FORMATION OF N-NITROSODIMETHYLAMINE (NDMA) DURING MONOCHLORAMINE DISINFECTION OF 8 SELECTED PHARMACEUTICALS

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submitted by **GÖKÇE SOMUN** in partial fulfillment of the requirements for the degree of **Master of Science in Environmental Engineering Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Faika Dilek Sanin
Head of Department, **Environmental Engineering** _____

Dr. Barış Kaymak
Supervisor, **Environmental Engineering Dept., METU** _____

Examining Committee Members:

Prof. Dr. Ülkü Yetiş
Environmental Engineering Dept., METU _____

Asist. Prof. Dr. Barış Kaymak
Environmental Engineering Dept., METU _____

Asist. Prof. Dr. Tuba Hande Ergüder
Environmental Engineering Dept., METU _____

Dr. Robert Murdoch
Environmental Engineering Dept., METU _____

Asist. Prof. Dr. Nuray Ateş
Environmental Engineering Dept., Erciyes University _____

Date: May 23, 2014

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Gökçe SOMUN

Signature:

ABSTRACT

FORMATION OF NITROSODIMETHYLAMINE (NDMA) DURING MONOCHLORAMINE DISINFECTION OF 8 SELECTED PHARMACEUTICALS

Somun, Gökçe

M.S., Department of Environmental Engineering

Supervisor: Dr. Barış Kaymak

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In the last decade, traces of pharmaceuticals and personal care products (PPCPs), mostly at levels in the ng/L to low µg/L range, have been reported in the water cycle, including surface waters, wastewater, groundwater, and drinking water and this has been a major concern in recent years. Simultaneously, formation of N-Nitrosodimethylamine (NDMA) as a disinfection by-product (DBP) during chloramine disinfection has become another important concern for drinking water quality because of its potent carcinogenicity. This study investigated NDMA formation potential of eight amine based PPCPs namely, ranitidine, doxylamine, diltiazem, sumatriptan, caffeine, diclofenac, atrazine and sulfamethoxazole during disinfection with 2-2.5 mg/L monochloramine and mutagenicity of PPCP and monochloramine mixture after 24 hours of reaction time. Water samples spiked with these PPCPs were subjected to disinfection process individually with monochloramine and NDMA formation was observed after 24 hours. NDMA concentrations were measured by gas chromatography/mass spectrophotometry (GC/MS). Four of the selected PPCPs, namely ranitidine, doxylamine, diltiazem and sumatriptan formed NDMA after reaction with monochloramine. The molar conversion rate for ranitidine, doxylamine, diltiazem and sumatriptan were average 123.3 %, 0.4 %, 0.6 % and 0.5 %, respectively. In the other four PPCPs, namely caffeine, diclofenac, atrazine and sulfamethoxazole, NDMA formation was observed

but they were lower than the calibration range of GC/MS readings. The magnitude of NDMA formation potential from reaction between amine groups and monochloramine were dependent on molecular properties of PPCPs. In mutagenicity tests *S.typhimurium* TA100 strain showed higher mutation than *S.typhimurium* TA98 strain, in other words, TA100 strain seemed to be more sensitive to mutagenic effects of chemicals than that of TA98 strain. Mutations observed in doxylamine, samples were thought to be due to PPCPs. No conclusive NDMA mutagenicity was observed in samples tested without metabolic activation.

Keywords: PPCPs, Nitrosodimethylamine, NDMA, Monochloramine, Disinfection, DBPs

ÖZ

SEÇİLMİŞ SEKİZ TIBBİ İLAÇ ÜRÜNÜNÜN MONOKLORAMİN DEZENFEKSİYONU SONRASI NİTROSODİMETHYLAMİN (NDMA) OLUŞUMU

Somun, Gökçe
Yüksek Lisans, Çevre Mühendisliği Bölümü
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Son on yılda, tıbbi ilaç ve kozmetik ürünlerinin bulguları, ng/L seviyelerinden düşük µg/L seviyeleri aralığında, yüzey suları, atık su, yeraltı ve içme suyu da dahil olmak üzere su döngüsünde bildirilmiş ve son yıllarda endişe nedeni olmuştur. Aynı zamanda, kloramin dezenfeksiyonu sırasında dezenfeksiyon yan ürünü olan N-Nitrosodimetilamin (NDMA) oluşumu güçlü kanserojen olması nedeniyle içme suyu kalitesi için bir diğer önemli sorun haline gelmiştir. Bu çalışma, sekiz amin bazlı ve ranitidine, doxylamine, diltiazem, sumatriptan, caffeine, diclofenac, atrazine ve sulfamethoxazole olarak adlandırılan tıbbi ilaç ve kozmetik ürünlerinin, 2-2.5 mg/l monokloramin ile dezenfeksiyonu sonucu NDMA oluşturma potansiyelini ve 24 saat reaksiyon süresinden sonra tıbbi ilaç ve kozmetik ürünlerinin ve monokloramin karışımının mutajenik etkisi incelemiştir. Bahsedilen tıbbi ilaç ve kozmetik ürünlerinin eklendiği su örnekleri ayrı ayrı monokloramin ile dezenfeksiyon prosesine tabi tutulmuş ve 24 saat sonra NDMA oluşumu gözlemlenmiştir. NDMA konsantrasyonları gaz kromatografisi/kütle spektrometresi ile ölçülmüştür. Seçilen ranitidine, doxylamine, diltiazem ve sumatriptan olarak adlandırılan dört tıbbi ilaç ve kozmetik ürünü NDMA oluşturmuştur. Ranitidine, doxylamine, diltiazem ve sumatriptan ortalama molar dönüşümleri sırasıyla % 123.3, % 0.4, % 0.6 ve % 0.5'dir. Diğer dört tıbbi ilaç ve kozmetik ürünlerinde, caffeine, diclofenac, atrazine ve sulfamethoxazole, NDMA oluşumu gözlemlenmiş fakat gaz kromatografisi/kütle spektrometresi kalibrasyon aralığının altındadır. Amin grubu ve monokloraminin reaksiyonu sonucu NDMA oluşum potansiyelinin büyüklüğü tıbbi ilaç ve kozmetik

ürünlerinin moleküler yapısına bağlıdır. NDMA mutajenite testlerinde, *S. typhimurium* TA100, TA98'den daha yüksek mutasyon gösterdi, diğer bir değişle, TA100'ün kimyasalların mutajen etkisi için TA98'den daha hassas olduğu görülmüştür. Doxylamine, örneklerinde gözlemlenen mutasyonlar tıbbi ilaç ve kozmetik ürününün kendisinden kaynaklı olduğu düşünülmektedir.

Anahtar Kelimeler: Tıbbi İlaç ve Kozmetik Ürünleri, Nitrosodimethylamine, NDMA, Monokloramin, Dezenfeksiyon, Dezenfeksiyon Yan Ürünü

To My Family

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LIST OF ABBREVIATIONS

CDHS	California Department of Health Services
CNS	Central Nervous System
CPSC	Consumer Product Safety Commission
DBPs	Disinfection Byproducts
DMA	Dimethylamine
DWTPs	Drinking Water Treatment Plants
EBPI	Environmental Bio-detection Products Inc.
FDA	Food and Drug Administration
GC	Gas Chromatograph
GC/MS	Gas Chromatography/Mass Spectrophotometry
GERD	Gastro-Esophageal Reflux Disease
HAAs	Haloacetic Acids
ICR	Information Collection Rule
IRIS	Integrated Risk Information Service
IUPAC	International Union of Pure and Applied Chemistry
LFSM	Laboratory Fortified Sample Matrix
LRB	Laboratory Reagent Blank
MOE	Ministry of the Environment
MS	Mass Spectrometer
NDEA	Nitrosodiethylamine
NDMA	Nitrosodimethylamine
NDBA	N-nitroso-di-n-butylamine
NDPA	N-nitroso-di-n-propylamine
NMEA	N-nitrosomethylethylamine
NOM	Natural Organic Matter
NPYR	N-nitrosopyrrolidine
NTP	National Toxicology Program
OSHA	Occupational Safety & Health Administration
OEHHA	Office of Environmental Health Hazard Assessment

QA/QC	Quality Assurance/Quality Control
PPCPs	Pharmaceuticals and Personal Care Products
PUD	Peptic Ulcer Disease
SIM	Selected-Ion Monitoring
SPE	Solid Phase Extraction
THMs	Trihalomethanes
USEPA	U.S. Environmental Protection Agency
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) include pharmaceutical drugs, cosmetics, foods, and components in other consumer products. Historically, the use of these products has increased their presence in the environment causing significant environmental issues. However, it was not until recent years that these issues have grown in popularity because overuse and existence in the environment specifically in the amount of ng/L to mg/L (Calamari *et al*., 2003; Conley *et al*., 2008; Godfrey *et al*., 2007; Jasim *et al*., 2006; Kasprzyk- Hordern *et al*., 2008; Kolpin *et al*., 2002, 2004; Metcalfe *et al*., 2003; Servos *et al*., 2007; Zuccato *et al*., 2005). Although there are studies related to detection of PPCPs in the environment and these studies only cover a small portion of all PPCPs. There are a number of studies focusing on removal of PPCPs from water bodies by applying different treatment techniques, but there are still unknowns remaining related to degradation processes and byproducts resulting from these treatments (Shen and Andrews, 2011).

The other significant environmental health issue is the formation of nitrosamines, which are potential carcinogen products during chloramine disinfection. When compared with free chlorine, chloramines maintain a much more stable residual in the distribution system and chloramines can form less regulated disinfection by-products (DBPs), namely trihalomethanes (THMs) and haloacetic acids (HAAs). Although, there are thought to be advantages in using chloramines instead of free chlorine, chloramine has a potential to form nitrosamine DBPs more than free chlorine (Shen and Andrews, 2011). Up until the 2000s, there were no regulations and limit values for nitrosamines. Currently, there are some regulating authorities that have set limits for nitrosamines in drinking water such as the Ontario Ministry of the Environment (MOE) and the California Office of Environmental Health Hazard Assessment (OEHHA) (OEHHA, 2006). The U.S. Environmental Protection Agency

(USEPA) has also named 5 different nitrosamines as chemical contaminants in the "Contaminant Candidate List 3".

In recent studies, scientists researched on the precursors of nitrosodimethylamine (NDMA). The common NDMA precursors investigated for both treatment systems of water wastewater are dimethylamine (DMA) (Mitch *et al*., 2003), tertiary and quaternary amines with dimethylamine groups (Kemper *et al*., 2010; Lee *et al*., 2007), natural organic matter (NOM) (Dotson *et al*., 2007; Chen and Valentine, 2007; Mitch and Sedlak, 2004; Gerecke and Sedlak, 2003; Krasner *et al*., 2008;), polyelectrolytes in treatment systems (Kohut and Andrews, 2003; Mitch and Sedlak, 2004; Wilczak *et al*., 2003), and some pesticides and herbicides (Chen and Young, 2008; Graham *et al*., 1995; Schmidt and Brauch, 2008).

The objective of this study is to investigate nitrosamine formation potential of a number of PPCPs that contain amine groups during disinfection process. The selected PPCPs containing amine groups are ranitidine, doxylamine, diltiazem, sumatriptan, caffeine, diclofenac, atrazine and sulfamethoxazole. In addition, formation potential of any other mutagenic disinfection by-product will be investigated by conducting mutagenicity test on samples after PPCPs have reacted with monochloramine for 24 hours. The mutagenicity test results would not directly show the by-products formed, but rather would prove the presence of mutagenic by-products once these PPCPs react with monochloramine.

CHAPTER 2

LITERATURE REVIEW AND THEORETICAL BACKGROUND

This study examines the formation potential of NDMA during disinfection of water contaminated with PPCPs. Therefore, in this chapter, a literature review on properties, sources, and exposure pathways of PPCPs used in this study are discussed. Next, disinfection by chlorine and chloramines are explained. Finally, a comprehensive introduction on chemical properties, applications, occurrences, related regulations, available analytical methods, and current studies on NDMA formation in waters are provided.

2.1 Pharmaceuticals and Personal Care Products (PPCPs)

2.1.1 Definition, Sources and Exposure Pathways

Pharmaceuticals & Personal Care Products (PPCPs) are defined as a very broad, diverse collection of thousands of chemical substances, including prescription and over-the-counter therapeutic drugs, cosmetics, sun-screen agents, diagnostic agents, nutraceuticals, biopharmaceuticals, and many others (Daughton and Ternes, 1999). These substances are basically any product used by individuals for personal health or cosmetic reasons (Roig, 2010). PPCPs are divided into two parts as indicated by its name; pharmaceuticals and personal care products. Pharmaceuticals are divided into two parts as prescription drugs and over the counter drugs. Prescription drugs are a licensed medicine that is regulated by legislation to require a medical prescription for use like antibiotics and hormones. Over-the-counter drugs not valid in Turkey are medicines that may be sold directly to a consumer without a prescription. Personal care products are cosmetics, shampoo and soaps and others. Figure 2.1 represents components of the PPCPs (Polimeni, 2008).

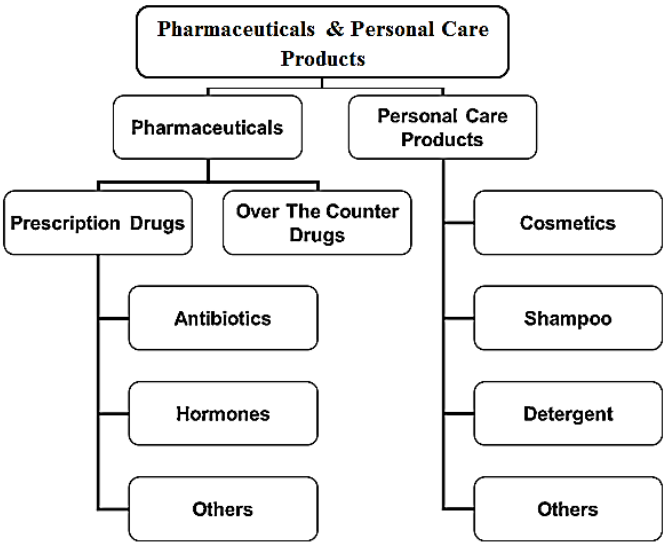


Figure 2.1 Components of the Pharmaceuticals & Personal Care Products (Polimeni, 2008)

Although pharmaceuticals in the environment are not a recent issue, it has become an important concern for public in recent years. Occurrence of PPCPs in the environment has been studied since the 1990s in Europe and other parts of the world. Figure 2.2 shows the exponential expansion of scientific publications in the topic of pharmaceuticals in the environment since 1991 (Roig and Touraud, 2010).

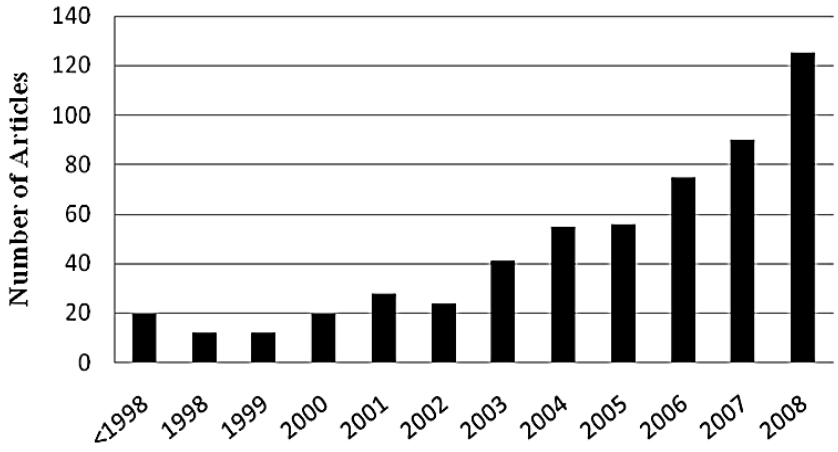


Figure 2.2 Evolution of the Scientific Production Concerning Pharmaceuticals in the Environment Between 1991 and 2008 (approximately 550 articles) (Roig and Touraud, 2010)

Actually, the presence of active pharmaceutical substances in water bodies is an unwanted side effect of their normal usage. This issue has two different results, the first one is the active compounds are not metabolized completely in the human body, but excreted primarily via urine and thus reach domestic wastewater; second, the desired stability of the molecules hinders their biological degradation in conventional sewage treatment plants. To maintain their desired effects on the personal health, adequate intact molecules should reach the target cells before they are degraded by the body's biochemical processes. To reach this goal, pharmaceuticals should be optimized for their stability (Roig and Touraud, 2010).

The effluent of wastewater treatment plants reaches surface waters. Untreated PPCPs forge a route from surface water to drinking water. Similar to wastewater treatment plants, conventional drinking water treatment plants have limited capability to remove PPCPs from water. This means that these compounds will ultimately reach humans. It is thought that the main sources of PPCPs are pharmaceutical production areas and hospitals or medical care services; however, actually, domestic wastewater, in other words, wastewater resulting from human activities, is the main source of PPCPs in the environment (Roig and Touraud, 2010).

The main sources of the PPCPs in the environment are listed below:

- Residues from hospitals, nursing homes, pharmacy and healthcare facilities;
- Veterinary drug use, especially antibiotics and steroids;
- Livestock wastes;
- Residues from pharmaceutical manufacturing (well defined and controlled); and,
- Excreted metabolites entering wastewater.

In the last decade, traces of pharmaceuticals, mostly at levels in the ng/L to low μg/L range, have been found in the water cycle, including surface waters, wastewater, groundwater, and drinking water (WHO, 2011). Figure 2.3 shows the exposure pathway of PPCPs in the environment. The main sources of exposure are human drugs, veterinary drugs, and feed additives. Via excretion, these drugs disperse into

sewage and finally into sewage treatment plants. These treatment plants are not well designed to eliminate PPCPs; therefore, PPCPs are discharged to the surface water via treatment plant effluent. Also, via land application of treatment plants sludge, soil and ground water are affected. In addition, surface run-off at these contaminated sites also carries PPCPs to surface water. Some of the PPCPs are also wasted by consumers and these wastes go into landfills. By way of the leachate or runoff, they can reach to other surface and groundwater resources. Veterinary drugs and feed additives also affect soil, ground and surface water via the same routes as human drugs (Ternes, 1998).

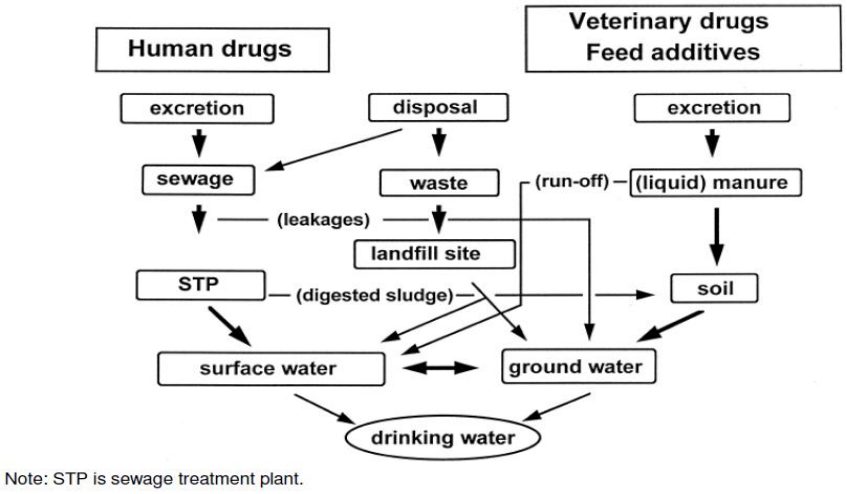


Figure 2.3 Exposure Pathways of the PPCPs in the Environment (Ternes, 1998)

2.1.2 Properties of PPCPs Used

Eight PPCPs and endocrine disrupters were used in this study, namely ranitidine, doxylamine, diltiazem, sumatriptan, caffeine, diclofenac, atrazine and sulfamethoxazole. The common feature is that they all contain amine groups as potential precursors for NDMA. In presence of oxidants, these chemicals have potential to break into smaller NDMA precursors. In other words, they might cause the formation of NDMA during drinking water disinfection. Moreover, the selection of these compounds was because of their common use in the market and their

presence in the environment (Shen and Andrews, 2011). Their molecular structures and molecular formulas are detailed in Table 2.1.

Table 2.1 Names, Formulas and Molecular Structures of Chemicals Used

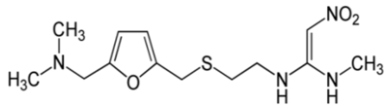
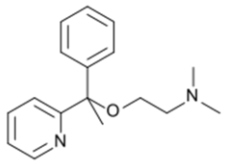
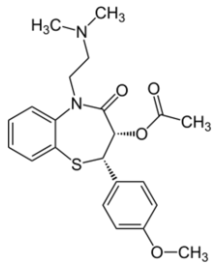
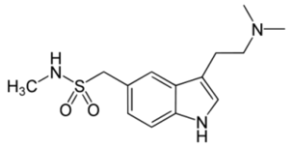
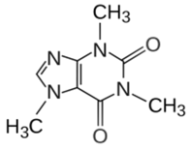
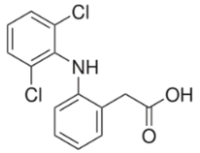
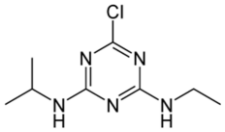
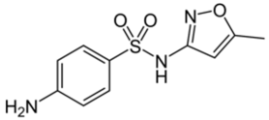
Compound	Therapeutical Group	CAS Number	Elemental Formula	Structure	Molecular Weight
Ranitidine	Anti-Allergic Agents, Auxiliaries	66357-59-3	$C_{13}H_{22}N_4O_3S$		314.41 (Boleda *et al.*, 2013)
Doxylamine	Antiemetic, Histamine H_1 Antagonists	562-10-7	$C_{17}H_{22}N_2O$		270.37 (Reungoat *et al.*, 2012)
Diltiazem	Antihypertensive Agents, Calcium Channel Blockers	33286-22-5	$C_{22}H_{26}N_2O_4S$		414.52 (Fontela *et al.*, 2011)
Sumatriptan	Antimigraine, Serotonin Receptor Agonist, 5-HT1	103628-48-4	$C_{14}H_{21}N_3O_2S$		295.40 (Shen & Andrews,2013)

Table 2.1 (Continued)

Compound	Therapeutical Group	CAS Number	Elemental Formula	Structure	Molecular Weight
Caffeine	Cardiac and respiratory stimulant, diuretic	58-08-2	$C_8H_{10}N_4O_2$		194.19 (Daneshvar, 2012)
Diclofenac	Anti-inflammatory	15307-79-6	$C_{14}H_{11}Cl_2NO_2$		296.15 (Boleda *et al.*, 2013)
Atrazine	Herbicides	1912-24-9	$C_8H_{14}ClN_5$		215.68 (Daneshvar, 2012)
Sulfamethoxazole	Antibacterial	723-46-6	$C_{10}H_{11}N_3O_3S$		253.28 (Boleda *et al.*, 2013)

Some detailed information, usage areas and side effects of these chemicals are set forth in the following sections.

2.1.2.1 Ranitidine

The International Union of Pure and Applied Chemistry (IUPAC) systematic name of ranitidine is N-(2-[(5-(dimethylaminomethyl) furan- 2-yl) methylthio] ethyl) - N-methyl - 2-nitroethene- 1,1-diamine**.** Its trade name is Zantac and commonly used to treat mild heartburn related to gastro-esophageal reflux disease (GERD) and peptic ulcer disease (PUD). It is a histamine H_2-receptor antagonist that inhibits stomach acid production. Doctors and pharmacists also recommend Ranitidine for colic caused by reflux. Ranitidine is effective for relieving food-triggered heartburn. It is long-lasting and relieves symptoms for 6-12 hours; therefore, it's effective for treating nighttime heartburn. There are some side effects of Ranitidine. It can cause dizziness, excessive fatigue, diarrhea and headache. Ranitidine may reduce the absorption of medications requiring an acidic stomach. Notably, regular use of an acid blocker reduces absorption of vitamin B_{12} (URL 1).

2.1.2.2 Doxylamine

The IUPAC systematic name of doxylamine is (RS)-N,N-dimethyl-2-(1-phenyl-1-pyridin-2-yl-ethoxy)- ethanamine**.** It is used for treatment of insomnia. Doxylamine is an antihistamine against depression. It is used as a short-term sedative and in connection with other drugs it is used for night-time allergy and cold relief. Some of the side effects of doxylamine are dizziness; drowsiness; dry mouth, throat, and nose; and, thickening of mucus in nose. The rare side effects are allergic reactions, convulsions, decreased alertness, excitability, fast heartbeat, hallucinations, tightness or pounding in the chest, tremor, and wheezing (URL 2).

2.1.2.3 Diltiazem

The IUPAC systematic name of diltiazem is cis-(+)-[2-(2-dimethylaminoethyl)-5-(4-methoxyphenyl)-3-oxo-6-thia-2azabicyclo[5.4.0]undeca-7,9,11-trien-4-yl] ethanoate. It is a nondihydropyridine (non-DHP) member of the class of drugs known as calcium channel blockers. It works by relaxing the muscles of your heart and blood vessels. Diltiazem is used to treat hypertension (high blood pressure), angina (chest pain), and certain heart rhythm disorders. It is also an effective preventive medication for migraines. Difficulty breathing, swelling of face, lips, tongue and throat are some of the side effects of Diltiazem. Less serious diltiazem side effects include headache, dizziness, weakness, upset stomach, nausea, sore throat, cough, stuffy nose or flushing (URL 3).

2.1.2.4 Sumatriptan

The IUPAC systematic name of sumatriptan is 1-[3-(2-dimethylaminoethyl)-1H-indol-5-yl]- N-methyl-methanesulfonamide**.** Sumatriptan is used to treat acute migraine headaches in adults. It relieves the pain from migraine headaches in the brain. Sumatriptan belongs to the group of medicines called triptans. There are some side effects of using Sumatriptan such as abdominal or stomach pain, anxiety, changes in patterns and rhythms of speech, chest pain or tightness, chills, confusion, dizziness and headache (URL 4).

2.1.2.5 Caffeine

The IUPAC systematic name of caffeine is 1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione. It is a popular central nervous system (CNS) stimulant. It is a well-known drug commonly used as a mild stimulant, found in dietary sources such as coffee, tea, and cocoa. It acts through adenosine receptors and monoamine neurotransmitters. It is an adenosine receptor antagonist and adenosine 3',5'-cyclic monophosphate (cAMP) phosphodiesterase inhibitor. It has been reported to affect cellular calcium levels, releasing calcium from intracellular stores. It overrides the cell cycle effects of various chemicals such as protease inhibitors, thereby preventing apoptosis; and it

has been shown to inhibit cellular DNA repair mechanisms. It is the world's most widely consumed psychoactive drug and has a modest protective effect against some diseases, including Parkinson's disease and certain types of cancer (URL 5).

2.1.2.6 Diclofenac

The IUPAC systematic name of diclofenac is 2-(2-(2,6-dichlorophenylamino) phenyl) acetic acid. It is a non-steroidal anti-inflammatory drug (NSAID). This medicine works by reducing substances in the body that cause pain and inflammation. Diclofenac is used to treat pain or inflammation caused by arthritis. Diclofenac powder (Cambia) is used to treat a migraine headache attack. It is also effective against menstrual pain and endometriosis and it is used to treat chronic pain associated with cancer (URL 6).

2.1.2.7 Atrazine

The IUPAC systematic name of atrazine is 1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine. It is a triazine herbicide for the control of grass and broadleaf weeds in crops such as sorghum, maize, sugarcane, lupins, pine and eucalypt plantations, and triazine tolerant (TT) canola. Potential human health and ecological impacts of atrazine is a controversial issue; therefore, there are some regulatory authorities on using of atrazine safely around the world. It is a controversial issue due to contamination of drinking water. It creates birth defects and menstrual problems when consumed by humans. It is banned in the EU but is still one of the most widely used herbicides in the world (URL 7).

2.1.2.8 Sulfamethoxazole

The IUPAC systematic name of sulfamethoxazole is 4-amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide**.** It is a sulfonamide bacteriostatic antibiotic. It is used to against susceptible forms of *Streptococcus, Staphylococcus aureus* (including MRSA), *Escherichia coli*, *Haemophilus influenzae*, and oral anaerobes. It is used to treat urinary tract infections, sinusitis and toxoplasmosis (URL 8).

2.2 Occurrence of PPCPs in Drinking Water Sources

There are several studies carried out by researches regarding the occurrence of PPCPs in drinking water and drinking water sources including the ones used in this study. Caffeine in tap water was reported at concentrations of 60 ng/L to 119 ng/L in the USA (Stackelberg *et al*., 2007 and 2004) and 22.9 ng/L in France (Togola and Budzinski, 2008), caffeine in surface water was found at 6 µg/l in USA. Sulfamethoxazole (antibiotic) was detected at a concentration of 1.9 µg/L in surface water in the USA (Kolpin *et al*., 2002). Ranitidine (anti-acid) was found at concentrations of up to 580 ng/L in surface waters in Italy (Kolpin *et al.*, 2002). Diclofenac was observed at concentrations of 6 ng/L to 35 ng/L in tap water in Germany (Heberer *et al*., 2004) and at a concentration of 2.5 ng/L in France (Togola and Budzinski, 2008). Diclofenac in surface water was found in the range of 0.4 ng/l to 15 µg/l in Germany, USA and UK (Jux, *et al.,* 2002; Moder *et al.,* 2007). Atrazine was found in drinking water at concentrations higher than federal drinking water standard of 3 ppb in various locations: Versailles, Indiana (4.60 ppb), Mt. Olive, Illinois (3.79 ppb) and Evansville, Illinois (3.20 ppb) (Wu *et al.*, 2009). According to experiments conducted in drinking water treatment plants in Spain, the raw water from the Llobregat River (NE-Spain) used for drinking water production contained diltiazem at concentrations lower than 10 ng/L (Fontela *et al*., 2011). Shen and Andrews reported the occurrence of sumatriptan and doxylamine in drinking water in significant amounts (Shen and Andrews, 2011).

2.3 Disinfection of Drinking Water

The main purpose of water disinfection is the inactivation of microorganisms, such as viruses, bacteria and protozoa causing negative health effects and deaths. Drinking water disinfection is generally achieved by applying of chemical agents and physical agents to the water. Chlorine and its compounds, iodine, ozone, phenol and phenolic compounds are commonly used chemical agents for the purpose of disinfection. Most of the disinfectants are oxidants and within oxidants, chlorine is the most commonly used disinfectant in the world. It reduces unpleasant tastes and odors by decomposing organic contaminants and oxidizes iron and magnesium. There are also

some disadvantages of using chlorine for disinfection processes. It requires safety procedure for transportation and storage since there is health risk in the case of a leakage. Additionally, chlorine forms disinfection by-products, such as trihalomethanes (THMs) and haloacetic acids (HAAs). The purpose of disinfecting drinking water is reduce harm to consumers by eliminating pathogens, and formation of harmful disinfection by-products would be conflicting with the intend of disinfection. Ideal disinfection would be effective in inactivating pathogen while not forming any harmful by-product chemicals.

Using sodium hypochlorite instead of chlorine gas is relatively safer during storage and use. Also, it does not require transportation and storage of hazardous chemicals when produced on site. However, it is ineffective in inactivation of cysts (*Giardia, Cryptosporidium*) and it loses its activity during long-term storage due to decay. Sodium hypochlorite also forms disinfection by-products similar to chlorine. The other chemical disinfectant, chlorine dioxide, works in small doses and does not react with oxidizable compounds to form trihalomethanes. It also destroys some THM precursors. However, chlorine dioxide requires on-site generation equipment and it forms chlorates and chlorites as by-products (Tchobanoglous *et al*., 2003).

The other common chemical agent used as a very effective disinfectant is ozone. Since ozone is very active oxidant, it has a short half-life and decays very quickly once it is applied to water. Although ozone does not leave any residual for the distribution system, usage of ozone as a disinfectant has increased all over the world. There are some disadvantages of ozone use as disinfectant. It produces disinfection by-products like aldehydes, ketones, and brominated by products. In order to remove these by-products, the use of biologically active filters is necessary. High initial expenses for equipment and high operation expenses are also problematic issues for using ozone (Tchobanoglous *et al*., 2003).

Other than chemical agents, physical disinfectants are also used, namely, heat, light and sound waves. Heating water to boiling point inactivates most of the microorganisms requiring little equipment; however, it is not a feasible means of disinfecting large quantities of water. This process is very energy intensive and

expensive. Sound waves inactivate microorganisms by vibration; however, it is also a cost prohibitive method. Sunlight is also used as physical agent for disinfection. Ultraviolet (UV) wavelength component of the sunlight damages the genetic components of microorganisms and inactivates them by destroying their capability to reproduce. Also, special mercury lamps emitting light at UV wavelength are used to disinfect drinking water. The efficiency of UV light disinfection depends on suspended solids, dissolved organic molecules and water characteristics which may absorb radiation and may impede UV light form reaching the microorganisms. UV disinfection systems do not require storage and transportation of chemicals and it do not form disinfection by-products. However, UV light has high maintenance requirement, high capital and operating costs (Tchobanoglous *et al.,* 2003).

Investigations in 1998 about disinfectant usage in the United States were carried out according to the Information Collection Rule (ICR) on water utilities. This database contains information from 527 community water systems. Table 2.2 shows types of disinfectants used those 527 community water systems. In some cases, the facility may use more than one disinfectant for different units of treatment systems; therefore, the 527 systems reported use of 740 disinfectants. The water systems utilizing more than one disinfectant generally use monochloramine to maintain adequate disinfectant residual in the distribution system. From Table 2.2, it can be seen that NH_2Cl usage is 31% and is one of the most commonly used disinfectant in water systems.

Table 2.2 Disinfectant Usage Numbers for Water Sources (EPA, 1999)

Flow, mgd	Cl_2	NaOCl	NH_2Cl	O_3	ClO_2	Total Use	Total Plants
0-5	11	3	3	0	0	17	16
6-10	26	3	3	0	0	32	29
11-50	200	12	65	10	20	307	215
51-100	113	6	49	6	11	185	119
>100	94	5	41	5	3	148	96
Unknown	40	7	4	0	0	51	52
Total	484	36	165	21	34	740	527
Percentage*	92%	7%	31%	4%	6%	140%	

*Percentage calculated as a fraction of 527 – the total number of systems. 740 disinfectants are used by the 527 systems.

Another survey related to disinfectant usage percentages was conducted in 2007 by the AWWA Disinfection Systems Committee. This survey was the Committee's fourth survey of drinking water disinfection practices. Figure 2.4 shows disinfectant usage from all four surveys. According to this chart, chlorine is the most popular disinfectant with 63% using chlorine gas, 31 % using bulk liquid hypochlorite, 8 % using onsite generated chlorine/hypochlorite, and 8% using dry forms of hypochlorite. Some systems used multiple forms of chlorine. Other disinfectants for free chlorine alternatives, such as chloramine (30 %); chlorine dioxide (8 %); ozone (9 %); and UV (2 %) were higher overall compared with previous surveys (AWWA Disinfection Committee, 2008).

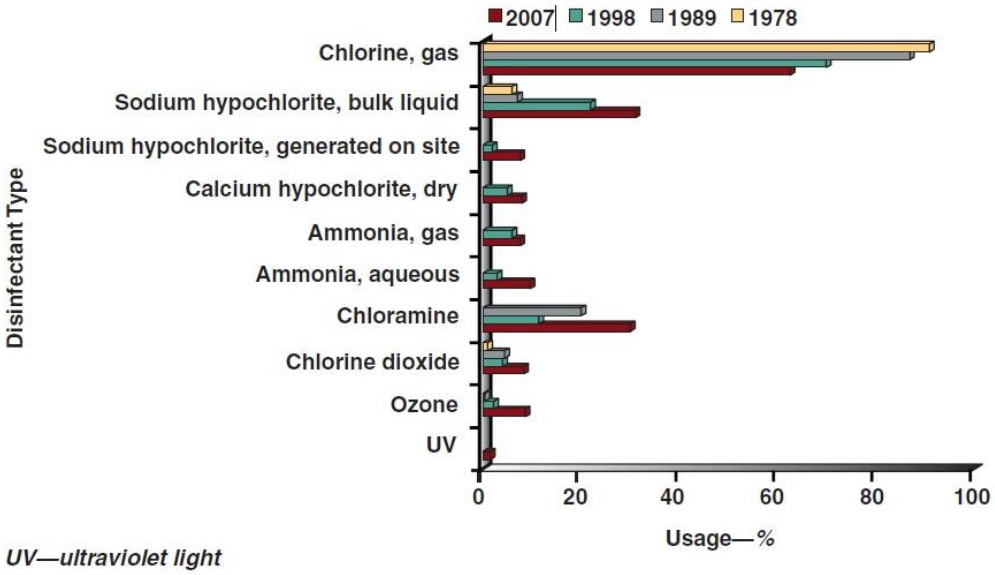


Figure 2.4 Disinfectants Use Identified in the Four AWWA Committee Surveys (AWWA Disinfection Committee, 2008).

