METFORMIN AND GUANYLUREA IN AQUATIC ENVIRONMENTS:

AN OVERVIEW AND IMPROVED ANALYSIS

By

© Yunwen Tao

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ABSTRACT

Metformin, a biguanide in chemical classification, is widely used as one of the most effective first-line oral drugs for type 2 diabetes. It is difficult to be metabolized by the human body and exists in both urine and faeces samples. Guanylurea is metformin's biotransformation product. Consequently, significant concentrations of metformin and guanylurea have been reported in wastewater treatment plants (WWTPs) and coastal aquatic environments.

In this thesis, a comprehensive overview is conducted to discuss the occurrence, impact, analysis and treatment of metformin and guanylurea in coastal aquatic environments of Canada, USA and Europe. The maximum concentrations of metformin and guanylurea in surface water samples were as high as 59,000 and 4,502 ng L^{-1}, respectively. Metformin can be absorbed in non-target organisms by plants and in Atlantic salmon (Salmo salar). Guanylurea has a confirmed mitotic activity in plant cells. Analysis methods of metformin are currently developed based on high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC-MS). The removal of metformin from aquatic environments in the target regions is summarized. The review helps to fill a knowledge gap and provides insights for regulatory considerations. The potential options for managing these emerging pollutants are outlined too.

To help better track the occurrence of the two non-volatile biguanide compounds in liquid samples, the improvement of existing GC-MS based methods for reliable metformin and guanylurea analysis is also conducted in this thesis. Derivatization of metformin and guanylurea is the key pre-treatment procedure before the associated GC-MS analysis. Four

selected factors affecting for the derivatization were evaluated, and the optimal factors include temperature (90ºC), reacting time (40 minutes), solvent (1,4-dioxane), and ratio (1.5:1) of reagent to target component. Buformin and N-methyl-bis(trifluoroacetamide) (MBTFA) were used as the internal standard (IS) and the derivatization reagent, respectively. Calibration curves were made based on the optimal conditions of derivatization for metformin and guanylurea with the R^2 values of calibration linearity achieved as 99.35% and 99.2%, respectively. The values of relative standard deviation (RSD%) of metformin and guanylurea based on seven repeated trails are 2.67% and 15.37%, respectively. The optimal conditions for enhancing the sensitization of metformin and guanylurea derivatization performance were obtained. The improved GC-MS analysis method was eventually applied for metformin and guanylurea analysis in real water samples. Detection of metformin and guanylurea in other types of real water samples could be conducted in the future like final effluents of wastewater treatment plants.

Keywords: anti-diabetic pharmaceutical, GC-MS, tap water, derivatization, DOE, north Atlantic

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LIST OF SYMBOLS AND ABBREVIATIONS

AOPs	advanced oxidation processes
BCF	bioconcentration factor
CCD	central composite design
CDC	Disease Control and Prevention
DDD	defined daily dosage
DOE	design of experiments
EF	electrochemistry
EPA	environmental Protection Agency
GAC	granular activated carbon
GC-MS	gas chromatography–mass spectrometry
HLB	hydrophilic-lipophilic-balance
HPLC	high-performance liquid chromatography
IDF	International Diabetes Federation
IS	Internal standard
LC	liquid chromatography
LLE	liquid–liquid extraction

LOD	limit of detection
LOQ	limit of quantitation
MBTFA	N-methyl-bis (trifluoroacetamide)
MGo	methylglyoxal
MIPs	molecularly imprinted polymers
MSTFA	N-methyl-N-(trimthylsilyl)trifluoroacetamide
MTBSTFA	N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide
OFAT	one-factor-at-a-time
PPCPs	pharmaceutical and personal care products
RSD	relative standard deviation
RSM	response surface methodology
SDS	sodium dodecyl sulfate
SPE	solid-phase extraction
TP	transformation product
UV	ultra violet
WHO	World Health Organization

CHAPTER 1 INTRODUCTION

1.1 Background

The World Health Organization (WHO) defines diabetes as a chronic and serious disease. According to the study of International Diabetes Federation (IDF) diabetes atlas (IDF, 2000, 2003, 2006, 2009, 2011, 2013, 2015, 2017), the populations of people with diabetes are increasing from 151 to 451 million with a rate at 44% (Fig. 1.1). One out of 11 people worldwide live with diabetes (IDF, 2015). Among the three main types of diabetes (i.e., type 1, type 2 and gestational diabetes), type 2 diabetes is the most common (WHO, 2016). Up to 91% of people with diabetes in high income countries have type 2 diabetes (IDF, 2017). The prevalence of people with diabetes in the coastal region of Atlantic regions including Canada, USA and Europe, is higher than that of the global average (WHO, 2016). Populations of adults (aged 20–79 years) with diabetes in the USA are the third highest in the top 10 countries in the world with large diabetic populations. In 2015, 30.3 million adults (age over 18 years old) in the USA including those in the coastal Atlantic region, lived with diabetes in 2015. It occurred in 9.4% of the population in the USA and type 2 diabetes cases accounted for 90-95% of all diabetes cases (National Center for Chronic Disease Prevention and Health Promotion, 2017). In the Atlantic region, 9.3% of the Canadian population lived with diabetes in 2015 (Diabetes Canada, 2018), and 8.8% of the European population lived with diabetes in 2017. The prevalence rates are not lower than the global diabetes prevalence rate (8.8%). It indicates that the increasing population of diabetic people will cause a rise of antidiabetic drugs usage.

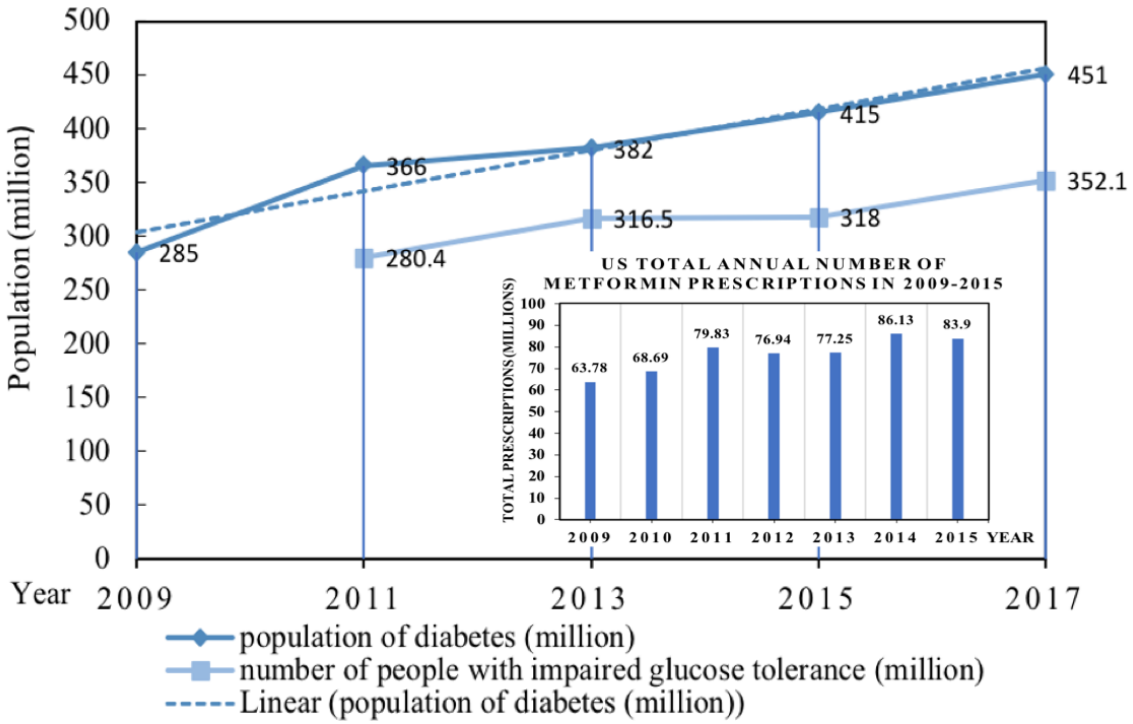


Figure 1.1 The trend of global populations of diabetes development (2009-2017) including total annual number of metformin prescriptions in the USA in 2009-2015

Source: (Statista, 2018) Based on the data from IDF diabetes atlas (IDF, 2009, 2011, 2013, 2015, 2017)

Metformin ($C_4H_{11}N_5$) belongs to the biguanide class of oral antidiabetic drugs and can improve insulin action without weight gain (Chaudhury et al., 2017). As one of the most effective medications for type 2 diabetes (Cho et al., 2018; IDF, 2015), metformin has been used to treat adult diabetes since 1957 (Bailey, 2017). The daily dose of metformin is generally 2 g according to the data from the Anatomical Therapeutic Chemical classification system (WHO Collaborating Center for Drug Statistics Methodology, 2016). It is the only antidiabetic drug that has been conclusively shown to prevent the cardiovascular complications of diabetes. As a first-line oral medication for controlling glucose, metformin is generally available in 119 countries of the world and in over 90% of countries of America and Europe (WHO, 2016). More than 200 million people worldwide are taking metformin as an antidiabetic drug (Kyzas et al., 2015). A 5-year type 2 diabetes mellitus treatment indicated that almost 90% of diabetic patients underwent metformin treatment in five studied European countries (Overbeek et al., 2017). In the USA, the total prescriptions of metformin increased from 40.89 to 83.9 million between 2004 and 2015 (Statista, 2018).

Antidiabetic drugs including metformin are newly identified emerging pollutants (Doujet and Arukwe, 2016; Meador et al., 2018) that have been widely released to the environment. Gabr et al. (2017) studied the pharmacokinetics of metformin in rats and indicated that 92-100% of metformin taken by rats was discharged out of the body after usage. It is therefore assumed that most of the metformin could be discharged into the environment in its active form, through the excrement of patients (Niemuth and Klaper, 2015). A correlation between the consumption data of metformin and its release to German surface waters has been

identified (Scheurer et al., 2009). The release of metformin to the environment has also been reported in many other countries, such as Netherlands, Portugal, Belgium, Canada and USA (Briones et al., 2016). Metformin has been identified as the top pharmaceutical being discharged into the aqueous environment (Niemuth and Klaper, 2018). Its ubiquitous release to the environment including coastal marine creatures thus has drawn the attention of environmental scientists, owing to their potential endocrine-disrupting activity (Doujet and Arukwe, 2016; Meador et al., 2018). There were studies indicating that metformin was an endocrine disruptor in juvenile fathead minnows (Crago et al., 2016; Niemuth and Klaper, 2015). As a pharmaceutical pollutant, metformin could affect the food chain through the discharge of wastewater treatment plants (WWTPs) into the aquatic systems (Eggen and Lillo, 2012).

The incomplete treatment of metformin in WWTP and its biodegradation in the environment have led to a high concentration of guanylurea, a by-product of metformin, in WWTP effluent and surface waters. Guanylurea ($C_2H_6N_4O$) is the main transformation product (TP) and is formed from metformin by a twofold dealkylation and an oxidative deamination (Richardson and Ternes, 2014). Resistant to further degradation, guanylurea is considered as the only biodegraded transformation product and metabolite of metformin (Tisler and Zwiener, 2018). A primary biodegradation of metformin to dead-end product guanylurea was detected (Markiewicz et al., 2017a, b). Guanylurea could have an anti-mitotic effect to inhibited root growth of onions (Turno et al., 1960). To assess the environmental persistency of metformin and guanylurea, Markiewicz et al. (2017a, b) recommended the implementation of monitoring programmes, and occurrence of

metformin and guanylurea in aquatic environments has been frequently reported with increased concentrations. Methods for the detection and removal of metformin and guanylurea have been developed in recent years. Liquid chromatography (LC) method was generally used to examine metformin in human plasma (da Trindade et al., 2018) or aqueous samples (USEPA, 2007). High-performance LC with ultra-efficiency, ultraviolet absorption spectroscopy and diffuse infrared spectroscopy has also been developed to detect metformin in samples (da Trindade et al., 2018). Gas chromatography-mass spectrometry (GC-MS) method is another analytical option for metformin detection (Goedecke et al., 2017; Majidano and Khuhawar, 2012; Uçaktürk, 2013). Goedecke et al. (2017) indicated that the performance (expressed by limit of detection (LOD), limit of quantitation (LOQ) and linear correlation coefficient) of GC-MS was better than that of LC-MS/MS. Advanced oxidation processes (AOPs), activated carbon filtration, phytoremediation and molecularly imprinted polymers, were regarded as popular methods for metformin removal from aqueous samples. Among them, the AOPs are the commonly used technique to remove pharmaceutical pollutants.

1.2 Statements of Problems

(1) Lack of a comprehensive overview on occurrence, impact, analysis and treatment of metformin and guanylurea in coastal aquatic environments in the Atlantic region

The occurrence and distribution of metformin and guanylurea, their treatment and analysis, have been progressively reviewed in wastewater, surface water and drinking water in recent years (Blair et al., 2013a, b; Bradley et al., 2016, 2017; Briones et al., 2016; de Solla et al.,

2016; Ghoshdastidar et al., 2015; Kim et al., 2014; Lacorte et al., 2018; Meador et al., 2016; Moermond and Smit, 2016; Petrie et al., 2016; Scheurer et al., 2009; Tisler and Zwiener, 2018). Nevertheless, few of them focused on the Atlantic region (e.g., Bradley et al., 2017; Briones et al., 2016; Ghoshdastidar et al., 2015; Kim et al., 2014; Lacorte et al., 2018; Moermond and Smit, 2016; Petrie et al., 2016; Scheurer et al., 2009; Tisler and Zwiener, 2018), which has an above average usage and an ever-increasing population of diabetes (WHO, 2016). A comprehensive literature review is thus desired to identify research gaps and support future works.

(2) Lack of effective GC-MS based methods for metformin and guanylurea detection

Methods for the detection of metformin and guanylurea have been developed in recent years based on chromatography (Brack et al., 2015; Goedecke et al., 2017; Majidano and Khuhawar, 2012; Trindade et al., 2018; Uçaktürk, 2013; USEPA, 2007). Goedecke et al. (2017) reported that the GC-MS method could detect low concentrations of metformin in water samples. Derivatization of metformin and guanylurea is the key pre-treatment procedure before the analysis of metformin and guanylurea by GC-MS. Since metformin and guanylurea are non-volatile biguanides, the direct volatilization during GC-MS analysis could degrade these non-volatile biguanides if without proper derivatization treatment. However, the derivatization performance still needs to be enhanced to achieve sensitive and accurate detection of low concentrations of metformin and guanylurea in water samples by GC-MS.

Various studies have implemented the derivatization of metformin using one-factor-at-a-time (OFAT) (Goedecke et al., 2017; Majidano and Khuhawar, 2012; Uçaktürk, 2013), but

extremely limited studies focused on system optimization by using design of experiments (DOE) methodologies. DOE can help to evaluate the configurations of basic experimental design and material alternatives, to select parameters of design for working well with different field conditions, to obtain the key parameters influencing performance of design, and to optimize the ideal results of models. Thus, designing experiments is helpful to effectively achieve computer simulations or laboratory experiments (Lye, 2002). There are many different types of experimental statistical design techniques (Lye, 2002) and the response surface methodology (RSM), has been identified as one of the most useful design for optimizing the ideal conditions of a process. RSM containing mathematical and statistic techniques is used to model and analyze a response of interest affected by several variables and to optimize the response. Based on RSM, the optimization function could be obtained including the maximum, minimum, or target to a set of results. Curvature response surface can be determined under the RSM. Central composite design (CCD) is a popular RSM design to fit the second order model. CCD is generally constructed with a fractional factorial part of Resolution V following star or axial points with couple center points (Lye, 2018). Therefore, derivatization of metformin and guanylurea can be potentially optimized via CCD.

1.3 Research Objectives

Based on the research gaps identified in previous sections, this thesis tackles a comprehensive overview of metformin and guanylurea in Atlantic coastal aquatic

environments and generation of an improved GC-MS based method for metformin and guanylurea detection in water samples. It entails the following tasks:

1) The occurrence of metformin and guanylurea in the aquatic environments of coastal Atlantic regions including Canada, USA and Europe is investigated. Their physiochemical properties and environmental impacts on aqueous environments are evaluated. The analytical methods for tracing these two targeted emerging pollutants and the treatment methods for removing them from the aqueous environments are also summarized.

2) An improved GC-MS based method is generated and optimized for simultaneous analysis of both metformin and guanylurea in water samples. Parameters include the cartridge condition, volume of extraction solvents, and the extraction flow rate are determined to achieve an acceptable extraction of metformin and guanylurea from water samples. CCD is employed to develop a model for evaluating factors influencing the derivatization of metformin and guanylurea. The four factors included within the CCD modelling are temperature (70-90ºC), reacting time (40-70 minutes), solvent (acetonitrile, 1,4-dioxane), and ratio (0.5-1.5:1) of reagent to target component (Goedecke et al., 2017; Majidano and Khuhawar, 2012; Uçaktürk, 2013). Through evaluating the impact of each factor, the optimal conditions for enhancing the sensitization of metformin and guanylurea derivatization performance are obtained. The performance of the developed method including LOD and LOQ was evaluated, followed by detection of metformin and guanylurea in a real water sample.

1.4 Structure of the Thesis

Chapter 1 introduced the background of this research on metformin and guanylurea and proposed the statement of problems. Also, the objectives of this study were clarified. Chapter 2 focused on the occurrence and distribution of metformin and guanylurea, their treatment and analysis in the Atlantic region which few of studies focused on, while this region has an above average usage and an ever-increasing population of diabetes. Chapter 3 focused on development of improved GC-MS analytical method for metformin and guanylurea detection in water samples. Chapter 4 concluded this thesis with summarized findings, contribution and recommendations for future research.

CHAPTER 2 OCCURRENCE, IMPACT, ANALYSIS AND TREATMENT OF METFORMIN AND GUANYLUREA IN COASTAL AQUATIC ENVIRONMENTS OF CANADA, USA AND EUROPE[1]

[1] *This chapter has been used to generate a journal publication:*

Tao, Y. et al. 2018. Occurrence, Impact, Analysis and Treatment of Metformin and Guanylurea in Coastal Aquatic Environments of Canada, USA and Europe. Advances in Marine Biology, 81, 23-58

Role: Yunwen Tao solely worked on this study and acted as the first author of this manuscript under the guidance of Dr. Baiyu Zhang and Dr. Bing Chen.

2.1 Occurrence

The chemical structures of metformin and guanylurea are presented in Fig. 2.1. The molecular weight of metformin and guanylurea is 165.63 and 102.10 g mol^{-1}, respectively (Medicine, 2005). Metformin has a low octanol-water partition coefficient (K_{ow}) which is -1.3 or -1.43 (Bailey, 2017; Meador et al., 2016). Metformin hydrochloride is freely soluble in water and slightly soluble in 95% ethanol but insoluble in acetone, chloroform, dichloromethane and ether (Sharma et al., 2010). The authors identified that metformin hydrochloride can be decomposed up to 10% within 208 h at 30°C, and metformin follows zero order kinetics. The occurrence of metformin and guanylurea has been frequently reported with increased concentrations in the Atlantic regions, namely eastern coast of Canada and USA, and western coast of European countries including Belgium, Germany, Denmark, Spain, France, Ireland, Portugal, Netherlands and UK (Mieszkowska et al., 2009) (Fig. 2.2). These are reviewed in detail.

