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Discovery and Characterisation of Antimicrobial Peptides Using Genome-Wide *In-Silico* Screening and Peptidomics

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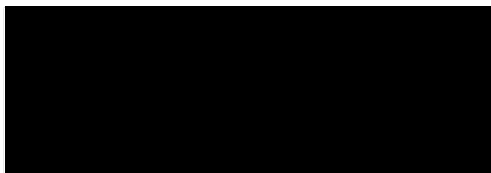
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Thesis submitted for the Degree of Doctor of Philosophy

2022

Declaration

I, Nathan Simpson, declare all work presented in this thesis as my own unless clearly stated otherwise.



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Abbreviations

Acronyms

AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ASL	Airway surface liquid
CF	Cystic fibrosis
CFU	Colony forming units
dH ₂ O	Demineralised H ₂ O
DIC	Diisopropylcarbomide
DMEM	Dulbecco-s modified eagle medium
DMF	Dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
Fmoc	Fluorenylmethyloxycarbonyl
HEK	Human embryonic kidney
HOBt	Hydroxybenzotriazole
HPLC	High-performance liquid chromatography
MDR	Multidrug resistant or multidrug resistance
MH	Mueller-Hinton
MHb	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
OD ₆₀₀	Optical density measured using light of wavelength 600 nm
PBS	Phosphate-buffered saline
QPI	Quantitative phase imaging
RBC	Red blood cells
relIC ₇₅	Relative IC ₇₅
SERCA	Sarco/endoplasmic reticulum Ca ²⁺ -ATPase
SPPS	Solid phase peptide synthesis
TFA	Trifluoroacetic acid
UNC	University of North Carolina
VRE	Vancomycin-resistant <i>enterococci</i> or Vancomycin-resistant <i>Enterococcus faecium</i>
WHO	World Health Organisation

Organism Names

<i>A. baumannii</i>	<i>Acinetobacter baumannii</i>
<i>B. anthracis</i>	<i>Bacillus anthracis</i>
<i>B. pseudomallei</i>	<i>Burkholderia pseudomallei</i>
<i>C. lupus familiaris</i>	<i>Canis lupus familiaris</i>
<i>C. lupus lupus</i>	<i>Canis lupus lupus</i>
<i>C. porosus</i>	<i>Crocodylus porosus</i>
<i>D. plexippus plexippus</i>	<i>Danaus plexippus plexippus</i>
<i>E. cloacae</i>	<i>Enterobacter cloacae</i>
<i>E. coli</i>	<i>Eschericia coli</i>
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
<i>E. faecium</i>	<i>Enterococcus faecium</i>
<i>F. peregrinus</i>	<i>Falco peregrinus</i>
<i>G. gallus</i>	<i>Gallus gallus</i>
<i>G. lucidum</i>	<i>Ganoderma lucidum</i>
<i>G. mellonella</i>	<i>Galleria mellonella</i>
<i>G. sulphuraria</i>	<i>Galdieria sulphuraria</i>
<i>H. anuus</i>	<i>Helianthus anuus</i>
<i>K. pneumoniae</i>	<i>Klebsiella pneumoniae</i>
<i>M. Martensii</i>	<i>Mesobuthus martensii</i>
<i>M. sedula</i>	<i>Metallosphaera sedula</i>
<i>O. hannah</i>	<i>Ophiophagus hannah</i>
<i>O. orca</i>	<i>Orcinus orca</i>
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
<i>P. tigris altaica</i>	<i>Panthera tigris altaica</i>
<i>R. typus</i>	<i>Rhincodon typus</i>
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>T. rubripes</i>	<i>Takifugu rubripes</i>
<i>T. saltator</i>	<i>Talitrus saltator</i>

Units of Measurement

µg/mL	Micrograms per millilitre
°C	Degrees Celsius
µL	Microlitre
µmol/L	Micromoles per litre
AU	Arbitrary units
CFU/mL	Colony forming units per millilitre
cm	Centimetre
cm ²	Square centimetres
g	Grams
g/mol	Grams per mole
kb	Kilobase
mol/L	Moles per litre
mL	Millilitre
mL/min	Millilitres per minute
mmol/L	Millimoles per litre
nm	Nanometres
RCF	Relative centrifugal force
RFU	Relative fluorescent units
RLU	Relative luminescent units
rpm	Revolutions per minute
USD	United states dollars
v/v	Volume percent

Abstract

Antimicrobial resistance is widely recognised as one of the greatest threats to global healthcare. Its increasing prevalence necessitates the development of novel therapeutics with mechanisms of action that are still effective against multidrug resistant pathogens. This is particularly relevant in the context of the ESKAPE pathogens, a group of pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and the *Enterobacter* species) known to cause virulent and multidrug resistant hospital-acquired infection across the globe.

Antimicrobial peptides (AMPs) are attractive candidates for therapeutic development due to their exhibited activity against an array of pathogens with differing profiles of resistance. This project explored the viability of two sources of AMPs. Peptides were isolated from human lung airway surface liquid (ASL) samples or identified within 1 of 20 organism genomes using a metagenomic screening and prediction software. The software identified over 4000 sequences with predicted activity, 2000 of which were selected and synthesised on cellulose membranes. These were then screened against *E. coli* in 10% human serum solution, with candidates showing high antimicrobial activity being selected for synthesis on resin and subsequent purification.

Purified candidates were then tested for activity against the ESKAPE pathogens in 0, 10, and 25% serum solutions. In addition to serum tolerance, haemolytic and cytotoxic capacities of the candidates were investigated, and their mechanisms of action were characterised following generation of dose-response and time-kill curves. Lastly, selected candidates were assessed for *in vivo* activity using a *G. mellonella* infection model.

Peptides isolated from the lung peptidome were also investigated for antimicrobial activity; these peptides were then screened further for inhibition or stimulation of cell growth and calcium signalling. Candidates were then further characterised through investigations into the dose-response relationships of the observed effects.

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1. Introduction

1.1 Antimicrobial Resistance

Impact of Antimicrobial Resistance

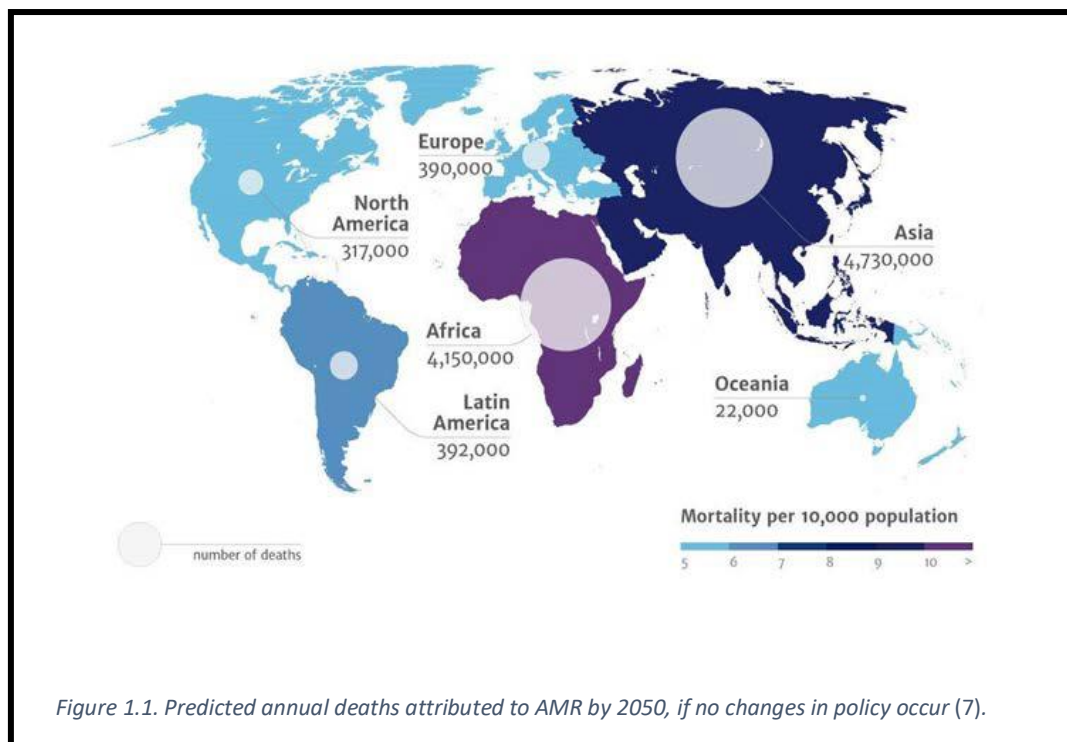
The discovery of antibiotics stands as one of the most significant events in the history of medicine, with the development of arsphenamine – regarded as the first modern chemotherapeutic agent – paving the way for the ‘golden age’ of antibiotics half a century later (1). This age saw the discovery of numerous antimicrobial agents whose efficacy and selectivity lead to their use across the globe; today, antimicrobials have abundant applications as both therapeutic and prophylactic agents. However, their widespread use has given rise to an increasing ability of a microorganism to survive exposure to a previously effective antimicrobial agent; a phenomenon referred to as antimicrobial resistance (AMR) (2).

While the first antimicrobial agents were put to use in the early 20th century (3), resistance was reported as early as a decade later (4). The increasing prevalence of antimicrobial resistance since then is one that is well documented, as are the primary facets of its impact (5,6). The oft-cited O’Neill report – a compilation of research conducted by both the Research AND Development (RAND) Europe Corporation and Klynveld Peat Marwick Goerdeler (KPMG) – elucidated both the current effects of antimicrobial resistance worldwide, as well as the potential turmoil that has yet to come (7). To elaborate, because strains resistant to conventional methods of treatment are becoming more common, it has become more necessary to employ ‘last line’ antimicrobials: therapeutic agents whose use is reserved due to the severity of their side effects, or because there would be no further means of treatment if resistance to them arose (8). Thus, AMR has both a direct and indirect influence on the rise of infection-related mortality, with an estimated 1.3 million deaths associated with bacterial resistance in 2019 alone (9).