2.3.1 Disinfection by Chlorine and Chloramines

The most prevalent applicable disinfectant in the world is free chlorine due to its power to kill most microorganisms, its ability to maintain a residual in distribution systems and its practical use compared to other disinfectants such as combined chlorine (chlorine combined with ammonia), chlorine dioxide and ozone. Also, investment and operation costs of free chlorine are lower than other disinfectants (Water Works Association, 1997; Montgomery Watson Harza, 2005). Although using free chlorine has many advantages, there are some disadvantages, especially, related to the production of disinfection by-products (DBPs), when there organic substances are present in the water. Among the DBPs formed, trihalomethanes (THMs) and haloaceticacids (HAAs) are regulated under Stage 2 Disinfectant and Disinfection By-Products Rule (D/DBP Rule) by the United States Environmental Protection Agency (USEPA, 2006) and by WHO Environmental Health Criteria 216 - Disinfectants and Disinfectant By-Products (WHO, 2000). In Turkey, the quantity of THMs in drinking water is regulated under TS 266 Water Intended for Human

Consumption. Due to the lower formation potential of THMs and HAAs with chloramine, chloramination has gained high popularity in recent years. Chloramine is formed in the water by adding chlorine and ammonia compounds separately to water. Chloramines maintain more stable residual than free chlorine in distribution systems and form less regulated DBPs (Desiderio and Nibbering, 2010).

As a result of some chemical reactions, monochloramine is formed. Free chlorine refers to the total of hypochlorous acid (HOCl) and hypochlorite ions (OCl^-) produced from chlorine hydrolysis.

$$Cl_2 + H_2O \rightarrow HOCl + HCl$$

When ammonium is present in water, chlorine reacts successively with ammonia to form three chloramine species as more chlorine is added.

Monochloramine formation : $NH_3 + HOCl \rightarrow NH_2Cl + H_2O$
Dichloramine formation : $NH_2Cl + HOCl \rightarrow NHCl_2 + H_2O$
Trichloramine formation : $NHCl_2 + HOCl \rightarrow NCl_3 + H_2O$

The total of these three reaction products (chloramines) is referred to as combined chlorine. The NH_3-N concentrations in water are usually below 1 mg/L and the type of chloramine formed depends on the pH (Pressley, *et al*. 1972). Spectrophotometric analyses (Czech *et al*., 1961; Moore, 1951; Palin, 1952) show that monochloramine is formed in the pH range of 7-8.5. When pH decreases below 7, dichloramine is formed and the amount of dichloramine increases when pH decreases. In the pH range of 4.5- 5.0, dichloramine is the dominant product. Trichloramine is the predominant product below pH 4.0. Studies indicate that monochloramine concentrations reach a maximum at the 5:1 weight ratio of Cl:NH_3-N (Yutaka, 1967; Pressley *et al*., 1972). As this weight ratio increases, the disproportionation of monochloramine takes place and forms dichloramine and ammonia (Morris, 1967; Pressley *et al*., 1972).

$$2NH_2Cl \rightarrow NHCl_2 + NH_3$$

The dichloramine concentration reaches a maximum at the $Cl:NH_3$-N weight ratio of about 7.5:1 when pH is lower than 7.0. In water with less than 1 mg/L of NH_3-N, this reaction proceeds in competition with monochloramine formation until the chlorine dosage reaches the breakpoint at approximately a 10:1 weight ratio of $Cl:NH_3$-N (Griffin and Baker, 1941; Pressley *et al*., 1972). At this point, monochloramine is also believed to be oxidized to nitrogen gas by excess chlorine under slightly alkaline conditions (Cole and Taylor, 1956; Griffin and Chamberlain, 1956; Palin, 1952; Pressley *et al*., 1972). Other end products including nitrate are also suggested (Chapin, 1931; Corbett *et al*., 1953; Griffin and Baker, 1941; Palin, 1952; Pressley *et al*., 1972).

$$2NH_2Cl + HOCl \rightarrow N_2 + 3HCl + H_2O$$

The rate constants from previous studies (Morris, 1967; Moore, 1951; Taras, 1953; Pressley *et al*., 1972) indicate the formation of monochloramine and dichloramine to be completed well in 1 minute. In practice, monochloramine has more disinfection power than the other forms of chloramine and due to its oxidative power; monochloramine is preferred in drinking water treatment plants.

NDMA formation is typically higher in distribution systems that use chloramines compared to chlorine (Barrett *et al.,* 2003). Moreover, chlorination is generally accepted to form lower NDMA and higher THM whereas chloramination can lead to high NDMA formation (Shaw and Knight, 2009). As discussed in earlier in section 2.3, use of monochloramine in the distribution system is becoming more preferred due to lower THM and HAAs formation potential; however, there are studies showing formation of other unregulated DBPs (Richardson, 2003; Wilczak *et al.,* 2003). Due to this need of further research on disinfection by-products formation with use of monochloramine, chloramination process (monochloramine as a disinfectant) was chosen in this study. Main physicochemical properties of monochloramine are represented in the Table 2.3.

Table 2.3 Physicochemical Properties of Monochloramine (WHO, 2004)

Property	Value
Physical state	Colorless, unstable liquid
Melting point	-66 °C
Water solubility	Soluble

Although chloramination has more advantages than free chlorine regarding the reduction of THM levels, formation of other unregulated by-products, such as haloketones, chloropicrin, cyanogen chloride, haloacetic acids, haloacetonitriles, aldehydes and chlorophenols, has been reported (Trussell & Montgomery, 1991; Krasner, 1989). NDMA is known to be a disinfection by-product resulting from disinfection with monochloramine. The U.S. EPA has placed six nitrosamines on the Unregulated Contaminant Monitoring Rule List 2, namely N-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA), N-nitroso-di-n-butylamine (NDBA), N-nitroso-di-n-propylamine (NDPA), N-nitrosomethylethylamine (NMEA), and N-nitrosopyrrolidine (NPYR) (U.S. EPA, 2006). In this study, the formation of N-nitrosodimethylamine (NDMA) was due to use of monochloramine as disinfectant investigated.

Chloramine concentrations are typically 0.5–2 mg/L in drinking-water supplies where chloramine is used as a primary disinfectant (Bull, 1991). Chloramine residuals in the USA changes between 0.6 to 5.0 mg/L; finished water in almost all utilities have chloramine residual levels between 1.0 and 3.0 mg/L in the distribution system (Kirmeyer, 1993). Moreover, a maximum acceptable level for chloramines in drinking water is 3 mg/L according to World Health Organization (WHO, 1996).

2.4 Nitrosamine Compounds

N-Nitrosamines are compounds having the general structure as shown in Figure 2.5, where R^1 and R^2, are alkyl or aryl groups.

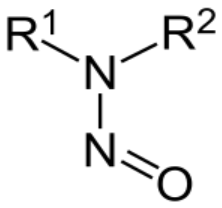


Figure 2.5 General Structure of Nitrosamines (URL 9)

Nitrosamines are found in water, soil and air. They can be present in farm animal feed, drugs, cosmetics, and pesticides (Rostkowska *et al.*, 1998). Nitrosamines are absorbed by skin, airways and the alimentary tract (Rostkowska *et al.*, 1998). There is evidence that nitroso compounds may be generated *in vivo* from nitrites or nitrates and primary, secondary and tertiary amines in organs of people not exposed to these compounds (Rostkowska *et al.*, 1998).

2.4.1 *N*-Nitrosodimethylamine (NDMA)

N-Nitrosodimethylamine (NDMA) is also known as dimethylnitrosoamine, dimethyl-nitrosamine, N,N-dimethylnitrosoamine, N-methyl-N-nitrosomethanamine, N-nitroso-N,N-dimethylamine, DMN and DMNA. Its chemical structure is shown in Figure 2.6.

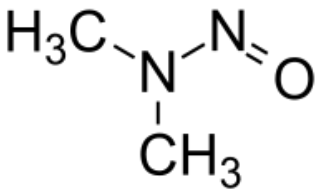


Figure 2.6 Chemical Structure of NDMA (URL 10)

N-Nitrosodimethylamine is a nitrosamine compound. It is a yellow liquid with a faint characteristic odor at room temperature. It is very soluble in water, alcohol, and ether, miscible with dichloromethane and vegetable oils, and soluble in lipids, chloroform, and most other organic solvents (HSDB, 2000; Xianghua, 2006). It is stable in the dark in neutral or alkaline solutions for at least 14 days; on the other hand, it is less stable in more acidic solutions or in light, especially under ultraviolet light (Tate and Alexander, 1975). The physical-chemical properties relevant to the environmental fate of NDMA are demonstrated in Table 2.4.

Table 2.4 Physical and Chemical Properties of NDMA (Siddiqui, 2004)

Properties	Values/Definitions
CAS Number	62-75-9
Molecular Formula	$C_2H_6N_2O$
Molecular Weight	74.08 g/mol
Melting Point	-50^oC
Boiling Point	151-154^oC
Vapor Pressure	1080 Pa at 25^oC
Water Solubility	Miscible
Henry's Law Constant	3.34 Pa m^3/mol at 25^oC
Specific Gravity	1.00059

2.4.1.1 Effects of NDMA on Living Organisms

N-Nitrosodimethylamine (NDMA) is a member of a family of extremely potent carcinogens (U.S. EPA, 2002). It is classified as reasonably anticipated to be a human carcinogen (also known as suspect human carcinogen) by the National Toxicology Program (NTP), Department of Health and Human Services. It was first listed in the Second Annual Report on Carcinogens in 1981 by the International Agency for Research on Cancer. The Integrated Risk Information Service (IRIS) of the U.S. EPA database also classifies NDMA as probably carcinogenic to humans (2002). The U.S. Occupational Safety & Health Administration (OSHA) Health Code and Health Effects list the principal effects of exposure to NDMA as cancer

and reproductive hazards (teratogenesis or other reproductive impairment) of the organs such as liver, kidney, and lungs (OSHA, 2006).

2.4.1.2 Sources of NDMA

NDMA is a by-product formed in industries where they use nitrates, nitrites and amines in suitable pH conditions (WHO, 2002). NDMA formation during these processes is unintentional. In these processes, NDMA may be formed because of two reasons: the first one is when alkylamines, especially DMA (dimethylamine) and trimethylamine, react with nitrogen oxides, nitrous acid, nitrite salts; and, the second one is when trans-nitrosation via nitro or nitroso compounds occurs (ATSDR, 1989). Because of these reasons NDMA may be found in discharges of rubber, pesticides or dye manufacturing, leather tanning, food processing and also found in sewage treatment plant effluent.

NDMA may also occur during drinking water treatment processes (OME, 1994). DMA is a precursor of NDMA and it is discharged into water resources from agricultural run-offs since it is found in the feces of dairy cattle (Van Rheenan, 1962). Thus, NDMA can be formed as a disinfection by-product in some drinking water treatment plants applying chlorination process (such as sodium hypochlorite and chloramine) for disinfection of water (Richardson, 2003).

NDMA may be present in water resources due to run-off from agricultural fields where pesticides are applied, discharges from medical services, and discharges from certain industries that form NDMA during manufacturing processes or storage (Pancholy, 1978).

The pH is one of the important parameter affecting NDMA formation potential and kinetics of the reactions. NDMA formation potential is the highest in the pH range of 7-8. At lower pH, the reaction is limited because of the deficiency of non-protonated amines. At higher pH, NDMA formation potential is limited because of the deficiency of chloramine compounds (Shen and Andrews, 2013). Therefore, in this study, pH was adjusted to 8.0 for better NDMA formation potential.

2.4.1.3 Environmental Exposure of NDMA

N-nitrosodimethylamine is exposed in three ways to humans; namely, ingestion, inhalation and dermal contact (Xianghua, 2006). There are some higher risk groups for possible exposure to NDMA due to their jobs such as workers in laboratories, copolymers, lubricants, and pesticide workers. Additionally, people may get exposed to NDMA from foods and beverages, tobacco smoke, herbicides, pesticides, drinking water and industrial pollution In these instances, quantities of the exposure are unknown. In previous studies related to the NDMA exposure, NDMA was found in most of foods like cheeses, soybean oil, canned fruit, various meat products, bacon, various cured meats, frankfurters, cooked hams, fish and fish products, spices used for meat curing, apple brandy, beverages, beer (Scanlan *et al*., 1980) and tobacco smoke (Spincer and Westcott, 1976). Scientists estimate the NDMA exposure of a human from air, diet and smoking at levels of a few micrograms per day (Xianghua, 2006). Concentrations of N-nitrosodimethylamine in the food stuffs mentioned above have been measured to be between 0 and 85 μg/kg. For example, NDMA concentrations are approximately 90 to 100 ng/L for whole milk, 2600 to 2700 ng/kg for bacon, and 300 to 800 ng/kg for cheese (Cerutti and Airoldi, 1996). The U.S. Food and Drug Administration (FDA) and U.S. Consumer Product Safety Commission (CPSC) have explained that NDMA is mostly formed in rubber processing and may be found as a contaminant in the final rubber product. NDMA has been also found in most of the drugs formulated with aminopyrine such as tablets, suppositories, injections, drops, and syrups, at concentrations ranging from < 10 to 371 μg/kg (Kobylinski and Peterman, 1979; Poocharoen *et al*., 1992). In tobacco smoke, NDMA has been detected at concentrations of 0 to 140 ng/cigarette (Xianghua, 2006).

2.4.1.4 Occurrence of NDMA in Drinking Water Sources

There are numerous studies related to the occurrence of NDMA in drinking water and drinking water sources. According to a study, NDMA was found in samples taken from both raw and finished water samples in drinking water treatment plants (DWTPs) in Japan. NDMA was detected in 15 of 31 raw water samples collected

during the summer at concentrations up to 2.6 ng/L, and in 9 of 28 raw water samples collected in winter at concentrations up to 4.3 ng/L. The population was higher in the areas where NDMA was found at higher concentration in water samples. NDMA was detected in 10 of 31 finished samples collected in summer at concentrations of up to 2.2 ng/L, while 5 of 28 finished samples collected in winter demonstrated NDMA concentrations up to 10 ng/L. The samples taken from Yodo River basin DWTP had higher NDMA concentrations (Asami *et al.*, 2009). Another study related to the occurrence of nine nitrosamines in drinking water following different water treatment processes in three cities and tap waters in one city was performed in China. Among other nitrosamines, NDMA was identified in raw water, disinfecting water, finished water and tap water samples, ranging from 0.8 ng/L to 21.6 ng/L, 0.12 ng/L to 24.2 ng/L, not detected to 8.8 ng/L, and not detected to 13.3 ng/L, respectively. From this study showed it was observed that using chloramine and chlorine as a disinfectant caused the most considerable amounts of NDMA. Using additional disinfection processes such as UV radiation and ozonation reduced NDMA amount (Luo *et al.*, 2012).

There are some standards regulated some authorities for NDMA. EPA established a cleanup concentration of 0.7 ng/L for NDMA in groundwater based on 10^{-6} life time excess cancer risk in drinking water (EPA, 2001), EPA has calculated 0.42 ng/L as the no enforceable screening level for NDMA in tap water, based on a 1 in 10^{-6} life time excess cancer risk (EPA, 2011), The Ontario Ministry of the Environment (MOE) has regulated a maximum acceptable level of 9 ng/L for NDMA in drinking water (MOE, 2003), The California Department of Health Services (CDHS) has regulated a notification level of 10 ng/L of NDMA, The California Office of Environmental Health Hazard Assessment (OEHHA) has set up a public health goal for NDMA at 3 ng/L in drinking water, based on a 1 in 10^{-6} life time excess cancer risk (OEHHA,2006).

2.4.1.5 NDMA Precursor Studies

There are many researches to observe potential precursors of NDMA. The common NDMA precursor related to water treatment are dimethylamine (DMA; Mitch *et al*.,

2003), tertiary and quaternary amines with DMA groups (Kemper *et al.,* 2010), natural organic matter (NOM) (Chen and Valentine, 2007; Dotson *et al.,* 2007; Gerecke and Sedlak, 2003; Mitch and Sedlak, 2004), polyelectrolytes in water treatment plants (Kohut and Andrews, 2003; Mitch and Sedlak, 2004; Najm and Trussell, 2001; Wilczak *et al.,* 2003), some pesticides and herbicides (Chen and Young, 2008; Graham *et al*., 1995; Schmidt and Brauch, 2008) and pharmaceuticals (Shen and Andrews, 2011). For example, ranitidine has showed a high conversion rate to NDMA during chloramination proses (Schmidt *et al*., 2006). Krasner (2008) has suggested the possibility of amine-based pharmaceuticals to be part of the NDMA precursor in wastewater effluent. In the study carried by Shen and Andrews (2011) demonstrated that 20 PPCPs including ranitidine, doxylamine, sumatriptan and diltiazem were nitrosamine precursors during chloramine disinfection. In the study carried by Selbes *et al.,* (2013) observed NDMA formation from chloramine disinfection of 21 selected amines including DMA, ranitidine, trimethylamine (TMA), dimethylethanolamine (DMEA), dimethylbuthylamine (DMBA), dimethylaniline (DMAN). Another study carried by Lee *et al.* (2007) showed that dimethylamine (DMA), trimethylamine (TMA), dimethylethanolamine (DMEA), dimethylformamide (DMFA), dimethyldithiocarbamate (DMDC), dimethylaminobenzene (DMAB), 3-dimethylamiomethyl indole (DMAI) and 4-dimethylaminoantipyrine (DMAP) are NDMA precursors during disinfection with ozone and chlorine. The chemical structures of some precursors studied previously are close to PPCPs used in this study.

2.5 Use of Genotoxicity Tests on DBP Studies

In a research carried by Richardson *et al.* (2007) complied previous studies related to the occurrence, genotoxicity, and carcinogenicity of DBPs, currently regulated U.S. EPA, and unregulated such as halonitromethanes, iodo-acids, THMs, haloamides, tribromopyrrole, aldehydes, and N-nitrosodimethylamine (NDMA) and other nitrosamines. Studies showed that DBPs resulting from chlorinated waters are more mutagenic than DBPs from alternative disinfectants (primarily ozone or chloramines). However, use of ozone and chloramines increased the level of emerging DBPs, such as nitrosamines and some of these emerging DBPs are more

genotoxic than some of the regulated ones. According to a study by Richardson *et al.* (2007), nitrosamines (up to 180 ppt; probable human carcinogens) increased with chloramination. Nitrosamines are emerging non-halogenated DBPs. A study carried by Wagner *et al.* (2012) investigated five nitrosamine DBPs for genotoxicity namely, NDMA, N-nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR), N-nitrosomorpholine (NMOR) and N-nitrosodiphenylamine (NDPhA). They used *S.typhimurium* strain YG7108 and order of mutagenicity was ranked in the order of highly mutagenic to less mutagenic as NDMA, NPIP, NMOR and NPYR; NDPhA was not mutagenic. Moreover, the rank order for genotoxicity was NDMA, NPIP and NMOR. NDPhA was genotoxic only at one concentration and NPYR was not genotoxic.

Also, according to WHO Guidelines for Drinking-Water Quality (2008), NDMA is found as genotoxic both *in vivo* and *in vitro*. Activation by liver microsomal S9 fractions is necessary for a positive *in vitro* result. The recent observation that human S9 fractions are much more active in promoting genotoxicity in the Ames test than rat S9 fractions which suggests that humans may be especially sensitive to the carcinogenicity of NDMA (WHO, 2008).

CHAPTER 3

MATERIAL AND METHODS

3.1 Chemicals and Reagents

3.1.1 Laboratory Grade Water

The laboratory grade water used in the experiments was processed by Millipore RiOs Essential Water Purification System (Merck Millipore, Cat. No: ZR055016Y). The purification process included pretreatment and reserve osmosis.

3.1.2 Stock Phosphate Buffered Solution

Phosphate buffer solution (pH=8.0) was prepared by dissolving 34 g Potassium Phosphate (KH_2PO_4, Merck KGaA Company, Cat. No. 104871) in 500 ml laboratory grade water. Then the pH was adjusted to 8.0 with 1 N of sodium hydroxide (NaOH, Merck KGaA Company, Cat.No. 106498). 1 N of NaOH was prepared by dissolving 12 g NaOH into 300 ml of laboratory grade water.

3.1.3 Stock Sodium Hypochlorite Solution

Stock sodium hypochlorite solution was prepared by dissolving 3 ml of sodium hypochlorite (NaOCl, Sigma Aldrich, Cat.No. 425044-250ML, available chlorine 10-15% stored at 2-8^{o}C) in one liter stock phosphate buffered solution. Stock solutions were stored in brown glass bottles in a dark refrigerated condition at 4^{o}C.

3.1.4 Stock Ammonium Chloride Solution

Stock ammonium chloride solution was prepared by the addition of 150 mg ammonium chloride (NH_4Cl, Sigma Aldrich, Cat.No. 11209) to one liter of stock phosphate buffered solution.

3.1.5 Stock Monochloramine Solution

Stock monochloramine solution was prepared fresh daily by mixing stock ammonium chloride and stock sodium hypochlorite solutions buffered at pH 8.0 approximately 40 minutes before each use. Each stock solution was prepared at 150 ± 10 mg/l concentration and stock monochloramine solution was prepared by mixing equal volumes of stock sodium hypochlorite and ammonium chloride solutions to yield 3:1 Cl_2/N weight ratio.

3.1.6 Sodium Thiosulfate Solution

The sodium thiosulfate solution was prepared by the mixing of 132 g/l sodium thiosulfate pentahydrate ($Na_2O_3S_2.5H_2O$, Sigma Aldrich, Cat.No. 106516) and 3 g/l potassium iodide (KI, Sigma Aldrich, Cat.No. 03124) in an equal volume ratio.

3.1.7 Stock PPCP Solutions

According to the solubility of PPCPs in liquids, stock solutions were prepared either in laboratory grade water or dichloromethane (CH_2Cl_2, Sigma-Aldrich, Cat. No. 34856). Stock solutions of doxylamine, diltiazem, sumatriptan, ranitidine and caffeine were prepared in laboratory grade water and diclofenac, atrazine and sulfamethoxazole were prepared in dichloromethane. After reviewing the literature, stock solution concentrations were determined.

Doxylamine: Doxylamine succinate salt ($C_{17}H_{22}N_2O.C_4H_6O_4$) was purchased as 5 g in solid phase (Sigma-Aldrich, Product No. D-3775). 5×10^6 µg/l stock solution was prepared by dissolving 5 g of doxylamine succinate salt in 1000 ml of laboratory

grade water. The stock solution was stored in dark glass bottle in refrigerated conditions at 4^{o}C until use in the experiments.

Diltiazem: (+)-cis-diltiazem hydrochloride ($C_{22}H_{26}N_2O_4S.HCl$) was purchased as 1 g in solid phase (Sigma-Aldrich, Product No. D2521), 10^6 µg/l stock solution was prepared by dissolving 1 g of (+)-cis-diltiazem hydrochloride in 1000 ml of laboratory grade water. The stock solution was stored in dark glass bottle in refrigerated conditions at 4^{o}C until use in the experiments.

Sumatriptan: Sumatriptan succinate ($C_{14}H_{21}N_3O_2S.C_4H_6O_4$) was purchased as 10 mg in solid phase (Sigma-Aldrich, Product No. S-1198), 10^6 µg/l stock solution was prepared by dissolving 10 mg of sumatriptan succinate in 10 ml of laboratory grade water. The stock solution was stored in dark glass bottle in refrigerated conditions at 4^{o}C until use in the experiments.

Ranitidine: Ranitidine hydrochloride ($C_{13}H_{22}N_4O_3S.HCl$) was purchased as 500 mg in solid phase (Sigma-Aldrich, Product No. R-101), 5 x 10^7 µg/l stock solution was prepared by dissolving 500 mg of ranitidine hydrochloride in 10 ml of laboratory grade water. The stock solution was stored in dark glass bottle in refrigerated conditions at 4^{o}C until use in the experiments.

Caffeine: Caffeine ($C_8H_{10}N_4O_2$) was purchased as 250 mg in solid pahse (Dr. Ehrenstorfer GmbH Company, Cat. No. C11693000), 25 x 10^5 µg/l stock solution was prepared by dissolving 250 mg of caffeine in 10 ml of laboratory grade water. The stock solution was stored in dark glass bottle in refrigerated conditions at 4^{o}C until use in the experiments.

Diclofenac: Diclofenac sodium salt ($C_{14}H_{10}C_{l2}NNaO_2$) was purchased as 1 g in solid phase (Fluko Analytical, Product No. PHR-1144), 10^6 µg/l stock solution was prepared by dissolving 1 g of diclofenac sodium salt in 10 ml of dichloromethane. The stock solution was stored in dark glass bottle in refrigerated conditions at 4^{o}C until use in the experiments.

Sulfamethoxazole: Sulfamethoxazole ($C_{10}H_{11}N_3O_3S$) was purchased as 1 g in solid phase (Fluko Analytical, Product No. PHR-1126), 10^6 µg/l stock solution was prepared by dissolving 1 g of sulfamethoxazole in 10 ml of dichloromethane. The stock solution was stored in dark glass bottle in refrigerated conditions at 4°C until use in the experiments.

Atrazine: Atrazine ($C_8H_{14}ClN_5$) was purchased as 100 mg in solid phase (Supelco Analytical, Product, No. 4-9085), 10^6 µg/l stock solution was prepared by dissolving 100 mg of atrazine in 10 ml of dichloromethane. The stock solution was stored in dark glass bottle in refrigerated conditions at 4°C until use in the experiments.

3.1.8 NDMA Stock Standard Solutions

Analytical standard N-nitrosodimethylamine solution was obtained as 5000 µg/mL in 1 mL methanol (Sigma-Aldrich, Cat. No. 40059). In order to prepare NDMA stock solutions, 1000 ml of dichloromethane was added to the NDMA solution which was purchased as 5000 µg in solvent methanol. Thus, a 5000 ppb stock solution was formed.

From the 5000 ppb stock solution and using dichloromethane for dilution, 500 ppb with 1:10 dilution, 100 ppb with 1:50 dilution, 50 ppb with 1:100 dilution, 25 ppb with 1:200 dilution and 10 ppb with 1:500 dilution were prepared. This procedure is illustrated in Figure 3.1. These stock solutions were stored in a freezer at -18°C. After preparation of these stock solutions, they were analyzed using GC/MS and these results were used to for calibration curve of GC/MS abundance readings.

Figure 3.1 Analysis Procedures on Non-Extracted NDMA Solutions

3.1.9 Solutions for Mutagenicity Test

Mutagenicity Tests were performed using test kits from Environmental Bio-detection Products Incorporated (EBPI, The Muta-ChromoPlateTM).

The test kits included the following prepared reagents;

- A: Davis-Mingioli salts (concentrate),
- B: D-glucose,
- C: Bromocresol Purple,
- D: D-Biotin,
- E: L-Histidine,
- F: Sterile laboratory grade water,
- G: Growth Medium,

Standard mutagens included in the kit were as follows:

- Sodium azide (NaN_3, 0.5 µg/100 µl), a direct-acting mutagen, for *S.typhimurium* TA100 bacterial strain ; and,
- 2-Nitrofluorene (2-NF, 30 µg/100 µl), a direct-acting mutagen, for *S.typhimurium* TA98 bacterial strain.

S.typhimurium TA98 and TA100 strains have been successfully used in a number of studies to investigate genotoxicity of disinfection by-products (Richardson *et al*., 2007; Guzzella *et al*., 2004).

3.2 Analytical Methods

3.2.1 Monochloramine Analytical Methods

Free chlorine, monochloramine and total chlorine concentrations were determined using the DPD Colorimetric Method (APHA, 1995). The spectrophotometer, (Hach-Lange, Model: DR2800) used has a built-in library for chlorine measurement with

kits provided by the same manufacturer (Hach-Lange, Cat. 21055-69). The range for this method was 0.02 to 2.00 mg/L Cl_2. Therefore, necessary dilutions were done for concentrations above 2.00 mg/L Cl_2 before measurements.

Sample cells were filled with 10 ml of diluted sample. It was inserted into the spectrophotometer and pushed to zero. A DPD, free chlorine powder pillow was added to each cell and swirled to mix. The free chlorine concentrations were read. Free chlorine exists in the samples in very low amount due to unreacted free chlorine with ammonia. Due to low free chlorine in sample cells, the color of sample did not turn to pink color; but rather, was close to transparent color.

In order to measure monochloramine concentrations one crystal of potassium iodide (KI) was added (APHA, 1995). The color of each sample turned to a pink color as shown in Figure 3.2 and next the sample was placed in the spectrophotometer to measure light absorbance. The result concentration shows the total of chloramine and free chlorine concentrations. In order to measure the total chlorine in each sample, an extra amount of potassium iodide was added and read using the spectrophotometer. Again, there may be very low concentrations of dichlormine ($NHCl_2$), and nitrogen trichloride (NCl_3) and with the measurement of total chlorine other forms of chloramines can be detected. This procedure was repeated three times for each sample in order to minimize errors.

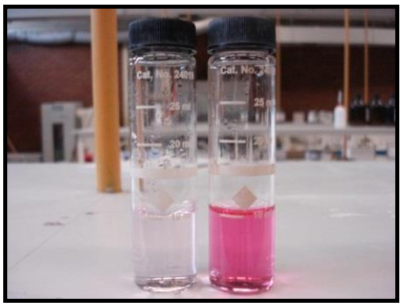


Figure 3.2 Free Chlorine (1st Sample Cell) &Monochloramine (2nd Sample Cell)

3.2.2 NDMA Analytical Methods

3.2.2.1 Instrumentation Conditions

Samples were analyzed with an Agilent 6850 Network Gas Chromatograph (GC) System coupled with Agilent 5975C VL MSD (mass spectrometer) and a 7683B Series Injector shown in Figure 3.3.

Figure 3.3 Agilent 6850 gas chromatograph (GC) coupled with and Agilent 5975C mass spectrometer (MS).

In order to find the reliable program for analyses of the samples, four different programs from the literature were applied to this specific model of GC/MS. Three of GS/MS programs from the literature did not read NDMA accurately, fluctuations were formed and there were no peaks according to the concentration increases. One program, the control parameters demonstrated in Table 3.2, showed peaks for different concentrations, the abundance of which increased with the concentration increases (Park, 2008). Instrument control parameters of the program found as the most reliable are shown in Table 3.1 and Table 3.2.

Instrument Control Parameters

Sample Inlet	:	GC
Injection Source	:	GC ALS
Mass Spectrometer	:	Enabled

Table 3.1 Gas Chromatography Control Parameters

Oven:			
Parameters		**Values**	
Initial temperature		35 °C (On)	
Maximum Temperature		325 °C	
Initial Time		1.00 min	
Equilibrium Time		0.50 min	
Ramps:			
Number	**Rate**	**Final temp.**	**Final time**
1	10.00	70	0.00
2	2.00	72	5.50
3	15.00	240	2.40
4	0.0 (off)		
Post temp	0 °C		
Post time	0.00 min		
Run time	24.60 min		
Inlet:			
Parameters		**Values /Types**	
Mode		Splitless	
Initial temperature		250 °C (On)	
Pressure		6.73 psi (On)	
Purge flow		25.0 mL/min	
Purge time		2.00 min	
Total flow		28.6 mL/min	
Gas saver		On	
Saver flow		20.0 mL/min	
Saver time		2.00 min	
Gas type		Helium	
Column:			
Parameters		**Values/Types**	
Type		Capillary Column	
Column Inventory Phase		DB-5ms	
Model Number		J&W 122-5533E	
Max temperature		325 °C	
Nominal length		30.0 m	
Nominal diameter		250.00 um	
Nominal film thickness		1.00 um	
Mode		constant flow	
Initial flow		1.0 mL/min	
Nominal init pressure		6.73 psi	
Average velocity		36 cm/sec	
Source		Inlet	
Outlet		MSD	
Outlet pressure		vacuum	

Table 3.1 (Continued)

GC Injector	
Front Injector	
Sample Washes	3
Sample Pumps	3
Injection Volume	2.00 microliters
Syringe Size	10.0 microliters
PreInj. Solvent A Washes	3
PreInj. Solvent B Washes	3
PostInj. Solvent A Washes	3
PostInj. Solvent B Washes	3
Viscosity Delay	0 seconds
Plunger Speed	Fast
PreInjection Dwell	0.00 minutes
PostInjection Dwell	0.00 minutes
Back Injector	

Table 3.2 MS Acquisition Control Parameters

MS Information (Acquisition Mode : SIM)		
Parameters	**Values/Types**	
Solvent Delay	6.00 min	
EMV Mode	Gain Factor	
Gain Factor	7.00	
Resulting EM Voltage	1965	
SIM Parameters for NDMA		
Ions/Dwell In Group		
(Mass, Dwell)	(Mass, Dwell)	(Mass, Dwell)
(42.00, 100)	(43.00, 100)	(55.00, 100)
(74.00, 100)	(74.10, 100)	
MS Zones		
MS Source : 230°C maximum 250°C		
MS Quad : 150°C maximum 200°C		

3.2.2.2 Calibration of GC/MS

After determination of the appropriate instrument control parameters and selected-ion monitoring (SIM) values for NDMA, non-extracted NDMA solutions prepared as stock solution (10 ppb, 25 ppb, 50 ppb, 100 ppb and 500 ppb) were analyzed by GC/MS. Areas under each concentration peaks were calculated using either the manual integration or autointegration function of the GC/MS computer program (MSD Enhanced Chem Station E.02.02.1431, Agilent Technologies) illustrated in

Appendix A and B. According to the correlation between stock solution concentrations and areas under the chromatography curve, a calibration curve was drawn as shown in Figure 3.4, on March of 15th, 2013. Detailed analysis results for these five concentrations are shown in Appendix C.

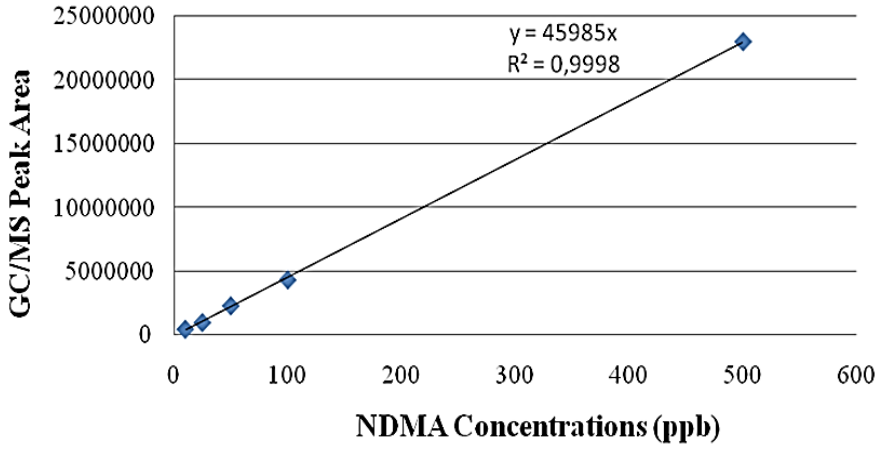


Figure 3.4 Calibration Curve (prepared on March of 15th, 2013)

During experiments, the carrier gas, helium, of GC/MS was consumed up to limit values. Therefore, a new tank of helium gas was installed and the gas tube was filled. Due to the gas exchange, the calibration curve was prepared again. Stock standard solutions of NDMA (10 ppb, 25 ppb, 50 ppb, 100 ppb and 500 ppb) were read again in GC/MS on May of 25th, 2013 illustrated in Figure 4.8. Detailed analysis results for these five concentrations are shown in Appendix C.

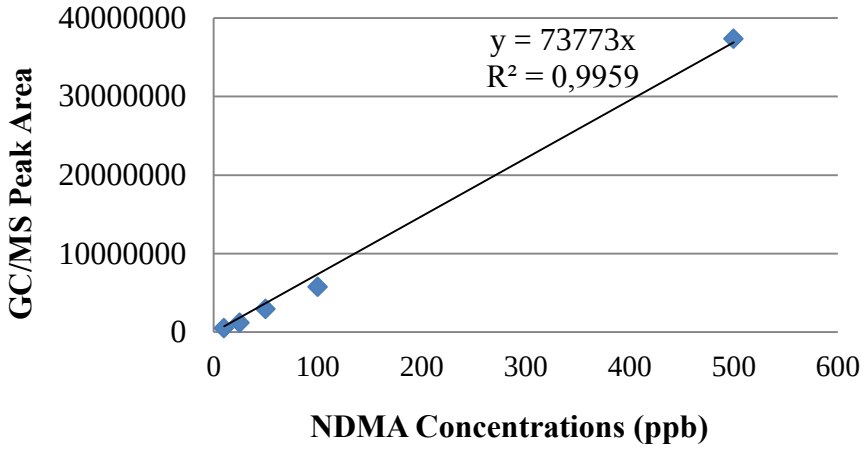


Figure 3.5 Second Calibration Curve (prepared on May of 25th, 2013)

In order to calculate recovery percent of samples a third calibration curve was prepared and it is shown in Figure 3.6.

