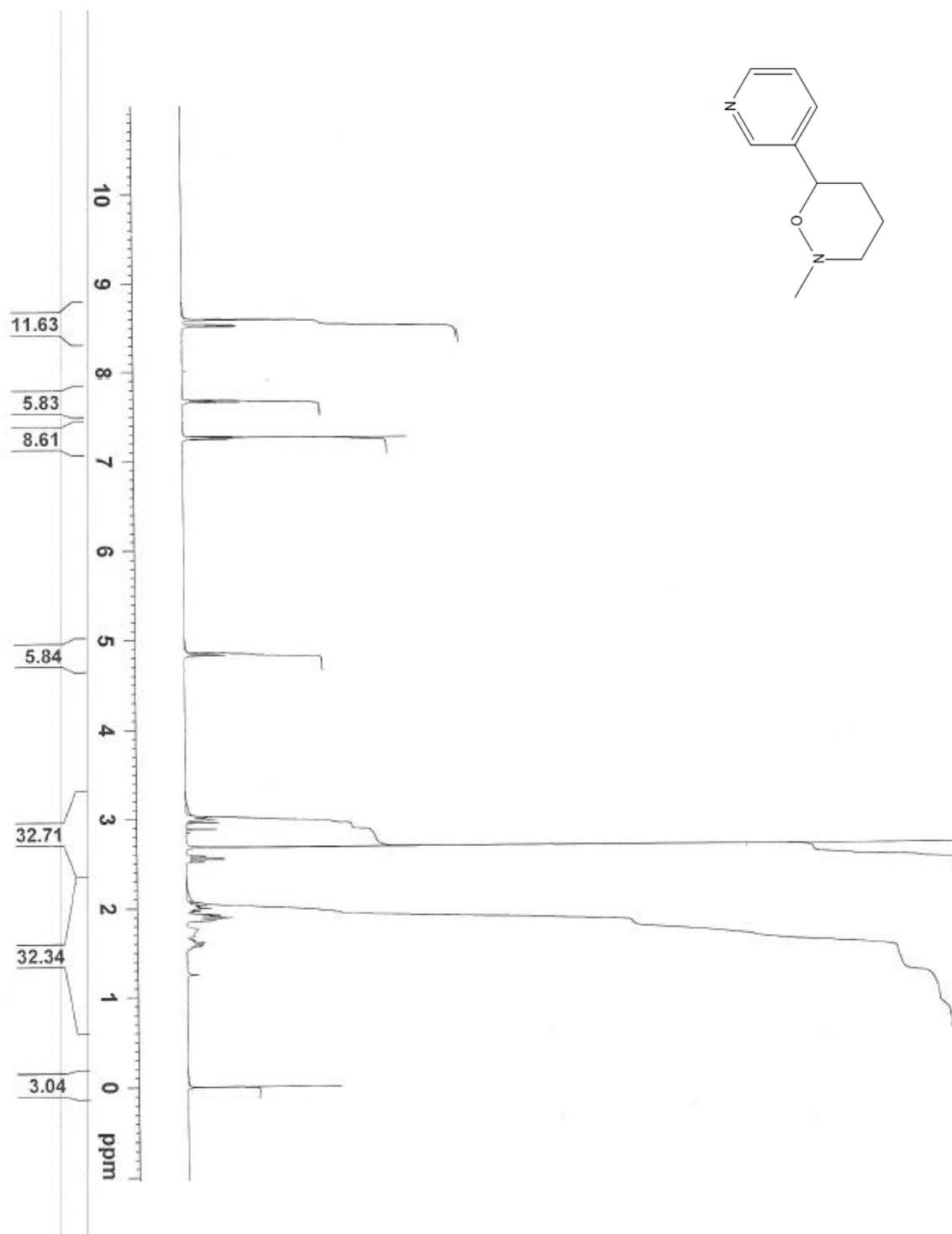**Figure 11: HNMR of 2-methyl-6-(3-pyridyl)tetrahydro-1,2-oxazine (oxazine).**

Figure 12: HNMR of 2-methyl-6-(3-pyridyl)tetrahydro-1,2-oxazine (oxazine) used to make the N-oxide calibration curve.