If an infection is not responsive to treatment – as is often the case with resistant strains – it is inevitably coupled with increased length of treatment, which escalates cost of treatment in turn. This can also coincide with lost days of labour, depending on the nature and severity of the infection. A 2018 study investigating the clinical burden of AMR in Europe reported that the disability-adjusted life year impact of resistance-associated infection that year was comparable to influenza, tuberculosis, and HIV combined (10). To clarify, this study focused solely on eight prominent bacterial species within the region, suggesting that the total burden of AMR is considerably greater. As a result, the impact of AMR is also economical, such that resistance-related infections are believed to cost 20 billion USD annually in the US alone (11).

Arguably more concerning is the notion that this burden will continue to grow if left unconstrained. While the path of AMR progression cannot be definitively stated, it is a trend

that has shown few signs of slowing down (12,13). To corroborate, a 2016 update to the J. O'Neill report states that if we extrapolate the progression of resistance of six prevalent pathogens, the rise of AMR globally is predicted to bring about an estimated 10 million deaths annually by the year 2050 – coupled with a cumulative cost of 100 trillion USD globally to economic output - if no policy changes are made (**Figure 1.1**) (11).



It should be noted here that the depletion of effective antimicrobials has implications beyond the therapeutic context; a number of treatments rely on their prophylactic use to minimise inherent risks. For example, the risk of infection during invasive surgeries is mitigated through prophylaxis. Similarly, as organ transplantation and cancer chemotherapy compromise the immune system of the recipient, prophylactic antimicrobials are used to prevent infection. This highlights another considerable facet of the AMR burden, and one whose influence on the most fundamental levels of healthcare is difficult to quantify (6,14).

The rapid progression of AMR has also given rise to the concerning trend of multidrug resistance (MDR) (15). An organism is deemed multidrug resistant if it is insensitive to three or more classes of antibiotics (16), which in turn inevitably escalates the morbidity, mortality, and cost of infection. This phenomenon has led to the emergence of ‘superbugs’ such as extremely drug-resistant tuberculosis (XDR-TB), or the various ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) that are the leading cause of hospital-acquired infections worldwide (17). MDR strains are most commonly associated with

healthcare settings, with transmission of MDR pathogens being most frequently reported in acute care facilities (18,19). This is of particular concern due to the high concentration of immunocompromised patients in these areas.

It is not without reason that the spread of AMR is regarded as one of the most pressing global issues of our time (20). In light of the prevalence of AMR and MDR, the urgent need for therapeutic agents with novel methods of action is apparent.

Causative Factors of Antimicrobial Resistance

The rapid propagation of AMR is the result of a collection of factors (3). Firstly, the availability of antimicrobial agents has grown significantly since their initial discovery, but has not been coupled with sufficient regulation in certain regions of the globe (21–24). There are many countries where patients are able to request antimicrobials from their doctors or buy them over the counter, without understanding the implications of their unnecessary use (25); studies have shown that misconceptions regarding the development and consequence of AMR are common, with many within the general public not being aware of what AMR is (26–28). In addition to this, there is much contention regarding the appropriate length of antimicrobial treatment, and whether poor patient adherence to that length is contributing to resistance (29,30). While it has long been stated that interruption of a prescribed antimicrobial course propagates resistance, consensus is currently shifting to the notion that - in certain cases – a course shorter than what is prescribed can be just as effective or more beneficial due to the reduced selection pressure (31–35). It can therefore be argued that current practices contribute to resistance and resistance-related mortality through increased length of treatment (and therefore increased selection pressure) and increased exposure to side effects.

Consideration must be given to the contributions of the agricultural sector. Although many animals do require therapeutic intervention, it is often the case that antimicrobial agents are used prophylactically or, in some instances, excessively in order to promote growth (36). It is reported that a number of drugs considered ‘last-line’ for humans are being used much more liberally for animals. This practice, coupled with the large number of animals that are kept in close proximity or non-hygienic conditions, provides the perfect conditions for drug-resistant strains to propagate.

Lastly, an arguably ignored means of AMR development is the release of antimicrobials into the environment through waste: animal, human, and industrial. It is reported that up to 90 percent of tested antibiotics were excreted unmetabolized after being fed to animal subjects, allowing biologically active ingredients to enter sewage systems with impunity (37). From there, these ingredients act as contaminants for soil and water, fostering resistance amongst the pathogens that they come into contact with. This is particularly relevant in the context of pharmaceutical

manufacturing; a considerable portion of antimicrobial production occurs in India or China, where disposal of biologically active ingredients is relatively unmonitored (38–40).

The Spread of AMR

Within a microbial population, there may be variants of an organism with an intrinsic resistance to certain mechanisms of antimicrobial action (41). This resistance can be caused by an innate feature of the organism or can occur due to spontaneous genetic mutations; exposing this population to antibiotics using those resisted mechanisms will halt the spread of susceptible strains, leaving the resistant variants to grow and spread in the absence of competition. Once established, mobile genetic elements carrying these resistance-conferring features can be transferred to previously susceptible organisms, allowing them to acquire the resistance-conferring genotype (42). It should be noted that the progression of this ‘conventional’ resistance is a natural process that began long before the first antimicrobials were applied (3); it is therefore possible for resistance-conferring genes to pre-date the affected antibiotics by any number of years.

Typically, resistant strains will make up the minority of a microbial population, as many resistance mechanisms are associated with a loss of fitness (43). The prevalence of many resistance mechanisms is therefore heavily dependent on selection pressures, a fact that becomes particularly relevant within the context of antimicrobial agents and their widespread use. The increased selection pressure applied by the heavy global use – and arguable misuse – of antimicrobials has escalated the development and spread of resistance by a considerable degree. Additionally, there are instances of resistant species able to persist and propagate even in the absence of antimicrobial selection pressure (44,45).

The exact mechanisms of resistance are numerous, with the organisms from which many antimicrobials were isolated employing means of protection from their own effects (46). It is often the case that these organisms possess multiple mechanisms for the sake of self-defence, with the genetic determinants of these resistance mechanisms typically clustered together under the control of a biosynthesis regulator to facilitate co-expression (47).

However these mechanisms arise, it is clear that their increasing spread represents a threat to public health and infrastructure on a global scale. The effort to combat this phenomenon is one that has persisted for almost a century, requiring constant adaptation to the agents that propagate it.

Our continual reliance on established antimicrobial mechanisms is due to a lack of available agents with novel mechanisms; this in turn is the result of a lack of incentive for novel pharmaceutical development (48). A considerable majority of the antibiotics of natural origin in use today were derived from the *Actinobacteria* phylum, and of those antibiotics, approximately

80% are produced by single genus (49,50) (**Figure 1.2**). While this implies a vast breadth of antimicrobial sources beyond what has already been discovered, progress into these new areas is slow (48). An unfortunate truth of antimicrobial research and development is that it is not cost effective; the process from drug discovery to clinical trials to review and approval is lengthy and expensive, meaning that a single product can take a decade or more to develop and an additional decade to turn a profit (51). So long as there is a misalignment between the health of the public and commercial incentive, innovation of short-course antimicrobial treatment will continue to stagnate despite its evident necessity.



1.2 The ESKAPE Pathogens & Clinically Relevant Bacteria

The acronym ESKAPE refers to the names of six highly resistant pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and the *Enterobacter* species. This group of pathogens is singular in its contribution to global infection, as it is the leading cause of hospital-acquired infection across the world (52). As is possible with the acquisition of resistance, ESKAPE pathogen infection is associated with greater burden of disease due to increased cost, mortality, and treatment failure; it is therefore unsurprising that this group was designated a global priority by the WHO in 2017 (53).

The organisms collectively showcase a variety of different resistance mechanisms, which can broadly be separated into one of four modes of action: modification of the antimicrobial target site, modification of the antimicrobial agent, reduced accumulation of the antimicrobial agent, and formation of bacterial biofilms (54). Through these methods the ESKAPE pathogens have exhibited resistance to a number of currently used classes of antibiotics including β -lactams and β -lactam- β -lactamase inhibitor combinations, the relatively novel oxazolidinone antibiotics, and several last line antibiotics such as polymyxins and glycopeptides (55–57). Conversely, resistance to the action of lipoglycopeptides has only been recently documented in the ESKAPE pathogens; this may be attributed to the two-fold action of this antibiotic group, as they both inhibit peptidoglycan synthesis and destabilise the membrane of bacterial cells (58). Should this be the case, this occurrence would showcase both the efficacy of multiple mechanisms of action and the peril of an expanding spectrum of resistance within the ESKAPE group.

***Enterococcus faecium* and Vancomycin-resistant enterococci (VRE)**

Within the *enterococcus* genus, *E. faecium* is one of the main contributors to human infection, with the other major causative agent being *E. faecalis* (59). *E. faecium* is a gram-positive commensal organism, found in the gastrointestinal (GI) tract of humans and animals (60). Despite its innocuous colonisation of the GI tract, clinical relevance of the bacterium has increased considerably in tandem with its rising levels of resistance., as this has facilitated greater occurrence of opportunistic infection in hospitalised patients. *E. faecium* infections can cause an array of symptoms dependent on the site of infection; this includes urinary tract infections, meningitis arising from bacteraemia, and wound infections arising from surgery (61).

The establishment of *E. faecium* as a nosocomial pathogen – a pathogen responsible for hospital-acquired infection – is largely attributed to the acquisition of resistance to glycopeptide antibiotics such as vancomycin, as glycopeptide antibiotics are considered the ‘last resort’ of treatment for *enterococcus* infection (62). These vancomycin-resistant *enterococci* (VRE) variants have acquired mobile genetic elements containing *van* operons. Regulatory genes carried within these operons control the expression of resistance-conferring ligase genes, with

vanA and *vanB* being the most common contributors to human infection (63). Current understanding of the *van* genes is considerable, with much information having been gathered regarding their carriers, locations, and expression mechanisms. However, the set of known *van* gene clusters is expanding, with three additional operons (*vanL*, *vanM*, and *vanN*) discovered relatively recently (64). The primary mechanism of glycopeptide resistance – including resistance to vancomycin - involves the alteration of the peptidoglycan synthesis pathway; by binding to the D-alanine-D-alanine dipeptide terminus of peptidoglycan precursors, glycopeptides prevent bacterial cell wall synthesis from taking place (65). *Van* operons allow bacteria to overcome this mechanism, through the substitution of D-Alanine-D-Alanine (D-A-D-A) to either D-Alanine-D-Lactate (D-A-D-Lac) or D-Alanine-D-Serine (D-A-D-S).