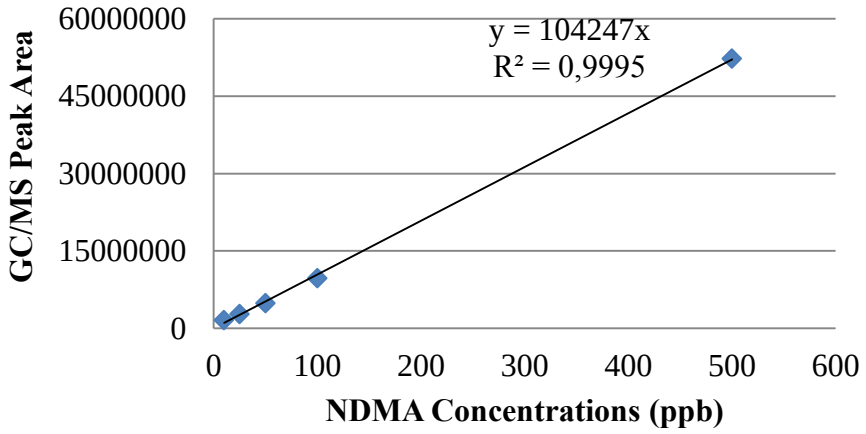


Figure 3.6 Third Calibration Curve

3.2.2.3 NDMA Measurement with GC/MS

NDMA analyses were performed in accordance with EPA Method 521 of Determination of nitrosamines in drinking water by solid phase extraction and capillary column gas chromatography with large volume injection and chemical ionization tandem mass spectrometry (MS/MS) (Munch & Bassett, 2004).

Before GC/MS measurements, solid phase extraction (SPE) was applied to extract the NDMA dissolved in water and elute NDMA with solvent that can be analyzed in GC/MS according to EPA Method 521. In order to apply the solid phase extraction method, extraction cartridge (coconut charcoal) with 6 ml polypropylene tubes, vacuum extraction manifold, and the needles of nitrogen evaporator were required. The schematic experiment installation is illustrated in Figure 3.7.

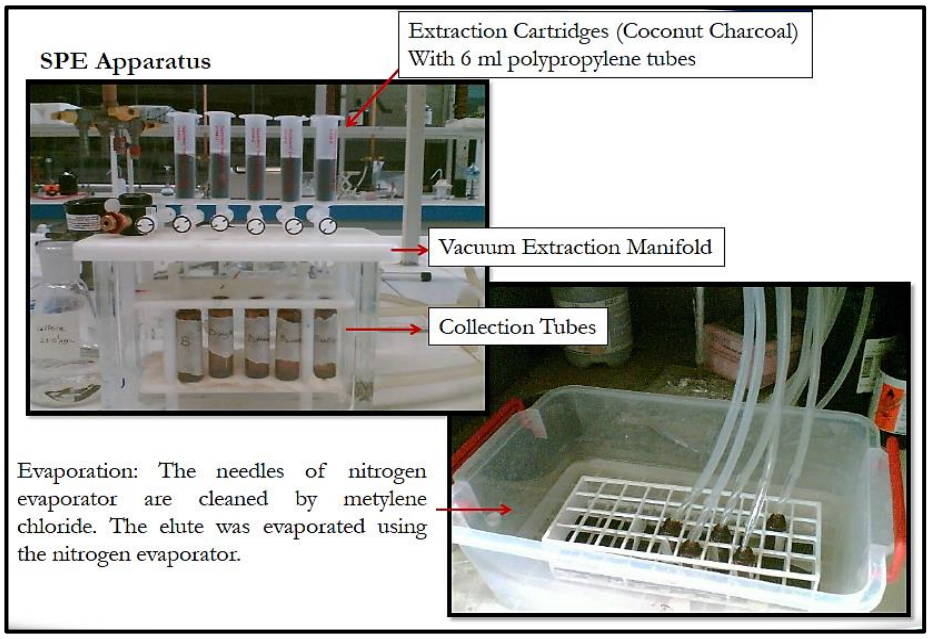


Figure 3.7 Solid Phase Extraction Experiment

Solid phase extraction was done in four parts. The first stage was cartridge conditioning. Cartridges were filled with approximately 3 mL dichloromethane and the vacuum was turned on to pull the solvent through. Cartridges were aspirated completely. This process was repeated once again. Next, same procedure was applied for methanol. Cartridges were filled with approximately 3 mL methanol and the vacuum was turned on, pulling the solvent was through. Cartridges were also aspirated completely. This process was repeated once again. Cartridges were filled again with approximately 3 mL methanol and eluted with the vacuum to just above the top frit - not allowing the cartridge to go dry at the end. From this point forward, the cartridge was not permitted to dry. The elution process was repeated once again. Next, the cartridge was filled with approximately 3 mL laboratory grade water. The

vacuum was turned on to pull the water through the cartridge. This process was repeated five times without allowing the cartridge to dry between washes or at the end.

The second part was sample extraction. A transfer tube was attached from each sample bottle to each cartridge and then the vacuum was turned on. The flow rate of the vacuum was adjusted to 10 mL/min. 100 ml of sample passed through the cartridges. After all the samples passed through SPE cartridges, full vacuum was applied for approximately 10 minutes continuously.

The third part was cartridge elution. The extraction manifold was lifted to the top and a rack was inserted with collection tubes into the extraction tank in order to collect the extracts as they were eluted from the cartridges. Each cartridge was filled with dichloromethane and at low vacuum the sorbent was soaked with dichloromethane. Next, the vacuum was turned off and the system was vented approximately 1 minute. The sorbent was allowed to soak in the dichloromethane. A low vacuum was then applied and 12 ml of dichloromethane was added to the cartridge and collected into the tubes. By this process the sorbent was extracted from the SPE cartridge and ready for GC/MS analysis.

The fourth part was concentration of the eluted samples. The extracted samples were further concentrated to 1 mL in a water bath near room temperature (20 to 25°C) under a gentle stream of nitrogen. This process was applied only for PPCPs that have not formed detectable NDMA in the preliminary NDMA formation potential analysis.

After application of SPE process, 1 ml of samples was poured into the silanized amber wide opening screw to vials (2 ml, Agilent Technologies, Part No. 5183-2072) and according to GC/MS instrumentation conditions NDMA measurements were completed. Areas under each concentration peaks were calculated using either the manual integration or autointegration function of the GC/MS computer program. After calculation of areas under the NDMA chromatograms, calibration curves were used to calculate concentrations in terms of areas.

3.2.3 Mutagenicity Tests

All stages of the mutagenicity test were carried out under aseptic conditions. The first stage of the mutagenicity test, rehydration of the dried bacteria and preincubation, was a preparatory step performed on the day prior to the assay. Using the aseptic technique, two nutrient broth vials (Bottle G) were transferred separately to the vials of lyophilized (dried) bacteria (*S. typhimurium* TA 100 and *S. typhimurium* TA 98) and mixed with the vortex mixer for approximately 30 seconds. A rubber stopper was used to cover the bottles which were then incubated in a 37°C incubator overnight for approximately 16 to 18 hours. After that period of incubation, turbidity (bacterial growth) was observed as expected.

In the next stages, sample preparation and setting up the test plates, were performed on the day of the assay. Samples to be tested (7.5 ml) were filter-sterilized using the 0.22 μm sterile membrane filters (Sartorius Minisart, Cat. No. 16534). Necessary dilutions were done with sterile laboratory grade water. The quantities of sample to be tested with the sterilized laboratory grade water (to achieve the appropriate dilution to be tested) were adjusted to 8.75 ml in the sterile tubes labeled with contents and dilutions. A reaction mixture was prepared with components "A" to "E" set forth in section 3.1.9 Solutions for Mutagenicity Test. The reaction mixture included 21.62 mL from bottle (A), 4.75 mL from bottle (B), 2.38 mL from bottle (C), 1.19 mL from bottle (D), and 0.06 mL from bottle (E), totaling 30 ml of mixture. This reaction mixture was prepared daily before starting mutagenicity experiments. 1.25 ml of reaction mixture was dispensed to each sterile tube containing 8.75 ml of a sample to be tested and mixed thoroughly. The tube containing a total volume of 10 ml bacterial suspension (either TA98 or TA100) that was grown over night were dispensed each of the sample tubes by 2.5 μL and then mixed with the vortex mixer for approximately 30 seconds. In addition to the mutagenicity test of samples, 2 backgrounds, and 1 standard mutagen test were performed. In the background tests, 7.5 ml of laboratory grade water was used instead of 7.5 ml of the sample. In the standard mutagen test, 7.45 ml of laboratory grade water and 0.05 ml of standard mutagen were used instead of 7.5 ml of the sample. Standard mutagens used were sodium azide (NaN_3) and 2-Nitro fluorence (2-NF) for TA 100 and TA 98,

respectively. The mixture in each tube was poured into an each sterile multichannel pipette reagent boats. The liquots of the mixture taken as 200 µL were dispensed into each well of a 48-well microtitration plate using a multichannel pipette. Background was prepared for both TA 98 and TA 100. In background plate, instead of the sample, 8.75 ml reagent water and TA 98 and TA 100 bacterial test strains were added. The aim of backgrounds is to eliminate the environmental factors affecting the bacterial strain. The plate was covered with a lid and sealed in air tight plastic bags to prevent evaporation. The plate containing Sodium Azide was stored in a separate bag due to potential contamination for surrounding plates. The plates in the air tight plastic bag were incubated at 37^{o}C for 5 days.

3.3 Performance of Experiments

The basic and brief summary scheme of performance of the experiments is shown in Figure 3.8.

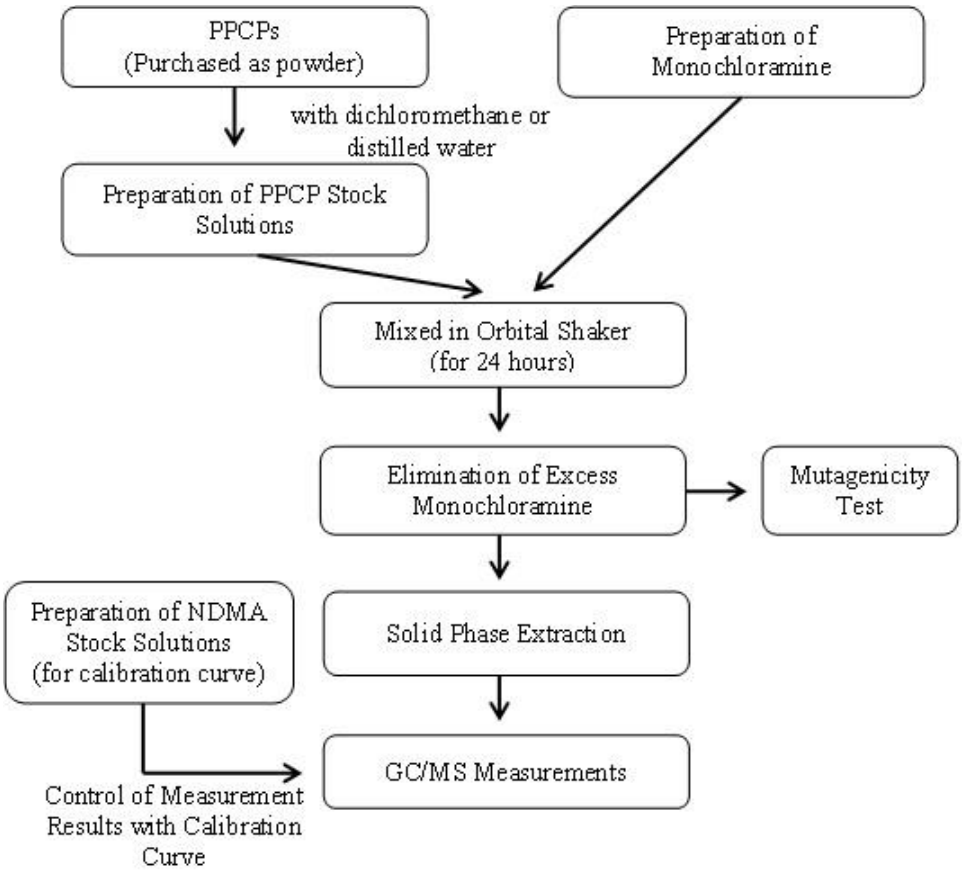


Figure 3.8 Summary Scheme of Experiments

On the day of the experiments, stock monochloramine solutions were prepared as described in Section 3.2.1 Monochloramine Analytical Methods. Once the stock monochloramine concentration was determined, the following formula was used to find volume amount of monochloramine mixed with PPCPs.

$C_1 \times V_1 = C_2 \times V_T$

C_1 : monochloramine concentration acquired

V_1 : monochloramine volume added

C_2 : monochloramine concentration intended (2 or 2.5 mg/l)

V_T : total volume

$V_T - V_1$: chemical volume added

The total volume (V_T) of experimental reactors was determined as 200 ml. The desired monochloramine concentration in mixture was determined as approximately 2-2.5 mg/l. Therefore, the monochloramine concentration intended (C_2) value was identified as 2.5 mg/l. The monochloramine concentration from the stock ammonium chloride and stock sodium hypochlorite mixture was measured with spectrophotometer and it was calculated according to the dilution ratio as mentioned above. Thus, the monochloramine concentration acquired (C_1) value was determined for this formula and the only unknown value of this formula, the monochloramine volume added (V_1), was found. The total volume was 200 ml; therefore, the chemical volume added was found with the subtraction of the monochloramine volume from 200 ml.

For each PPCP experiment the stock monochloramine and PPCP stock solution and monochloramine were mixed in appropriate volumes to achieve final the monochloramine concentration of 2.0-2.5 mg/l and the PPCP concentration determined previously for the specific PPCP being tested. The volume of reactors in each experiment was adjusted to 200 ml. Once the PPCP solution was mixed with monochloramine, in 250 ml erlenmayer flasks, the flasks were placed in an orbital shaker set at 110 rpm and 20-25^{o}C for the duration of 24 hours. The reason of the 24 hours selection as a contact time is that monochloramine becomes relatively stable during the 24 hours reaction period (Choi and Valentine, 2001). Moreover, a study

by Choi and Valentine (2003) demonstrated that NDMA formation due to reaction with monochloramine was continuous over 24 hours and the maximum NDMA formation occurred during 24 hours.

Before mixing in the orbital shaker, the monochloramine concentrations of each flask were measured in a spectrometer at t_0 and recorded. After 24 hours, the monochloramine concentrations were measured again. The monochloramine decayed during the 24 hour period and these results are shown in Chapter 4. Before analyzing samples with GC/MS, residual monochloramine was quenched with the addition of sodium thiosulfate solution. For 200 ml of monochloramine and PPCP mixture, 1 ml of sodium thiosulfate solution was sufficient to eliminate all of the residual chlorine compounds. Thus, while preparing for GC/MS measurements, the PPCPs did not react with excess monochloramine in vials. After elimination of the residual monochloramine in the samples, the GC/MS measurement procedures were applied. This procedure was explained in detail in Section 3.2.2 NDMA Analytical Methods.

PPCP + monochloramine measurements were done three times for quality assurance and quality control.

3.4 Quality Assurance and Quality Control (QA/QC)

Two different sets of control experiments were applied for GC/MS measurements. In the first one, a phosphate buffer solution at pH 8.0 was used without addition of any PPCP stock solution. In these solutions, the monochloramine was added and the standard experimental procedures were followed. In the second set of control reactors, predetermined amount of one of the PPCP stock solutions was added to phosphate buffered solution, but this time no monochloramine was added in the reactors. Next, the standard experimental procedures were followed. These two control reactors are crucial in order to understand if the sole source of NDMA formation in our reactors is reaction of PPCPs with monochloramine. All analyses were done three times in order to eliminate error and GC/MS reads were performed two times for each sample. In total, each sample was read six times.

Another important QA/QC parameter in analysis of NDMA formation is success of the extraction of NDMA from water samples. The recovery of NDMA forms water samples were tested in accordance with EPA 521 Method. Quantification of NDMA was attained through internal calibration using stock standard solutions. The calibration standards (10 ppb, 25 ppb, 50 ppb, 100 ppb and 500 ppb) were subjected to the same extraction (SPE) process as the samples in order to account for recovery named in EPA 521 Method as Laboratory Fortified Sample Matrix (LFSM). A calibration curve was prepared before analyzing the calibration standards and interferences from background of the samples was accounted for using a blank (Laboratory Fortified Blank - LFM) control sample. All samples and blanks were prepared in duplicate. Percent Recovery was calculated using following formula:

$R = [(A-B)/C]* 100$

R : percent recovery

A : measured fortified concentration

B : background concentration

C : fortified concentration

For fortified at or above native concentration, recoveries should range between 70 and 130% for all method analytes.

CHAPTER 4

RESULTS & DISCUSSIONS

Experiment schedules are shown in Table 3.1. The first control experiment, laboratory grade water + monochloramine without PPCP, was repeated three times and the second control experiment, laboratory grade water + PPCPs without monochloramine, was applied one time for each PPCP. For quality assurance and quality control, each experiment, monochloramine + PPCP, was repeated three times. Experiments were divided into five days and each day included seven measurements. In total, 35 measurements were conducted as shown in Table 4.1.

Table 4.1 Experiment Schedule for GC/MS Measurements

	Experiment Dates				
Measurements	**June 2nd, 2013**	**June 9th, 2013**	**June 16th, 2013**	**June 30th, 2013**	**July 7th, 2013**
1	Lab. Grade Water + Monochloramine (K_1)	Lab. Grade Water + Ranitidine (4-K_2)	Lab. Grade Water + Monochloramine (K_1)	Monochloramine + Sumatriptan (3-D)	Monochloramine + Diltiazem (2-D)
2	Lab. Grade Water + Doxylamine (1-K_2)	Monochloramine + Ranitidine (4-D)	Lab. Grade Water + Atrazine (7-K_2)	Monochloramine + Ranitidine (4-D)	Monochloramine + Sumatriptan (3-D)
3	Monochloramine + Doxylamine (1-D)	Lab. Grade Water + Caffeine (5-K_2)	Monochloramine + Atrazine (7-D)	Monochloramine + Caffeine (5-D)	Monochloramine + Ranitidine (4-D)
4	Lab. Grade Water + Diltiazem (2-K_2)	Monochloramine + Caffeine (5-D)	Lab. Grade Water + Sulfamethoxazole (8-K_2)	Monochloramine + Diclofenac (6-D)	Monochloramine + Caffeine (5-D)
5	Monochloramine + Diltiazem (2-D)	Lab. Grade Water + Diclofenac (6-K_2)	Monochloramine + Sulfamethoxazole (8-D)	Monochloramine + Atrazine (7-D)	Monochloramine + Diclofenac (6-D)
6	Lab. Grade Water + Sumatriptan (3-K_2)	Monochloramine + Diclofenac (6-D)	Monochloramine + Doxylamine (1-D)	Monochloramine + Sulfamethoxazole (8-D)	Monochloramine + Atrazine (7-D)
7	Monochloramine + Sumatriptan (3-D)	Lab. Grade Water + Monochloramine (K_1)	Monochloramine + Diltiazem (2-D)	Monochloramine + Doxylamine (1-D)	Monochloramine + Sulfamethoxazole (8-D)

4.1 Quality Assurance/Quality Control (QA/QC) Results

Two different sets of control experiments results are shown in Figure 4.1 and Figure 4.2. It is seen that there were not any peaks in the time of NDMA peak existing between 7.00 to 7.50 minutes. Existing peaks were solvents used during GC/MS measurements.

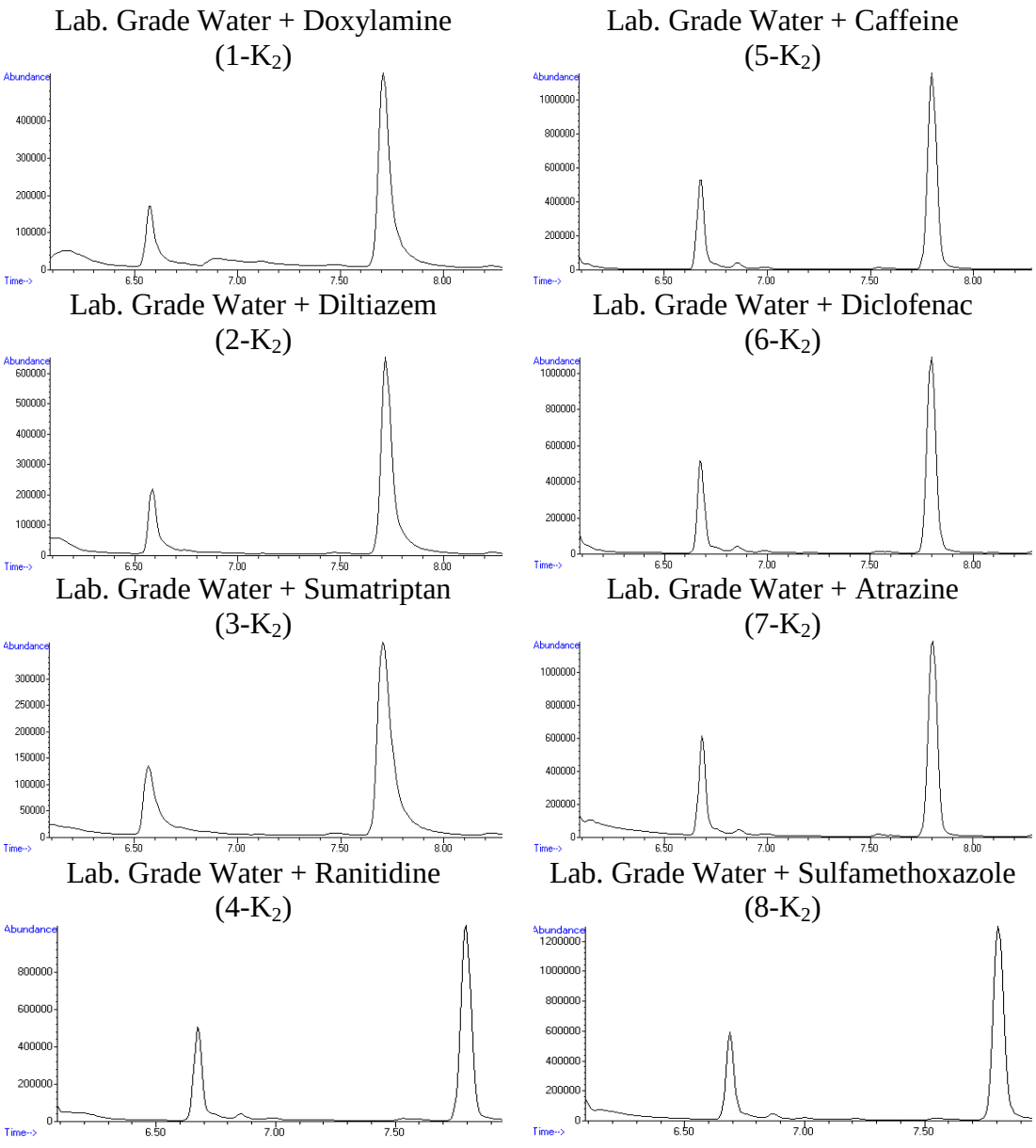


Figure 4.1 Control Experiment Results (PPCP + Laboratory Grade Water)

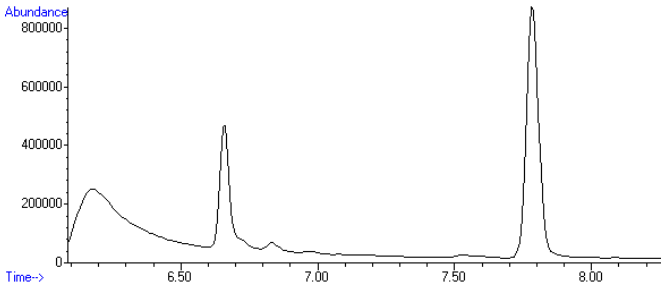


Figure 4.2 Control Experiment Result (Monochloramine + Laboratory Grade Water)

The Laboratory Fortified Sample Matrix (10 ppb, 25 ppb, 50 ppb, 100 ppb and 500 ppb) were subjected to the same solid phase extraction (SPE) process and calculated with recovery percent formula mentioned in materials and methods chapter. For fortified at or above their native concentration, recoveries should range between 70 and 130% for all method analytes (Munch and Bassett, 2004). Recovery percent for standard curve concentrations are shown in Table 4.2. Percent recovery for NDMA related to PPCPs was calculated with interpolation approach according to NDMA standard curve concentrations.

Table 4.2 Recovery Percent for Standard Curve Concentrations

Repl. No.	10 ppb		25 ppb		50 ppb		100 ppb		500 ppb	
	Area	**Conc.(ppb)**	**Area**	**Conc.(ppb)**	**Area**	**Conc.(ppb)**	**Area**	**Conc.(ppb)**	**Area**	**Conc.(ppb)**
R1-1	750486	7.20	1956133	18.76	3990772	38.28	8385082	80.43	43639933	418.62
R1-2	792834	7.61	1883993	18.07	3933380	37.73	8881266	85.19	43933673	421.43
R2-1	801354	7.67	1898833	18.21	3994833	38.32	8557097	82.08	42226570	405.06
R2-2	746441	7.17	1943164	18.64	4065278	38.99	8708794	83.54	42573484	408.39
Average	**773031**	**7.40**	**1920531**	**18.42**	**3996066**	**38.33**	**8633059**	**82.81**	**43093415**	**413.38**
Recovery (%)*	**74.02**		**73.67**		**76.66**		**82.81**		**82.68**	

*In recovery calculations, background NDMA concentration (B) was equal to zero due to lack of interferences during blank control prepared with deionized water.

4.2 Preliminary NDMA Formation Potential Analysis

After preparation of the first calibration curve (prepared on March of 15th, 2013) preliminary analyses were performed with test chemicals (ranitidine, doxylamine, sumatriptan, diltiazem, caffeine, diclofenac, atrazine and sulfamethoxazole) in order to determine whether NDMA is formed as a DBP when monochloramine reacts with each of these chemicals. First, ranitidine with higher potential formation for NDMA was chosen of all chemicals. After research of the literature, for ranitidine, 10^3 µg/l as initial concentration was chosen. The GC/MS measurement of NDMA at the end of this experiment is shown in Figure 4.3.

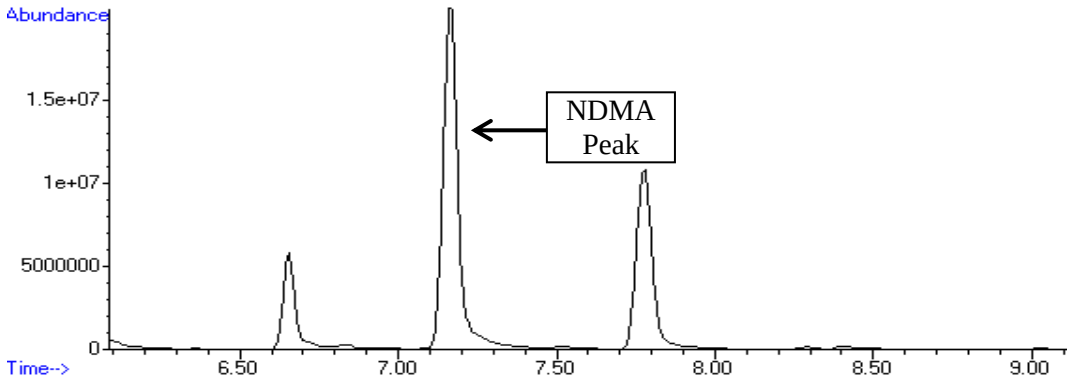


Figure 4.3 NDMA Concentration Peak Resulting from Ranitidine – Monochloramine Reaction

The NDMA peaks due to ranitidine – monochloramine reaction and 500 ppb NDMA peak (the highest concentration in calibration curve), are plotted on the same graph as shown on Figure 4.4. It is seen that the NDMA peak resulting from 10^3 µg/l ranitidine – monochloramine reaction is significantly higher than 500 ppb. It is above the calibrated concentration range (10-500 ppb). Therefore, in order to get an NDMA reading between the calibrated concentrations' range, initial ranitidine concentration needed to be reduced.

It should be noted that the NDMA readings in GC/MS for extracted samples are concentrated at a ratio of 100:12 during solid phase extraction process in preliminary NDMA formation potential analysis experiments. The actual concentration of

NDMA formed during the chloramination was 100:12 times less then GC/MS reading.

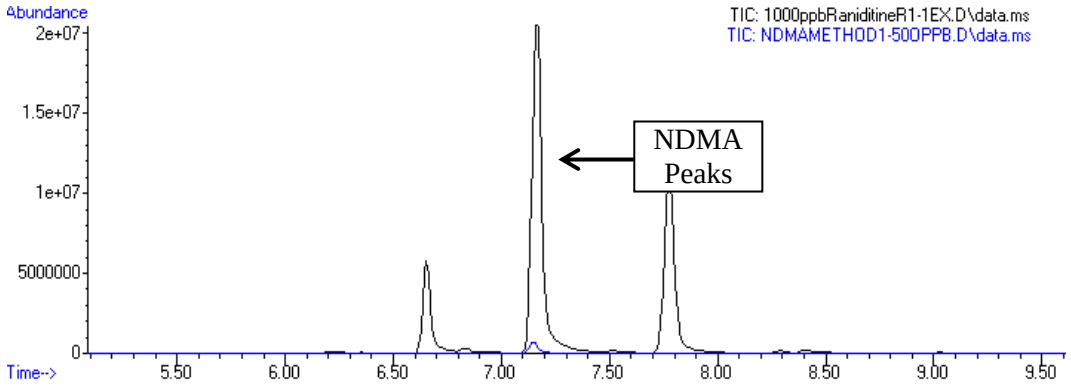


Figure 4.4 Presentation of NDMA Concentration Peaks (Concentrated by 100:12) Resulting from Ranitidine – Monochloramine Reaction and 500 ppb on a Graph

The same procedure was applied for the chemicals doxylamine, sumatriptan, and diltiazem. Initial concentrations, selected for doxylamine, sumatriptan and diltiazem were 5 x 10^3 µg/l, 10^3 µg/l and 10^3 µg/l respectively. The results of NDMA formed when these chemicals reacted with monochloramine for 24 hours are illustrated on Figure 4.5.

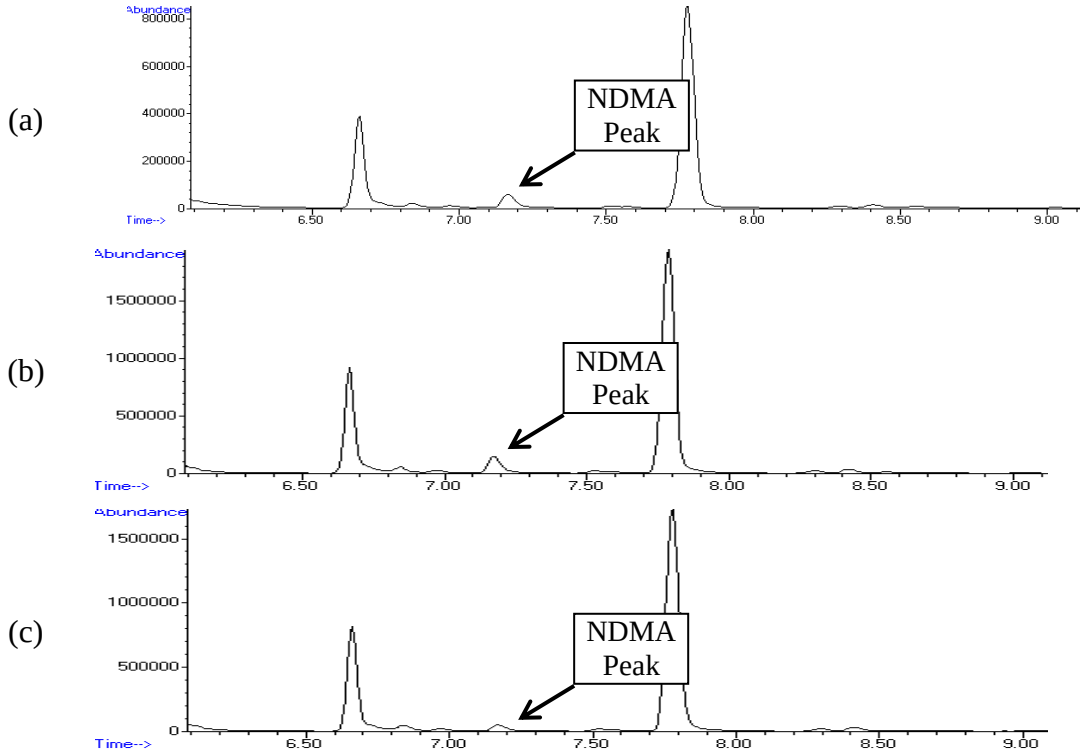


Figure 4.5 NDMA Peaks (Concentrated by 100:12) Resulting from Reaction with Monochloramine and (a) Doxylamine, (b) Sumatriptan and (c) Diltiazem

NDMA peak due to doxylamine – monochloramine reaction and the highest and lowest concentrations in calibration curve (10 ppb and 500 ppb, respectively) are shown on the same graph on Figure 4.6. It is observed that the NDMA peak resulting from 5 x 10^3 µg/l doxylamine – monochloramine reaction is between 10 ppb and 500 ppb. Area under the NDMA peak was calculated and the corresponding concentration of the extracted sample was found as 40.5 ppb using the calibration curve. This value fell directly into the calibration curve concentrations range. Thus, use of 5 x 10^3 µg/l doxylamine concentration in other analyses performed was suitable.

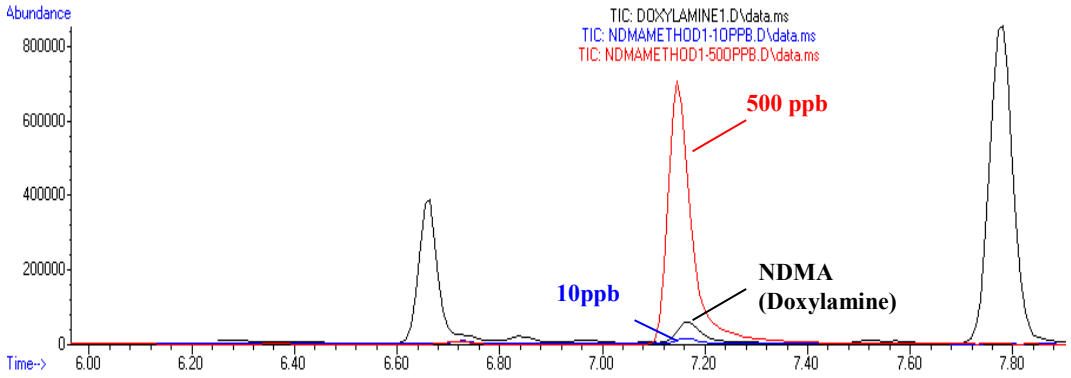


Figure 4.6 Presentation of NDMA Concentration Peaks (Concentrated by 100:12) Resulting from 5 x 10^3 µg/l Doxylamine – Monochloramine reaction and Calibration Curve Peaks

The NDMA peak due to sumatriptan – monochloramine reaction and the highest and lowest concentrations in calibration curve (10 ppb and 500 ppb, respectively) are shown on the same graph on Figure 4.7. It is discerned that the NDMA peak resulting from 10^3 µg/l doxylamine – monochloramine reaction is between 10 ppb and 500 ppb. Area under the NDMA peak was calculated and the corresponding NDMA concentration in the extracted sample was established as 98.5 ppb using the calibration curve. This value was in the calibration curve concentrations' range. Thus, using of 10^3 µg/l sumatriptan concentration in other analyses performed was suitable.

