Detection of Deoxyribonucleic Acid Damage Induced by Metal Ion Interactions and Repair of Metal Ion Deoxyribonucleic Acid Cross-links

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Thesis submitted for the Master of Science degree in the Faculty of Science and Environmental Studies

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Declaration:

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgment has been made in the text.

Neal Esau

Acknowledgements

I would like to first and foremost thank Dr. Carney Matheson for his assistance and guidance in this research. The drive and passion you possess for the field of DNA research is inspirational and pivotal to keeping my interest and focus on this field as strong as it is today.

I would also like to acknowledge my graduate committee of Dr. Joseph Carney, Dr. Greg Pyle, and my external examiner Dr. Ryan Parr for reviewing this thesis.

Furthermore, thank you to the staff of the Paleo DNA Laboratory, the Lakehead University Instrumentation Lab, and ALS Laboratory Thunder Bay for the equipment training and use, reagents and supplies, and knowledgeable input into the data collected.

Finally, thank you to my family and friends for the unending moral support and genuine interest in the progress of this research. I am humbled by your words of encouragement and am overcome by your pride for what I have accomplished. Thank you for your unconditional love and sincerity when I needed it the most.

Dedicated to my grandmother, Pearl Mary Behman.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

A	Adenine	HPLC	High performance liquid chromatography
aDNA	Ancient DNA	LC-MS	Liquid chromatography-mass spectrometry
AGE	Advanced glycation end product	M-DNA	Metal-DNA complex
AP	Abasic site	$MgCl_2$	Magnesium chloride
BER	Base excision repair	MMR	Mismatch repair
bp	Base pair	mtDNA	Mitochondrial DNA
BSTFA	Bis(trimethylsilyl) trifluoroacetamide	MW	Molecular weight
C	Cytosine	m/z	Mass to charge ratio
C8	Carbon 8	NAD+	Nicotinamide adenine dinucleotide
CpG	Copper-phosphate-guanine	N7	Nitrogen 7
Cu-GMP	Copper-guanine monophosphate	NER	Nucleotide excision repair
$CuCl_2$	Copper chloride	np	Nucleotide position
dATP	Deoxyadenosine triphosphate	PCR	Polymerase chain reaction
dCTP	Deoxycytidine triphosphate	*Pfu*	*Pyrococcus furiosus*
ddH_2O	Double distilled water	phen	1, 10-phenanthroline
dGTP	Deoxyguanosine triphosphate	PTB	Phenacylthiazolium bromide
DNA	Deoxyribonucleic acid	py	Pyridine
dNTP	Deoxynucleoside triphosphate	ROS	Reactive oxygen species
DR	Direct reversal repair	SDS	Sodium dodecyl sulphate
DSB	Double strand break	SSB	Single strand break
dsDNA	Double stranded DNA	SSC	Saline-sodium citrate
DTT	Dithiothreitol	ssDNA	Single stranded DNA
dTTP	Thymidine triphosphate	STR	Short tandem repeat
EDTA	Ethylenediaminetetraacidic acid	T	Thymine
E. coli	*Escherichia coli*	T_m	Melting temperature
en	Ethylenediamine	*Taq*	*Thermus aquaticus*
EtBr	Ethidium bromide	TBE	Tris-borate-EDTA
G	Guanine	Tris	Tris(hydroxymethyl) aminomethane
GC-MS	Gas chromatography-mass spectrometry	UVB	Untraviolet B

Abstract

There are many forms of DNA damage. One of these, DNA-metal cross-links are particularly problematic as they not only affect the tautomeric structure of DNA, but can also inhibit enzymatic activity and block amplification with DNA polymerases. Since the inability to amplify DNA can affect many fields of genetic research, it is necessary to find ways to identify this damage and repair the DNA. Copper is one metal that can form metal-DNA cross-links and can seriously affect the recovery and analysis of degraded DNA from forensic or archaeological material. In this research DNA-copper cross-links were generated for the development of a repair method and applied to an archaeological sample. The DNA-copper adducts were identified through the use of gas chromatography-mass spectrometry on several templates of different complexity: single deoxyribonucleotides, a synthetic 22 base pair double stranded DNA fragment, modern amplified DNA, and an ancient extract with naturally occurring copper cross-links. A number of chemicals were considered for direct reversal repair of copper-DNA cross-links of which ethylenediamine was successful. Treatment of all the templates with ethylenediamine resulted in the repair of the nucleobase, specifically guanine which is the most susceptible to copper cross-link formation. The success of the direct reversal repair was verified using GC-MS based on expected retention time and the identification of ion fingerprints. The amount of copper-DNA adducts measured in each template varied greatly as did the success of the direct reversal repair although repair was evident in all samples.

1.0 Introduction

The study and understanding of DNA has been a major step forward in the world of molecular biology. As we become more and more familiar with its complete purpose in the function of life, we are also discovering it's potential to be manipulated and studied *in vitro*. Advances in DNA research have lead not only to a better understanding of the structure and function, but also the necessary processes required for *in vitro* analysis. With the ongoing development of extraction, amplification, and purification protocols, it is now possible to work with low yield or even highly degraded sources of DNA that were once thought to be unusable. Our understanding has now allowed us to manipulate the "genetic blueprint" for not only ancient degraded populations, but in the medical and forensic fields as well.

1.1 DNA Structure and Amplification

In order for DNA to be manipulated and studied with any success, certain aspects of DNA needed to be understood and method's developed for *in vitro* research. First and foremost, an understanding of the structure of DNA needed to be established. Once this was understood, the next logical step was the ability to manipulate the nucleic acid. In order to work with DNA in a laboratory setting, the amount of DNA present, especially in older degraded samples, was insufficient for research purposes. The ability to study DNA could not be possible without the advent of an artificial amplification process, this was developed by Kary Mullis and became known as the polymerase chain reaction (PCR) (Saiki et al. 1988). Since all these steps of our understanding of DNA have developed, it is now possible to analyze DNA.

1.1.1 Watson and Crick

Although the presence of DNA has been known of for some time, the ability to study DNA did not begin to move in full stride till after 1953, when Dr. James Watson and Dr. Francis Crick proposed a structure for these nucleic acids, the double helix (Watson and Crick 1953). Although previous models had been proposed, they had all been found to have major flaws or were inconsistent with previously collected evidence. This inconsistency caused their proposal of two helical chains coiled around the same axis to be a more likely structure for DNA (Watson and Crick 1953). This proposed arrangement coincided with x-ray diffraction studies of the structure of DNA (Franklin and Gosling 1953). Previous research also indicated that the ratio of adenine to thymine and cytosine to guanine were very close implying a possible pairing system (Zamenhof et al. 1952). With their model of DNA structure, they were able to lay out a basic structural design, based on the purine and pyrimidines in their most probable tautomeric form. This also offered a valid explanation for the method of replication. Watson and Crick's model in essence contained two templates for replication due to the double helix (Watson and Crick 1953). The assumption was made that these complementary strands could separate in order to be replicated *in vivo* and, conversely, that this function could be accomplished *in vitro*. Although this assumption did prove to be true, their interstrand bond layout was not entirely accurate. It wasn't until 1956 that the specific bonds were studied and it was discovered that the cytosine-guanine structure was shown to have three hydrogen bonds, not two as stated in Watson and Cricks model (Pauling 1956). For this reason, it is much easier for the cell to begin DNA replication at an adenine-thymine rich area where it is

easier to break the two hydrogen bonds as opposed to a cytosine-guanine site rich of triple hydrogen bonds.

1.1.2 Polymerase Chain Reaction

Invariably, this new found understanding of the building blocks of life would need to be harnessed *in vitro* in order for further study to occur. It was not until 1986 that Dr. Kary Mullis and team published a paper on enzymatic amplification of DNA. By incorporating a set of oligonucleotides in the presence of single stranded DNA (ssDNA) that overlap in their extension direction and repeating the cycles, Dr. Mullis created an *in vitro* method of amplifying specific DNA regions exponentially (Mullis et al. 1986). He dubbed this process the polymerase chain reaction (PCR), and with it, changed the course not only of the study of DNA, but essentially established the model of molecular biology. The process was simple yet elegant: by mixing optimized concentrations of DNA template, each of the four deoxynucleoside triphosphates (dNTP), and magnesium chloride ($MgCl_2$) to a buffered solution and raising the temperature to 95°C, they were able to first denature the double stranded DNA (dsDNA) template to single strands (Mullis et al. 1986). The temperature was then brought back down to 30°C at which point the Klenow fragment DNA polymerase harvested from *Escherichia coli* was added and the solution was held at the temperature for 2 minutes to allow the DNA polymerase to extend the template (Mullis et al. 1986). The initial use of the Klenow fragment for amplification was soon replaced by the more thermostable DNA polymerase harnessed from *Thermus aquaticus*, which opened the door to not only the use of other thermostable enzymes, but genetically modified DNA polymerases as well (Saiki et al. 1988).

Success of this procedure hinged on the reagents included and their concentrations. The buffer was included in order to maintain an optimal pH condition for the DNA polymerase to replicate the target strand in the 5′ to 3′ direction. It was also optimal to have equal concentrations of each dNTP in order to maintain the equilibrium of purines to pyrimidines, the building blocks of DNA. The concentration of primers also needed to be optimized in order to avoid producing primer dimers. Primer dimer occurs when the excess primers act as their own template, binding to create a short double stranded fragment (Brownie et al. 1997). The PCR would not operate without a proper metal ion cofactor concentration of $MgCl_2$. Divalent metal cations are an essential cofactor for DNA polymerase and for the dNTPs (Springgate et al. 1973). Although several cations can be used, the most common used for *in vitro* replication is Mg^{2+}. Since it is required by both the DNA polymerase and the dNTPs, the total concentration of the Mg^{2+} must exceed the total concentration of the dNTPs in order to have enough left over to initiate the DNA polymerase. If this concentration is not optimized, the fidelity of the enzyme could be jeopardized, leaving a final product that is less than expected in both amount and quality.

The flexibility of PCR has resulted in it becoming a diverse tool for DNA analysis, even being made able to overcome any inherent shortcomings such as lack of specificity in amplification. Since it is capable of replicating DNA from very small amounts of source material with high fidelity, its applications are vast. These include not only DNA sequencing and forensic applications, but also genetic fingerprinting that has proven useful in identifying genetic relationships between individuals for such studies as population genetics, phylogenetics, and species identification. PCR has made the

quantification of DNA a reality with the introduction of real-time PCR, a procedure allowing the simultaneous amplification and quantification of DNA by using fluorescently labeled oligonucleotides. This allows results to be obtained based on quantifiable data as opposed to the qualitative band size discrimination used in standard PCR.

Standard PCR can also exhibit low sensitivity regarding certain samples as well as being plagued by poor precision. This spurred researchers to adapt the protocol to their specific needs. These slight changes create a new specified purpose for PCR with seemingly endless possibilities. One variant created to increase sensitivity is Hot Start PCR. This design reduces the occurrence of non-specific amplification by inhibiting the DNA polymerase activity until a certain temperature is reached. This is accomplished by covalently binding inhibitory agents to the DNA polymerase that will only dissociate after a high temperature is reached. Since the DNA polymerase is inert until that temperature occurs, it is not able to amplify random non specific sequences, resulting in a higher yield of specific amplification. Another well known variant of PCR is Multiplex-PCR. This PCR uses several pairs of primers that anneal to different sequences allowing multiple target analyses from a single sample. This protocol is the one commonly used in studying DNA fingerprinting as it allows the discrimination of multiple targets to be compared to other samples. Although this variant is widely used, it does have certain limitations. The design of complementary primer sets that will not interfere with one another is critical to a successful Multiplex-PCR. These are just two of the many variations that have been developed over the years. Although they all differ in approach

and application, the one common aspect of all of them is the foundation of Kary Mullis' original design. Without PCR, the field of DNA research could not be where it is today.

1.2 Metal Inhibition

Metal inhibition of DNA is a common problem for genetic analysis. Not only are metal ions excellent inhibitors of enzymatic activity, they have also been shown to interfere with the DNA extraction process (Iachetta and Matheson 2008). This is particularly frustrating in the field of ancient DNA research, where environmental exposure to compounds such as metals can be common place (Wilson 1997). The inhibitory effects of metal ions are particularly effective in the form of divalent cations. While monovalent cations can also inhibit and damage DNA, the presence of divalent cations appears to bind preferentially (McFail-Isom et al. 1999). However some earth metals have been shown to act as a preservative of the strand of DNA against thermal and acid degradation (Zimmer et al. 1974).

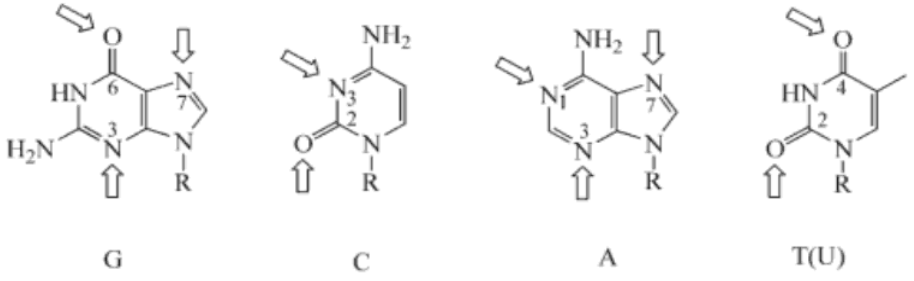


Figure 1: Potential divalent cation binding sites of the 4 common nucleobases in their neutral, preferred tautomeric structures
(image taken from (Hud 2009))

Many metals have been studied in attempts to understand the metal-DNA interaction (M-DNA), these include Sr^{2+} , Ba^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+} (Duguid et al. 1995). Although many metal divalent cations have the potential to inhibit,

the one metal with the highest affinity to induce cross-links is copper. The interaction of Cu^{2+} and DNA has been a recurring theme in M-DNA papers due to its affinity to bind covalently to the DNA (Geierstanger et al. 1991; Kagawa et al. 1991; Liu et al. 1999).

Although Cu^{2+} has a higher attraction to the purines (adenine and guanine) there are potential binding sites available on all four of the nitrogenous bases (Figure 1). This preference for purines is logical since they contain more potential binding sites than the pyrimidines. Further M-DNA studies revolving around specific purine interaction also noted a further penchant for guanine that in some cases was 3 or 4 times higher than that for adenine (Arjmand et al. 2006). The susceptibility of guanine and adenine to form covalently bonded modifications with Cu^{2+} is most prevalent on the N7 atoms (Geierstanger et al. 1991).

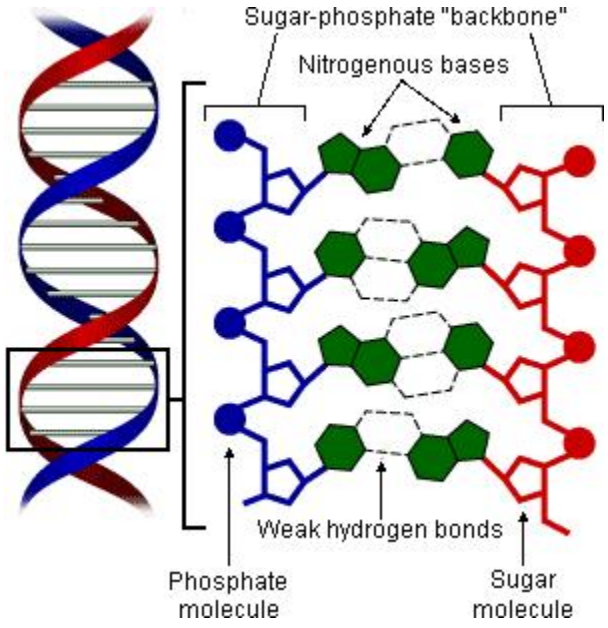


Figure 2: Sectional view of the chemical structure of DNA.
(image taken from http://evolution.berkeley.edu/evosite/history/dna2.shtml)

While metal inhibition is probable on the nitrogenous base, there are also other potential sites of binding found directly on the DNA backbone. Studies have shown that

the metal interaction may also occur at the phosphate-sugar portion of DNA (Perrin 1960; Tu and Friederich 1968). This interaction is not limited to the backbone unit, but has also shown binding potential to sugars and phosphates individually (Angyal and Davies 1971). As phosphates are inherently negative, the positively charged cations would have a natural electrostatic charge attraction. Due to this, it is possible that a metal ion may crosslink-with the phosphate portion of the DNA back bone (Granot et al. 1982; Granot and Kearns 1982a; Granot and Kearns 1982b).

1.3 Proteinase K-Metal Interaction

The proteinase K extraction is a common extraction used for DNA isolation through cellular digestion. This enzyme was first discovered and isolated from fungus extracts where it was noted for its ability to digest keratin (Ebeling et al. 1974). The first application to molecular biological study was in 1974 when it was discovered that proteinase K in the presence of DNA has the ability to digest cellular protein components which in turn can free the DNA from any holding proteins (Hansen 1974). It was also discovered by Hansen (1974) that the proteinase K was required to be present in order to lyse the cell and release the DNA (Hansen et al. 1974). It also appeared to be able to hydrolyse nucleases thus rendering them unable to degrade the DNA being extracted (Hansen 1974). Proteinase K's abilities are further improved in the presence of sodium dodecyl sulphate (SDS) which is a detergent that also causes protein denaturation and disruption, including disruption of the cell wall and other intracellular materials (Weber and Kuter 1971). In ideal conditions, the utilization of this enzyme can be an excellent DNA extraction method however it has been shown to be susceptible to metal inhibition. Proteinase K exposure to metals compromises the enzyme's active site thus leaving it

unable to perform its digestion (Muller and Saenger 1993). The active site of the enzyme is a catalytic triad of Asp39 - His69 - Ser224 which must maintain its integrity in order for the enzyme to function (Betzel et al. 1988). Proteinase K also contains two Ca^{2+} binding sites which can be easy binding points to inhibit (Betzel et al. 1988). The introduction of an inhibitory metal to the proteinase K structure will cause deformity in the folding and jeopardize the stability of its structure. This is evident in Muller and Saenger's (1993) work in which it was discovered that proteinase K exposure to mercury (Hg) ions caused the metal to covalently bond to a cysteine amino acid that was nearby the hystidine of the active site. Although the Hg ions bound in places other than the cysteine, they were deemed only to be minor structural changes. Some metals have varying degrees of protein inhibition potential but this damage can be irreversible if concentrations are high enough (Nishina et al. 2004). Further research into metal inhibition of proteinase K has revealed that inhibition is not limited to specific metal groups. Inhibition has been shown to occur with alkali, transition, and post transition metals (Iachetta and Matheson 2008).

1.4 DNA Polymerase

DNA polymerase is a fundamental component in DNA replication. Through the functions of this enzyme, a single strand of DNA can be read and replicated, producing a double stranded product. Due to the base-pairing DNA exhibits, this synthesized strand is identical to the original complementary strand that was bound to the template thus resulting in semi-conservative replication (Meselson and Stahl 1958). This process is the key to the *in vitro* success of PCR as well, allowing replication to increase the yield exponentially.

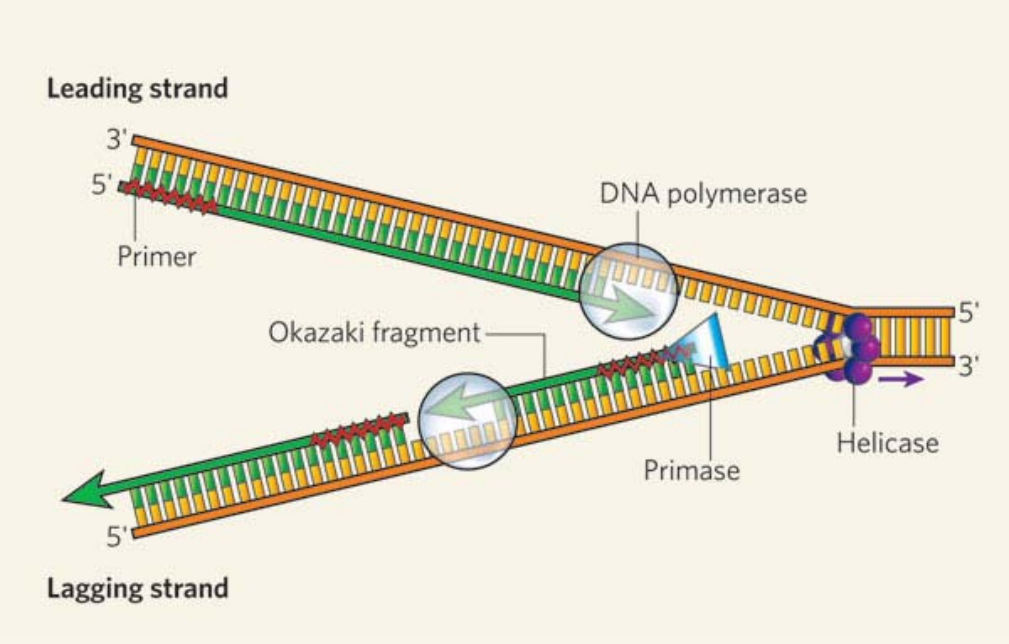


Figure 3: Simplified schematic of double stranded DNA being replicated.
(image taken from http://www.nature.com/nature/journal/v439/n7076/fig_tab/439542a_F1.html)

DNA polymerases were first discovered and studied in 1955 from the bacterial species *Escherichia coli* (*E. coli*) (Lehman 2003). With the enzyme's biological function understood, research went underway to conclude what conditions were necessary to manipulate DNA polymerase *in vitro*. It was discovered that in order for the DNA polymerase to replicate there were several requirements: a certain fraction of the DNA present had to be polymerized, Mg^{2+} had to be present, and the deoxynucleotide triphosphates of thymine (T), cytosine (C), guanine (G), and adenine (A) had to be available in equal concentrations (Bessman et al. 1958). Through further investigation, it was also discovered that these single deoxynucleotides were forming strong covalent 3′,5′-phosphodiester linkages (Adler et al. 1958). It was also determined that the ratio of guanine to cytosine and adenine to thymine was about the same just as was seen during *in vivo* DNA formation (Lehman et al. 1958).

All DNA polymerases extend in the 5′ to 3′ direction (Figure 3). In order to accomplish this, the dsDNA must be denatured into single ssDNA (Kornberg 1988). In order for the DNA polymerase to attach to the ssDNA however, it needs a double stranded starting point of replication. To account for this, single stranded primers were designed in order to bind to the ssDNA before and after the area of replication. Once the DNA is unwound, the DNA polymerase is able to bind to the ssDNA at the site of the annealed primer and begin the replication process. Although the enzyme is fairly reliable for this process, it does occasionally incorporate errors leading to some DNA polymerases adapting a proof-reading function in order to lower the chances of a misincorporation. This proofreading function is an exonuclease enzyme that is responsible for cleaving phosphodiester bonds at the end of a chain. The presence of this exonuclease activity is an excellent biological advantage since it significantly lowers the chances of incorporating potentially lethal mutations.

1.4.1 Polymerase-Metal Interaction

DNA polymerase is an enzyme that is vital to the amplification and repair of DNA template. When metal inhibition of the DNA polymerase activity occurs it not only impedes the replication process, but also impedes the cell's attempt to repair the damage *in vivo* (Hartwig 1998). Due to the relatively low copy numbers of DNA polymerase found *in vivo*, potential inhibition can be detrimental to the cell's biological activity (Lehman 2003). The possible sources for these contaminants are also quite vast. A previous study by Esau and Matheson (2009) has shown that direct exposure of extracted DNA to copper, nickel, cobalt, mercury, and calcium all show varying degrees of DNA polymerase activity inhibition. Certain metals, such as copper, have been observed to

cause a discoloration of the sample material (such as bone) making this a good indicator of its presence (Baby 1961). These metals are commonly associated with the presence of burial artifacts such as necklaces, medallions, and other personal belongings. The amount of time of metal exposure in these samples parallels the amount of DNA inhibition witnessed through metal interactions. Although there has been research done showing that size exclusion chromatography columns can remove free metal ions causing inhibition from contaminated samples, it does not mean there is no residual metal remaining and this size exclusion chromatography method cannot remove the metal ions cross-linked to the DNA (Matheson et al. 2009). The ease with which divalent cations can interrupt the DNA polymerase makes the removal of any ions crucial to the success of DNA amplification.

The enzymatic sensitivity of DNA polymerases in the presence of metal ions has been well established. It has been found that certain transition metals (such as Mg) have a tendency to bind to the phosphate group, increasing the melting temperature (T_m) and stabilizing the double helix while others (such as Cu) will bind to the nucleobases, lowering the T_m and destabilizing the double helix (Eichhorn and Shin 1968). It is this destabilization of the double helix that leads to DNA polymerase inhibition. A weakened double stranded template produced *in vitro* can be detrimental to replication. In order to operate smoothly, the DNA polymerase must stay bound to the strand being replicated. This is especially vital in amplifying long fragments of DNA, since a low fidelity DNA polymerase has a tendency to dissociate quickly and reattach elsewhere to continue replicating. This stability is accomplished through a clamp protein which is tightly bound to the DNA polymerase, allowing it to slide along the DNA strand (Alberts 1994). If a

metal adduct has formed in the path of replication this will inhibit the protein clamp, causing it to release the polymerase from the strand and halt the replication process.

1.4.2 The 3′ → 5′ Exonuclease Activity

An exonuclease is an enzyme that is capable of cleaving single nucleotides from the polynucleotide chain. If an incorrect base has been inserted into the strand, the exonuclease enzyme will sense this error and cleave the phosphodiester bond, allowing the base to be removed. Some DNA associated enzymes can have an exonuclease activity. These exonuclease activities can be performed in either the 5′ → 3′ or the 3′ → 5′ direction. Some but not all DNA polymerases have a 3′ → 5′ exonuclease activity whereby it is able to recognize an incorrect base insertion directly 3′ to its position. At this point the DNA polymerase is able to excise this nucleotide and insert the correct nucleotide. This error correction system is known as a proofreading ability in DNA polymerases. Those DNA polymerases that have this ability have been shown to improve their error rate from 10^{-4} to 10^{-6} (Kornberg 1988). In some cases, even a slight deficiency in the mismatch repair system can lead to mutation accumulation amongst the replicated product (Vanderstraeten et al. 1998). Since it has been shown that the exonuclease enzyme has a 0.14% chance of removing a properly base paired nucleotide by mistake, this must be taken into consideration when discussing the full potential of exonuclease repair (Johnson and Johnson 2001). If the mismatch repair system is not running at peak performance, mutation rates will increase.

1.4.3 Types of DNA Polymerases

Although DNA polymerases are highly conserved by nature, there are still variations amongst them. As of 2002, there were at least 19 different eukaryotic DNA polymerases alone (Hubscher et al. 2002). Although there are many eukaryotic DNA polymerases available, most research is done with prokaryotic DNA polymerases. These DNA polymerases are more ideal for PCR due to the high thermostability of some species. This thermostability was a result on their environmental adaptation to living in hot springs or hot thermal vents. As mentioned earlier, the DNA polymerase used with PCR was harvested from *E. coli* and was known as *E. coli* DNA polymerase 1 (Klenow Fragment) (Mullis et al. 1986). This fragment was obtained by enzymatically cleaving *E.* coli DNA polymerase to isolate the DNA polymerase from its 5′ to 3′ exonuclease enzyme (Klenow and Henningsen 1970). Although the DNA polymerase was successfully isolated, the low melting temperature of this Klenow fragment DNA polymerase combined with the variable high temperatures required to induce replication lead to it being completely destroyed after each cycle. With no DNA polymerase remaining, the replication would cease at the end of that this temperature cycle. The only way to combat this was to reintroduce more DNA polymerase after each high temperature cycle was complete, allowing for another cycle of amplification to occur. This method was not efficient and it was soon realized that a DNA polymerase would be needed with a much higher tolerance to heat.

Thermus aquaticus is a bacterium found in hot springs. To thrive in this environment, its heat tolerance had to be much higher than that of *E. coli*. It was discovered that the thermal stable DNA polymerase harnessed from *T. aquaticus* (*Taq*)

had a temperature optimum of 80°C (Chien et al. 1976). This made it an excellent alternative DNA polymerase to use in the PCR. It was soon realized that there were other microorganisms that could also be harnessed for their thermophilic enzymes.

Table 1: DNA polymerases harvested from different species of thermophilic bacteria and their novel traits

Manufacturer	DNA Polymerase	Harvested Species	Novel Trait
Fermentas	*Pfu* DNA polymerase	*Pyrococcus furiosus*	- 3′ to 5′ exonuclease activity - highly thermostable - 8X fidelity of *Taq*
Sigma-Aldrich	Klen*Taq* LA DNA polymerase	Klenow Fragment + proof-reading enzyme	- analogous to the Klenow fragment - proof-reading done by proof-reading enzyme - 4X fidelity of *Taq*
New England Biolabs	$Vent_R$ DNA polymerase	*Thermococcus litoralis*	- 3′ to 5′ exonuclease activity - highly thermostable 5-15X fidelity of *Taq*
New England Biolabs	Deep $Vent_R$ DNA polymerase	*Pyrococcus* species GB-D	- 3′ to 5′ exonuclease activity - more thermostable than $Vent_R$ - 5X fidelity than *Taq*
Invitrogen	Platinum® *Taq* DNA polymerase	*Thermus aquaticus* + proprietary antibody	- antibody inhibits polymerase activity until 94°C - ideal choice for ancient/highly degraded samples

Several other species DNA polymerases have since been developed for their use in the polymerase chain reaction. *Pyrococcus furiosus* (*Pfu*) has been used as a DNA polymerase source since its optimal growth is around 100°C (Fiala and Stetter 1986). *Thermococcus litoralis* (*Tli*) also contained potential as a polymerase well, with an optimal growth around 85°C (Tóth 2002). An increase in fidelity was achieved through not only the acquisition of DNA polymerases from different species of bacteria, but also through manipulation of the enzyme itself (Table 1). There are now many genetically engineered DNA polymerases that have been created to serve in the PCR amplification process. Alterations to the enzyme itself commonly increase the observed fidelity, which

invariably leads to increased specificity. Most importantly, these improvements lead support the DNA polymerases ability to repair DNA damage such as modified bases.

1.5 DNA Damage

Although DNA damage can be intrinsic in nature (such as mismatch errors induced by enzymes) there are also many extrinsic sources of DNA damage. Damage to DNA occurs structurally, with most damage being done to the bases, the sugar, the backbone, or a combination of all three. Damage to any of these portions can lead to structural instability through such means as chemical modification, breaks or the addition of adducts such as those created by metal exposure. If this damage is allowed to accumulate without repair the DNA would cease to function properly, leading to uncontrolled cellular growth and/or death.

1.5.1 DNA Damage Types

As the location of DNA damage varies, so do the types of damage that can occur. DNA damage has the potential to inhibit genetic activity through mutation or apoptosis. Although mutations are rare with a frequency of 1 per 10^9 to 10^{10}, if they are allowed to incorporate into either strand, they will multiply exponentially as the DNA replicates (Iyer et al. 2006). For this reason, the types of damage that can lead to these outcomes must be understood in order to avoid genetic disaster. There are several different types of DNA damage that can be studied however there are three groups that are the most detrimental: modification, strand breaks, and cross-linking. A modification is any chemical alteration to the standard sugar, phosphate or nitrogenous base within a nucleotide that make up DNA (A, G, T and C). Breaks within the DNA molecule have

disastrous effects on the integrity of DNA and can be caused by many factors. Cross-linkage is the formation of a bond between the DNA and proteins, metals, lipids, nucleic acids, hydrocarbons or sugars.