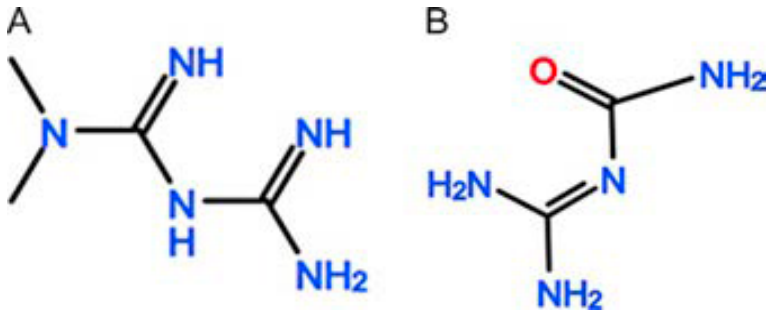


Figure 2.1 Chemical structures of (A) metformin and (B) guanylurea.

Figure 2.2 Atlantic coastal regions in Canada, USA and Europe.

2.1.1 Canada

The Canadian population was 36.29 million in 2016 and 11 million Canadians were diabetic or at the risk of diabetes (CDA, 2016a). The population with diabetes was 3.5 million in October of 2016. Approximately 90% of Canadians living with diabetes are type 2 diabetes (CDA, 2017). There were 116,000 diabetics in Newfoundland and Labrador in 2016 (CDA, 2016b). In 2017, Newfoundland and Labrador had 528,817 inhabitants (Newfoundland and Labrador Statistic Agency, 2017). Thus, 19.74% of Newfoundlanders probably were living with type 2 diabetes and the predicted number of type 2 diabetic cases was 104,400. Based on the metformin consumption in USA in 2012 (Markiewicz et al., 2017a, b), the predicted metformin consumption in the province of Newfoundland in Canada was 131.91 kg day^{-1}. The population number of St. John's, Mount Pearl and Paradise (the central communities in the province) was 153,206 in 2017 (Wikipedia, 2017). The estimated wastewater production was approximately 120 million liters per day from these communities and was discharged into the WWTP (City of St. John's, 2017; Jing et al., 2017). Hence, the predicted concentration of metformin might be approximately 286.87 μg L^{-1} per day in influents of the WWTP. In addition, Newfoundland and Labrador had the highest prevalence of overweight and obesity in adults of all the provinces (CDA, 2016b). Thus, metformin may have a more serious impact in the North Atlantic region of Canada than other regions of Canada.

The Environmental Protection Agency (EPA) *Method 1694* (USEPA, 2007) was used to analyze 99 pharmaceutical and personal care products (PPCPs), among which 49 PPCPs were detected in the influent, final effluent and biosolid samples of a Canadian full-scale

WWTP (Kim et al., 2014). With reporting limit concentrations of metformin in influents, final effluents and biosolids at 12, 3 and 6 ng L^{-1}, respectively, the concentration of metformin ranged from 88,800 to 95,300 ng L^{-1} in the influents, from 842 to 968 ng L^{-1} in the effluents and from 2630 to 2960 ng L^{-1} in the biosolids. Metformin was one of the pharmaceuticals having high concentrations in all the influent, final effluent and biosolid samples.

There were 11 pharmaceuticals and 2 metabolites in the Canadian top prescribed drugs list examined in samples that were collected from outflows between 100 and 300 meters away of the final effluents of 16 WWTPs in Southwest Nova Scotia, Canada (Ghoshdastidar et al., 2015). They found that the concentrations of metformin in the effluent outflows were from 0 to 10,600 ng L^{-1}. Metformin had high concentrations in effluent or downstream samples collected from 9 of the 16 WWTPs. Metformin was one of the pollutants which was highly frequently detected in effluent and downstream samples in this study. The fact that metformin was the only pharmaceutical that was found to be ubiquitous in the effluents among the list indicated that the efficiency of the secondary treatment for breaking down metformin was low (Ghoshdastidar et al., 2015).

In the past couple of years, metformin has been frequently detected in Canadian sewage systems. This situation of metformin increasing in WWTPs conforms to the rise of diabetic populations in the past decade. However, only limited surveys focused on the bioaccumulation of metformin in Canadian halobios and freshwater organisms. In addition, there has been little investigation of the occurrence of metformin in the estuarine and coastal environments in Canada, especially in the eastern coast of Canada where high

percentages of diabetic populations have been reported. Furthermore, there has been no study on the detection of guanylurea occurrence in influents and effluents of WWTPs and aquatic environments in Canada.

2.1.2 USA

In 2011, 63.3% of adult diabetes were taking pills in USA (CDC, 2013). The population percentage of diabetes on the USA eastern coasts of the Atlantic region ranged from 8.3% to 16.5% in 2015, at an average of 9.56% (CDC, 2015). Around 12,913,313 t of metformin were used to treat type 2 diabetes in the USA in 2012, which represented 99.84% consumption of oral type 2 diabetes drugs in the country (Markiewicz et al., 2017a, b). The U.S. population in the North Atlantic coastal region is 58 million residents (NOAA, 2017), resulting in the associated the predicted metformin consumption of approximate 7 t day^{-1} in the region.

A study by the U.S. Geological Survey evaluated the concentrations of pharmaceutical pollutants in 59 wadeable headwater streams in the highly urbanized Piedmont ecoregion of the Southeastern USA during 2014 (Bradley et al., 2016). This ecoregion is not interfered by sources of pharmaceutical pollutants from WWTPs. In this survey, 108 pharmaceuticals were examined in 5 aqueous samples collected once in every 2 weeks from these 59 wadeable headwater streams. The concentrations of metformin were from about 1.4 to almost 2000 ng L^{-1}. The frequency of metformin detection was 89% of samples and 97% of sites. Environmental health in wadeable streams should thus cause concern based on the results of pharmaceutical pollutants in this study (Bradley et al., 2016). The authors also

stated that the impact of antidiabetic pharmaceutical pollutants highlighted the connection between human health and aquatic health.

From 2013 to 2015, Bradley et al. (2017) collected 72 water and sediment samples for analyzing targeted organic contaminants from 16 sites in Congaree National Park in South Carolina, USA. These sites were contaminated with multiple urban and agricultural pollutants. In water samples, there were 110 targeted pharmaceutical analytes examined and 49 of 110 targeted pharmaceutical analytes were frequently detected in most samples (Bradley et al., 2017). Metformin was discovered in 62% of these samples and was the only pharmaceutical that was observed to be widespread at every site in this national park. The detected concentrations of metformin were from about 2.1 to 220 ng L^{-1}. Therefore, the frequency of detecting metformin was very high in all the samples. Metformin was thus considered to be a frequently and widespread detected endocrine targeted pharmaceutical (Bradley et al., 2017). As an endocrine-active anthropogenic contaminant, the biodegradation of metformin was recognized by the model ^{14}C substrates, and its biodegradation potential was observed in the sediments of this national park. Metformin showed efficient mineralization under aerobic and anaerobic conditions in all the minimization treatments on the sediment samples of Congaree National Park.

Acquiring increasing attention, metformin has thus become a frequently recurring compound in aqueous sample analysis across USA as shown in the surveys and studies reviewed. Metformin in aquatic environments is increasingly being regarded as a serious problem in the USA. Metformin has already been included in the list of targeted pharmaceutical pollutants in pollutant analysis (Bradley et al., 2017). The bioaccumulation

of metformin in halobios and freshwater organisms was detected by Bradley et al. (2017). A greater attention to metformin removal is needed for protecting these coastal environments. In addition, there is also limited study on detecting guanylurea in influents and effluents of WWTPs or aquatic environments.

2.1.3 Europe

Single person sales rates of metformin are from 5.9 to 12.1 g day^{-1} in Europe (Oosterhuis et al., 2013). In Denmark, metformin consumption was 40,832,000 defined daily dosage (DDD) in 2016 (Statens Serum Institut, 2016). Depending on the current situation of metformin as a first-line antidiabetic drug, it has received a lot of attention from European environmental scientists. Metformin monotherapy is the most commonly used medicine in the first and third stages of treatment in European countries (Overbeek et al., 2017). As the first country that detected metformin in WWTPs, German researchers contributed a lot of studies regarding metformin and guanylurea (Scheurer et al., 2009). In 2011, a study by Trautwein and Kummerer was published illustrating that metformin can be transferred to a dead-end transformation product, guanylurea, through an aerobic biodegradation process in sewage. They tested the aquatic samples from sewage treatment plants on metformin and guanylurea. The results showed that the guanylurea concentration in the effluent (1.86 μg L^{-1}) was over four times its concentration in the influent (0.40 μg L^{-1}). The metformin concentration in the influent (56.8 μg L^{-1}) was almost 75 times its concentration in the effluent. The presence of unmetabolized metformin in the environment is highly undesirable because some of its transformation products retain their pharmacological activity. The results of assessing the aerobic biodegradability of metformin indicated that

approximate 50% of metformin could be degraded in high bacterial density and activated sludge samples under aerobic condition. However, guanylurea was detected in these test samples as a prevalent and stable aerobic bacterial degradation product. The detection of metformin and guanylurea concentrations in the influent and effluent of the WWTP detected by Scheurer et al. (2009) indicated that guanylurea is a stable dead-end transformation product of metformin (Trautwein and Kummerer, 2011). Biodegradation is considered as one of the main routes to eliminate metformin from wastewater or surface water.

The concentrations of metformin were from 101 to 129 ng L^{-1} in influents and from 2.2 to 21 ng L^{-1} in effluents (Scheurer et al., 2009) in samples collected from a WWTP and several major rivers in Germany. Moreover, the metformin concentrations in surface water samples were from 130 to 1,700 ng L^{-1} (Scheurer et al., 2009). In 2014, samples were collected from influent and effluent of a WWTP, a German rivers Elbe and Weser, German Bight and North Sea, and Lake Constance and river Rhine (Trautwein et al., 2014). The mean concentrations of metformin and guanylurea in the influent of the WWTP were 111,800 and 1300 ng L^{-1} while the mean concentrations of metformin and guanylurea in the effluent of the WWTP were 4,800 and 44,000 ng L^{-1} (Trautwein et al., 2014). In addition, metformin and guanylurea were both detected in surface and seawater samples in this 2014 study. The mean concentrations of metformin and guanylurea were from102 to 472 ng L^{-1} and from 9 to 137 ng L^{-1} in surface water samples, respectively. In seawater samples, the mean concentrations of metformin and guanylurea were 13 and 11 ng L^{-1}, respectively. Recently, Tisler and Zwiener (2018) collected water samples from influents and effluents of two

WWTPs and three rivers in Germany. The concentrations of metformin and guanylurea were from 14,000 to 95,000 ng L^{-1} and from 0.031 to 4.678 μmol L^{-1} in the influents of the WWTPs. Moreover, the concentrations of metformin and guanylurea were from 700 to 6500 ng L^{-1} and from 0.179 to 2.401 μmol L^{-1} in the effluents of the WWTPs. Metformin was detected at the concentrations between 67 and 87 ng L^{-1} in the surface samples collected at the upstream of the WWTPs and at the concentrations between 103 and 234 ng L^{-1} in the downstream surface samples of the WWTPs. The metformin concentrations raised up to 470 ng L^{-1} in the downstream surface samples collected 15 km away from outflows of WWTPs. Guanylurea was detected at concentrations up to 4502 ng L^{-1} of all surface samples. Some recent investigations also showed that metformin was found in surface water and groundwater at concentrations over 100 ng L^{-1} in France (Lopez et al., 2015).

According to the European Water Framework Directive Policy (European Commission, 2000), a Dutch surface water study was instituted on the surface water standards which could be used for the future water quality standard policy (Moermond and Smit, 2016). This study, based on the methodology regarding to the European Water Framework Directive guidance document, evaluated the data of emerging pollutants collected in 2012 to obtain the maximum acceptable concentrations of emerging pollutants and annual average concentrations of emerging pollutants for establishing the future water quality standard policy. Three pharmaceutical pollutants including metformin were selected for research. The analysis of metformin indicated the environment quality standard of metformin on acute effects was 780 μg L^{-1} considered as a maximum acceptable

concentration of metformin for Daphnia magna, a species of Crustacea. However, there is a lack of the data on saltwater organisms. This concentration of metformin was not taken by the Dutch government as an environment quality standard because the global monitoring data of metformin were lower than this concentration (Moermond and Smit, 2016).

The global population aged over 60 has doubled since 1980. The senior population is 35% in Europe and 28% in North America (United Nations, 2017). A prediction of pharmaceutical pollution in river water related to senior residences was made by Lacorte et al. (2018). The predicted environmental concentrations of pharmaceuticals were estimated based on the hypothesis that senior residences as a source of pharmaceutical pollutants. This study based on the situation of effluents from WWTPs serving five health institutions in Portugal, Spain and France. The pharmaceutical consumptions of four institutions were over 1000 mg day^{-1} and the consumption of another was over 400 mg day^{-1}. The surveyed results revealed that metformin was the only common medicine used to treatment type 2 diabetes in these five institutions and its consumption totaled from 4,355 to 17,466 mg day^{-1}. The predicted concentrations of metformin in sewerage systems were from 1,388 to 3,675 ng L^{-1} and the predicted concentrations in river waters were from 17 to 139 ng L^{-1}. Comparing the predicted concentrations with other selected target pharmaceuticals, metformin occurred in higher levels of concentrations in river waters. The European Medicine Agency threshold for risk assessment decided that 10 ng L^{-1} should be the maximum allowable concentration of pharmaceutical pollutants in environments (European Medicines Agency, 2006). The predicted metformin concentrations should be considerably over 10 ng L^{-1}, indicating it was a serious contaminant.

Petrie et al. (2016) detected 90 emerging contaminants including metformin in wastewater, final effluent, digested sludge and river water samples in Southwest England. The minimum detection limit and minimum quantification limit of metformin were from 156 to 1,509 ng L^{-1} because metformin was not extracted by SPE while directly injected for analysis. The maximum metformin concentrations in the influents, final effluents and river water were 45,104, 20,041 and 2,381 ng L^{-1}, respectively, which were high concentrations among these target emerging contaminants. The removal rate for metformin was 55%. However, the metformin concentration in digested sludge was not available.

Lindim et al. (2016) estimated pharmaceutical contamination in Swedish river basins based on the consumption data of pharmaceuticals, human metabolic rates and removal rates in WWTPs. The metformin consumption in the basins was 113,804 kg $year^{-1}$ in 2011 which was the second highest consumed drug, and the human excretion rate unchanged at 41%. The WWTPs discharge was 334×106 m^3 of effluent to the Baltic Sea in 2014. The removal rate of metformin was a maximum 93.8% in WWTPs in 2011, while the high emissions of metformin in water and soil were 2,895.9 and 43,812.6 kg $year^{-1}$ in 2011. Moreover, metformin showed high measured concentrations in 2006-2014. Based on these data, the predicted metformin concentrations in the major rivers of Swedish were in a range of 0.4387-8.4072 ng L^{-1}. In addition, the emissions of metformin in soil were predicted at 43,812.6 kg $year^{-1}$. Therefore, metformin was identified as a high emission drug to surface water and soil.

Lindim et al. (2017) predicted pharmaceutical contaminations in Swedish surface water based on the STREAM-EU model. This model could be used to simulate the transport and

fate of any organic contaminant in any European river basin. The amount of metformin flushed into the Baltic Sea was 27 t $year^{-1}$ predicted by STREAM-EU model, with a higher than 50% rate emitting into the Baltic Sea and Danish Strait. The predicted metformin concentration in surface water was over 1,000 ng L^{-1} with a half-life over 1,500 days in aqueous matrix. The prediction inferred that metformin had the highest annual flow rate to the sea of 54 selected studied drugs.

There were 33 pharmaceuticals and 7 additives of personal care products detected in samples collected from the sub-arctic area of the Faroe Island, Iceland and Greenland (Huber et al., 2016). As one of the most frequently detected pharmaceuticals, metformin was reported at a concentration up to 59,000 ng L^{-1}, detected from hospital influent. Furthermore, metformin was firstly analyzed in fjord water, sediment and sludge samples collected from Nordic countries. The risk assessment of these targeted pharmaceuticals did not indicate acute or chronical risks to aquatic organisms in the effluent of water pipe outlets.

The European Water Framework Directive Policy (European Commission, 2000) and Commission Directive 2014/80/EU (European Commission, 2014) helped to declare a watching list of substances including pharmaceutical pollutants. However, there are no relevant policies concerning emerging pharmaceutical pollutants in Canada and USA. Studies of metformin and guanylurea occurrence in coastal aquatic environments indicated that the concentrations of the two chemicals have been increasing in the recent years. Metformin has been commonly observed in European aqueous and aquatic environments. Guanylurea has not been considered a target of research except in Germany. These

concentrations of metformin and guanylurea in the effluents of WWTPs and aquatic samples, which were represented in the reviewed studies, indicated generally ineffective removal rates of the WWTPs. Due to the increasing global populations of diabetes (Fig. 1.1), the consumption of metformin will keep rising in the future. Future studies might expect rising concentrations of metformin and guanylurea in aquatic environments. Precautionary environmentalism would advise more rigorous study of coastal and ocean metformin and guanylurea.

2.2 Impacts

There have been studies indicating that metformin is an endocrine disruptor to juvenile fathead minnows (Crago et al., 2016; Niemuth and Klaper, 2015). The characteristics and occurrence of metformin and guanylurea indicate that the two chemicals have already contaminated natural environments and spread to animals and plants. The adverse effects of metformin on coastal marine creatures are of mounting concerns to environmental scientists. There is some research focusing on impacts of metformin and guanylurea on halobios, freshwater organisms including plants living in the contaminated areas.

Eggen and Lillo (2012) noticed that pharmaceutical pollutants could affect land food chain through the WWTPs effluents discharged into aquatic systems. Metformin was selected as the target contaminant to test the uptake, translocation and bioconcentration factor (BCF) of metformin in edible plant species. BCF represents the contaminant concentration in the plant over initial concentration in soil (all in dry weight). Oily seed rape (*Brassica napus cv*. Sheik and *Brassica rapa cv*. Valo) showed a high absorption for metformin with the

BCF value of 21.72. Moreover, metformin was also transferred into the cereals wheat, barley, oat, tomato, squash and bean, but the average BCF in these vegetables was lower than the BCF in oily seed rape. In addition, guanylurea was detected in barley grains, bean pods, potato peel and small potatoes. Although organic cation transporters (OCTs) in mammals are known to actively transport of polar chemicals, the mechanisms for the transport of metformin and guanylurea in plants are still unknown (Eggen and Lillo, 2012).