It should be noted that the specific profile of antimicrobial resistance will differ depending on the operons possessed by any given strain (**Table 1.1**). While this does highlight that certain VRE variants – including those possessing solely *vanB*, a significant contributor to human infection – still retain susceptibility to conventional therapeutics, it also draws attention to the fact that the number of these effective agents is dwindling, with this ESKAPE pathogen presenting as a clear and present clinical threat all the while.

Table 1. 1. Summary of phenotypic and genotypic characteristics of known *van* operons (307)₂

<i>Van-operon</i>	<i>Common carrier spp.</i>	<i>Degree level of resistance vancomycin vs. teicoplanin</i>
<i>vanA</i>	<i>E. faecium</i> , <i>E. faecalis</i>	High for both
<i>vanB</i> ; <i>vanB1</i> , <i>B2</i> , <i>B3</i>	<i>E. faecalis</i> , <i>E. faecium</i>	High-variable to vancomycin Susceptible to teicoplanin
<i>vanC</i> ; <i>vanC1</i> , <i>C2</i> , <i>C3</i> , <i>C4</i>	<i>E. gallinarum</i> , <i>E. casseliflavus</i> , <i>E. flavescens</i>	Low to vancomycin Susceptible to teicoplanin
<i>vanD</i> ; <i>vanD1</i> , <i>D2</i> , <i>D3</i> , <i>D4</i> , <i>D5</i>	<i>E. faecium</i>	Low to high for both
<i>vanE</i>	<i>E. faecalis</i>	Low-moderate to vancomycin Susceptible to teicoplanin
<i>vanG</i> ; <i>vanG1</i> , <i>G2</i>	<i>E. faecalis</i>	Low to vancomycin Susceptible to teicoplanin
<i>vanL</i>	<i>E. faecalis</i>	Low to vancomycin Susceptible to teicoplanin
<i>vanM</i>	<i>E. faecium</i>	High for both
<i>vanN</i>	<i>E. faecium</i>	Low to vancomycin Susceptible to teicoplanin

***Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA)**

S. aureus is a gram-positive, round bacterium that is commonly found on the skin and upper respiratory tract of humans. Like *E. faecium*, *S. aureus* is a commensal organism and can colonise human hosts asymptomatically, with approximately 30% of the human population carrying the bacteria nasally (66). Infection can be spread through inhalation of droplets or direct contact with an infected person or contaminated object, leading to opportunistic infections that give rise to skin infections, respiratory infections, and food poisoning (67).

Methicillin resistance was first documented in *S. aureus* in 1961; 32 years after the published discovery of penicillin, and only 2 years after methicillin was introduced to treat penicillin-resistant infection (68). However, the burden of MRSA today can vary considerably when considered globally, with substantial presence in the Asia-Pacific region, and a declining one in Europe (69,70). Regardless, it should be noted that even in instances where MRSA infection is relatively rare, incidence of multidrug resistance when infections do occur is very common (71). To complicate matters further, consideration must be given to both healthcare-associated and community-associated MRSA strains, which differ in pathology, distribution, and susceptibility to antibiotics (72).

Methicillin shares the mechanism of other β -lactams: inhibition of bacterial cell wall synthesis by binding to the penicillin binding proteins (PBPs) responsible for peptidoglycan cross-linking (73). The methicillin resistance observed in *S. aureus* is attributed to the SCCmec gene cassette, a mobile genetic element that can be acquired via horizontal gene transfer. This cassette houses the *mecA* gene, which produces a PBP analogue (PBP2a) with a significantly reduced binding affinity for β -lactams (74). In this way, MRSA is granted a broad-spectrum resistance to β -lactams including penicillins, cephalosporins, and carbapenems.

Persistent MRSA infections are treated most commonly with vancomycin and similar glycopeptides (72); however, greater tolerance of vancomycin has been observed in a number of *S. aureus* strains since the 1990s (75). While this is often due to what is known as vancomycin intermediate-resistant *S. aureus* (VISA) – where the minimum inhibitory concentration (MIC) of vancomycin versus the strain equals or exceeds 4.8 $\mu\text{g/mL}$ – there have been reports of vancomycin resistant *S. aureus* (VRSA) strains that are tolerant of vancomycin at considerably higher concentrations (MIC $\geq 16 \mu\text{g/mL}$), once again due to the acquisition of *van* operons (76). The threat of methicillin and vancomycin resistance among *S. aureus* is further compounded by the fact that despite much research and development, no vaccine for *S. aureus* has been approved.

Klebsiella pneumoniae

Klebsiella pneumoniae is a gram-negative microorganism commonly found in soil, water, and plants, as well as in humans and other organisms (77).

In humans, *K. pneumoniae* infection is known to manifest in the respiratory or urinary tracts, as well as in the blood; this pathogen is in fact the second most common cause of bloodstream infection across gram-negative bacteria, outmatched only by *Escherichia coli* (78). Furthermore, these infections can have notably dire outcomes; bloodstream *K. pneumoniae* infections have an estimated case mortality rate of 20-30% (79). These infections are often opportunistic, and are commonly treated with antibiotics known to be effective against *Enterobacteriaceae*, such as β -lactams (80). However, genetic variation within the population of this species has seen the emergence of both hypervirulent and multidrug-resistant strains (81). To elaborate, a bacterial species will typically contain a collection of conserved genes referred to as the core genome while genes that vary between species isolates are known as the accessory genome. It is the accessory genome of *K. pneumoniae* that divides its various strains into opportunistic, hypervirulent, and MDR strains (77).

Infections of opportunistic variants typically occur in the immunocompromised – these *K. pneumoniae* are thus commonly involved with healthcare associated infections but can be routinely treated with the antimicrobial agents mentioned previously. Contrarily, hypervirulent strains are able to infect otherwise healthy individuals in the general population. These strains will go on to cause considerably more severe infections which can present as endophthalmitis (inflammation of the intraocular fluids), meningitis, and pyogenic liver abscess (82). As with the opportunistic variants, hypervirulent *K. pneumoniae* strains remain susceptible to antibiotics. However, hypervirulent strains exhibiting resistance to carbapenems have been isolated in China, which may facilitate the spread of *K. pneumoniae* variants that both cause severe infection and are highly difficult to treat (83).

It should be noted that *K. pneumoniae* possesses intrinsic resistance to some β -lactams that would otherwise be used to treat them and has incorporated further β -lactams and extended-spectrum β -lactams (ESBLs) into the accessory genome as well (77). Possibly as a result of this - and the selection pressure caused by treating these resistant strains with carbapenems – a third group of *K. pneumoniae* have acquired genetic elements that allow them to express carbapenemases, resulting in considerable resistance to antibiotics (84). Infections of these MDR strains are opportunistic but are unsurprisingly difficult to overcome with therapeutics. Moreover, prevalence of this multidrug resistance is such that *K. pneumoniae* is the most common carbapenem-resistant *Enterobacteriaceae* known (85). Concerningly, protocols for optimal treatment of MDR *K. pneumoniae* have yet to be fully established, with many combinations of currently available antibiotics proving suboptimal (86).

Acinetobacter baumannii

A. baumannii is a gram-negative coccobacillus most commonly isolated from hospital environments (87). The organism is predominantly known for infections at breaches of anatomical barriers, with symptoms varying depending on the site of infection; ventilator-associated pneumonia and central-line associated bloodstream infections are most common, but infections of skin and urinary tract following catheterisation have been known to occur as well (88). Prevalence of *A. baumannii* infection across the globe is considerable, with *A. baumannii* accounting for roughly 2% of all healthcare associated infections across Europe and the United States as of 2017, as well as 80% of all nosocomial *Acinetobacter* infections (89,90). Notably, roughly 45% of *A. baumannii* isolates exhibit multidrug resistance, a much higher proportion than many other g-negative pathogens, including fellow ESKAPEs such as *K. pneumoniae* and *P. aeruginosa* (91).

It is widely asserted that the ability of *A. baumannii* to thrive in the hospital environment can be attributed to both its environmental persistence and antimicrobial resistance (92). While the extent of this persistence has yet to be fully characterised, it is known that *A. baumannii* exhibits tolerance to desiccation – with some isolates able to retain viability in dry conditions for as many as 100 days – as well as disinfectants such as ethanol and chlorhexidine, which are extensively used in hospitals and other health-care settings (93,94). As with many AMR mechanisms, these environmental are attributed to genetic elements that facilitate the expression of protective proteins. These include the protein RecA that mitigates DNA damage induced by desiccation–rehydration, or the AceI efflux protein that is known to actively pump chlorhexidine out of the cell (93,95). In addition, *A. baumannii* is known to form biofilms on skin, soft tissue, and abiotic surfaces within the hospital, providing a greater tolerance to many forms of extracellular stress (96,97).

With regards to its antimicrobial resistance, *A. baumannii* possesses intrinsic resistance to several antimicrobial agents. Known contributors to this intrinsic resistance are the AmpC and OXA-51 genes that facilitate the production of potent β -lactamases, but the pathogen also exhibits resistance to carbapenems and fluoroquinolones (98). In addition to this, genomic sequencing of susceptible and resistant strains has identified acquired genes believed to be associated with resistance to antibiotics, as well as antiseptics and heavy metals; 45 of these genes were clustered within an 86kb region, referred to as the AYE resistance island (99,100). Currently the last line of treatment for MDR *A. baumannii* strains is colistin, but colistin resistant isolates have been reported within the last 10 years (101,102).

Pseudomonas aeruginosa

P. aeruginosa is a gram-negative pathogen known to cause opportunistic infections in humans; these infections typically involve the respiratory tract and are particularly relevant for sufferers of cystic fibrosis, who are at high risk of morbidity and mortality following *Pseudomonas aeruginosa* infection (103). Additionally, *P. aeruginosa* infection is associated with increased mortality in patients with chronic obstructive pulmonary disease, accounting for over 5% of infectious exacerbations in sufferers (103). With its ubiquity across a variety of environments – both terrestrial and aquatic – it is arguably unsurprising that this pathogen accounts for roughly 10% of all recorded nosocomial infections (104). While the pathogen is distributed across the globe, the prevalence of AMR *P. aeruginosa* strains will vary according to region, with the highest rates reportedly occurring across Europe and Central Asia as of 2021 (105).