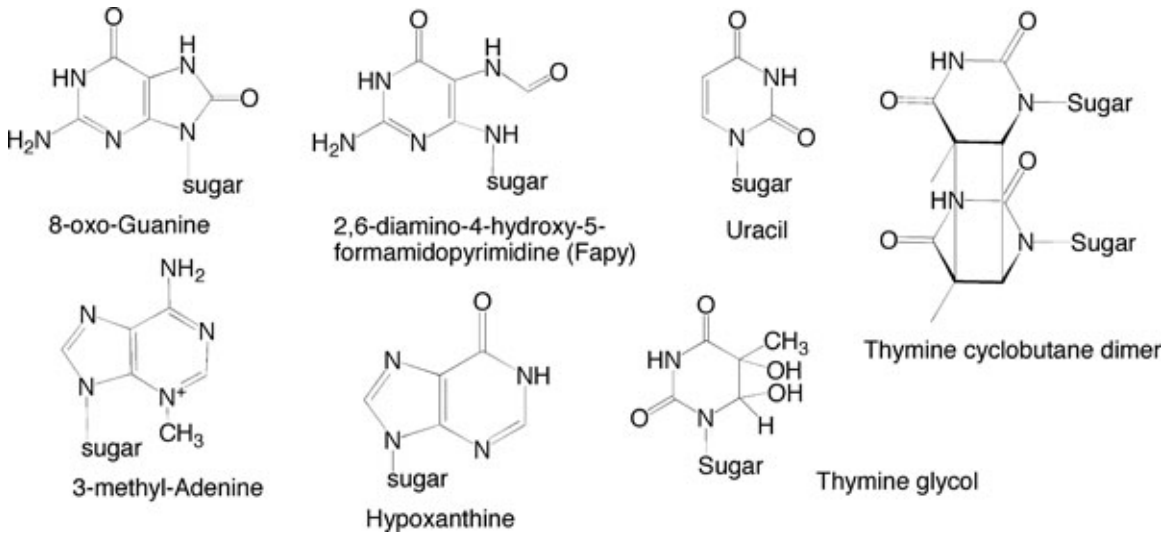


Figure 4: Several examples of modified bases and DNA damage including 3-methyl-adenine (methylation), 8-oxoguanine (oxidative damage), and thymine cyclobutane dimer (DNA-DNA cross-link)
(image taken from http://www.mtholyoke.edu/~menunez/ResearchPage/lesions.jpg)

Modification to DNA, especially base modification, can occur with relative ease under certain conditions. There is a vast range of modifications that can occur to DNA (a small number is shown in Figure 4). There is a greater range of damage causing modification to the nitrogenous bases than there is to the sugar and phosphate. These modifications can be generated in several ways, including hydrolysis, methylation and oxidation. Hydrolysis is the addition of a water molecule which breaks an existing bond. This can occur with many of the bonds within DNA causing deamination, demethylation, depurination and depyrimidination. Deamination can occur to cytosine, guanine and adenine bases which is the loss of an amine group ($-NH_2$). Demethylation can occur to

thymine and is the loss of a methyl group ($-CH_3$). Depurination and depyrimidination is the loss of the entire nitrogenous base by hydrolysis. These empty sites on the backbone are known as abasic sites (AP). Depurination affects adenine and guanine, while depyrimidination affects cytosine and thymine. Methylation (a form of alkylation) is the addition of a methyl group by an alkylating agent at one of the active sites on the nucleobase (Figure 4) (McMurry and McMurry 2004). This typically occurs in CpG sites *in vivo* (cytosine-phosphate-guanine) and are relatively common in mammals, occurring at rates up to 80% (Jabbari and Bernardi 2004). Although this is a high percentage, methylation seems to be limited to nonpromoting regions, leaving the high GC rich gene promoters relatively non-methylated. If allowed to occur in vital areas of the genome, methylation can interrupt DNA amplification and has been known to cause detrimental gene splicing as well as transcriptional mutations (Razin and Riggs 1980). These effects are also observed when methylation occurs in ancient, damaged or degraded DNA that has been exposed to an exogenous methylating agent.

Oxidative damage can cause the greatest range of DNA damage due to the ubiquitous nature of oxygen. This damage is particularly harmful to DNA in the form of reactive oxygen species (ROS). ROS are small free radicals that are highly reactive due to the presence of unpaired valence shell electrons. The generation of ROS, and thus oxidative damage, is an unavoidable form of DNA damage, fueled by the process of cellular metabolism. Although exposure to oxidative stress can induce many variations of damage to the structure of DNA, the major product of this is 8-hydroxyguanine (Cheng et al. 1992). This modified base occurs when an oxygen molecule is introduced and binds to carbon 8 (C8) in the heterocyclic ring of guanine (Figure 4). This double

bonded oxygen causes a lesion that is susceptible to mismatched binding with adenine, as opposed to cytosine (Fromme et al. 2004). Oxidative damage from these modified bases has been linked to many human diseases such as cancer, Parkinson's, and even aging (Alam et al. 1997; Collins 1999; Fraga et al. 1990). Oxidative damage is a potent form of damage, causing structural instability leading to mismatch DNA replication and eventual mutation (Wiseman and Halliwell 1996). In ancient, damaged and degraded DNA, oxidative damage will generate errors in the sequence analysis and can form blocking lesions that will prevent the recovered DNA from being amplified (Pääbo et al. 1989).

Base modification is not the only damage accumulated by DNA. Strand breaks can also have detrimental effects on genetic stability. There are two types of strand breaks; single strand breaks (SSB) affecting only one strand of DNA; and double strand breaks (DSB) affecting both strands of DNA.

The SSB can be formed in a number of ways, when the backbone is weakened in the presence of an AP site or modified base, when the sugar is modified through oxidation or when the phosphate group is affected by hydrolytic attack. These SSB can be easily repaired by the cell due to the presence of the intact complementary strand serving as a template for repair processes.

The DSB can be caused by two processes, the presence of two SSB in close proximity, and by physical damage of the DNA. A common cause of DSB by physical damage is from continuous freeze-thaw cycles of double stranded template. As the temperature is lowered, the molecules in the DNA change conformation. This constant change in conformation weakens the molecular bonds, increasing the likelihood of a DSB occurring. When a double stranded break occurs, the effects can be disastrous. Since a

DSB involves damage to both strands of the double helix, it is not able to be repaired in the same fashion as SSBs (van Gent et al. 2001). Without the use of the complementary strand as a template, the attempted repair of this damage can result in life threatening genome rearrangements. Supplementary to this, the terminal nucleobases at the sites of DSBs are often damaged, meaning that the break cannot even be addressed by the repair pathways until they are repaired (Jackson 2002). Inability to repair DSBs by the cell will eventually lead to apoptosis or tumor production, however their frequency is much lower than that of SSBs (Siddiqi and Bothe 1987).

Another concerning damage type that can affect the function of DNA is cross-link or adduct formation. There are a number of different types of adducts that can be generated. These include DNA-DNA, DNA-protein and DNA-metal cross-links. DNA-DNA cross-links have the potential to occur in the same strand, known as intrastrand cross-linking, or between both strands, which is known as interstrand cross-linking. These cross-links are such effective inhibitors of replication, that their formation is commonly used in chemotherapy treatments in order to interrupt tumor growth (Dronkert and Kanaar 2001). DNA-DNA cross-links are commonly induced by ultraviolet B (UVB) exposure which is directly absorbed by the DNA and causes adjacent thymine bases to bond covalently to each other (Figure 4) (Parrish et al. 1982). The presence of these thymine dimers will completely inhibit DNA replication from occurring. DNA-protein cross-links are covalently bonded adducts that can have just as detrimental effect as DNA-DNA cross-links (Singh 2000). These are where the proteins are bound to the nitrogenous base, sugar or phosphate group of the nucleotide. DNA-metal adducts have been studied as anticancer agents and have shown success at inhibiting tumor cell

replication. Although this application is beneficial to the cell, the damage induced by these adducts is not limited to malignant cellular growth. DNA metal adducts are induced by metal ion exposure that overtime can bind to the nucleobases, hindering amplification of viable DNA.

1.6 Metal-DNA Cross-links

The interaction between DNA and metals in a biological system is necessary for some biological functions such as the helix stabilization by magnesium in replication. However, the presence of certain metals can cause inhibition to everyday function or lead to DNA damage. Since metal-DNA complex cross-links inhibit the ability to replicate DNA, they have been utilized in the field of chemotherapy as anti-tumor treatments, particularly the use of platinum based drugs (such as cisplatin). There is also the ever growing concern of metal ions present in environmental samples, particularly ancient DNA recovered from archaeological sites in areas of high metal concentrations. Since metal ion interactions can both help or hinder the genetic stability, it is crucial that any possible effects be studied and understood so that damage to the DNA can be recognized, identified, and repaired.

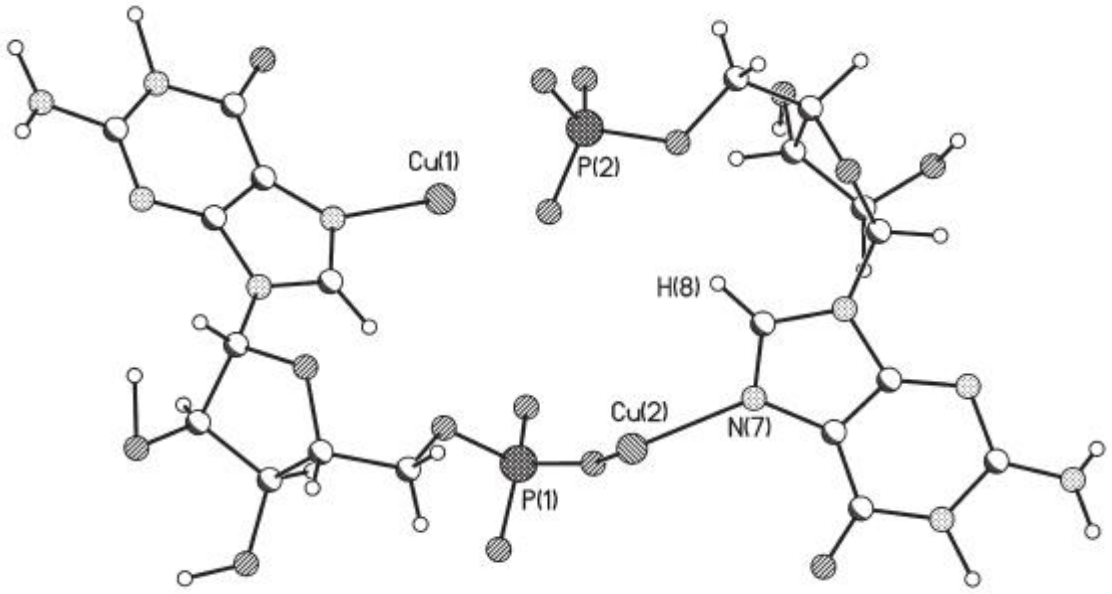


Figure 5: The nucleobase metal phosphate linkage as seen in copper-guanine monophosphate (Cu-GMP). Divalent cations are responsible for the inner-sphere binding of guanine to phosphate.

The metal-DNA interaction can occur through several different binding mechanisms. Since both the DNA and the introduced metal are hydrated, diffuse binding is a common form of interaction. In this type of binding, the metal and DNA are not directly linked, but interact through each other's water molecules (Manning 1978). This interaction is the first step in M-DNA site bonding leading to cross-link formation.

There are two modes of site binding that can occur with M-DNA known as outer-sphere electron transfer and inner-sphere electron transfer. The outer-sphere binding mode is attained through water molecule interactions and is analogous to diffuse binding. The difference between inner sphere and outer sphere binding is that in outer-sphere binding only the inner most hydration layer is retained in the metal ion, thus the M-DNA complex will share these electrons and are never connected by a direct chemical bridge.

In order to generate a covalent bond between the molecules, inner-sphere binding

must occur (Porschke 1979). Inner-sphere electron transfer is a stepwise process. The metal and nucleotide must first bind to each other by diffuse binding. Next, the metal ion and DNA must form an outer-sphere complex, resulting in a hydration layer remaining between the metal ion and the DNA. There are several factors to consider before this outer-sphere binding can even occur, such as electrostatic attractions and hydrogen bonds (Black and Cowan 1994). If these factors are not ideal for the metal ion involved, the binding process most likely will not continue. In order to complete the process and create the inner sphere complex, dehydration must occur at both the metal ion and the nucleic acid binding site. Once this has occurred, the inner-sphere binding can occur as long as there are no steric limitations and that the binding site has a high nucleophilic attraction to the metal ion **(**Hadjiliadis and Sletten 2009**)**.

As mentioned previously, there are several factors that have an impact on whether or not a covalent M-DNA complex can occur. Most importantly for cross-link formation is the presence of divalent cations. If the duplex DNA is to be cross-linked to inhibit its genomic functions, the metal ion involved must be divalent in order to obtain an electron from both strands (Figure 6). This makes Cu^{2+} an excellent metal ion to use for this study since it is a divalent cation and its high affinity for nucleobase binding (particularly the N7 binding site of guanine) over direct phosphate interaction (Figure 5). However, creating metal adducts between purified nucleotide bases and double stranded DNA creates a new set of conditions to consider. For instance, the steric interference from the hydrogen bonds holding the complementary bases together could prove to be more limiting in regards to available active sites on the nucleobases. This is especially evident in GC rich areas of the genome due to the presence of a third hydrogen bond between

them. There is also the additional constraint of adjacent nucleobases influencing the production of potential cross-links.

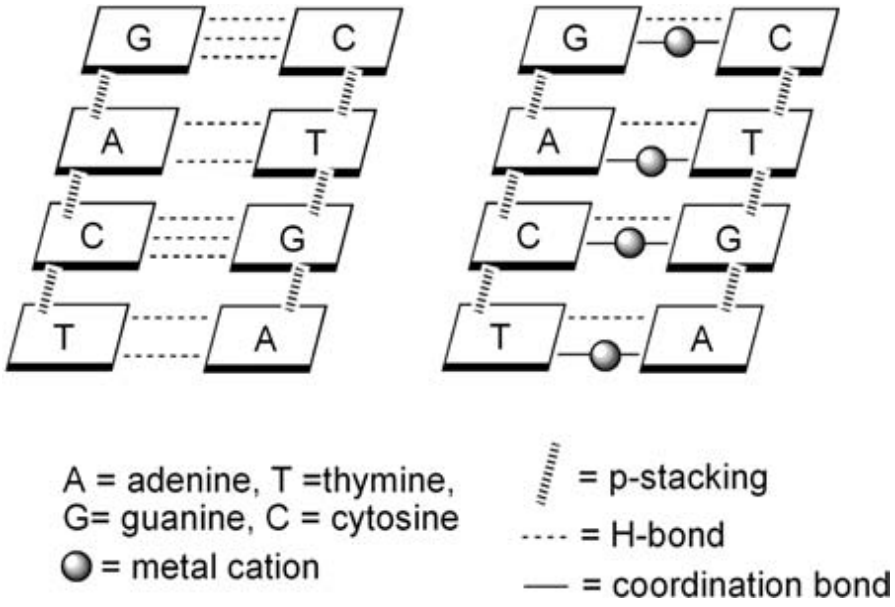


Figure 6 : A simplified proposed structure for the formation of M-DNA complexes involving divalent metal cations with nucleobase affinity.

1.7 Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (GC-MS) is a reliable method for separating and positively identifying specific compounds. It's ability to analyse compounds as opposed to individual elements makes it unique compared to ICP-MS. It does this by involving lower temperature throughout the analysis so that molecular integrity is maintained. As the name suggests, it is in fact two machines working in tandem. When a mass spectrometer is used as a detector for a gas chromatograph, a large increase in sensitivity occurs that makes it an excellent analytical tool (Abian 1999).

In gas chromatography the volatile compounds are able to separated and analysed (Eiceman et al. 1998). These separated peaks are then graphed, as a chromatograph, with detector response (y-axis) against the retention time (x-axis). This detector response is in

standard counts that may be shortened by prefixes such as giga, mega, and kilo counts (G counts, M counts and K counts respectively). Even though this method is excellent for separation, there are limitations to its analytical abilities. Although the substances are separated, the lack of a detector makes positive identification of the molecules impossible. It is common for two substances to share the same time of elution therefore making conclusive identification of either one impossible. For this reason, a mass spectrometer is attached to detect the molecular weights of these substances leading to a positive identification.

A mass spectrometer is used to positively identify molecular compounds based on their respective masses (McMurry and McMurry 2004). It does this by first ionizing the compounds causing the formation of positive ions. These ions are then guided to the detector through the quadrupole. The quadrupole oscillates the electric field to selectively stabilize the compound being identified. The quadrupole then accelerates the compounds allowing the mass to charge ratio (m/z) to be determined based on the details of motion. These compounds then enter the detector where they are identified by the induced charge of current caused by collision with the primary dynode. This computer analysis ultimately leads to an isotopic fingerprint of the compound, making positive identification possible.

The applications of this technique has been evident in many and varied fields of research. For DNA analysis however, there are a few aspects that needed to be altered for analytical identification. The two major hurdles for DNA analysis were the low volatility of the DNA in solution and the high thermostability required to maintain the molecular integrity during column oven exposure. Dizdaroglu and Senturker (1999)

addressed these issues through silylation, opening the door for DNA analysis on the GC-MS (Senturker and Dizdaroglu 1999). Silylation requires the samples to be derivatized in order to increase volatility and thermostability in the GC-MS. This is done, first by formic acid treatment, hydrolyzing the glycosidic and phosphodiester bonds within the DNA then silylation. Silylation is the introduction of silyl groups to a molecule which will bind to available binding spots. The introduction of these silyl units resulted in not only increasing the thermostability of the derivatives to be analysed, but also increasing the volatility, making analysis much easier. It became possible for GC-MS to positively identify derivatives of a DNA sample, however the additional weight of the silyl units has to be taken into consideration in order to make a proper identification.

1.8 DNA Repair

No matter what the source, any damage to the genetic code may be detrimental to an organism's survival. For this reason, the cell has evolved several different repair mechanisms to correct these errors. These repair processes include Direct Reversal repair (DRR), base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR). Our understanding of these mechanisms within the last decade has allowed *in vitro* methods to be researched and optimized, permitting these methods to be used to repair the many types of damage that DNA can accumulate.

1.8.1 Direct Reversal Repair

Direct reversal repair is the use of a chemical or enzymatic treatment to reverse the damage, usually by cleavage, without any modification of the nucleotide. These methods are particularly applicable to *in vitro* studies of DNA damage and repair as it can

be easily applied under specific laboratory conditions. The direct reversal repair of DNA damage is the simplest form of DNA repair but is limited to DNA damage caused by cross-links (DNA-DNA, DNA-metal, DNA-protein, DNA-sugar, DNA-alkyl groups). It involves the repair of damaged or modified nucleobases by direct chemical interaction without disrupting the phosphodiester bonds. Since the backbone is not disrupted by direct reversal, it does not require a complementary strand to function properly, making it an ideal form of repair if the involvement of enzymes is to be of limited use.

An enzymatic example of direct reversal repair would be the use of demethylases and DNA methyltransferases. These enzymes are able to remove the methyl groups (the simplest alkyl groups) in 5-methylcytosine. Methylation of the bases in DNA involves the addition of a methyl group to an available bonding site on the DNA nucleobase. It is a commonly used strategy in the cell for gene suppression, since a methylated base in a coding region would not allow gene expression to occur. The damage can also have a negative impact on the DNA if allowed to get out of control. If the methylation were to occur in a gene coding region for a tumor suppression gene, this could lead to cancerous growth or tumor formation. In order to remove this damage, demethylase is used to remove the methyl group without breaking the phosphodiester bond, thus maintaining the DNA structure.

The most common chemical treatment used in degraded DNA research would be the use of phenacylthiazolium bromide (PTB). PTB is a reagent known to cleave sugar-derived protein cross-links. Since DNA has the potential to be involved in the formation of acylglycosylation end (AGE) product cross-links with proteins, it was postulated by Poinar et al. (Poinar et al. 1998) that the addition of PTB may help in retrieving viable

DNA that was being inhibited by these protein-(sugar)-DNA adducts. Application of PTB proved to be a success, resulting in detectable DNA in samples that could not be extracted prior to PTB treatment, thus leading to an increase in viable DNA.

1.8.2 Base Excision Repair

Base excision repair (BER) is an enzymatically driven repair process that is capable of removing a damaged base and repairing it by using the complementary strand as a template. When a damaged nucleobase exists, DNA glycosylase is able to detect the damaged base and remove it creating an AP site. An AP site is the name given to a location on the DNA where there is neither a purine (apurinic) nor pyrimidine (apyrimidinic) base attached to the sugar phosphate backbone (Figure 7). The DNA glycosylase is responsible for hydrolyzing the glycosidic bond between the sugar and the nucleobase itself (Krokan et al. 1997). Once the AP site is created, AP endonuclease is able to cleave the phosphate backbone, creating both a 3′ hydroxyl and 5′ phosphate termini that are vital to permit DNA polymerase I to fill the gap (Norbury and Hickson 2001). Once the DNA polymerase has synthesized the new strand, DNA ligase will seal the backbone to complete the BER process.

Base excision repair can be used *in vitro*, especially in the analysis of ancient or degraded samples. The method for the repair of degraded DNA involves the use of the

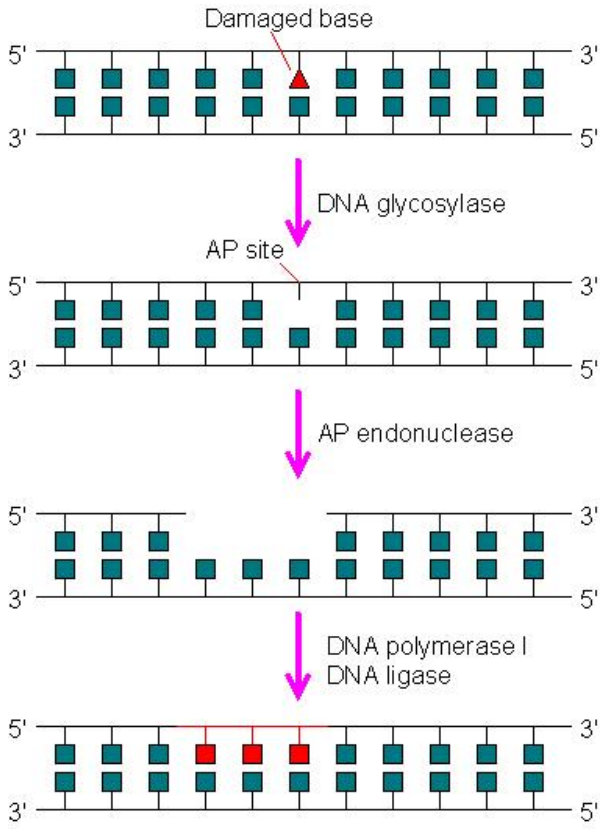


Figure 7: Simplified diagram of base excision repair including the major enzymes involved.
(http://www.web-books.com/MoBio/Free/images/Ch7G1.gif)

enzymes DNA polymerase I and T4 DNA ligase. The low fidelity of DNA polymerase I makes it a suitable amplification enzyme as it is not inhibited by nicks found in the DNA backbone (Pusch et al. 1998). These gaps are then closed by the T4 DNA ligase, increasing the chances of amplification. This method met with limited success however, until Di Bernardo et al. (Di Bernardo et al. 2002) redesigned the method to improve its success rate. They proposed that the presence of interstrand cross-links was a vital component to the success of *in vitro* BER, allowing the removal of the damaged base without causing the duplex to denature. To isolate samples with interstrand cross-links, they incorporated an initial denaturation step to the Pusch et al. (Pusch et al. 1998) protocol. Since the ancient DNA samples that they were using were of extremely low

concentrations, this meant that the only template that would have a chance at reannealing after this step was that being held together by interstrand cross-links (Bernardo 2002). By utilizing this hypothesis, the rate of successful rescue was increased from 22% to almost 80%.

Although base excision repair is necessary *in vivo* in order to avoid potentially life threatening mutations, it is limited in its ability to only handle single nucleobase damages. When DNA accumulates strong helix distorting damages such as thymine dimerization, an alternative repair mechanism must be utilized.

1.8.3 Nucleotide Excision Repair

DNA damage can not only alter the nucleobase but in some cases can distort the entire helix leading to severe genetic impairment. When damage occurs that a glycosylase cannot identify and remove by BER, nucleotide excision repair (NER) is used. Nucleotide excision repair is unique in that it identifies helix distortions as opposed to damaged nucleobases. These helix distortions usually involve pyrimidine dimers and other photoproducts, however NER can be used to eliminate bulky chemical adducts as well. It accomplishes this by scanning the DNA through the use of a multi-enzyme complex. Once a damaged site is located, this complex then cleaves the phosphodiester backbone on both sides of the distortion and this section is removed from the double helix. This large gap in the DNA is then repaired by DNA polymerase and sealed by DNA ligase (Alberts 1994).

While the process of NER seems undemanding, the mechanism of use is the complete opposite. The process involves several protein factors, and also includes a multitude of necessary sub-units to be present in order to complete the process (de Laat et

al. 1999). In prokaryote's such as *E. coli*, the multi-enzyme complex may not be as complicated, but the integrity of the NER system remains intact (Figure 8). The length of DNA cut must also be taken into consideration. In prokaryotes, the section removed is around 12 bp in length, but can be more than double that length in human DNA (Alberts 1994). The larger section of DNA removed inherently requires a larger intact template in order to maintain stability within the double helix.

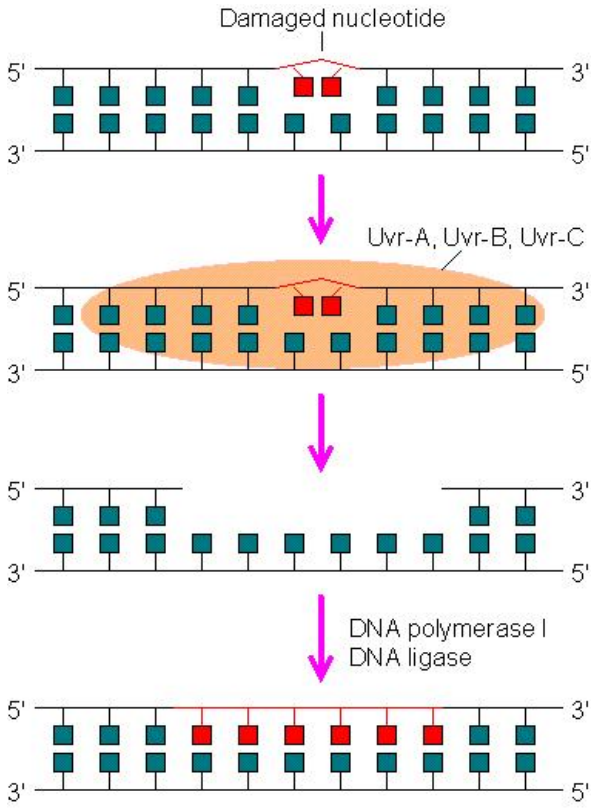


Figure 8: Simplified diagram of nucleotide excision repair as it occurs in *E. coli*. This repair mechanism is much more complicated in eukaryotes.
(http://www.web-books.com/MoBio/Free/images/Ch7G2.gif)

1.9 Project

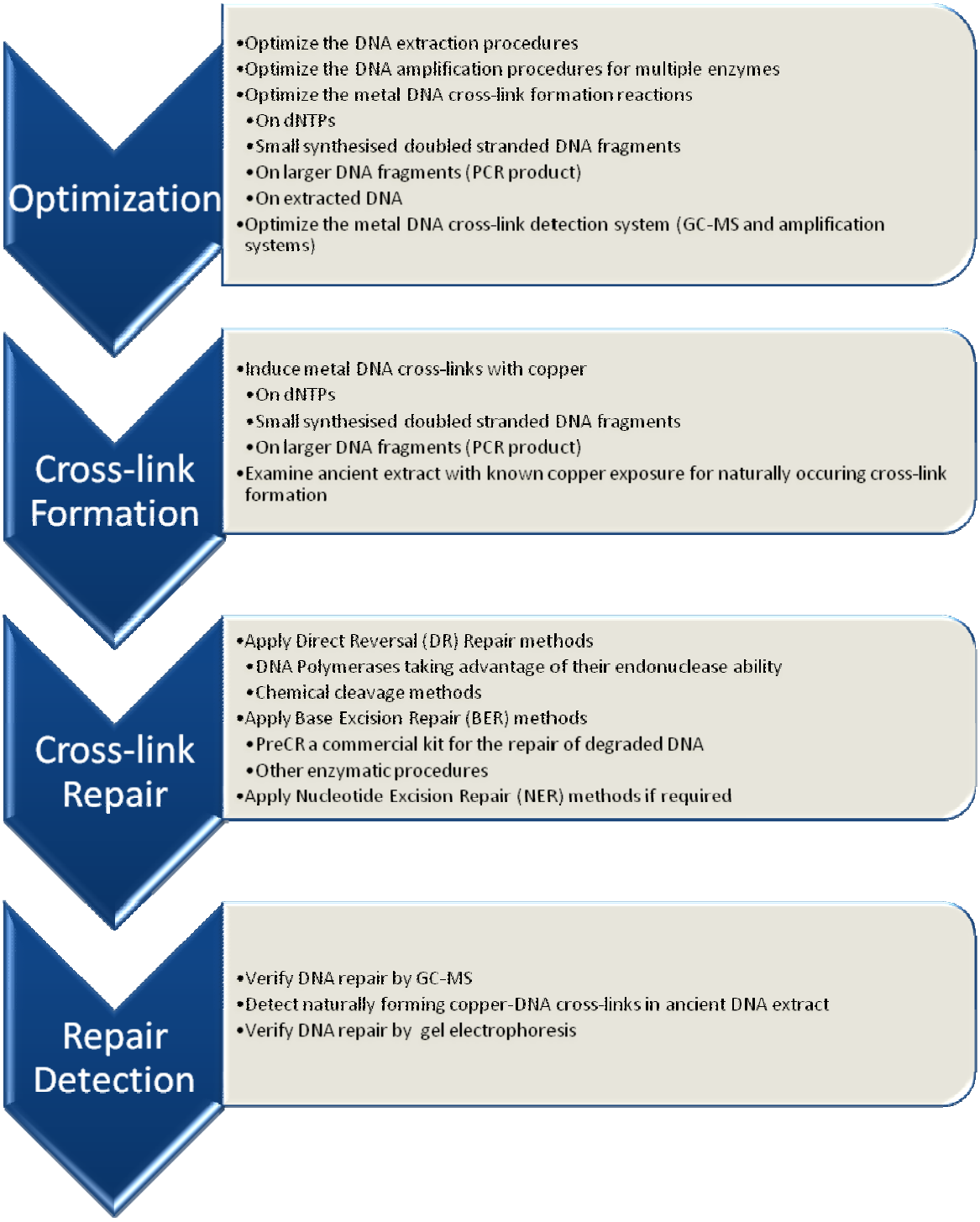
Hypothesis: Metal ion cross-links with deoxyribonucleic acid can be removed to allow molecular analysis.

Null Hypothesis: Metal ion cross-links with deoxyribonucleic acid cannot be removed to allow molecular analysis.

Justification: The presence of metal ions can inhibit molecular analysis such as DNA amplification (PCR). If this metal ion inhibition is not removed the affected samples cannot be analyzed. The types of samples that are affected are any that have been exposed to high concentrations of metal ions especially in the environment. So any samples that are found in the soil can be inhibited. These include environmental microbiological samples, forensic samples, archaeological samples and paleontological samples. If a method can be developed to repair the damage generated by metal ions then these samples will be available for future molecular analysis.

Background: There are many types of damage that can occur to DNA, such as abasic site production, modified bases, single stranded nicks, double strand breaks, and cross-linking. There are two mechanisms that can lead to inhibition of DNA analyses by metal ions, 1) inhibition of the activity of the DNA polymerase and 2) inhibition from interaction with the template. The interaction that metals ions can have with the template includes: 1) induce and catalyze oxidation and free radical damage and 2) cross-linking with the DNA molecule. I have already investigated the enzymatic inhibition of DNA polymerases as an independent topic project. This thesis will focus on the detection of metal DNA cross-links (focusing on copper) and the removal or repair of these metal DNA cross-links.

Methodology: The methodological approach in this thesis project can be summarized stepwise as follows:



The repair of the cross-linked product will be attempted sequentially based on availability of resources. The easiest method of removing the cross-links involves direct reversal by means of chemical treatment. If chemical attempts are unsuccessful, an enzymatic approach will be used involving DNA polymerases of different species origins. If this cannot be accomplished, *in vitro* base excision repair (BER) will be attempted followed by nucleotide excision repair (NER) if no other methods are successful in repairing damage.