Given the fact that cattail (*Typha latifolia*) and reed (*Phragmites australis*) is frequently utilized in phytoremediation, the roots of these plants were selected to test the uptake mechanism of metformin (Cui et al., 2015). Quinidine was used as an inhibitor to assess the role of OCTs in the uptake of metformin by cattail. The results revealed that the uptake processes of metformin were independent of initial concentrations of intake metformin in both cattail and reed roots. Moreover, metformin uptake could be significantly affected by quinidine in cattail roots with inhibition ratios of 70-74%. This study indicated that metformin could be taken up by plant roots and had the potential for subsequent translocation. Moreover, OCTs conduit could be the important pathway of metformin uptake into plants (Cui et al., 2015).

Metformin can cause endocrine disruption at an environmentally relevant concentration (40 μg L^{-1}) (Niemuth and Klaper, 2015). This study exposed fathead minnows (Pimephales promelas) to a concentration of metformin at 40 μg L^{-1} relevant to the concentration evaluated by Blair et al. (2013a, b) in wastewater effluent. The exposure period was over 360 days with a light cycle of 16:8 h light/dark photoperiod. This experiment indicated that metformin caused the development of intersex gonads in males, reduced size of treated

male fish and reduction in fecundity for treated pairs by observing gonad histology. The results demonstrated that metformin acted as an endocrine disruptor at environmentally relevant concentrations.

The mRNA of vitellogenin (VTG), oestrogen receptor-alpha (ERα), gonadotropin releasing hormone (GnRH3) and cytochrome P450 3A4-like isoform (CYP3A126) were examined to comprehend the effects of metformin on adult and juvenile FHMs. Cytochrome P450-dependent monooxygenases (CYPs) can defend xenobiotics in metabolism (Christen et al., 2010). About 50% all pharmaceuticals can be metabolized by human CYP3A enzymes in daily use. Moreover, a family gene of CYP3A from FHMs, which is designated as CYP3A126 by P450 nomenclature committee, was cloned and studied by Christen et al. (2010). Authors stated that a CYP3A isoform was identified as CYP3A126 in FHM cells liver tissue. Moreover, it was confirmed that a CYP3A enzyme can be activated in FHM cells by altering pharmaceuticals (Christen et al., 2010). Thus, there was another study conducted about whether adult and juvenile FHMs would be affected by various levels of environmentally relevant concentrations of metformin at different stages of its biosynthesis, clearance and activation (Crago et al., 2016). The male and female adult and juvenile FHMs were separated into different beakers and exposed to different levels of metformin concentrations, such as 0 μg L^{-1} (purified water), 1 μg L^{-1} (low metformin), 10 μg L^{-1} (medium metformin) and 100 μg L^{-1} (high metformin). The temperature was stable at 25±1 °C. The light cycle of 16:8h light/dark photoperiod was 7 days because metformin would probably degrade 10% over a period of more than 8 days. Hence, the evaluation of activity of CYPs in FHMs exposing under metformin is significant. This research

substantiated that oestrogen-associated VTG, ERα and expression of GnRH3 were increased by metformin in juvenile FHMs compared with adult male FHMs. Moreover, the same alteration in mRNA expression of VTG, ERα, GnRH3 and CYP3A126 occurred at concentrations of metformin from 1 to 10 μg L^{-1} (in waterways). This study demonstrated that metformin could change endocrine function in juvenile FHMs with the concentrations measured in the aquatic environment (1-10 μg L^{-1}). In addition, the concentration of metformin and exposure period on this study was much lower than the one reported by Niemuth and Klaper (2015). This lower concentration of metformin might be a reason that the adult male FHMs did not show significant changes on hepatic VTG, ERα and CYP3A126.

Metformin can be absorbed by juvenile Atlantic salmon (*Salmo salar*). Five juvenile Atlantic salmon were exposed to three different concentrations of metformin at 5, 50, 500 μg L^{-1} through 3, 7 and 10 days in lab tanks (Doujet and Arukwe, 2016). This study determined the concentration of metformin in gills of the fish, which were relatively low. The gene expression of StAR and P450scc was increased by absorbing metformin but there was no change in the protein expression from the StAR and P450scc gene. Exposure time could affect two biotransformation enzymes (e.g., cyp1a1 and cyp3a) inhibited or increased. However, there was no observed toxicity by accumulation of exogenous and endogenous compounds related to increased gene expression of StAR and P450scc, no effects on the OCTs and no oxidative stress induced upon metformin treatment. Overall, the authors believed that metformin could cause some biological response in juvenile Atlantic salmon but no potential biological effects and neurotoxicity on juvenile Atlantic salmon.

Meador et al. (2018) researched effects of emerging contaminants on juvenile Chinook salmon (*Oncorhynchus tshawytscha*) and staghorn sculpin (*Leptocottus armatus*) collected from two estuaries in Washington State. These feral fishes were treated with 16 common emerging contaminants including metformin assess the potential impacts. The fish in laboratory were raised at 12°C. After 32 days, liver, plasma and whole-body were collected to analyze the effects of emerging contaminants on these fishes. The concentration of metformin was up to 2.9 ng g^{-1} in the tested fish bodies and 27.8 ng g^{-1} in the wild staghorn sculpin bodies. The authors believed that metformin could inhibit growth and alter metabolic pathways.

MacLaren et al. (2018) exposed the Siamese fighting fish (*Betta splendens*) into the concentrations of metformin at 40 and 80 μg L^{-1} for 4 and 20 weeks. The authors stated that the aggression of the Siamese fighting fish was reduced by chronic waterborne exposure to metformin. Aggression as a significant character of male Siamese fighting fish has impacts on offspring surviving to adulthood. If the aggression level of male Siamese fighting fish was reduced, survival of male Siamese fighting fish would be critically affected.

There is limited research on the impact of guanylurea on organisms. Turno et al. (1960) believed that guanylurea could be formed in soil during the transformation of $CaCN_2$, and $CaCN_2$ had an anti-mitotic effect on root growth of onions beginning at a concentration of 0.00625% with sulphate solution and at 0.0125% or higher with phosphate. For wheat, maize, barley and vetch, growth inhibition began at 0.025%, 0.00125%, 0.00625% and 0.00156%, respectively, with sulphate and at 0.05%, 0.025%, 0.0125% and 0.0031%,

respectively, with phosphate. At concentrations below the anti-mitotic level guanylurea was a good nitrogen source (Turno et al., 1960).

2.3 Analytical Methods

Analytical methods of metformin are currently developed based on the usage of high-performance liquid chromatography (HPLC) and GC-MS after sample pre-treatment of metformin contaminated aqueous samples through extraction. The US EPA recommended the standard method *1694* (USEPA, 2007) for analyzing pharmaceuticals and personal care products in water, soil, sediment and biosolids by HPLC-MS/MS using isotope dilution and internal standard (IS) quantification techniques. Although the method *1694* was developed for the Clean Water Act programme as a standard method, there are continuous efforts to develop more effective LC-MS methods. Moreover, the GC-MS method was developed for metformin analysis from human plasma to aqueous samples (Goedecke et al., 2017; Majidano and Khuhawar, 2012; Uçaktürk, 2013).

2.3.1 Sample Pre-Treatment

Liquid-liquid extraction (LLE) is a traditional extraction method based on the varied solubility of chemicals in two different liquid solvents (Peake et al., 2016). However, this method is less commonly used for environmental analysis due to its large solvent usage and low target recoveries (Peake et al., 2016). Matin et al. (1975) examined 1,2-dichloromethane to extract these three biguanide compounds (phenformin, buformin and metformin) from the solvent after derivatization reaction but the performance of the

extraction of phenformin was the best of all. Chloroform was selected to extract the metformin after derivatization reaction by Majidano and Khuhawar (2012).

Solid-phase extraction (SPE) was developed to effectively extract many contaminants together in these studies, as conducted by Martin et al. (2012) and Goedecke et al. (2017). This development aimed to conveniently determine various contaminants by a single extraction. The SPE method is generally used to preconcentrate metformin from aqueous samples because the high polarity of metformin can cause affinity of sorbent of the SPE cartridge. The SPE method has been used to extract the spiked metformin and the IS buformin from surface water samples with an autotrace SPE workstation (Goedecke et al., 2017; Martin et al., 2012). The pH value of the samples would be adjusted to about 7 before the solution passes through the SPE cartridge. The cartridge (Strata-X-CW) performed a weak cation exchanger as a stationary phrase. The sorbent type of this cartridge is polymeric based. SPE has been used as a pre-treatment for determining antidiabetic drugs in environmental samples, followed by high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (Martin et al., 2012). Several SPE materials, such as Chromabond Tetracycline, Oasis HLB, Supelclean C18, Strata-X and Strata-XCW cartridges, were selected to extract these targets. The selected classic and new antidiabetic drugs include metformin and other antidiabetic drugs such as glibenclamide, vildagliptin, sitagliptin and pioglitazone. The best extraction recoveries were achieved with Chromabond Tetracycline and Oasis HLB cartridges. However, there was no single cartridge which could offer optimum extraction to all the antidiabetic drugs.

2.3.2 GC-MS

The GC-MS method was used to determinate the metformin in biological samples in the 1970s (Brohon and Noel, 1978; Matin et al., 1975). GC with electron capture detection was utilized to detect the quantitation of phenformin, metformin and buformin from plasma samples by Matin et al. (1975). They considered that the plasma concentration of phenformin could be measured through this method of 24-h single therapeutic doses to diabetic patients. This research proved that the derivatization reaction is important to these biguanides to make them suitable for the GC quantification. The reason is that direct volatilization involved in GC-MS analyzing procedure could degrade these non-volatile biguanides without proper derivatization. Moreover, the authors found that buformin and metformin have very similar to the gas chromatograms. Brohon and Noel (1978) used a nitrogen detector with gas-liquid chromatography to identify and quantify metformin in plasma samples. This method was considered to be a sensitive way to quantitatively analyze metformin from plasma and tissue samples using a linear IS and a nitrogen detector.

Majidano and Khuhawar (2012) developed a capillary GC following nitrogen gas procedure to determinate four pharmaceuticals (such as famotidine, ranitidine, cimetidine and metformin) of medicine tablets from deproteinized serum after derivatization with methylglyoxal (MGo). This method could be used to detect these pharmaceutical preparations and plasma samples for quality control. Preventing drug toxicity and optimizing drug therapy in patients have been the goal of the determination of these pharmaceuticals in biological fluids. This study also examined the efficiency of derivatization reaction under a range of pH from 1 to 10 and different warming times. The

better derivatization reaction was presented in Table 2.1. After derivatization reaction, sample solution (1 μL) was injected into GC system with a split ratio of 10:1. The column and all the parameters of GC-MS process where are represented in Table 2.1. The LOD of this method was 17-25 ng mL^{-1}, and the LOQ for metformin was 75 ng mL^{-1}. The relative standard deviation (RSD) of separation and elution was 2.3-3.7%. This method proved that these four pharmaceuticals could be simultaneously detected and separated by GC-MS with low cost after a derivatization reaction. The reported HPLC and capillary electrophoresis procedures were used to compare this analysis method for these four drugs in terms of sensitivity, selectivity and analysis time. The GC connected to a flame ionization detector probably has lower calibration range, and the LOD of this method was not better than other two analysis methods except for famotidine.

Table 2.1 The GC-MS methods described in the literature for the determination of metformin

Derivatization reagent	Derivatization condition	Condition	Total run time (min)	Sample matrices	Reference
methylglyoxal	90°C, 30 min, pH 7.5 buffer	1 μL sample injected, HP-5 column (30 m × 0.32 mm id, 0.25 μm film thickness), initial temp. at 90°C for 2 min, then up to 265°C with a rate of 25°C min^{-1}. The nitrogen flow was 2.5 mL min^{-1}. The injector and detector temperatures were 270°C and 275°C.	11	plasma samples	Majidano and Khuhawar (2012)
MBTFA, MTBSTFA, MSTFA, MSTFA/ imidazole	80°C, 60 min	2 μL sample injected, a 5% phenylmethylpolysiloxane capillary column (15 m × 0.25 mm id with 0.33 μm film thickness), initial oven temp. at 100°C then up to 210°C with a rate of 20°C min^{-1}. The flow rate of helium at 1	5.5	plasma samples	Uçaktürk (2013)

		mL min^{-1}. The injector and ion source temperatures were 280°C and 230°C.			
MBTFA	60°C, 60 min, acetonitrile	2 μL sample injected, a VF-1ms column (30 m × 0.25 mm id with 0.25 μm film thickness), initial temp. at 100°C, up to 140°C with a rate of 5°C min^{-1}, then the temp. increased to 170°C with a rate of 20°C min^{-1}, finally up to 310°C with a rate of 30°C min^{-1}. The constant flow rate of helium at 1.0 mL min^{-1}. The injector and detector temp. were set at 250°C. The source and the transfer-line were maintained at 230°C and 310°C.	14.2	water samples	Goedecke et al. (2017)

A simple GC-MS method was developed by Uçaktürk (2013) for the determination of metformin in human plasma samples. This study also compared different derivatization reagents and reactions for metformin prior to GC-MS. Silylation, methylation and acylation were considered as derivatization reactions for metformin analysis prior to GC-MS, in which the silylation was better than other derivatization procedures such as methylation and acylation. The author stated that there was no derivatization product after the metformin methyl iodide reaction. The derivatization reaction temperature and time were examined as parameters, and the effective derivatization reaction is displayed in Table 2.1. The N-methyl-bis (trifluoroacetamide) (MBTFA) was selected as derivatization reagent after comparing with N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), N-methyl-N-(trimthylsilyl)trifluoroacetamide (MSTFA) and MSTFA/imidazole. A 2 μL sample was injected with a split rate of 10:1 into column (Table 2.1). The LOD and LOQ were 40 and 100 ng L^{-1}. To ensure the precision and accuracy, the studies were duplicated for five batches and the RSD did not exceed 20%. The stability of metformin in plasma was examined under room temperature and freezing condition, and metformin was stable under these different storage conditions. Uçaktürk (2013) considered that this novel derivatization reaction of metformin with MBTFA could offer a short run time as a high-through put analysis of metformin in clinical labs. However, this novel derivatization reaction does not happen in polar environments thus the pH values could not be adjusted. The rate of derivatization reagent with metformin and the yield rate of this derivation reaction were not mentioned in this paper.

A GC-MS-based method was developed to quantify the metformin in surface water samples at low concentration (Goedecke et al., 2017). 1-Butylbiguanidee (buformin) was the IS and MBTFA was the derivatization reagent. Different reaction temperatures and volumes of reagent of the derivatization were evaluated. The ideal derivatization, the column and all the parameters of GC-MS process were exhibited in Table 2.1. All the parameters of the GC-MS process were detailed in Table 2.1. The LOD and LOQ of this method were 3.9 and 12 ng L^{-1} in surface water samples (Goedecke et al., 2017) which were lower than the values of previous studies on GC-MS method. However, this study did not examine the precision, accuracy and stability of the real samples.

Most GC-MS methods were developed and used to analyze the metformin samples in human plasma. The derivatization reaction was interfered before injecting the samples into the GC. Metformin can react with reactive α-dicarbonyls such as MGo or glyoxal (Ruggiero-Lopez et al., 1999). Thus, MGo and MBTFA were selected as derivatization regents to react with metformin for forming a cyclic compound. The difference between these two reagents is the chemical reaction conduction. Metformin can react with MGo in a polar environment but cannot react with MBTFA in the same environment. MGo reacts with metformin to form an imidazolone adduct as advanced glycation end products. This formation can contribute to pathophysiologies associated with ageing and long-term complications of diabetes (Majidano and Khuhawar, 2012; Uchida et al., 1997). MBTFA was selected as another derivatization reaction reagent because by-products of MBTFA or excess MBTFA are volatile. Therefore, there will be less impurity interfered into GC analysis and form a trifluoroacetamide derivative of metformin (Uçaktürk, 2013). To all of

these GC-MS methods, although the duplicate analysis was done in four or five batches, the precision and accuracy varied among different studies. Therefore, an IS is needed to assist to GC-MS analysis though obtaining the absolute values of metformin from the peak areas/peak height each time. However, there is little GC-MS use to examine metformin in wastewater and surface water samples.

When compared with the GC-MS method, the LC-MS/MS method does not need the derivatization reaction after the pre-treatment of samples. Moreover, the sample preparation of metformin for the LC-MS/MS method is simpler than that for the GC-MS method. The HPLC/MS/MS method is used to identify PPCPs in water samples in the US EPA *Method 1694* (USEPA, 2007). However, according to the study of Goedecke et al. (2017), the total run time of HPLC/MS/MS method was more than the total run time of GC-MS method, even when the total run time of GC-MS method in this study is the maximum time in all the GC-MS methods used to analyze metformin in Table 2.1. The results of this study showed that the performance of the GC-MS method, such as linear correlation coefficient, LOD and LOQ, was better than the performance of HPLC-MS/MS method. This study indicated that the GC-MS method was sufficiently reliable to quantify metformin in surface water when compared to the HPLC/MS/MS method.

2.4 Treatment

There are some general treatment methods that have been used to remove metformin in recent years, such as advanced oxidation processes (AOPs), activated carbon filtration, flocculation, phytoremediation and adsorption methods. Adsorption methods used to

remove metformin from water samples are molecular imprinting and graphene oxide. AOPs occupy the major part of removal applications in the WWTPs.

2.4.1 Advanced Oxidation Processes

AOPs are based on the in-situ generation of a powerful oxidizing agent such as hydroxyl radicals (•OH). The oxidizing agent can achieve a sufficient concentration of radicals to effectively decontaminate waters (Oturan and Aaron, 2014). Various chemical, photochemical, sonochemical or electrochemical reactions are used to develop different types of AOPs. Chemical AOPs include Fenton, peroxidation, photolysis of H_2O_2 (H_2O_2/UV), photolysis of O_3 (O_3/UV), photo-Fenton (H_2O_2/Fe^{3+}/UV) and heterogeneous photocatalysis (TiO_2/UV). Electrochemical AOPs consist anodic oxidation, indirect electrochemistry (EF) process and EF-related processes coupling of EF with other AOPs (Jing et al., 2015; Oturan and Aaron, 2014).

Metformin, as one of the 32 selected target pollutants, was removed from the effluent of a WWTP by AOPs methods such as ultraviolet (UV)-light alone, dark Fenton ($Fe^{2+,\ 3+}/H_2O_2$) and photo-Fenton ($Fe^{2+,\ 3+}/H_2O_2$/light). The photo-Fenton combined two light sources, namely UV at 245 nm and sunlights (De la Cruz et al., 2012). All these treatment methods were processed in laboratory-scale experiments at neutral pH. The UV lamps (Table 2.2) were set into two devices, a 400 mL beaker to treat the wastewater sample by stirring and an artificial solar-light device containing a cylindrical pyrex reactor (128 mm diameter and 74 mm height) to treat wastewater with a magnetic stirrer. The lower degradation of metformin in this study (6%) was achieved with UV at 245 nm after 10 min. UV_{245}/H_2O_2

treatment was seen for the metformin with a 34% removal rate after 10 min. After 30 min of treatment, the degradation increased up to 11% under the same conditions, remaining over metformin LOQ (15 ng L^{-1}). In addition, the removal rate of metformin by dark Fenton ($Fe^{2+, 3+}/H_2O_2$) was 43% after 30 min. The wastewater sample was treated by photo-Fenton ($Fe^{2+, 3+}/H_2O_2/UV_{245}$) with the rate of $Fe^{2+, 3+}/H_2O_2$ at 5/50 mg L^{-1} for 10 and 30 min. Its removal rates were 43% and 88% respectively, and it could not be detected after 90 min. The removal rate of the sample treated by photo-Fenton ($Fe^{2+, 3+}/H_2O_2$/artificial sunlight) was 75% in 90 min (De la Cruz et al., 2012).