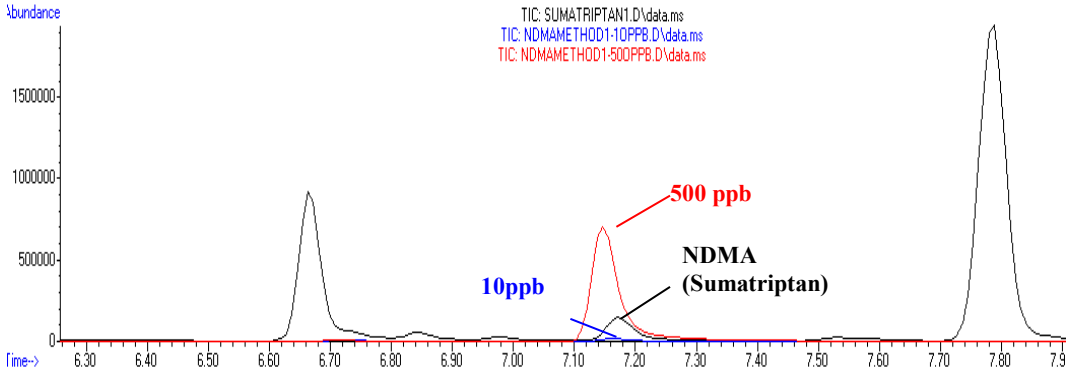


Figure 4.7 Presentation of NDMA Concentration Peaks (Concentrated by 100:12) Resulting from 10^3 µg/l Sumatriptan – Monochloramine reaction and Calibration Curve Peaks

NDMA peak due to diltiazem – monochloramine reaction and the highest and lowest concentrations in calibration curve (10 ppb and 500 ppb, respectively) are shown on the same graph on Figure 4.8. It is seen that the NDMA peak resulting from 10^3 µg/l diltiazem – monochloramine reaction is between 10 ppb and 500 ppb. Area under the NDMA peak was calculated and the corresponding NDMA concentration in the extracted sample was found as 30.4 ppb using calibration curve. This value was in the calibration curve concentrations' range. Thus, using of 10^3 µg/l doxylamine concentration in other analyses performed was suitable.

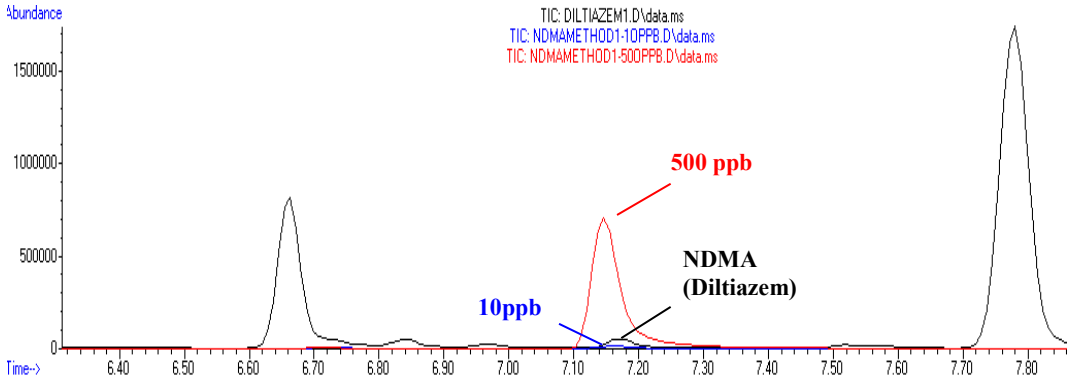


Figure 4.8 Presentation of NDMA Concentration Peaks (Concentrated by 100:12) Resulting from 10^3 µg/l Diltiazem – Monochloramine reaction and Calibration Curve Peaks

The same procedure was applied for the chemicals atrazine, caffeine, diclofenac and sulfamethoxazole. Two different measurements on two different days were performed for these four chemicals. In the first measurement, for each chemical and as initial concentrations, the concentration of the 10^3 µg/l was chosen. However, clear peaks for NDMA were not observed in the measurement results. In the second measurement, there was less dilution of the chemicals. For each chemical and as their initial concentrations, the concentration of the 10^6 µg/l was chosen. NDMA peaks resulting from reactions of monochloramine with each chemical are graphed in Figure 4.9 through Figure 4.12. A clear NDMA peak was not observed in either of these samples. Therefore, as mentioned in the solid phase extraction method explanations in the Materials and Methods Chapter, samples containing these four PPCP were further concentrated to 1 ml as later described in following sections.

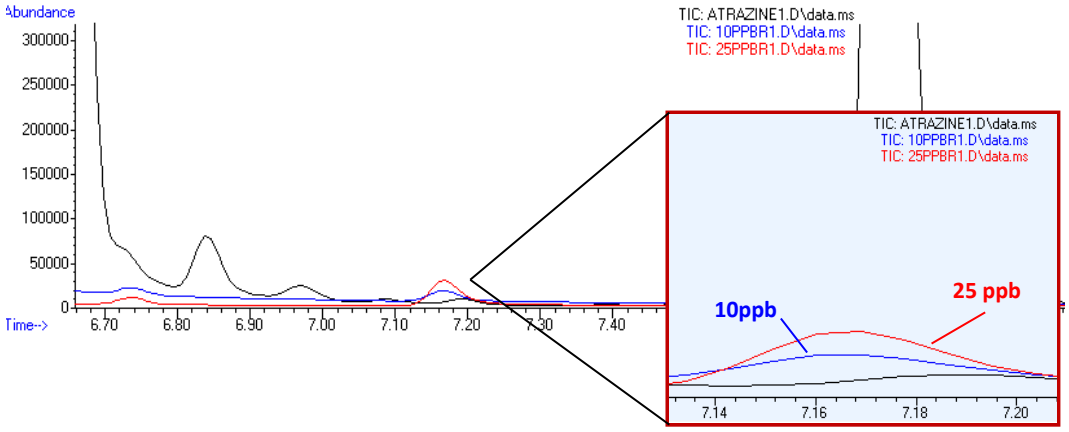


Figure 4.9 Measurement Result for Atrazine (Concentrated by 100:12)

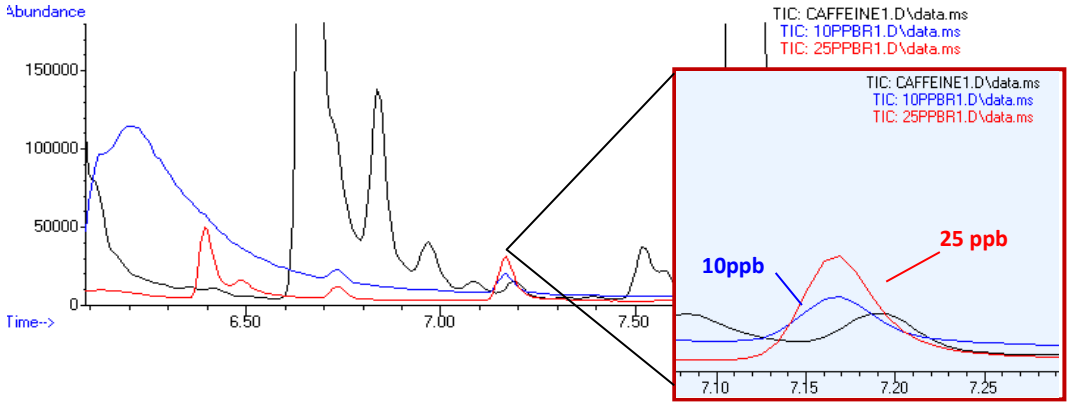


Figure 4.10 Measurement Result for Caffeine (Concentrated by 100:12)

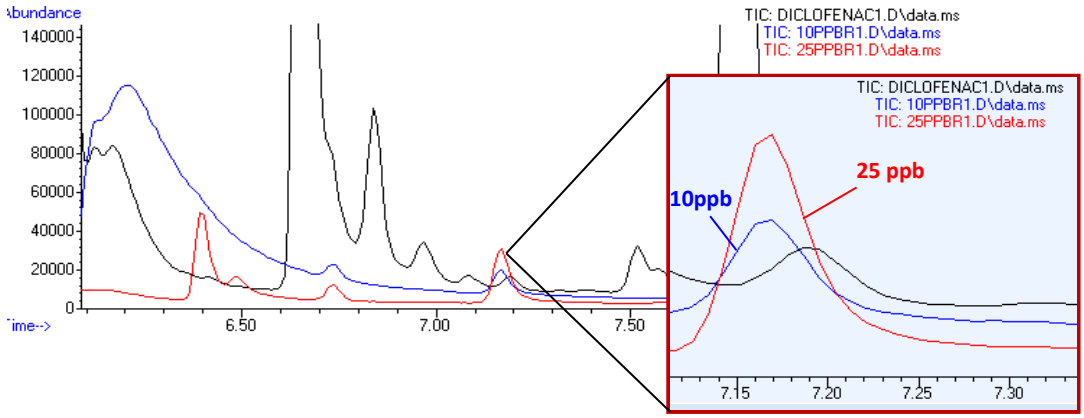


Figure 4.11 Measurement Result for Diclofenac (Concentrated by 100:12)

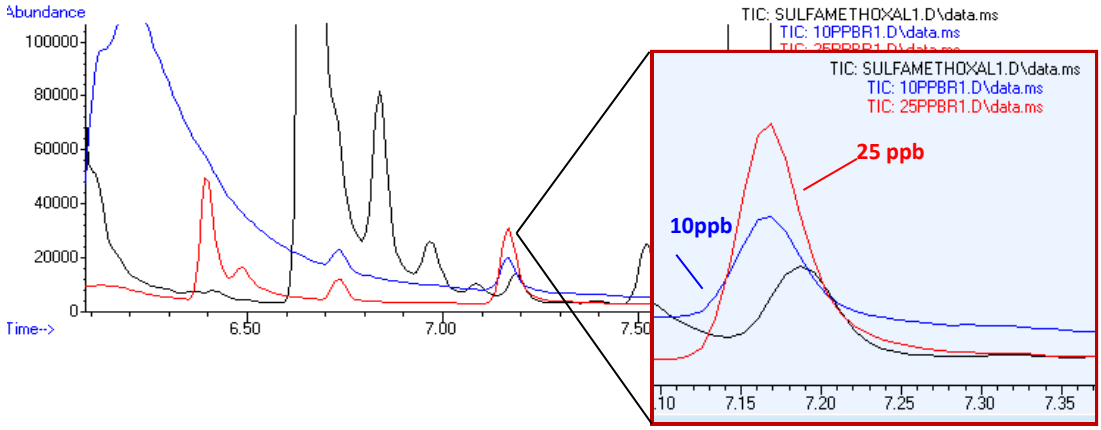


Figure 4.12 Measurement Result for Sulfamethoxazole (Concentrated by 100:12)

4.3 Analysis of NDMA Formation with Chloramination

Experiments for the NDMA formation with chloramination were performed at 5 different dates. Since we were capable of measuring 7 samples using GC/MS as time, we were able to conduct 7 experiments in parallel in one day. In total, 35 experiments were performed. In the experiments, two types of control reactors were run. In one of them, none of the PPCP chemicals were added to the phosphate buffered experimental water and only chloramine was added. In the second, one of the PPCP chemicals was added as usual, but no chloramine was added. In these reactors, instead of chloramine, the same volume of phosphate buffered experimental water was added.

In the analysis of NDMA formation during chloramination, NDMA concentrations were obtained for all chemicals from GC/MS measurements. Percent recoveries calculated as explained in section 3.4 Quality Assurance/Quality Control and concentration ratios (100:12 or 100:1 depending on PPCP tested) were used in order to calculate the concentrations of NDMA formed for each PPCPs. NDMA molar concentrations were calculated as the ratio of NDMA concentration to molecular weight. Same procedure was applied for PPCPs and PPCPs molar concentrations were calculated. In calculation of molar conversions, it was assumed that all PPCPs amount were consumed during monochloramine and PPCPs reactions. Thus, NDMA molar conversion was calculated as the ratio of molar concentration of NDMA formed to molar concentration of spiked PPCPs.

$$Molar\ Conversion = \frac{Molar\ Concetration\ of\ NDMA\ formed}{Molar\ Concetration\ of\ PPCP\ spiked}$$

4.3.1 Stock Monochloramine Analysis

In order to determine stock monochloramine volume to be added to reactors, three measurements were executed for each day. Measurements were performed on 1:30 diluted samples due to limitation of DPD kits to 0.02-2.00 mg/l. Volume of stock monochloramine solution to be added was determined and represented in Table 4.3. According to monochloramine concentrations determined, monochloramine and PPCP stock solution mixtures were analyzed after applying required experimental procedures mentioned in Materials and Methods section of this study.

Table 4.3 Stock Monochloramine Measurements for Each Date

Experiment Date	Free Chlorine (mg/l)	Monochloramine (mg/l)	Total Chlorine (mg/l)
June 2nd, 2013	6.3 ± 2.7	58.2 ± 9.3	66 ± 13.5
June 9th, 2013	2.1 ± 0.3	49.2 ± 1.2	57.9 ± 1.2
June 16th, 2013	3.6 ± 2.1	38.1 ± 0.6	40.5 ± 1.2
June 30th, 2013	3.0 ± 1.2	63.6 ± 0.6	68.4 ± 1.2
July 7th, 2013	2.4 ± 0.00	57.3 ± 0.9	63.6 ± 1.5

4.3.2 NDMA Analysis Results for Doxylamine (1-D)

Doxylamine was investigated as a potential for NDMA precursor formation in the experiments conducted on June 2nd, 16th and 30th as indicated in Table 3.1 - Experiment Schedule for GC/MS Measurements. In the Preliminary NDMA Formation Potential Analysis section, the doxylamine concentration was determined as 5 x 10^3 µg/L; therefore, 5 x 10^3 µg/L of initial doxylamine concentration was used for all experiments. In SPE, samples related to doxylamine were concentrated by 100:12. NDMA analyses results for doxylamine experiments are shown in Figure 4.13 through Figure 4.15. These figures demonstrate concentration peaks of NDMA formed due to doxylamine and monochloramine reaction. NDMA peak of the

extracted sample and calibrated NDMA concentration peaks are plotted on the same graph. Areas under NDMA peak were determined by the GC/MS program.

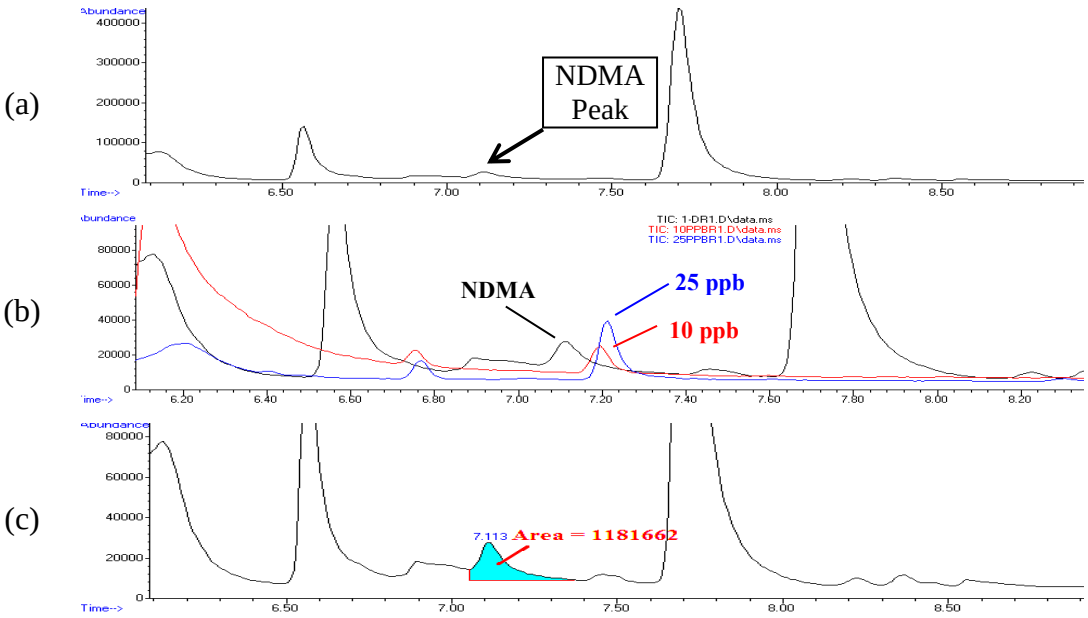


Figure 4.13 Presentation of Doxylamine Experiment Results on June 2nd, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentrations (10 ppb and 25 ppb), (c) Area under NDMA Peak

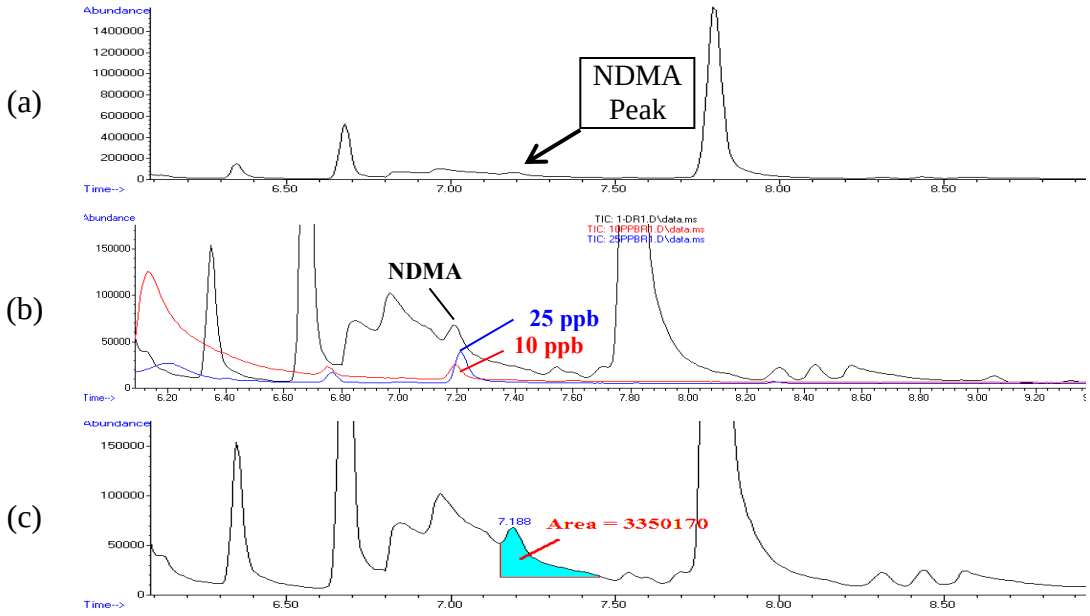


Figure 4.14 Presentation of Doxylamine Experiment Results on June 16th, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentrations (10 ppb and 25 ppb), (c) Area under NDMA Peak

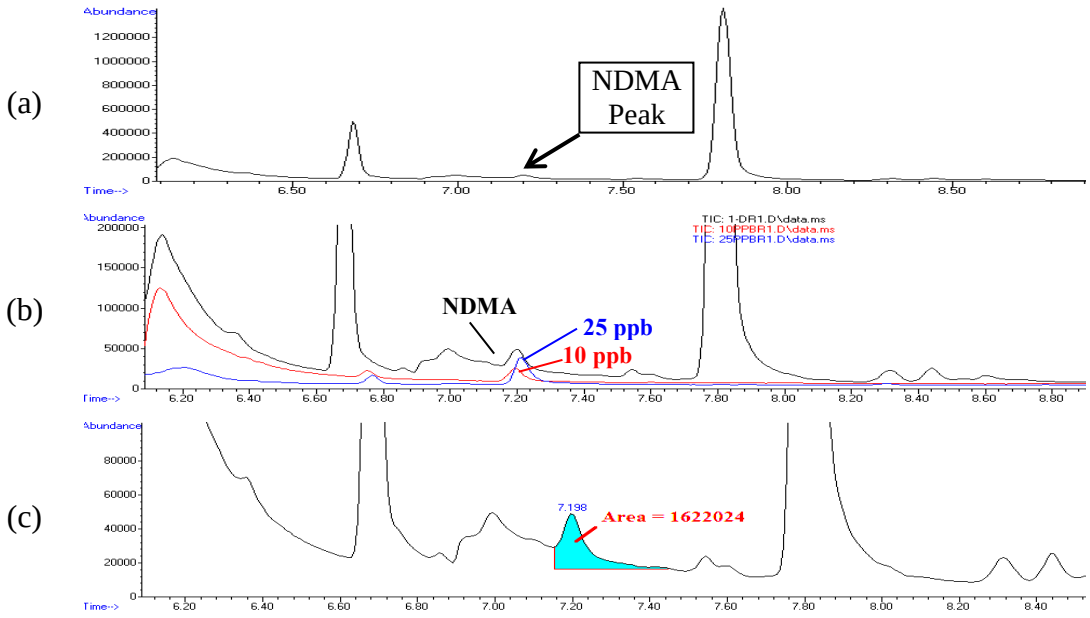


Figure 4.15 Presentation of Doxylamine Experiment Results on June 30th, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentrations (10 ppb and 25 ppb), (c) Area under NDMA Peak

For all experiments, doxylamine concentrations were chosen as 5 x 10^3 µg/L. Free chlorine, monochloramine and total chlorine were measured at t_0 and t_{24} and are summarized in Table 4.4. For three experiments, monochloramine was consumed but it is seen that the reaction is not limited by the availability of monochloramine, in other words, monochloramine amounts were not consumed completely during experiments according to t_{24} measurements. In section 3.4 Quality Assurance/Quality Control, percent recoveries for calibration curve concentrations were calculated. Recovery percent for NDMA in the extracted sample related to doxylamine was calculated by the interpolating between the upper and lower tested concentrations according to NDMA standard curve concentrations. Using recovery percent and concentration ratio, 100:12, the concentration of NDMA formed was calculated. NDMA molar conversion was calculated as the ratio of molar concentration of NDMA formed to molar concentration of spiked PPCP. Results of doxylamine experiments are summarized in Table 4.5. In Figure 4.16, NDMA concentrations formed and molar conversions, doxylamine to NDMA due to reaction with monochloramine are demonstrated in a graph.

Table 4.4 Summary Table of Disinfectant Concentration in Doxylamine Experiment

Experiment Dates	Free Chlorine (mg/l)		Monochloramine (mg/l)		Total Chlorine (mg/l)	
	t_0	t_{24}	t_0	t_{24}	t_0	t_{24}
June 1st, 2013	0.24±0.03	0.03±0.03	1.95±0.23	0.54±0.08	2.18±0.02	0.58±0.04
June 16th, 2013	0.16±0.07	0.05±0.02	1.30±0.02	0.51±0.02	1.32±0.05	0.52±0.03
June 30th, 2013	0.08±0.01	0.05±0.02	2.15±0.02	0.85±0.03	2.32±0.04	0.89±0.03

Table 4.5 Summary Table of NDMA in Doxylamine Experiment

Experiment Dates	Initial Doxylamine Conc. (µg/l)	NDMA Concentration in Extracted Sample (µg/l)	Concentration of NDMA Formed (12:100 of Measurement) (µg/l)	Recovery Percent (%)*	Concentration of NDMA Formed with Recovery Percent (µg/L)	Molar Conversion (%)
June 1st, 2013	5×10^3	16.02	1.92	74.73	3.36	0.24
June 16th, 2013	5×10^3	45.41	5.44	76.11	9.60	0.70
June 30th, 2013	5×10^3	21.98	2.64	73.55	4.58	0.34

* Percent Recovery was calculated with interpolation approach according to NDMA concentration range.

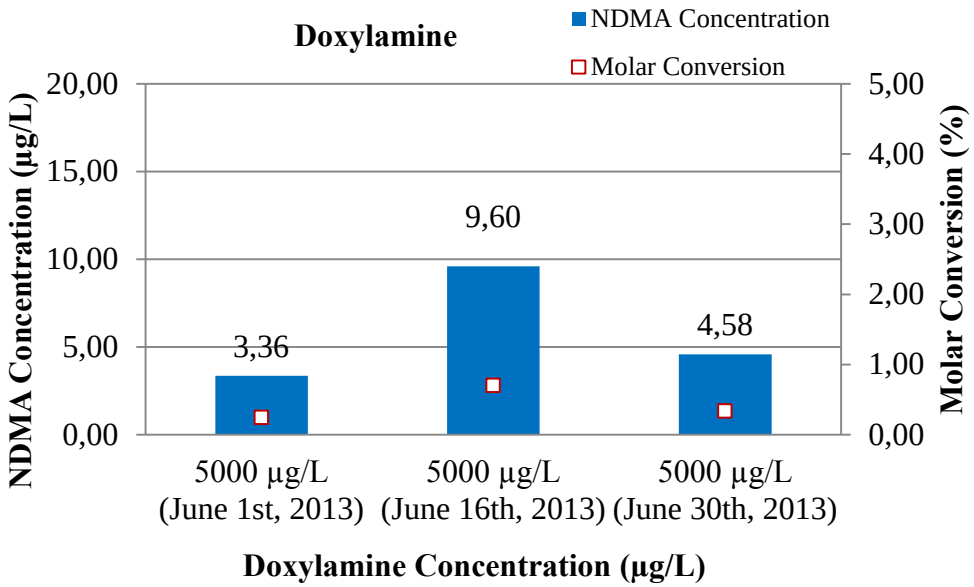


Figure 4.16 NDMA Concentrations Formed and Molar Conversion Related to Doxylamine – Monochloramine Reaction

In a study conducted by Shen and Andrews (2011), NDMA molar conversion in doxylamine experiment was about 10 % in experimental conditions where, chloramine dosage was 2.5 mg/L ± 0.2 mg/l, incubation time was 24 h, temperature was 21 °C and PPCP molar concentration was 25 nM. In this study, NDMA molar conversions in doxylamine experiments were 0.24 %, 0.70 % and 0.34 %. The only difference in these two studies was initial doxylamine molar concentrations. In this study, doxylamine molar concentration was 18.49 µM and in Shen and Andrews' study this value was 25 nM. The reason for differences between molar conversions between these two studies might be due differences initial PPCP concentrations. When analyzed monochloramine concentrations after 24 hours, it is seen that the reactions for doxylamine experiments is not limited by the availability of monochloramine. Therefore, if lower initial doxylamine concentration was used, the molar conversions would be higher. The impact of initial pharmaceutical concentration was examined by Shen and Andrews. For doxylamine, NDMA molar conversion for 5 nM of initial concentration was higher than NDMA molar concentration for 25 nM of initial concentration (Shen and Andrews, 2011).

4.3.3 NDMA Analysis Results for Diltiazem (2-D)

Diltiazem was used for determination of NDMA formation in the experiments conducted on June 2nd, 16th and July 7th as indicated in Table 3.1 - Experiment Schedule for GC/MS Measurements. In section 4.2 Preliminary NDMA Formation Potential Analysis, initial diltiazem concentration of 10^3 µg/L yielded detectable levels of NDMA formation; however, in our experiments, 5 x 10^3 µg/L of initial diltiazem concentration was chosen. In SPE, samples related to diltiazem were concentrated by 100:12. NDMA analyses results for diltiazem experiments are shown in Figure 4.17 through Figure 4.19. These figures demonstrate concentration peaks of NDMA formed due to diltiazem and monochloramine reaction. NDMA peak of the extracted sample and calibrated NDMA concentration peaks are plotted on the same graph. Areas under NDMA peak were determined by the GC/MS program.

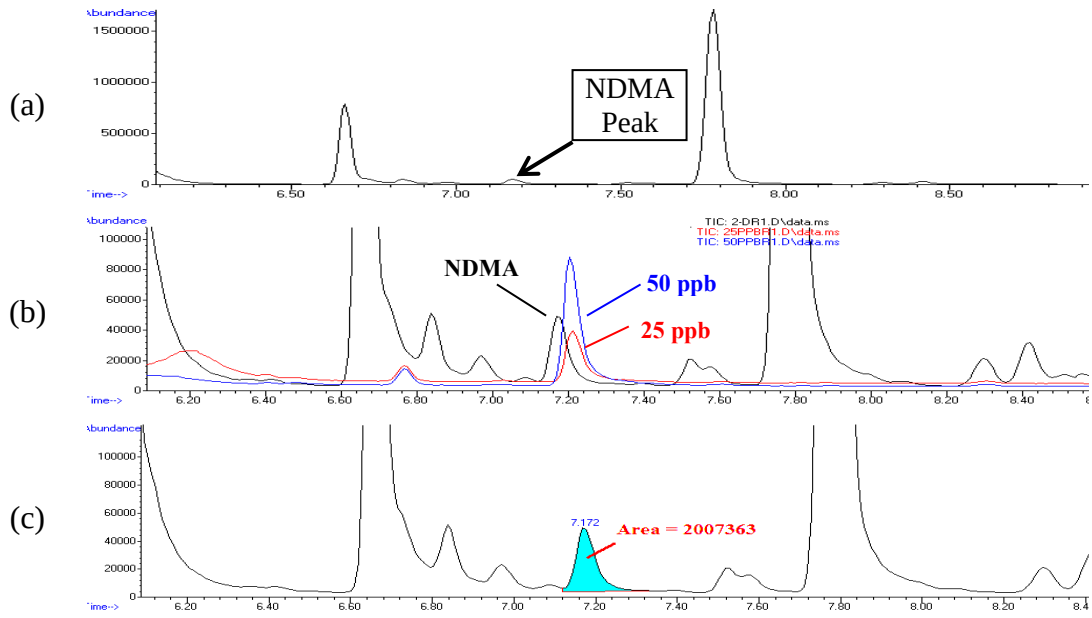


Figure 4.17 Presentation of Diltiazem Experiment Results on June 2nd, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentrations (25 ppb and 50 ppb), (c) Area under NDMA Peak

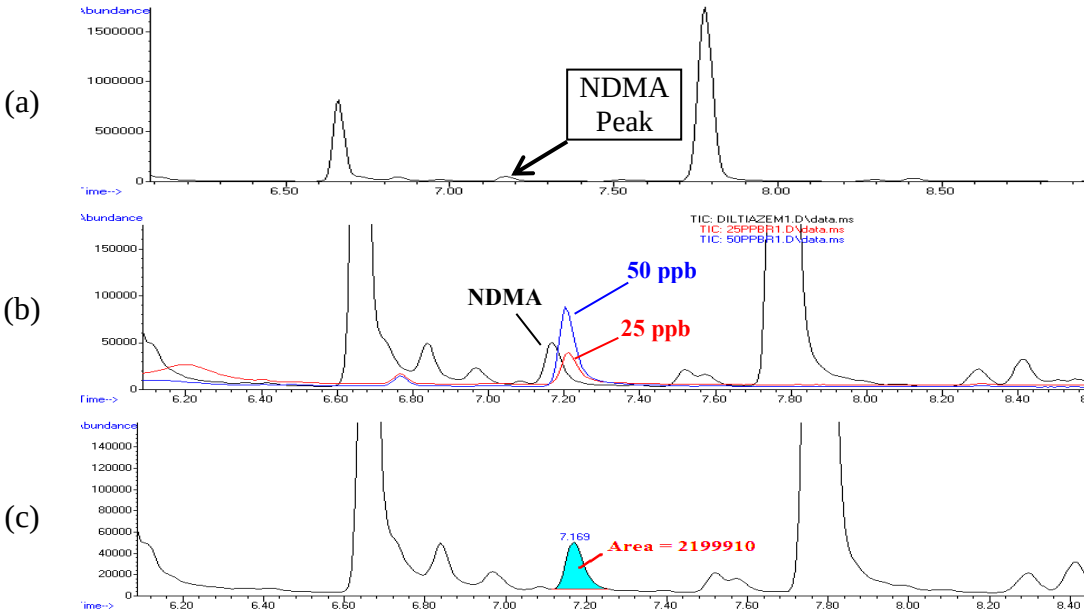


Figure 4.18 Presentation of Diltiazem Experiment Results on June 16th, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentrations (25 ppb and 50 ppb), (c) Area under NDMA Peak

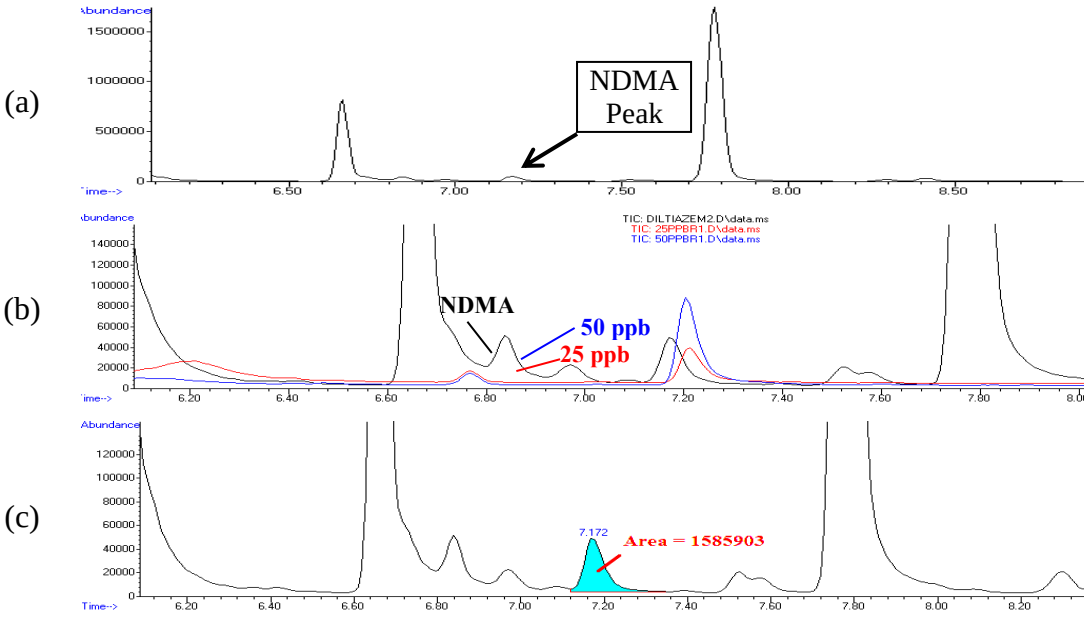


Figure 4.19 Presentation of Diltiazem Experiment Results on July 7th, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentrations (25 ppb and 50 ppb), (c) Area under NDMA Peak

For all experiments, diltiazem concentrations were chosen as 5×10^3 μg/L. Free chlorine, monochloramine and total chlorine were measured at t_0 and t_{24}. Diltiazem results are summarized in Table 4.6. For three experiments, monochloramine amounts were not consumed completely during experiments according to t_{24} measurements. Recovery percent for NDMA in the extracted sample related to diltiazem was calculated by the interpolating between the upper and lower tested concentrations according to NDMA standard curve concentrations. Using recovery percent and concentration ratio, 100:12, concentration of NDMA formed was calculated. NDMA molar conversion was calculated as the ratio of molar concentration of NDMA formed to molar concentration of spiked PPCP. Results of diltiazem experiments are summarized in Table 4.7. In Figure 4.20, NDMA concentrations formed and molar conversions, diltiazem to NDMA due to reactions with monochloramine are shown.

Table 4.6 Summary Table of Disinfectant Concentration in Diltiazem Experiment

Experiment Dates	Free Chlorine (mg/l)		Monochloramine (mg/l)		Total Chlorine (mg/l)	
	t_0	t_{24}	t_0	t_{24}	t_0	t_{24}
June 2nd, 2013	0.22±0.09	0.04±0.01	2.01±0.11	1.01±0.03	2.75±0.20	1.09±0.03
June 16th, 2013	0.17±0.06	0.04±0.01	1.29±0.02	0.46±0.04	1.36±0.04	0.48±0.04
July 7th, 2013	0.08±0.01	0.06±0.01	1.88±0.03	0.48±0.03	2.15±0.05	0.49±0.03

Table 4.7 Summary Table of NDMA in Diltiazem Experiment

Experiment Dates	Initial Diltiazem Conc. (µg/l)	NDMA Concentration in Extracted Sample (µg/l)	Concentration of NDMA Formed (12:100 of Measurement) (µg/l)	Recovery Percent (%)*	Concentration of NDMA Formed with Recovery Percent (µg/L)	Molar Conversion (%)
June 2nd, 2013	5×10^3	27.21	3.27	73.93	5.68	0.64
June 16th, 2013	5×10^3	29.82	3.58	74.25	6.24	0.70
July 7th, 2013	5×10^3	21.49	2.59	73.55	4.48	0.50

* Percent Recovery was calculated with interpolation approach according to NDMA concentration range.