2.0 Methods and Materials

2.1 DNA Template

The template used in this study included single nucleotides, synthetic dsDNA fragments, amplified PCR product and extracted DNA for the optimization of the cross-link formation. The single nucleotides were commercially available nucleotides in separate tubes (dATP, dGTP, dCTP, dTTP) (Fermentas). The synthesized fragments of DNA were small oligonucleotides matching nucleotide position (np) 16301 to 16322 of the human mitochondrial genome. These synthesized oligonucleotides were complementary single stranded pieces of DNA annealed into a double stranded template of 22 bp in length (Operon). The PCR product was 425 bp of amplified mtDNA extracted from human epithelial cells (details in section 2.1.3). The DNA samples were extracted from buccal swabs (MedPro). The swabs were used to extract the mtDNA from human epithelial cells lining the cheek wall. To achieve this, sterilized single use cotton tipped applicators were acquired and used to swab the inner cheek for approximately 30 seconds. This applicator is then allowed to air dry for 10 minutes and stored in a sterile tube until DNA extraction was required.

In addition to the DNA template used for the optimization in this project a bone sample was also analyzed to confirm the validity of the method developed. This bone sample is approximately 90 years old and was found in close association with metal objects containing copper. The copper of the metal objects have permeated the bone over time, possibly leading to cross-link formation with the DNA. This was verified by scanning electron microscope elemental x-ray analysis.

2.1.1 DNA Extraction

The DNA extraction was performed by using modified proteinase K extraction protocol (Hansen 1974). The swab samples are cut and placed into a 1.5 mL sterile tube with 358 mL of the extraction solution (0.5 M NaCl, 0.5 M EDTA, 1 M Tris-HCl pH 8.0, 0.2% SDS, 0.039 M DTT) then vortexed for 15 seconds. To this solution, 2 µL of proteinase K (20 mg/mL) (Fermentas) was added and this solution (final concentration 0.1 mg/ml proteinase K) was once again vortexed quickly. The extraction was then incubated at 55°C for 3 hours.

After the 3 hour incubation an ethanol precipitation is performed as a purification step within the extraction process. The ethanol precipitation was prepared in a sterile tube. First, the aqueous portion from the proteinase K extraction tube was transferred to this sterile tube and the volume attained was recorded. Secondly, 10% v/v of sodium acetate was added to this solution and vortexed for 1 minute. After vortexing, cold ethanol was added in a volume of 2.5X the total volume. The tube is then immediately placed on ice for 30 minutes. After 30 minutes, the samples were centrifuged for 5 minutes at 13,000 rpm. After the sample has been spun down, a white or beige pellet would become noticeable in the bottom of the tube. It is this pellet that contains the DNA from the extraction. In order to complete the purification step, the supernatant must be carefully removed via pipette (being careful not to dislodge the pellet) and the pellet allowed to air dry. Once dry, the pellet is then resuspended in 150 µL of double distilled H_2O (ddH_2O) and incubated at 37°C for 15 minutes. This final solution can then be stored in -20°C for future use.

2.1.2 DNA Quantification

The DNA extracts were quantified using the Invitrogen Qubit™ Quantification system. This procedure relies on a fluorescent dye that binds to dsDNA, allowing the product to fluoresce and read by the fluorometer. This reagent must be diluted to 1:200 with the supplied buffer to make the working solution (proprietary). The assay tubes are then prepared by putting 198 μL of the working solution (proprietary) into a tube with 2 μL of sample. The tube was then vortexed for 2-3 seconds and allowed to incubate at room temperature for 2 minutes. After this incubation period, the fluorometer was used to read the tubes and calculate the sample concentration of the extracted template. Each sample set was read at least 5 times immediately following one another and the amounts were then averaged to determine the amount of DNA present.

2.1.3 Templates

To determine the success of M-DNA cross-link repair, several forms of DNA product will be tested, including single nucleotides, synthetic dsDNA fragments, amplified PCR product, and extracted DNA. The first, and most simple is the study of deoxynucleotide triphosphates (dNTPs). These are easily obtained as purified individual bases (dGTP, dATP, dTTP, and dCTP) in dNTP sets from Fermentas.

Oligonucleotides are short nucleic acid polymers that when used in PCR are referred to as primers. Primers are inherently short (usually no more than 20-30 bases) and can be designed to be complementary, turning single stranded into double stranded product. This is accomplished by obtaining complementary primers form Lakehead University's PaleoDNA laboratory and mixing equal aliquots of each in a single sterile tube. The two primers selected for this research were 16301 forward and 16322 reverse,

each with a length of 22 base pairs. When combined, this synthetic double stranded DNA has a mitochondrial sequence of CAGTACATAGTACATAAAGCCA. This primer mix was then allowed to incubate at room temperature overnight to ensure maximum annealing. The binding of these complementary primers is shown by quantifying the product with the Invitrogen Qubit™ Quantification Platform by the protocol described above.

The next template that will need to be manipulated is PCR amplified product. The preparation for this sample involves a proteinase K extraction, ethanol precipitation, and then amplification in the polymerase chain reaction. This extract will then be amplified using mitochondrial primers 14724 Forward and 15149 Reverse, resulting in a 425 base pair amplicon. The concentrations used in PCR in order to amplify this fragment were 1X buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3 at 25°C), 0.2 mM dNTP, 0.2 µM of forward and reverse primer, 2 mM $MgCl_2$, 2.5U *Taq* DNA polymerase, 34.5 µL of H_2O, 5 µL of the buccal swab DNA extract (5 ng), and double distilled water added to a total reaction volume of 50 µL. Optimized amplification settings for *Taq* DNA polymerase began by heating to 105°C to begin an initialization step of 94.0°C for 2 minutes. This was followed by a denaturation step also at 94.0°C for 30 seconds. The annealing step was run at 50.0°C for 1 minute and then elongated at 72.0°C for 1 minute. The cycle from denaturation to elongation was then allowed to repeat 25 times before being held at 4.0°C. The final template that repair was attempted was on extracted DNA template. This DNA extract was from the same source and extraction methods as the previous template.

2.1.4 Copper (II) Chloride

Copper (II) Chloride ($CuCl_2$) is an ionic compound that is highly soluble in water. Although many divalent cations have inhibitory potential, copper is commonly used in DNA inhibition studies because it has the highest affinity to base alteration (Geierstanger et al. 1991). Copper (II) chloride dihydrate was obtained in its solid form. A 1 M solution was then prepared using ddH_2O. The pH of this solution was found to be slightly acidic, so the solution was buffered with solid tris (hydroxymethyl) aminomethane (Tris) to a final pH of approximately 7. Tris is a primary amine and a common buffer used in nucleic acid preparations. This 1 M buffered solution was then vortexed for 2 minutes to ensure it was thoroughly mixed and then stored in -20°C for long term storage.

2.1.5 Cross-Link Formation

The production of M-DNA cross-links will constitute a small percentage of the total ions present in each sample. For this reason, it is very important to optimize their production to ensure the maximum amounts of cross-links are produced. In order to verify cross-link formation, the data collected will be compared to unaltered control samples based on retention times and isotopic fingerprints.

Once the 1 M solution of $CuCl_2$ is prepared, it is combined with a volume of the template at a ratio of 1:1 by volume. This solution is then incubated on a dry bath at 50°C overnight, or approximately 15 hours. Once the incubation has taken place, the samples were ran on the gas chromatography-mass spectrometer (GC-MS) and compared to the chromatographs from the undamaged positive controls.

2.2 Polymerase Optimization

In order to have optimal DNA amplification from each polymerase in Table 1, the reagents and temperature settings had to be optimized for each individually. Ideal optimization was based on maximizing amplification specificity as well as quantity. If optimization is not achieved, then the amplification results may not be clear enough to infer success or failure.

The Fermentas *Pfu* DNA polymerase was optimized by including 1X buffer (200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% Triton X-100, 1 mg/mL BSA, 20 mM $MgSO_4$), 0.2 mM dNTP, 0.2 µM of forward and reverse primer, 1.25U of *Pfu* DNA polymerase, 5 µL of DNA template (5 ng), and double distilled water for a total volume of 50 µL. The temperature settings for PCR amplification were optimized as follows. After heating the lid to 105°C, an initial denaturation temperature of 94.0°C was held for 2 minutes. This step was followed by the denaturation step at 94.0°C for 30 seconds. The annealing temperature used was 50.0°C for 1 minute leading into an extension temperature of 72.0°C for 1 minute. The cycle was then repeated from the annealing step to the extension step for 25 cycles and held at 4°C for storage.

Sigma-Aldrich Klen*Taq* LA DNA polymerase was optimized by including 1X buffer (400 mM Tricine-KOH (pH9.2 at 25°C), 150 mM KOAc, 35 mM $Mg(OAc)_2$, 750 µg/mL bovine serum), 0.2 mM dNTP, 0.2 µM of both primers, 0.1U of Klen*Taq* LA DNA polymerase, 5 µL of DNA template (5 ng), and double distilled water added for a total volume of 50 µL. Optimized amplification settings for Klen*Taq* DNA polymerase began with heating the lid to 105°C to begin an initialization step of 94.0°C for 2 minutes. This was followed by a denaturation step also at 94.0°C for 30 seconds. The

annealing step was ran at 50.0°C for 1 minutes and allowed to elongate at 72.0°C for 1 minutes. The cycle from denaturation to elongation was then allowed to cycles 25 times before being held at 4.0°C.

The New England Biolabs $Vent_R$ DNA polymerase optimization required concentrations of 1X buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1 % Triton X-100, pH 8.8 at 25°C), 0.2 mM dNTP, 0.1 µM of forward and reverse primer, 0.5U of $Vent_R$ DNA polymerase, 5 µL of DNA template (5 ng), and double distilled water added to a total volume of 50 µL. The PCR amplification temperatures were slightly altered from the previous settings. After heating the lid to 105°C, an initialization step was held at 94.0°C for 2 minutes. This was followed by a denaturation step at 94.0°C for 30 seconds. The annealing temperature needed to be increased for this polymerase and was held at 60.0°C for 30 seconds followed by an elongation step at 72.0°C for 1 minute. The amount of cycles also needed to be increased and was found to be optimized at 28 cycles from denaturation to elongation. Once complete, the sample was held at 4.0°C.

New England Biolabs Deep $Vent_R$ DNA polymerase had optimal running conditions at concentrations of 1X buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1 % Triton X-100, pH 8.8 at 25°C), 0.1 mM dNTP, 0.4 µM of both primers, 4 mM $MgSO_4$, 1U of Deep $Vent_R$ DNA polymerase, 5 µL of DNA template (5 ng), and double distilled water added to a total volume of 50 µL. This high fidelity polymerase also required a more specialized temperature protocol for optimal DNA amplification. After heating the lid to 105°C, an initial denaturation step was held at 95.0°C for 3 minutes. This was followed by a denaturation held at 95.0°C for 30

seconds. The sample was then annealed at a temperature of 60.0°C for 30 seconds followed by an extension at 72.0°C for 1 minute. These settings were then repeated (from denaturation to extension) for a total of 25 cycles. After the completion of the 25 cycle, a final elongation to ensure any remaining single stranded DNA was fully extended. This elongation was also held at 72.0°C for 5 minutes and finally, the sample was held at 4.0°C.

The final polymerase, Invitrogen Platinum *Taq* DNA polymerase, was optimized by including 1X buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3 at 25°C), 0.2 mM dNTP, 0.2 μM of the forward and reverse primer, 2 mM $MgCl_2$, 2.5U of Platinum *Taq* DNA polymerase, 5 μL of DNA template (5 ng), and double distilled water to a total volume of 50 μL. The temperature settings for PCR amplification was optimized as follows. After heating the lid to 105°C, an initial denaturation temperature of 94.0°C was held for 2 minutes. This step was followed by the denaturation step at 94.0°C for 30 seconds. The annealing temperature used was 50.0°C for 1 minute leading into an extension temperature of 72.0°C for 1 minute. The cycle was then repeated from the annealing step to the extension step for 25 cycles and held at 4°C for storage.

2.3 DNA Repair

The *in vitro* repair of damaged DNA can be accomplished through several different methods. Success depends on the extent of the damage and the type of damage that has been induced. For the purpose of this study, three DNA repair approaches will be utilized: Direct reversal (DR) repair, base excision repair (BER), and nucleotide excision repair (NER).

2.3.1 Direct Reversal Repair

A number of chemicals were considered for their possible ability to cleave the M-DNA adducts. Ethylenediamine was found to be the most likely chemical capable of cleaving these M-DNA cross-links. Ethylenediamine was used to attempt to repair the copper cross-links by direct reversal. This was accomplished by adding ethylenediamine in an equal volume to a copper-DNA template solution. This solution was then allowed to incubate at room temperature overnight.

These samples were then purified via size exclusion chromatography. BioRad Micro Bio-Spin P-30 spin chromatography columns were first inverted sharply several times in order to resuspend the settled gel and remove any bubbles. The tip of the column was then snapped off and the column was placed in a 2 mL tube with its lid removed. The column was the centrifuged for 4 minutes at approximately 1000 rpm in order to ensure that as much packing buffer is removed from the columns as possible. The 2 mL tube was then discarded and the column was placed in a fresh 1.5 mL tube.

The entire sample volume was added to the spin column and the tube was spun down again for 4 minutes at approximately 1000 rpm. This final solution was kept and the repair was verified by comparison to undamaged template via agarose gel visualization and gas chromatography-mass spectrometry.

2.3.2 Base Excision Repair

In order to utilize base excision repair *in vitro*, a pre-optimized proprietary enzyme cocktail, known as PreCR, was obtained from New England Biolabs. In this kit, the PreCR repair and the PCR reactions are performed in the same tubes. In order to test this method, a 50 μL volume reaction must be prepared as follows. At room temperature,

1X ThermoPol Buffer, 100 μM dNTPs, 1X NAD^+, damaged template DNA and H_2O ware combined to total volume of 46 μL. Next, 1 μL of the PreCR Repair Mix is added and the solution is vortexed gently. The repair solution was then be incubated for 20 minutes on an Eppendorf Mastercycler at 37°C. After incubation is complete, the reaction is kept on ice while the primers, a second aliquot of dNTPs (another 100 μM) and the *Taq* DNA polymerase are added directly to the repair reaction mix. This reaction is then amplified by the PCR on the Eppendorf Mastercycler. Once the lid is heated to 105°C, the initial denaturation is set at 94.0°C and held for 2 minutes. This first step will then be followed by the denaturation step, which will also be held at 94.0°C for a time of 30 seconds. The sample is then annealed at 50.0°C for 1 minute, leading to an extension period set at 72.0°C for an additional 1 minute. This setting is then repeated for 25 cycles followed immediately by incubation at 4°C for storage.

2.3.3 Nucleotide Excision Repair

Nucleotide excision repair was considered as an approach for DNA damage repair. However due to the number of nucleotides (~30 bp) required for this repair process it was not considered feasible for ancient or degraded DNA. For this study, nucleotide excision was not attempted.

2.4 DNA Repair Detection

There were two methods for detecting potential repair used in this research. The easiest method of cross-link repair detection is agarose gel visualization. If the damage has been removed, then the DNA will be able to be amplified and visualized on the gel as a solid band around the 425 bp molecular marker. If repair is minimal or a cross-linked

metal is still bound to the DNA, a faded band may be observed, indicating partial inhibition. There is also the possibility that no band could appear at all which would lead to one of two conclusions: that complete inhibition has occurred or PCR has failed. Although this method is able to discern between complete inhibition and no inhibition, its partial inhibition detection is far too subjective. For this reason, gas chromatography-mass spectrometry (GC-MS) will also be used to detect M-DNA repair. By comparing results of copper induced damage to repair product, the ionic ratio and mass to charge ratio (m/z) will give a more exact value to any repair that may have successfully occurred.

2.4.1 Agarose Gel Visualization

To verify DNA repair through the use of agarose gel electrophoresis, a 2% gel was produced using agarose powder and TBE buffer (Tris-Borate-EDTA). The agarose and TBE mixture was prepared in an Erlenmeyer flask and covered in tinfoil to avoid moisture loss. This solution was then heated slowly by hotplate in order to dissolve the agarose and to retain the moisture content. Once the agarose was completely dissolved, the solution was allowed to cool for 2 to 4 minutes. After slight cooling, 2 μL of ethidium bromide (EtBr) solution (1%) was added and the flask was swirled to ensure even mixing. Once mixed, the liquid gel was then poured into the apparatus, removing any residual bubbles with a pipette in order to maintain unobstructed migration. A 10 lane comb was placed in the apparatus to form the wells into which the sample will be placed for migration. The gel was then allowed to sit at room temperature for approximately 30 minutes in order to ensure it had solidified. Once the gel had solidified, it was placed in the electrophoresis apparatus. The apparatus was then filled

with TBE buffer until the gel was completely submerged about 1 centimeter below the surface.

The samples were prepared for loading in a mixing tray by adding 5 μL of sample and 3 μL of loading dye (6X). This 8 μL was then injected into the assigned well of the gel. Once all the lanes were loaded, the apparatus was covered up and plugged into an EC-105 voltage apparatus. To allow definitive migration to occur, the voltage was set to 110V and the gel was allowed exposure to this voltage for approximately 45 minutes. The gel was then removed and visualized under UV light, allowing the ethidium bromide to fluoresce and reveal if DNA is present.

2.4.2 Gas Chromatography-Mass Spectrometry

To prepare the samples for GC-MS analysis, the samples were first hydrolyzed by combining 0.5 mL of 60% formic acid with the sample in an autosample vial. These vials were then evacuated with nitrogen and heated to 140°C for 30 minutes. The samples were then lyophilized for 48 hours and 0.4 mL of BSTFA with 1% trimethylchlorosilane and 0.1 mL of acetonitrile were added to the vial (Senturker and Dizdaroglu 1999). The samples were purged with nitrogen, sealed and incubated at 120°C for 30 minutes. Once derivitization was complete, an additional 0.5 mL of acetonitrile was added to dilute the sample for GC-MS. These samples were then loaded onto a Varian Quadrupole Gas Chromatograph-Mass Spectrometer immediately after derivitization. In order to avoid overloading the column, the split inlets had to be adjusted to compensate for sample size. When the purified nucleotides and the anneal primers were run, a 1:25 split was used for ideal detection. When the larger 425 bp fragment was analyzed, the split had to be bumped to 1:50 in order to allow for a clean chromatograph.

3.0 Results

In order to grasp the full potential of the repair of metal-DNA cross-links, several templates were analyzed. By including all aspects of DNA from single nucleotides to complete extractions in a stepwise fashion, the ability of these repair methods can be assessed.

3.1 Nucleobase Cross-Link Formation and Repair

The first template used in attempt to induce copper cross-links to the DNA was dGTP. Since this template consists of a single purified nucleotide, it was not possible to confirm inhibition through the use of agarose gel visualization. Due to this limitation, the presence of guanine was verified by gas chromatography-mass spectrometry analysis.

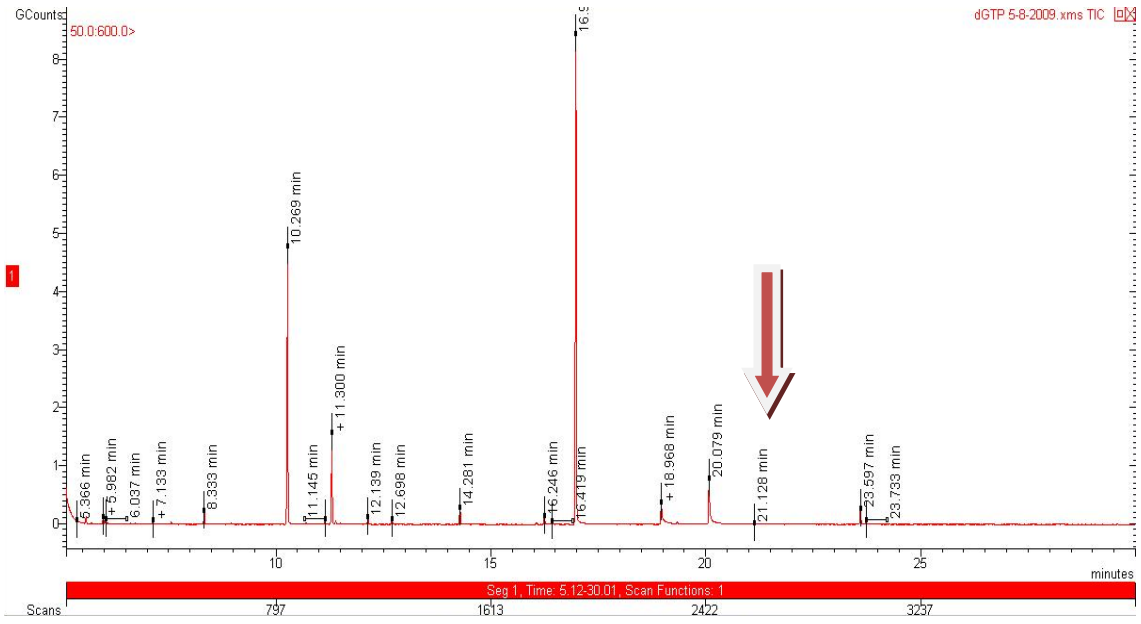


Figure 9: Gas chromatography spectra of derivatized deoxyguanosine triphosphate. The arrow shows the expected retention time for derivatized guanine (N,9-bis(Trimethylsilyl)-6-[(trimethylsilyl)oxy]-9H-purin-2-amine). The other large peaks at 16.983 minutes and 10.269 minutes represent the derivatized ribose sugar and the monophosphates respectively. The other peaks that have eluded are other products formed as a result of the sample preparation and are not pertinent to the experiment.

With all the potential binding sites contained in dGTP, it was necessary to perform a derivitization reaction to transform the molecule down to its basic components. In Figure 9, the spectrum is shown of the major GC peaks found, including an arrow pointing to the peak representing guanine. This was positively identified by the MS Data Review MAINLIB search (version 6.9.1) (Figure 10).

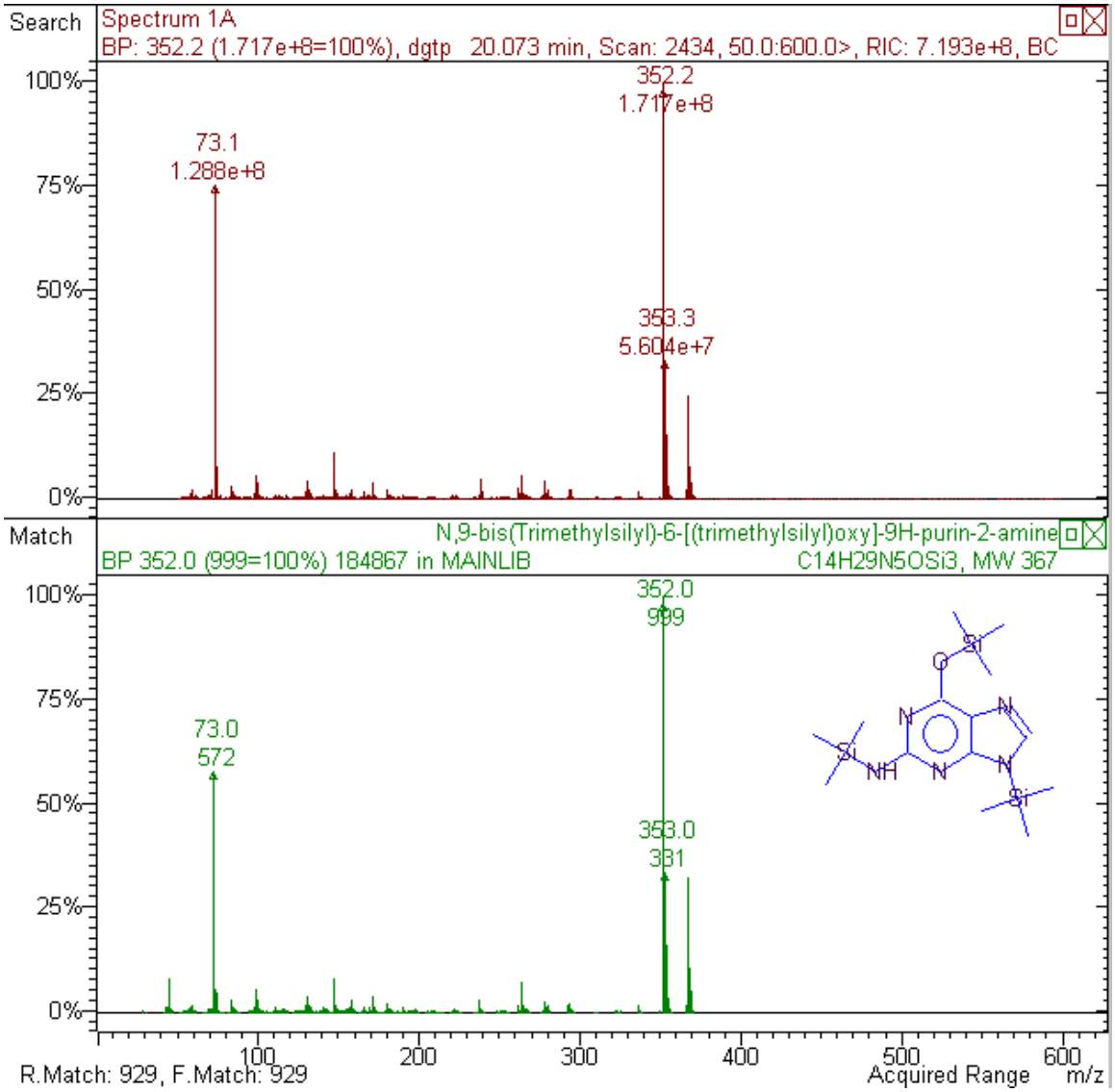


Figure 10: Library search of the active spectrum using MS Data Review MAINLIB. The eluted compound at 20.079 minutes was positively identified through its isotopic fingerprint as belonging to silylated guanine.

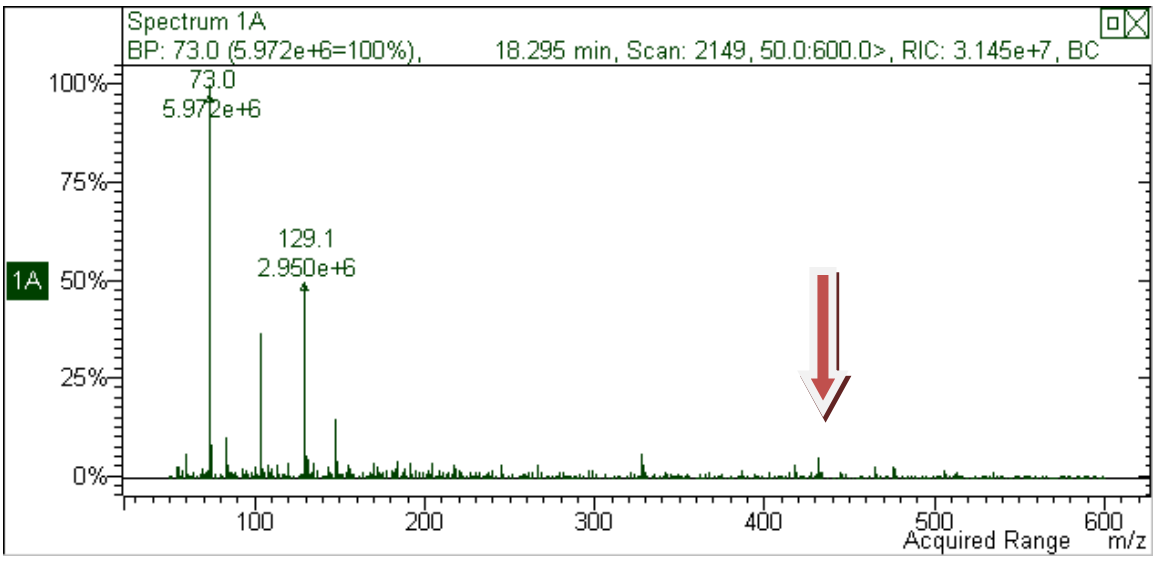


Figure 11 : A mass spectrum of a definitive peak located at a retention time of 18.295 minutes. This peak contained the expected molecular weight of a silylated guanine with a bound copper, a mass of 431. The extraneous peak of 73 belongs to unbound silyl groups. The other peaks are the result of derivitization and potentially other forms of damage such as oxidation.

Once the base retention time was determined for unaltered guanine, the guanine-copper cross-linked sample was analyzed to verify cross-link presence. Upon performing an ion search on the damaged template, it was found that derivatized guanine was no longer detected (MW 367). Upon further analysis, it was noted that a search for derivatized guanine with an attached copper (MW 431) produced an ion peak at a retention time of 18.295 minutes (Figure 11). Further analysis indicated that this 431 peak had a relative abundance of 5.2%, which is within the expected percent of cross-link formation (Figure 12). It must be noted that the mass spectrometer results did not yield a positive identification for any substance found in the software's database.

Since promising results were obtained with guanine, the other three nucleobases were also used as templates. These results proved inconclusive to crosslink production, even for adenine, the other pyrimidine. While adenine did show that damage had occurred since it was no longer identifiable, this was most likely due to the catalytic

affects of the metal ion to the formation of DNA damage rather than by cross-link formation. This catalytic effect makes the samples prone to oxidative damage which can occur due to oxygen exposure during the derivitization process. The observed cross-link formation with the purines was also negligible. Both cytosine and thymine were identified separately by GC-MS by the same method as the purines. Once retention time's and identifying peaks were determined, analysis was completed upon cross-linked and repaired product by ethylenediamine. The purines did not seem to be affected by the presence of copper as their spectra were easily identified in the unaltered control and the damage exposed template. Exposure to copper did not affect the relative abundance of the ions, maintaining high percentage of probable identity.

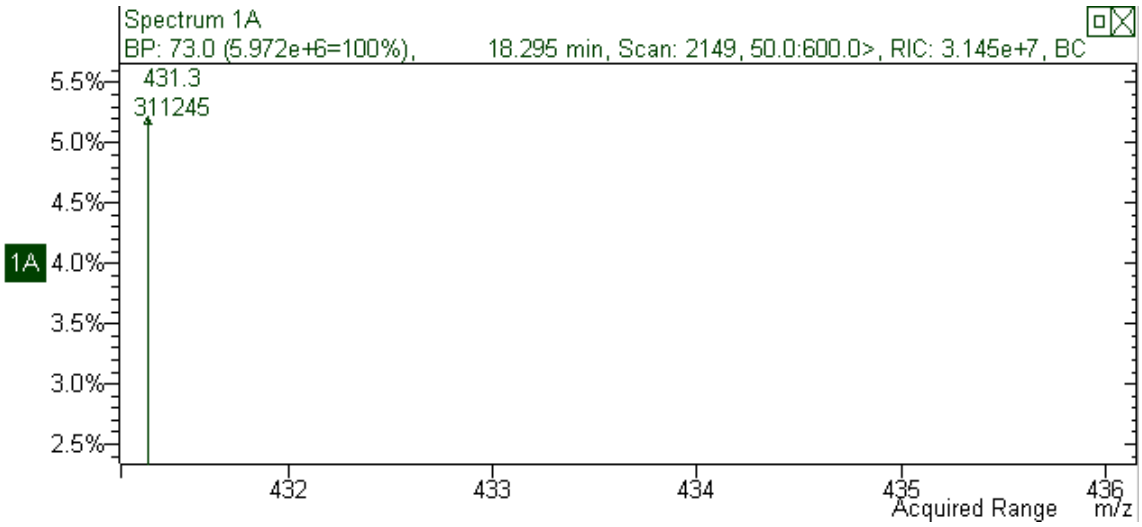


Figure 12: A zoomed in view of the peak seen in Figure 11. At a mass to charge ratio of 431.3, an approximate relative abundance of this ion can be seen to equal 5.2%.

Once it was determined that copper had cross-linked to guanine, a direct reversal of this cross-link was attempted by treating the sample with ethylenediamine. This resulted in successful retrieval of the guanine spectrum where it had previously been unrecognizable (Figure 13). Upon analyzing the mass spectrum, the 431 peak is no

longer present, and the identifying peaks of guanine have returned. Not only has the 431 peak been eliminated at the predicted retention time of approximately 20 minutes, but a further ion search of the repaired guanine sample indicates that peak 431 is not present at any viable intensity anywhere near the expected retention time.