De la Cruz et al. (2013) used the UV, UV/H_2O_2 and UV/H_2O_2/ Fe^{3+} methods to remove 22 selected contaminants including metformin in wastewater effluent samples, and the samples were pumped as a continuous flow in a 37 L tank. Comparing with the last study by De la Cruz et al. (2012), this study was conducted as a pilot-scale laboratory experiment. The UV system included five efficient, high-performance, low pressure mercury lamps (254 nm, 150 W each), and the treatment time was 20 min at least. UV_{254}/H_2O_2 method was used to treat wastewater samples by pumping H_2O_2 into the reactor with a maximum flow rate of 3.9 L s^{-1}. The removal rate of metformin by this method was decreased from 56% to 47% with an increasing concentration of H_2O_2 from 20 to 30 mg L^{-1}. $FeCl_3$ was used as Fenton catalysis in UV_{254}/H_2O_2/Fenton method, and the mixed maximum flow rate was 2.8 L s^{-1}. The removal rates of metformin were from 48% to 63% by the $UV_{254}/H_2O_2/Fe^{3+}$ method (De la Cruz et al., 2013), lower than the rates of the study by De la Cruz et al. (2012). Moreover, the researchers stated that added iron (Fe^{3+}) did not enhance the effects of the treatment of UV/H_2O_2 in wastewater samples because the iron dissolved in water could

restrain the light transmission through the reactor. The economic flow of wastewater flow rate with H_2O_2 dose was 14 $m^3 h^{-1}$ with 50 mg L^{-1} to treat the selected contaminants depending on the study (De la Cruz et al., 2012).

Wols et al. (2013) reported a study on treating 40 pharmaceutical pollutants including metformin by UV_{254} and UV_{254}/H_2O_2 methods at laboratory scale. A monochromatric low pressure and a polychromatic medium pressure lamp were selected as UV source, and the flow rate of H_2O_2 was 10 mg L^{-1}. The treatment time was not available. A 100mL sample was treated each time in this study. The LOD of metformin was 50 ng L^{-1}. Water samples were the stock solution (the pharmaceutical concentrations in the stock were 100 times of LOD) spiked in MilliQ water and collected from tap water and pre-treated water from the river Meuse. There was no metformin detected in tap water samples. The degradation rates of metformin in MilliQ water and river samples were from 1% to 4% by UV treatment method while the rates of metformin were raised up to 55% by UV with 10 mg L^{-1} H_2O_2. The effect of polychromatic medium pressure lamps was better than the one with monochromatric low pressure one for removing metformin from water matrix samples.

Table 2.2 Performance of AOPs for metformin removal

Methods	Process	Application	Removal rate	Reference
UV_{254}	Five mercury lamps (254 nm, 150 W each), treating 20 mins, at neutral pH	Wastewater effluent samples	28%	De la Cruz et al. (2013)
	Five mercury lamps (254 nm, 150 W each), treating 10 mins, at neutral pH	Wastewater effluent samples	6%	De la Cruz et al. (2012)
	A monochromatric lamp (249-360 nm) and a polychromatic lamp (254 nm)	Stock solution, river samples	4%	Wols et al. (2013)
UV_{254}/H_2O_2	Five mercury lamps (254 nm, 150 W each), a flow rate of H_2O_2 at 50 mg L^{-1}, treating 10 and 30 mins, at neutral pH	Wastewater effluent samples	34%, 11%	De la Cruz et al. (2012)

	Five mercury lamps (254 nm, 150 W each), a maximum flow rate of H_2O_2 at 3.9 L s^{-1}, treating 20 mins, at neutral pH	Wastewater effluent samples	56%	De la Cruz et al. (2013)
	A monochromatric lamp (249-360 nm) and a polychromatic lamp (254 nm), a flow rate of H_2O_2 at 10 mg L^{-1}	Stock solution, river samples	55%	Wols et al. (2013)
UV_{254}/H_2O_2/Fenton	UV at 254 nm, flow rates of Fe^{2+} and H_2O_2 at 5 and 50 mg L^{-1} respectively, treating 10, 30 and 90 min	Wastewater effluent samples	43%, 88%, 97%	De la Cruz et al. (2012)
	UV at 254 nm, flow rates of Fe^{2+} and H_2O_2 at 5 and 25 mg L^{-1} respectively, treating 10 and 30 min		46%, 88% 47%	
	UV at 254 nm, flow rates of Fe^{2+} and H_2O_2 at 5 and 10 mg L^{-1} respectively, treating 10 min			

	Five mercury lamps (254 nm, 150 W each), a maximum flow rate of H_2O_2 at 3.9 L s^{-1}, the mixed maximum flow rate of $FeCl_3$ at 2.8 L s^{-1}, treating 20 min	Wastewater effluent samples	63%	De la Cruz et al. (2013)
O_3	The concentrations of O_3 are from 0.5 to 1 mg L^{-1}, treating 30 min	Surface water	50-55%	Scheurer et al. (2012)
	The concentration of O_3 at 8 mg L^{-1}, treating 30 min	Aqueous solutions	60%	Quintao et al. (2016)
Photolysis (UV-C)	One UV-C lamp (model TUV PL-S, 9 W) with a total nominal power of 25.7 W	Aqueous solutions	9.2%	Quintao et al. (2016)
Photocatalysis (TiO_2/UV-C)	One UV-C lamp (model TUV PL-S, 9 W) with a total nominal power of 25.7 W; the concentration of TiO_2 at 120 mg L^{-1}	Aqueous solutions	31%	Quintao et al. (2016)

The photocatalysis method contained UV-C radiation and TiO_2, and one UV-C lamp (9W) with a total nominal power of 25.7 W. The UV lamp was immersed in glass jars which received 350 mL of metformin aqueous solution. Forty-two mg of TiO_2 (anatase) were used resulting in a dosage of about 120 mg L^{-1} in the solution. The treatment times were 0, 5, 10, 15 and 30 min (Quintao et al., 2016). Phototdegradation was used to treat guanylurea but guanylurea negligibly reacted on photolysis. Trautwein and Kummerer (2011) spiked guanylurea at a mean concentration of 9.41 mg L^{-1} into the reactor underling irradiation for 120 min. Then, the final concentration of guanylurea was 9.17 mg L^{-1}. The removal rate was 2.5% which may due to analytical inaccuracies. Therefore, guanylurea was considered not to be removed in surface waters by natural sunlight or technical processes in advanced water treatment (Trautwein and Kummerer, 2011).

Based on the discussed AOPs method used to treat metformin from aquatic samples, the highest removal rate of metformin is 97% (Table 2.2). This removal method is UV_{254}/H_2O_2/Fenton method on the flow rates of Fe^{2+} and H_2O_2 at 5 and 50 mg L^{-1}, treating 90 min (De la Cruz et al., 2012). This experiment was processed in a small-scale laboratory experiment with a volume of 400mL samples in this study (De la Cruz et al., 2012). However, in the pilot-scale laboratory experiment (De la Cruz et al., 2013), the removal rate of UV_{254}/H_2O_2/Fenton method was 63% achieved in 20 min with a continuous flow in a 37 L tank. The 90min treating time was not tested in this study (De la Cruz et al., 2013). The removal rate of other AOPs methods on removing metformin in aqueous samples was all lower than 63% (Table 2.2). For industrial practicality, there still is a gap in the AOPs methods to improve the removal rate economically.

2.4.2 Other Methods

A first systematic study of the environmental fate and the effectiveness of treatment techniques used in water treatment plants of metformin and guanylurea were performed by Scheurer et al. (2012) in laboratory batch tests. In this study, the efficiency of metformin from water matrix by four treatment techniques, namely chlorination, ozonation, activated carbon filtration and flocculation, were compared. The ozonation method was to inject known concentrations of ozone into the stock solutions and stirred for a certain time. The flocculation method is to flocculate targets with flocculants such as polyaluminium chloride and iron chloride. To achieve a rapid distribution, it is necessary to stir the solution with a high velocity to form flocs in a short time. Hypochlorite or chlorine dioxide was used as a chlorination method to treat drinking water in Germany (Scheurer et al., 2012). Hypochlorite (OCl^-) was used in a chlorination test at a flow of 0.2 and 1 mg L^{-1} at a range of 5h in 22-23°C. The removal rate of metformin was 99%. Although hypochlorite was considered as a redox potential chemical to oxidize metformin, chlorination is not permitted for the intended oxidation of water contaminants (Scheurer et al., 2012). Another experiment of chlorination (NaOCl) was used to treat an aqueous solution with a flow of 10 mg L^{-1} NaOCl treated at a range of 30 min in 22-23°C, when the removal rate of metformin was 60% (Quintao et al., 2016).

Activated carbon filtration is used to remove metformin and guanylurea from drinking water. The activated carbon filtration is filled with granular activated carbon (GAC). The GAC filter unit includes eight filters. The removal rates of metformin and guanylurea by a coarse filter were 56% and 29%, respectively (Scheurer et al., 2012). Sixty-eight per cent

of metformin could be retained by the subsequent GAC filter whereas the removal rate was negligible for guanylurea. In the ozonation (O_3) test, the concentrations of O_3 were 0.5-1 mg L^{-1}, and the time was at least 30 min (Scheurer et al., 2012). The removal rate of metformin and guanylurea is around 50-55% and 68-75%, respectively. Another study by Quintao et al. (2016) also used ozone gas generated using an ozone generator of electrical discharge with production capacity of 3 g O_3 per hour, and the details of the removal method of metformin are listed in Table 2.2.

Molecularly imprinted polymers, according to molecular imprinting techniques based on selective binding or isolation of target species from a mixture, are used to absorb metformin and guanylurea in aqueous matrix (Kyzas et al., 2015). The authors used two novel molecularly imprinted polymers (MIPs) to remove metformin and its transproduct guanylurea in aqueous media. MIPs have remarkable recognition properties, and these polymers can be reused five times to adsorb the metformin and guanylurea. The two types of MIPs are metformin-MIP and guanylurea-MIP. The removal rates for metformin and guanylurea were 60% and 73% by metformin-MIP and guanylurea-MIP respectively.

Phytoremediation, as an absorption method, is used to study on removal of metformin from soil and water. This method based on the characteristic of plant roots uptake the chemicals during phytotreatment on wastewater. *P. australis* and *T. latifolia* are used to plant in the matrix including metformin (Cui et al., 2015; Cui and Schroder, 2016). Metformin was uptaken by both *P. australis* and *T. latifolia* roots independently on the initial concentrations (Cui et al., 2015). The authors found that quinidine as an inhibitor affected the ability of *T. latifolia* roots to adsorb metformin. *T. latifolia* was used as a

phytoremediation method to assess, uptake and translocation of metformin in hydroponic solutions (Cui and Schroder, 2016). The removal processes of metformin followed first-order kinetics. Removal efficiencies were from 74.0 (± 4.1) to 81.1 (± 3.3) % after 28 days. Metformin concentration in roots increased during the first 2 weeks of the experiment then decreased. However, metformin concentration continuously increased in rhizomes and leaves. Bioaccumulation of metformin in leaves and rhizomes was not as high as in roots. Methyl biguanide was detected as degradation product of metformin in the plant but guanylurea was not detected. Moreover, methyl biguanide concentration in roots increased with exposure time. The degradation rate of methyl biguanide was higher than metformin based on an enzymatic degradation experiment (Cui and Schroder, 2016).

Graphene oxide, as an atomic layer material, can be obtained through oxidizing graphite. It has high performance on adsorption, photocatalytic degradation and sensor because of its physical-chemical properties and extraordinary surface area (Zhu et al., 2017). The removal rate of metformin was almost 80% within 20 min of treatment with graphene oxide at 288K and pH 6.0 in water matrix. In this experiment, 3 mg graphene oxide was added in 10 mg L^{-1} metformin solution. There were inhibiting effects of both anions and cations in the adsorption process (Zhu et al., 2017). Anions, such as Cl^-, $SO_4^{2-}$and PO_4^{3-}, could cause a decreased metformin adsorption on graphene oxide. The authors stated that graphene oxide, as a potential adsorbent, could be used to remove metformin. Overall, the studies involved the treatment of guanylurea has been very limited.

2.5 Summary

This study first reviewed the occurrence, impact, analysis and treatment of metformin and guanylurea in Atlantic coastal regions including Canada, USA and Europe. Metformin is a first-line pharmaceutical medicine to treat type 2 diabetes and has been widely detected in the reviewed regions. Guanylurea is the biodegraded transformation product and metabolite of metformin. Its occurrence, impact, analysis and treatment have been much less reported compared to those of metformin. New transformation products of metformin, other than guanylurea, should be kept monitored (Tisler and Zwiener, 2018).

In terms of the occurrence of metformin and guanylurea, most existing data were collected based on analysis of the aquatic and sediment samples collected in coastal communities/cities and estuaries of Atlantic regions. Extremely limited studies focusing on their occurrence in ocean environments were conducted, especially in Canada and the USA. Their distribution, transport and fate in marine and coastal regions need to be further investigated. The effects of metformin on biological system and biodiversity have gained growing attention. However, there is a lack of sufficient data regarding the acute and chronic ecotoxicological impacts of metformin on bacteria, fishes, algae and cyanobacteria.

LC-MS-based methods have been widely used for metformin analysis in liquid samples. GC-MS-based methods recently became alternatives and suitable independent references due to their lower LOD and LOQ values, although the standard protocols have not yet been established. No GC-MS method has ever been applied for guanylurea analysis. Recommendations for future research include developing an IS used in GC-MS to

accurately measure the concentration of metformin and a GC-MS method quantifying guanylurea and metformin simultaneously in aqueous samples. Metformin removal from liquid phase has been extensively studied. Satisfactory treatment efficiency has been achieved using technologies such as AOPs. However, technology scale-up and field applications are still challenging since much lower removal rates have been observed in the field than in the laboratory. Guanylurea removal from the coastal aquatic environments has been less well tackled in Atlantic regions.

CHAPTER 3 IMPROVED ANALYSIS OF METFORMIN AND GUANYLUREA IN LIQUID SAMPLES BY USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)[2]

[2] *This chapter has been used to generate the following publications:*

Tao, Y. et al. 2019. Central Composite Design for Optimizing Derivatization of Metformin and Guanylurea in Water Samples Detected by Gas Chromatography-Mass Spectrometry. Proceeding of the Canadian Society for Civil Engineering (CSCE) 2019 Annual General Meeting and Conference, June 12-15, Laval, Canada. 15: 1-10

Tao, Y. et al. Enhanced gas chromatography-mass spectrometry (GC-MS) based analysis of metformin and guanylurea in water samples. Journal of Water, Air, & Soil Pollution. Accepted in July 2020

Role: Yunwen Tao solely worked on the study and acted as the first author of this manuscript under the guidance of Dr. Baiyu Zhang, Dr. Bing Chen and Dr. Yuming Zhao.

3.1 Methodology

3.1.1 Standards and Reagents

Metformin, buformin, guanylurea, and N-methyl-bis(trifluoroacetamide) (MBTFA) were purchased from Sigma-Aldrich (Canada). Methanol, formic acid, and sodium dodecyl sulfate (SDS) solution (20% in H_2O) were of analytical reagent grade purchased from Sigma-Aldrich (Canada). 1,4-dioxane, acetonitrile and ultra-pure water were of analytical reagent grade purchased from Fisher company (Canada). Metformin, guanylurea, and buformin were accurately weighed and then dissolved in methanol for preparing stock solutions (1 mg mL^{-1}). Working standard solutions were obtained through diluting the stock solutions to 0.1 mg mL^{-1}. All stock solutions were kept at -20°C, and working standard solutions were kept at 4°C.

3.1.2 Sample Extraction

Solid phase extraction (SPE) is an extraction method used to obtain targeting organic chemicals from liquid samples (Martin et al., 2012; Tao et al., 2018). The polarity of metformin and guanylurea can cause the affinity of the SPE cartridge sorbent, based on which SPE can be used to extract them from water samples (Tao et al., 2018). The visiprep DL from Supelco Inc. (USA) was used for SPE treatment in this study. Extraction tubes and all other glassware were primarily washed with detergent and then seriously followed the steps in EPA *Method 1694* (USEPA, 2007) to remove all types of fingerprints, rusts and other contaminants before usage.

The extraction protocol of metformin and guanylurea was adjusted based on EPA *Method 1694* (USEPA, 2007) and Martin et al. (2012). Metformin and guanlyurea standard solutions were spiked into ultra-pure water to obtain samples with certain mass (0.5 μg and 1 μg, respectively). SPE treatment was then performed by using hydrophilic-lipophilic-balance (HLB) extraction cartridges (Oasis® HLB solid-phase cartridges, 6 cc/200 mg, Waters Corporation, Milford, Massachusetts, USA). Before sample extraction, the HLB cartridges were conditioned by using 20 mL methanol (HPLC grade) followed by usage of 6 mL ultra-pure water (HPLC grade); 5 mL SDS solution (2 mM) was then used as ion pair reagent. After conditioning, each 100 mL spiked ultra-pure water sample went through the HLB cartridge with a stable sample flow rate at 5 mL min^{-1}. The cartridge was vacuumed for 5 minutes then dried in an oven under 60°C for 10 minutes. The cartridge was then put in a desiccator for cooling, followed by a further dry-up underneath a gentle nitrogen gas flow for one hour. Each cartridge was transferred to the visiprep DL for elution. Methanol (4.5 mL) was used to elute the target compounds off the sorbent following 6 mL of mix elution (methanol: acetonitrile (50:50) + 2% formic acid). The eluate was collected by a 15-mL glass tube and stored in refrigerator under 4°C overnight before evaporation. Buformin, as IS (internal standard), should be spiked constant volume (10 μL) into the eluate before the evaporation step. The eluate was fractionally evaporated in a v-vail glass tube (5 mL) for six times. The v-vail glass tube was treated underneath a gentle nitrogen gas flow with a warm bath (45±5°C) at the bottom until the eluate was completely dried. The v-vail glass tube with concentrated target chemicals was then ready for the derivatization reaction.