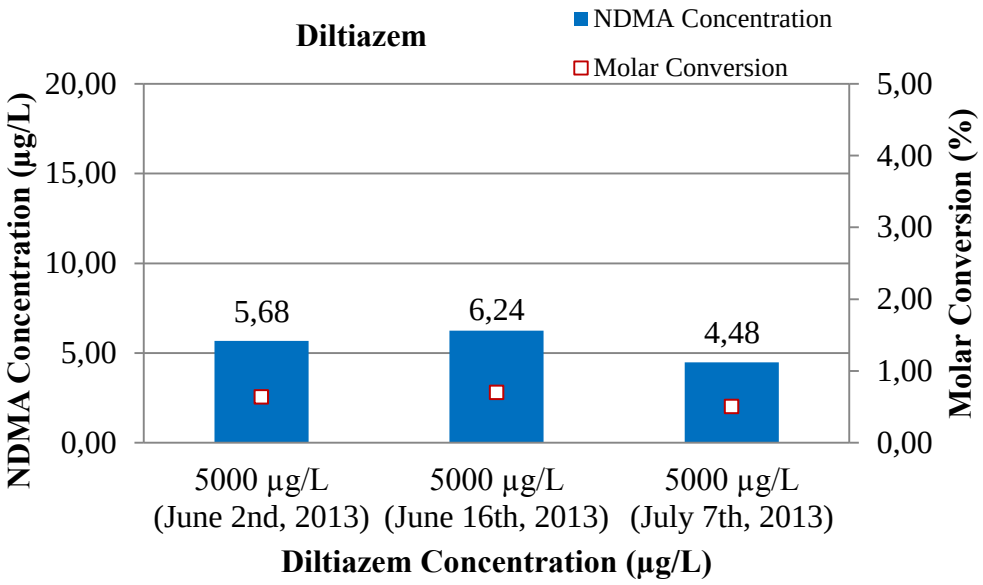


Figure 4.20 NDMA Concentrations and Molar Conversion Related to Diltiazem – Monochloramine Reaction

In a study conducted by Shen and Andrews (2011), NDMA molar conversion in diltiazem experiment was about 1.5 % in experimental conditions where, chloramine dosage was 2.5 mg/L ± 0.2 mg/l, incubation time was 24 hr, temperature was 21 °C and PPCP molar concentration was 25 nM. In this study, NDMA molar conversions in diltiazem experiments were 0.64 %, 0.70 % and 0.50 %. The only difference in these two studies was initial diltiazem molar concentrations. In this study, diltiazem molar concentration was 12.06 µM and in the mentioned study this value was 25 nM. The reason for differences between molar conversions between these two studies might be the differences in initial PPCP concentrations. When analyzed monochloramine concentrations after 24 hours, it is seen that the reactions for diltiazem experiments is not limited by the availability of monochloramine. Therefore, if lower initial diltiazem concentration was used, the molar conversions would be higher.

4.3.4 NDMA Analysis Results for Sumatriptan (3-D)

Sumatriptan was used for determination of NDMA formation in the experiments performed on June 2nd, 30th and July 7th as indicated in Table 3.1 - Experiment Schedule for GC/MS Measurements. In section 4.2 Preliminary NDMA Formation Potential Analysis, sumatriptan concentration was determined as 10^3 µg/L; however, for analyses, 5 x 10^3 µg/L and 10^4 of initial sumatriptan concentrations were chosen. In SPE, samples related to sumatriptan were concentrated by 100:12. NDMA analyses results for sumatriptan experiments are shown in Figure 4.21 through Figure 4.23. These figures demonstrate concentration peaks of NDMA formed due to sumatriptan and monochloramine reaction. NDMA peak of the extracted sample and calibrated NDMA concentration peaks are plotted on the same graph. Areas under NDMA peak were determined using the GC/MS program.

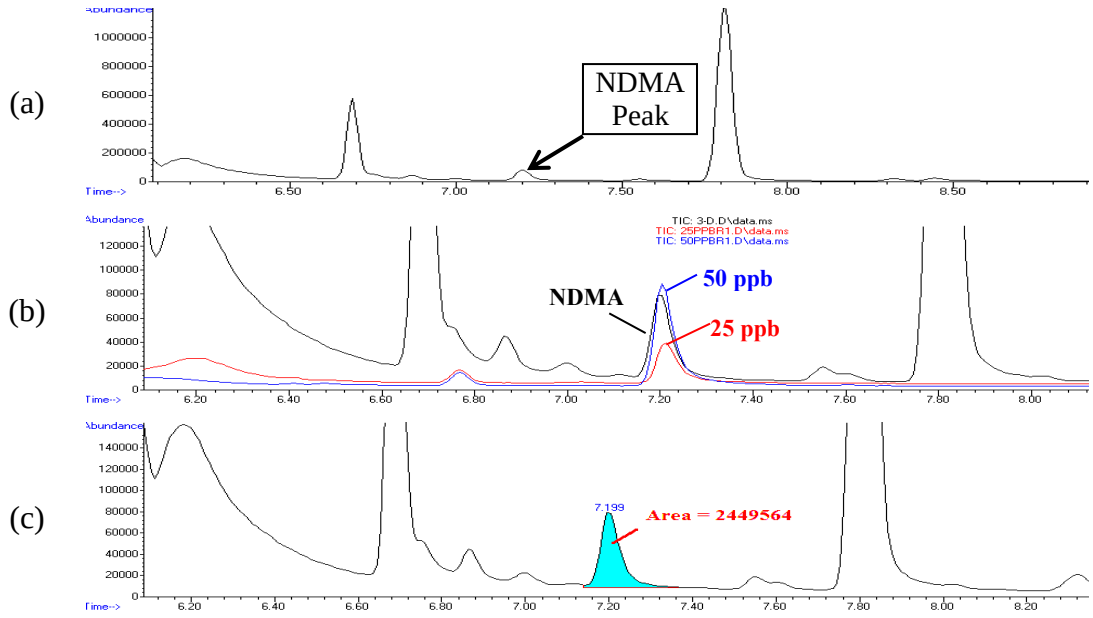


Figure 4.21 Presentation of Sumatriptan Experiment Results on June 2nd, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentrations (25 ppb and 50 ppb), (c) Area under NDMA Peak

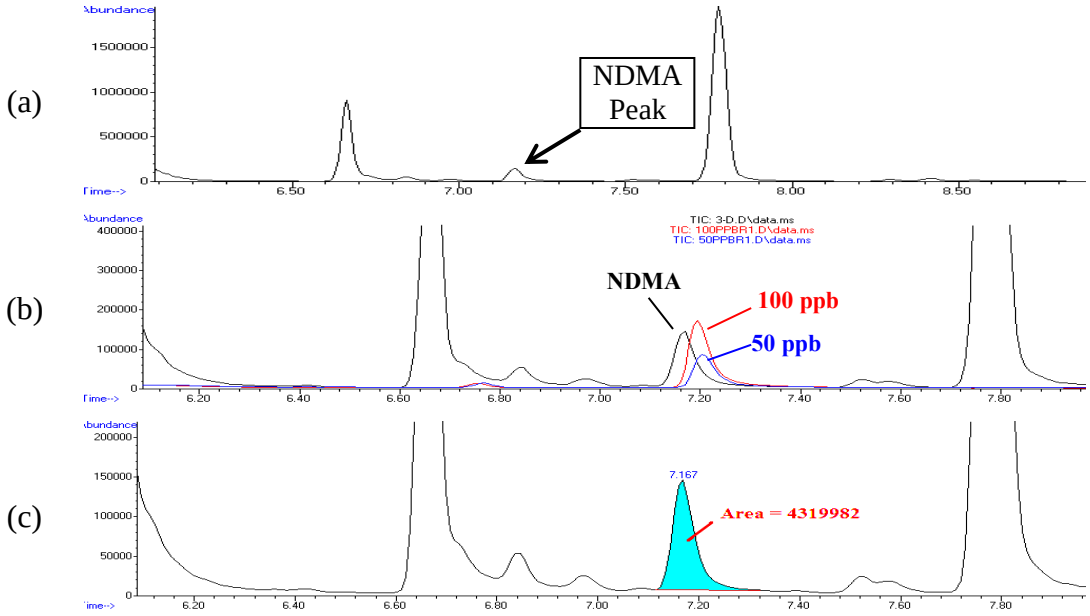


Figure 4.22 Presentation of Sumatriptan Experiment Results on June 30th, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentrations (50 ppb and 100 ppb), (c) Area under NDMA Peak

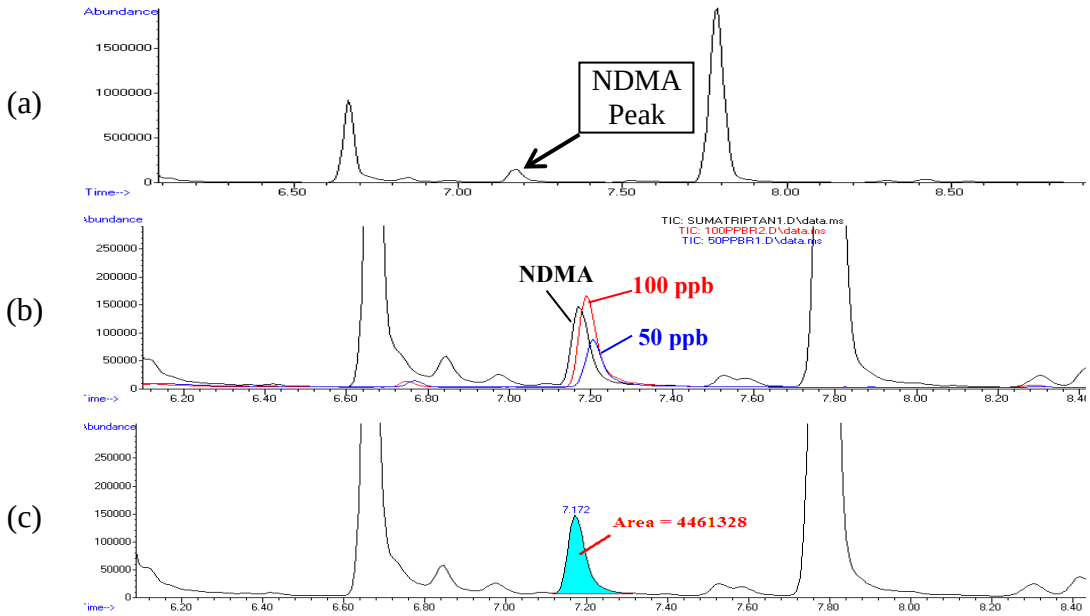


Figure 4.23 Presentation of Sumatriptan Experiment Results on July 7th, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentrations (50 ppb and 100 ppb), (c) Area under NDMA Peak

Sumatriptan concentrations were chosen as 5 x 10^3 µg/L in the experiments conducted on June 2nd and 10^4 µg/L in the experiment conducted on June 30th and July 7th. Free chlorine, monochloramine and total chlorine were measured at t_0 and t_{24}. Sumatriptan results are summarized in Table 4.8. For three experiments, monochloramine amounts were not consumed completely during experiments according to t_{24} measurements. Recovery percent for NDMA in the extracted sample related to sumatriptan was calculated by the interpolating between the upper and lower tested concentrations according to NDMA standard curve concentrations. Using recovery percent and concentration ratio, 100:12, concentration of NDMA formed was calculated. NDMA molar conversion was calculated as the ratio of molar concentration of NDMA formed to molar concentration of spiked PPCP. Results of sumatriptan experiments are summarized in Table 4.9. In Figure 4.24, NDMA concentrations formed and molar conversions, sumatriptan to NDMA due to reactions with monochloramine are shown.

Table 4.8 Summary Table of Disinfectant Concentration in Sumatriptan Experiment

Experiment Dates	Free Chlorine (mg/l)		Monochloramine (mg/l)		Total Chlorine (mg/l)	
	t_0	t_{24}	t_0	t_{24}	t_0	t_{24}
June 2nd, 2013	0.24±0.09	0.02±0.01	1.96±0.21	0.39±0.02	2.32±0.20	0.40±0.01
June 30th, 2013	0.10±0.05	0.08±0.00	2.18±0.09	0.91±0.03	2.26±0.04	0.96±0.01
July 7th, 2013	0.09±0.03	0.08±0.01	1.92±0.03	0.61±0.02	2.16±0.01	0.65±0.01

Table 4.9 Summary Table of NDMA in Sumatriptan Experiment

Experiment Dates	Initial Sumatriptan Conc. (µg/l)	NDMA Concentration in Extracted Sample (µg/l)	Concentration of NDMA Formed (12:100 of Measurement) (µg/l)	Recovery Percent (%)*	Concentration of NDMA Formed with Recovery Percent (µg/L)	Molar Conversion (%)
June 2nd, 2013	5×10^3	33.20	3.98	74.65	6.96	0.55
June 30th, 2013	10^4	58.56	7.02	77.70	12.49	0.50
July 7th, 2013	10^4	60.47	7.26	77.94	12.91	0.51

* Percent Recovery was calculated with interpolation approach according to NDMA concentration range.

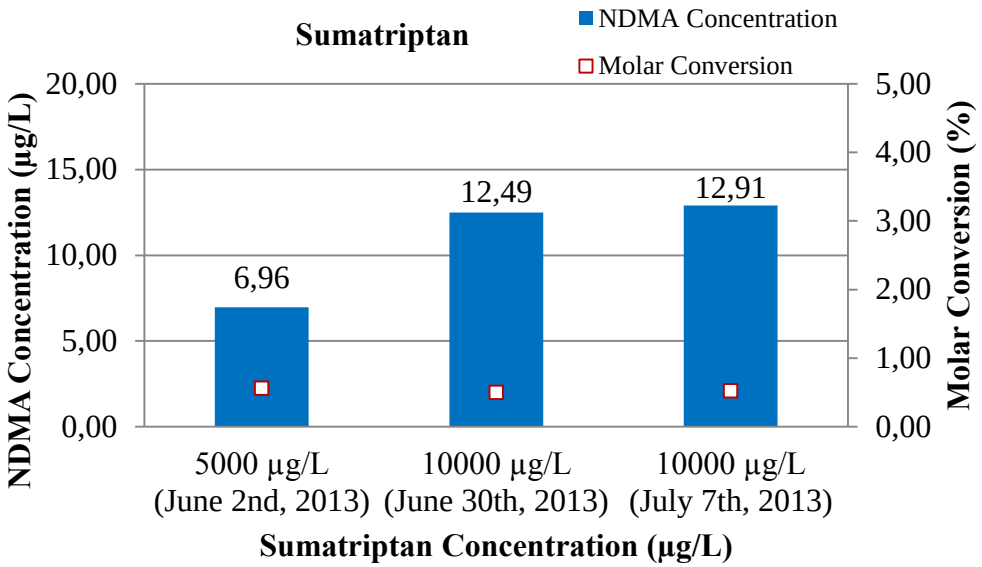


Figure 4.24 NDMA Concentrations and Molar Conversion Related to Sumatriptan – Monochloramine Reaction

In a study conducted by Shen and Andrews (2011), NDMA molar conversion in sumatriptan experiment was about 2 % in experimental conditions where, chloramine dosage was 2.5 mg/L ± 0.2 mg/l, incubation time was 24 hr, temperature was 21 °C and PPCP molar concentration was 25 nM. In this study, NDMA molar conversions in sumatriptan experiments were 0.55 %, 0.50 % and 0.51 %. The only difference in these two studies was initial sumatriptan molar concentrations. In this study, doxylamine molar concentration was 16.93 µM in the experiment conducted on June 2nd and 33.85 µM in the experiment conducted on June 30th and July 7th and in the above mentioned study this value was 25 nM. The reason for differences between molar conversions between these two studies is might be the initial PPCP concentrations. When analyzed monochloramine concentrations analyzed after 24 hours, it is seen that the reactions for sumatriptan experiments is not limited by the availability of monochloramine. Therefore, if lower initial sumatriptan concentration was used, the molar conversions would be higher.

4.3.5 NDMA Analysis Results for Ranitidine (4-D)

Ranitidine was used for determination of NDMA formation in the experiments conducted on June 9th, June 30th and July 7th as indicated in Table 3.1 - Experiment Schedule for GC/MS Measurements. In section 4.2 Preliminary NDMA Formation Potential Analysis, it was seen that NDMA peak resulting from 10^3 µg/l ranitidine – monochloramine reaction is significantly higher than 500 ppb. Therefore, initial ranitidine concentrations were chosen as 5 x 10^2 µg/L for the experiment dates of June 9th and July 7th and 10^2 µg/L for the experiment date of June 30th. In SPE, samples related to ranitidine were concentrated by 100:12. NDMA analyses results for ranitidine experiments are shown in Figure 4.25 through Figure 4.27. These figures demonstrate concentration peaks of NDMA formed due to the reaction with ranitidine and monochloramine. NDMA peak of the extracted sample and calibrated NDMA concentration peaks are plotted on the same graph. Areas under NDMA peak were determined using the GC/MS program.

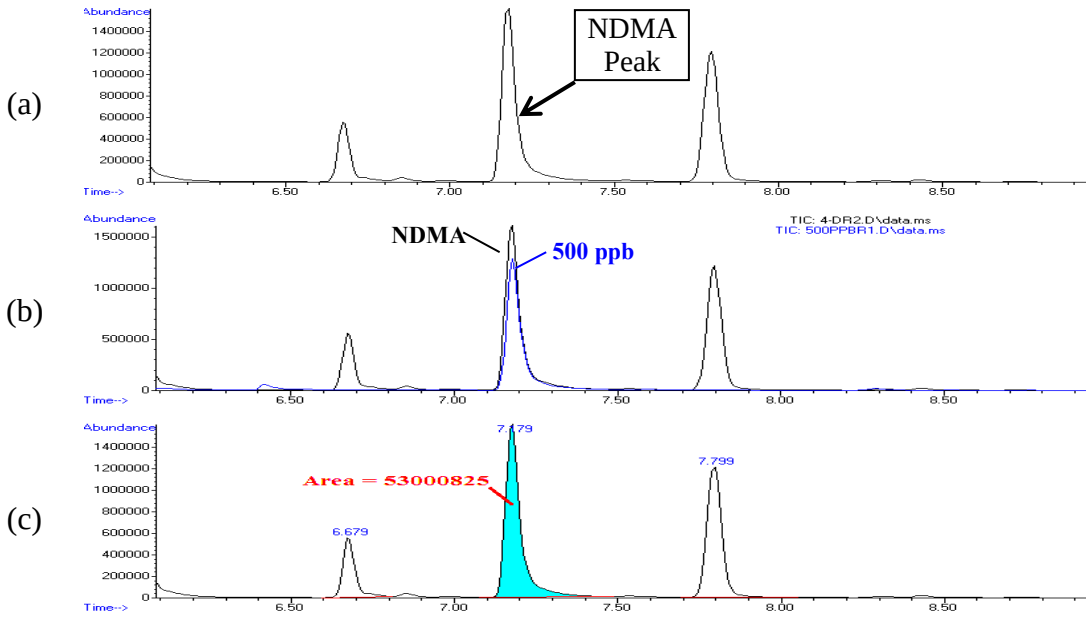


Figure 4.25 Presentation of Ranitidine Experiment Results on June 9th, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentration (500 ppb), (c) Area under NDMA Peak

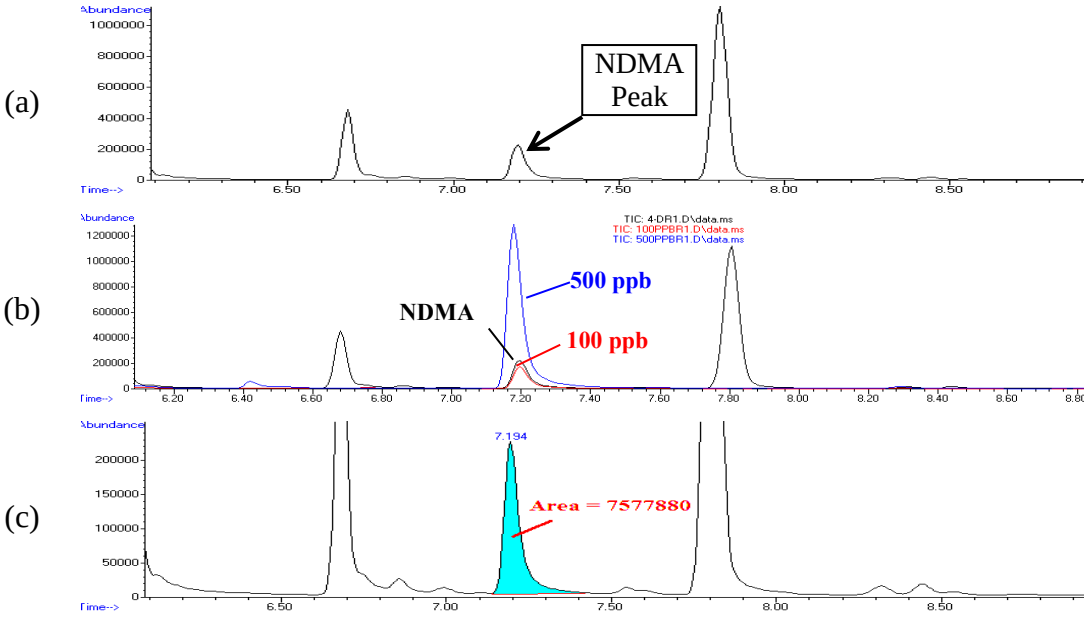


Figure 4.26 Presentation of Ranitidine Experiment Results on June 30th, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentrations (100 ppb and 500 ppb), (c) Area under NDMA Peak

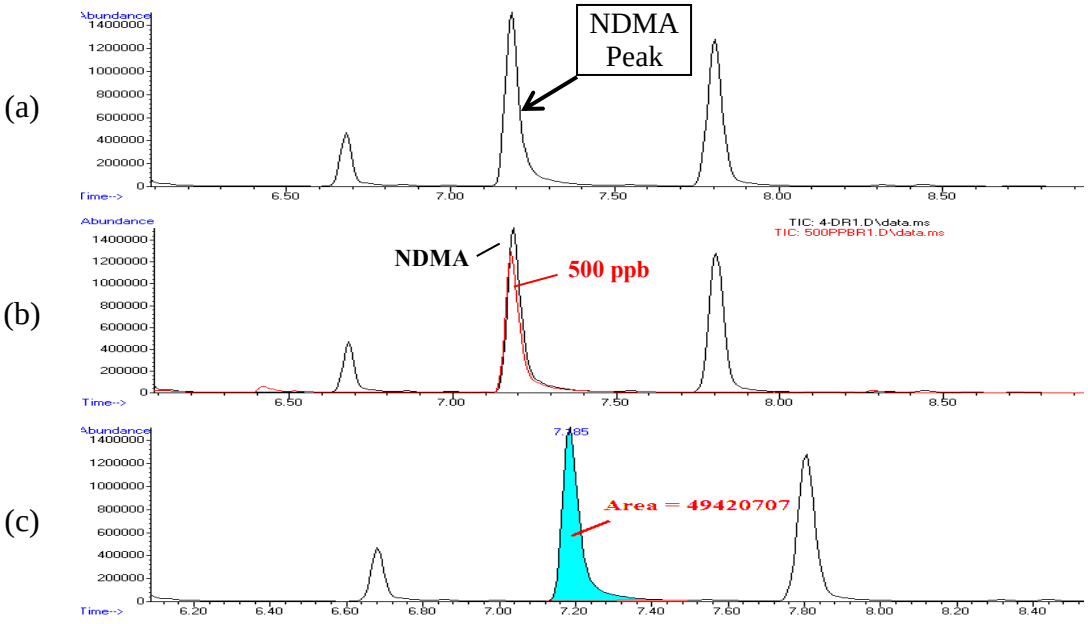


Figure 4.27 Presentation of Ranitidine Experiment Results on July 7th, 2013 (a) NDMA Concentration Peak, (b) NDMA Peak and Calibrated Concentration (500 ppb), (c) Area under NDMA Peak

Ranitidine concentrations were chosen as 5 x 10^2 μg/L in the experiments conducted on June 9th and July 7th and 10^2 μg/L in the experiment conducted on June 30th. Free chlorine, monochloramine and total chlorine were measured at t_0 and t_{24}. Ranitidine related results according to experiment dates are summarized in Table 4.10. For three experiments, monochloramine amounts were not consumed completely during experiments according to t_{24} measurements. Recovery percent for NDMA in the extracted sample related to ranitidine was calculated by the interpolating between the upper and lower tested concentrations according to NDMA standard curve concentrations. Using recovery percent and concentration ratio, 100:12, concentration of NDMA formed was calculated. NDMA molar conversion was calculated as the ratio of molar concentration of NDMA formed to molar concentration of spiked PPCP. Results of ranitidine experiments are summarized in Table 4.11. In Figure 4.28, NDMA concentrations formed and molar conversions, ranitidine to NDMA due to reactions with monochloramine are shown.

Table 4.10 Summary Table of Disinfectant Concentration in Ranitidine Experiment

Experiment Dates	Free Chlorine (mg/l)		Monochloramine (mg/l)		Total Chlorine (mg/l)	
	t_0	t_{24}	t_0	t_{24}	t_0	t_{24}
June 9th, 2013	0.07±0.01	0.03±0.01	1.65±0.04	0.67±0.02	1.96±0.04	0.68±0.03
June 30th, 2013	0.09±0.01	0.05±0.02	2.15±0.02	0.88±0.03	2.30±0.03	0.94±0.01
July 7th, 2013	0.08±0.02	0.05±0.02	1.89±0.04	0.59±0.03	2.11±0.03	0.64±0.02

Table 4.11 Summary Table of NDMA in Ranitidine Experiment

Experiment Dates	Initial Ranitidine Conc. (µg/l)	NDMA Concentration in Extracted Sample (µg/l)	Concentration of NDMA Formed (12:100 of Measurement) (µg/l)	Recovery Percent (%)*	Concentration of NDMA Formed with Recovery Percent (µg/L)	Molar Conversion (%)
June 9th, 2013	5 x 10^2	708.61	85.03	70.00**	144.56	136.40
June 30th, 2013	10^2	102.72	12.33	82.80	22.53	104.80
July 7th, 2013	5 x 10^2	669.00	80.28	70.00**	136.48	128.80

* Percent Recovery was calculated with interpolation approach according to NDMA concentration range.
** NDMA Concentrations for the experiment dates on June 9th and July 7th were higher than the highest calibration curve concentration (500 ppb); therefore, the recovery percent was assumed to be approximately 70%.

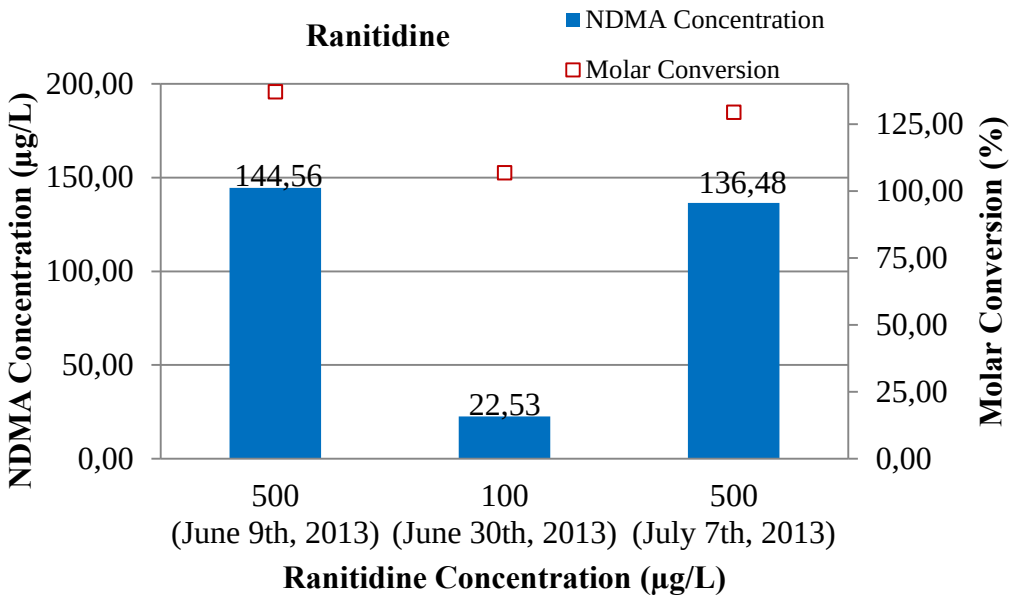


Figure 4.28 NDMA Concentrations and Molar Conversion Related to Ranitidine–Monochloramine Reactions

In a study conducted by Shen and Andrews (2011), NDMA molar conversion in ranitidine experiment was about 85 % in experimental conditions where, chloramine dosage was 2.5 mg/L ± 0.2 mg/l, incubation time was 24 hr, temperature was 21 °C and PPCP molar concentration was 25 nM. In this study, NDMA molar conversions in ranitidine experiments were 136.4 %, 104.8 % and 128.8 %. The reason of higher than 100 % molar conversion of NDMA is that during monochloramine and ranitidine reaction, ranitidine was chemically degraded in more than one point and two different NDMA precursors were formed. The only difference in these two studies was initial ranitidine molar concentrations. In this study, ranitidine molar concentration was 1.43 µM for the experiment on June 9th and July 7th and 0.29 µM for the experiment on June 30th and in Shen and Andrews' study this value was 25 nM. The reason for differences between molar conversions between these two studies is initial PPCP concentrations. When the impact of initial pharmaceutical concentration was examined, for ranitidine, NDMA molar conversion for 5 nM of initial concentration was approximately equal to NDMA molar concentration for 25 nM of initial concentration.

4.3.6 NDMA Analysis Results for Caffeine (5-D)

NDMA formation potential of caffeine was investigated in the experiments conducted on June 9th, June 30th and July 7th as indicated in Table 3.1 - Experiment Schedule for GC/MS Measurements. In section 4.2 Preliminary NDMA Formation Potential Analysis, no NDMA peak was observed at 10^3 µg/l and 10^6 µg/l caffeine – monochloramine reaction. Three further tests were performed where initial caffeine concentration was set to 5 x 10^4 µg/l, due to available stock chemical. It should be noted that the NDMA readings for caffeine in GC/MS for extracted samples are concentrated at a ratio of 100:12 during solid phase extraction process in preliminary NDMA formation potential analysis experiments; however, NDMA concentrations were observed under the detection limits. Therefore, samples were further concentrated by 100:1 during the SPE process. NDMA analyses results for caffeine monochloramine reactions are shown in Figure 4.29.

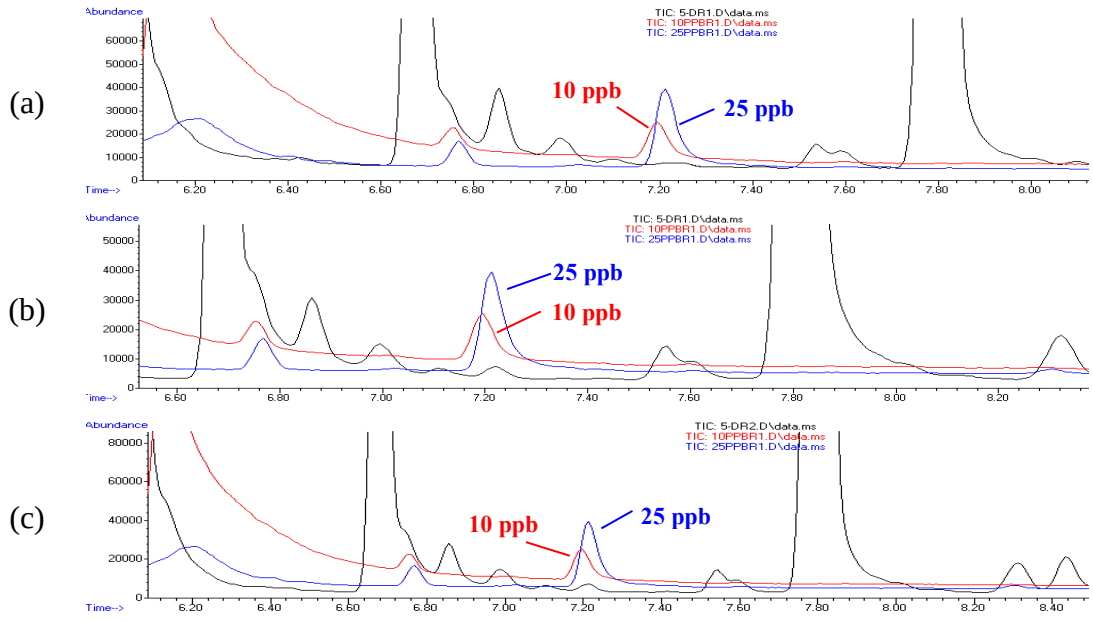


Figure 4.29 Presentation of NDMA out of the range of the calibration curve due to Caffeine Experiment Results on (a) June 9th, (b) June 30th, (c) July 7th, 2013

Caffeine concentrations were chosen as 5 x 10^4 µg/L for all experiments. Free chlorine, monochloramine and total chlorine were measured at t_0 and t_{24}. Caffeine experiment results are summarized in Table 4.12. As seen in Figure 4.37, some peaks

under 10 ppb were observed. These peaks were not detected as NDMA in GC/MS program. However, with manual integration, the areas under these peaks were calculated and concentrations were found based on calibration curve for the range of 10 ppb – 500 ppb. The estimated concentrations are given in Table 4.12 as peak concentration. The concentrations of NDMA formed due to reaction of 5 x 10^4 µg/L caffeine with monochloramine were estimated to be in the range of 1.1 to 1.3 µg/L noted that the estimated concentration are out of the range of the calibration curve.

Table 4.12 Summary Table of Caffeine Related Measurements

Experiment Dates	Chemical Conc. (µg/l)		Free Chlorine (mg/l)	Monochloramine (mg/l)	Total Chlorine (mg/l)	Peak Conc. (µg/l)
June 9th, 2013	5 x 10^4	t_0	0.06±0.01	1.71±0.04	1.94±0.03	1.30
		t_{24}	0.04±0.02	1.12±0.01	1.14±0.04	
June 30th, 2013	5 x 10^4	t_0	0.09±0.01	2.16±0.02	2.24±0.03	1.14
		t_{24}	0.06±0.01	0.98±0.01	1.01±0.04	
July 7th, 2013	5 x 10^4	t_0	0.08±0.02	1.94±0.03	2.17±0.03	1.17
		t_{24}	0.07±0.01	1.05±0.02	1.09±0.04	

Our literature search on NDMA formation potential of caffeine failed to find any previous studies. There are no previous studies that have investigated the NDMA formation due to reaction with monochloramine and caffeine to the best of author's knowledge. The concentrations of NDMA formed due to reaction of caffeine and monochloramine cannot be confirmed since it was out of the calibrated range, but it can be confirmed that caffeine is an NDMA precursor for chloramination.

4.3.7 NDMA Analysis Results for Diclofenac (6-D)

NDMA formation potential of diclofenac was investigated in the experiments conducted on June 9th, June 30th and July 7th as indicated in Table 3.1 - Experiment Schedule for GC/MS Measurements. In section 4.2 Preliminary NDMA Formation Analysis, no NDMA peak was observed resulting from 10^3 µg/l and 10^6 µg/l diclofenac – monochloramine reaction. Three further tests were performed where initial diclofenac concentration was 10^4 µg/l. It should be noted that the NDMA readings for diclofenac in GC/MS for extracted samples are concentrated at a ratio of

100:12 during solid phase extraction process in preliminary NDMA formation potential analysis experiments; however, NDMA concentrations were observed under the detection limits. Therefore, samples were further concentrated by 100:1 during the SPE process. NDMA analyses results for diclofenac – monochloramine reactions are shown in Figure 4.30.

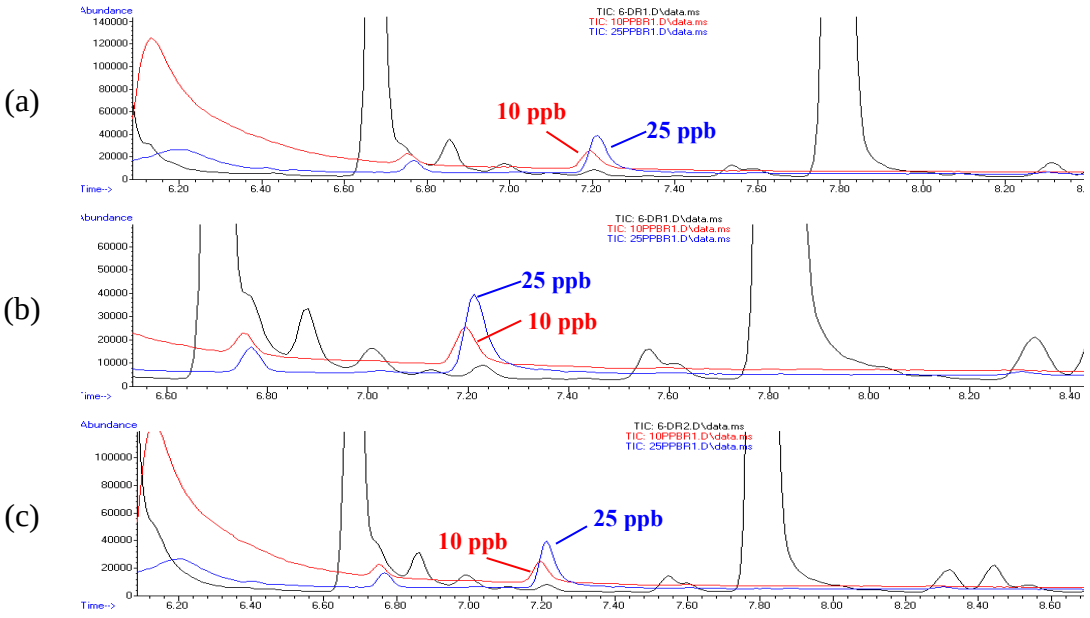


Figure 4.30 Presentation of NDMA out of the range of the calibration curve due to Diclofenac Experiment Results on (a) June 9th, (b) June 30th, (c) July 7th, 2013

Diclofenac concentrations were chosen as 10^5 µg/L for all experiments. Free chlorine, monochloramine and total chlorine were measured at t_0 and t_{24}. Diclofenac experiment results are summarized in Table 4.13. As seen in Figure 4.30, some peaks under 10 ppb were observed. These peaks were not detected as NDMA in GC/MS program. However, with manual integration, the areas under these peaks were calculated and concentrations were found based on calibration curve for the range of 10 ppb – 500 ppb. The estimated concentrations are given in Table 4.13 as peak concentration. The concentrations of NDMA formed due to reaction of 10^5 µg/L diclofenac with monochloramine were estimated to be in the range of 1.8 to 2.0 µg/L noted that the estimated concentrations are out of the range of calibration curve.