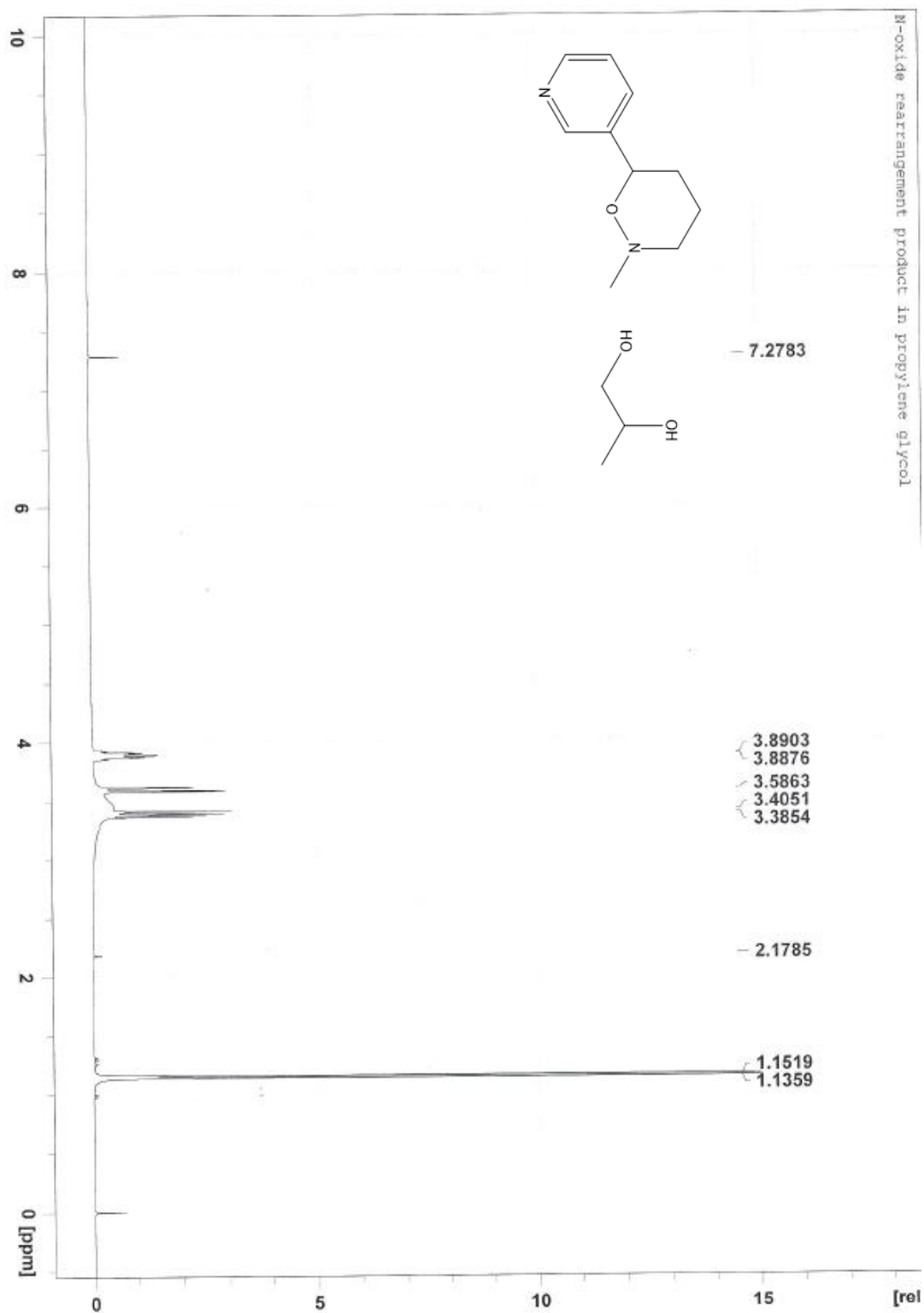
Figure 13: HNMR of 2-methyl-6-(3-pyridyl)tetrahydro-1,2-oxazine (oxazine) in propylene glycol