Treatment of *P. aeruginosa* is difficult due to both its intrinsic and acquired resistance to conventional antibiotics; this has manifested as multidrug resistance across a number of its strains (**Table 1.2**). In addition, the ability of *P. aeruginosa* to colonise moist environments facilitates its biofilm formation in healthcare settings, particularly in the context of wounds and respiratory or urinary tract devices; as previously mentioned, the presence of biofilm predisposes for environmental persistence and antimicrobial resistance of the pathogen (106).

Development of novel therapeutic avenues for *P. aeruginosa* infection is a topic of much interest, with a number of antibiotics with novel modes of action being explored, as well as novel routes of administration (107,108). While the *in vitro* efficacy of these potential candidates is encouraging, it also serves to highlight another menacing facet of AMR: even novel therapeutics are susceptible to the development of resistance. It is common for therapeutic approaches to employ multidrug or combination therapies to curtail resistance to any one antimicrobial agent, but while this is effective, it also emphasises the need to continually develop antimicrobial agents to be used in tandem.

Table 1. 2. Summary of AMR across different strains of *P. aeruginosa* (308).

Strains of <i>Pseudomonas aeruginosa</i>	Showing resistance to antibiotic class	Mode of action
PA40, PA43	Amikacin	Multi-drug-resistance (MDR)
ATCC 27853, P2284	Ticarcillin/clavulanate	Production of β -lactamase
K385	Chloramphenicol and norfloxacin	Overexpression of <i>mexC-MexD-OprJ</i> operon
PA-M4	Ciprofloxacin	Overexpression of <i>MexEF-OprN</i> operon
OCR1	Gentamicin	Overexpression of <i>MexAB-OprM</i> operon
PAO4222	Carbapenem (imipenem and meropenem)	Loss of porin channels in the outer membrane, expression of OprD and secreting carbapenem-hydrolyzing metalloenzyme
PAO4098E	Carbenicillin and tobramycin	Inactivation of aminoglycosides enzyme, ribosomal methyl group transferase enzyme
PAO1	Tigecycline	Inhibition of <i>MexXY-OprM</i> activity
KG3002	Ofloxacin	Inactivation of <i>MexC</i> operon
KG3000	Ciprofloxacin	Expression of <i>MexC-MexD-OprJ</i> operon
PAO1	Fluroquinolones	DNA gyrase topoisomerase IV activity
PA1109	Polymyxin E (colistin)	Modification in the LPS layer
PA124	Tetracyclines	Activation of <i>MexXY-OprM</i> efflux pump
PAO1	Quinolones	Expression of <i>MexEF-OprN</i> efflux pump due to mutation of NfxB, NfxC and NalB
ATTC 27853, K1178	Cephalosporin	Overexpression of <i>MexAB-OprM</i> efflux pump due to the NalB mutation

***Enterobacter* Species**

The *Enterobacter* genus houses various gram-negative bacilli; to date over 20 species have been discovered: most commonly from isolates found in soil and water, although species within this genus are also commensal organisms of the gut microbiota in humans and other animals (109). Of the species discovered, 3 have been most frequently isolated in nosocomial outbreaks: *E. aerogenes*, *E. cloacae*, and *E. homaechi* (110,111). Infection of these three species can present a host of symptoms, including but not limited to bacteraemia and septicaemia, pneumonia, cerebral abscesses and various abdominal cavity infections (112,113). As with many of the pathogens within the ESKAPE group, these outbreaks primarily affect immunocompromised patients due to their susceptibility to opportunistic infection. This - combined with the considerable prevalence of multidrug resistance – result in the *Enterobacter* species being associated with a very high risk of mortality (114,115).

The presence of and mechanisms facilitating resistance in these organisms has been extensively studied. *Enterobacter* species such as *E. cloacae* have shown intrinsic resistance to cephalothin, cefoxitin, ampicillin, and amoxicillin-clavulanic acid; this resistance is conferred through the low-level production of an inducible natural cephalosporinase and has been documented for over 20 years (116). In addition, much is known about *Enterobacter* resistance to β -lactams, attributed to the pathogens' ability to produce various β -lactamases. Like *A. baumannii*, these *Enterobacter* species are able to produce AmpC cephalosporinases that contribute to resistance against first and third -generation cephalosporins (113,117). Finally, while carbapenems and fourth-generation cephalosporins were considered plausible options for treatment, resistance to both classes of antimicrobials has been documented within the last 10 years (118,119).

However, therapeutic options do still exist with regards to *Enterobacter* infection.

Aminoglycoside activity against these bacteria is well-documented, with reported resistance remaining relatively scarce in literature (120,121). Additionally, efforts have been made to develop new antibiotics to combat *Enterobacter* infection (118,122,123). However, use of these agents as a primary means of treatment is discouraged to limit the emergence of resistance.

Escherichia coli

E. coli, a gram-negative rod-shaped bacterium, is commonly found in the gut of humans and other animals (124). Despite not being recognised as one of the ESKAPE pathogens, *E. coli* stands as a considerable burden with regards to human health much like its fellow members of the *Enterobacteriaceae* family, *K. pneumoniae* and the *Enterobacter* species. *E. coli* has significant clinical relevance as a causative agent of bloodstream and urinary tract infections, both in healthcare settings and community settings across the world, and once stood as the most common cause of gram-negative nosocomial and community acquired infection (10,125–128).

E. coli can be non-pathogenic – as is the case with its commensal variants - or pathogenic; these two populations differ in their possession of various genetic traits that facilitate pathogenicity (129). Pathology caused by *E. coli* infection is collectively referred to as “colibacillosis”, but the presentation of this infection can vary considerably, such that pathogenic *E. coli* isolates are often categorised into separate pathovars describing their primary symptoms (130). These begin with the broad classifications of “intestinal” and “extraintestinal” *E. coli*, before diverging further. Within extraintestinal pathogenic *E. coli* (ExPEC) are uropathogenic *E. coli* (UPEC) and meningitis-associated *E. coli* (MNEC); these pathovars combined act as one of the leading causes of UTIs, bacteraemia, and neonatal meningitis in humans after migrating from the intestines to other regions of the body (131–133). Intestinal pathogenic *E. coli* are broken down into the 6 categories of enteroaggregative, enterohaemorrhagic, enteroinvasive, enteropathogenic, enterotoxigenic, and diffusely adherent *E. coli*: EAEC, EHEC, EIEC, EPEC, ETEC, and DAEC, respectively (134). Infection of these pathovars commonly manifests as vomiting, fatigue, and diarrhoea that may be watery or bloody.

While *E. coli* is typically susceptible to many classes of antibiotics, it displays considerable capacity to acquire resistance genes from other members of the *Enterobacteriales* family, often through horizontal gene transfer (135). Resistance mechanisms exhibited by *E. coli* are typically due to the expression of ESBLs and carbapenemases that allow it to withstand the effects of broad-spectrum cephalosporins and carbapenems (136). In addition, *E. coli* strains have been observed with plasmid-mediated quinolone resistance (PMQR) genes and *mcr* genes that confer resistance to fluoroquinolones and polymyxins, respectively (136). This particular occurrence is one of great concern, as strains of *E. coli* with resistance to the last-resort polymyxin E (colistin) have been reported (137). As a result, while therapeutic agents are still available for MDR *E. coli* infection – azithromycin and ciprofloxacin, for example - their use is not currently recommended as a first line of treatment (138).

1.3 Antimicrobial Peptides

The Nature of Antimicrobial Peptides

The term ‘antimicrobial peptides (AMPs)’ – also known as host defence peptides - refers to a diverse group of antimicrobial agents that play a role in the physiology of numerous forms of life. They are prominent components of the innate immunity of higher organisms, and are produced by microbes to combat competition within a chosen ecological niche (139). The variability of AMPs means that separating them into distinct categories is difficult – AMPs are often categorised based on source, structure, nature of activity, or predominance of specific amino acid residues if these are present (**Figure 1.3**).

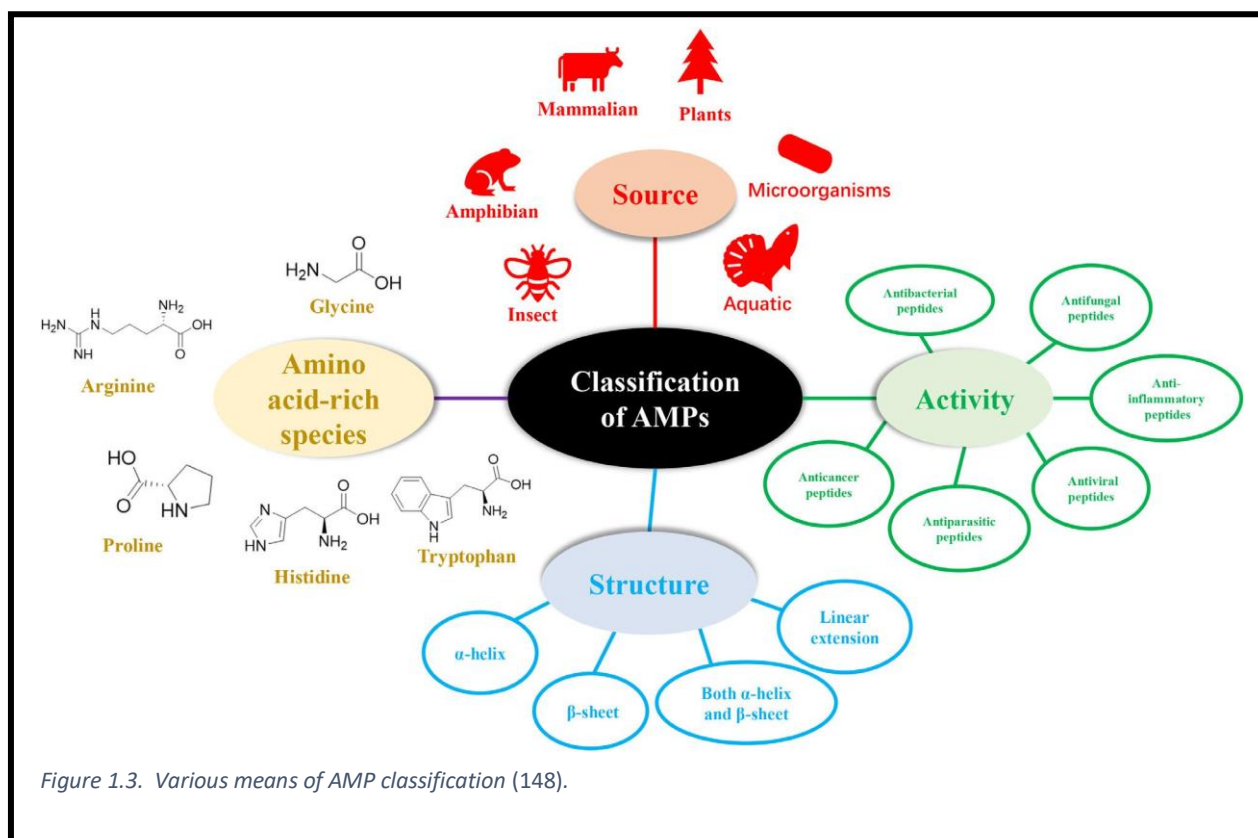


Figure 1.3. Various means of AMP classification (148).