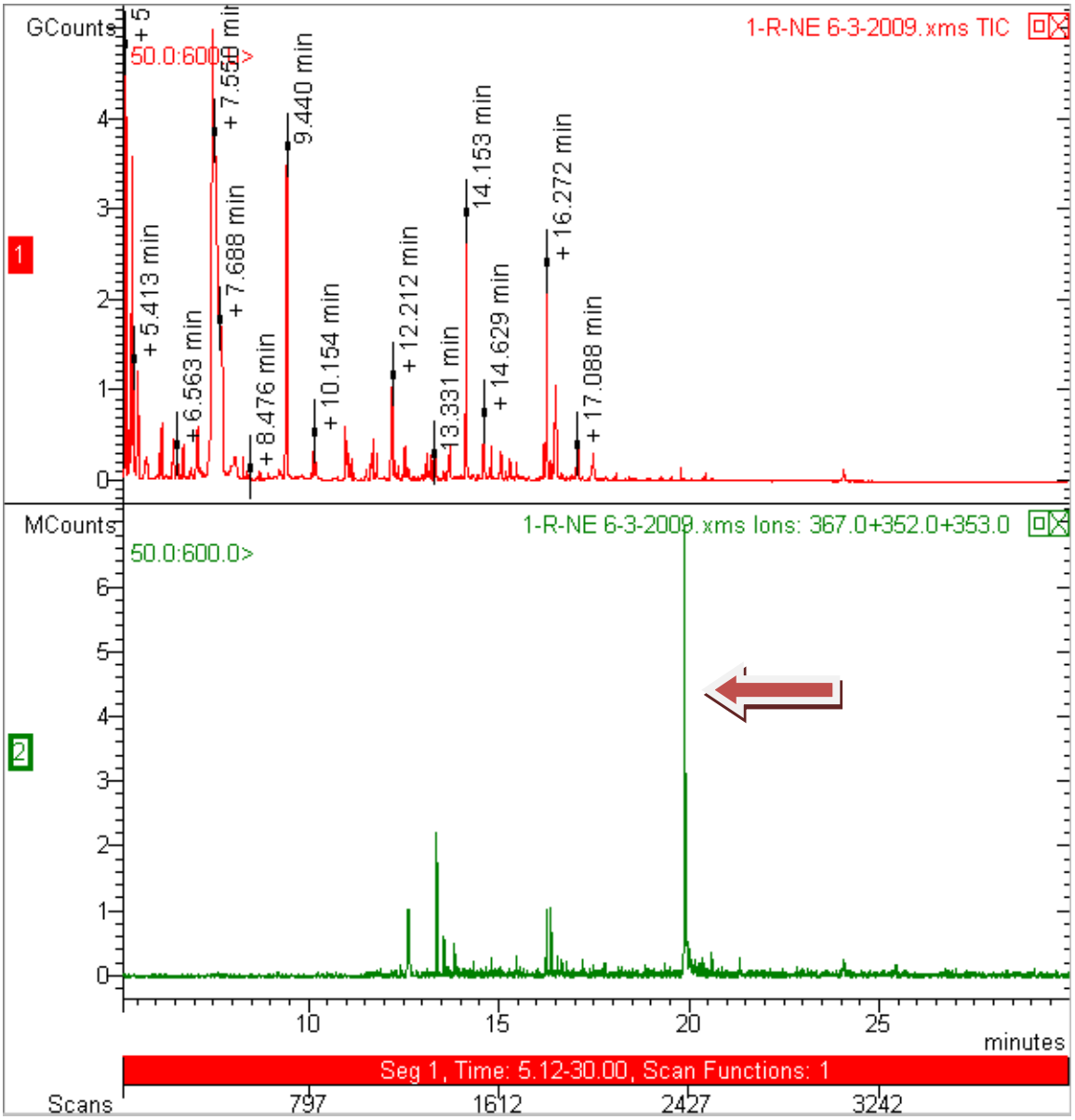


Figure 13: GC-MS analysis of copper cross-linked guanine treated with ethylenediamine. The direct reversal repair has resulted in the return of derivatized guanine at a retention time of 19.887 minutes.

Further inspection of this retention time peak resulted in a positive identification in the ion library search engine. Although the ion search was able to recognize the peak, a noticeable reduction was also noticed in the molecular ion as compared to the unaltered guanine control (Figure 14).

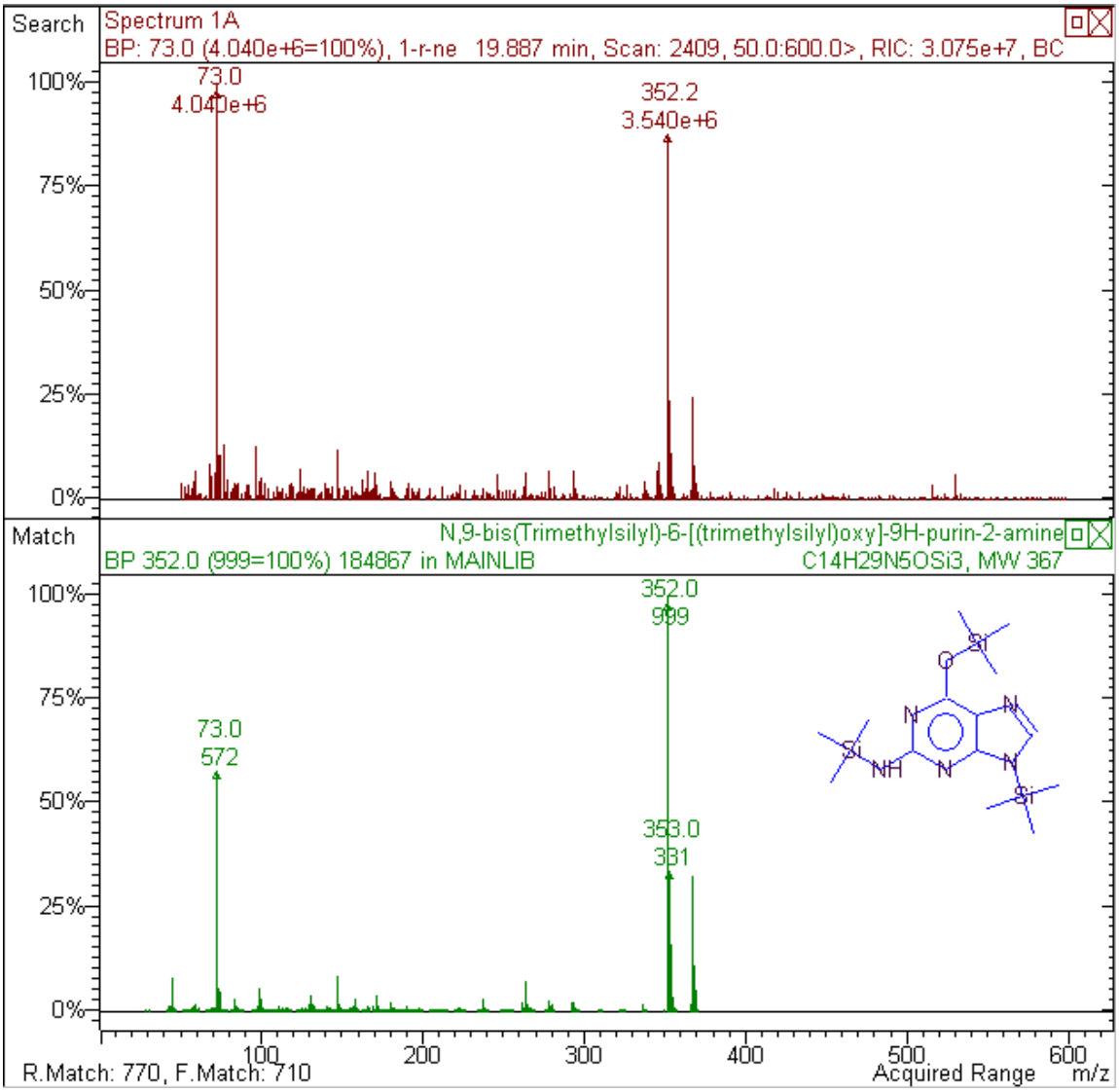


Figure 14: Library search of the mass spectrum found at retention time 19.887. The ion search resulted in a positive identification of silylated guanine with an 83.73% probability.

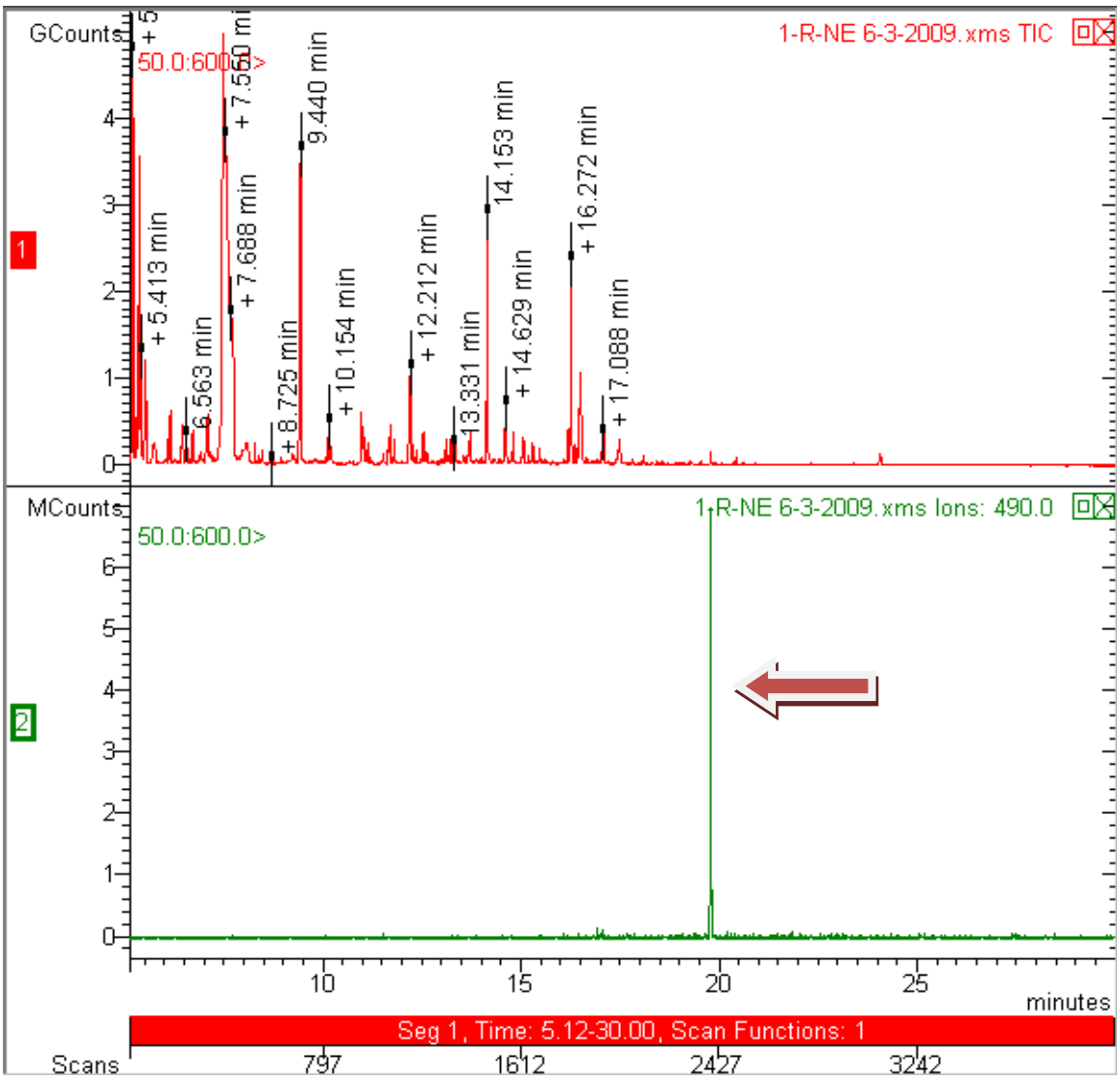


Figure 15: Ion search for the intermediary molecule of guanine bound to copper bound to ethylenediamine. This molecular weight of this intermediary compound is 490 and an ion search for this weight turned up one very distinct peak.

Not only was guanine repaired in this sample, but the intermediary of direct reversal repair was also located. This intermediary is the compound of copper bound to guanine with ethylenediamine bound to the copper atom. When copper is the central atom bound to a derivatized guanine and an ethylenediamine, it has an expected molecular weight of 490. A search for this ion finds a definitive 490 peak at 19.789 minutes retention time with a relative intensity of 20.7% (Figure 15); (Figure 16).

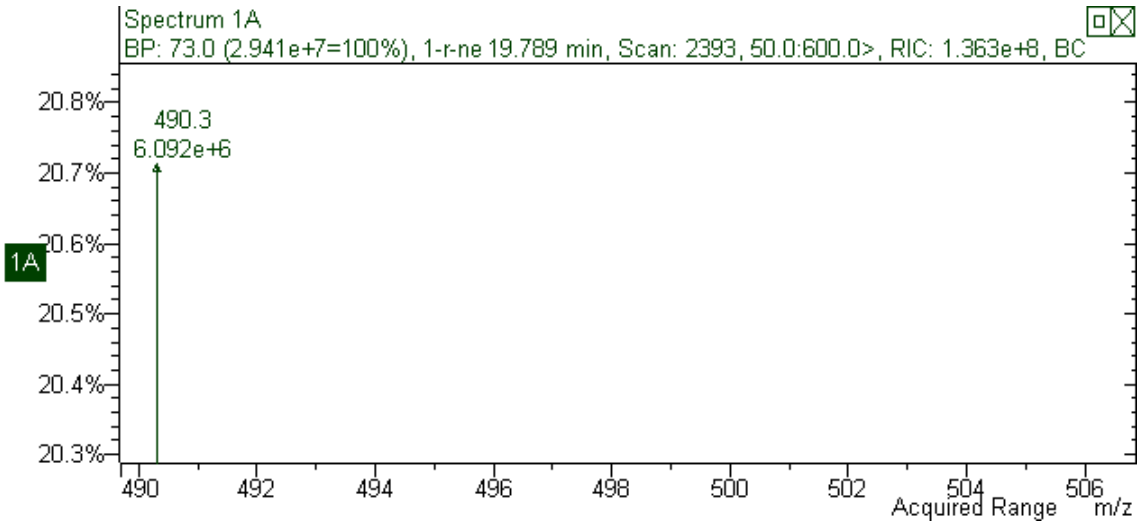


Figure 16: A mass spectrum of the 490.3 peak located at the intermediary molecule retention time of 19.789 minutes. The relative abundance of this molecular ion is approximately 20.7%.

Inspection of the purines after ethylenediamine treatment resulted in very little change in the relative intensities of both nucleobases. The retention times were also unchanged by the ethylenediamine exposure.

3.2 Synthetic dsDNA Cross-Link Formation and Repair

The next template used for analysis was the synthetic dsDNA. This introduced a much more difficult spectrum for interpretation since the sample contains all four nucleobases as well as other constituents that can interfere with the ion detection in the mass spectrum. Since several marker ions, as well as expected retention times, were determined for each purified nucleobase, it was relied upon to detect the presence or absence of damaged and undamaged nucleobases (Table 2). With a known retention time

Table 2: Summary of the expected retention times and the identifying mass spectrum peaks used to positively identify the presence of the four nucleobases.

Nucleobase	Expected Retention Time	Identifying Mass Spectrum Peaks
Guanine	20 minutes	367, 352, 353
Adenine	17 minutes	279, 264
Cytosine	13-14 minutes	255, 240
Thymine	12 minutes	270, 255

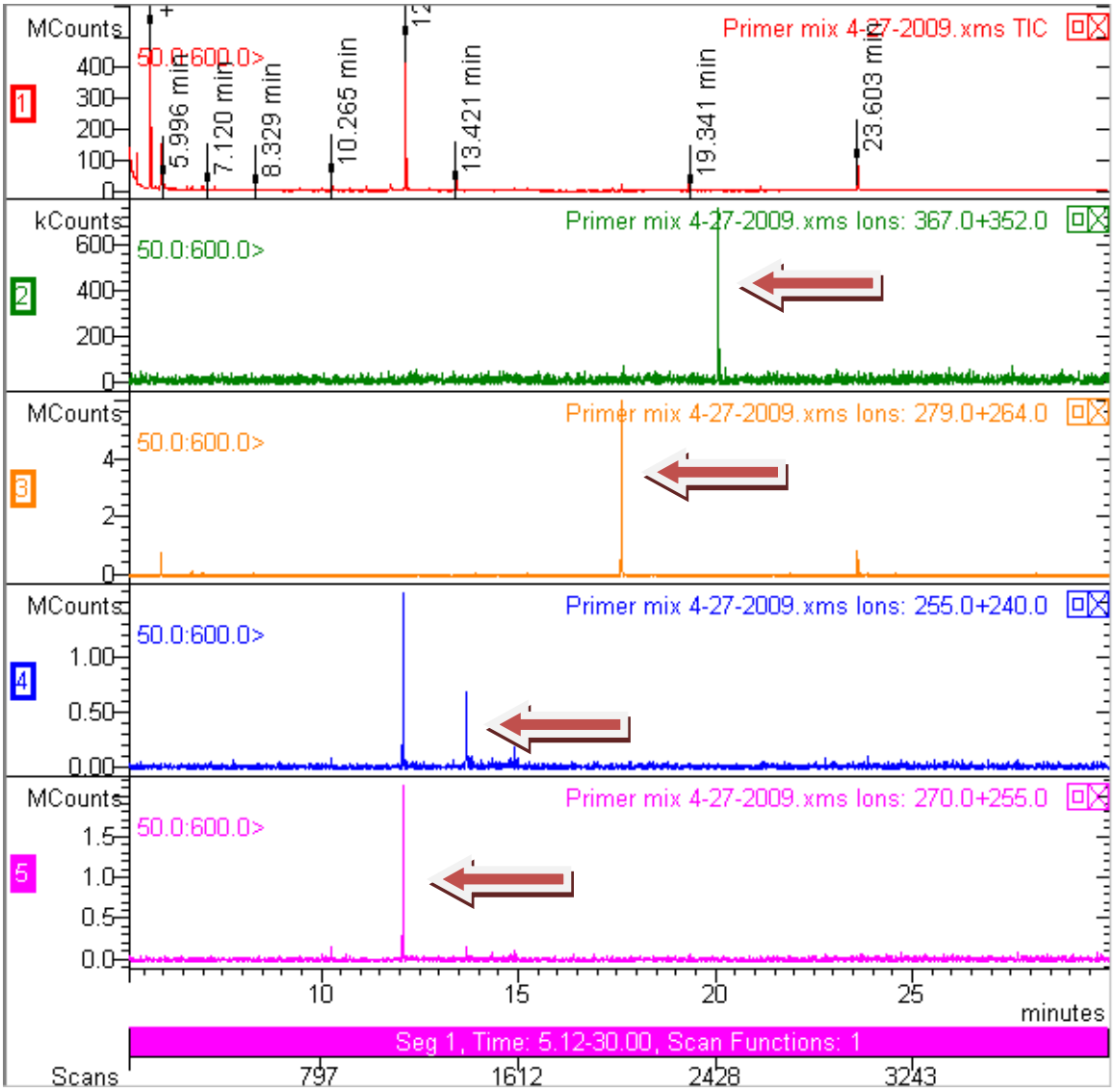


Figure 17: Gas chromatograph results for the unaltered primer mix (red). A search of the isotopic fingerprint for each base was performed in order to positively identify their presence. The bases in descending order that were positively identified based on their ions and expected retention times were guanine (green), adenine (orange), cytosine (blue), and thymine (purple). Cytosine (blue) can be seen to contain a peak for both cytosine and thymine. This is most likely caused by cytosine's natural instability, making conversion to thymine a common occurrence.

and the identifying mass spectrum peaks, an isotopic fingerprint can be searched for to show presence or absence of our unaltered nucleobases.

The mass spectrum for the unmodified synthetic dsDNA was found to contain all the identifying mass spectrum peaks at the expected retention times, positively identifying the presence of guanine, adenine, cytosine, and thymine (Figure 17). Due to spectral interference, a positive ion search was not possible using the software's internal identification library (Figure 18).

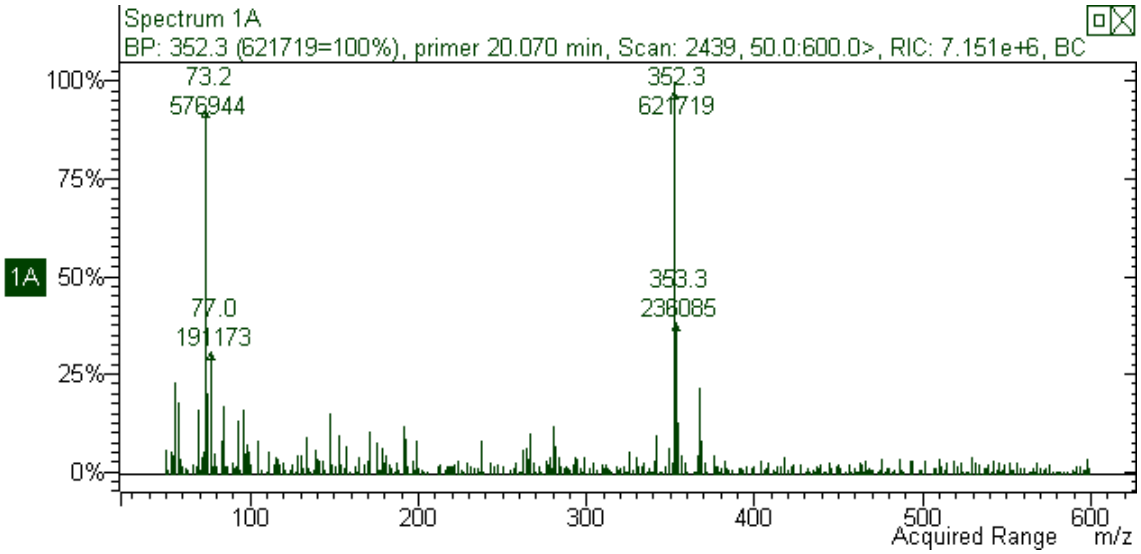


Figure 18: Mass spectrum of a peaked located at a retention time of 20.070 minutes in the annealed primer mix. The presence of the three ions that make up guanine's isotopic fingerprint combined with a retention time are the expected 20 minute mark indicates positive identification. The extraneous peaks along the base line make identification through the MS Data Review software not possible. These peaks are once again by products of other damages occurring during the derivitization process.

With the combination of increased background noise and the success of guanine-copper binding, the 431 marker indicating a copper-guanine crosslink at the N7 position was the continued focus for the double stranded templates.

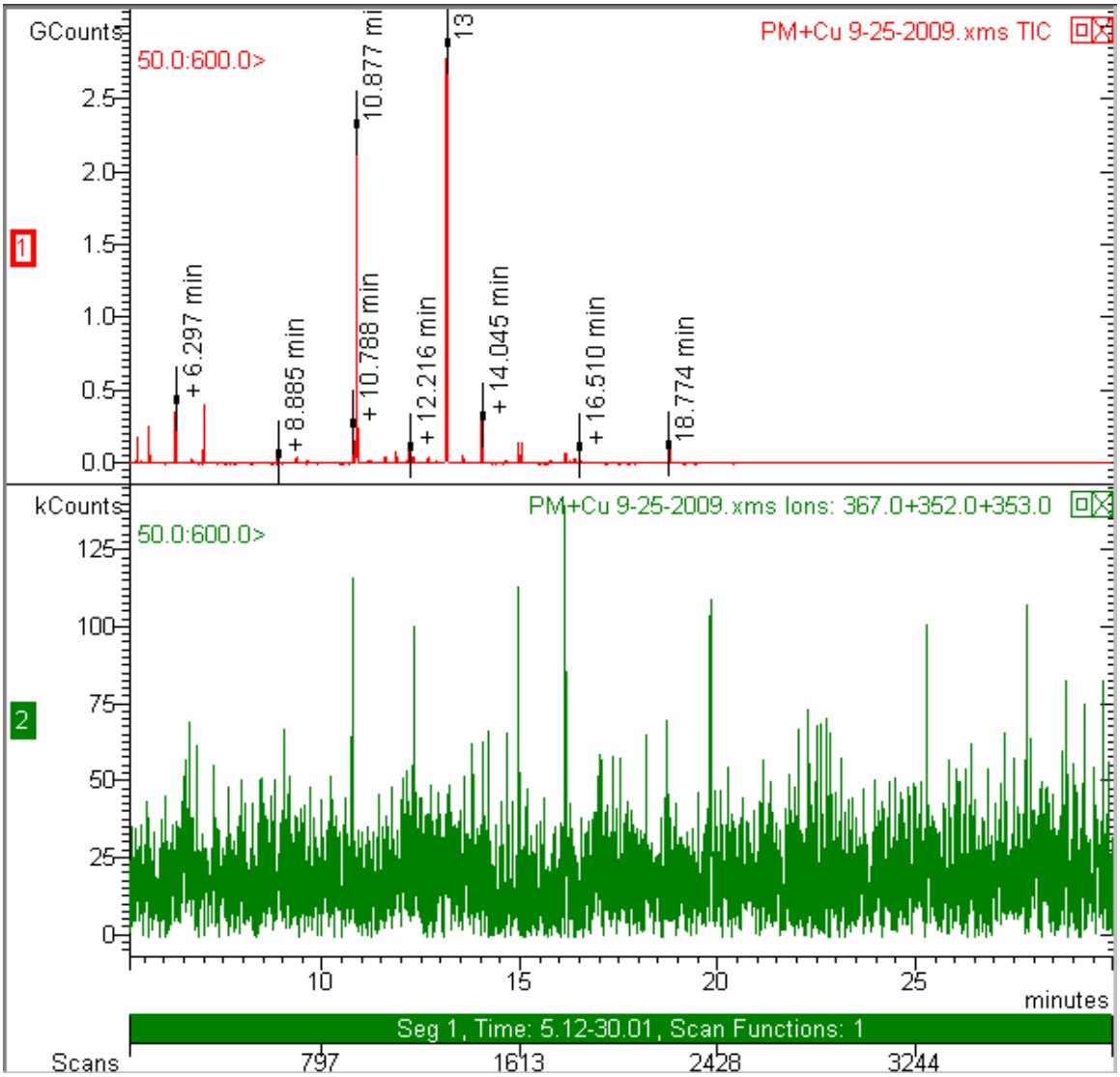


Figure 19: Gas chromatograph (red) of the annealed primer mix with copper induced cross-link formation. The mass spectrum data (green) shows a search for the isotopic fingerprint of guanine (367, 352, and 353). There is a thick peak located around the 20 minutes retention time, however investigation of this peak found that it completely lacked the 367 ion.

Once the annealed primer template was treated with copper chloride, an ion search was performed to identify if a reduction in unaltered guanine was present. An ion search was performed for the identifying peaks of guanine (367, 352, and 353). The search resulted in no definitive peaks. Any peaks which occurred in the expected areas were off the expected retention time or do not contain enough of the identifying peaks to

enable a positive identification of guanine's presence. The strongest peak in the general area was found to contain 353 but the molecular ion of 367 was not present in any intensity (Figure 19).

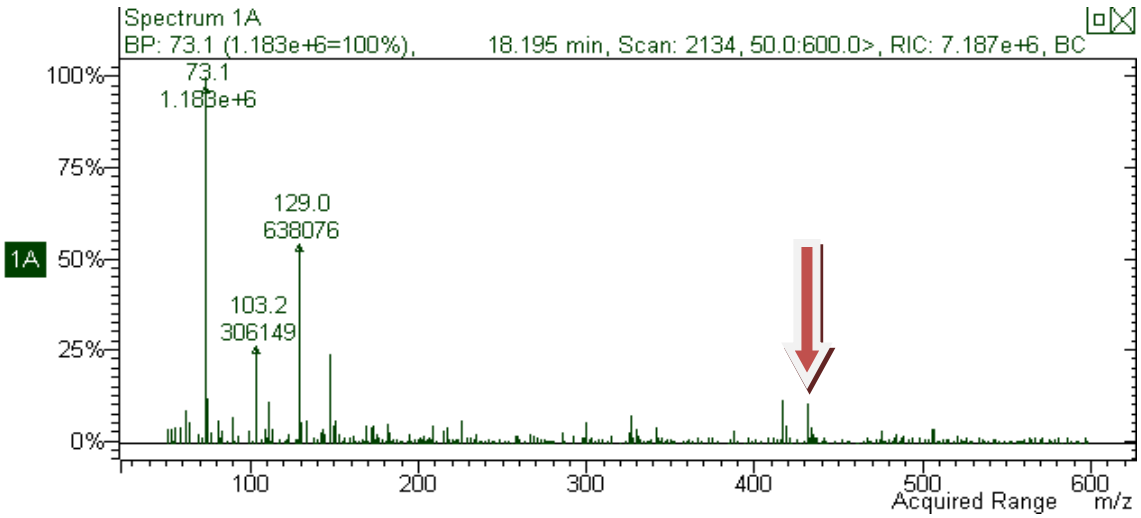


Figure 20**: Mass spectrum of the annealed primer template with copper induced cross-links. At a retention time of 18.195 minutes, a peak with a molecular weight of 431 was located. This weight coincides with the calculated weight of a silylated guanine cross-linked to copper.**

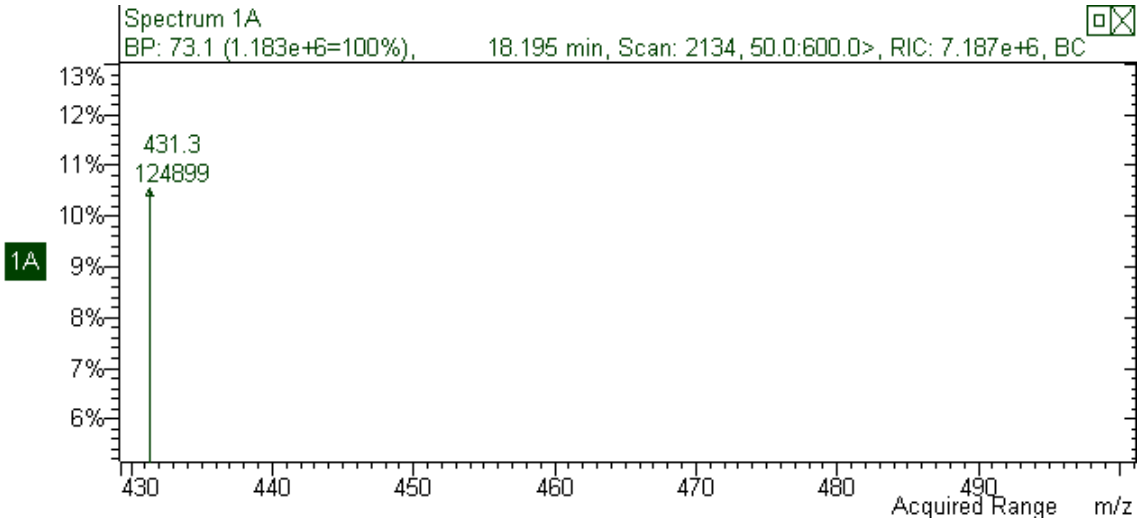


Figure 21: A closer look at the 431.3 peak present in the annealed primer mix copper cross-link. At this retention time of 18.195 minutes, the 431.3 peak is seen to have a relative abundance of approximately 10.5%.

Upon performing an ion search for the 431 ion in the annealed primer template with copper exposure, it was once again located at a retention time of approximately 18

minutes with a relative abundance of approximately 10.5% (Figure 20); (Figure 21). This peak was not discernable in the unaltered annealed primer mix.