3.1.3 Optimization of Derivatization Reaction

Design Expert® software version 11 and Python version 3 were employed to perform optimization of derivatization reactions of metformin and guanylurea. In total, 40 runs were conducted using DOE to determine the impact of four factors on derivatization. There four factors included three numeric factors (i.e., temperature, reaction time, ratio of the reagent buformin and target chemicals) and one categoric factor (i.e., solvents: 1,4-dioxane and acetonitrile). After optimization, desired derivatization conditions were determined. The data visualization was performed using the Python's Matplotlib Library and the data processing was performed using Python data analysis and manipulation tool called Panadas. The data was filtered, and the four factors were mapped on a contour surface plot.

3.1.3.1 Determination of the Factor Levels

The factors selected include temperature, reaction time, solvents and ratio between the reagent and target chemical (Table 3.1). The reagent is important to metformin achieve derivatization reaction for preparing procedure of GC-MS method. The reagents applied (Goedecke et al., 2017; Majidano and Khuhawar, 2012; Uçaktürk, 2013) were methylglyoxal (MGo), N-methyl-bis(trifluoroacetamide) (MBTFA), N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), N-methyl-N (trimthylsilyl)trifluoroacetamide (MSTFA), and MSTFA/imidazole. Buformin and diphenylamine were respectively used as IS for derivatization (Goedecke et al., 2017; Uçaktürk, 2013). Based on the references (Goedecke et al., 2017; Majidano and Khuhawar, 2012; Uçaktürk, 2013), MBTFA and buformin were selected as the derivatization reagent and IS, respectively, in this study.

Guanylurea has never been detected with metformin using the GC-MS method. At the initial exploratory stage, OFAT experiment was used to determine the range of three numeric factors. Four chemicals were involved in the derivatization reaction, which are two emerging pollutants (metformin and guanylurea), one IS for indicating derivatization reaction (buformin), and one reagent (MBTFA).

Different temperatures, ratios, and solvents were tested by OFAT. The data of OFAT is not shown (available upon request). The selected high and low levels of the four factors are illustrated in Table 3.2. In addition, the value of alpha was set as 1.5 instead of 1.68179 ($k<6$) because the ratio of reagent and each target chemical should be geometric progression. (categoric)

Table 3.1 Range of factors in previous derivatization reactions

Derivatization reagent	Temperature (°C)	Time (minutes)	Solvent	Reference
MGo	90	30	pH 7.5 buffer	Majidano and Khuhawar (2012)
MBTFA, MTBSTFA, MSTFA, MSTFA/imidazole	80	60	none	Uçaktürk (2013)
MBTFA	60	60	acetonitrile	Goedecke et al. (2017)

Table 3.2 High and low levels of four examined factors

Name	Factor	High	Low	Center	-alpha	+alpha
Temperature (°C)	A	90	70	85	65	95
Time (minutes)	B	70	40	55	32.5	77.5
Ratio*	C	0.5:1	1.5:1	0.75:1	0.25:1	1.75:1
Solvent	D	1,4-dioxane	Acetonitrile	NA	NA	NA

Ratio = the volume of the reagent to the total volume of target chemicals

3.1.3.2 DOE Procedure

Preparation of stock solutions is the initial procedure of the experiment. The standard solutions of buformin, metformin and guanylurea were prepared by dissolving an appropriate amount of the substances in methanol to reach a concentration of about 1 mg mL^{-1}. The standard solutions were stored at 4°C in the dark. During the derivatization reaction, there are several steps involved. Firstly, 10 µL standard solutions of buformin, metformin and guanylurea should be added into a vial. The solutions are dried by nitrogen gas. A certain amount of reagent MBTFA (ratio of reagent to target chemicals from 0.5:1 to 1.5 :1) will be added into the vial following 100 µL solvent to the residue. Water bath is set at a certain temperature (70-90°C) for a defined time interval minute (40-70 minutes). Afterwards, the vial is taken out of the water bath and a certain amount of solvent is added up to 1 mL for GC-MS measurement.

Design-Expert® (State-Ease, 2018) software in version 11 was employed to design and analyze the data. Using the CCD design, 40 runs were conducted to determine the effect of the four factors on performance of derivatization. For three numeric factors, 20 runs including 6 runs of center point were involved. Then, one categoric factor will double the 20 runs to 40 runs.

3.1.4 GC-MS Instrumentation

An Agilent GC 7890 equipped with a 5975C mass triple-axis detector was used in this study. DB-5ms of Agilent (30m × 0.25µm × 0.25mm) column was applied. The volume of each injected sample was 1 µL. The initial temperature in GC oven was set at 80°C, then

raised to 110°C at a rate of 6°C min^{-1}. Then following temperature roused to 210°C with a rate of 15°C min^{-1}. Finally, the temperature was up to 230°C with a rate of 20°C min^{-1}. The flow rate of helium was at 1.0 mL min^{-1}. The injector and detector temperatures were 240 and 300°C. The total run time of the GC-MS analysis was 12.67 minutes. A ratio of peak areas between guanylurea and metformin generated by GC-MS analysis was treated as the response for quantification of guanylurea and metformin, as well as optimization of the derivatization reaction. The addition of buformin and the associated ratios of peak areas (i.e., metformin vs. buformin and guanylurea vs. buformin) were used for GC-MS based method calibration.

3.1.5 GC-MS Performance Evaluation

Performance of the GC-MS based metformin and guanylurea analysis was evaluated using parameters including linearity, recovery, repeatability, LOD and LOQ. The linearity of the method was assessed by making calibration curves over the mass range of 100-2000 ng. The calibration curves were performed at seven different weights of metformin (1, 0.8, 0.5, 0.4, 0.25, 0.2, 0.1 μg) and seven different weights of guanylurea (2, 1.6, 1, 0.8, 0.5, 0.4, 0.2 μg) with constant weight of buformin (1 μg). The working solutions (0.1 mg mL^{-1}) of metformin were diluted to 0.8, 0.5, 0.4, 0.25, 0.2, and 0.1 μg mL^{-1}, and the stock and working solutions (1 and 0.1 mg mL^{-1}) of guanylurea were diluted to 0.2, 0.16, 0.1, 0.8, 0.5, 0.4, and 0.2 μg mL^{-1}.Then, constant volume (10 μL) of metformin and guanylurea at each concentration were added into 10.5 mL mixture solvents with 10 μL buformin (0.1 mg mL^{-1}) as same as eluents in glass v-vials (5 mL), following with the derivatization reaction and GC-MS analysis. The calibration curves were constructed by plotting the peak area ratios

(metformin/IS or guanylurea/IS) versus the nominal concentrations of metformin. The coefficient of determination (R^2) and the back-calculating concentration were evaluated for each calibration point. Replicate analyses of the samples were performed seven times to ensure reproducibility (variation of ≤ 6.0%) in GC-MS quantification. Duplicate samples were prepared and analyzed to ensure the reproducibility of results. The acceptable precision and accuracy of the LOQ should be 30% and 50%-149%, respectively. To perform the precision and accuracy test, metformin and guanylurea standard solutions at different lowest concentration points (0.1 and 0.2 μg mL^{-1} respectively) were conducted and repeated in seven times.

The chromatographs obtained and calculations were conducted with the ions m/z 303, 288 and 274 for metformin, 302 and 288 for buformin, and 182 and 69 for guanylurea. Quantitative analysis of metformin and guanylurea were performed based on the GC-MS responses (peak area) relative to that of internal standard (buformin) with a known concentration. A blank sample was included in each set of 10 samples. The area responses of the characteristic m/z against concentration for two target chemicals and an internal standard were analyzed and response factors (RFs) for metformin and guanylurea were calculated using Eq. (1) (USEPA, 2007; Fan, Zhang, & Morrill, 2017).

$$\mathrm{RF} = \frac{(\mathrm{A_s})(\mathrm{C_{is}})}{(\mathrm{A_{is}})(\mathrm{C_s})} \quad (1)$$

where:

A_s = Area of the characteristic m/z for the parameter to be measured.

A_{is} = Area of the characteristic m/z for the IS.

C_{is} = Known concentration of the IS.

C_s = Known concentration of the parameter to be measured.

Then the RF value over the working range can be obtained and the concentration in the sample was calculated using the determined RF and Eq. (2).

$$\text{Concentration} = \frac{(A_s)(C_{is})}{(A_{is})(RF)} \quad (2)$$

where:

A_s = Area of the characteristic m/z for the parameter or surrogate standard to be measured.

A_{is} = Area of the characteristic m/z for the IS.

C_{is} = Concentration of the IS.

3.1.6 Water sample analysis

Tap water (100 mL) was collected in the laboratory and spiked with 1 μg of metformin and 2 μg of guanylurea. The spiked water samples were used to demonstrate the applicability of the developed analytical method. All sample bottles were treated 15 minutes by ultrasonic cleaning. Then, the samples were pre-treated according to Sections 3.1.2. After the extraction and derivatization, samples were analyzed by GC-MS for quantification of metformin, guanylurea and buformin.

3.2 Results and Discussions

3.2.1 Optimization of Derivatization Reaction Results

3.2.1.1 CCD Results

The response equals to the peak area of guanylurea/the peak area of metformin (response=G/M). All the responses were illustrated in Table 3.3. The quadratic process was ordered with backwards selection. The ANOVA table was illustrated in Table 3.4. The effect A, B, C, D, and A^2 are significant because of their p-values less than 0.05. The values of R^2, adjusted R^2, and predicted R^2 are 0.8626, 0.8427, and 0.8143, respectively, (Table 3.4) and show both a good fit and satisfactory predictive accuracy.

Table 3.3 The design and input responses

Run	Factor 1 A: Temperature	Factor 2 B: Time	Factor 3 C: Ratio	Factor 4 D: Solvent	Response (G/M)
1	80	77.5	1.00	dioxane	0.46
2	90	40.0	1.50	dioxane	0.56
3	70	70.0	1.50	acetonitrile	0.33
4	70	70.0	0.50	dioxane	0.25
5	80	55.0	1.00	dioxane	0.33
6	80	55.0	1.00	dioxane	0.35
7	90	40.0	0.50	dioxane	0.45
8	80	55.0	1.00	acetonitrile	0.25
9	80	55.0	1.00	acetonitrile	0.21
10	70	70.0	1.50	dioxane	0.41
11	70	40.0	1.50	acetonitrile	0.31
12	80	55.0	1.00	dioxane	0.37
13	80	55.0	1.00	dioxane	0.37
14	80	55.0	1.75	dioxane	0.51
15	90	40.0	0.50	acetonitrile	0.28
16	80	55.0	1.00	acetonitrile	0.29
17	70	40.0	0.50	dioxane	0.23
18	65	55.0	1.00	dioxane	0.33
19	80	55.0	1.00	acetonitrile	0.28

20	70	40.0	0.50	acetonitrile	0.21
21	90	70.0	1.50	dioxane	0.57
22	80	32.5	1.00	acetonitrile	0.27
23	90	70.0	0.50	dioxane	0.53
24	80	55.0	1.75	acetonitrile	0.48
25	80	55.0	1.00	acetonitrile	0.36
26	70	40.0	1.50	dioxane	0.46
27	80	55.0	0.25	dioxane	0.19
28	90	70.0	1.50	acetonitrile	0.48
29	90	70.0	0.50	acetonitrile	0.36
30	80	77.5	1.00	acetonitrile	0.42
31	80	32.5	1.00	dioxane	0.32
32	90	40.0	1.50	acetonitrile	0.55
33	95	55.0	1.00	acetonitrile	0.50
34	65	55.0	1.00	acetonitrile	0.26
35	70	70.0	0.50	acetonitrile	0.19
36	80	55.0	1.00	dioxane	0.38
37	95	55.0	1.00	dioxane	0.64
38	80	55.0	1.00	acetonitrile	0.35
39	80	55.0	0.25	acetonitrile	0.12
40	80	55.0	1.00	dioxane	0.47

Table 3.4 The ANOVA Results

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	0.5070	5	0.1014	42.78	< 0.0001
A-Temperature	0.1962	1	0.1962	82.79	< 0.0001
B-Time	0.0102	1	0.0102	4.30	0.0457
C-Ratio	0.1918	1	0.1918	80.93	< 0.0001
D-Solvent	0.0706	1	0.0706	29.77	< 0.0001
A^2	0.0382	1	0.0382	16.11	0.0003
Residual	0.0806	34	0.0024		
Lack of Fit	0.0523	24	0.0022	0.7707	0.7134
Pure Error	0.0283	10	0.0028		
Cor Total	0.5876	39			
R^2	0.8628				
Adjusted R^2	0.8427				
Predicted R^2	0.8143				

In addition, all the assumptions of regression such as normality of residuals, constancy of variance, and lack of fit, etc. were fulfilled. The necessary diagnostic plots for verifying the ANOVA reliable were illustrated in Figure 3.1. The points scatter a linear trend around the indicate line in normal plot of residuals and predicted versus actual plot in Figure 3.1 a & b. In the normal plot of residual, although the points scattering shows a very slight "S-shape" pattern, the residuals actually appear fairly normal in this case. The points scatter randomly well in residual versus run plot (Figure 3.1 d) indicating the residuals conditions of this assumptions satisfied. The points randomly scatter close to the linear in residual versus predicted plot (Figure 3.1 c) indicating the good fit and no consistent under or over the predicted model.

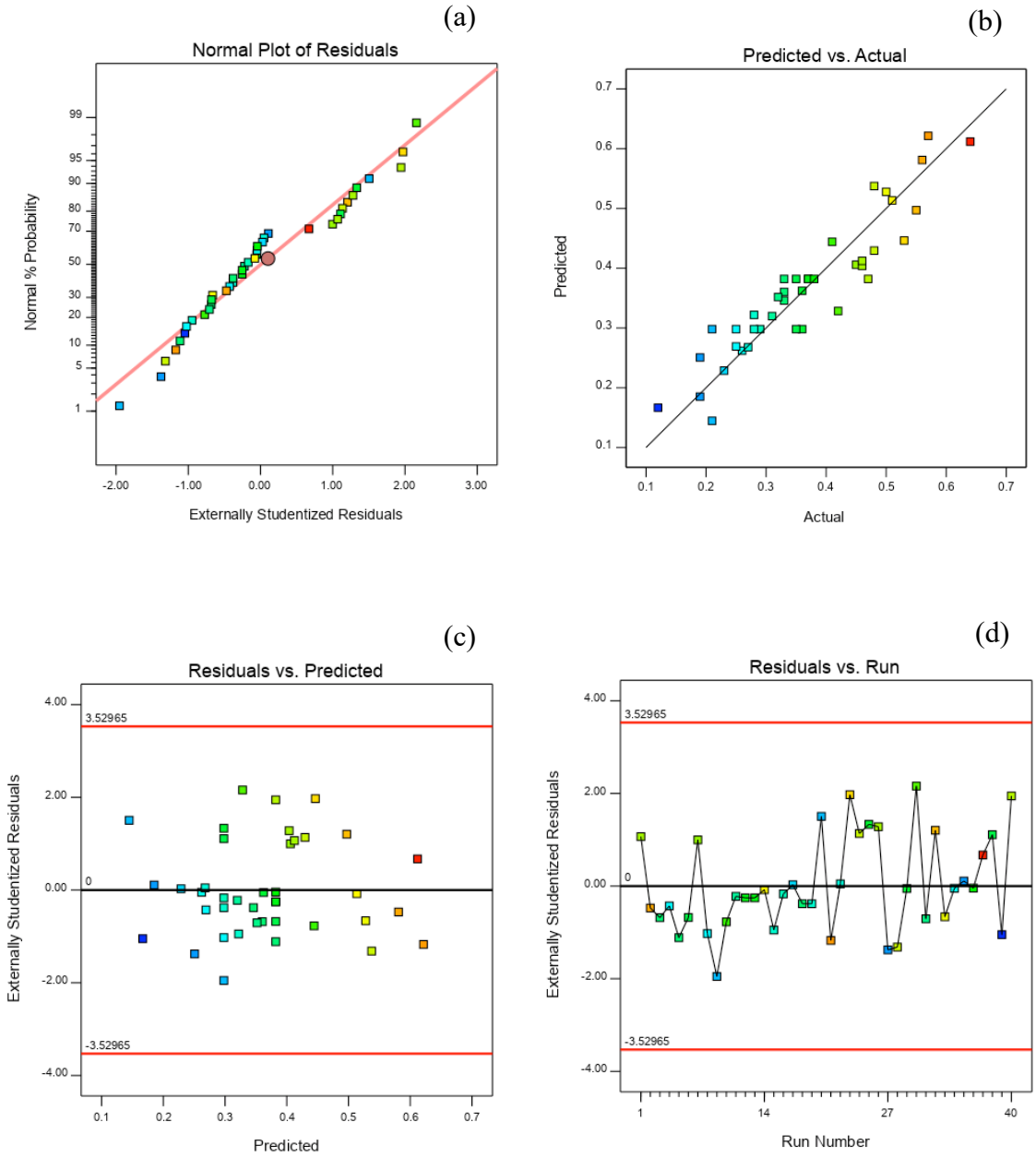


Figure 3.1 Diagnostic plots, (a) normal plots of residuals, (b) predicted and actual plot, (c) residuals and predicted plot, (d) residuals vs runs plot

All the ANOVA assumptions were reasonably fitted with the second-order polynomial model in form of coded factor scale is defined in Equation (3). The Equation 1 can be converted to actual factor scale to give Equation (4) & (5) due to different solvents. In the coded model, the temperature (A) and ratio (C) are similar magnitude effects on the efficiency of the response (G/M). High level of temperature and ratio can increase the responses obeying expected assumption.

$$G/M = 0.3401 + 0.0886A + 0.0202B + 0.0876C + 0.042D + 0.043A^2 \quad (3)$$

$$G/M = 2.09298 - 0.05998\ \text{Temperature} + 0.001347\ \text{Time} + 0.1752\ \text{Ratio} + 0.00043\ \text{Temperature}^2 \text{ (in solvent acetonitrile)} \quad (4)$$

$$G/M = 2.17798 - 0.05998\ \text{Temperature} + 0.001347\ \text{Time} + 0.1752\ \text{Ratio} + 0.00043\ \text{Temperature}^2 \text{ (in solvent 1,4-dioxane)} \quad (5)$$

This is the first study that discovers the relationships between the factors in derivatization reaction of metformin and guanylurea for the GC-MS method. In this design, it is found that the responses of G/M display linear with factor time and ratio and curvature shape with factor temperature. The reason is probably the selection of the levels of these numeric factors.