There are a number of well characterised AMPs in nature; the human peptide lactoferricin, for example, is the proteolyzed derivative of the iron-binding protein lactoferrin and has been the subject of study for over 25 years (140,141). Similarly, the applications of the cathelicidin LL-37, notably the only cathelicidin known to be discovered in humans, has been a topic of interest for a similar length of time (142,143). It should however be emphasised that many viable AMPs have been identified in organisms outside of the animal kingdom. For example, the peptide Nisin sourced from *Lactococcus lactis* or the fungal defensin Plectasin – derived from *Pseudoplectania nigrella* – to name a few (144,145). AMPs are typically between 5 and 50 amino acids long and are characteristically amphiphilic and positively charged, though it should be noted that this is not always the case, as exhibited by AMPs such as the peptide dermcidin which exhibits bactericidal activity via membrane permeabilization despite its overall negative charge (146).

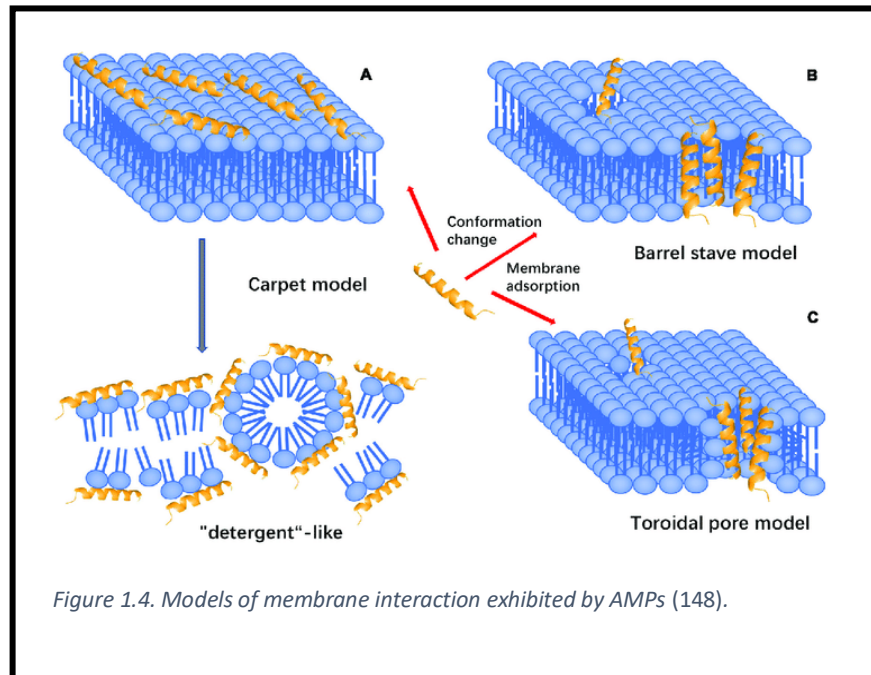
The linear sequence of amino acids comprising an AMP is referred to as the primary structure; the order in which these amino acids appear greatly influences a peptide's function, as the interactions between amino acid molecules - as well as amino acids and their surroundings - will go on to determine structures of higher order (secondary, tertiary, etc), ultimately defining their three-dimensional conformation (147). Secondary structures commonly observed among AMPs are α-helices, β-sheets, or a combination of the two; these arrangements may also exhibit structural "turns" that serve as connecting regions between the secondary structures of the peptide (148). Certain amino acids are associated with specific structural phenomena if present

within the primary structure, such as aspartate lending stability to the α -helix, or glycine contributing greater flexibility to structural turns (149,150). Particular attention must also be given to the amino acid cysteine, due to the presence of a sulfhydryl group within its structure that grants it the capacity to form disulphide bridges: a form of covalent bonding that can occur intra- and intermolecularly. While disulphide bridging can influence activity and structural stability, this does not always result in greater therapeutic viability for the peptide of interest; intermolecular bridging, for example, can allow peptide aggregation that negatively impacts AMP interaction with bacterial membranes (151,152).

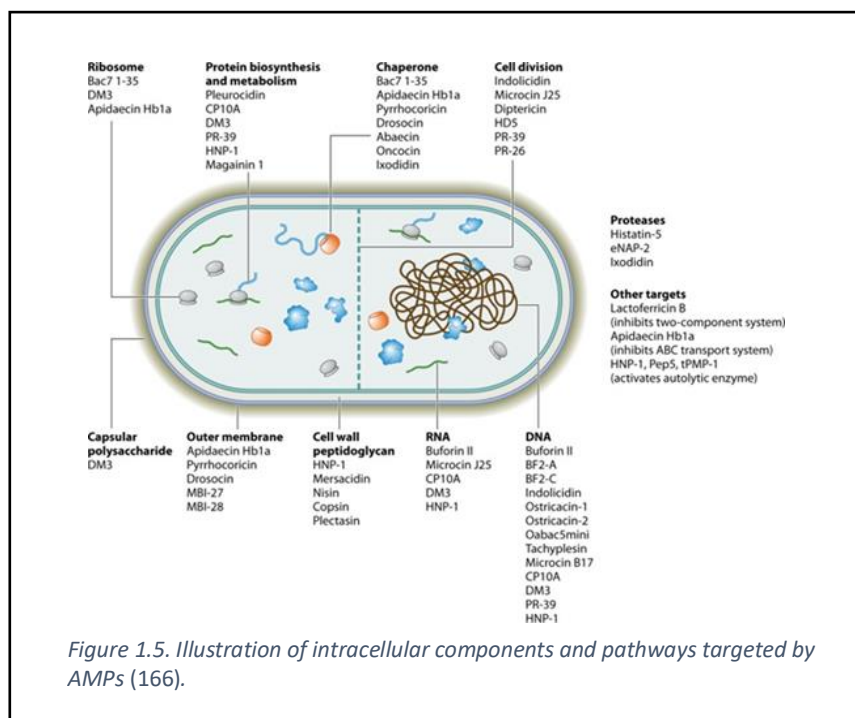
AMPs have displayed antimicrobial activity against a broad spectrum of microbes including fungi and viruses, as well as both gram-negative and gram-positive bacteria (153–156). Additionally, certain AMPs found in higher organisms exhibit immunomodulatory properties that allow for further promotion of pathogen clearance indirectly (157). Most notably, AMPs have been observed exhibiting efficacy against MDR bacteria (158,159); it is therefore unsurprising that AMPs have been studied extensively for the scope of their therapeutic potential with regards to infectious diseases for several decades. A number of AMP databases have been curated including the Antimicrobial Peptide Database (APD), which contains over 3000 naturally-occurring peptides with characterised antimicrobial activity (160). Despite this, there are currently only a small number of AMPs in clinical use, with a few more currently undergoing clinical or preclinical trials (161,162).

The factors influencing breadth and depth of an AMP's activity are numerous; observed efficacy has been reportedly altered by changes in amino acid sequence, structure, charge, hydrophobicity, and more (148,163). However, common determinants of AMP activity are peptide sequence, amphipathicity, hydrophobicity, and net charge, as these factors directly contribute to the selection and interaction of many AMPs and the membranes of their targets (163–165).

AMPs are known to the general scientific community for their ability to disrupt the integrity of the microbial membrane, resulting in cell death (148). There are three broadly accepted models of AMP-mediated membrane permeabilization: the barrel-stave model in which a pore or channel is formed across the depth of the membrane bilayer, the carpet model wherein peptide molecules accumulate across the surface of the bilayer to disrupt integrity and induce cell lysis, and the toroidal model where this surface accumulation of peptide instead reaches a critical concentration, inducing the formation of a toroidal pore comprised of both lipid and peptide (**Figure 1.4**).



While these models provide valuable elucidation of peptide-membrane interactions they are arguably an oversimplification of the full scope of AMP activity, despite the prevalence of these models in literature. Recent publications have given greater focus to the alternative mechanisms that AMPs can enact, including several that target intracellular structures following uptake into the microbial cytoplasm (163). This allows them to disrupt critical physiological processes, such as the synthesis of cell walls, nucleic acids, or proteins (**Figure 1.5**). A number of AMPs act on multiple intracellular targets in parallel, achieving high antimicrobial efficacy as a result (166,167).



Selective action of AMPs is enabled by the differences in composition between microbial and mammalian cell membranes. Mammalian cells possess a comparatively less negative transmembrane potential range, and their membranes are rich in zwitterionic phospholipids that contain an equal number of positively and negatively charged functional groups, leading to a neutral charge overall (168). On the other hand, bacterial membranes typically have negatively charged phospholipids; the resultant electrostatic attraction between negatively charged bacteria and cationic AMPs means that these interactions are highly favoured over those of AMPs and host cells. This serves to enforce the selectivity of AMP action, minimising haemolytic or otherwise toxic interactions (169).