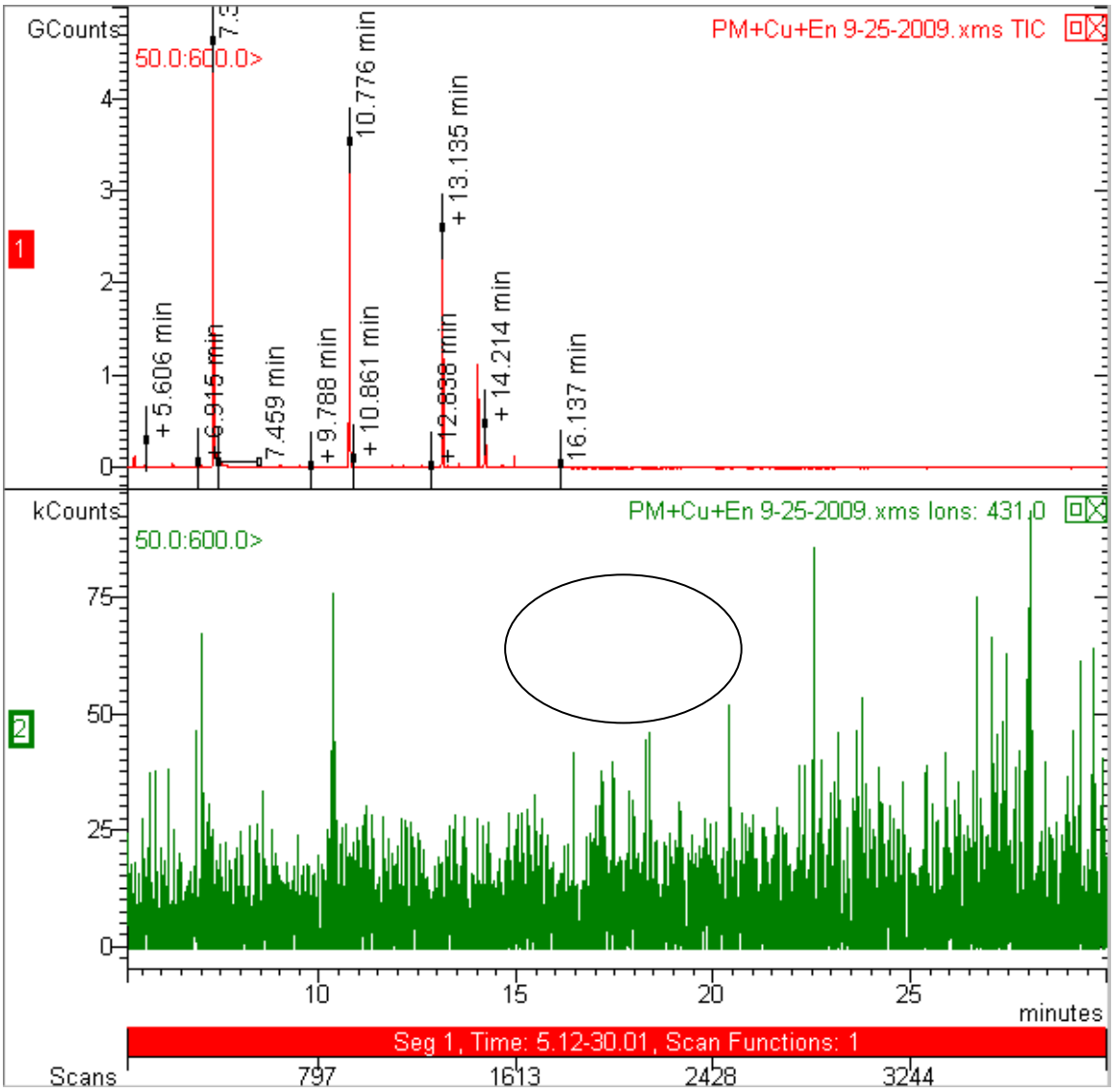


Figure 22: Cross-linked annealed primer treated with ethylenediamine. An ion search for the 431 ion that indicates the presence of guanine-copper cross-links is no longer present at the 18 minute mark. The other large peaks were inspected and ruled out as indentified cross-link formation.

Ethylenediamine also showed success in the double stranded templates as well. When the annealed primer template with copper-guanine cross-links was treated with

ethylenediamine the peak identified at 431.3 was found to no longer be discernable in the spectrum (Figure 22).

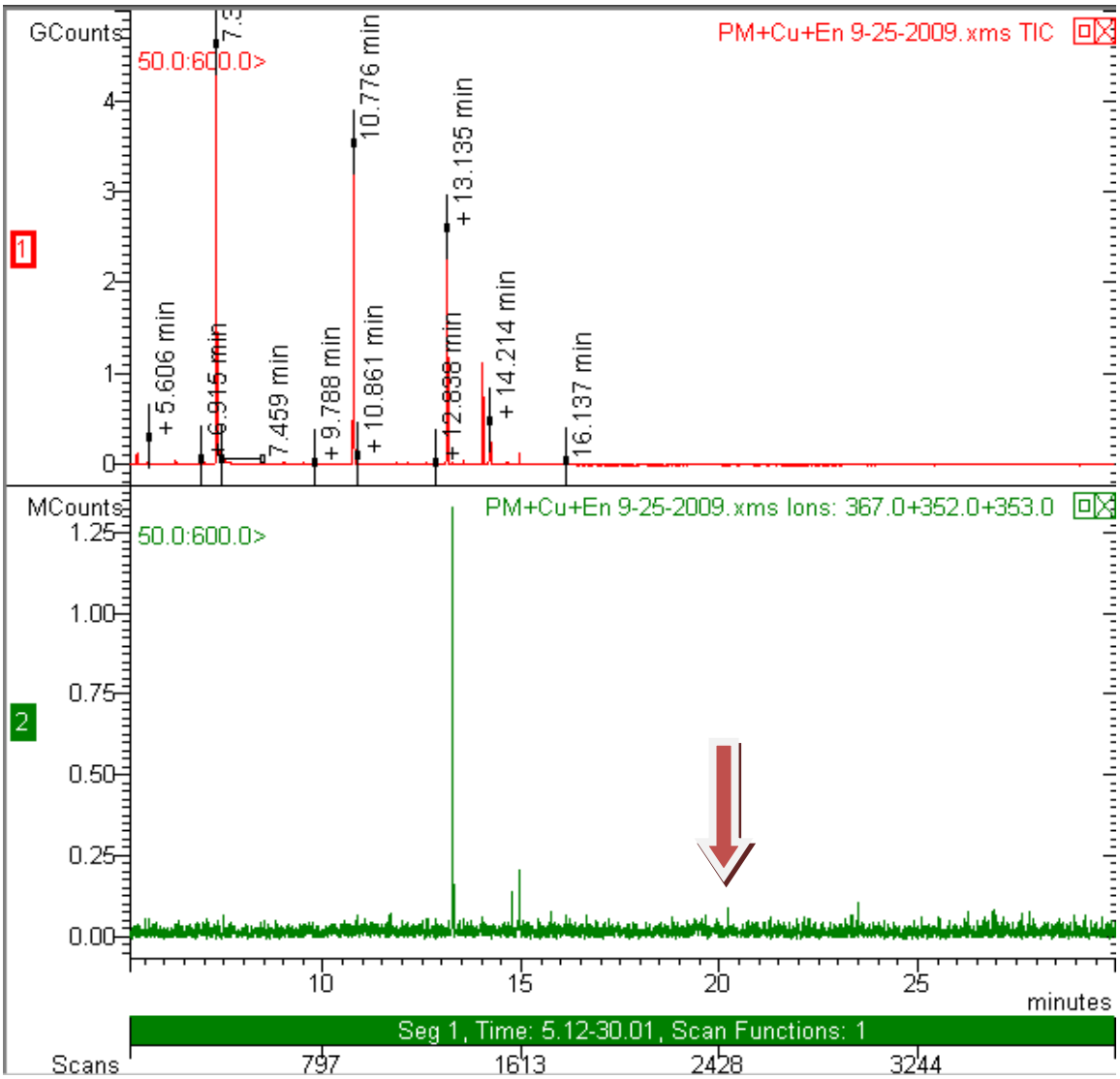


Figure 23: An ion search of the ethylenediamine repaired annealed primer template. Searching for the isotopic fingerprint of guanine found that a small peak had returned around the expected 20 minute retention time. The other large peaks were investigated and ruled out as representing guanine since they lacked one or more of the necessary ions and were not found at the known retention time.

Since the 431 peak was no longer present, an ion search was performed to see if the guanine spectrum had returned. A search for the identifying ions found a small peak at a retention time of 20.225 minutes (Figure 23). This peak was found to contain the

identifying ions or 352 and 368 amidst a significant amount of background noise (Figure 24).

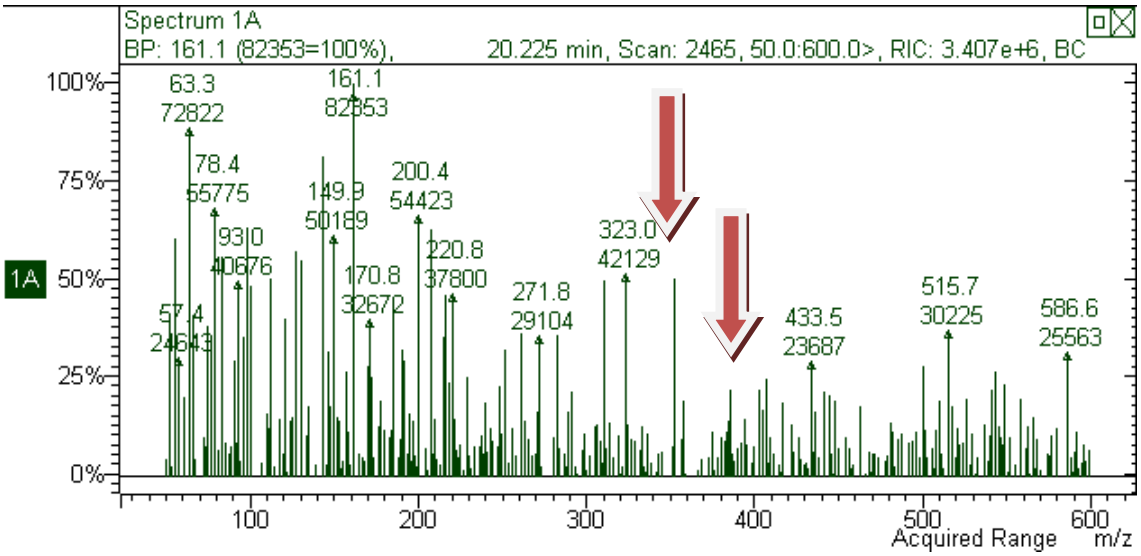


Figure 24: Mass spectrum of the peak that appeared at 20.225 minutes in Figure 23. 352 and 367 were found to be present amongst much background noise, caused by alternative DNA damage.

3.3 Modern dsDNA Cross-Link Formation and Repair

Testing of copper cross-link production and repair took place on a 425 base pair amplified product template. The sheer size of this template compared to previous templates made detecting peaks even more difficult. As was the case for the synthetic dsDNA, a copper-guanine cross-link was the main focus as it is the most willing nucleobase to cross-link to the copper.

Analysis of the unaltered amplified product resulted in being able to positively distinguish guanine, adenine, and thymine in the mass spectrum (Figure 25). However, cytosine was not as definitive, and its mass spectra could not be determined with any certainty.

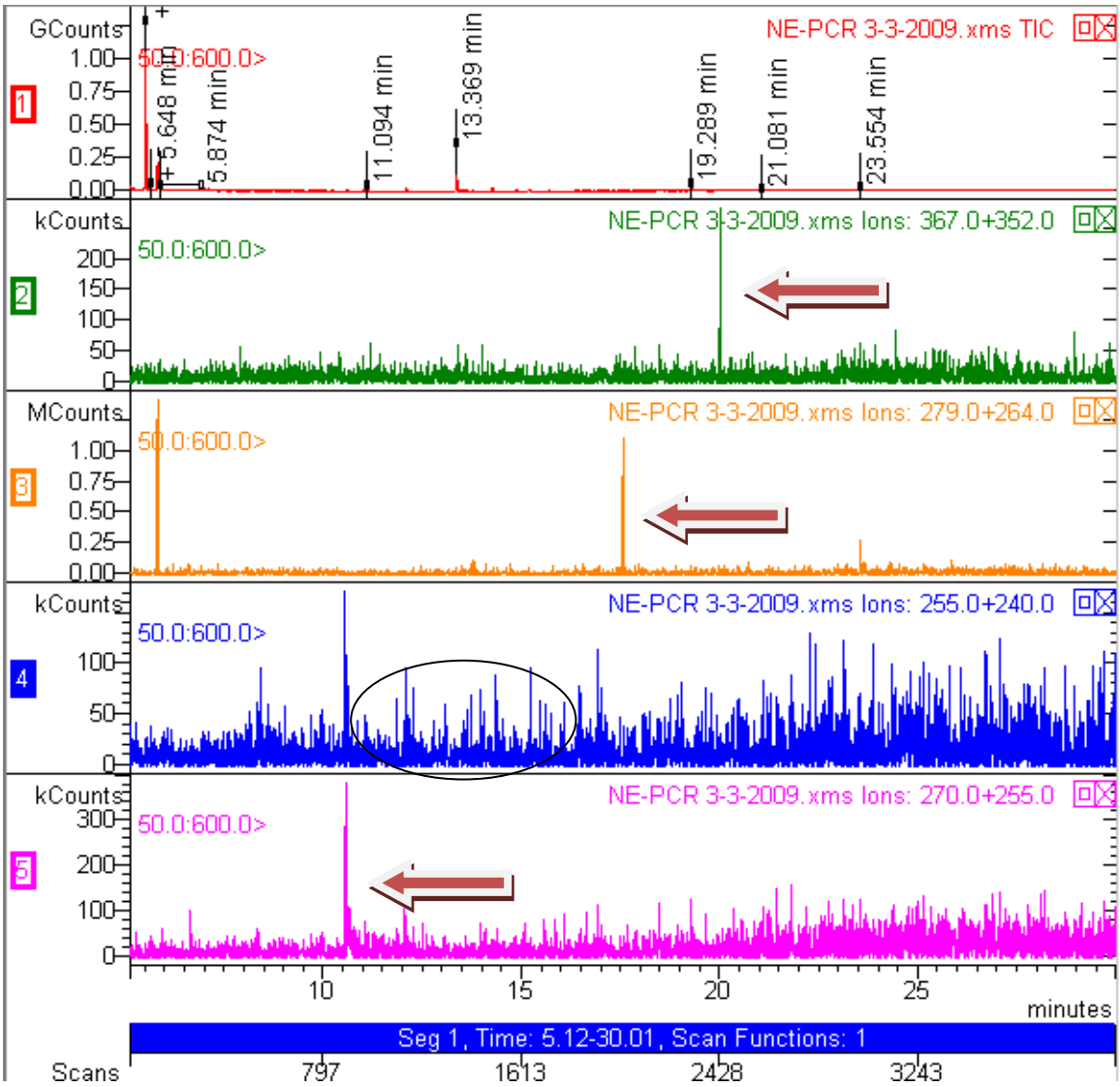


Figure 25: Mass spectrum of the unaltered amplified product of 425 base pairs (red) as well as the retention times for three of the four nucleobase: guanine (green), adenine (orange), and thymine (purple). Cytosine was inconclusive as to its mass spectrum results, once again due to its unstable nature.

With focus placed on guanine, the 431 peak was searched for and once again located at a retention time of 18.207 minutes (Figure 26). The relative intensity for the amplified product was also higher, measuring in at approximately 18% (Figure 27).

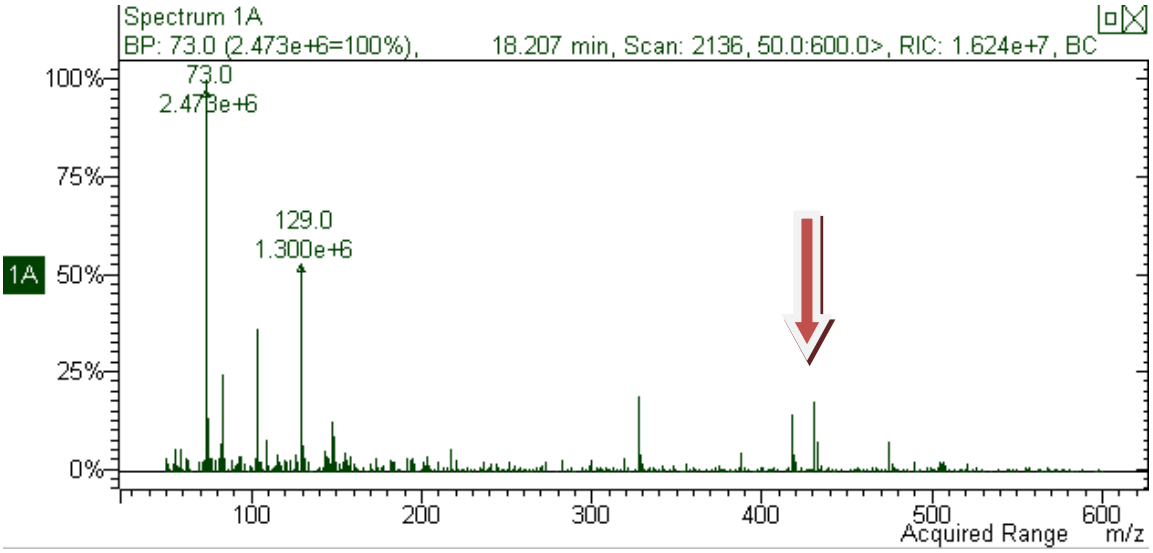


Figure 26: A mass spectrum search for the 431 ion of the double stranded amplified template with copper cross-links. At 18.207 minutes, a 431 ion peak was located in the same spot as found previously in the annealed primer-copper cross-linked template.

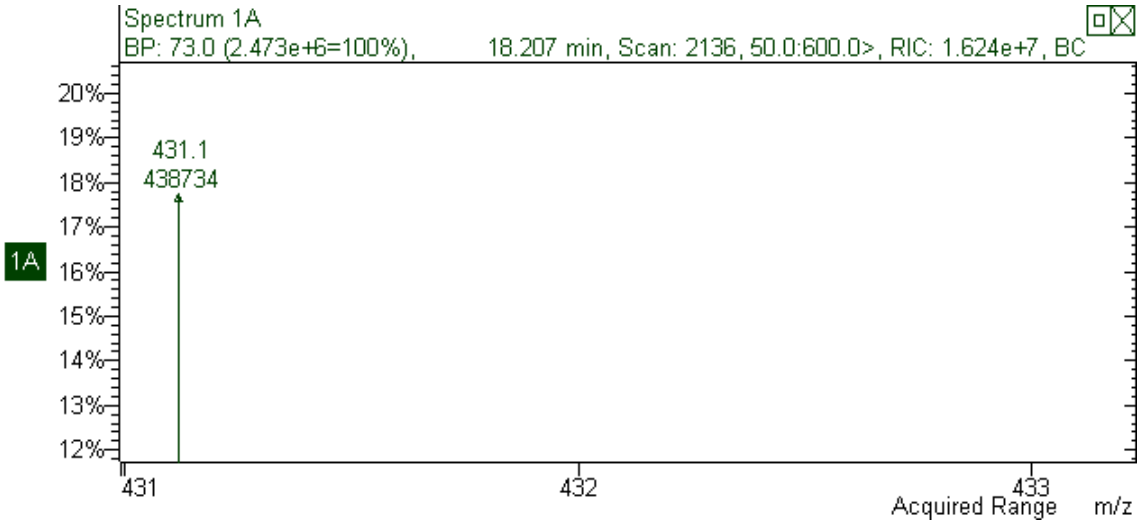


Figure 27: A closer look at the 431.1 peak present in the double stranded amplified template-copper cross-link. At this retention time of 18.207 minutes, the 431.1 peak is seen to have a relative abundance of approximately 17.9%.

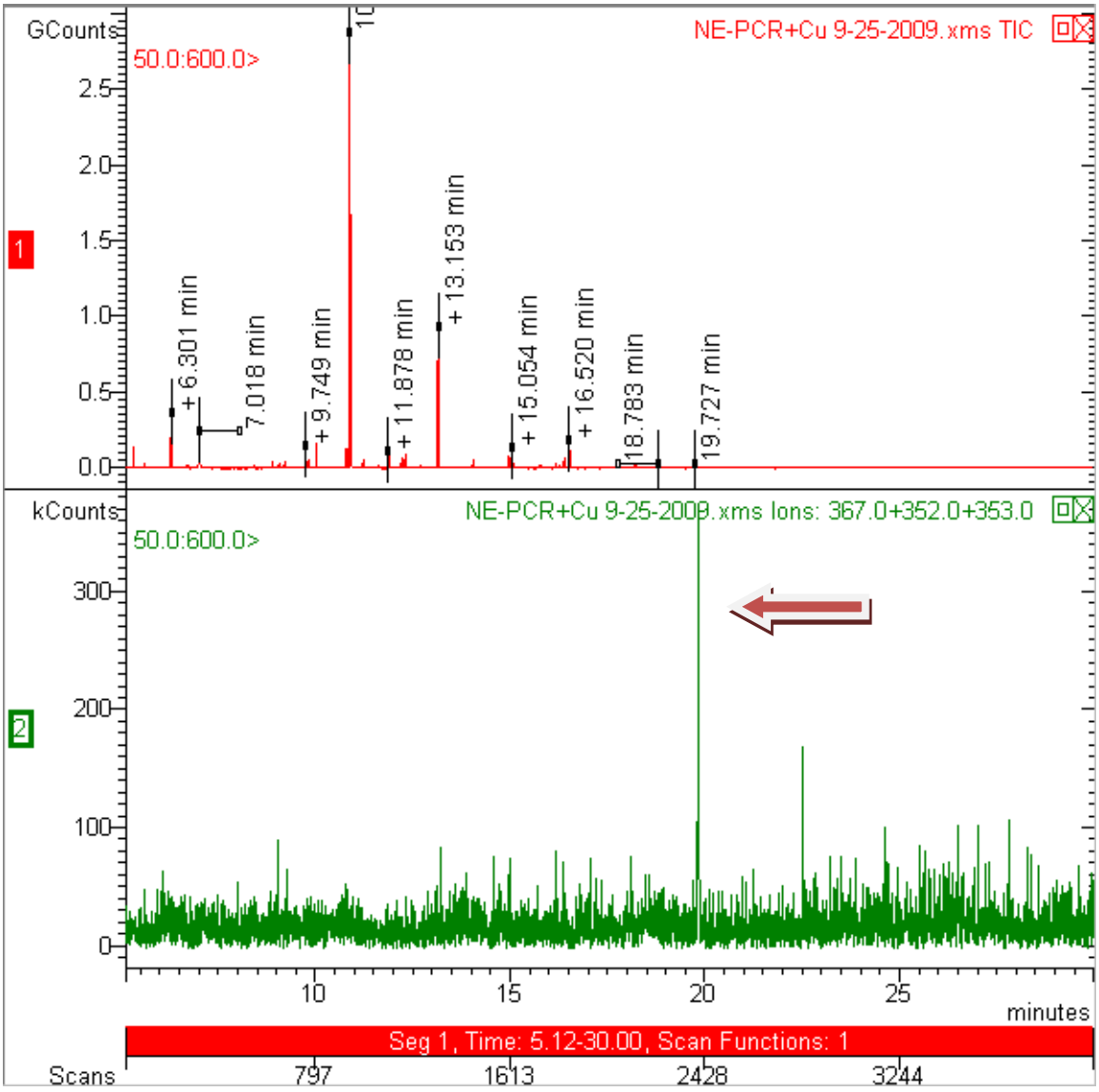


Figure 28: An ion search of the mass spectrum in the copper cross-linked double stranded template. A search for the isotopic fingerprint resulted in a definitive peak at the expected 20 minute retention time.

These results were further verified by searching for unaltered guanine in the copper exposed amplified template and finding that only one of the identifying peaks remained at the expected retention time for unaltered guanine (Figure 28). The absence of a molecular ion of 367 is a positive indicator that guanine has been damaged (Figure 29).

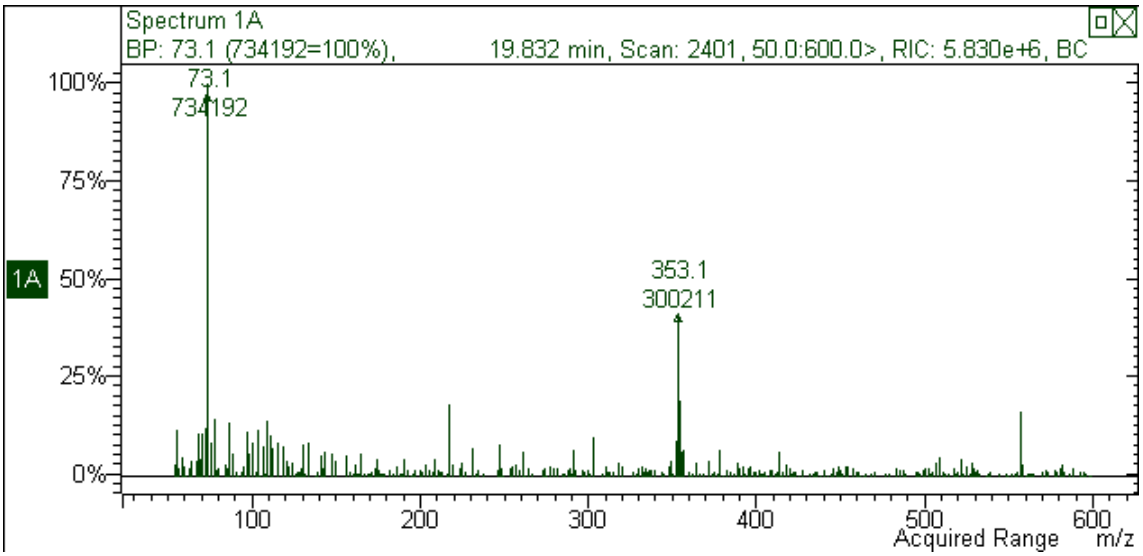


Figure 29: Mass spectrum of the expected guanine peak shown in Figure 28. Although all three ions were searched for, further investigation showed the only peak of the three remaining was 353, with 352 and 367 absent.

The direct reversal repair showed more definitive success in this 425 base pair amplified product. Although the 431 peak was still located at 18.207 minutes retention time (Figure 30), its relative abundance was reduced from approximately 18% down to 10.35% (Figure 31).

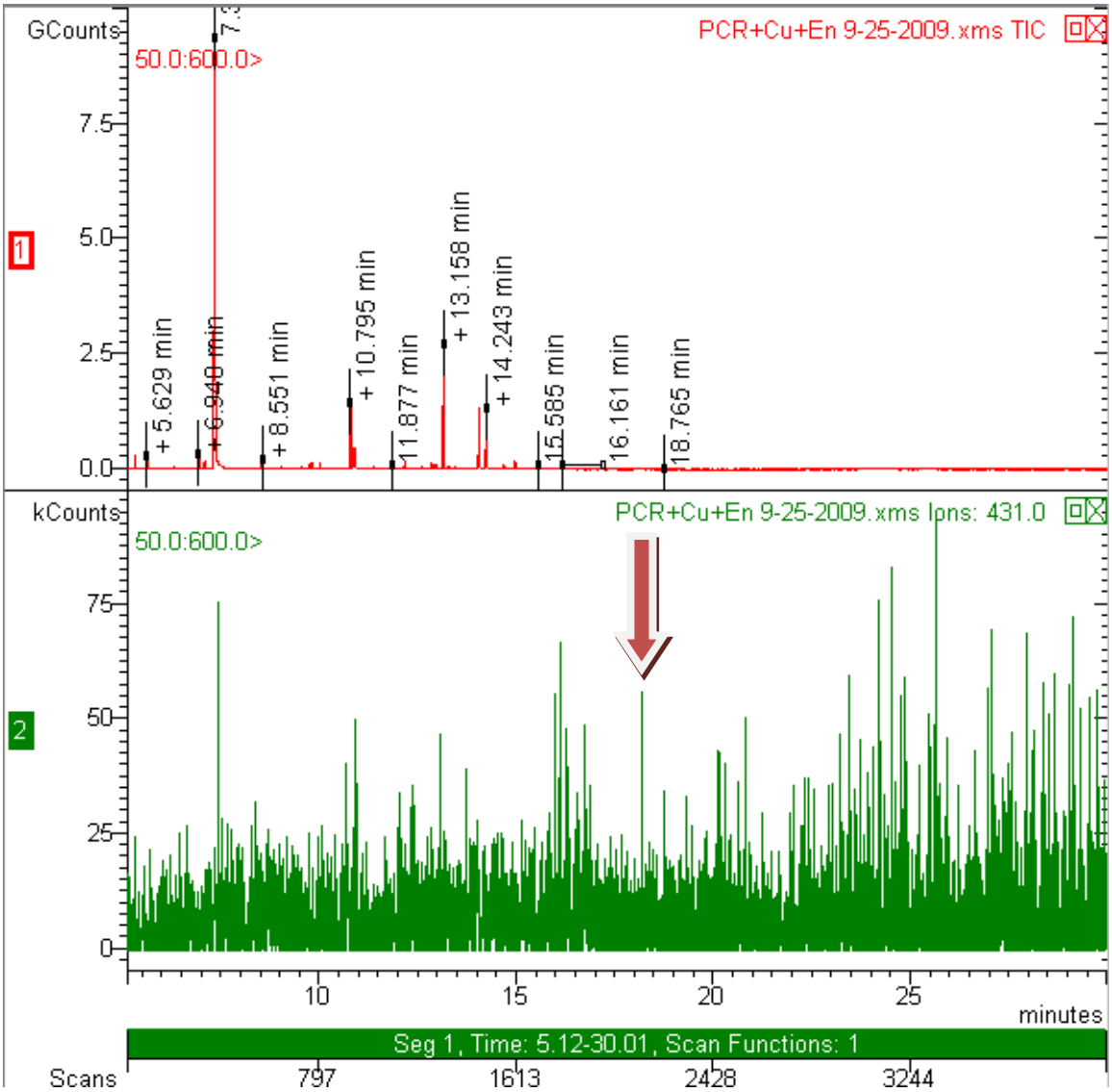


Figure 30: GC-MS of the amplified product template showing the presence of the 431 ion even after treatment with ethylenediamine. Although this peak remains present, its size and intensity are more than half of what they were before ethylenediamine treatment.

The success of the ethylenediamine in treating the copper damage and restoring guanine in the modern amplified template is also easily located upon searching for the identifying ions (Figure 32). Although this mass spectrum also contains a significant amount of interference, it can be verified that guanine is present due to the retention time, and presence of 352, 353, and 367 m/z (Figure 33).

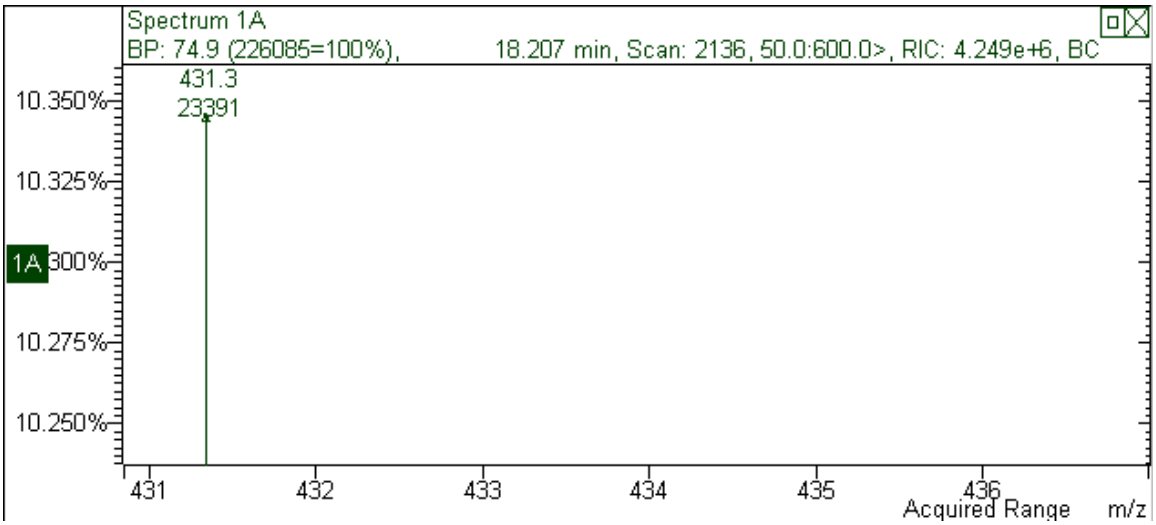


Figure 31: Mass spectrum showing the 431.3 peak present at 18.207 minutes. The relative intensity for this ion has dropped significantly after being exposed to ethylenediamine.