The temperature is the first important factor to the derivatization reaction because the reaction is sensitive to the temperature (Goedecke et al., 2017; Uçaktürk, 2013). The range of temperature was detected from 20 to 60°C for derivatization reaction of metformin by Goedecke et al. (2017) because the boiling point of acetonitrile is 82°C. The results showed that the responses of metformin were increased with increasing temperatures (Goedecke et

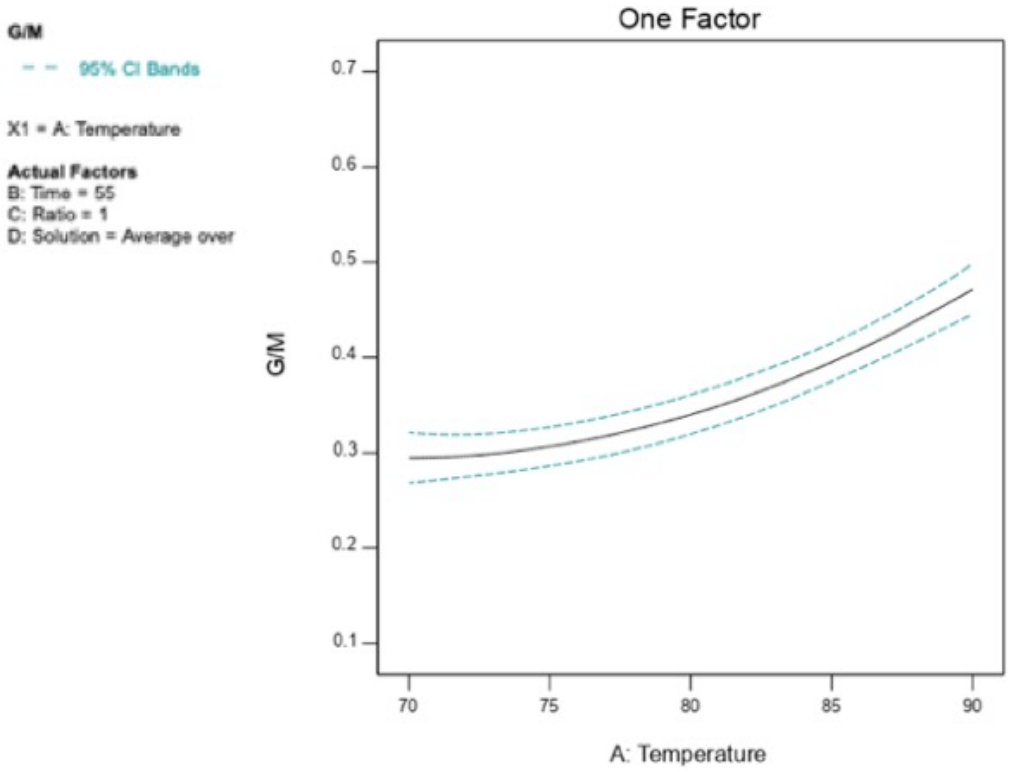
al., 2017). The temperature at 80°C was used to do the derivatization reaction of metformin without solvent by Uçaktürk (2013). In two reviewed studies (Goedecke et al., 2017; Uçaktürk, 2013), the range of temperature were tested from 20 to 80°C with or without solvent acetonitrile. Thus, temperature was tested from 60 to 90°C without solvents in the previous OFAT experiments because of the boiling point of acetonitrile. The results indicated that metformin had good performance with increasing temperatures no matter with or without the solvent. However, guanylurea did not perform well without a solvent, thus, solvent should be involved in the derivatization reaction. In addition, responses of metformin and guanylurea detectability increased with solvents due to increasing temperatures based on the results of previous OFAT experiments. Thus, the range of temperature has been confined with the following range of 70-90°C as documented in Table 3.2.

Due to the bad performance of guanylurea without a solvent in the derivatization reaction, the selection of solvents was operated through comparing the boiling point and chemical polarity. Acetonitrile has been used in reviewed study (Goedecke et al., 2017). Two more solvents were selected to test, such as 1,4-dioxane, and toluene. The boiling points of 1,4-dioxane and toluene are 101.1°C and 110.6°C, respectively (ATSDR, 2012). The results of previous OFAT experiment revealed that 1,4-dioxane performed good at responding to metformin and guanylurea in the derivatization reaction at 80°C with ratio at 1:1 for 60 minutes. Note that toluene does not solve polar chemical compound very well. Thus, 1,4-dioxane and acetonitrile are selected as solvents.

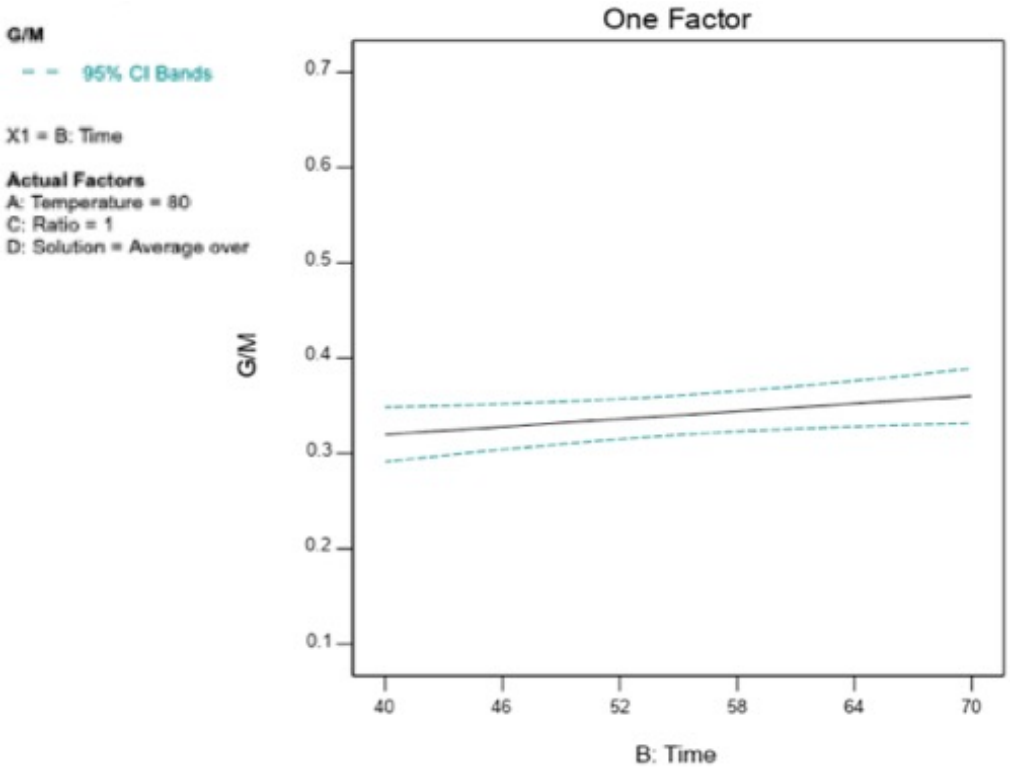
The ratio of reagent to target chemicals is another important factor to affect the response of derivatization reaction. The previous OFAT experiments tested the range of ratio at 80°C and 60 minutes with or without the solvent, acetonitrile. The range of ratio was selected from 0.5:1 to 1.5:1. The results of previous experiments showed that the high ratio could affect the derivatization reaction to obtain better responses of metformin and guanylurea. The reaction time has been detected at 30 minutes, 60 minutes, and 90 minutes by Uçaktürk (2013) for derivatization reaction of metformin. The highest response of metformin was obtained at 60 minutes (Uçaktürk, 2013). Hence, the range of reaction time is selected from 40 minutes to 70 minutes.

The effect of individual variances can be better understood visually through graphs illustrated in Figure 3.2. It is evident that temperature and ratio have higher increasing slopes indicating the importance of these two factors in determining the response of G/M. The significant curvature trend of factor A (Time) matches to the model. The slope of time is the flattest of all the slopes which means that time minimally affects the response of G/M. There is an obvious increase in the responses of G/M via changing solvents from acetonitrile to 1,4-dioxane showing that 1,4-dioxane displays a good performance in the derivatization reaction.

(a)

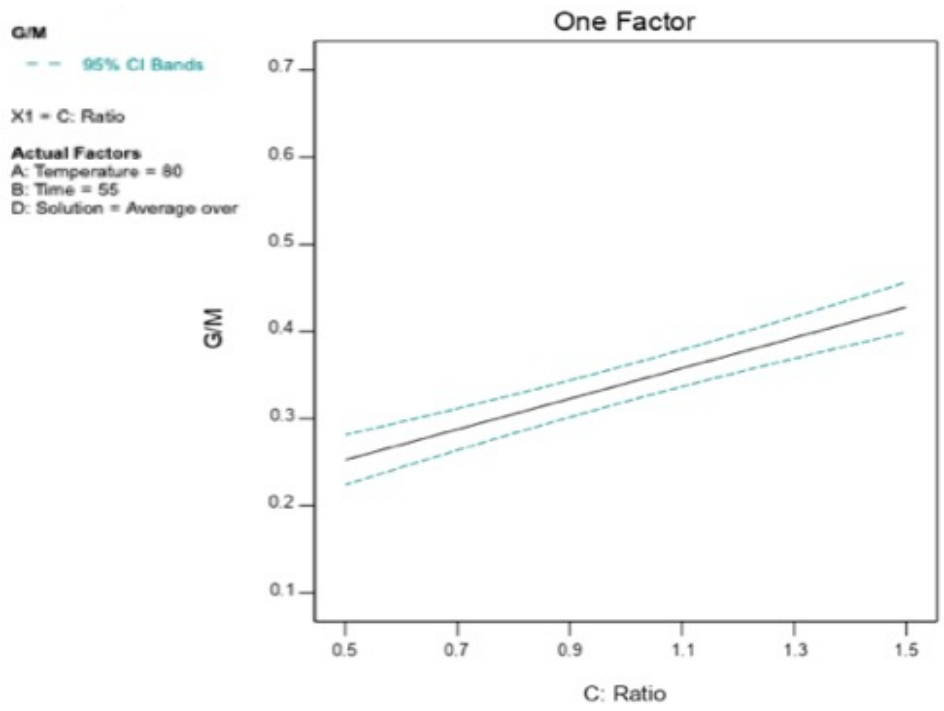

G/M
95% CI Bands
X1 = A: Temperature
Actual Factors
B: Time = 55
C: Ratio = 1
D: Solution = Average over
One Factor
G/M
0.7
0.6
0.5
0.4
0.3
0.2
0.1
70
75
80
85
90
A: Temperature

(b)


G/M
95% CI Bands
X1 = B: Time
Actual Factors
A: Temperature = 80
C: Ratio = 1
D: Solution = Average over
One Factor
G/M
0.7
0.6
0.5
0.4
0.3
0.2
0.1
40
46
52
58
64
70
B: Time

continue.

(c)



(d)

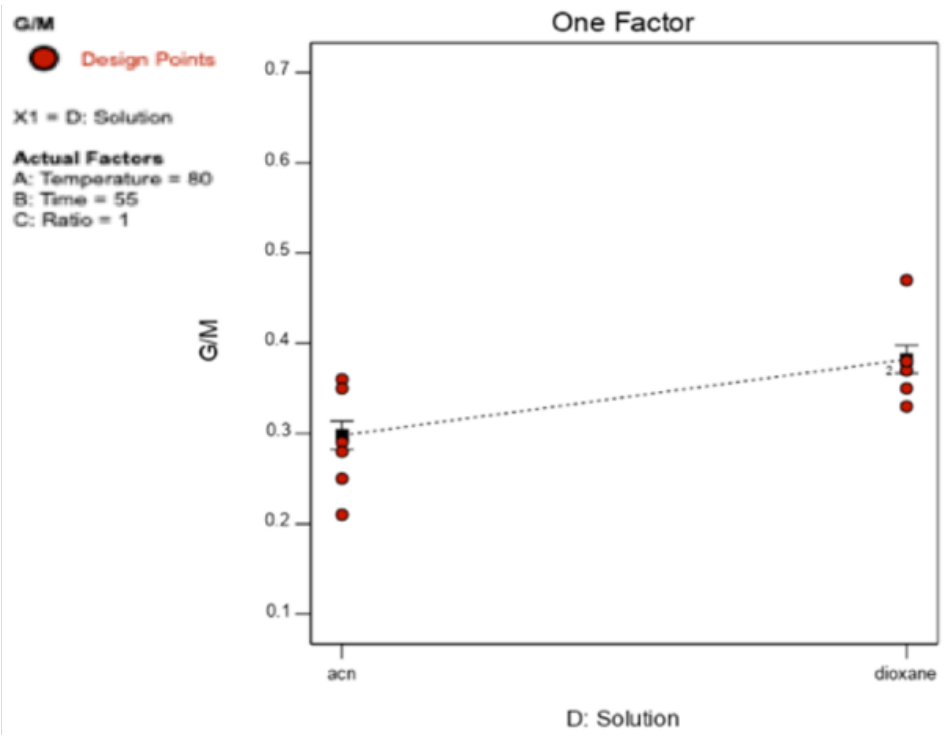
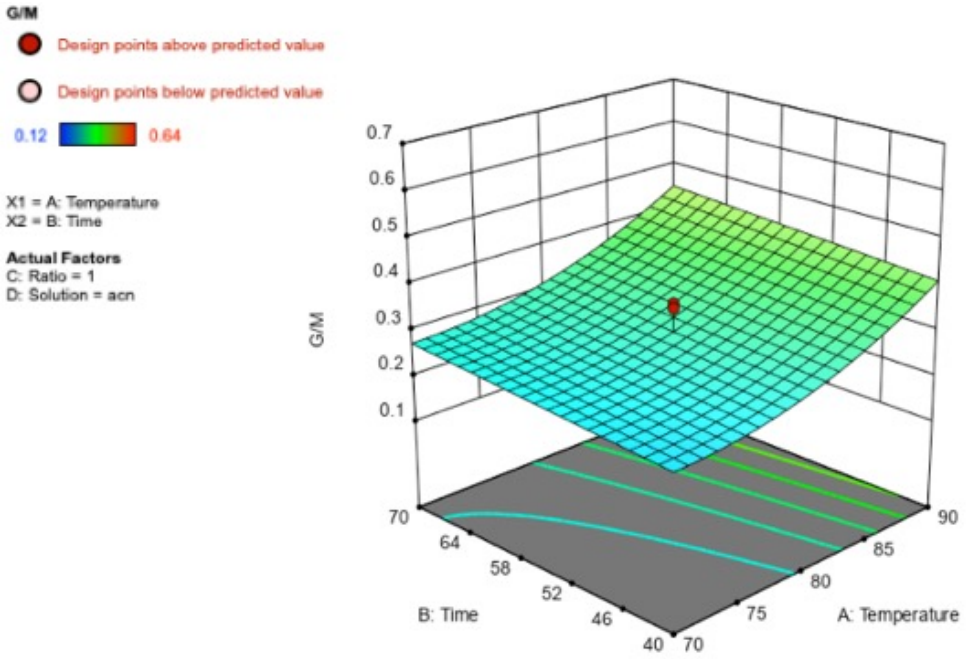


Figure 3.2 Model graphs of important factors, (a). factor A-temperature, (b). factor B-time, (c). factor C-ratio, (d). factor D-solution

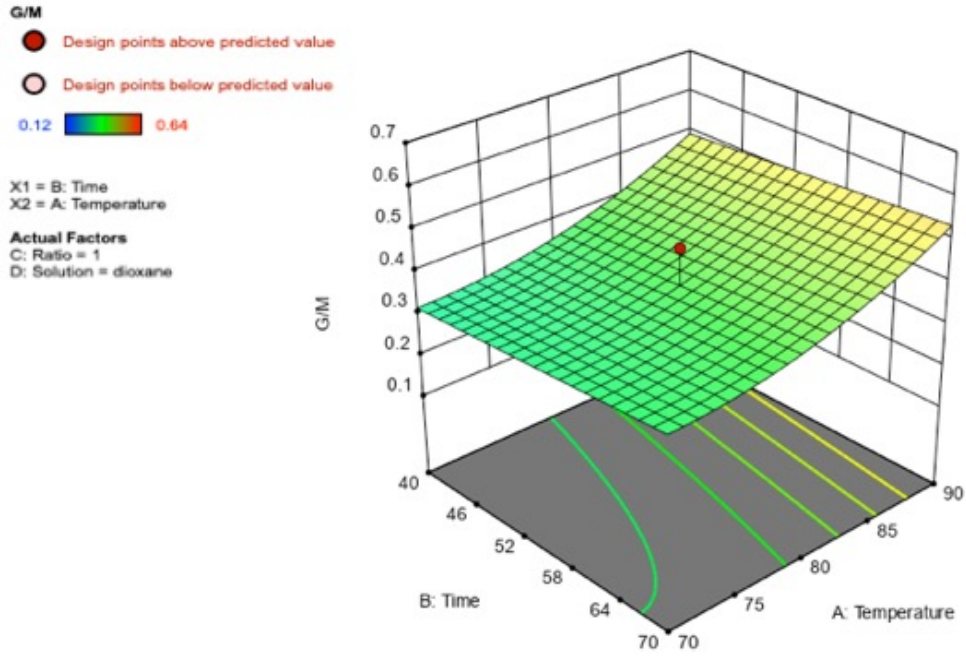
Overall, interaction plots are unavailable because there are no significant interactions between the four factors. The interactions between the four factors are respectively illustrated in three-dimensional response surface plots (Figure 3.3). In Figure 3.3 a & b, it is obvious that the responses of G/M in solvent 1,4-dioxane are much higher than the responses in solvent acetonitrile. In addition, the responses of G/M at high ratios are much higher than the responses at low ratios in Figure 3.3 c & d.

All the factors were set in range to optimize the maximum response. The maximum result of G/M was 0.622 with desirability at 0.964, and the temperature, time, ratio, and solvent were 90°C, 70 minutes, 1.5:1, and 1,4-dioxane respectively. In addition, the shorter reaction time, the better performance of GC-MS analysis. The factor of time was selected to be minimum while other factors were set in range. The optimal result with minimum time (40 minutes) was that G/M equals to 0.581 with desirability at 0.942, and temperature, ratio, and solvent were 90°C, 1.5:1, and 1,4-dioxane, respectively.

(a)

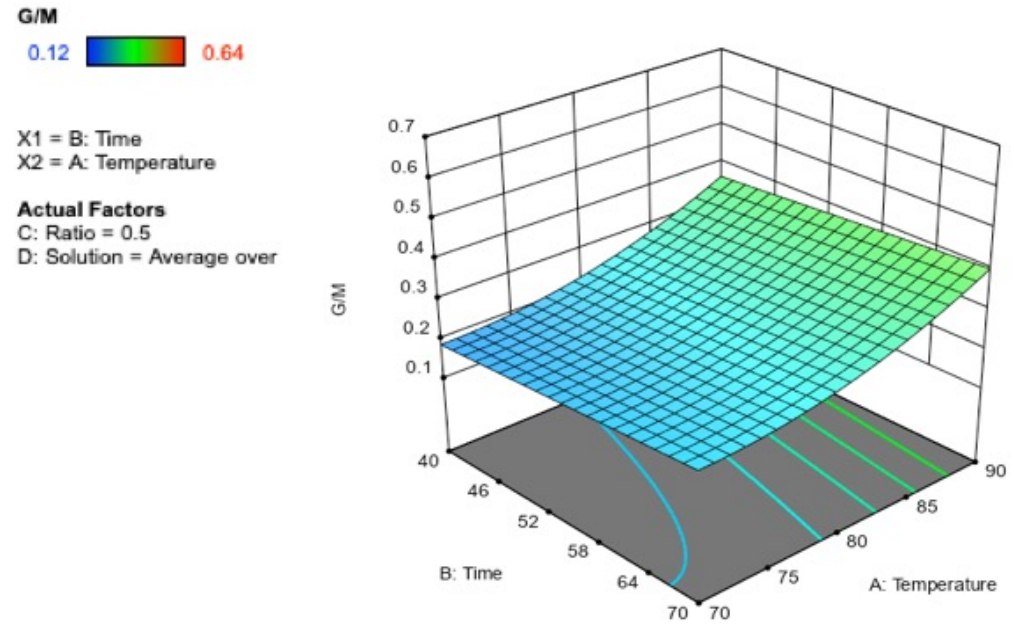

G/M
Design points above predicted value
Design points below predicted value
0.12
0.64
X1 = A: Temperature
X2 = B: Time
Actual Factors
C: Ratio = 1
D: Solution = acn
G/M
0.7
0.6
0.5
0.4
0.3
0.2
0.1
70
64
58
52
46
40
B: Time
70
75
80
85
90
A: Temperature

(b)


G/M
Design points above predicted value
Design points below predicted value
0.12
0.64
X1 = B: Time
X2 = A: Temperature
Actual Factors
C: Ratio = 1
D: Solution = dioxane
G/M
0.7
0.6
0.5
0.4
0.3
0.2
0.1
40
46
52
58
64
70
B: Time
70
75
80
85
90
A: Temperature

continue.