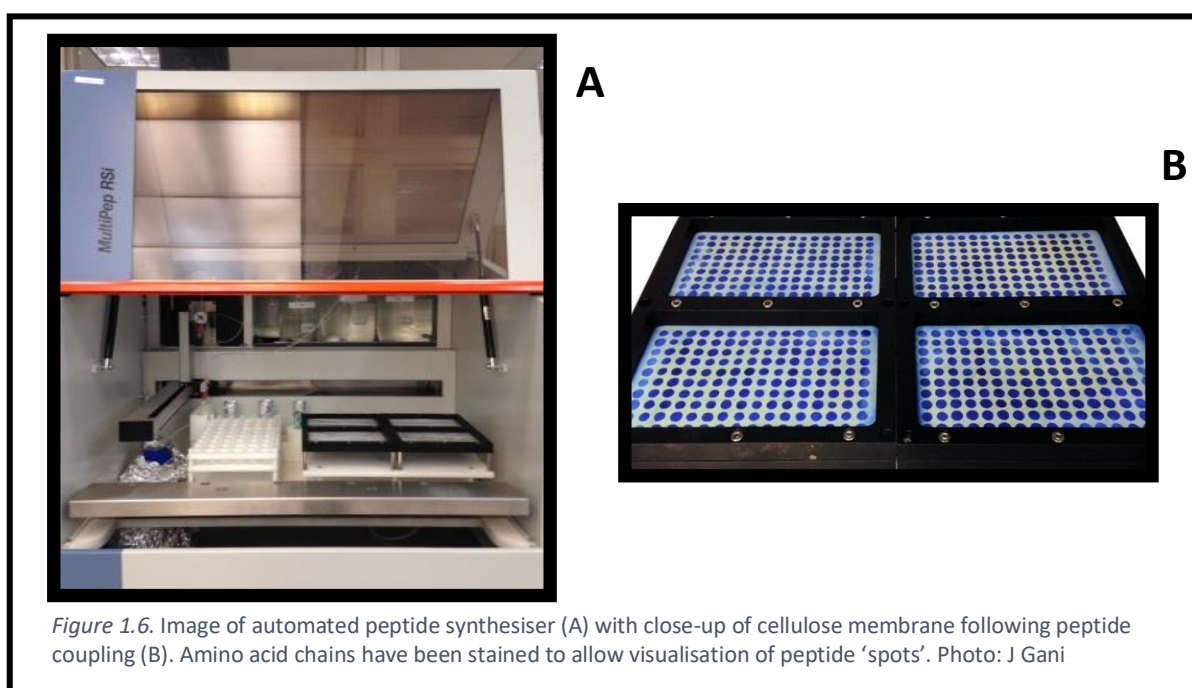
Attention has also been given to AMP properties that lie beyond direct antimicrobial action; literature reports a number of AMPs able to influence immune activity – as mentioned previously – as well as wound healing, cell proliferation, and bacterial biofilm integrity (170–173). For example, the well-characterised peptides LL-37 and lactoferrin have both exhibited inhibition of biofilm production in *S. aureus*, with lactoferrin displaying similar capacity against *P. aeruginosa* (170). This effect was improved when the AMP was administered alongside current antibiotics (174). Additionally, an array of peptides have exhibited promotion of cell proliferation through induction of macrophage differentiation or the expression of receptor-signalling cytokines and growth factors (175–178).

It is possible for the use of AMPs to place selection pressure on their target populations, bringing about a greater prevalence of resistance to their mechanisms as is the case with conventional therapeutics. A number of AMP resistance mechanisms have already been discovered, typically altering composition of the plasma membrane that many AMPs use to attach and enact their effects (173). Several gram-positive bacteria have exhibited modifications to polysaccharide and teichoic acid structures that contribute to the net negative charge that facilitates selective AMP action (180,181). Additionally, gram-negative bacteria such as the ESKAPE pathogen *P. aeruginosa* have displayed modifications to the pathogenic factor Lipid A that are associated with resistance to polymyxins and other cationic antimicrobial peptides (182,183). Other resistance mechanisms have been recognised, including neutralisation of AMPs via proteolytic degradation and protein binding, as well as the implementation of efflux pumps (184).

It should be noted, however, that the nature of AMPs as components of innate immunity means that these mechanisms – and resultant selection pressures – have existed for millennia, but AMPs remain efficacious against a broad spectrum of pathogenic species to this day. It has been suggested that this may be attributed to the multimodal nature of their bactericidal and immunomodulatory actions, the counteractive mechanisms to which are therefore less likely to arise than more direct antimicrobial processes (184).

Peptide Synthesis and Modification

The chemical synthesis of AMPs enables the development of peptides that would be difficult or impossible to acquire in nature, due to rarity of expression or incorporation of unnatural amino acids. While the chemical synthesis of peptides can be carried out using solution phase methods, the early 1960s saw a shift towards solid phase peptide synthesis (SPPS), due to the facilitation of automation and increased peptide length that the method provides (185). Originally developed in 1963 by Robert Bruce Merrifield (186), SPPS involves the elongation of a peptide chain via consecutive coupling reactions between amino acids on a solid support, with the chain then being cleaved off and isolated. There are two chemical approaches to SPPS that see the most use: *tert*-butoxycarbonyl (tBOC) and 9-fluorenylmethyloxycarbonyl (Fmoc), with Fmoc being generally preferred due to the relatively safer chemistry involved in the final cleavage step (187). These strategies can be appropriated by a method referred to as ‘spot synthesis’ wherein peptide chains are elongated on a cellulose membrane acting as solid support; a single membrane will contain up to 3000 ‘spots’, with each one corresponding to a unique peptide sequence (**Figure 1.6**) (188).



The automation of spot synthesis allows for mass production of peptide with relatively little labour when compared to other SPPS methods. Critically, the product yields of this method are sufficient for use in high throughput antimicrobial screens, allowing potential therapeutic candidates to be identified.

As components of innate immunity, many AMPs - both engineered and naturally occurring - are susceptible to enzymatic degradation or protein binding as a means of response regulation; this feature serves to limit *in vivo* efficacy of these antimicrobial agents, and therefore their therapeutic capacity. There have been many efforts to modify the structure of a peptide in order to increase resistance to enzymatic degradation, or optimise its therapeutic potential via direct increase of antimicrobial activity and/or reduction of host toxicity (189,190).

Selection and implementation of these modifications is not as straightforward as this summary would suggest. For example, it has been observed that the very properties associated with AMP efficacy – hydrophobicity, amphipathicity, and net positive charge – are also factors that contribute to toxicity (191,192). Adaptations to a peptide sequence that would reduce these qualities must therefore be introduced with caution to avoid reducing the peptide's efficacy. Similarly, strategies to increase peptide stability often implement structural changes: addition of disulphide bridges, or the addition of macromolecular structures that prevent the peptide from being acted upon by proteolytic enzymes and binding proteins. However, it is empirically evident that the alteration of peptide size, sequence, or secondary structure can impact peptide activity in unforeseen ways, regardless of whether the desired effect of greater *in vivo* stability is achieved (193,194). Thus, AMP optimisation is often a delicate process, requiring careful monitoring of peptide activity once a modification has been introduced.

Identification of Naturally Occurring Peptides

AMP amino acid sequences can be derived directly from living organisms, designed and generated in a laboratory, or a combination of the two wherein an AMP is sourced from an organism and then further synthetically modified using methods mentioned previously.

The study of the peptide population within an organism is referred to as 'peptidomics', with the peptide population itself being known as the 'peptidome'. While abundant across the kingdoms of life, AMPs only comprise a fraction of an organism's peptidome – isolation of AMPs therefore requires a rational approach to population selection and investigation. There are available tools that can be applied to peptidome characterisation following acquisition of a biological sample. For example, by combining size exclusion, liquid chromatography, and mass spectrometry, it is possible to identify mass profiles and sequences within a mixture of proteins and peptides, and isolate compounds of interest for further study. This thesis investigates the peptidome of the human lung, an environment of considerable potential for AMP derivation, particularly as various cathelicidins and β -defensins have already been found within airway surface liquid (195). The cellular population of lung tissue also contains a strong contingent of alveolar macrophages that play a critical role in host defence (196), and transcriptome analysis has found that protein expression within the lung showcases 76% of all known human protein

types (197,198). Due to this broad diversity, it can be reasoned that peptides isolated from this peptidome may also possess other therapeutically relevant properties.

However, the investigation of individual peptidomes for AMP discovery is a meticulous process, such that many naturally occurring peptides undoubtedly remain undiscovered to this day. Additionally, while peptide databases containing identified and characterised AMPs exist, selecting from these libraries leaves one unaware of the antimicrobial efficacy of peptides that have not been experimentally validated (199,200), further restricting the pool of potential therapeutic candidates.

Within a living organism, peptide synthesis occurs through the expression of associated genes and subsequent post-transcriptional assembly; the amino acids that comprise these peptides are therefore encoded within the organism's genome. Recent years have seen an increasing interest in the use of bioinformatic analysis for *in silico* prediction and identification of AMP sequences (201,202). This involves the generation or curation of biologically relevant data – such as genomic or transcriptomic data – and subjecting that data to analytical algorithms to identify patterns and properties associated with the desired AMPs.

With approximately 100,000 whole genomes having been sequenced to date, there is much interest in utilising these data as a source of novel AMP sequences (203–205). This approach necessitates the use of machine learning-guided software to parse the genomic output, identifying base sequences whose expression products are predicted to have antimicrobial properties. However, the application of an analytical algorithm - commonly referred to as a neural network - has the additional benefit of allowing prediction and preferential selection of desired properties, such as net charge, hydrophobicity, predicted activity, etc, prior to synthesis and pre-clinical research. Implementations of this strategy have thus far yielded many novel discoveries from previously untapped natural sources, from the human gut microbiome to the bacterial population of various oolong teas (206,207). As the speed and cost efficiency of sequencing technology continues to increase, so too will the reservoir of AMP sequences available to be discovered.