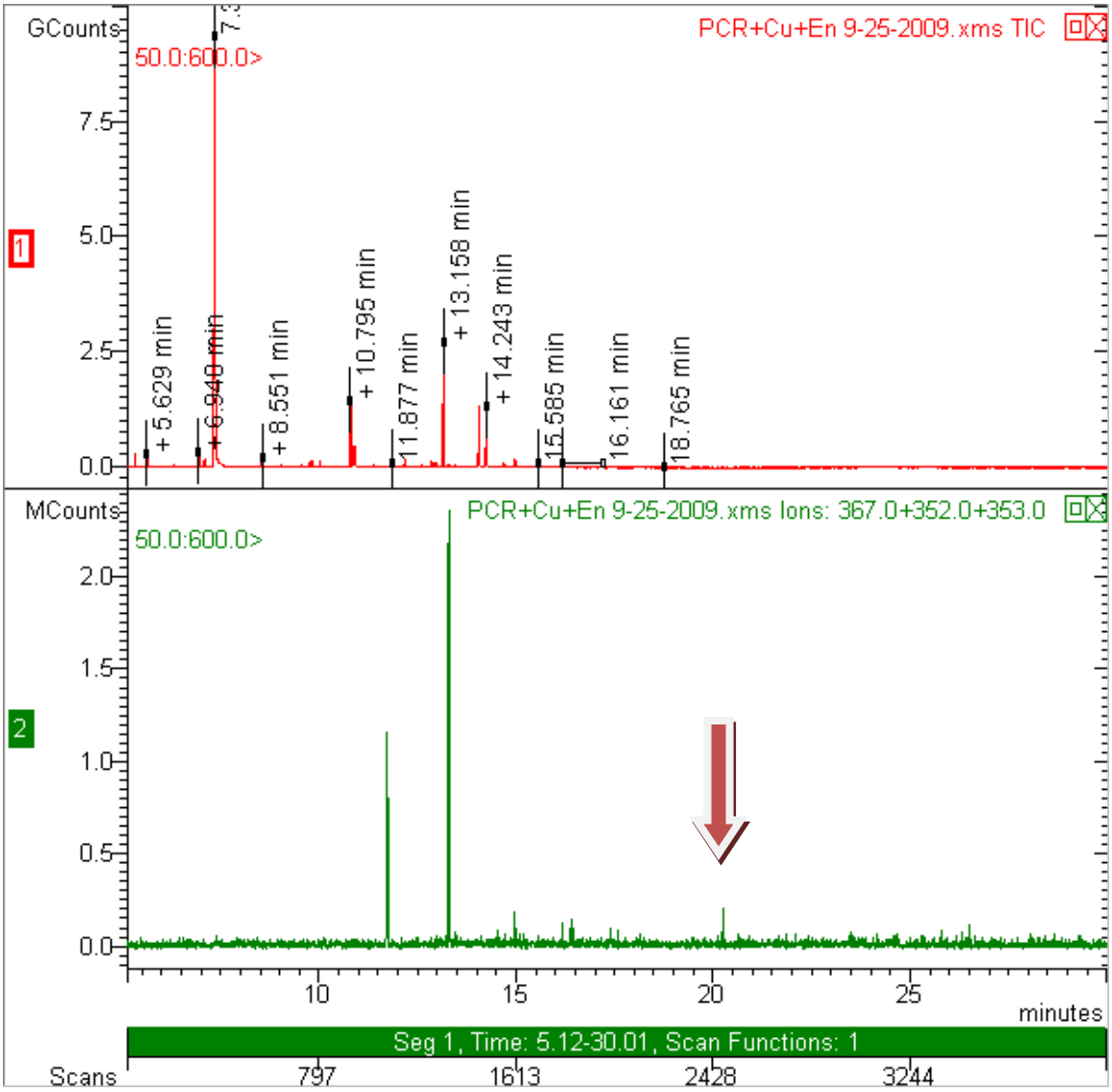


Figure 32: Ion search for ethylenediamine repaired guanine. A search for guanines isotopic fingerprint resulted in a small peak emerging aroudn the 20 minute retention time. The other definitive peaks were ruled out as representing guanine since they did not contain the proper ions and were not at the expected retention time.

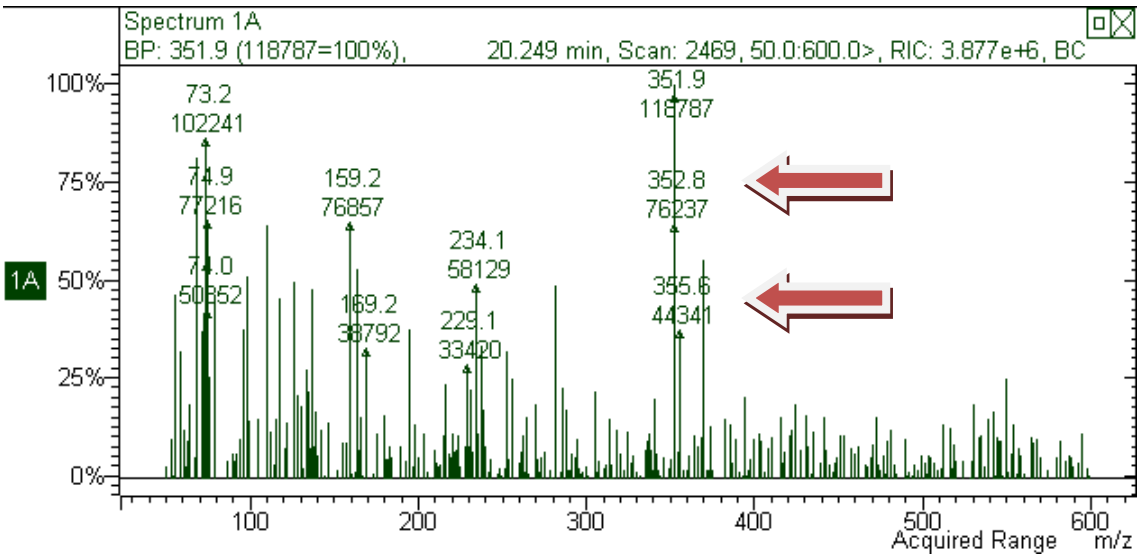


Figure 33: Mass spectrum of the ethylenediamine repaired amplified product at the retention time of 20.249 minutes. Even though extraneous ions are also visible, the presence of all three identifying peaks (352, 353, and 367) combined with the expected retention time for guanine is positive identification. The extraneous peaks are most likely a result of alternative damages that have occurred to the sample during the preparation process.

3.4 Ancient dsDNA Cross-Link Formation

Although there was success with cross-link production and repair with the previous templates, a naturally cross-linked sample of copper must be analyzed to compare to these synthetic cross-links. For this comparison, a bone sample extract known as Bone 201 was obtained. The sample was known to have been exposed to copper as an elemental analysis performed previously had shown copper to be present. Upon GC-MS analysis, the sample was found to contain detectable guanine and thymine (Figure 34). This was confirmed based on the retention time of 19.764 and the presence of all three identifying peaks (Figure 35).

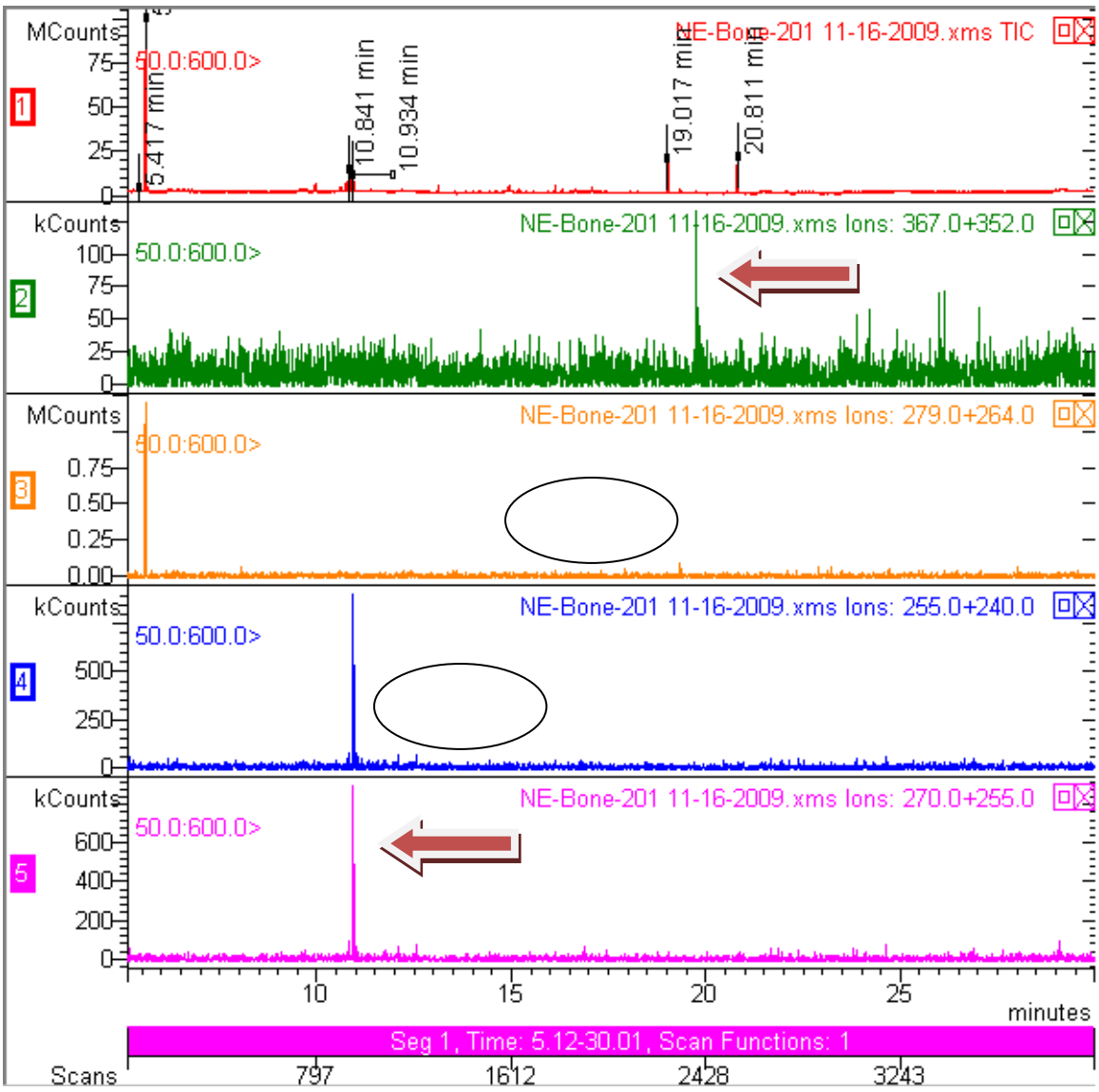


Figure 34: GC-MS of ancient bone DNA extract with natural copper cross-links due to exposure. An ion search results in a definitive retention time for derivitized guanine and thymine. Adenine and cytosine were not detected. The large peak in the adenine ion search (orange) at the beginning of the chromatographg was identified as tris(methylsilyl) borate and is most likely a result of contamination in the column of the GC-MS.

Although guanine was located, adenine and cytosine were not positively identified in this sample, indicating this sample is heavily damaged. Thymine was located using the identifying ions at its expected retention time. Thymine was also able

to be identified in the sample through the ion search library, being positively identified with a 74.69% probability.

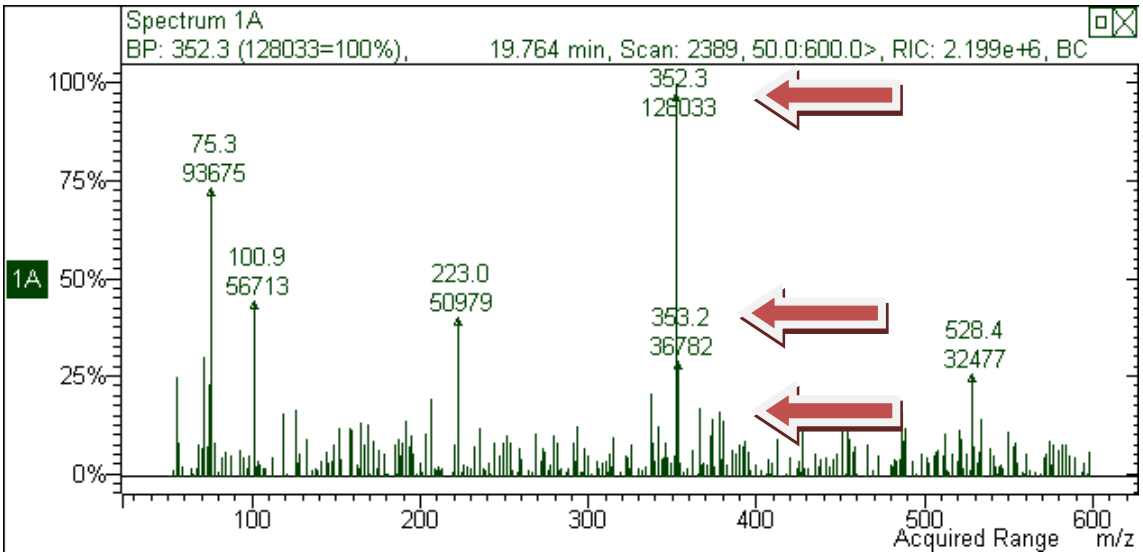


Figure 35: Mass spectrum of the peak at the retention time of 19.764 minutes in the ancient bone sample. The identifying peaks of 353, 352, and 367 are all present, indicating derivitized guanine. The extraneous peaks and background noise are to be expected with a sample this heavily damaged.

In order to find copper cross-linked damage to the guanine, a preliminary search was done for the ion peak 431 and 430. This search resulted in a peak of 429.7 being located at a retention time of 18.151 minutes (Figure 36).

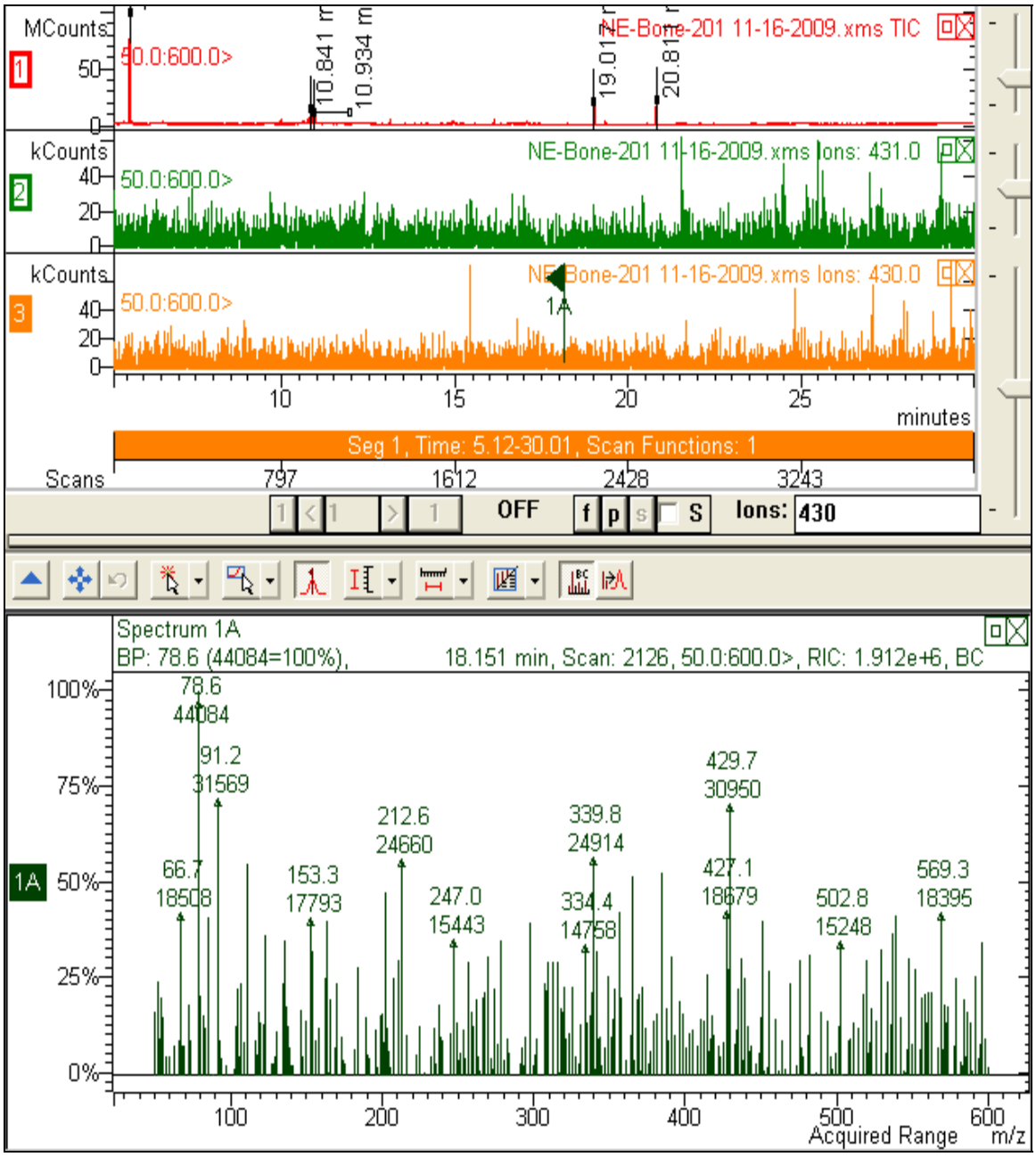


Figure 36: GC-MS of ancient bone sample. A 429.7 peak is located at a retention time of 18.151 minutes with a realtive abundance of close to 70%. This peak is surrounded by extraneous peaks of unknown identity, most likely caused by other types of damage the sample has been exposed to over time.

Since this sample was ancient and had been exposed to less than favourable conditions, the possibility of deamination occurring to the guanine should not be overlooked. Since the amine group on guanine is a binding point for one of the BSTFA

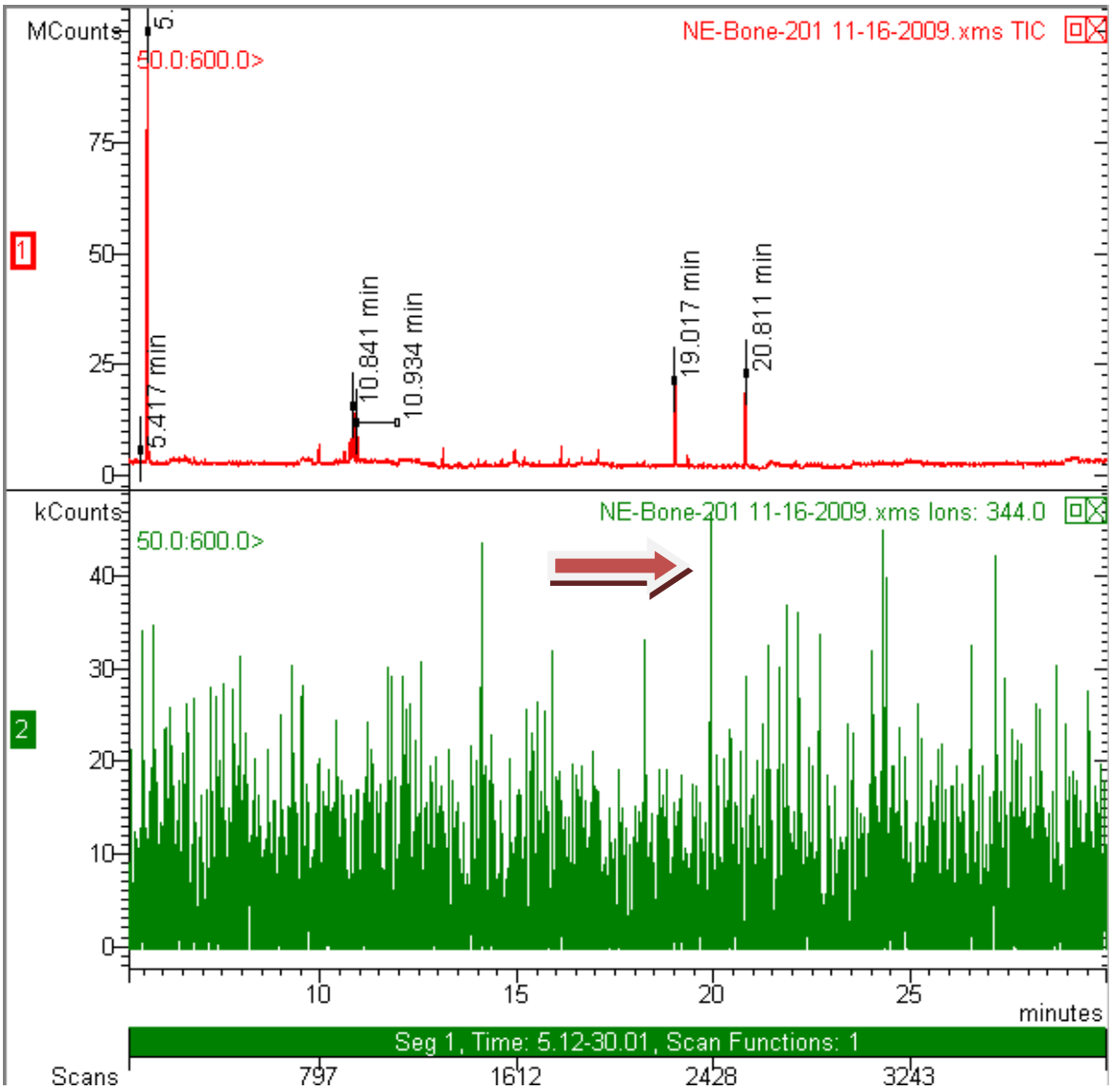


Figure 37: An ion search for deaminated guanine-copper cross-link. This compound's molecular weight is 344 so a search for this m/z was conducted. At the 19.917 minute retention time, a large peak was identified to contain this ion. The other large peaks in the chromatograph were also studied and determined to not be deaminated guanine-copper cross-links based on the lack of the molecular ion of 344.

silylation units, both the weight of the amine and one BSTFA unit must be subtracted, while adding a copper ion. The m/z of this damaged purine is around 344 units. Upon searching the ancient bone extract for this ion with the GC-MS, it was found that a strong ion occurs at a retention time of 19.917 minutes (Figure 37). This peak contains the 344 m/z ion at a relative abundance of close to 100% (Figure 38).

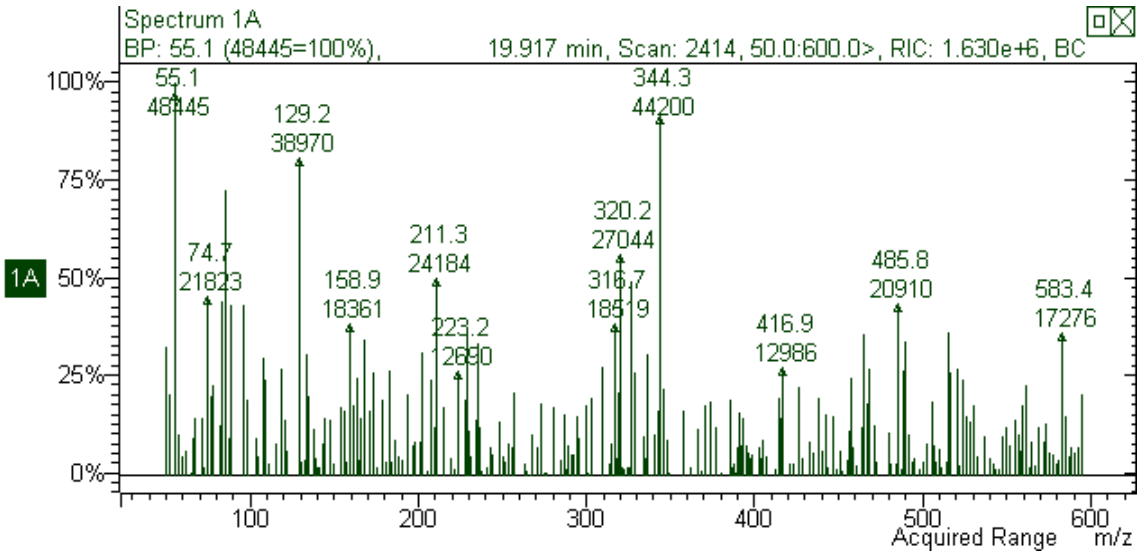


Figure 38: Mass spectrum of ancient bone sample at the 19.917 minute retention time. The peak at 344.3 m/z may represent derivatized and deaminated guanine cross-linked to copper.

3.5 Agarose Gel Cross-Link Detection

In order to determine if gel electrophoresis is a suitable method to detect damage and repair, it must first be determined what effect the presence of both copper and the ethylenediamine may have on the sample itself.

It was found that PCR template incubated with copper chloride was unable to migrate through the gel, and appeared to stay in the well. Results were further complicated when it was noticed that the presence of ethylenediamine as a repair agent caused the loading dye to migrate in the opposite direction. In the interest of this result, the gel was removed and visualized to determine whether or not a DNA band was visible migrating in the opposite direction. It was found that even with treatment of ethylenediamine, the DNA was unable to migrate out of the well. To determine if this was a result of the copper chloride inhibiting the migration, a final sample was run in which PCR template (as a positive control) as well as 1 M copper chloride solution as

added to the same well immediately before electrophoresis was initiated. It was found that even without the required incubation time, the DNA was unable to migrate through the agarose gel into any type of discernable band (Figure 39).

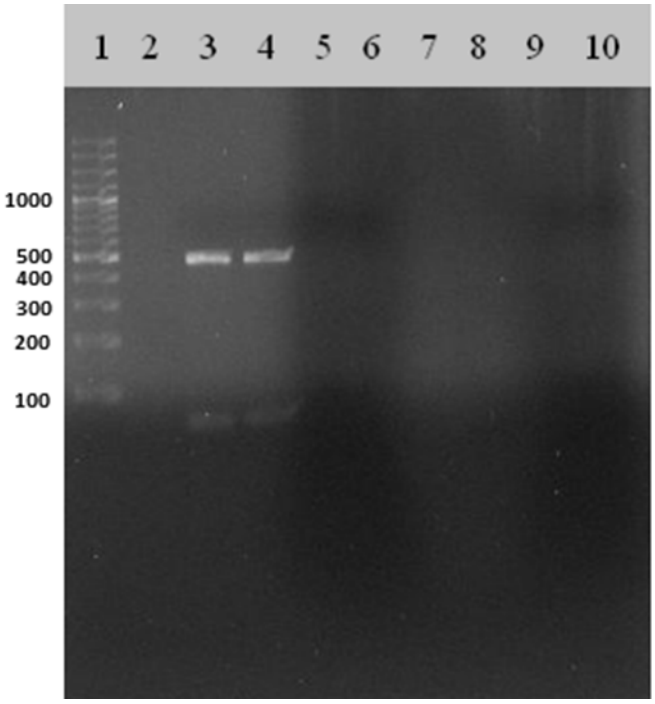


Figure 39: Agarose gel electrophoresis test on the inhibitory effects of copper and ethylenediamine on DNA migration with each sample run in duplicate to verify the results. The expected location for the amplified template is between the 400 and 500 bp marker.
Lane 1 contains a 100 base pair ladder. Lane 3 and 4 are the 425 bp fragment of PCR template used in this experiment. Lane 5 and 6 are the PCR template and copper chloride solution incubated at 50°C for a period of approximately 15 hours. Lane 7 and 8 are PCR template that have been incubated with copper chloride overnight then treated with ethylenediamine overnight at room temperature. Lane 9 and 10 contain PCR template and copper chloride added to the well simultaneously without any incubation period.

Although the copper was shown to inhibit, the possibility of inhibition occurring due to the presence of ethylenediamine was also examined. Gel electrophoresis of a short dsDNA template showed that the migration and visualization of a positive sample with equal volume of ethylenediamine introduced into the gel resulted in no inhibitory effects

(Figure 40). This gel also verified the inhibition seen in Figure 39 as once again the presence of copper resulted in no visible migration through the gel.

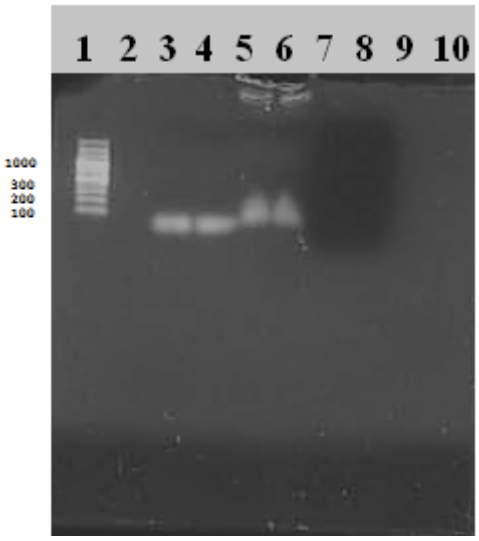


Figure 40: Agarose gel electrophoresis showing the lack of inhibition due to the presence of ethylenediamine with each result ran in duplicate to ensure accurate results. The small size of the dsDNA template used means the ladder did not get as much time to expand. However, since the band is seen below the 100 bp marker, it is the expected location for the band to elute based on its size.
Lane 1 contains a 100 base pair ladder. Lane 3 and 4 are a short dsDNA template used as a positive control. Lane 5 and 6 are the dsDNA template and ethylenediamine premised and introduced into the well. Lane 7 and 8 are dsDNA template that has been premixed with copper chloride and injected into the well. Lane 9 and 10 are the negative PCR controls.

With the copper chloride resulting in clear inhibition of any potential DNA migration, size exclusion chromatography was performed in order to discover if the excess copper could be purified out of the sample, allowing the DNA present to migrate, permitting a measure of band intensity that could allow for a quantitative measure of repair.

To determine the number of size exclusion chromatography columns that would be need to remove the copper inhibition allowing for DNA migration, 5 µL of Proteinase K extracted and ethanol purified template was combined with 5 µL of 1 M copper

chloride solution for a total of 5 aliquots. Each aliquot was then spun down with an increasing number of Bio-Rad Micro Bio-Spin 30 size exclusion chromatography columns, from 1 per aliquot up to 5 per aliquot. These extracts were immediately amplified with *Taq* DNA polymerase and ran on an agarose gel. The size exclusion chromatography resulted in unsuccessful amplification of the DNA template even after the application of 5 size exclusion chromatography columns (Figure 40).

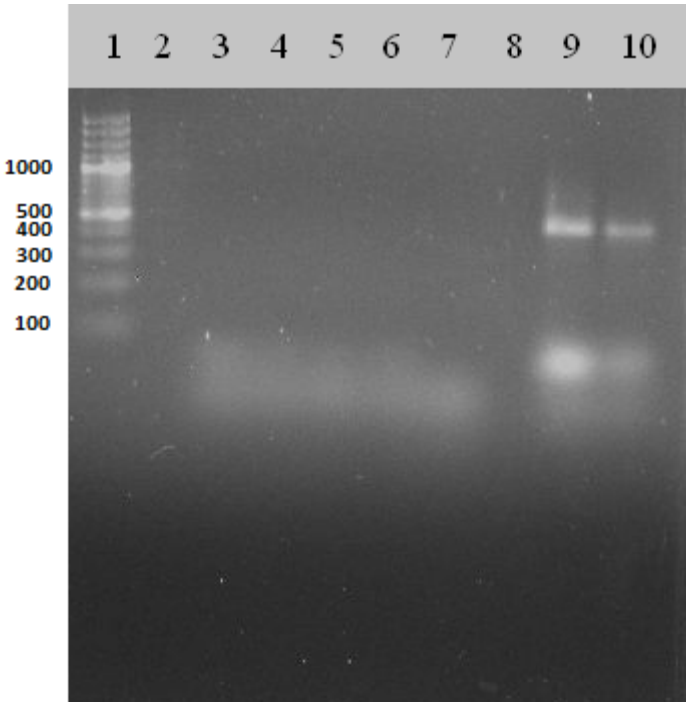


Figure 41: Size exclusion chromatography test using Bio-Rad Micro Bio-Spin 30 columns. The location of the band between the 400 and 500 bp maker in the 100bp ladder represents the expected weight of the amplified template.
DNA extract was combined with an equal volume of 1M copper chloride. Lane 1 contains a 100 bp ladder for size comparison. Lane 3 contains DNA template-copper aliquot spun through 1 P-30 column. Lane 4 contains DNA template-copper aliquot spun through 2 columns. Lane 5 contains DNA template-copper aliquot spun through 3 columns. Lane 6 contains DNA template-copper aliquot spun through 4 columns. Lane 7 contains DNA template-copper aliquot spun through 5 columns. Lane 9 and 10 are both positive controls to ensure the template was viable.