Table 4.13 Summary Table of Diclofenac Related Measurements

Experiment Dates	Chemical Conc. (µg/l)		Free Chlorine (mg/l)	Monochloramine (mg/l)	Total Chlorine (mg/l)	Peak Conc. (µg/l)
June 9th, 2013	10^5	t_0	0.06±0.01	1.62±0.03	1.94±0.06	1.94
		t_{24}	0.03±0.00	1.20±0.04	1.20±0.03	
June 30th, 2013	10^5	t_0	0.12±0.01	2.17±0.04	2.28±0.04	2.06
		t_{24}	0.09±0.00	1.28±0.03	1.30±0.04	
July 7th, 2013	10^5	t_0	0.09±0.01	2.06±0.04	2.21±0.06	1.84
		t_{24}	0.09±0.00	1.18±0.02	1.22±0.04	

Our literature search on NDMA formation potential of Diclofenac failed to find any previous studies. There are no previous studies that have investigated the NDMA formation due to reaction with monochloramine and diclofenac to the best of author's knowledge. The concentration of NDMA formed due to reaction of diclofenac and monochloramine cannot be confirmed since it was out of the calibrated range, but it can be confirmed that diclofenac is an NDMA precursor for chloramination.

4.3.8 NDMA Analysis Results for Atrazine (7-D)

NDMA formation potential of atrazine was investigated in the experiments performed on June 16th, June 30th and July 7th as indicated in Table 3.1 - Experiment Schedule for GC/MS Measurements. In section 4.2 Preliminary NDMA Formation Potential Analysis, no NDMA peak was observed resulting from 10^3 µg/l and 10^6 µg/l atrazine – monochloramine reaction. Three further tests were performed where initial atrazine concentrations were set to 10^4 µg/L for the experiment date on June 16th and 10^5 µg/L for the experiment dates on June 30th and July 7th. It should be noted that the NDMA readings for atrazine in GC/MS for extracted samples are concentrated at a ratio of 100:12 during solid phase extraction process in preliminary NDMA formation potential analysis experiments; however, NDMA concentrations were observed under the detection limits. Therefore, samples were further concentrated by 100:1 during the SPE process. NDMA analyses results for atrazine – monochloramine reactions are shown in Figure 4.31.

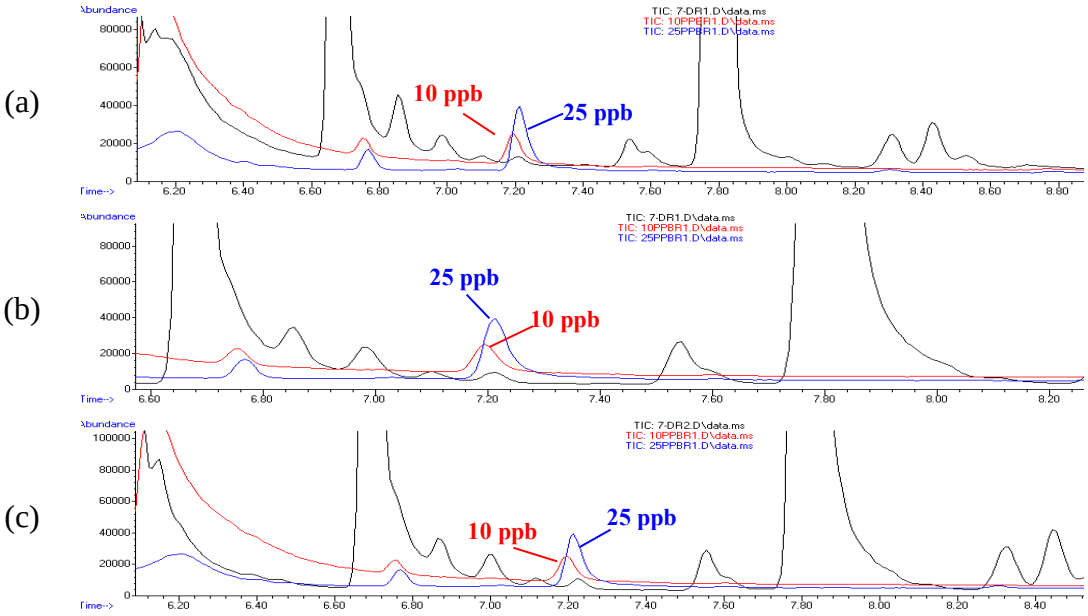


Figure 4.31 Presentation of NDMA out of the range of the calibration curve due to Atrazine Experiment Results on (a) June 16th, (b) June 30th, (c) July 7th, 2013

Free chlorine, monochloramine and total chlorine were measured at t_0 and t_{24}. Atrazine experiment results are summarized in Table 4.14. As seen in Figure 4.31, some peaks under 10 ppb were observed. These peaks were not detected as NDMA in GC/MS program. However, with manual integration, the areas under these peaks were calculated and concentrations were found based on calibration curve for the range of 10 ppb -500 ppb. The estimated concentrations are given in Table 4.14 as peak concentration. The concentrations of NDMA formed due to reaction of 10^4 and 10^5 µg/L atrazine with monochloramine were estimated to be in the range of 1.4 to 2.3 µg/L noted that the estimated concentrations are out of the range of calibration curve.

Table 4.14 Summary Table of Atrazine Related Measurements

Experiment Dates	Chemical Conc. (µg/l)		Free Chlorine (mg/l)	Monochloramine (mg/l)	Total Chlorine (mg/l)	Peak Conc. (µg/l)
June 16th, 2013	10^4	t_0	0.08±0.07	1.29±0.03	1.37±0.04	1.43
		t_{24}	0.04±0.07	1.01±0.03	1.07±0.04	
June 30th, 2013	10^5	t_0	0.08±0.07	2.15±0.04	2.29±0.07	2.34
		t_{24}	0.07±0.01	1.21±0.04	1.22±0.06	
July 7th, 2013	10^5	t_0	0.07±0.07	2.01±0.02	2.15±0.05	1.82
		t_{24}	0.06±0.01	1.16±0.02	1.20±0.06	

Our literature search on NDMA formation potential of Atrazine failed to find any previous studies. There are no previous studies that have investigated the NDMA formation due to reaction with monochloramine and atrazine to the best of author's knowledge. The concentrations of NDMA formed due to atrazine and monochloramine cannot be confirmed since it was out of the calibrated range, but it can be confirmed that atrazine is an NDMA precursor for chloramination.

4.3.9 NDMA Analysis Results for Sulfamethoxazole (8-D)

NDMA formation potential of sulfamethoxazole was investigated in the experiments performed on June 16th, 30th and July 7th as indicated in Table 3.1 - Experiment Schedule for GC/MS Measurements. In section 4.2 Preliminary NDMA Formation Potential Analysis, no NDMA peak was observed resulting from 10^3 µg/l and 10^6 µg/l sulfamethoxazole – monochloramine reaction. Three further tests were performed where initial sulfamethoxazole were 10^3 µg/L for the experiment date on June 16th, 5 x 10^4 µg/L for the experiment date on June 30th and July 7th. It should be noted that the NDMA readings for sulfamethoxazole in GC/MS for extracted samples are concentrated at a ratio of 100:12 during solid phase extraction process in preliminary NDMA formation potential analysis experiments; however, NDMA concentrations were observed under the detection limits. Therefore, samples were further concentrated by 100:1 during the SPE process. NDMA analyses results for atrazine – monochloramine reactions are shown in Figure 4.32.

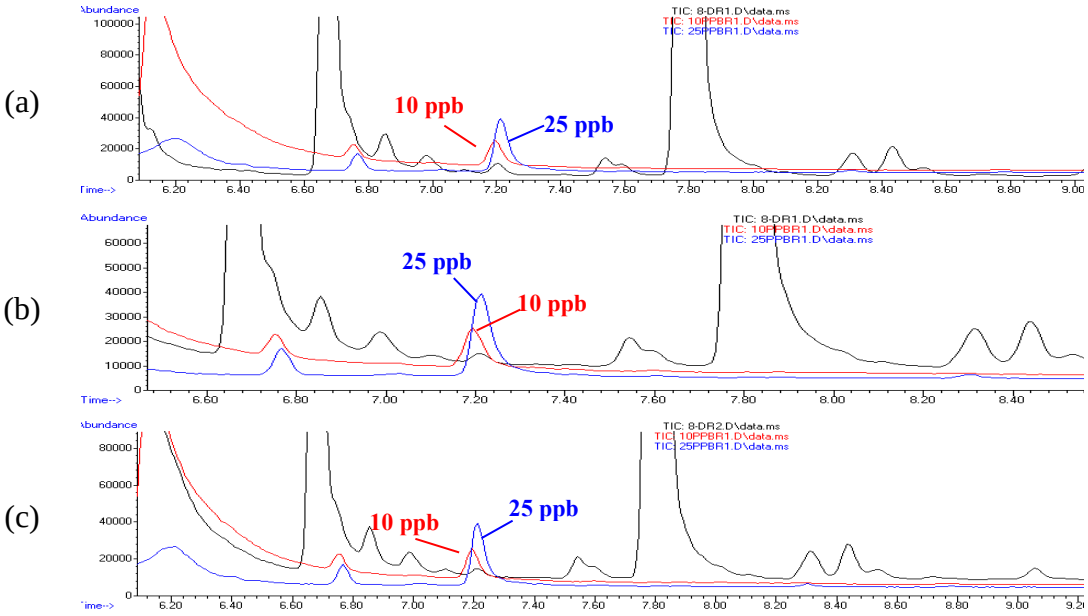


Figure 4.32 Presentation of NDMA out of the range of the calibration curve due to Sulfamethoxazole Experiment Results on (a) June 16th, (b) June 30th, (c) July 7th, 2013

Free chlorine, monochloramine and total chlorine were measured at t_0 and t_{24}. Sulfamethoxazole experiment results are summarized in Table 4.15. In all three experiments, some amounts of monochloramine were consumed during experiments according to t_{24} measurements. As seen in Figure 4.32, some peaks under 10 ppb were observed. These peaks were not detected as NDMA in GC/MS program. However, with manual integration, the areas under these peaks were calculated and concentrations were found based on calibration curve for the range of 10 ppb – 500 ppb. The estimated concentrations are given in Table 4.15 as peak concentration. The concentration of NDMA formed due to reaction of 10^3 and 5 x 10^4 µg/L sulfamethoxazole with monochloramine was estimated to be in the range of 1.8 to 3.1 µg/L noted that the estimated concentrations are out of the range of calibration curve.

Table 4.15 Summary Table of Sulfamethoxazole Related Measurements

Experiment Dates	Chemical Conc. (µg/l)		Free Chlorine (mg/l)	Monochloramine (mg/l)	Total Chlorine (mg/l)	Peak Conc. (µg/l)
June 16th, 2013	10^3	t_0	0.10±0.09	1.27±0.05	1.35±0.01	2.88
		t_{24}	0.05±0.01	1.08±0.01	1.09±0.02	
June 30th, 2013	5 x 10^4	t_0	0.09±0.08	2.14±0.07	2.35±0.05	1.79
		t_{24}	0.09±0.06	1.52±0.02	1.54±0.08	
July 7th, 2013	5 x 10^4	t_0	0.09±0.09	1.95±0.06	2.15±0.01	3.06
		t_{24}	0.07±0.02	1.22±0.02	1.28±0.08	

Our literature search on NDMA formation potential of sulfamethoxazole failed to find any previous studies. There are no previous studies that have investigated the NDMA formation due to reaction with monochloramine and sulfamethoxazole to the best of author's knowledge. The concentrations of NDMA formed due to the reaction of sulfamethoxazole and monochloramine cannot be confirmed since it was out of the calibrated range, but it can be confirmed that sulfamethoxazole is an NDMA precursor for chloramination.

4.4 Assessment of NDMA Analysis Results for All PPCP Used

NDMA analysis results and NDMA molar conversions are summarized in Table 4.16. Four selected PPCPs, namely ranitidine, doxylamine, diltiazem and sumatriptan formed detectable levels of NDMA when reacted with monochloramine. Ranitidine has showed the highest NDMA molar conversion in this study generally in good agreement with the literature (Shen and Andrews, 2011). Doxylamine, diltiazem and sumatriptan showed similar NDMA molar conversions (~ 0.3 – 1 %). In the study carried by Shen *et al.* (2011), sumatriptan and diltiazem showed similar NDMA conversions (~ 1.5 – 2 %); on the other hand, doxylamine showed higher NDMA molar conversion (~ 10%) than sumatriptan and diltiazem.

The selected PPCPs, ranitidine, doxylamine, diltiazem, sumatriptan, caffeine and diclofenac are tertiary amines and atrazine is a secondary amine containing DMA functional groups and sulfamethoxazole is primary aromatic amine. Tertiary amines have been degraded to form nitrosamines. Chlorine attaches from chloramine to the

nitrogen atom in the tertiary amines. Nitrosamine can be formed by electrophilic attack on the N-atom of the DMA group (Shen and Andrews, 2011). In this study, precursor responsible for nitrosamine formation was dependent on the electrostatic potential of the amine group. Molecules having negative electrostatic potential can be considered potential for electrophilic attack. Atomic partial charges for PPCPs are shown in Table 4.17. In general, the N-atom on the DMA group has a negative atomic partial charge which is related to a negative electrostatic potential and this means that it is a potential for electrophilic attack. Diclofenac and atrazine have lower negative potential charge than ranitidine, doxylamine, diltiazem and sumatriptan. The lower negative potential charge explains the reason of no formation of NDMA from diclofenac – monochloramine and atrazine – monochloramine reactions. Moreover, sulfamethoxazole has a positive atomic partial charge on N-atom of the DMA group. This is also an indication of no NDMA formation during sulfamethoxazole – monochloramine reaction.

Table 4.16 Summary Table for Chemicals Forming NDMA

Chemical Names	Experiment Dates	Initial PPCP Conc. (µg/l)	NDMA Concentration in Extracted Sample (µg/l)	Recovery Percent (%)*	Concentration of NDMA Formed (12:100 of Measurement) with Recovery Percent (µg/L)	Molar Conc. of NDMA (µM)	Molar Conc. of PPCP (µM)	Molar Conversion (%)
Doxylamine	June 2nd	5×10^3	16.02	74.73	3.36	0.045	18.49	0.24
	June 16th	5×10^3	45.41	76.11	9.60	0.130	18.49	0.70
	June 30th	5×10^3	21.98	73.55	4.58	0.062	18.49	0.34
Diltiazem	June 2nd	5×10^3	27.21	73.93	5.68	0.077	12.06	0.64
	June 16th	5×10^3	29.82	74.25	6.24	0.084	12.06	0.70
	July 7th	5×10^3	21.49	73.55	4.48	0.060	12.06	0.50
Sumatriptan	June 2nd	5×10^3	33.20	74.65	6.96	0.094	16.93	0.55
	June 30th	10^4	58.56	77.70	12.49	0.169	33.85	0.50
	July 7th	10^4	60.47	77.94	12.91	0.174	33.85	0.51
Ranitidine	June 9th	5×10^2	708.61	70.00**	144.56	1.951	1.43	136.4
	June 30th	10^2	102.72	82.80	22.53	0.304	0.29	104.8
	July 7th	5×10^2	669.00	70.00**	136.48	1.842	1.43	128.8

* Percent Recovery was calculated with interpolation approach according to NDMA concentration range.

** NDMA Concentrations related with Ranitidine for the experiment dates on June 9th and July 7th were higher than the highest calibration curve concentration (500 ppb); therefore, the recovery percent was assumed to be approximately 70%.

Table 4.17 Charges on N-amine of PPCPs (URL 11)

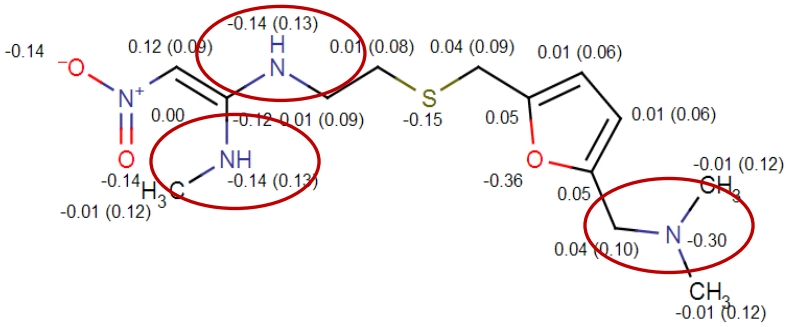
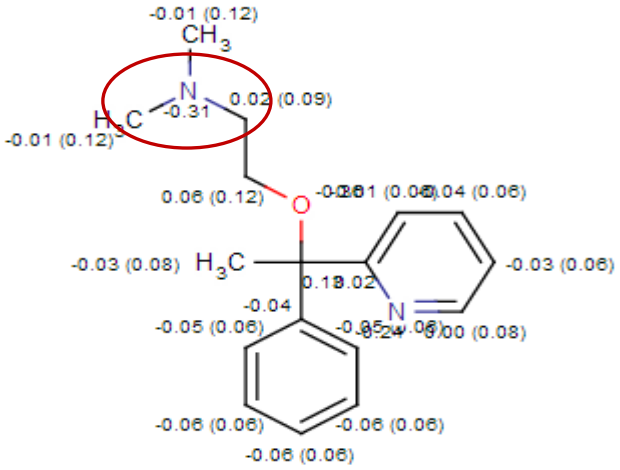
Compounds	Charge on N-amine
Ranitidine	**(- 0.30) and (-0.14)** Electron donating group near to the DMA group: CH_3 
Doxylamine	**- 0.31** Electron donating group near to the DMA group: CH_3 

Table 4.17 Continued

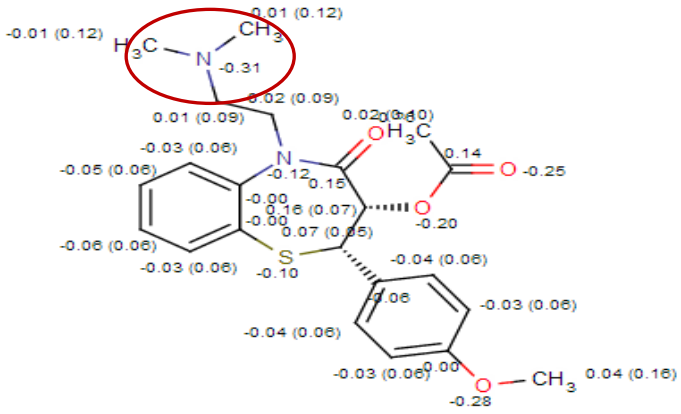
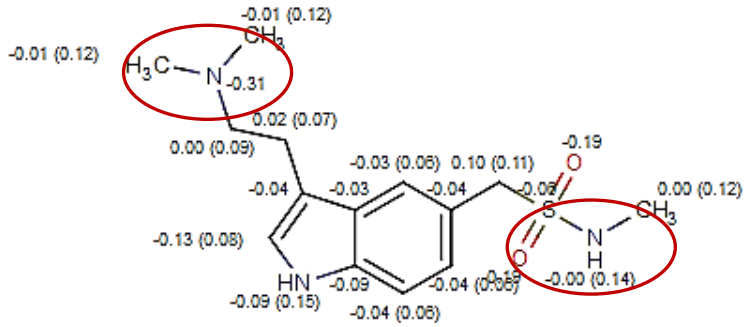
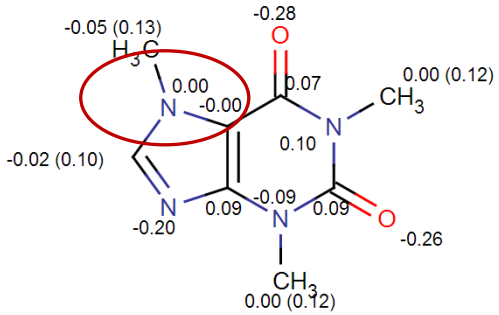
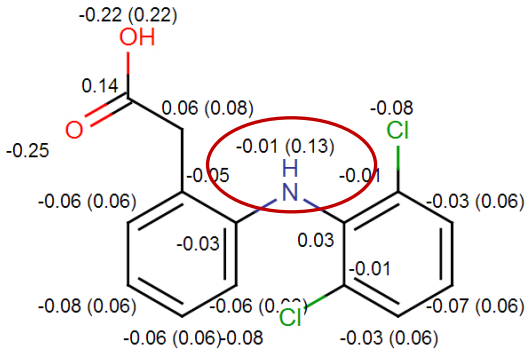
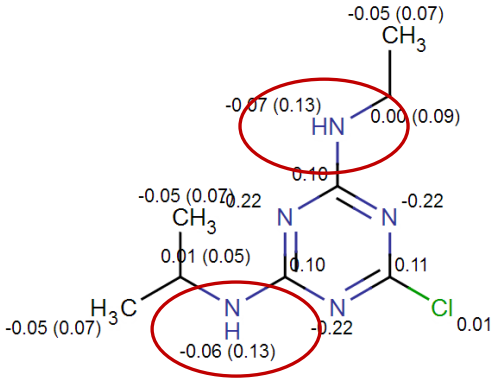
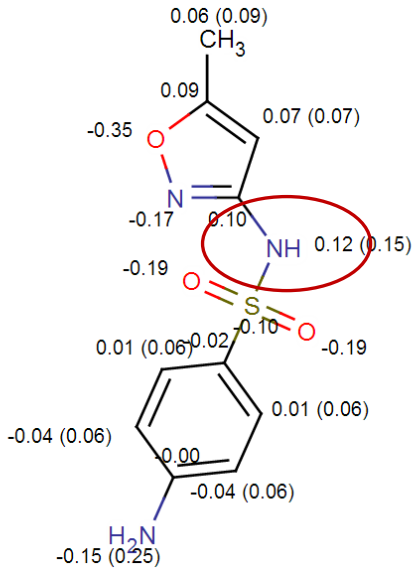
Diltiazem	**- 0.31** Electron donating group near to the DMA group: CH_3 
Sumatriptan	**- 0.31** Electron donating group near to the DMA group: CH_3 
Caffeine	**0.00** Electron donating group near to the DMA group: CH_3 

Table 4.17 Continued

Diclofenac	**- 0.01** There is no electron donating group near to the DMA group. 
Atrazine	**- (0.06 or 0.07)** There is no electron donating group near to the DMA group. 
Sulfamethoxazole	**+ 0.12** There is no electron donating group near to the DMA group. 

Electron density on the N-atom of DMA group is higher if an electron donating group near to the DMA group and the reaction with chlorine become easier (Shen and Andrews, 2011). DMA groups for all eight PPCPs seemed to be close to the aromatic ring system increasing electron density on the N-atom. However, double bonds near to DMA group may decrease NDMA formation due to their electron-withdrawing effect. This structure was observed in diclofenac, atrazine and sulfamethoxazole. For these three PPCPs, N-atom of DMA is near the aromatic ring and double bonds, and for caffeine, N-atom for DMA group is a part of the aromatic ring. On the other hand, ranitidine has the DMA group bound furan ring which is a heterocyclic organic compound and having five elements in its aromatic ring, four carbon elements and one oxygen element. Furan is a symmetric aromatic ring and electrophilic structure; therefore, ranitidine has the highest molar conversion among other PPCPs. When molecular properties of doxylamine are examined, it has a methyl group which is an inductive electron donating group increasing the electron density of the DMA group. This indicates higher potential forming of nitrosamines. Moreover, the molecular electrostatic potential is an effective factor for electrophilic attack for compounds. Higher negative minimal electrostatic potential indicates that the amine group of compound has higher reactive on for an electrophilic attack. Doxylamine has higher negative minimal electrostatic potential among other compounds. Sumatriptan and diltiazem have an aromatic ring system adjacent to the DMA group that likely increases the electron density on the N-atom of DMA.

Other than molecular properties of PPCPs, disinfection type is an important factor for NDMA formation potential. In this study, only using monochloramine as a disinfectant has been ineffective to rapture of bonds for some of the NDMA precursors on PPCPs. In drinking water treatment plants monochloramine is generally used as a secondary disinfectant after the primary disinfection, a pre-oxidation process such as Cl_2, or ozone. The pre-oxidation may partially destruct molecular structures of PPCPs, and forms amine groups lead to reaction with subsequent chloramine to form NDMA. For example, there was no observation of NDMA for sulfamethoxazole experiments in this study; however, a study carried by Albellon *et al.,* (2008) showed that ozone attacks sulfamethoxazole via aniline ring amine group, gives increase to nitro-aromatic compounds. If sulfamethoxazole and

other PPCPs with no observation of NDMA were subjected to ozone or other stronger oxidants as a pre-disinfectant and monochloramine was used as a second disinfectant, then NDMA formation may be observed.

There are several studies carried out on the occurrence of PPCPs in drinking water and drinking water sources. For example, ranitidine in surface water was found up to 580 ng/L in Italy (Kolpin *et al.*, 2002). When molar conversion of NDMA in ranitidine experiment for this study is used; the potential NDMA formation in Italy can be predicted as 500 ng/l, whereas the regulatory for NDMA is 10ng/L in Commonwealth of Massachusetts and State of California. Moreover, according to experiments conducted in drinking water treatment plants in Spain, the raw water used for drinking water production consisting of surface water from the Llobregat River (NE-Spain), diltiazem in raw water was found at concentrations lower than 10 ng/L (Fontela *et al*., 2011). When molar conversions found in this study are used for diltiazem, it can be predicted that NDMA concentration will be 7 ng/l in drinking water. This value is higher than 10^{-6} Cancer risk levels of 3ng/L as stated by State of California OEHHA's public health goal (PHG) for NDMA (URL 11).

Since the NDMA formed due to reaction with monochloramine occurs at the end of drinking water treatment plant, there will be no further treatment to remove NDMA. There are some approaches to prevent of NDMA formation. The first one is precursor removal and oxidation. Identification of NDMA precursors including tertiary and quaternary amines is difficult in water sources (Kemper *et al.,* 2010; Mitch *et al.,* 2003). Treatment plant design for NDMA precursor removal is also complicated. However, studies showed that peroxidation prior to chlorination/chloramination reduce the NDMA formation (Charrois and Hrudey, 2007; Chen and Valentine, 2007). For example, a study carried by Lee *et al*. (2007) showed that ozone and chlorine dioxide oxidation prior to chloramination in water sources minimized NDMA formation by 32-94%.

4.5 NDMA Mutagenicity Test Results

The experiment schedule for mutagenicity test is shown in Table 4.18

Table 4.18 Experiment Schedule for Mutagenicity Test

Samples	No.	Test A - 48 Well						No.	Test B - 48 Well					
		Standard (ml)	Sample (ml)	H_2O (ml)	Reaction Mixture (ml)	Bacterial Strain	Bacterial Strain (2.5 µl)		Standard (ml)	Sample (ml)	H_2O (ml)	Reaction Mixture (ml)	Bacterial Strain	Bacterial Strain (2.5 µl)
Background	**1A**	0	0	8.75	1.25	TA100	+	**1B**	0	0	8.75	1.25	TA98	+
Background	**2A**	0	0	8.75	1.25	TA100	+	**2B**	0	0	8.75	1.25	TA98	+
Standard Mutagens	**3A**	0.05	0	8.7	1.25	TA100	+	**3B**	0.05	0	8.7	1.25	TA98	+
Control (Water-Chlm)	**9A**	0	7.5	1.25	1.25	TA100	+	**9B**	0	7.5	1.25	1.25	TA98	+
Ranitidine - NDMA Test 1	**10A**	0	7.5	1.25	1.25	TA100	+	**10B**	0	7.5	1.25	1.25	TA98	+
Ranitidine - NDMA Test 2	**11A**	0	7.5	1.25	1.25	TA100	+	**11B**	0	7.5	1.25	1.25	TA98	+
Doxylamine - NDMA Test 1	**12A**	0	7.5	1.25	1.25	TA100	+	**12B**	0	7.5	1.25	1.25	TA98	+
Doxylamine - NDMA Test 2	**13A**	0	7.5	1.25	1.25	TA100	+	**13B**	0	7.5	1.25	1.25	TA98	+
Doxylamine - NDMA Test 3	**14A**	0	7.5	1.25	1.25	TA100	+	**14B**	0	7.5	1.25	1.25	TA98	+
Sumatriptan - NDMA Test 1	**15A**	0	7.5	1.25	1.25	TA100	+	**15B**	0	7.5	1.25	1.25	TA98	+
Sumatriptan - NDMA Test 2	**16A**	0	7.5	1.25	1.25	TA100	+	**16B**	0	7.5	1.25	1.25	TA98	+
Sumatriptan - NDMA Test 3	**17A**	0	7.5	1.25	1.25	TA100	+	**17B**	0	7.5	1.25	1.25	TA98	+

Table 4.18 (Continued)

Samples	No.	Test A - 48 Well						No.	Test B - 48 Well					
		Standard (ml)	Sample (ml)	H_2O (ml)	Reaction Mixture (ml)	Bacterial Strain	Bacterial Strain (2.5 µl)		Standard (ml)	Sample (ml)	H_2O (ml)	Reaction Mixture (ml)	Bacterial Strain	Bacterial Strain (2.5 µl)
Diltiazem - NDMA Test 1	**18A**	0	7.5	1.25	1.25	TA100	+	**18B**	0	7.5	1.25	1.25	TA98	+
Diltiazem - NDMA Test 2	**19A**	0	7.5	1.25	1.25	TA100	+	**19B**	0	7.5	1.25	1.25	TA98	+
Diltiazem - NDMA Test 3	**20A**	0	7.5	1.25	1.25	TA100	+	**20B**	0	7.5	1.25	1.25	TA98	+
Ranitidine - Control (No Chlm)	**21A**	0	7.5	1.25	1.25	TA100	+	**21B**	0	7.5	1.25	1.25	TA98	+
Doxylamine - Control (No Chlm)	**22A**	0	7.5	1.25	1.25	TA100	+	**22B**	0	7.5	1.25	1.25	TA98	+
Sumatriptan - Control (No Chlm)	**23A**	0	7.5	1.25	1.25	TA100	+	**23B**	0	7.5	1.25	1.25	TA98	+
Diltiazem - Control (No Chlm)	**24A**	0	7.5	1.25	1.25	TA100	+	**24B**	0	7.5	1.25	1.25	TA98	+

The samples from the experiments were placed into plates using the mutagenicity test kits and incubated at 37^{o}C for 5 days as described in section 3.2.3 Mutagenicity Tests. On the third, fourth and fifth days, the plates were observed and scored abased on color change from purple to yellow or partially yellow. The results of the mutagenicity test carried out on samples are shown in Table 4.19 and well plate figures are shown in Appendix D. Test A had 48 well plates for bacterial strain, TA100 and Test B had 48 well plates for bacterial strain TA98. In background plate, there were no standard solutions and samples; however, for both Test A and Test B, some wells showed positive in treatment plate. This showed the background or spontaneous mutation of the TA100 and TA98. Treatment plates of standard mutagens containing sodium azide (NaN3, 0.5 µg/100 µl) for TA100 and 2-Nitrofluorene (2-NF, 30 µg/100 µl) for TA98 were expected to have high scores. For TA100, all wells in the positive treatment plants showed positive score, in other words, all bacterial strains mutated in the positive treatment plate. On the other hand, for TA98, half of the wells in the positive treatment plates scored as positive. In control plate, where the phosphate buffered laboratory grade water was chloraminated for 24 hours and at the end, residual total chlorine was quenched with sodium thiosulfate, no mutations were observed. This proves that the residual monochloramine was successfully eliminated and there were no mutagenic effects of sodium thiosulfate.

When treatment plates containing PPCP controls and are examined, it is seen that the mutation of samples treated with only PPCPs were not significantly any different then background samples, except doxylamine. This shows that there was no mutagenic effect of ranitidine, diltiazem and sumatriptan. On the other hand, in samples containing doxylamine only, there was significantly higher number of positively scored wells containing TA100. In TA98, the positive scored wells were not similar as the background scores. According to a study by Jurado *et al*., (1993), in general, the genetic background of strain TA100 seems to be more sensitive to the killing effects of chemicals than that of TA98. This shows the mutagenic effect of doxylamine. According to best of author's knowledge, there are studies showing intoxication of doxylamine (Tiefenbach *et al.,* 1999), but no prior studies investigating mutagenic effects of doxylamine. When results are examined for

samples from monochloramine disinfection of PPCPs, sumatriptan did not show any difference in mutation compared to background or control samples. For the case of ranitidine, one of two experiments showed some positive values for TA100, the sample from the other experiment had same level of mutation as the background sample. TA98 was not affected by NDMA formed in the ranitidine experiments. These mutations might be related to NDMA genotoxicity for TA100. For doxylamine and diltiazem control plates, mutations were observed in TA100 and NDMA treatment plates for these PPCPs showed approximately same numbers of positive wells with controls. This means that these PPCPs might have a mutagenic effect for TA100. On the other hand, doxylamine, sumatriptan and diltiazem control plates did not show any mutations for TA98 and NDMA treatments plates for these PPCPs. This means that TA98 was not affected from these PPCPs and NDMA as a result of reaction of PPCPs with chloramine.

As part of this study mutagenicity test were also going to be performed for samples spiked with known concentrations of stock NDMA solutions. However, the NDMA stock solution were prepared in dichloromethane and due to reaction between the dichloromethane and filters, we were not able to filter sterilize samples prior to mutagenicity tests.

Table 4.19 The Results of Mutagenicity Test

		Test A - 48 Wells (Bacterial Strain – TA100)						Test B - 48 Wells (Bacterial Strain – TA98)					
No.	**Treatment Plate**	**Standard (ml)**	**Sample (ml)**	**H_2O (ml)**	**No. Wells Positive in Treatment Plate**			**Standard (ml)**	**Sample (ml)**	**H_2O (ml)**	**No. Wells Positive in Treatment Plate**		
					3rd Day	**4th Day**	**5th Day**				**3rd Day**	**4th Day**	**5th Day**
1	Background	0	0	8.75	2	2	3	0	0	8.75	3	3	3
2	Background	0	0	8.75	2	3	3	0	0	8.75	5	8	9
3	Standard Mutagens	0,05	0	8.7	22	43	48	0.05	0	8.7	0	3	21
9	Control (Water-Chlm)	0	7.5	1.25	0	0	0	0	7.5	1.25	0	0	0
10	Ranitidine - NDMA Test 2	0	7.5	1.25	6	7	7	0	7.5	1.25	0	1	1
11	Ranitidine - NDMA Test 3	0	7.5	1.25	2	3	3	0	7.5	1.25	0	0	0
12	Doxylamine - NDMA Test 1	0	7.5	1.25	5	7	10	0	7.5	1.25	1	1	1
13	Doxylamine - NDMA Test 2	0	7.5	1.25	9	10	10	0	7.5	1.25	1	1	1
14	Doxylamine - NDMA Test 3	0	7.5	1.25	0	1	5	0	7.5	1.25	3	1	3
15	Sumatriptan - NDMA Test 1	0	7.5	1.25	2	3	3	0	7.5	1.25	0	0	0
16	Sumatriptan - NDMA Test 2	0	7.5	1.25	4	4	4	0	7.5	1.25	0	0	0
17	Sumatriptan - NDMA Test 3	0	7.5	1.25	5	5	5	0	7.5	1.25	1	1	1
18	Diltiazem - NDMA Test 1	0	7.5	1.25	2	6	7	0	7.5	1.25	2	2	2
19	Diltiazem - NDMA Test 2	0	7.5	1.25	0	0	2	0	7.5	1.25	0	0	0
20	Diltiazem - NDMA Test 3	0	7.5	1.25	5	6	7	0	7.5	1.25	1	1	1
21	Ranitidine - Control (No Chlm)	0	7.5	1.25	0	0	0	0	7.5	1.25	0	0	0
22	Doxylamine - Control (No Chlm)	0	7.5	1.25	3	4	12	0	7.5	1.25	0	0	0
23	Sumatriptan - Control (No Chlm)	0	7.5	1.25	1	4	8	0	7.5	1.25	1	1	1
24	Diltiazem - Control (No Chlm)	0	7.5	1.25	1	3	7	0	7.5	1.25	1	1	1

CHAPTER 5

CONCLUSIONS

PPCPs have gained significant attention in recent years because of the contamination of aquatic environment and drinking water sources with them. Simultaneously, formation of nitrosamines during chloramine disinfection of drinking water has become another important issue because of carcinogenic effects of NDMA. In this study, a group of PPCPs with amine groups as nitrosamine precursors were subjected to disinfection process with monochloramine and formation potential of NDMA was observed. Moreover, relationships between NDMA molar conversions and PPCPs molecular properties were examined.