1.4 Hypothesis, Aims, and Objectives

This project hypothesised that both the human lung peptidome and the genomes of selected living organisms would be suitable sources of therapeutically relevant antimicrobial peptides. The project had two primary aims: to determine whether these efficacious antimicrobial peptides could be isolated from the human lung peptidome, and to evaluate the feasibility of peptide identification via *in silico* metagenomic analysis of 20 separate organism genomes. Thus, the following objectives were devised and effected:

- Synthesise peptides using sequences derived from *in silico* metagenomic analysis and isolated from lung fluid samples via SPOT SPPS methodology to facilitate high-throughput screening.
- Evaluate *in vitro* antimicrobial activity of selected crude synthesised peptides against a selection of gram-negative and gram-positive bacteria.
- Investigate therapeutic potential of selected lung-derived peptides by investigating influences on cell proliferation and calcium signalling.
- Purify peptide candidates that exhibit sufficient antimicrobial activity or therapeutically relevant bioactivity.
- Further characterise the antimicrobial efficacy of selected candidates through MIC testing against a selection of gram-negative and gram-positive bacteria, including the ESKAPE pathogens.
- Determine therapeutic viability of these candidates through evaluation of haemolytic and cytotoxic properties.

2. Materials and Methods

2.1 Generation of Peptide Sequences via AMPLY In Silico Analysis

Whole genome sequences were derived from publicly available sources (208–210) and mined for AMP sequences using the AMPLY analysis software developed by Dr Ben Thomas, Prof Chris Creevey, and Prof Sharon Huws of Queen's University Belfast (211).

2.2 Synthesis of Peptides via SPOT

2.2a – Functionalisation of cellulose membrane

The SPOT synthesis protocol involved the addition of an Fmoc-glycine spacer molecule. Four cellulose membranes (15 cm by 10 cm) were cut from hardened low Ash filter paper (Whatman grade 50) and placed into a non-reactive container. A reaction mixture was prepared, comprised of 0.4 mol/L (5.94 g) Fmoc-glycine (Bachem, Germany), 2.4 mol/L (3.4 mL) diisopropylcarbomide (DIC) and 0.8 mol/L (3.18 mL) N-methylimidazole (NMI) split evenly across two 50 mL Falcon tubes. The solutions were then topped up to 50 mL each using dimethylformamide (DMF). Upon addition of the solutions to the cellulose membranes, care was taken to remove air bubbles. The cellulose membranes were then incubated in the reaction mixture overnight with shaking (25 rpm).

The reaction solution was then discarded, and the membranes were subjected to a series of incubation steps carried out at room temperature and under shaking (25 rpm) for the purposes of washing, staining, and Fmoc deprotection (**Table 2.1**).

Washed membranes were then left exposed under an S&B 1550 Wide Fume Cupboard (S+B UK, England) overnight to dry.

Table 2.1 – Outline of washing protocol following the addition of Fmoc-glycine spacer to cellulose membrane. Solutions were prepared using DMF unless otherwise stated.

Wash Step	Reagent	Number of Washes	Incubation Time (Per Wash)
1	DMF	3	2 min
2	Ethanol	2	2 min
3	20% Piperidine (v/v)	1	20 min
4	DMF	3	2 min
5	Ethanol	3	2 min
6	0.01% Bromophenol Blue (v/v)	1	5 min
7	DMF	3	2 min
8	Ethanol	3	2 min

2.2b – Automated SPOT synthesis

Synthesis was carried out using Fmoc solid phase peptide chemistry, as previously described (187), using amino acids described in [Appendix A](#). Briefly, synthesis occurs through the formation of peptide bonds between the deprotected membrane glycine and the appropriate amino acid via a coupling reaction ([Figure 2.1](#)). The amino acid chain can then be extended through a series of deprotection-coupling cycles. This process was facilitated by an Intavis MultiPep RSi automated synthesizer (INTAVIS Bioanalytical Instruments AG, Germany) with a SPOT synthesis module and dedicated computer software.

In preparation for automated synthesis, the following reagents were prepared:

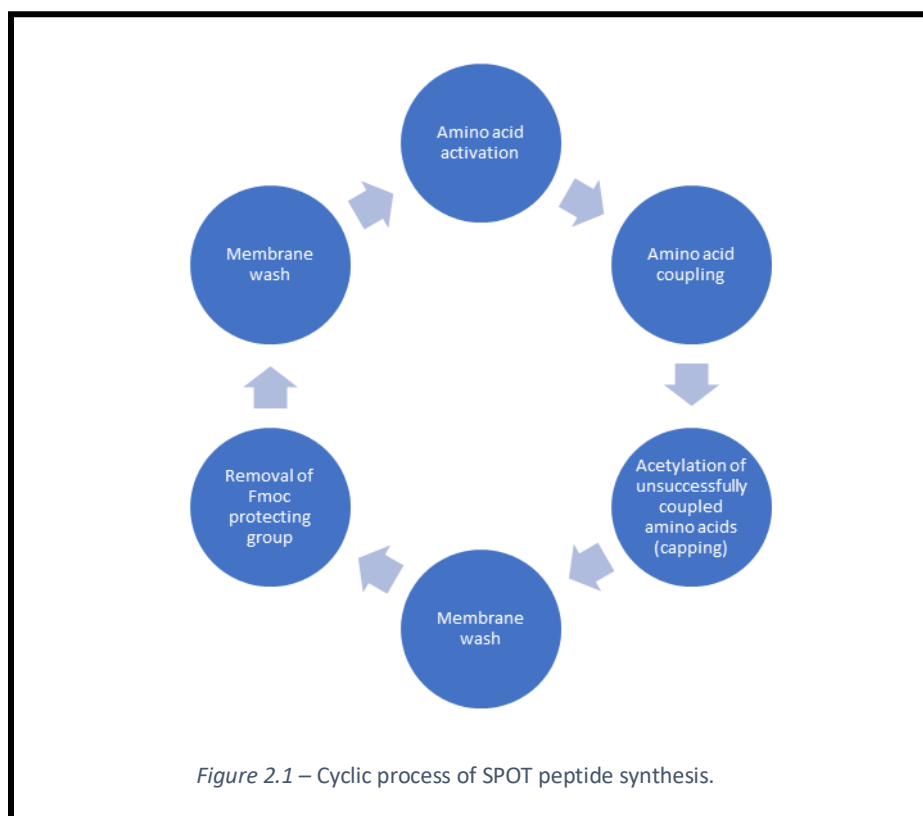
- 0.5 mol/L amino acid solutions were prepared in volumes determined by Intavis software. Amino acids were dissolved in N-methyl-2-pyrrolidone (NMP) with the exception of (trt)cysteine, which was dissolved in DMF.
- 1.1 mol/L DIC and hydroxybenzotriazole (HOBt) solutions (dissolved in NMP).
- 20% piperidine solution (v/v) in DMF.
- 5% acetic anhydride ‘capping’ solution (v/v) in DMF.

Reagents were placed into the automated peptide synthesizer as described by manufacturer instruction.

The synthesis protocol ([Figure 2.2](#)) consisted of a single ‘cycle’ for each amino acid, during which the Fmoc protecting group was removed via piperidine incubation, allowing the coupling reaction step to be carried out twice to increase yield. These cycles were repeated until all desired peptides had been synthesised. The prepared amino acid solutions were used to create activated coupling mixtures during the automated cycles, comprised of 50% amino acid solution (v/v), 20% 1.1 mol/L HOBt in NMP (v/v), 20% 1.1 mol/L DIC (v/v), and 1% NMP. Roughly 0.9 μ L of this coupling solution was aliquoted onto a designated ‘spot’ on the cellulose membrane for each coupling step of a cycle, using an automated pipetting needle.

Washing steps were incorporated to remove unwanted side products, reducing the occurrence of side reactions and further increasing peptide yield. In addition, the cellulose membranes were incubated in acetic anhydride ‘capping’ solution after double-coupling to acetylate unsuccessfully coupled peptide chains. It should be noted that capping steps were only carried out where completed – and thus Fmoc-deprotected – peptide chains would not be exposed to the capping solution, to prevent unwanted acetylation.

Synthesis protocols also included a “pause” after 4 to 5 cycles, during which the membranes could once again be stained with roughly 25 mL 0.01% bromophenol blue solution (v/v), allowing for visualization of the peptide spots, which are stained blue due to their free amino groups. The position of each peptide spot could subsequently be marked by a graphite pencil.



Cycle: 1	Cycle: 2-12	Cycle: Final
Rinse Pipetting Needle	Deprotection: Piperidine	Deprotection: Piperidine
Membrane Wash: EtOH x2	Wash x2	Washx2
Amino Acid Activation	5 Min Incubation	10 Min Incubation
Amino Acid Coupling 1	Deprotection: Piperidine	Deprotection: Piperidine
10 Min Incubation	Wash	Wash
Amino Acid Coupling 2	5 Min Incubation	10 Min Incubation
15 Min Incubation	Membrane Wash: DMF x5	Deprotection: Piperidine
Rinse Pipetting Needle		Wash
Acetylation: 'Capping' Solution Wash	Membrane Wash: EtOH x4	10 Min Incubation
5 Min Incubation	BPB Stain (Cycle 5 Only)	Membrane Wash: DMF x8
Membrane Wash: DMF x5	Amino Acid Activation	Membrane Wash: EtOH x8
Membrane Wash: EtOH x4	Amino Acid Coupling 1	Rinse Pipetting Needle
Rinse Pipetting Needle	10 Min Incubation	
Begin Next Cycle	Amino Acid Coupling 2	
	15 Min Incubation	
	Rinse Pipetting Needle	
	Acetylation: 'Capping' Solution Wash	
	5 Min Incubation	
	Membrane Wash: DMF x5	
	Membrane Wash: EtOH x4	
	Rinse Pipetting Needle	
	Begin Next Cycle	

Figure 2.2 – Typical SPOT method for the synthesis of 12-mer peptides. Solvent extraction steps (not included here) were carried out between repeated washing steps to prevent build-up.

2.2c – Side group deprotection of membrane-bound peptides

The included amino acids possessed side chain protecting groups ([Appendix A, Table 1](#)) in order to minimize the impact of side reactions. These protecting groups were acid-sensitive, and so could be selectively removed upon completion of the synthesis. To achieve this, a deprotection solution was prepared, consisting of 95 mL trifluoroacetic acid (TFA), 3 mL triisopropylsilane (TIPS), and 2 mL demineralised H₂O (dH₂O). The cellulose membranes were removed from the automated synthesizer and placed individually into four polypropylene wash boxes (Intavis). The membranes were then incubated in approx. 25 mL of the prepared deprotection solution for 30min, with care being taken to prevent prevalence of air bubbles.