Since the copper was unable to be removed by size exclusion chromatography and still inhibited the migration of the amplified DNA through the gel, it is not worthwhile to

continue on with agarose gel electrophoresis as a viable method of measurement. Band intensities of damaged template as compared to potentially repaired template cannot be measured if the DNA is unable to migrate out of the well.

4.0 Discussion

In order to evaluate the repair of cross-link damaged DNA, ideal conditions had to be maintained throughout the study starting with the extraction and purification of the initial templates. In the case of the dNTP's and the annealed primers, extraction and purification was not necessary since these items are purchased in a purified state. In the case of the amplified 425 bp template however, extraction and purification had to be performed in lab. The optimization of the modified proteinase K extraction had already been demonstrated to be ideal for extracting DNA from epithelial cells (Laird et al. 1991). Proteinase K extractions are so common they are considered the standard for most forensic testing purposes. Although this extraction method is enzymatic and has the potential to be susceptible to metal inhibition, the samples extracted by this method were never exposed to the copper chloride solution. This exposure would occur at a later step, beyond the proteinase K activity period. Ethanol purification was chosen to further purify this extract since it was easy to acquire and perform. The low polarity of pure ethanol does not shield the negative polarity of the phosphate backbone, leading the DNA to form an ionic bond to a positive ion which is supplied by the sodium acetate. This will precipitate the DNA from solution which sometimes forms a white or off-white mass in the ethanol. The second wash of diluted ethanol is to wash away any excess salts present as well as remove the salts that have attached to the DNA. Both the extraction and purification methods used in this study were successful, as the extract was amplified via PCR with no sign of inhibition.

With the templates ready to be analyzed, an optimized method of detection by GC-MS was required in order to positively identify the success or failure of cross-link formation. Upon research of the topic it was found that a method had already been developed for detection of multiple nucleic acid damage types. Senturker and Dizdaroglu's (1999) method has been used many times by researchers to detect several different types of damage. With the isotopic fingerprint known for each derivatized nucleotide combined with a known retention time, identification can be determined with high certainty. This isotopic fingerprint must not include the 73 m/z peak seen in all the results, since this peak represents unbound silyl units left over from the derivitization. Using GC-MS for the cross-linking study also proved to be successful since components from each template studied were positively identified based on ions present and known mass to charge ratios. This optimized detection method was best demonstrated on the cross-link detection of the ancient bone sample. The sheer amount of damage done to this sample through many years of poor preservation and natural degradation is evident when performing an ion search and visualizing the multiple peaks that showed up throughout the mass spectra.

Optimization of the cross-link formation was another important method to develop. In order to eliminate as many possible variables and keep the detection as simple as possible for the baseline readings, the cross-link formation method was optimized on the purified nucleobases. With the known contents and concentrations of each nucleotide, and limited exposure to inhibiting factors, any successful cross-link formation would be easily discernable in the GC-MS data. The first step to optimization of cross-link formations was the ratio of copper chloride to each dNTP. With the dNTP's

in a concentration of 100 mM and the $CuCl_2$ solution at 1 M, a 1:2 ratio in the volumes of nucleobase to copper chloride was more than adequate to produce copper cross-links. This concentration of copper chloride was much higher than what occurs naturally, however the purpose of the experiment on the synthetic templates was to induce cross-links that are known to occur in nature. The ancient bone sample examined in this study showed these cross-links can naturally occur over time. By introducing too much copper, other types of damage could also develop in the GC-MS data such as oxidative byproducts due to the catalytic effect of the metals presence. The incubation temperature was also an important factor in cross-link development. To find the optimal temperature, each dNTP was exposed to copper chloride at a 1:2 ratio and allowed to incubate at three different temperatures: room temperature, 36°C, and 54°C. Analysis of the expected 431 peak was the clearest to identify at an incubation temperature of 54°C, with 36°C also showing some success and room temperature not producing any definitive results. The length of incubation also had to be taken into consideration. To determine the ideal incubation period, two different time periods were tried, one overnight at approximately 15 hours and the other was incubated for a full 24 hours. Upon incubating for 24 hours, it was noticed that the GC-MS baseline becomes illegible, making any detectable peaks unreliable. The illegible results observed at the 24 hour incubation indicates that this length of incubation at 50°C is too much for the template, resulting in the sample being degraded beyond recognition. Therefore, based on these results, an optimal incubation was decided to be approximately 50°C with an overnight incubation of approximately 15 hours.

Implementing a direct reversal repair of cross-linked DNA requires an ideal chelating agent, one that would remove the copper from the guanine while at the same time not inhibiting the template in other ways, making its application useless. Phenacylthiazolium bromide has been used to disrupt glucose-derived protein cross-links successfully. Unfortunately, a protein cross-link is not the same as a metal cross-link. PTB cleaves advanced glycation end products (AGEs) produced from the Maillard reaction of sugars and proteins (Cooper et al. 2000). Since the guanine-copper complex is not a Maillard reaction, PTB is not expected to be a viable form of repair for metal cross-link formation and was not attempted in this study. Ethylenediaminetetraacetic acid (EDTA) was also another repair compound considered for study. This ligand may also have DNA repair potential since it has a high affinity for metal cations. DNA inhibition caused by EDTA is also very unlikely since it is a common ingredient in many different buffers used in amplification and a main ingredient in TBE buffer, used to create and run electrophoresis gels. Similar in affinity to metals as EDTA, several other compounds were considered due to their chelating or ligand properties. Pyridine is a simple aromatic heterocyclic ring with a molecular formula of C_5H_5N. It has been used as a ligand in chemistry and has a few known chelating derivatives. 1, 10-phenanthroline is a heterocyclic compound comprised of $C_{12}H_8N_2$. It is a bidentate ligand that forms strong complexes with most metal ions. Ethylenediamine is an organic compound with a molecular formula of $C_2H_4(NH_2)_2$. This bidentate ligand is known to produce chelate complexes with copper ions. All three of these compounds have been shown to act as nitrogen adducts to copper (II) complexes, however ethylenediamine has also been shown to displace the copper from these complexes (Boyd et al. 1981). It is also the main

source of synthesis of EDTA, being the amine contributor to the compound. Although EDTA also shows strong metal binding potential, the binding often occurs through the carboxylate groups as well as the amines. There is concern with the displacement of these carboxylate groups as they could allow a change in pH in the solution, not only potentially damaging the DNA further, but inhibiting enzymes involved in post repair amplification. Since ethylenediamine contains none of the carboxylate groups and has shown its ability to form strong bonds with copper (II) ions specifically, it has the most likely chance of successfully reversing the DNA-copper cross-links. Its reactive amine ends bound with copper (II) results in a five-membered ring neutralizing the metal ion from cross-linking with the DNA.

In order to study the direct reversal of DNA-copper cross-links by ethylenediamine, a repair method had to be optimized in order to show a significant increase in repaired DNA as compared to the damaged source template. Based on the work of Boyd et al. (1981) of copper binding to humic acid, it was found that a metal-ethylenediamine ratio of 1:2 resulted in displacement of the humic acid oxygen donors of the Cu^{2+} at room temperature (Boyd et al. 1981). With the success of this method in binding copper to ethylenediamine, the ratio and incubation temperature was duplicated. The incubation was also allowed to sit at room temperature overnight to ensure maximum copper removal.

With the incubation optimized, cross-links were attempted on all 4 dNTP's: guanine, adenine, cytosine, and thymine, even though previous research indicates that intrinsic properties of adenine, cytosine, and thymine makes them less susceptible to copper (II) binding than guanine (Geierstanger et al. 1991). This became evident upon

analyzing each dNTP for cross-link formation. As stated in previous research, the copper cross-linked to guanine at the N7 location was easily identifiable (Figure 11). However, there was an apparent lack of cross-link production for the other three nucleobases. The second most likely candidate, adenine, did show peaks when searching for the adenine-copper complex (342 m/z) however the size of the ion peaks made it difficult to detect. Cross-link development, it would seem, also ends here for adenine as the intrinsic interference makes copper (II) binding in double stranded template highly unlikely (Geierstanger et al. 1991). With cytosine and thymine, the lack of cross-link formation was shown in the relative abundance of the ions in both the unaltered and copper exposed samples with practically no loss in relative abundance, the identification of any possible cross-links would not be enough to create a distinctive peak. This is expected in thymine since it is limited in potential binding sites for silylation. The cross-link potential is even lower in these three nucleobases when the template is double stranded since the Van der Waal forces of the hydrogen bonds introduce a significant amount of steric interference. Due to these forces, guanine has been found to have the highest potential for being inhibited by divalent cations when bound in the double stranded form. Guanine not only contains the most potential binding sites of the nucleobases, but also the most active (N7). Since guanine is the most likely candidate for cross-linking and undoubtedly the easiest to manipulate, it was the focus for the rest of this study. Based on previous studies, the amount of guanine with a copper (II) molecule attached at the N7 location should be within the range of 3.5% to 20% (Kagawa et al. 1991). The relative abundance of this ion (431 m/z) was seen in all samples to occur within this expected range.

Reanalysis of guanine-copper cross-linked product that had been treated with ethylenediamine resulted in the identification of derivatized guanine (Figure 13). This shows that treatment was successful in returning guanine to its previously undamaged form with enough return to be recognized by the internal software for a probability of 83.73% (Figure 14). Further demonstration of successful repair was the detection of a repair intermediary (Figure 15). This intermediary of guanine-copper-ethylenediamine is a remnant of the first step of repair, eventually leading to complete copper dissociation.

Cross-link production on the synthetic dsDNA template also showed the most success with guanine. This template varied from the dNTPs since much more interference was noted in the mass spectrum. This is most likely a direct result of the catalytic effect of the metal but could also be a result of accidental oxygen exposure during the derivitization. If oxygen was still present in the nitrogen purged tubes it would lead to increases in oxidative damage. Even with additional interference, the 431 m/z was still located at the previously identified retention time of approximately 18 minutes (Figure 19). After ethylenediamine treatment of this sample, it was observed that the peak representing the guanine-copper complex was no longer present (Figure 22). The next logical step was to search for a return of guanine after treatment which was observed at retention time 20.225 minutes (Figure 24). Although only 2 of the peaks were identified, this is to be expected with more advanced samples as production of multiple side products is a common side effect of silylation or accidental oxidative damage. With the presence of ion peaks 352 and 368 combined with the expected retention time of 20.225 minutes, there is no doubt that guanine is present where it was not before ethylenediamine exposure.

The cross-link formation for the modern dsDNA template was also identified around the 18 minute retention time. This 431 m/z ion, representing the derivatized copper-guanine compound, was found to have a relative abundance of approximately 17.9% (Figure 27).

With the 425 bp amplified product, it is interesting to note that the 431.3 ion remained present in the sample after treatment with ethylenediamine (Figure 30). This is not of concern however, as the relative abundance of this ion has been cut almost in half, from 17.9% to 10.35% (Figure 31). It was then noted that at 20.249 minutes, a peak was recovered containing all three identifying peaks of guanine (352, 353, and 367) (Figure 33). This sample also contained many extraneous peaks attributed to the same factors of damage induced during derivitization or side products being formed. A cross-link reduction combined with a return of unaltered guanine shows the ethylenediamine treatment to be successful in reversing the induced cross-link damage for amplified double stranded template.

Direct reversal is a successful form of repair in synthetic cross-link formation, but in order to make this technique practical, natural cross-link formation due to copper exposure must be shown to occur in degraded samples collected in the field. For this, a bone sample known as Bone 201 was obtained and run on the GC-MS under the same conditions as the previous templates. This sample was collected and an elemental analysis was performed previous to this experiment. This analysis positively identified the presence of copper in the bone sample, making it an ideal template to examine as the chances of cross-link formation are high. To verify the sample did contain viable DNA template, a search was performed for the nucleobases even though guanine was the main

focus of the study. An ion search resulted in a positive identification of guanine at 19.764 minutes with ions 353, 352, and 367 present. A search for adenine resulted in no identifying peak of any intensity. Cytosine was also absent, however this was expected since cytosine can be easily oxidized into uracil with a molecular weight of 256. Thymine was once again easily identified because its strong stable formation does not allow for cross-linking to occur. Since this sample is ancient and has been exposed to severe damage inducing conditions, it is not surprising that adenine and cytosine were not located (Figure 34). Cytosine was also absent in the nucleobase search conducted on the modern dsDNA template (Figure 25). Once cytosine has been oxidized to a uracil state, its molecular weight and ion formation will mimic that of thymine. This is evident by the increase in intensity of thymine as the templates get progressively more complicated, even allowing thymine to be identified by an ion library search in the ancient template with a probability of 74.69%. With the presence of guanine confirmed, an ion search was conducted for the copper-guanine complex seen in the previous templates. At a retention time of 18.151 minutes, a dominant peak can be seen with a 429.7 m/z (Figure 36). The seemingly high relative abundance of this ion (approximately 70%) is to be expected in a sample this highly degraded. The ratio of time spent exposed to copper versus amount of cross-links formed is invariably linked. A sample as highly degraded as this would be expected to show higher levels and types of damage than a synthetically damaged sample that was only incubated for a 15 hour period. To investigate this further, the prospect of a guanine-copper complex that has been deaminated was also investigated. If the complex were deaminated, the loss of an amine group as well as the BSTFA unit bound to it would result in a new molecular weight of 344. At a retention time of 19.917 minutes, a strong

peak was identified with a m/z of 344.3 (Figure 38). With the state that this sample is in relative to DNA damage, it is highly likely that this peak represents a deaminated guanine-copper complex.

Although naturally occurring DNA-copper cross-links were shown by GC-MS analysis of the ancient bone extract, ethylenediamine treatment was not applied to this template. Without a reliable purification protocol that will not inhibit the extract, it will be unable to amplify even if the cross-links were removed. The inability to use base excision repair increases the difficulty in repairing the extract since it is evident that more damage is present than simple cross-links. A reliable purification method is an absolute requirement to investigate this topic further as the cross-link removal treatment has already been shown to be successful on the previous templates.

In order to test multiple repair types on cross-linked product, 5 different DNA polymerases were also optimized in order to test their efficacy for DNA repair. These polymerases were specifically selected for being isolated from different thermostable bacterial species. The variations in enzymatic fidelity when harnessed from different sources combined with the presence of a proofreading enzymatic subunit on the DNA polymerase could have potential to repair the DNA. DNA polymerase optimization was based on the data sheet insert acquired with each DNA polymerase and was tested multiple times with positive controls to ensure the enzyme was able to amplify. This approach was not followed up on however upon the discovery that $CuCl_2$ inhibits electrophoresis, causing any potential DNA to remain in the well (Figure 39). Further gel electrophoresis work was conducted to ensure that ethylenediamine would also not inhibit gel migration. It was found that template in the presence of ethylenediamine did

not show migration inhibition (Figure 40). This result was to be expected since ethylenediamine is the backbone of Ethylenediaminetetraacetic acid, a common additive in PCR buffers, and is not known to inhibit DNA amplification. Since the presence of copper was shown to inhibit, size exclusion chromatography was attempted to purify the sample post-copper chloride exposure. This also resulted in no visible migration, even when purification was attempted five times consecutively (Figure 41). A possible explanation for this failure to purify may lie in the storage buffer contained in the columns. The P-30 columns come with two standard storage buffers, saline-sodium citrate (SSC) and Tris. The columns used in this study were those containing the SSC buffer. The main constituent of this buffer, sodium citrate, has been shown to preserve DNA (Osterrieth 1962). This preservation ability makes it an ineffective form of purification and limits the potential repair value of any enzymatic approach, including the PreCR base excision repair. Although the BER treatment may repair the cross-links in the double stranded template, the required amplification of the sample post-treatment would fail due to the presence of any remaining copper chloride in solution. Nucleotide excision repair was previously ruled out as a feasible repair strategy due to the large fragment that it requires to remove in order to complete the repair process. NER in bacteria will excise and remove a section around 12 bp in length including the specific DNA damage being targeted which can be quite bulky. However the length required for NER is not definitive and can be over 20 bp in length. With the removal of such a large section of DNA from a small fragment of DNA, the risk of losing stability in the helix is greatly increased. If the damage were to be close to the end of the fragment, removal of a large section would not be repairable since the polymerase would not have a portion of

the double stranded template to attach and replicate from. The loss of double stranded stability in a 425 bp sample would be detrimental to its structure, leading to alternative damage and most likely leaving the sample unrecognizable. Since the use of a direct chemical reversal involved no enzymatic involvement and uses the GC-MS for detection, it was the most likely of the repair methods to show success using the available methods of detection.

Although successful detection was shown with GC-MS analysis, the effect the derivitization process has on the samples can make data interpretation difficult. It is necessary to derivitize the samples in order to increase thermostability and sample volatility in order to analyze DNA by GC-MS.

The significance of this research is understandable when considering the study of degraded DNA samples. In experimental cross-linking formation, a 10-20% yield was the maximum that could be produced. When DNA is exposed to copper over a long period of time, such as in the ancient bone template, this yield was seen to rise up to 70%. Removal of these copper cross-links, especially by a method such as direct reversal, would release template that was previously considered non-viable. These could greatly increase the chance of amplification on ancient and degraded samples. The importance of harnessing this repair and perfecting its use would be a vital tool for highly damaged samples from archaeological sites, forensic cases, and archived medical specimens that have been stored under suboptimal conditions.

5.0 Conclusion

The results of this research have shown that, when DNA is exposed to divalent cations, cross-link formation can inhibit the sample making it unviable for amplification or analysis. By introducing the bidentate ligand ethylenediamine, these cross-links can be removed, allowing previously unusable template to be theoretically viable for molecular analysis.

In order to detect the repair of any sample, GC-MS was found to be an adequate form of analytical detection. The attempts to use gel electrophoresis for detection had several issues. Further optimization and experimentation with this method could prove to be useful in the future, however at this point it is not a viable option for cross-link detection.

Although success was shown with the direct reversal method, there are still several other methods that need to be tested to compare their cross-link repair ability to that of ethylenediamine. Base excision repair (BER) and nucleotide excision repair (NER) are two enzymatic repair pathways that may also prove successful once a suitable purification method is discovered. With further research into a purification method, this method could become the standard protocol for dealing with metal-DNA cross-links in archaeology, forensics, or the field of medical research.

6.0 Future Considerations

Although successful detection was shown with GC-MS analysis, the effect the derivitization process has on the samples can make data interpretation difficult. It is necessary to derivitize the samples in order to increase thermostability and sample volatility in order to analyze DNA samples by GC-MS. Since the samples are in a liquid matrix, another form of detection that may be less destructive would be liquid chromatography-mass spectrometry (LC-MS). This analytical technique involves a physical separation by high performance liquid chromatography (HPLC) with the mass analysis of a mass spectrometer. If this technique could be optimized for DNA analysis, it would eliminate the need for derivitization, lowering the formation of side product formation as a result of the high temperature and formic acid exposure.

The most pressing issue for future consideration is researching an additional purification method to restore amplification. As mentioned earlier, there are P-30 size exclusion chromatography columns that are stored in a tris based buffer as opposed to SSC. This should be investigated since tris is known to not inhibit DNA amplification or migration. Once an alternate purification method is developed, this will make it possible to attempt cross-link repair by both BER and NER methods. With the excess $CuCl_2$ no longer present to inhibit, the enzymes involved in these two repair pathways should no longer be inhibited from amplification, nor will the DNA polarity be altered causing gel migration interference.

Further study must also be done on the ancient sample to assess the extent of the damage done by the copper ions. A thorough examination of the GC-MS data would allow for the identification of the ions being created and offer a better picture as to what

types of damage are occurring and to what extent. This may also help in quantifying the cross-link damage accumulation for future repair reference leading to *in vitro* metal cross-link repair being a standard protocol in the field of DNA research.

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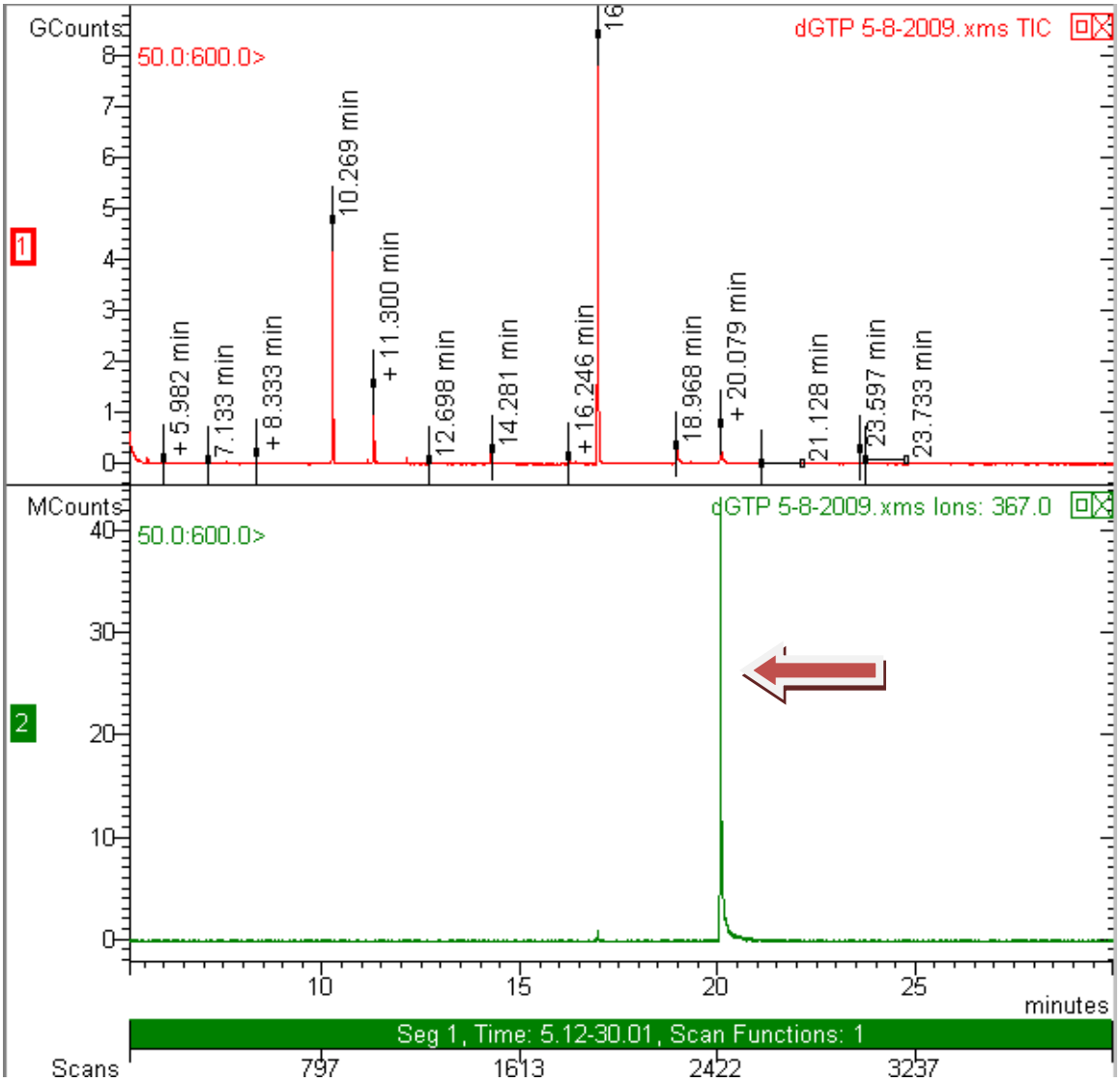
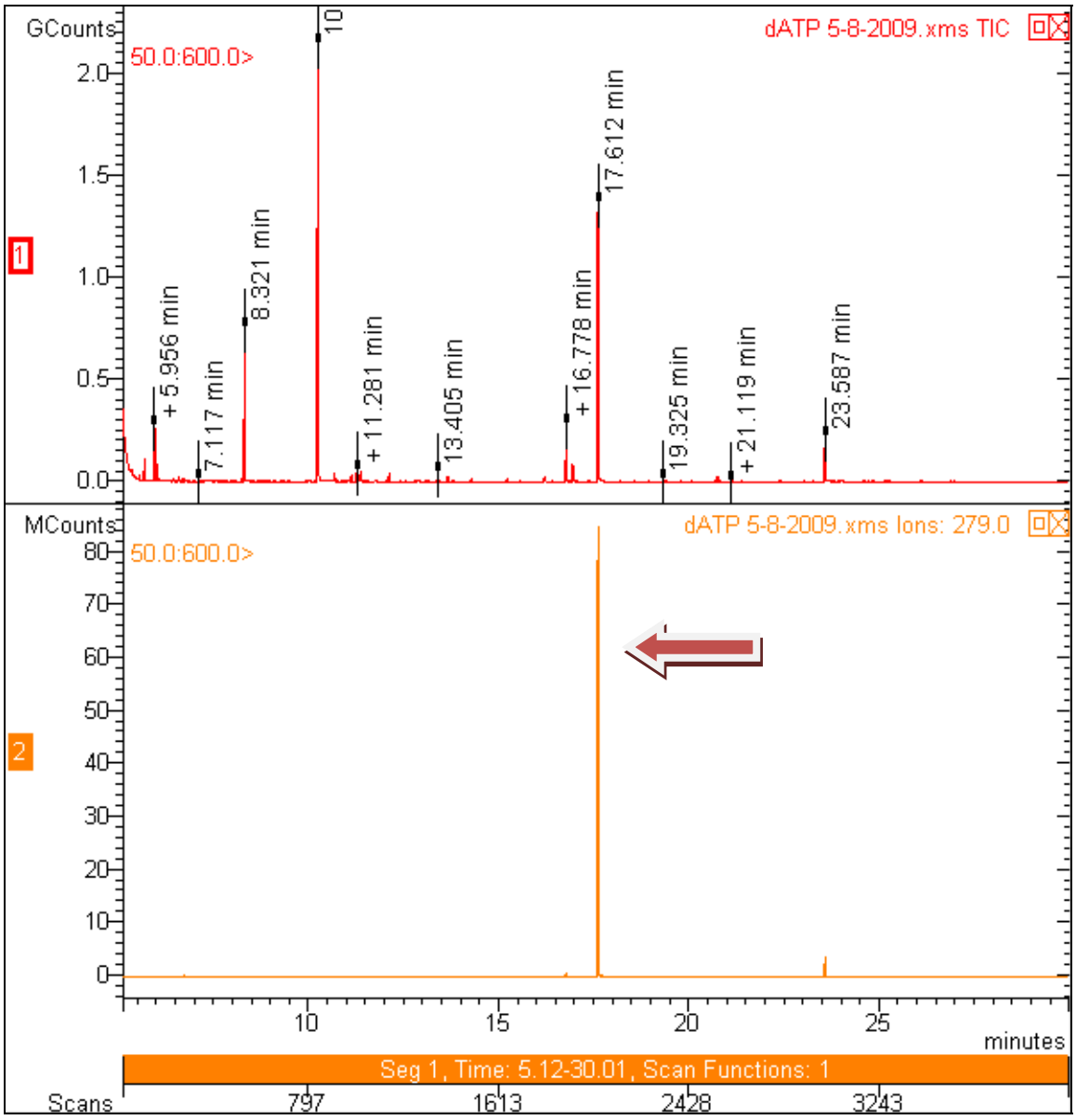
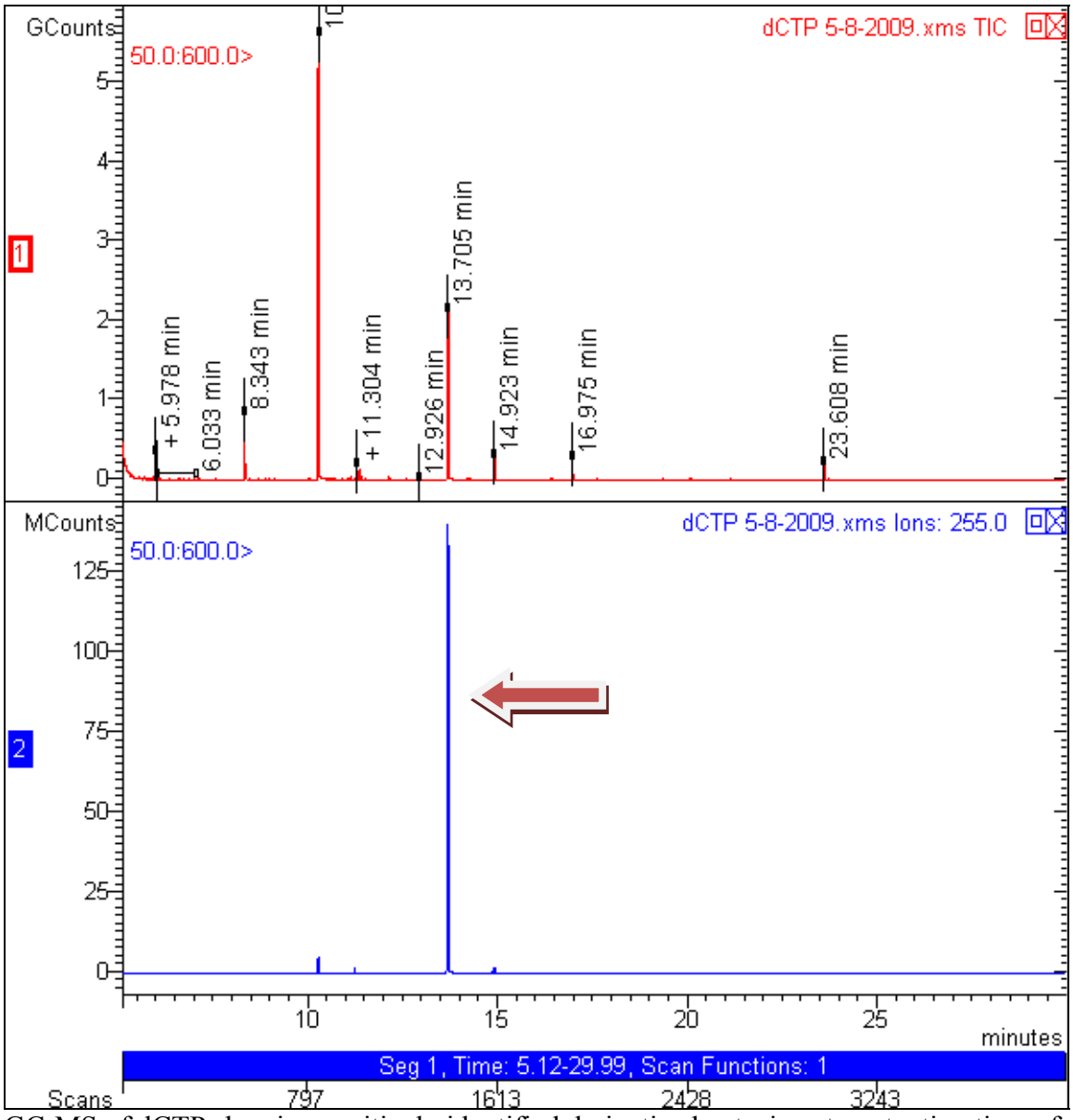
Appendix A: GC-MS Data



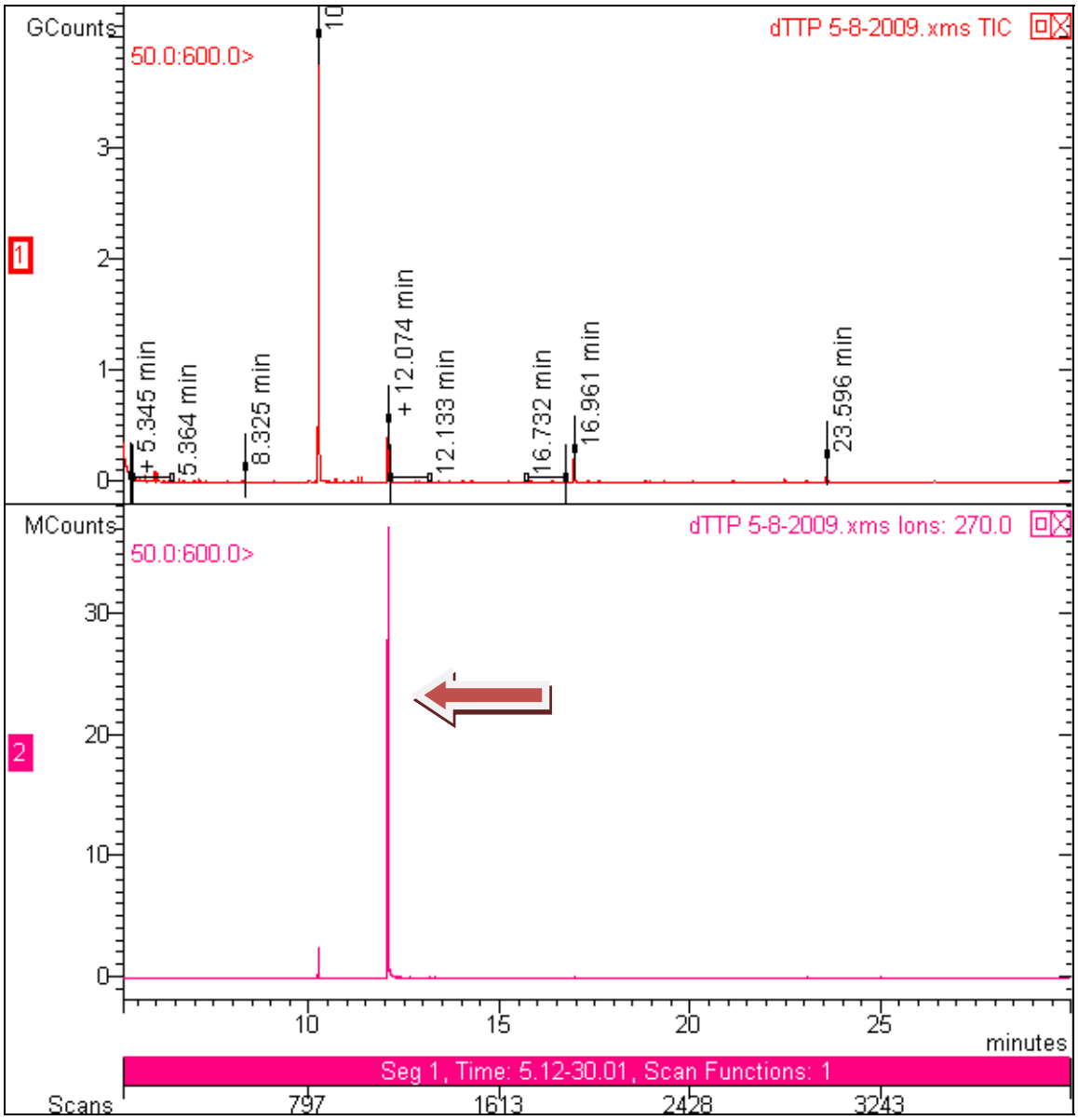
GC-MS of dGTP showing positively identified derivatized guanine at a retention time of 20.073 minutes.