In the scope of this study, eight amine-based PPCPs, namely ranitidine, doxylamine, sumatriptan, diltiazem, atrazine, caffeine, diclofenac and sulfamethoxazole were investigated whether NDMA would be formed due to monochloramine disinfection. The concentration of NDMA was measured by Gas Chromatography/Mass Spectrophotometry (GC/MS) and 4 PPCPs, namely ranitidine, doxylamine, diltiazem and sumatriptan showed potential to form NDMA. Ranitidine showed the highest NDMA molar conversion in this study and the results were in good agreement with those reported in other studies (Shen and Andrews, 2011; Mitch *et.al.,* 2009). Doxylamine, diltiazem and sumatriptan showed approximately same NDMA molar conversions (~ 0.3 - 1%). In the study carried by Shen *et al.* (2011), sumatriptan and diltiazem showed similar NDMA conversions (~ 1.5 – 2 %); on the other hand, doxylamine showed higher NDMA molar conversion (~ 10%) than sumatriptan and diltiazem.

In the other four PPCPs, namely caffeine, diclofenac, atrazine and sulfamethoxazole no detectable NDMA formation was observed. These four PPCPs were examined according to their molecular structures and molecular properties. Firstly, atomic

partial charges for PPCPs were considered and it was seen that diclofenac and atrazine have lower negative potential charge, which can explain the reason of no formation of NDMA from diclofenac – monochloramine and atrazine – monochloramine reactions. Moreover, sulfamethoxazole has a positive atomic partial charge on N-atom of the DMA group. This can be also an indication of no NDMA formation during sulfamethoxazole – monochloramine reaction. Secondly, electron densities on the N-atom of DMA groups were evaluated. Molecular structures of diclofenac, atrazine and sulfamethoxazole have double bonds adjacent to the DMA group; this decreases NDMA formation due to electron withdrawing effect. For caffeine, N-atom for DMA group is a part of aromatic ring. Thirdly, the effects of the disinfection type were considered for NDMA formation potential. From studies, chloramine has been used as a secondary disinfectant in drinking water treatment plants. In this study, monochloramine was used as a main disinfectant; therefore, oxidative power of monochloramine may be insufficient to react with tertiary amines of PPCPs to form NDMA.

In NDMA mutagenicity tests, NDMA related mutations were not clearly observed. This can be related to absence of metabolic activation of NDMA such like liver enzymes. In mutagenicity test, we have observed that Doxylamine causes mutation of TA100 cells with or without the presence of NDMA.

Overall, the results from this study have demonstrated that PPCPs containing amine groups can be potential NDMA precursors during monochloramine disinfection with transformation of PPCPs. Moreover, there is a potential additional risk related to NDMA toxicity for organisms on top of the health impacts of PPCPs.

CHAPTER 6

RECOMMENDATIONS

Actually, PPCPs are generally found in the form of mixtures rather than as single compounds in the water sources. In this study, sumatriptan, diltiazem and doxylamine have formed low levels of NDMA when compared to ranitidine. Other four PPCPs studied, caffeine, sulfamethoxazole, atrazine and diclofenac did potentially formed NDMA below detection limits; however, when present in the same water matrix together they may still affect the overall formation of NDMA. Therefore, to understand the potential effect of mixtures on the formation of NDMA via PPCPs, these PPCPs should be prepared in a mixture and subjected to monochloramine under the same conditions.

Moreover, instead of deionized water, the tap water and samples from drinking water sources should be analyzed with these PPCPs to examine formation potential of the NDMA in more complex matrices.

Further studies should be done on toxicity and genotoxicity of DBPs formed as a result of PPCPs reaction with monochloramine after metabolic activation such as liver enzyme activation.

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URL 2: http://www.drugs.com/cdi/doxylamine.html Accessed on July 10th, 2013.
URL 3: http://www.drugs.com/diltiazem.html. Accessed on July 10th, 2013
URL 4: http://www.drugs.com/cons/sumatriptan.html. Accessed on July 10th, 2013
URL 5: http://www.sigmaaldrich.com/etc/medialib/docs/Sigma-Aldrich/Product_Information_Sheet_/c0750pis.Par.0001.File.tmp/c0750pis.pdf Accessed on July 10th, 2013
URL 6: http://www.drugs.com/diclofenac.html. Accessed on July 10th, 2013
URL 7: http://www.apvma.gov.au/news_media/chemicals/atrazine.php#what_is Accessed on July 10th, 2013
URL 8: http://www.mayoclinic.com/health/drug-information/DR602685 Accessed on July 10th, 2013
URL 9: http://en.wikipedia.org/wiki/Nitrosamine Accessed on July 10th, 2013
URL 10: http://commons.wikimedia.org/wiki/File:NDMA.svg Accessed on July 10th, 2013
URL 11: http://www.chemicalize.org/ Accessed on May 13th,2014
URL 12: http://www.cdph.ca.gov/certlic/drinkingwater/pages/NDMA.aspx Accessed on May 16th, 2014

APPENDIX A

GC/MS INSTRUMENT CONTROL PROGRAM

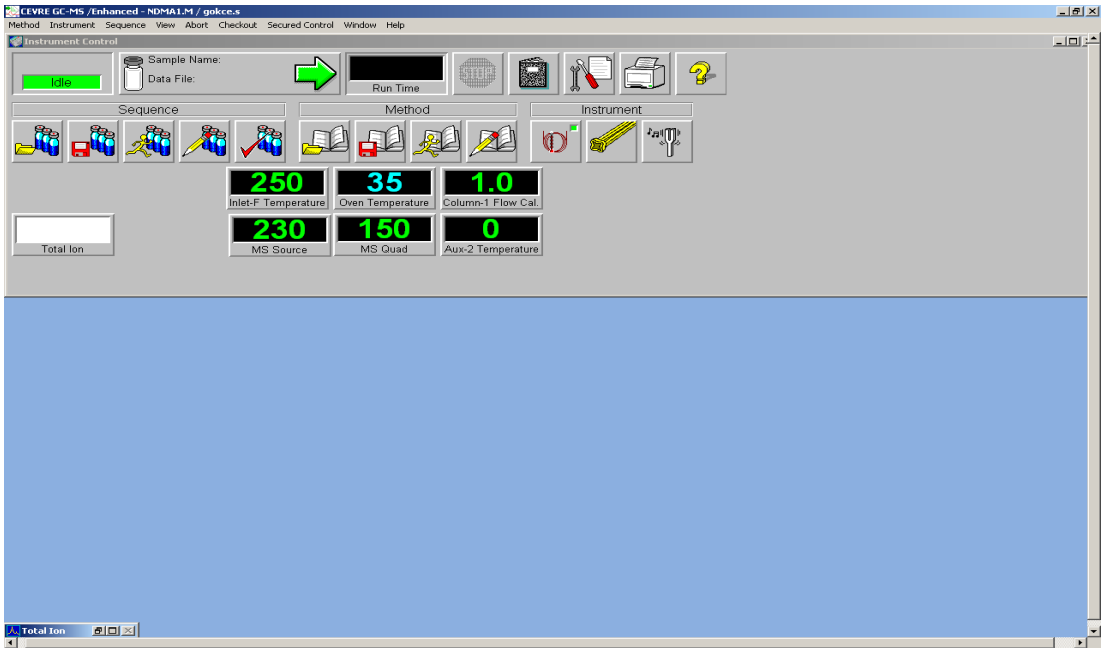


Figure A.1 MSD Enhanced Chem Station E.02.02.1431, Agilent Technologies

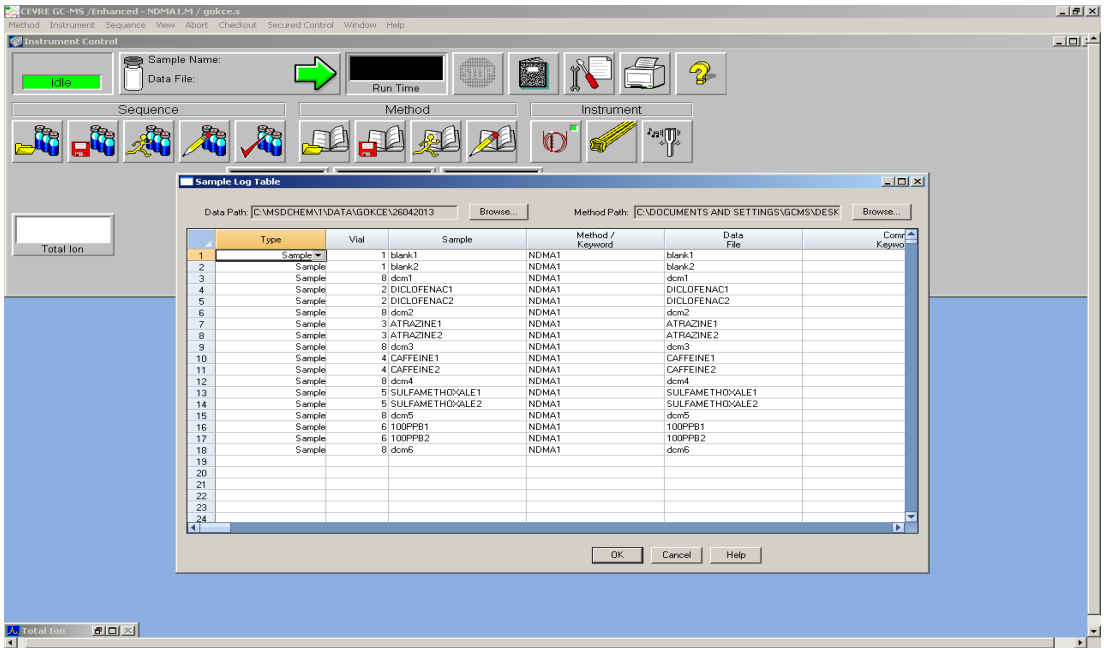


Figure A.2 Sample Log Table

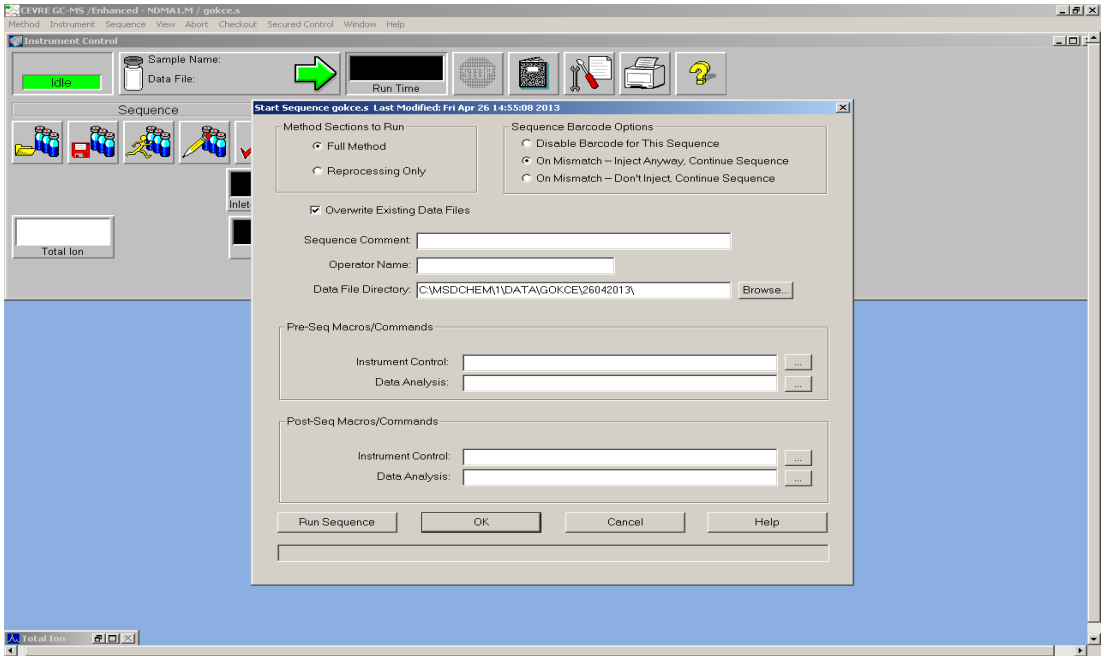


Figure A.3 Running of Sequence

APPENDIX B

GC/MS ANALYZING PROGRAM
(MSD Enhanced Chem Station E.02.02.1431, Agilent Technologies)

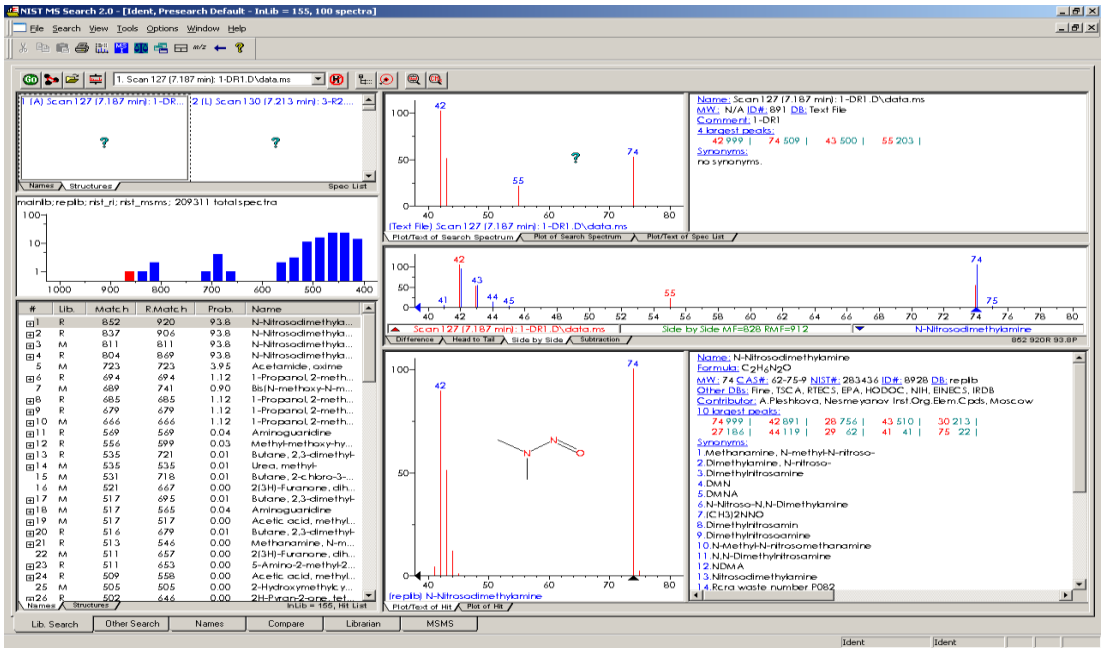


Figure B.1 Library of Chemicals

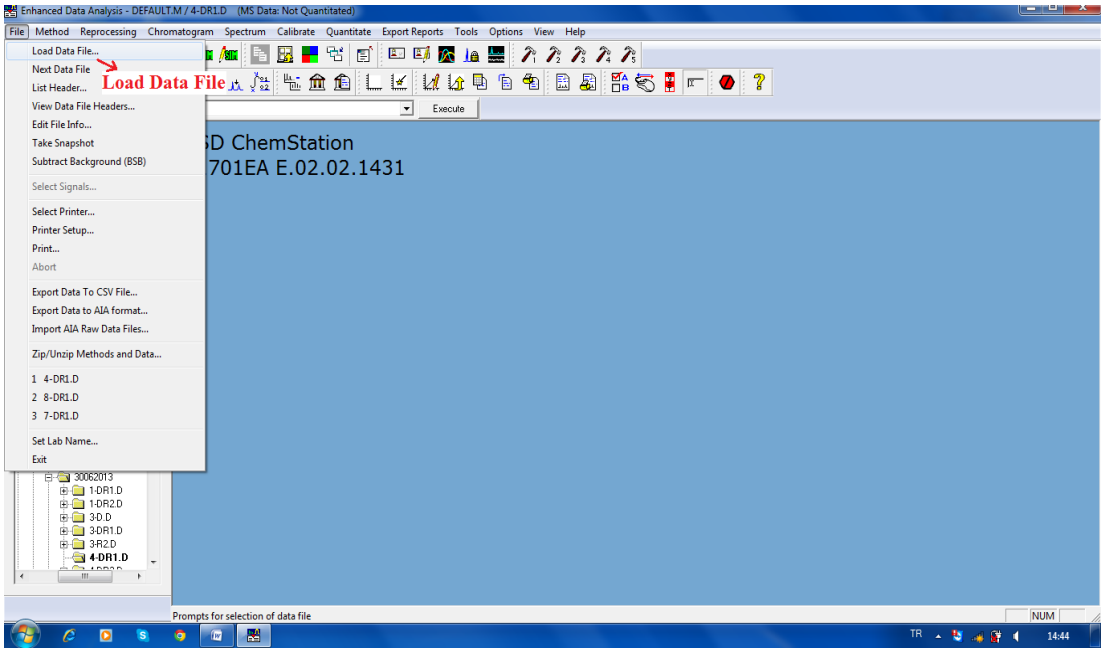


Figure B.2 Data Analysis Steps (Loading Data File)

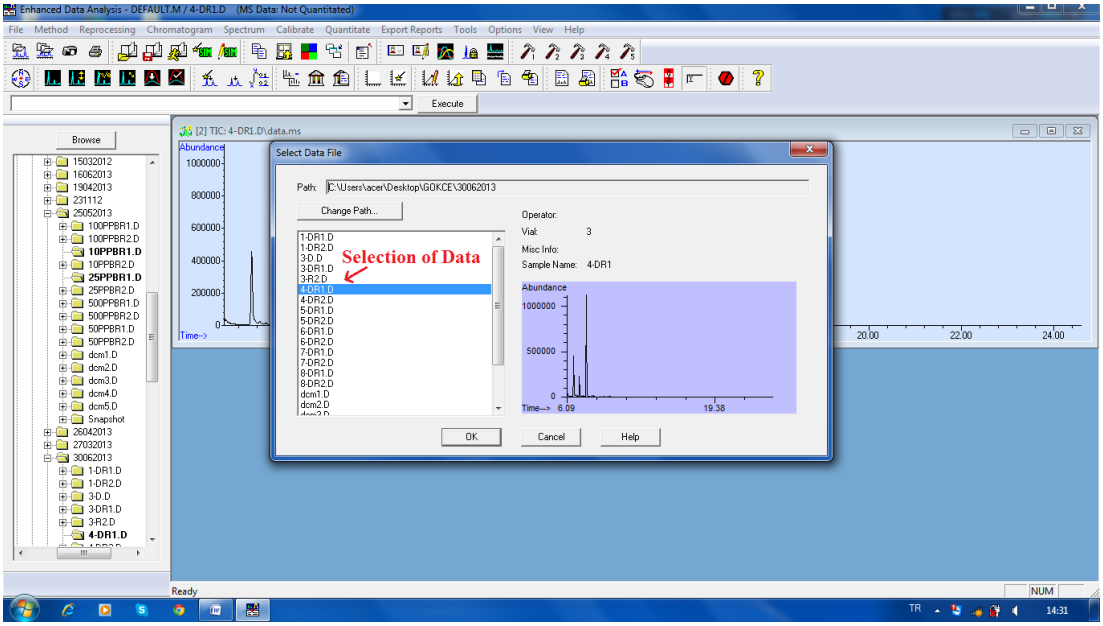


Figure B.3 Data Analysis Steps (Importing Data File)

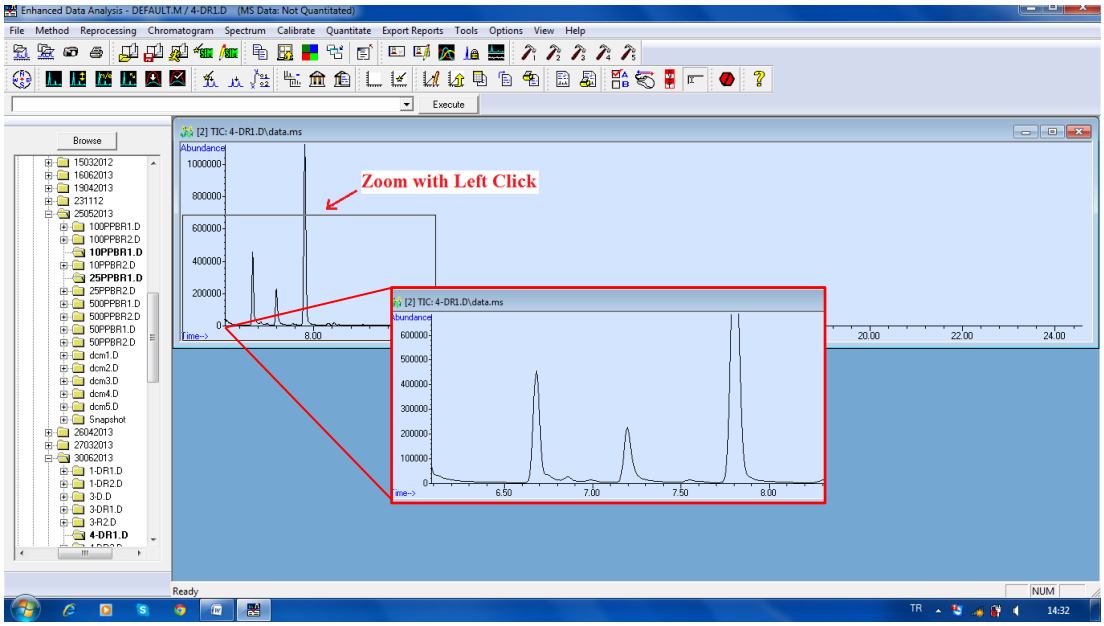


Figure B.4 Data Analysis Steps (Zoom in or Zoom out of Chromatograms)

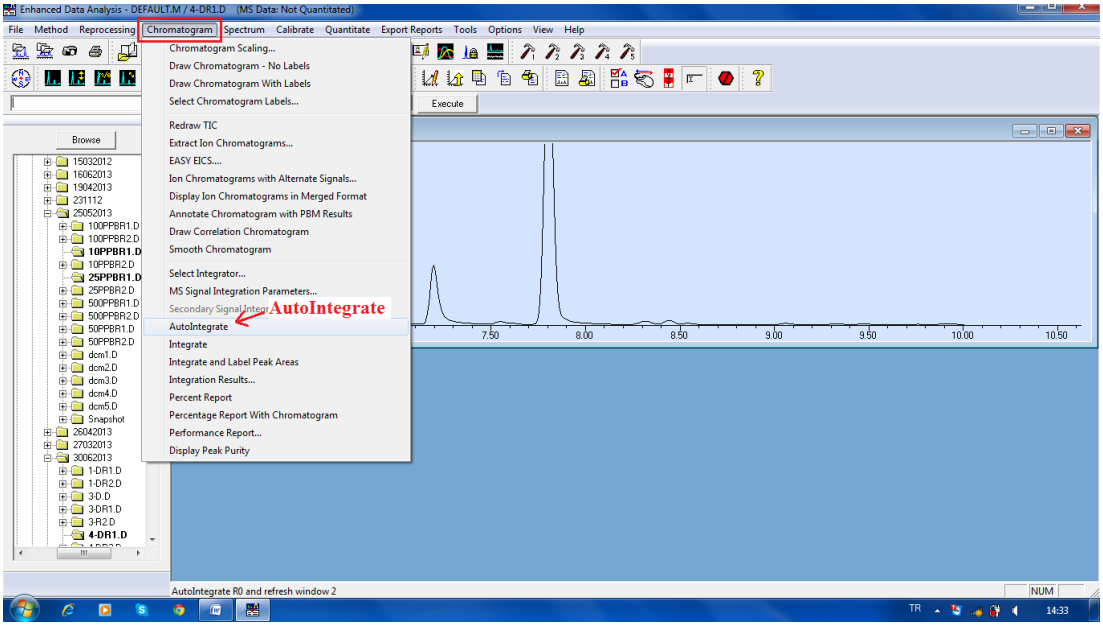


Figure B.5 Autointegration Method

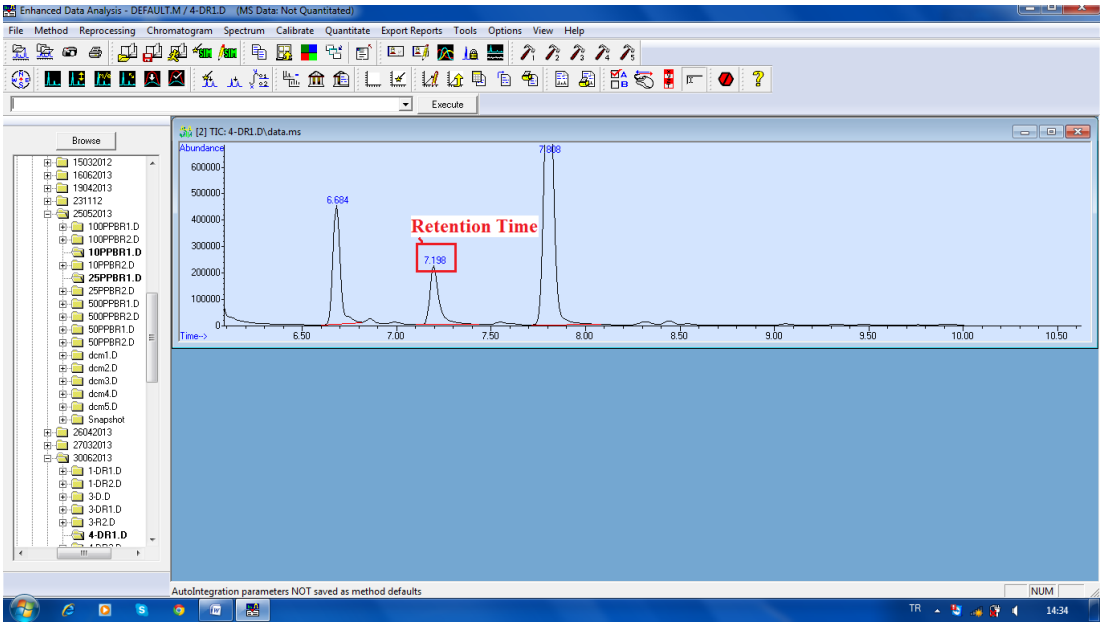


Figure B.6 Retention Times of Peaks

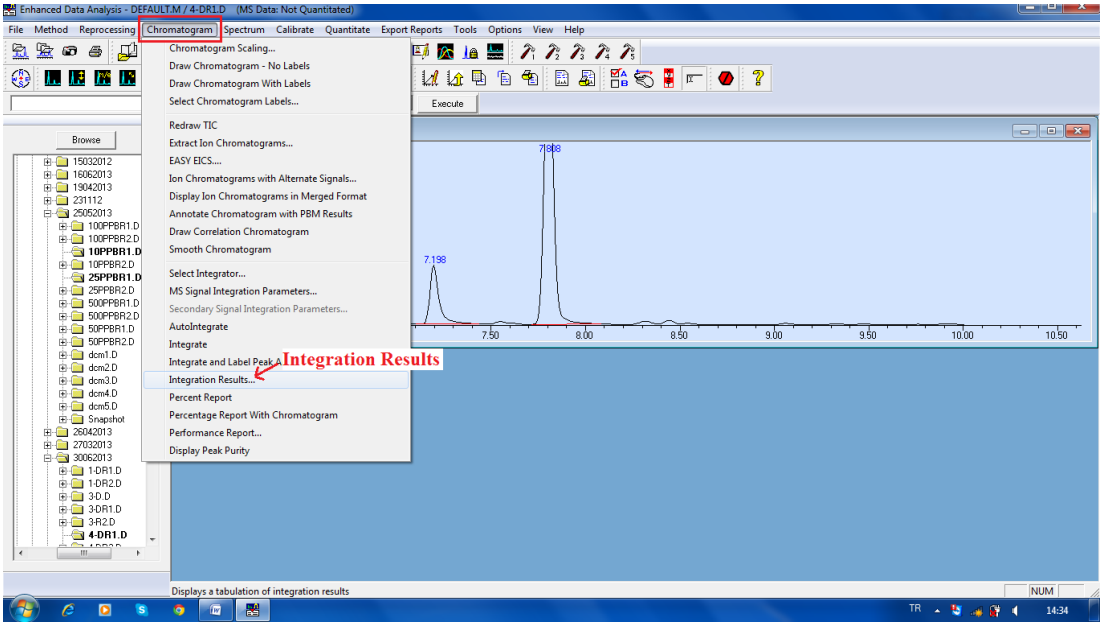


Figure B.7 Integration Results

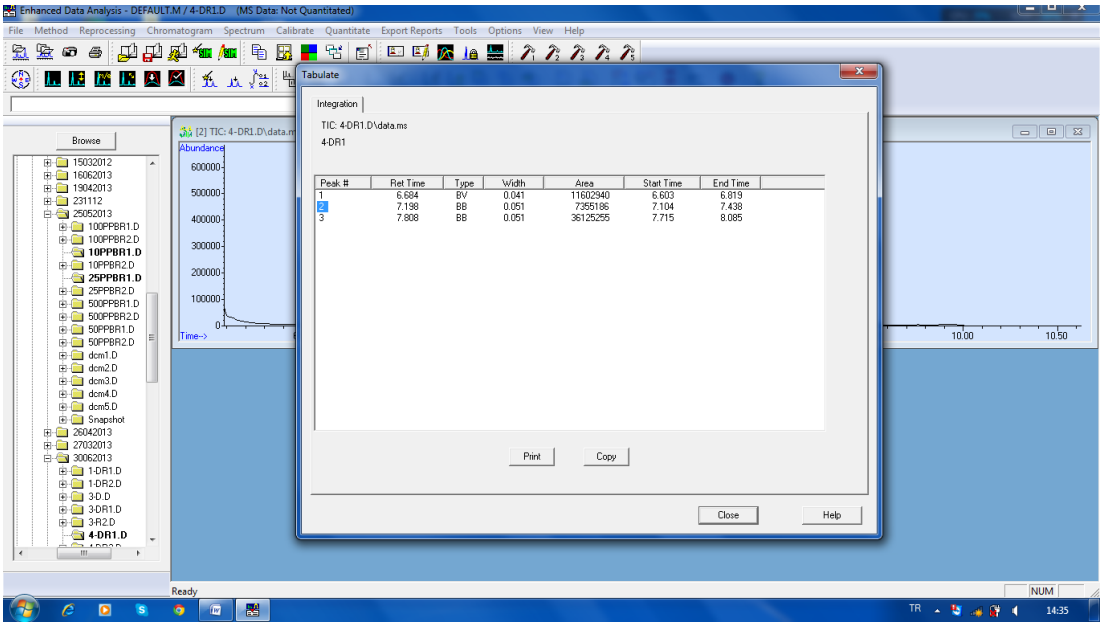


Figure B.8 Retention Time, Width, Area, Start and End Time of Peaks.

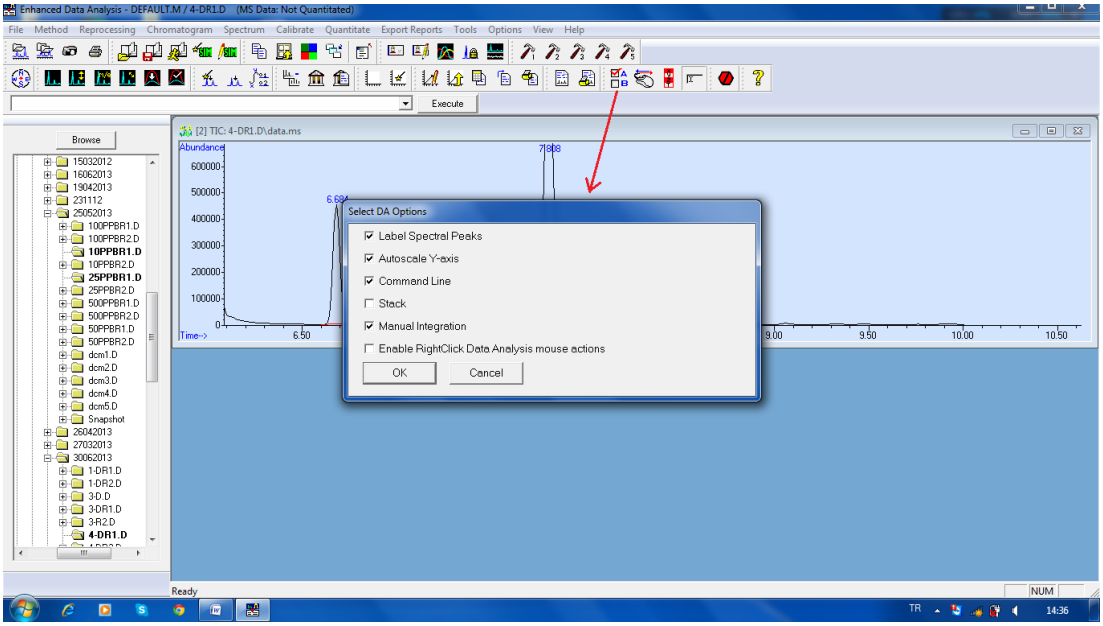


Figure B.9 Manual Integration

APPENDIX C

CALIBRATION CURVE RESULTS

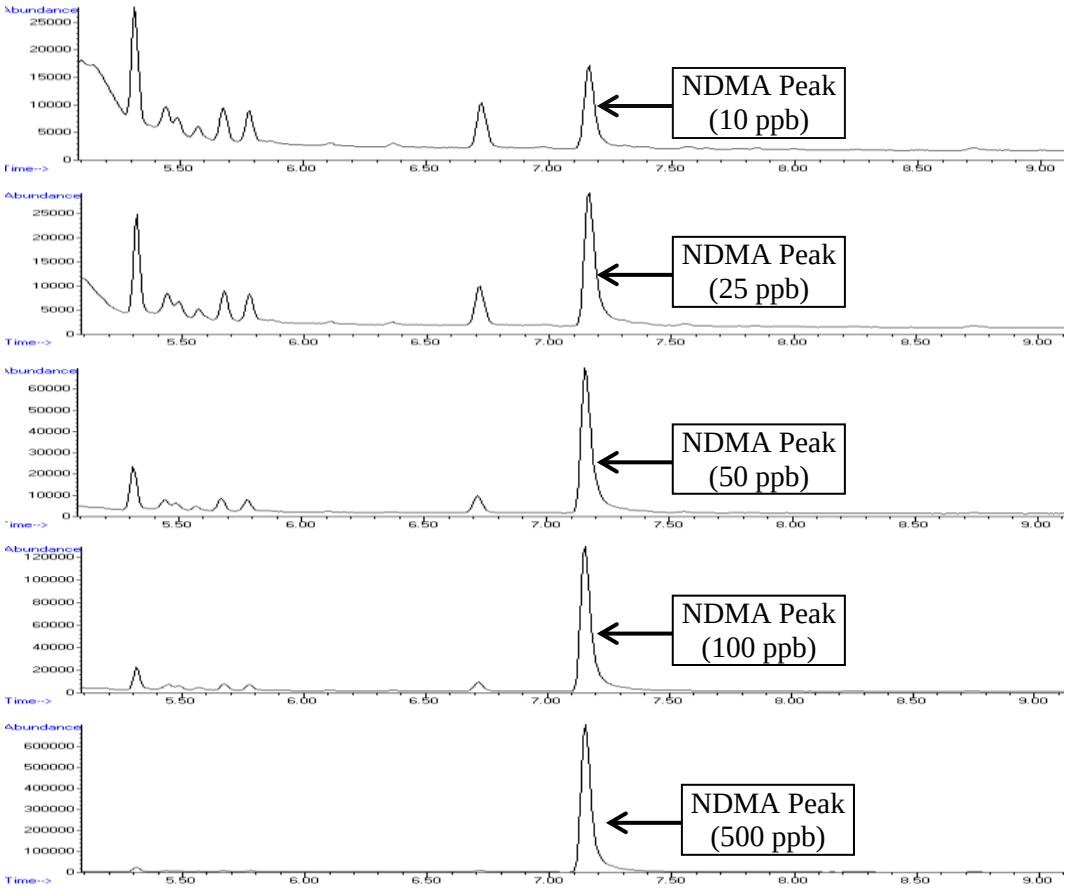


Figure C.1 GC/MS Readings of NDMA Stock Solutions (on March 15th, 2013) (10 ppb, 25 ppb, 50 ppb, 100 ppb and 500 ppb, respectively)