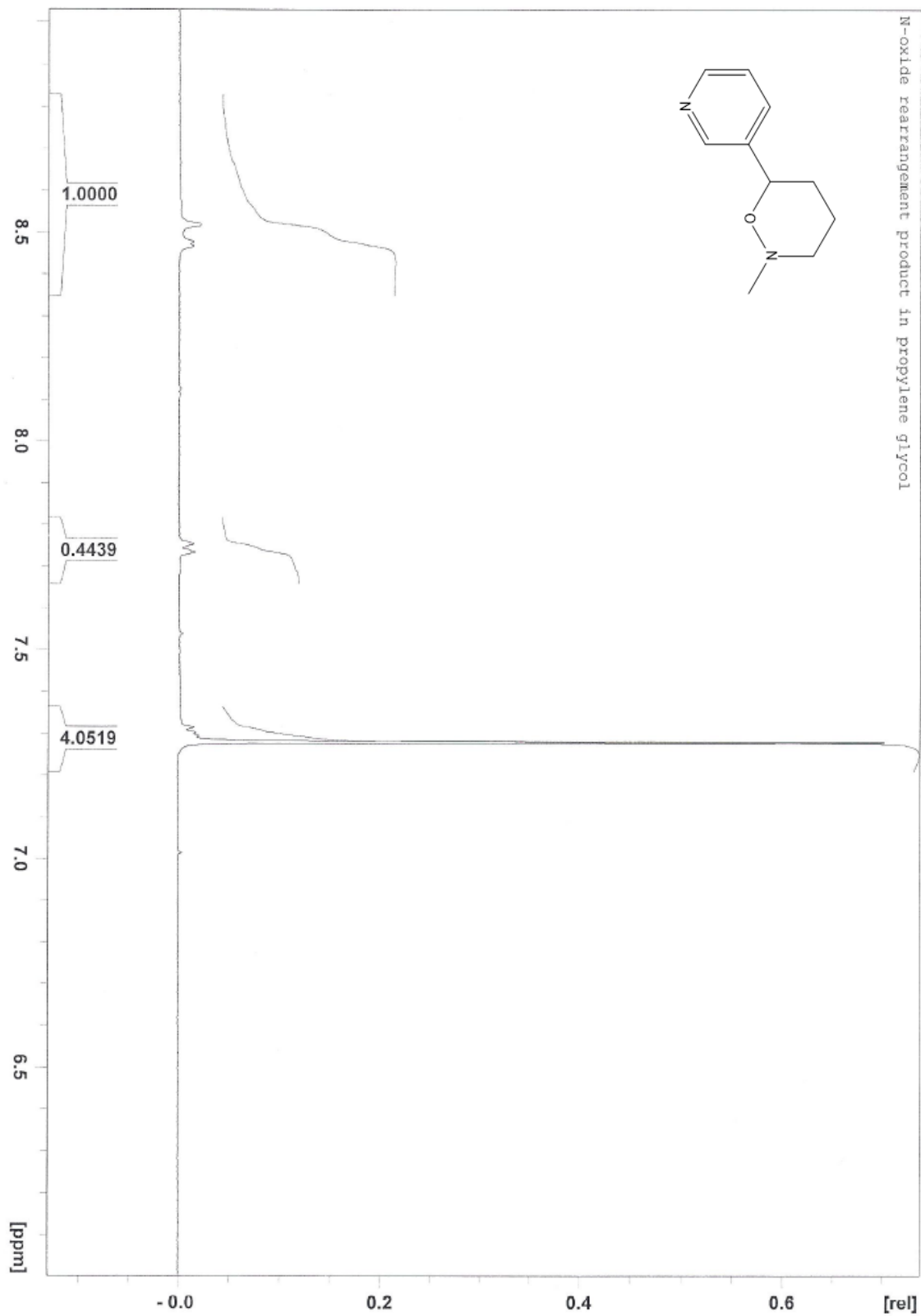
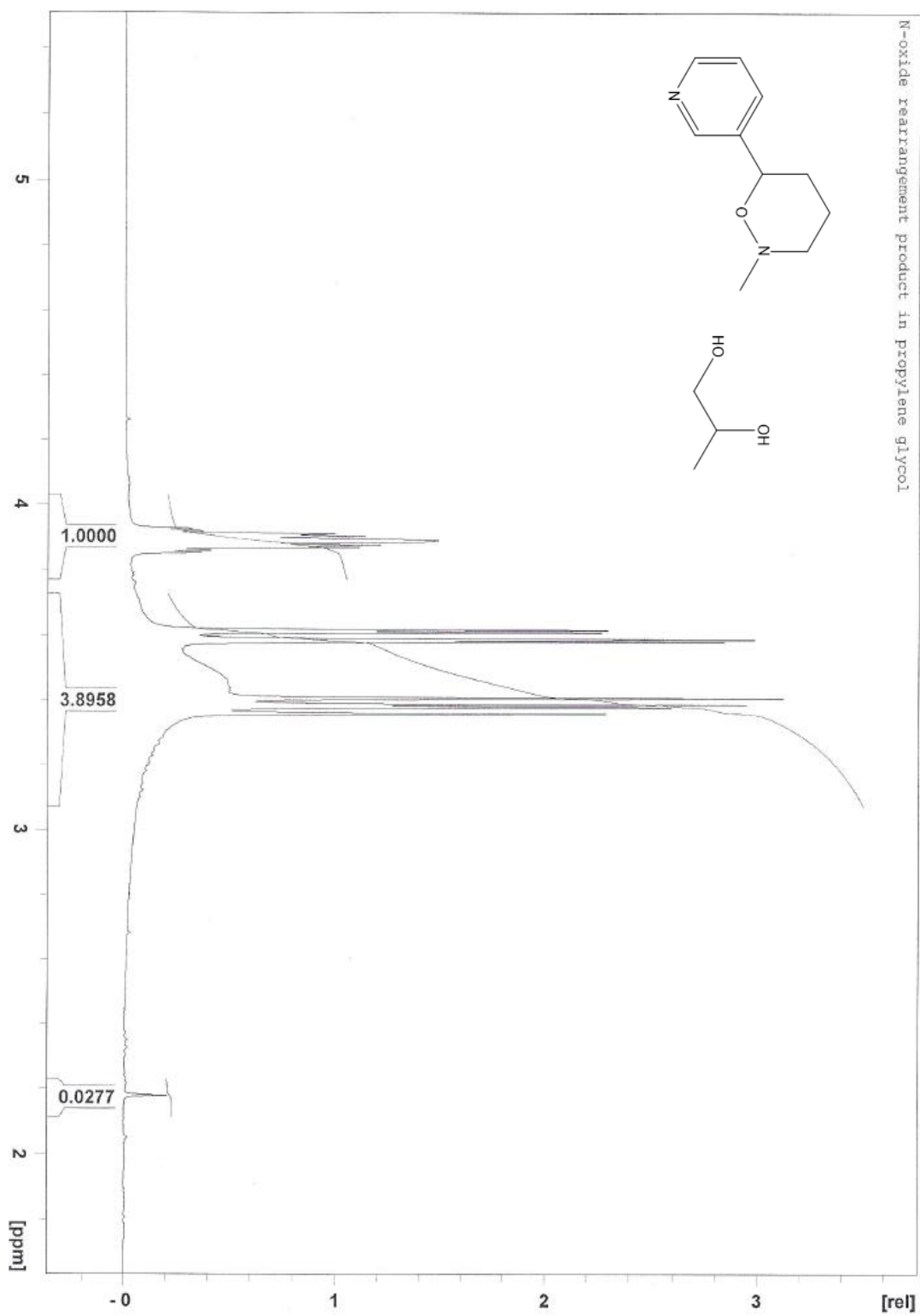**Figure 14: HNMR of 2-methyl-6-(3-pyridyl)tetrahydro-1,2-oxazine (oxazine) in propylene glycol.**

Figure 15: HNMR of 2-methyl-6-(3-pyridyl)tetrahydro-1,2-oxazine (oxazine) in propylene glycol.

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