(c)



(d)

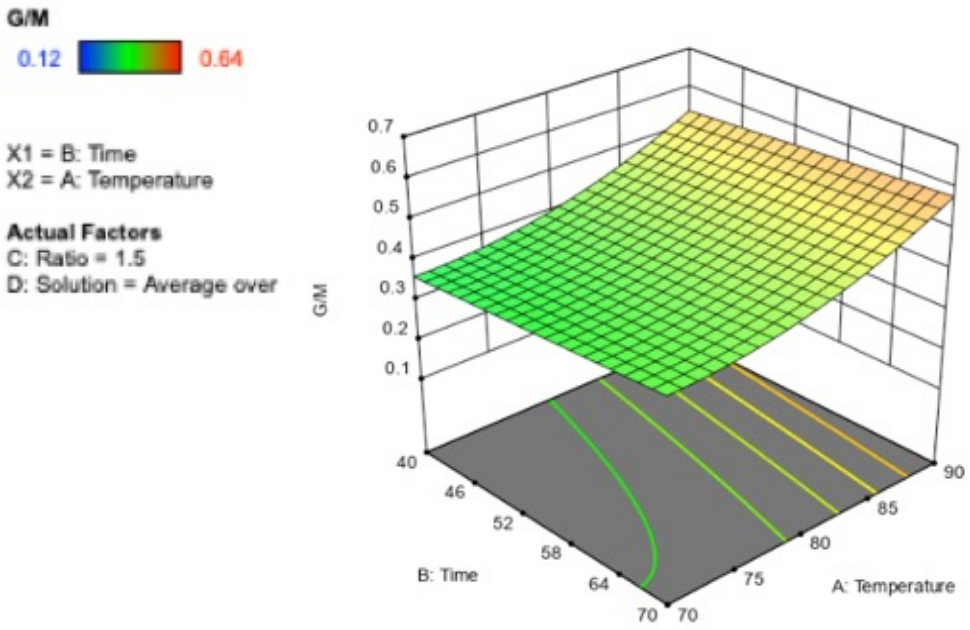


Figure 3.3 3-D response surface plot of temperature and time in different solvents and ratios, (a). acetonitrile, (b). 1,4-dioxane, (c). ratio=0.5:1, (d). ratio=1.5:1

3.2.1.2 Model Validation

Validation procedure is necessary to ultimately test a model, and in this procedure, points will be selected within the range of factors but not at specified test levels. Two additional samples have been used for model validation. In addition, four runs with optimal conditions of derivatization reaction have also been done to confirm whether the model is an adequate representation of the experiment. One replication of the optimal condition with all factors in range and three replications of the optimal condition with minimum time have been performed. All the predicted results for two additional runs and four optimized runs were illustrated in Table 3.5 which includes the predicted results as well. The results show that the responses of validation run, optimal run, and prediction agree with the error of the experiment. The values fall within the 95% prediction interval, indicating a valid model.

Table 3.5 Validation runs comparison

Run	Temperature (°C)	Time (minute)	Ratio	Solvent	Response	Prediction	95% PI Low	95% PI High
1	75	60	1.25:1	Acetonitrile	0.3078	0.3151	0.2122	0.4180
2	75	50	0.75:1	1,4-dioxane	0.2673	0.2980	0.1951	0.4009
Opt*-1	90	70	1.5:1	1,4-dioxane	0.5727	0.6220	0.5142	0.7289
Opt-2	90	40	1.5:1	1,4-dioxane	0.4803	0.5811	0.4738	0.6885
Opt-3	90	40	1.5:1	1,4-dioxane	0.4914	0.5811	0.4738	0.6885
Opt-4	90	40	1.5:1	1,4-dioxane	0.4909	0.5811	0.4738	0.6885

Opt: optimal

3.2.1.3 Extraction and Improved Optimization of Derivatization

The extraction rate of guanylurea was lower than the rate of metformin based on the results of DOE experiments. The same concentrations of standard solution of metformin and guanylurea were used to process the derivatization reaction while the peak areas of metformin were approximately two times of the peak areas of guanylurea from the graphs of GC-MS instrument. In the derivatization reaction, the reactivity of metformin with MBTFA is quicker than that of guanylurea. In addition, the derivative of guanylurea has a long chain which might be not as stable as derivative of metformin through the injection of GC-MS instrument. All of these factors affect the extraction rates of guanylurea compared to metformin.

Guanylurea has similar chemical property as metformin and could be extracted by the same elution solvents. The polarity of metformin and guanylurea is high and affects the efficiency of the extraction rate (Briones et al., 2016). The detection limit and elution time would be influenced. Thus, the mixture of elution (mentioned in Sentcion 3.1.2) is important to elute metformin and guanylurea from the cartridge. The mixture of methanol and acetonitrile buffer at 50:50 (v/v) with 2% formic acid is efficient to metformin extraction based on the EPA *Method 1694* (USEPA, 2007) and a previous review study (Tao et al., 2018).

Improving parameters of SPE method would be helpful to obtain more quantity of analytes from water samples. The composition of eluent, volume of the eluent, and sorbent mass were selected by several OFAT pre-experiments. The Thermo cartridges were used to extract metformin and guanylurea but compared to the HLB cartridges, these chemicals

had not been highly extracted from ultra-pure water samples. For achieving high derivatization reaction, it is necessary to dislodge water from the sorbent of the cartridges during extraction process due to analytes transferring from water phase samples to organic solvents. Analytes in organic solvents is obviously important and necessary to derivatization reaction and GC-MS measurement. Hence, the eluents should be total dried for dislodging water and preparing to process derivatization reaction.

The derivatization of metformin with MBTFA has been illustrated by Uçaktürk (2013). The derivatization reaction of guanylurea and buformin with reagent MBTFA are illustrated in Figure 3.4 a & b. The McLafferty rearrangement happened in the GC-MS after injecting the derivative of guanylurea with MBTFA. The derivative of guanylurea is a chain chemical which will be broke during in the EI mass detector of GC-MS instrument (Figure 3.4). The representative chromatograms of derivatized samples measured by the GC-MS instrument are illustrated in Figure 3.5. The corresponding mass spectra of derivatives of guanylurea, metformin, and buformin are shown in Figure 3.5, respectively.

The optimized four factors of derivatization were temperature, time, ratio, and solvent at 90°C, 40 minutes, 1.5:1, and 1,4-dioxane respectively. The values of R^2, adjusted R^2, and predicted R^2 of the DOE model are 0.8628, 0.8427, and 0.8143 respectively. The predicted R^2 is in reasonable agreement with the adjusted R^2 which the difference is less than 0.2. That means this mode is good fit and can offer a satisfactory predictive accuracy. The response equals to the peak area of guanylurea / the peak area of metformin (response=G/M). All the responses in two solvents (1,4-dioxane and acetonitrile) were illustrated in Table 3.3. The effect of individual variances could be better understood

visually through graphs illustrated in Figure 3.6. It was evident that the dots of high responses of G/M (G/M>0.45) were distributing at high temperatures above 85°C. The dots of responses of G/M distributed averagely at the time axis indicating the factor time minimally affected the response of G/M. There was an obvious increase in the responses of G/M via changing solvents from acetonitrile to 1,4-dioxane which shows that 1,4-dioxane displays a good performance in the derivatization reaction (Figure 3.2). The interactions between the four factors were respectively illustrated in three-dimensional response surface plots in Figure 3.6. The responses of G/M in solvent 1,4-dioxane were 33.33% higher than the responses in solvent acetonitrile. In addition, the average response of G/M at high ratios (1.5:1) was 46.8% higher than the average response at low ratios (0.5:1).

Based on the Figure 3.6, it was clear that increasing temperature and ratio would be helpful in obtaining effective derivatization reaction. There existed a limitation of the range of temperature due to the boiling point of solvents. The responses indicated that 1,4-dioxane was a better solvent than acetonitrile in the derivatization reaction because high temperature would be good to derivatization and 1,4-dioxane has the high boiling point compared to acetonitrile. In addition, the reaction time had been determined as the lowest effect to the derivatization reaction which was good to the preparing procedure of GC-MS method because the rapid sample preparation could reduce the sample detection time.

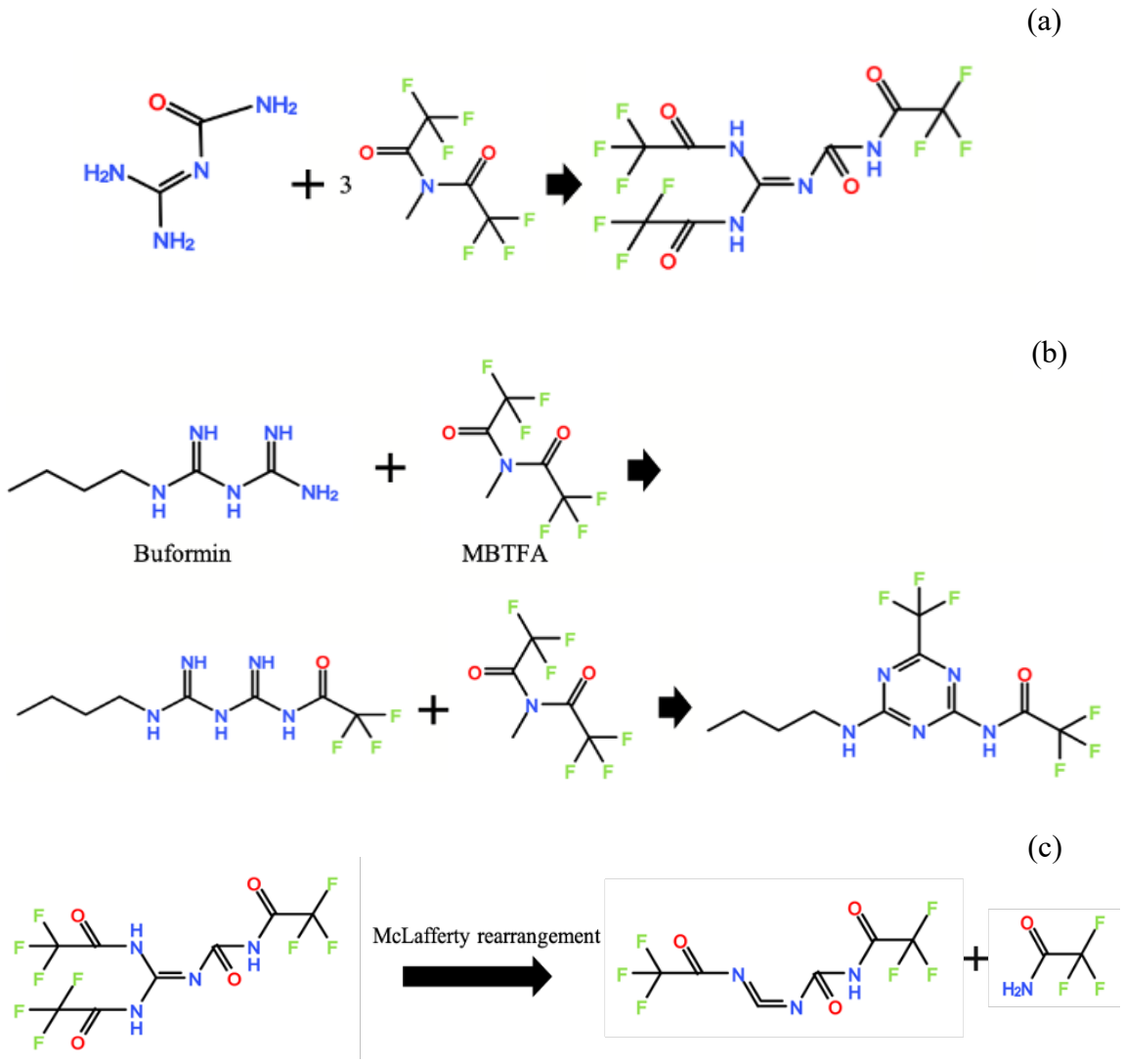


Figure 3.4 (a). The derivatization reactions of guanylurea with reagent MBTFA; (b). The derivatization reactions of buformin with reagent MBTFA; (c). The reaction of derivatized guanylurea happens in GC-MS

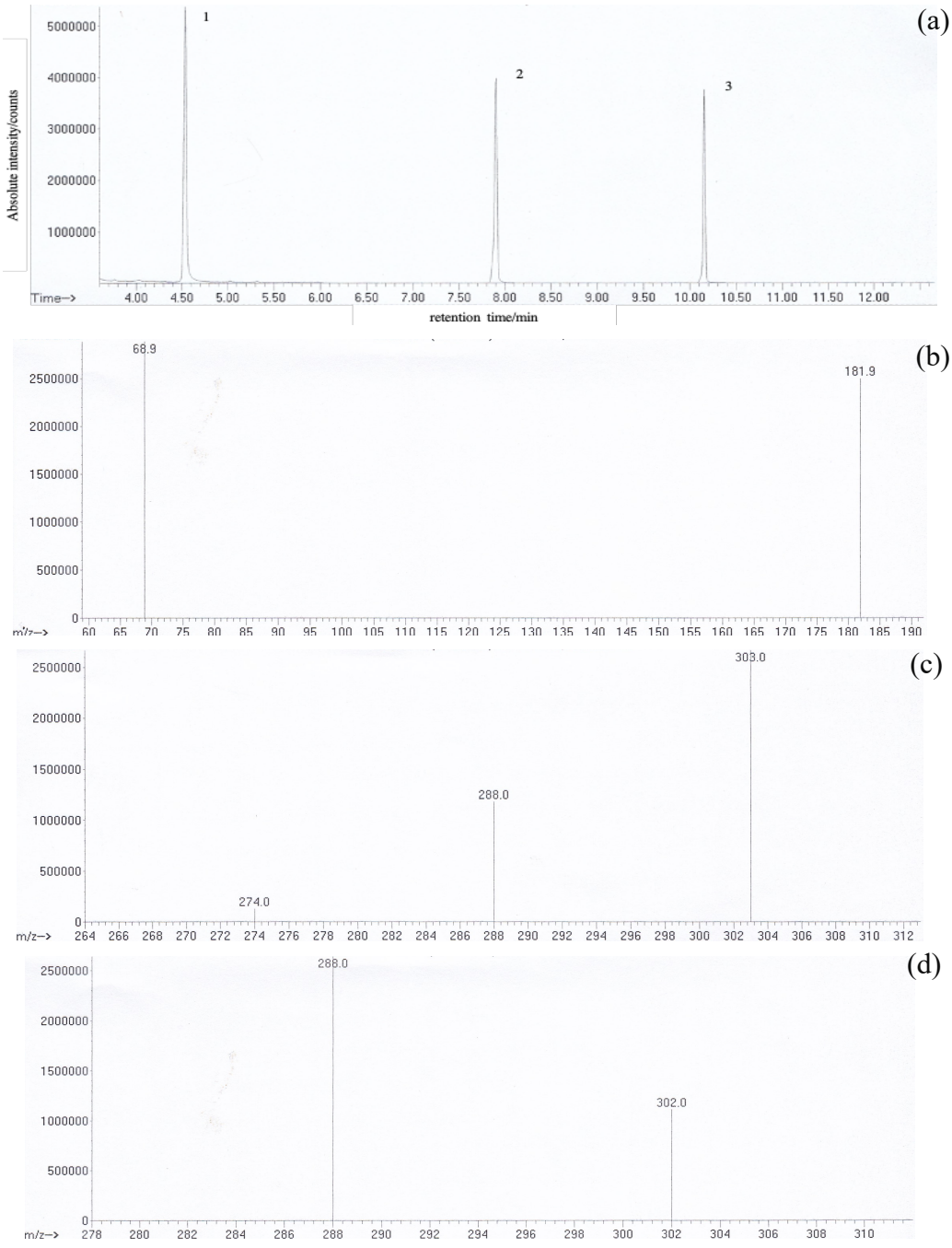


Figure 3.5 The chromatogram of guanylurea (1), metformin (2), and buformin (3) derivatized with MBTFA and analysis by GC-MS in SIM mode (a), as well as the mass spectra of (b) guanylurea, (c) metformin and (d) buformin with MBTFA as a derivatization agent

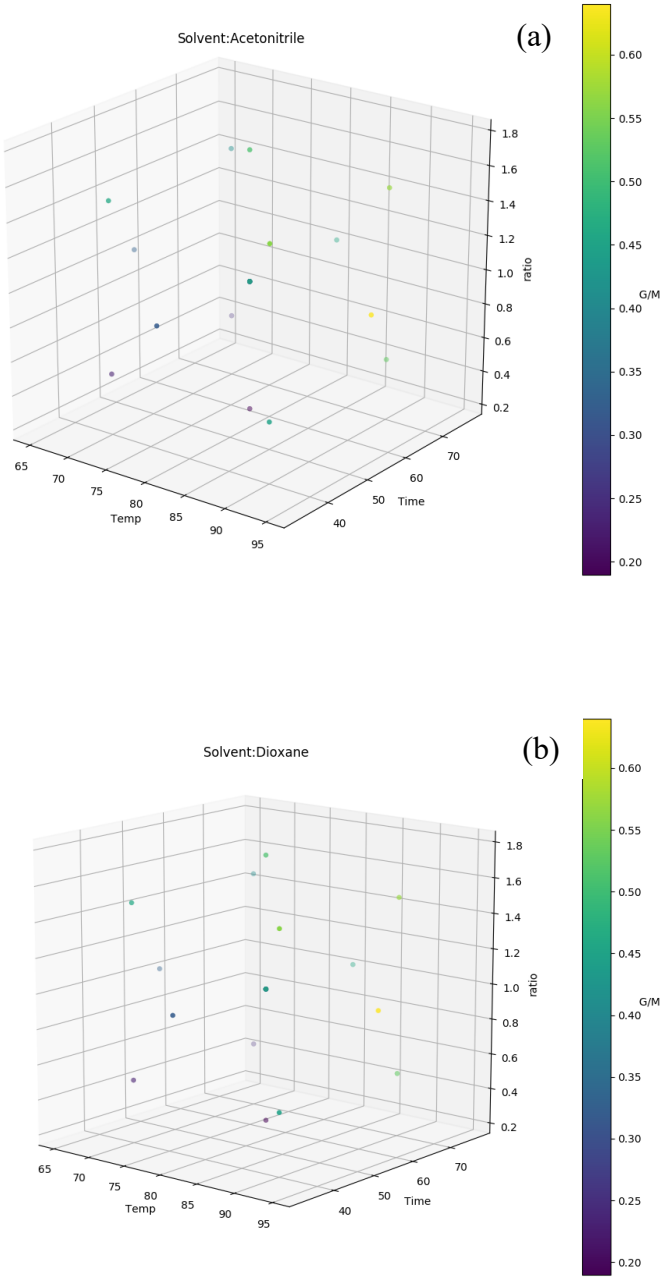


Figure 3.6 (a) 4D plot of 40 runs in solvent acetonitrile (X= temperature, Y= time, Z=ratio and colorful dots representing the response=G/M); (b) 4D plot of 40 runs in solvent 1,4-dioxane (X=temperature, Y= time, Z=ratio and colorful dots representing the responses=G/M)

3.2.2 Method Linearity and GC-MS Performance Analysis

The coefficients of determination (R^2) for the obtained calibration curves were accomplished at 0.9935 and 0.992 respectively. The LOD were determined based on the statistically calculated minimum concentration which could be measured with 99% confidence. The calculation equation was reported in EPA *Method 1694* (USEPA, 2007) and Fan et al. (2017). The LOD of metformin and guanylurea weight reacted in derivatization are 11 ng and 110 ng, respectively. The lowest concentrations of metformin and guanylurea in standard solutions were repeated seven times to obtain the precision and accuracy of calibration curves (Table A1 & A2). The values of precision and accuracy did not exceed 30% at the LOQ point or 15% at other concentration points. This method has low LOD of metformin, comparing with the LOD of metformin at 7.3 ng L^{-1} (Goedecke et al., 2017) and 14 ng L^{-1} (Martin et al., 2012) in real water samples.