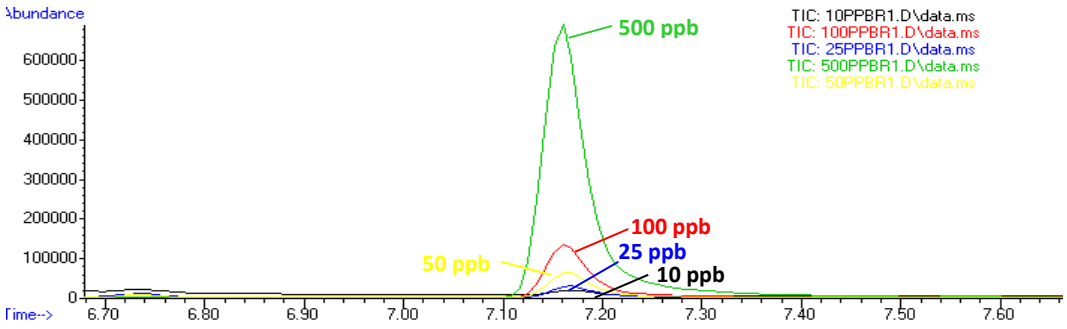


Figure C.2 Presentations of Concentrations as an Overlay (on March 15th, 2013)

(a)

(b)

(c)

(d)

(e)

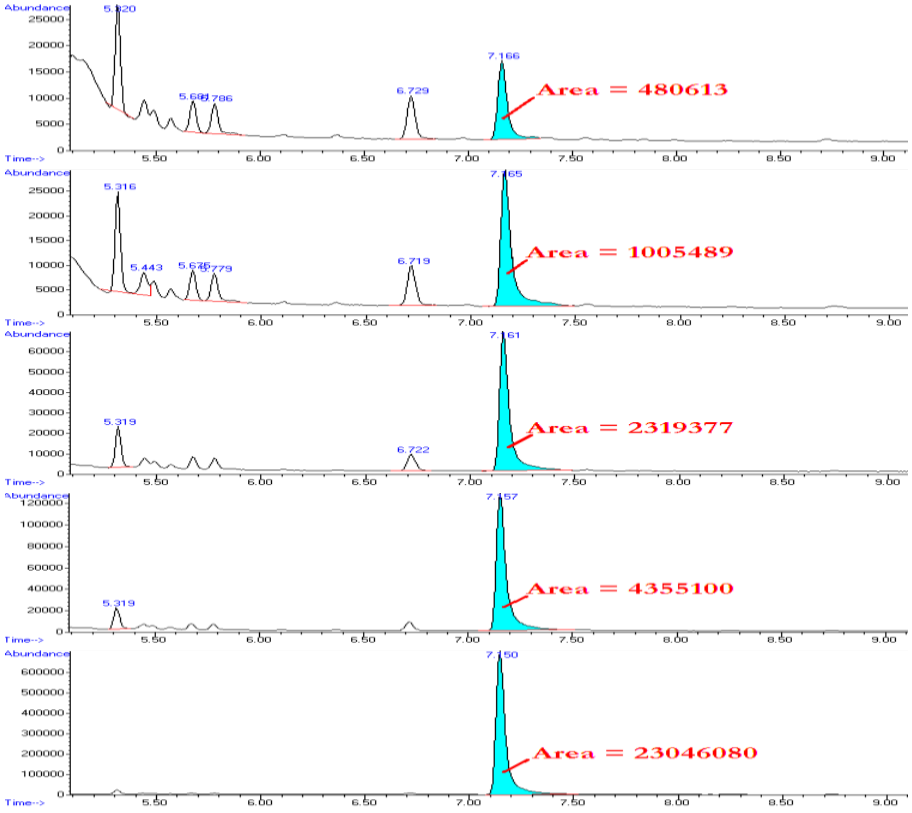


Figure C.3 Areas under NDMA Peaks: (on March 15th, 2013)

(a) 10 ppb, (b) 25 ppb, (c) 50 ppb, (d) 100 ppb, (e) 500 ppb

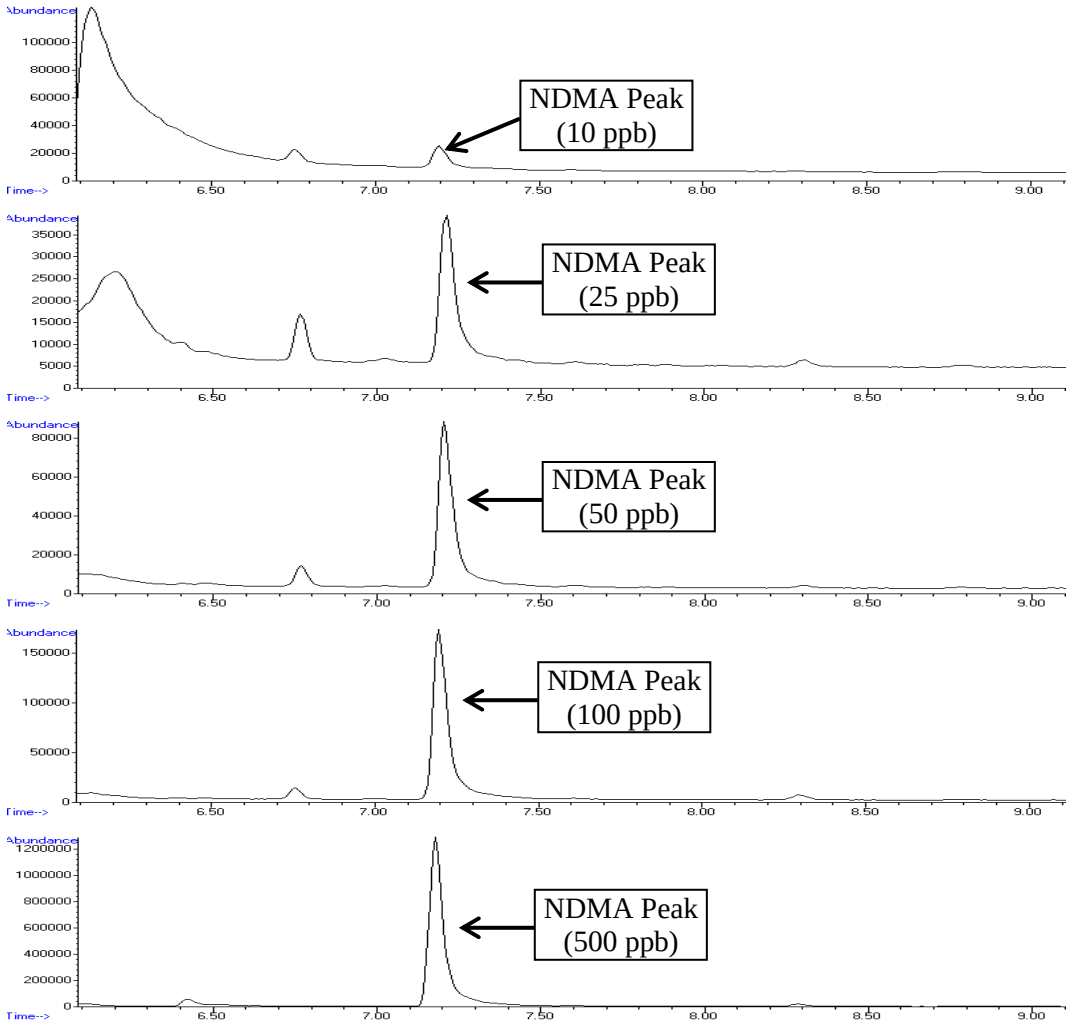


Figure C.4 Analysis Results of NDMA Concentrations (on May of 25th, 2013)

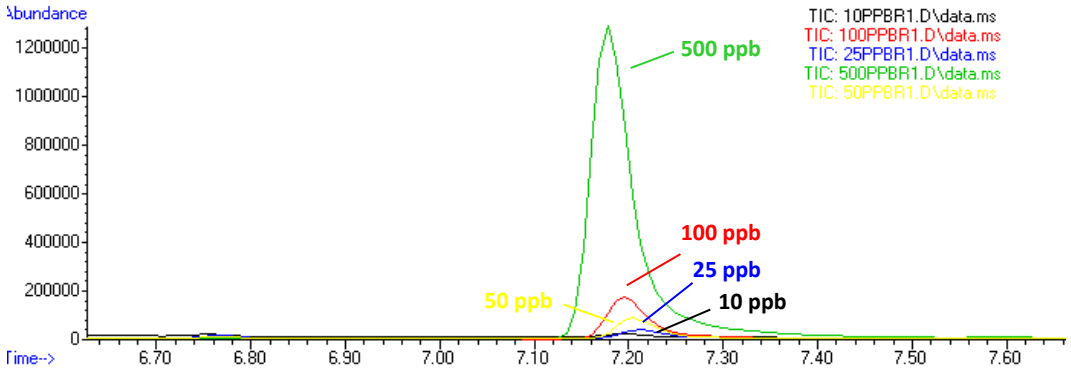


Figure C.5 Presentation of Concentrations as an Overlay (on May of 25th, 2013)

(a)

(b)

(c)

(d)

(e)

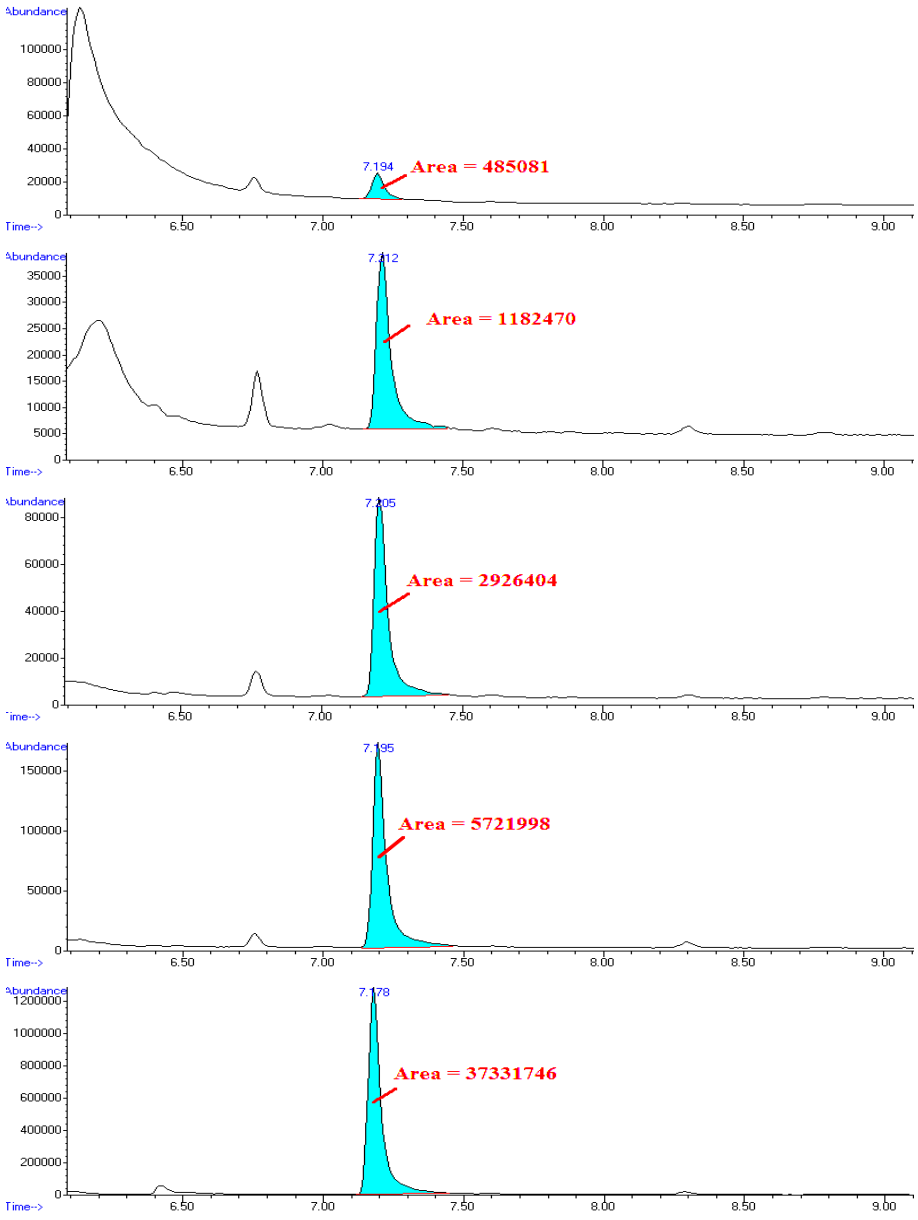


Figure C.6 Areas under NDMA Peaks: (on May of 25th, 2013)

(a) 10 ppb, (b) 25 ppb, (c) 50 ppb, (d) 100 ppb, (e) 500 ppb

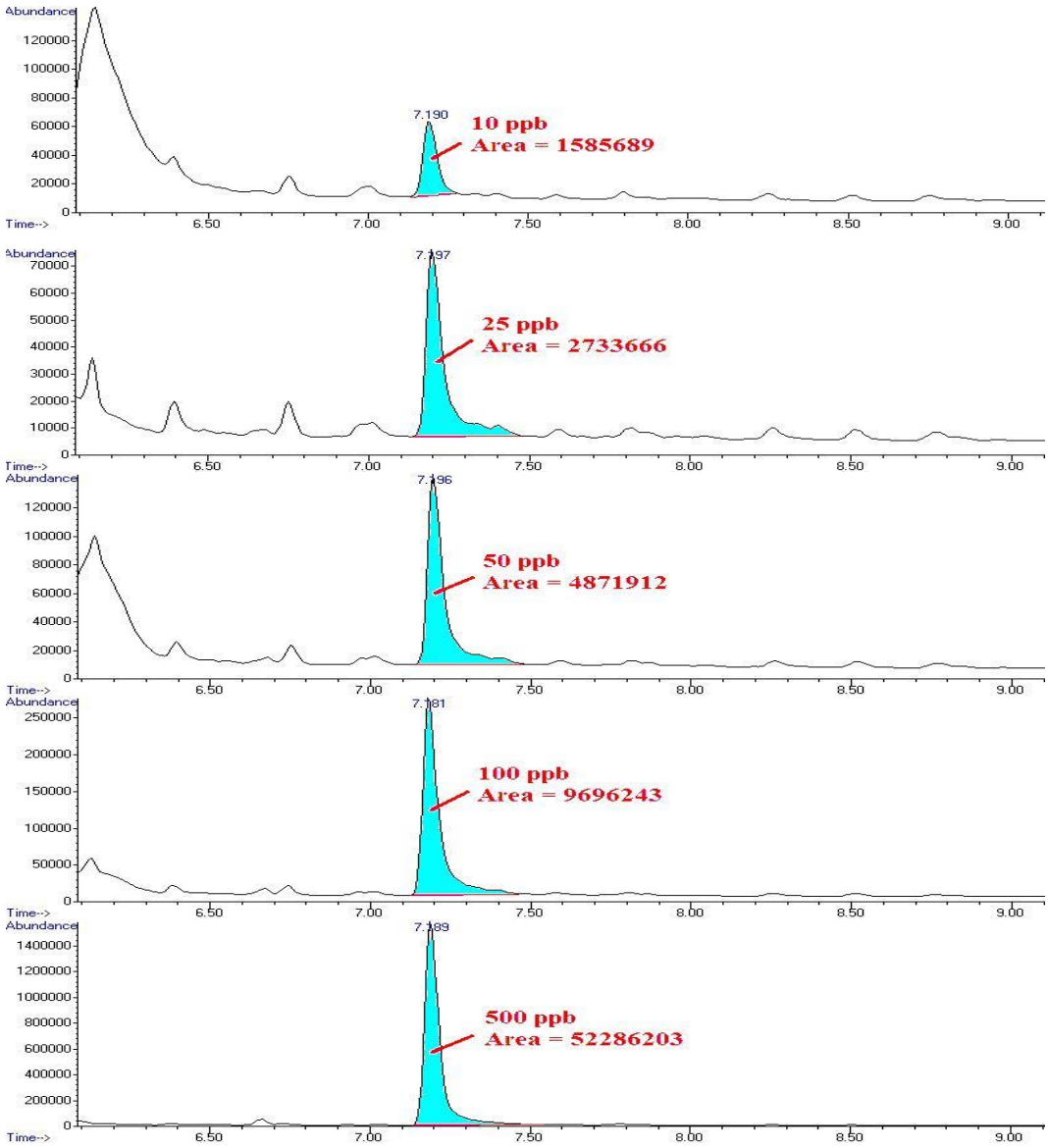


Figure C.7 Analysis Results of NDMA Concentrations (The Third Calibration) (10 ppb, 25 ppb, 50 ppb, 100 ppb and 500 ppb, respectively)

APPENDIX D

MUTAGENICITY TEST RESULTS

The results of mutagenicity test are given in the following pages as Figure D.1.

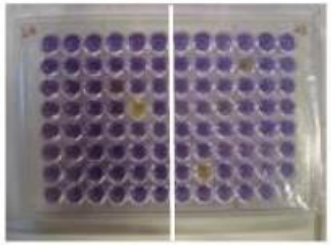
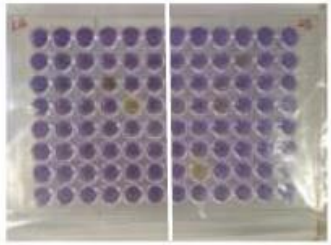
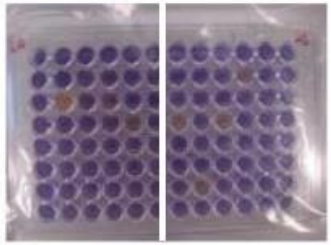
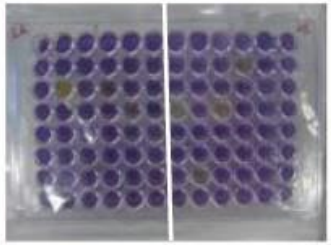
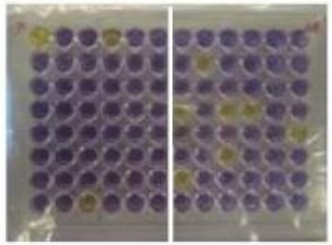
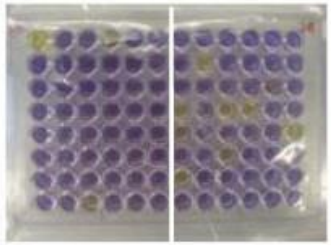
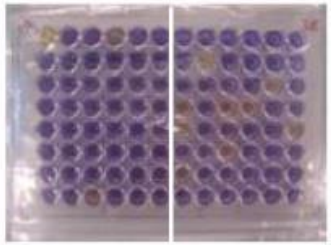
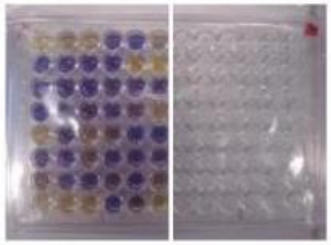
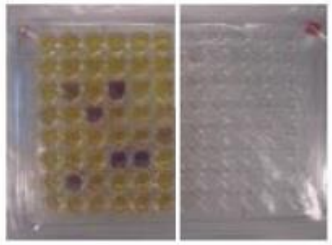
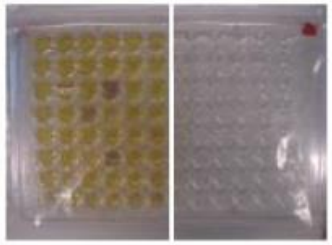
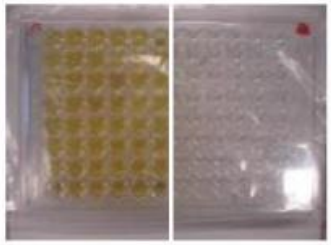
TA 100 / TA 98		3rd Day	4th Day	5th Day	6th Day
Background	1A/1B				
Background	2A/2B				
Standard Mutagens	3A				

Figure D.1 Mutagenicity Test Results

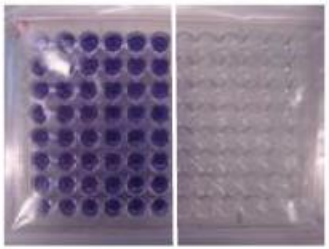
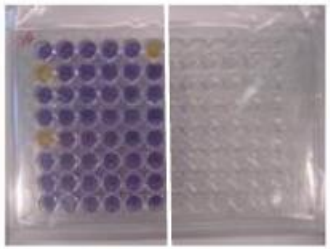
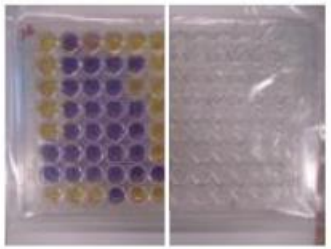
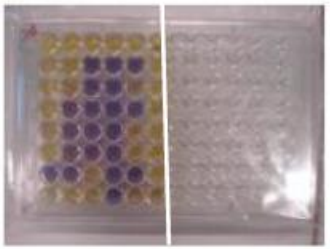
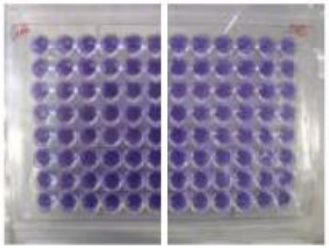
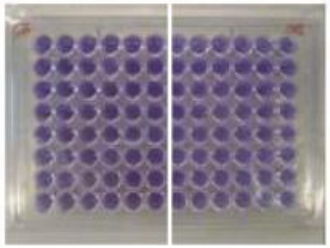
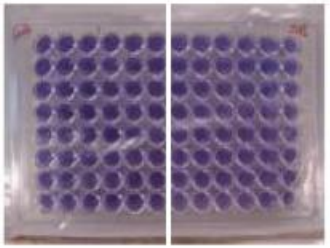
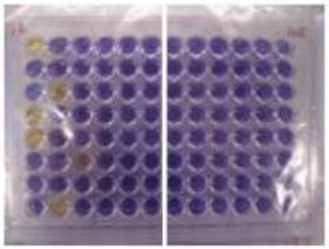
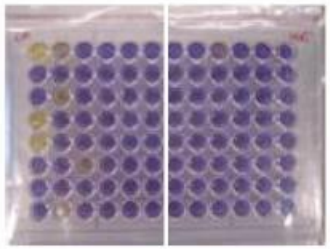
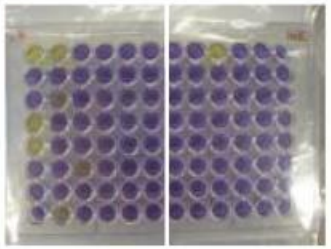
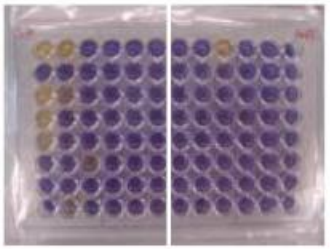
TA 100 / TA 98		3rd Day	4th Day	5th Day	6th Day
Standard Mutagens	3B				
Control (Water-Chlm)	9A/9B				
Ranitidine - NDMA Test 1	10A/10B				

Figure D.1 Continued

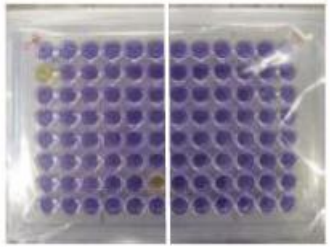
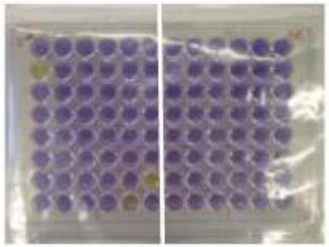
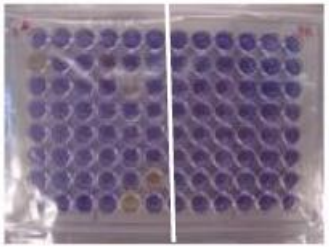
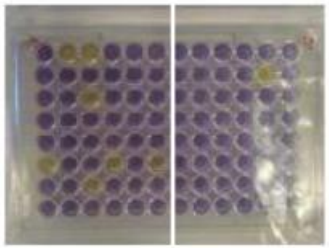
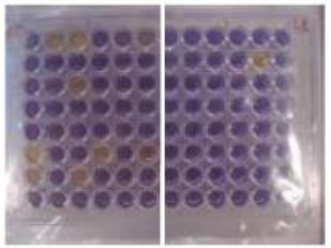
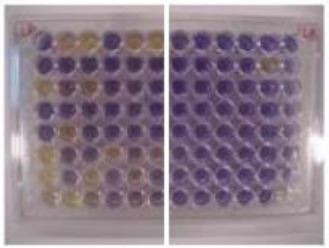
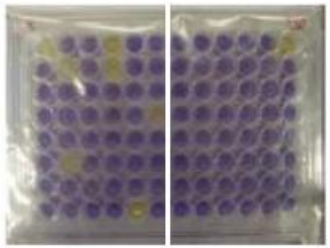
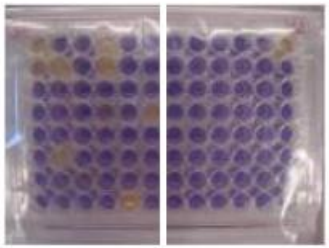
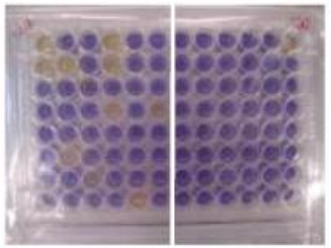
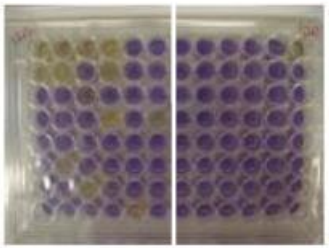
TA 100 / TA 98		3rd Day	4th Day	5th Day	6th Day
Ranitidine - NDMA Test 2	11A/11B				
Doxylamine - NDMA Test 1	12A/12B				
Doxylamine - NDMA Test 2	13A/13B				

Figure D.1 Continued

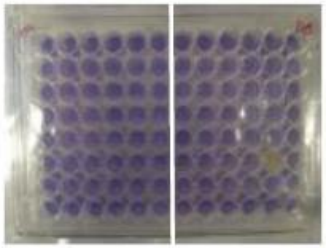
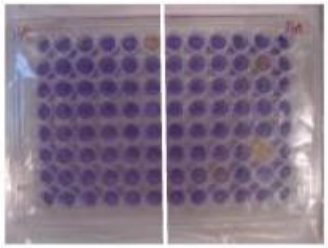
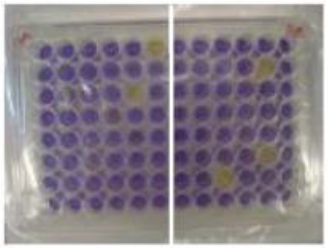
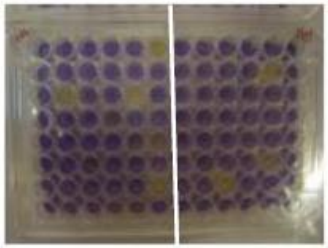
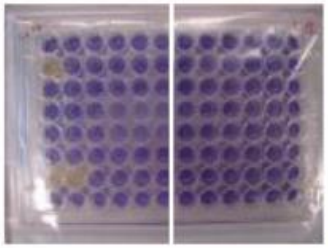
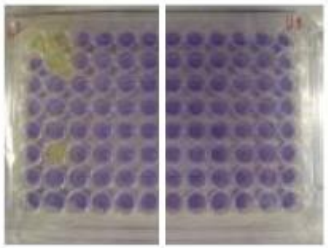
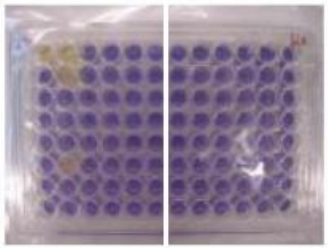
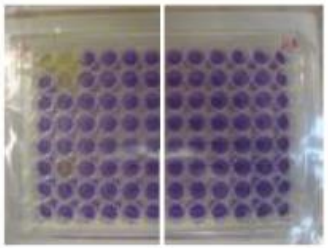
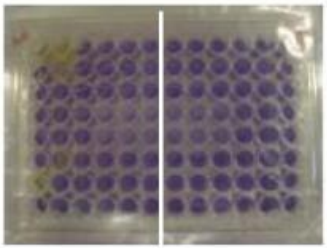
TA 100 / TA 98		3rd Day	4th Day	5th Day	6th Day
Doxylamine - NDMA Test 3	14A/14B				
Sumatriptan - NDMA Test 1	15A/15B				
Sumatriptan - NDMA Test 2	16A/16B				

Figure D.1 Continued

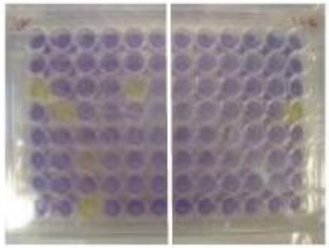
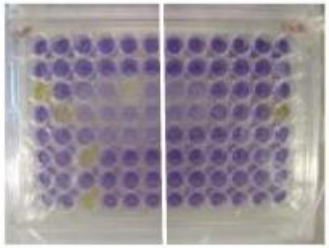
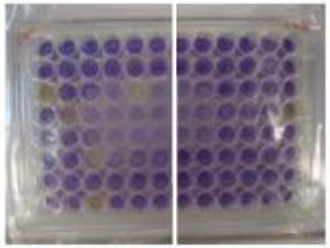
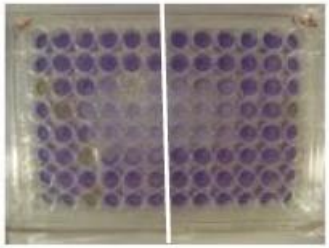
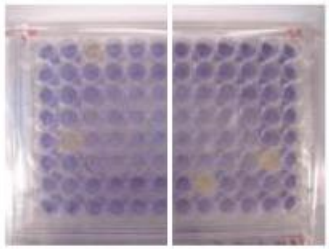
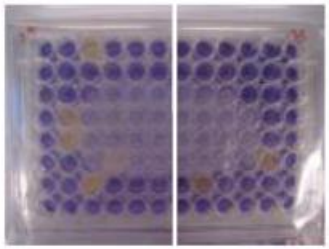
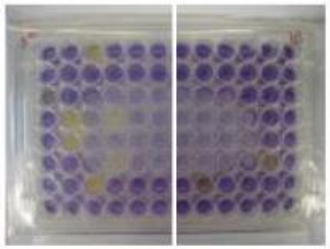
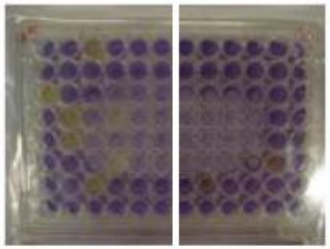
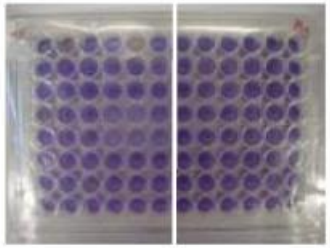
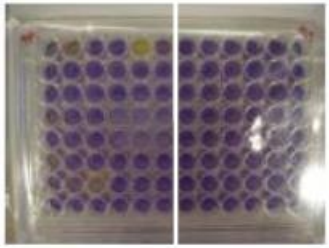
TA 100 / TA 98		3rd Day	4th Day	5th Day	6th Day
Sumatriptan - NDMA Test 3	17A/17B				
Diltiazem - NDMA Test 1	18A/18B				
Diltiazem - NDMA Test 2	19A/19B				

Figure D.1 Continued

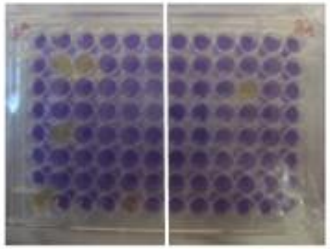
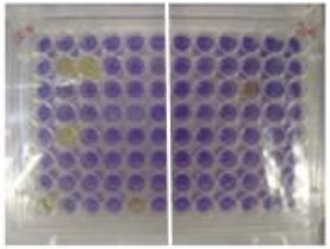
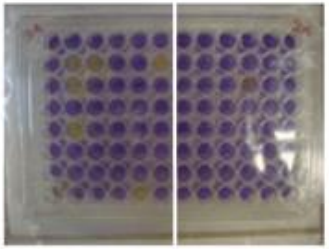
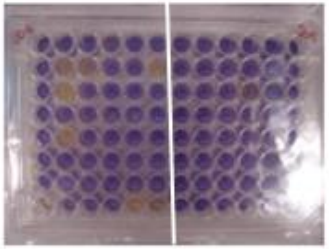
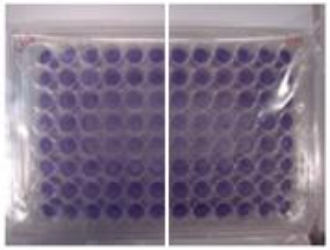
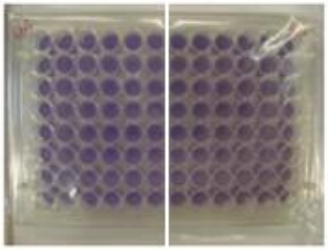
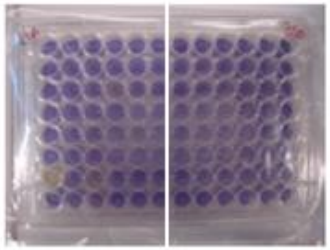
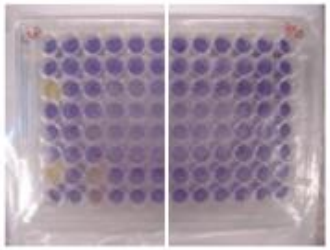
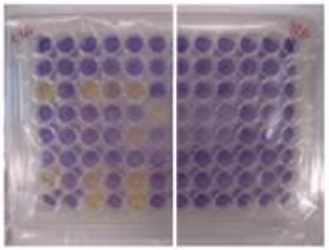
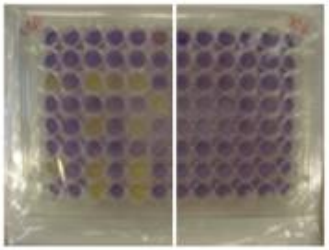
TA 100 / TA 98		3rd Day	4th Day	5th Day	6th Day
Diltiazem - NDMA Test 3	20A/20B				
Ranitidine - Control (No Chlm)	21A/21B				
Doxylamine - Control (No Chlm)	22A/22B				

Figure D.1 Continued

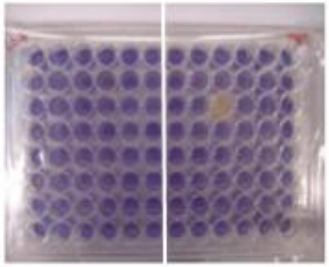
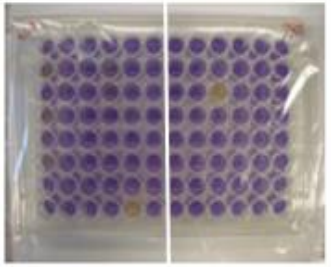
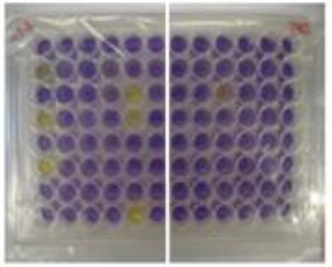
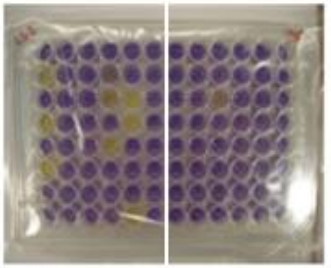
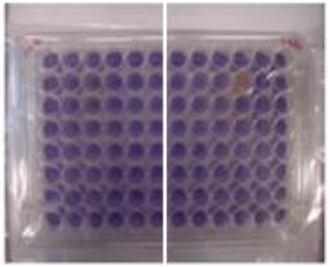
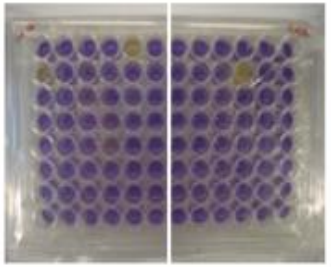
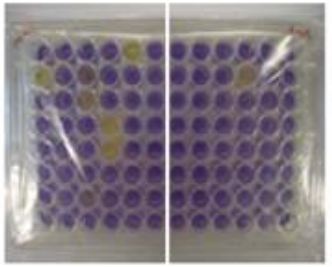
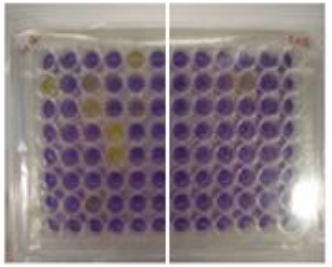
TA 100 / TA 98		3rd Day	4th Day	5th Day	6th Day
Sumatriptan - Control (No Chlm)	23A/23B				
Diltiazem - Control (No Chlm)	24A/24B				

Figure D.1 Continued