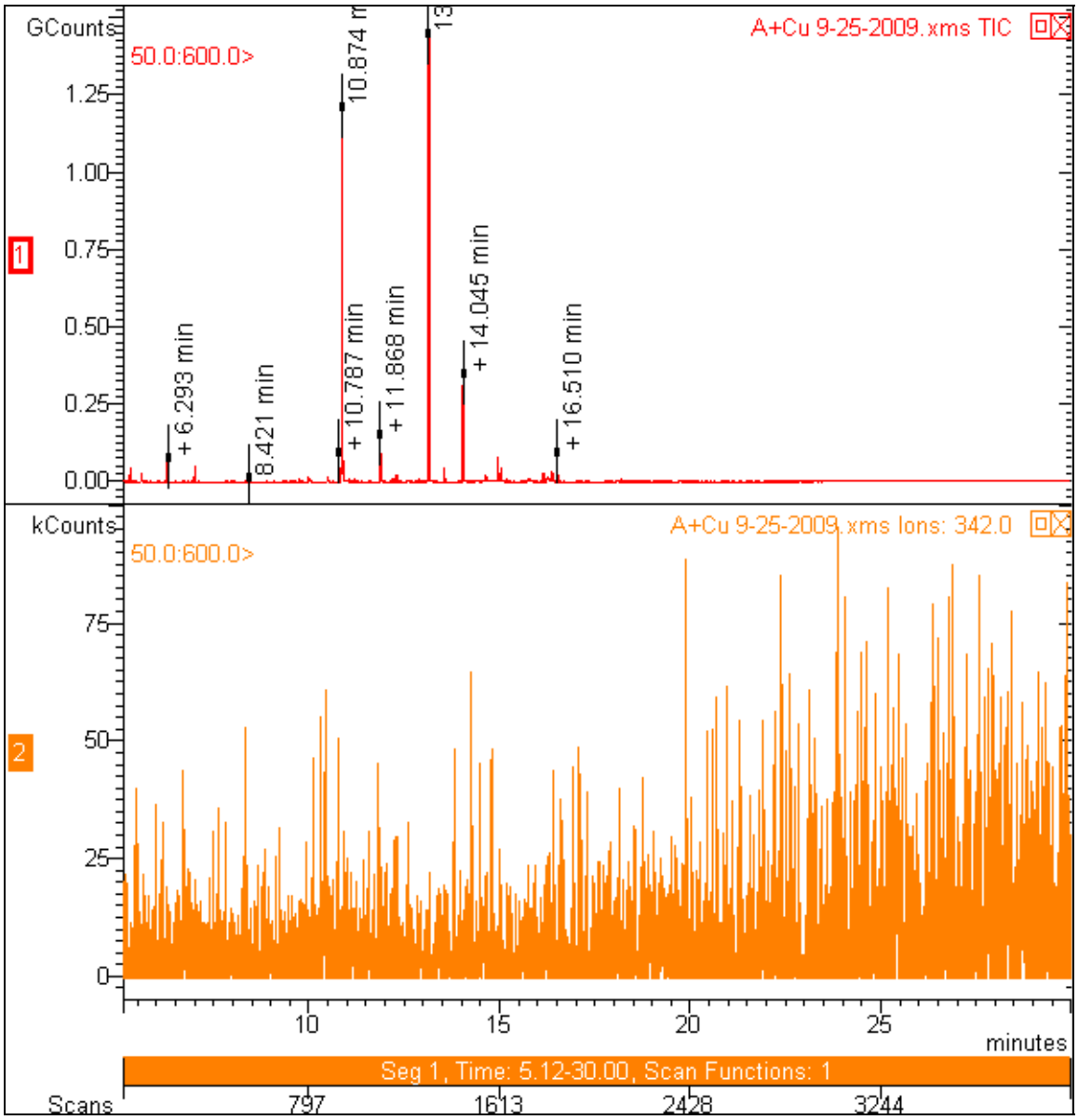
GC-MS of dATP showing positively identified derivatized adenine at a retention time of 17.619 minutes.



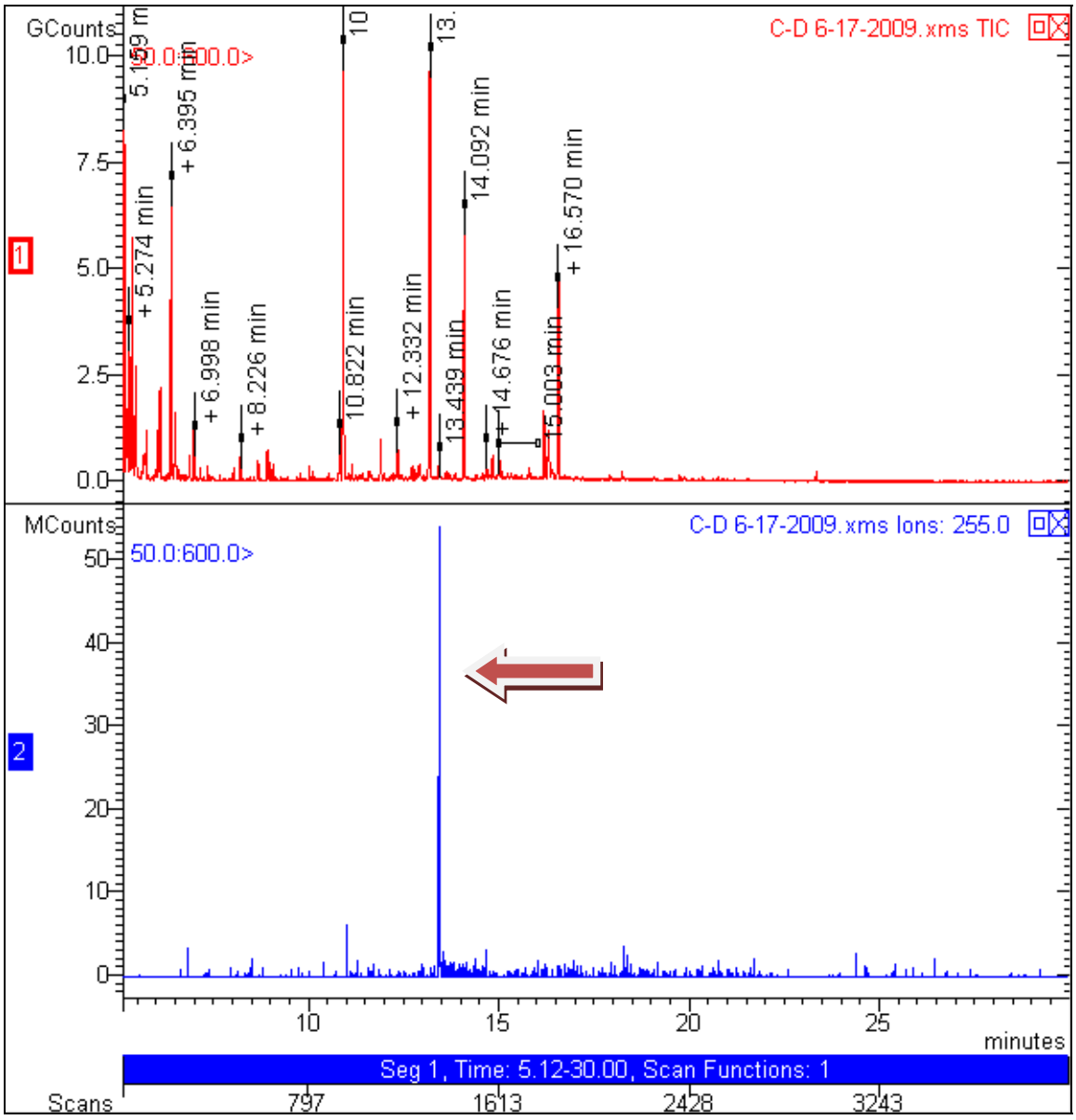
GC-MS of dCTP showing positively identified derivatized cytosine at a retention time of 13.702 minutes.



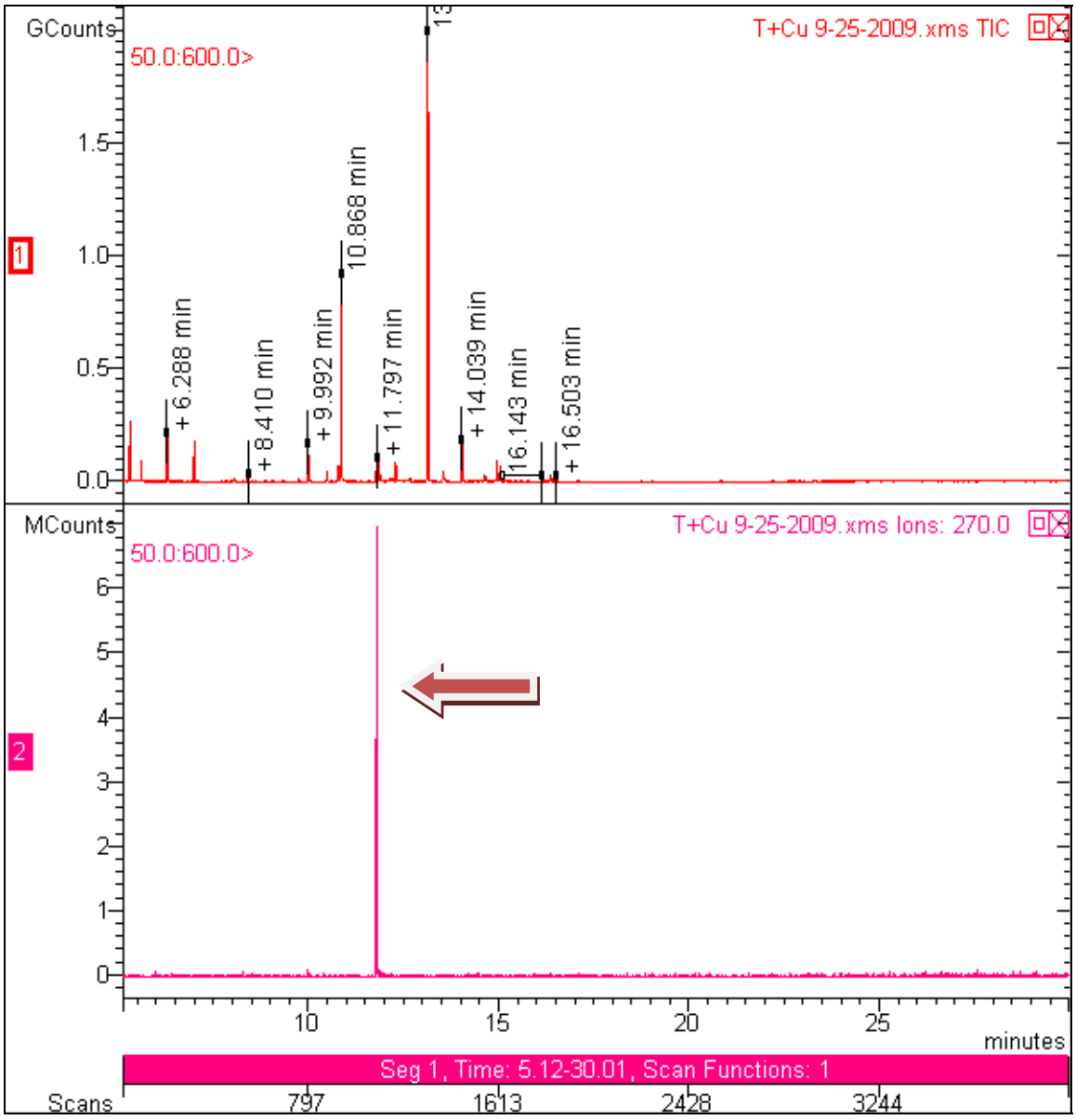
GC-MS of dTTP showing positively identified derivatized thymine at a retention time of 12.076 minutes.



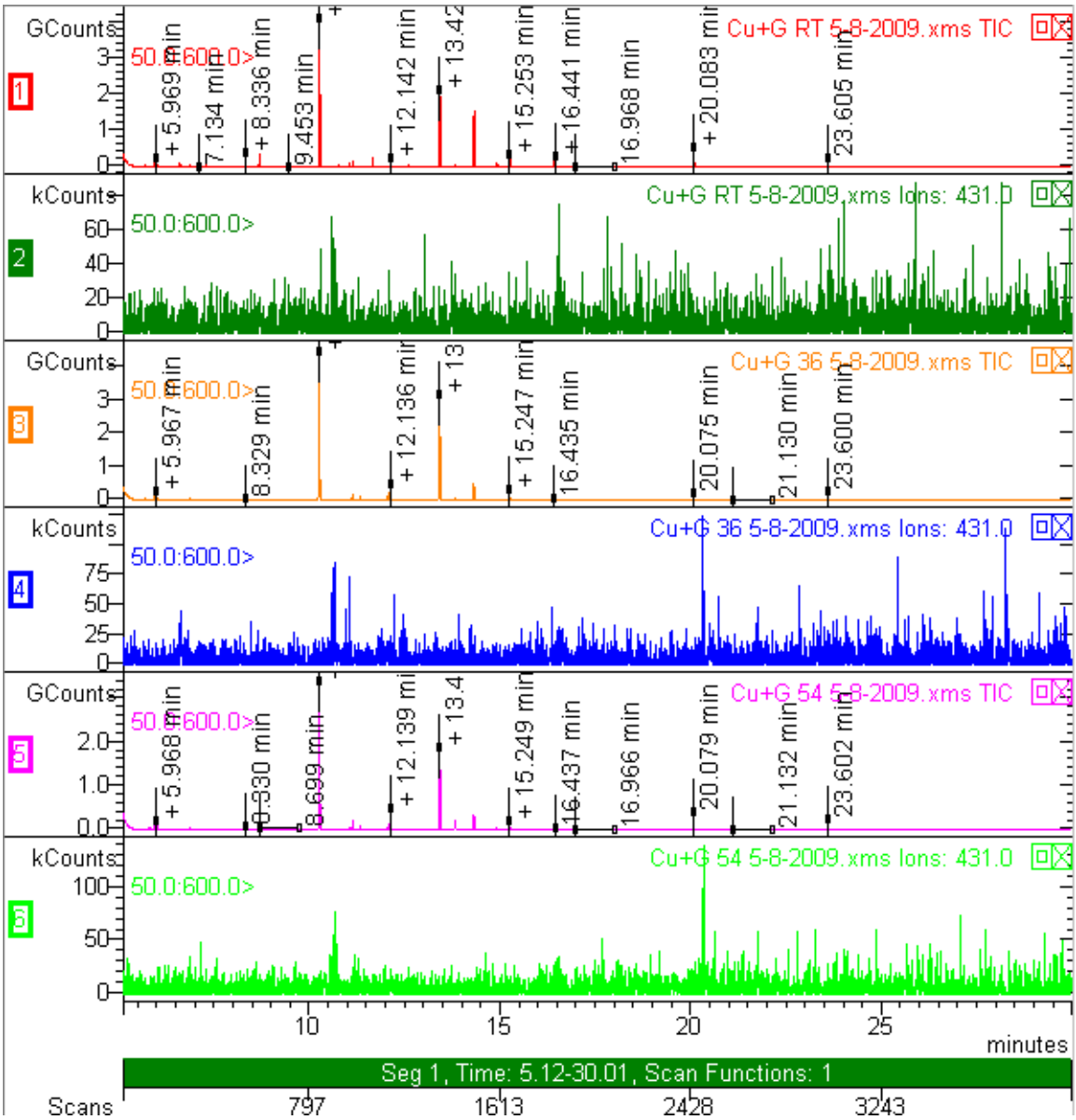
GC-MS of purified adenine exposed to copper chloride. The expected m/z of 342 (derivatized adenine-copper) did not result in an expected defined peak.



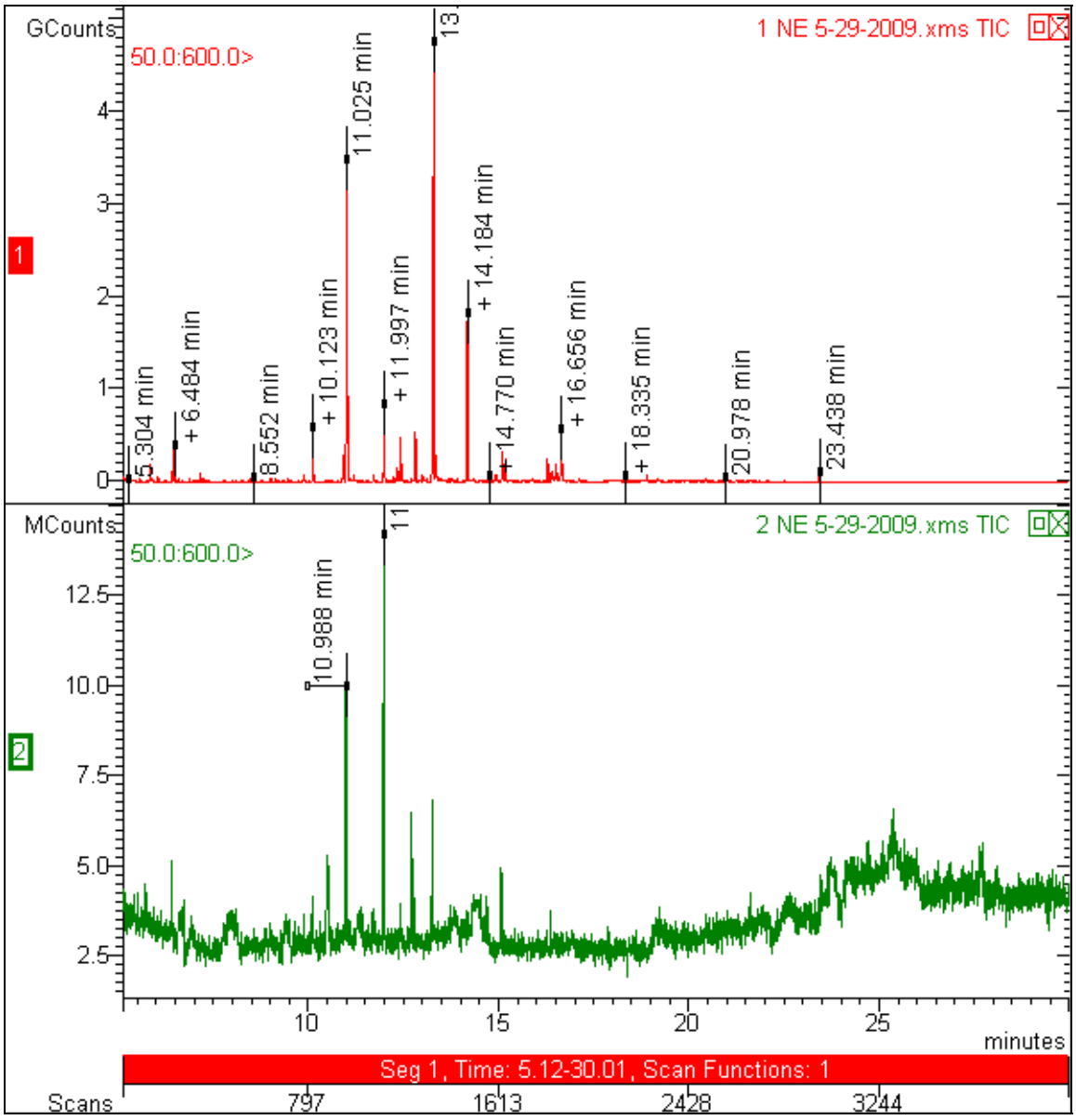
GC-MS of purified cytosine exposed to copper chloride. Any of the expected molecular weights of a copper bound cytosine resulted in no obvious peaks. Upon searching for cytosine itself, it was found that the relative abundance of the molecule still remained at 100% even after treatment with copper chloride (arrow).



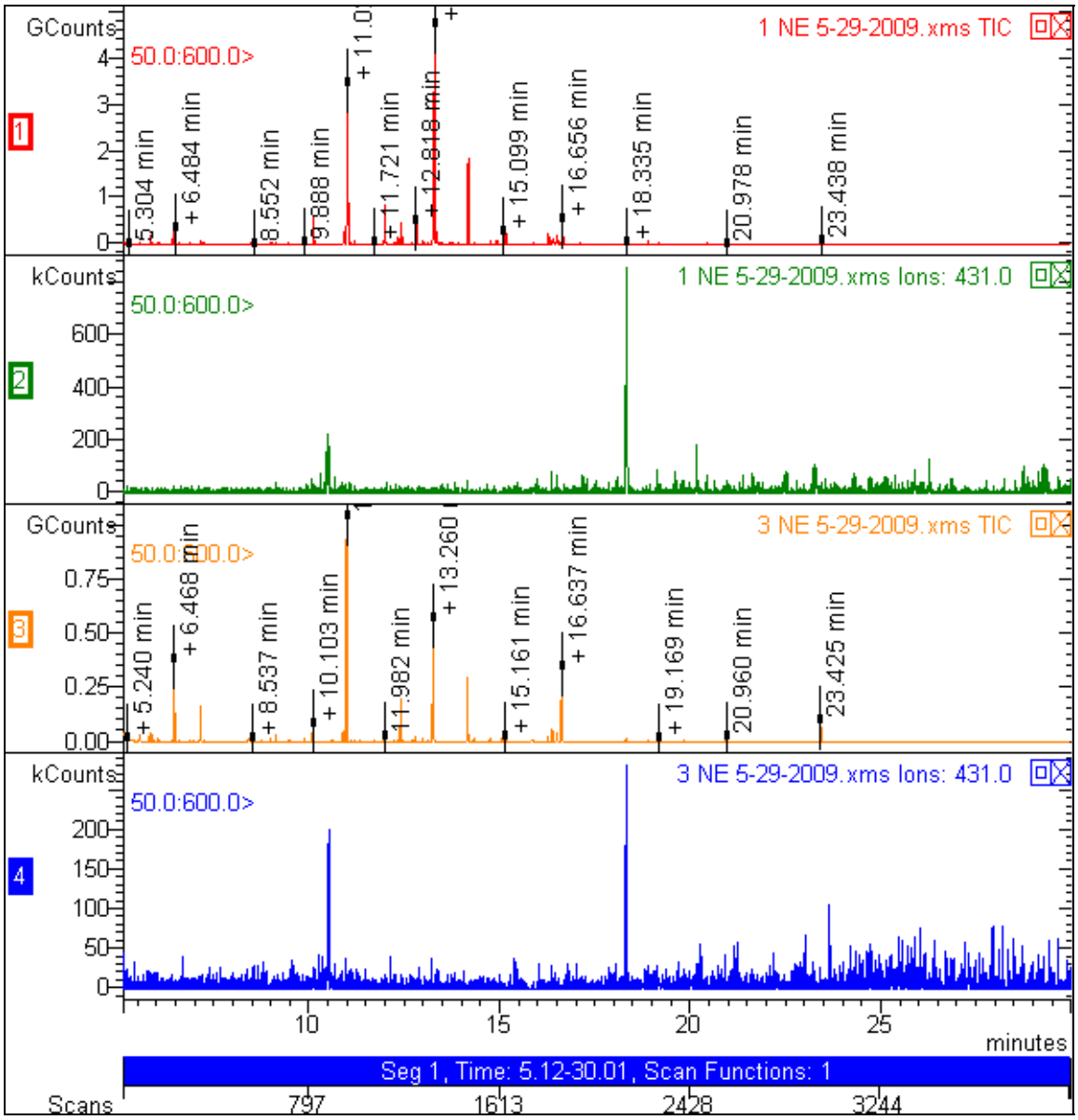
GC-MS of purified thymine exposed to copper chloride. A search of the spectrum resulted in no thymine-copper complexes found. Upon searching for unaltered thymine, it was found to have maintained its relative abundance even after being exposed to copper chloride.



GC-MS results for three different incubation temperatures: room temperature, 36°C, and 54°C. An ion search for the 431 m/z ion representing copper-guanine shows no definitive peak results at room temperature, slight peaks at 36°C and evens stronger results for 54°C. An incubation temperature of approximately 50°C was used for the incubation temperatures in this study.



Gas chromatography of samples incubated at 50°C for two time periods: 15 hours (1) and 24 hours (2). Incubation for 24 hours resulted in severe damage to the template resulting in no definitive data in the mass spectrum.



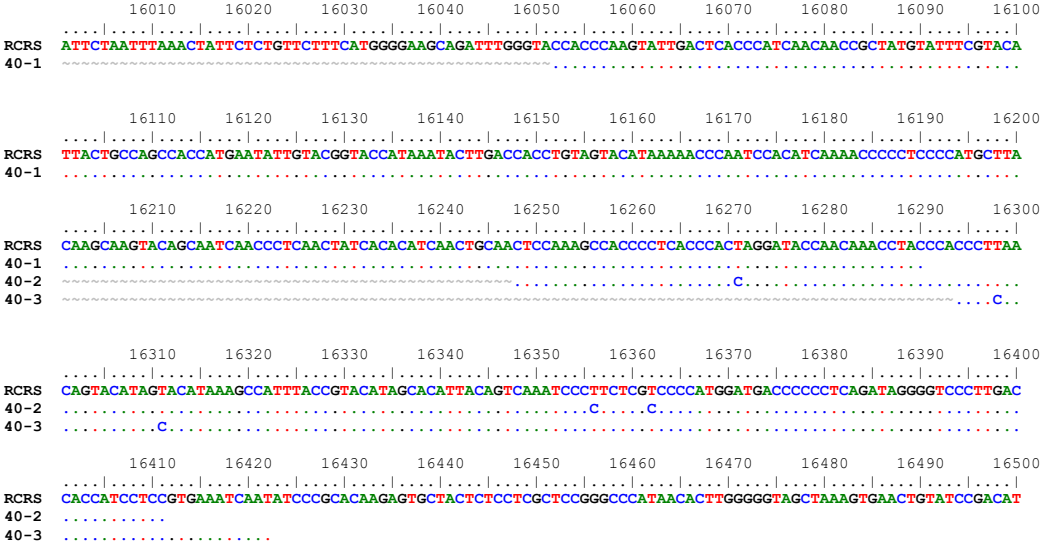
Side by side comparison of variations in copper concentration. Chromatograph 1 is a volumetric ratio of 1:1 (copper:guanine) while chromatograph 3 is a ratio of 2:1. Both concentrations produced expected 431 m/z cross-links around the 18 minute retention time, however the 1:1 ratio resulted in a cleaner mass spectrum with less interfering peaks.

Appendix B: Sequence Data

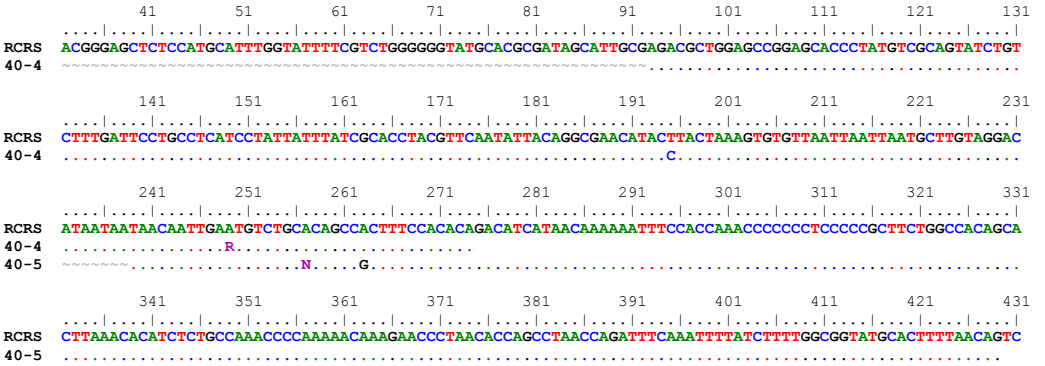
```
14701    caatgatatg aaaaaccatc gttGTATTTC AACTACAAGA ACACCAATGA CCCCAATACG
14761    CAAAACTAAC CCCCTAATAA AATTAATTAA CCACTCATTC ATCGACCTCC CCACCCCATC
14821    CAACATCTCC GCATGATGAA ACTTCGGCTC ACTCCTTGGC GCCTGCCTGA TCCTCCAAAT
14881    CACCACAGGA CTATTCCTAG CCATGCACTA CTCACCAGAC GCCTCAACCG CCTTTTCATC
14941    AATCGCCCAC ATCACTCGAG ACGTAAATTA TGGCTGAATC ATCCGCTACC TTCACGCCAA
15001    TGGCGCCTCA ATATTCTTTA TCTGCCTCTT CCTACACATC GGGCGAGGCC TATATTACGG
15061    ATCATTTCTC TACTCAGAAA CCTGAAACAT CGGCATTATC CTCCTGCTTG CAACTATAGC
15121    AACAGCCTTC ATAGGCTATG TCCTCCCGTG aggccaaata tcattctgag gggccacagt
```

Sequence of the amplified PCR product template (highlighted in green). This sequence was taken from the Revised Cambridge Reference Sequence of the Human Mitochondrial DNA.

HV1



HV2



BioEdit multiple sequence alignment for aDNA samples analyzed in this study (Bone 201).