Appropriate volumes of working standard solutions of metformin was spiked into 10.5 mL of blank elution solvent and then the derivatization reaction was performed. The samples were processed for GC-MS analysis and analyzed on the same day. The concentrations of metformin and guanylurea in each ultra-pure water sample was calculated using calibration curves, which were constructed daily. Precision was represented by percent relative standard deviation (RSD%), while the accuracy was expressed by bias. The values of precision and accuracy of metformin did not exceed 2.67% at the LOQ point or 4.55% at other concentration points, while the values of precision and accuracy of guanylurea did not exceed 15.37% at the LOQ point or 22.64% at other concentration points.

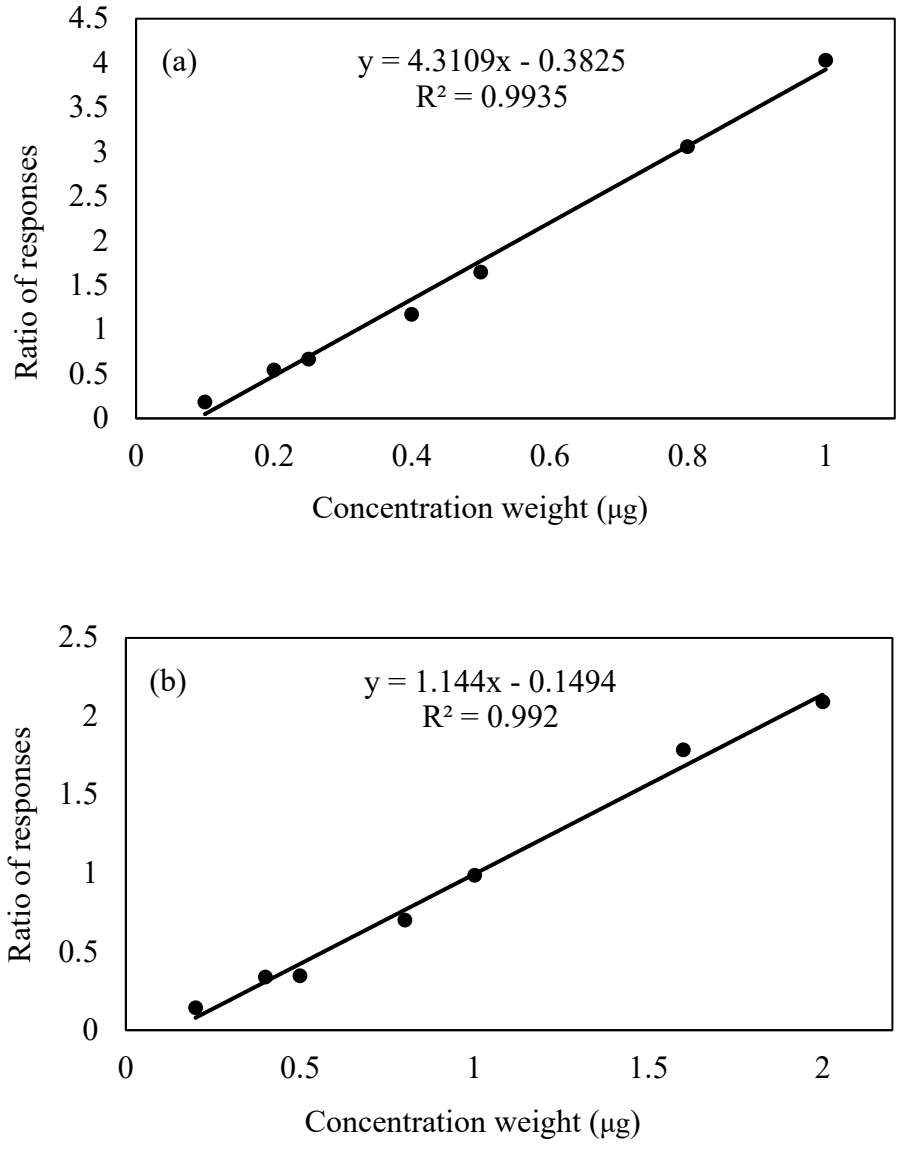


Figure 3.7 (a). Calibration curve of metformin concentration ranging from 0.1 to 1 µg; (b). Calibration curve of guanylurea concentration ranging from 0.2 to 2 µg

Table 3.6 Retention time, LODs, calibration range, linearity, recovery rates, and repeatability included in the analysis

Target chemical	Retention time (minutes)	LODs (ng)	Calibration range (ng)	Linearity (R^2)	Recovery rates (%)	Repeatability (n=7) RSD (%)
metformin	7.9	11(=t_{99}*S)	100-1000	0.9935	46.16	2.67
guanylurea	4.5	110(=t_{99}*S)	200-2000	0.992	68.31	15.37

The extraction of metformin and guanylurea was processed to estimate the extraction rate of both in ultra-pure water samples. The recovery rate was determined at two concentration points (i.e., spiked 0.5 and 1 μg into 100 mL (5 ppb and 10 ppb)). The mass weight of two target chemicals was used as unit to evaluate the performance of the GC-MS instead of using concentrations because the mass weight could be accurate and easy than concentration to measure the content of two chemicals due to the step of derivatization reaction involved. Appropriate volumes of working standard solutions of metformin and guanylurea were spiked into 100 mL of ultra-pure water samples and the control samples were prepared for GC-MS analysis. A constant amount of IS buformin was spiked into the elution solvent, then dried by a gentle nitrogen gas flow. For the 0.5 μg samples, the average percent recovery of metformin and guanylurea in 100 mL ultra-pure water samples were 46.16% and 68.31% respectively, and the rates were 42.42% and 53.33% respectively in 100 mL ultra-pure water with 1 μg samples. The RSD values of metformin in two concentrations (i.e., 5 ppb and 10 ppb) were 6.81% and 12.33% in two variables, but the RSD values of guanylurea were 41.59% and 22.43% in two variables. The performance of guanylurea recovery was not as stable as metformin in different concentrations of ultra-pure water samples. That is because the guanylurea derivative is a long chain chemical whose chains could be broke off during injecting the samples into GC-MS. This condition could cause the response of guanylurea derivative to be not very stable as metformin derivative. For achieving the high extraction rate in low concentration samples, the preparation of the experiments, special the glass tubes and other experiment equipment, should be absolutely clean. Also, it should be very careful to collect the eluate from the cartridges and to evaporate the eluate solution for concentrating the analytes.

3.2.3 Water Sample Analysis

The recovery rates of metformin and guanylurea in tap water were 36.13% and 11.99%, respectively. The extraction rates were same as the recovery rates. The RSD values of metformin and guanylurea were 1.75% and 3.58% respectively. For control samples, the same amounts of standard solutions were added to blank eluents and were prepared for GC-MS analysis. For blank samples, there were not any standard solutions of metformin and guanylurea spiked into tap water samples but IS buformin was spiked into the eluate (Section 3.1.2) for indicating the derivatization reaction.

The recovery rate of metformin in environmental samples varied substantially. The 36.13% recovery rates of metformin reported in this study were high compared with the 16% recovery rates of metformin detected by HPLC quadruple time-of-flight mass spectrometry in tap water samples (Martin et al., 2012). The recovery rates of metformin were 31% in river water samples, 54% in effluent wastewater samples (Martin et al., 2012) and 108% in canal water samples (Goedecke et al., 2017) by HPLC analysis. The recovery rates of metformin were 107% in canal water samples (Goedecke et al., 2017) and 69.83% - 84.12% in human plasma samples (Uçaktürk, 2013) in previous GC-MS analysis. Metformin generally has a lower recovery rate with the ones reported by other antidiabetics, which could be explained by the physical-chemical properties of metformin. Metformin is an aliphatic compound with a low molecular weight, and a high pKa value (Martin et al., 2012). Its octanol-water partition coefficient (Kow) is low (-1.3 or -1.43). This high polarity compound thus has a high solubility in water solution and low solubility in methanol. Therefore, metformin mainly presents as a double charged cation when dissolved in water.

It leads to poor recoveries with conventional analytical methods for trace analysis (Martin et al., 2012). In this study, SDS is used to condition the HLB cartridge as an ion-pair reagent in the SPE procedure which could make the matrix be less polar and easily eluted by methanol, the recovery rate still needs to be improved. Further, the recovery of metformin in tap water is different with the surface water, which might be explained by the matrix effect by using Oasis HLB cartridges during the SPE step. There is no referenced recovery rate of guanylurea analyzed by GC-MS method. Although the recovery rates of metformin and guanylurea are not high, the detecting both metformin and guanylurea has been achieved with the GC-MS analysis method.

3.3 Summary

Experimental results indicate that it is possible to design a model for performing the behavior of derivatization reaction. The CCD performs well with the application of DOE in this study. The developed quadratic model provides a reasonable fit and has successfully predicted the results of validation experiments. Based on these results, it is clear that increasing temperature and ratio will be helpful to obtain effective derivatization reaction. The reaction time has been determined as lowest effect to the derivatization reaction which is good to the preparing procedure of GC-MS method, because the rapid sample preparation can cut short the sample detection time. However, there exists a limitation of the range of temperature due to the boiling point of solvent acetonitrile this procedure a low point in the curvature of temperature factor plot. In addition, the model indicates that 1,4-dioxane is better than acetonitrile as a solvent in the derivatization reaction which means that in the

future work higher temperature can be used because of the high boiling point of 1,4-dioxane. The validation tests the quadratic prediction model. The responses of two validation trials and four optimization trial which have been predicted by the model equation to match up to the actual results of experiments.

This is the first study on analysis of metformin and guanylurea by using GC-MS method in water samples. As the biotransproduct of metformin, guanylurea is a key indicator of metformin in anaerobic environments (Tao et al., 2018). There is a lack of standard analysis method of guanylurea. Thus, it is necessary to develop another analysis method to detect both target chemicals in water samples in which GC-MS analysis can become a standard analysis method for the identification of these emerging contaminants. Derivatization reaction is the key pre-treatment process to both metformin and guanylurea for representing an independent reference method. It is important to achieve high efficiency of derivatization reaction of metformin and guanlyurea. Therefore, the factors of derivatization reaction exceptionally optimized were the following: temperature, time, ratio, and solvent at 90°C, 40 minutes, 1.5:1, and 1,4-dioxane. Based on the optimized factors, the GC-MS analysis method is successfully performed with derivatization reaction. The derivatization reaction time has been shortened up to 33.33% comparing with the reaction time in Table 3.1 based on the same derivatization reagent MBTFA. The calibration curves of metformin and guanylurea are obtained respectively at low concentrations for testing real samples. Tap water samples spiked with certain mass of metformin and guanylurea have been detected by the GC-MS analysis method. The HPLC-MS/MS method does not include derivatization in sample preparation steps for analysis of metformin and guanylurea

but it needs a longer time to condition the column and to rinse the column to prevent the buffer salts in the organic solvent. The GC-MS analysis method with derivatization of metformin and guanylurea is potential for the identification of possible transformation products because of the structural information of the mass spectra. The developed GC-MS method for detecting metformin and guanylurea can represent an independent reference method for using the HPLC-MS/MS method in water samples. This study is important to replenish the lack of certified reference materials of metformin and guanlyurea analyzed by GM-MS method in water.

CHAPTER 4 CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

Metformin is a first-line pharmaceutical medicine to treat type 2 diabetes and has been widely detected in the reviewed regions. Guanylurea is the biodegraded transformation product and metabolite of metformin (Tisler and Zwiener, 2018). Its occurrence, impact, analysis and treatment have been much less reported compared to those of metformin. This study filled the a few research gaps regarding these two emerging contaminates (metformin and guanylurea). The occurrence, impact, analysis and treatment of metformin and guanylurea in Atlantic coastal regions had been reviewed to summarize the condition of these two contaminates for further study. In addition, this research thesis was the first to analyze both metformin and guanylurea together by enhancing GC-MS method in water samples. The key findings have been summarized below:

(1) Literature overview: In terms of the occurrence of metformin and guanylurea, most existing data were collected based on analysis of the aquatic and sediment samples collected in coastal communities/cities and estuaries of Atlantic regions. Extremely limited studies focusing on their occurrence in ocean environments were conducted, especially in Canada and the USA. Their distribution, transport and environmental impact on fate in marine and coastal regions need to be further investigated. The effects of metformin on biological system and biodiversity have gained growing attention. However, there is a lack of sufficient data regarding the acute and chronic ecotoxicological impacts of metformin on bacteria, fishes, algae and cyanobacteria. Also, new transformation products of metformin, other than guanylurea, should be kept monitored.

(2) DOE based optimization of derivatization: An analytical method has been developed to detect both target chemicals in water samples in which GC-MS analysis served for the identification of these emerging contaminants. Derivatization reaction, as the key pre-treatment process before metformin and guanylurea analysis by GC-MS, has been optimized through generating a DOE based model. The developed quadratic model provides a reasonable fit and has successfully predicted the results of validation experiments. It is clear that increasing temperature and ratio were helpful to obtain effective derivatization reaction. The reaction time did not show same effective as temperature and ratio to the derivatization reaction. Thus, short time could fast sample preparation for the preparing procedure of GC-MS method, and short the sample detection time. The validation tested the quadratic prediction model and had been predicted by the model equation to match up to the actual results of experiments. Eventually, the optimized conditions of derivatization reaction were the following: temperature, time, ratio, and solvent at 90°C, 40 minutes, 1.5:1, and 1,4-dioxane, respectively.

(3) Improved GC-MS analysis: Based on the optimized conditions of the derivatization reaction, the GC-MS analysis was successfully performed and improved. The calibration of this metformin and guanylurea analytical method was conducted. Metformin and guanylurea in laboratory manufactured water samples were detected by using the developed GC-MS method to demonstrate its applicability. The research outputs replenished the lack of reliable GC-MS based analysis of metformin and guanlyurea in water and resulted in an effective analytical tool other than traditional HPLC-MS for aiding metformin and

guanlyurea studies. In regard to recommended future, manufactured water sample are to be used to validate the GC-MS method in non-laboratory settings.

4.2 Research Contributions

According to the research findings, this study can be summarized and highlighted by the following contributions:

1) This thesis, for the first time, documented an overview regarding the occurrence, impact, analysis and treatment of metformin and guanylurea in Atlantic coastal regions including Canada, USA and Europe.
2) Key research gaps have been identified based on the literature review to showcase the future prospective in the field. They include: (a) derivatization reaction, as the key pre-treatment process to both metformin and guanylurea for GC-MS method, has been developed; (b) analysis of both metformin and guanylurea by using GC-MS method in water samples has been developed.
3) This is the first study on simultaneous analysis of metformin and guanylurea in water samples using GC-MS method. Moreover, CCD methodology is for the first time adopted for optimization of GC-MS based analysis of metformin and guanylurea in water samples.

4.3 Recommendations for Future Research

The current research efforts focus on method development of GC-MS analysis of metformin and guanylurea. Further investigations can be carried out in the following aspects:

1) Detection of metformin and guanylurea in other types of real water samples, like final effluents of wastewater treatment plants, could be conducted in the future. There is a lack of data on metformin and guanylurea from non-laboratory-controlled water samples (i.e. municipal wastewater and marine bodies of water) to demonstrate the applicability of this newly improved GC-MS based analytical method.
2) Due to the high sensitivity of this enhanced GC-MS analytical method, it can be used to as an effective tool for testing metformin and guanylurea before and after the AOP treatment through using a micro-reactor. The mechanisms and performance of AOP for metformin and guanylurea reduction can be obtained in a micro-system.

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Appendix:

Table A1. The data of seven-time experiments of the lowest concentrations of metformin in standard solutions for the precision and accuracy of calibration curves

Number of the samples at 0.1 μg of metformin	Metformin response peak area	Buformin response peak area	Ratio	Values based on the calibration curve (μg)
1	3979	21885	0.18	0.13
2	3999	20276	0.20	0.13
3	2718.33	17365.67	0.16	0.13
4	3492.33	18661.33	0.19	0.13
5	3056	18128.33	0.17	0.13
6	3215.33	17992.33	0.18	0.13
7	3141.33	15835.67	0.20	0.13
Average			0.18	0.13
S.D.			0.015	0.0035

Table A2. The data of seven-time experiments of the lowest concentrations of guanylurea in standard solutions for the precision and accuracy of calibration curves

Number of the samples at 0.2 μg of guanylurea	Guanylurea response peak area	Buformin response peak area	Ratio	Values based on the calibration curve (μg)
1	2058.67	21885	0.094	0.21
2	1729.67	20276	0.085	0.21
3	1083.67	17365.67	0.062	0.19
4	3200.67	18661.33	0.171	0.28
5	2146.33	18128.33	0.118	0.23
6	2804.67	17992.33	0.156	0.27
7	2442	15835.67	0.154	0.27
Average			0.120	0.24
S.D.			0.041	0.036