The 95% TFA solution was then discarded, and a second deprotection solution was prepared, comprised of 50 mL TFA, 45 mL dichloromethane (DCM), 3 mL TIPS, and 2 mL dH₂O. The membranes were then incubated in this deprotection solution for 2hr. Following TFA deprotection, the membranes were subjected to a series of washing steps ([Table 2.2](#)). Washed membranes were then left under an S&B 1550 Wide Fume Cupboard (S+B UK, England) overnight to dry.

Table 2.2 – Outline of washing protocol following TFA deprotection of fully synthesised peptides.

Wash Step	Reagent	Number of Washes	Incubation Time (Per Wash)
1	DCM	3	2 min
2	DMF	3	2 min
3	Ethanol	4	2 min
4	10 mmol/L Tris (pH 7)	4	2 min
5	H ₂ O	3	2 min

2.2d – Peptide Cleavage and Dissolution

Deprotected peptides were cleaved from the cellulose membrane binding sites via incubation in ammonia gas – the resultant basic environment serves to break the ester bond that connects the peptide to the membrane. To facilitate this, dried membranes were transferred to a glass desiccator, and a vacuum was created within the desiccator using a vacuum pump. Ammonia was then introduced to the vacuum via a chemical-resistant balloon filled with ammonia gas, facilitating the breakage of ester bonds between peptide and membrane in the highly basic environment. After filling the internal volume of the desiccator with ammonia gas, a filled balloon was left attached to the desiccator overnight.

Individual peptide ‘spots’ – marked on the membranes with a graphite pencil – were punched out of the membranes using a single spot hole puncher. Each spot was transferred to a well of a polypropylene 96-well plate containing 150 μ L sterile dH₂O. The plates were then incubated at room temperature overnight with shaking (25 rpm). The peptides were then deemed to be solubilized, and the resultant solutions were stored at 4°C until use.

Each synthesis batch included a minimum of 16 peptide duplicates that would act as controls. Controls would be analysed via reverse-phase high performance liquid chromatography (RP-HPLC) and electrospray ionisation mass spectrometry (ESI-MS) in order to determine success of the synthesis and purity of product.

2.3 On-Resin Peptide Synthesis

Peptides sourced from the lung peptidome were synthesised on resin by Synpeptide (Shanghai, China), while high-yield SPPS of peptides derived from AMPLY was carried out on resin by Genscript Biotech (New Jersey, USA). Following synthesis, peptides were purified to a homogeneity greater than or equal to 85%, as determined via high performance liquid chromatography (HPLC) and mass spectrometry (MS).

2.4 Analysis of Peptide via Reverse-Phase HPLC

Synthesised peptides were dissolved in sterile demineralized water and injected into a Shimadzu LC2010 with a Shimpack VP-ODS column (Shimadzu, Japan). The system employed separate solvents of 0.1% TFA (v/v) in HPLC-grade water (solvent A) and 0.1% TFA in acetonitrile (ACN, solvent B), wherein the concentration of solvent B would rise along a linear gradient: a typical gradient was 5% to 70% solvent B over 37.5 min. Typical flow rate was 1 mL/min. Homogeneity of peptide samples was determined according to area and intensity of chromatogram peaks corresponding to the desired peptides, with absorbance of the eluent measured at 220 nm ([Appendix C](#)).

2.5 Peptide Analysis via Electrospray Ionisation Mass Spectrometry

The presence of desired peptides was confirmed via mass spectrometry, carried out on a Shimadzu LCMS-2020 (Shimadzu, Japan). Dissolved peptides were injected into a Jupiter Proteo 4u 90A column (Phenomenex, USA), presenting an increase in total ion count (TIC) when detected by the mass spectrometer. Observed molecular weight of a sample could then be calculated using the most abundant m/z signal in the sample’s mass profile. The presence of an observed mass that corresponded with the theoretical mass of the synthesized peptide was used to confirm successful peptide synthesis ([Appendix C](#)).

Sample fractions were collected based on expected RP-HPLC retention time and *m/z* signal. Pooled fractions could then be frozen at – 80°C and lyophilized using an Edwards Modulyo freeze dryer (Akribis Scientific Limited, England).

2.6 Storage and Preparation of Bacteria

Multiple bacterial strains were utilised over the course of this project (**Table 2.3**). Bacterial strains were stored in 7% dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) in Mueller-Hinton broth (MHb, v/v) at -80°C in cryogenic vials (Sigma, USA). Bacterial strains to be used for practical work were streaked on MH agar plates, prepared using 16.8 g MHb powder (Sigma-Aldrich, USA) and 10.8 g agar powder (Sigma-Aldrich, USA) autoclaved in 800 mL demineralized water. Agar plates – containing approx. 20 mL of this mixture – were stored at 4°C. Prepared streak plates were incubated overnight at 37°C.

Bacterial inoculum consisted of 3 morphologically similar bacterial colonies picked from the streak plate using a sterile loop (VWR, USA) and inoculated into approx. 3 mL of MHb under the cover of a sterilized class II microbiological safety cabinet (Walker, England). The culture was incubated overnight in an Incu-shake Mini (SciQuip, England) at 37°C with shaking (225 rpm).

Table 2.3 – Bacterial strains utilised in the project.

Bacterium	Strain	Purpose
<i>Staphylococcus aureus</i>	Epidemic methicillin-resistant strain #15 (EMRSA-15) - Clinical isolate from St. George's University of London	Preliminary antimicrobial screening
		Minimum inhibitory concentration tests
		Absorbance assays
<i>Pseudomonas aeruginosa</i>	H174 – Modified isolate with <i>lux</i> gene inserted into plasmid	Preliminary antimicrobial screening
<i>Pseudomonas aeruginosa</i>	PA01 – Obtained from Dr Robert Hancock of the James Hutton Institute	Minimum inhibitory concentration tests
		Absorbance assays
<i>Enterococcus faecium</i>	Clinical isolate from St. George's University of London	Minimum inhibitory concentration tests
		Absorbance Assays
<i>Klebsiella pneumoniae</i>	Clinical isolate from St. George's University of London	Minimum inhibitory concentration tests
		Absorbance Assays
<i>Acinetobacter baumannii</i>	Clinical isolate from St. George's University of London	Minimum inhibitory concentration tests
		Absorbance Assays
<i>Enterobacter cloacae</i>	Clinical isolate from St. George's University of London	Minimum inhibitory concentration tests
		Absorbance Assays
<i>Escherichia coli</i>	Strain K12 – Commercially available	Minimum inhibitory concentration tests
		Absorbance Assays
<i>Escherichia coli</i>	Strain #15 – Clinical isolate from St. George's University of London	Evaluation of antimicrobial activity in the presence of human serum
		Time-kill assays

2.7 Determining Optical Density via Spectrophotometry

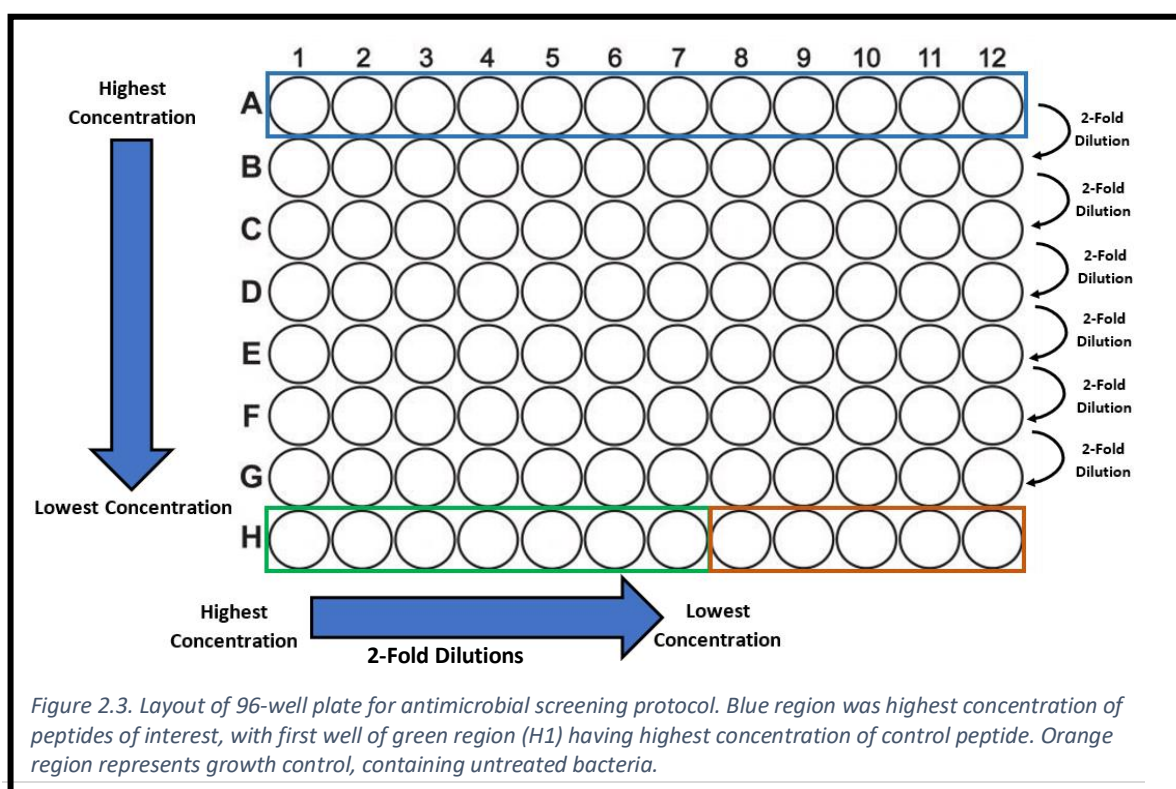
Optical density of bacterial cultures was assessed by measuring absorbance of light at wavelength 600 nm. Readings were gained from an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, England). Measurements were compared to a previously determined organism standard, allowing colony forming units (CFU) per mL to be estimated; an absorbance of 0.1 equated to approximately 1×10^9 CFU/mL for a bacterial culture.

2.8 Antimicrobial Screening of Synthesized Peptides

A bacterial solution cultured overnight was diluted 1:40 in MHb (250 μ L in 10 mL) and incubated at 37°C with shaking (225 rpm) for 2 hours. The culture was then diluted to a concentration of 5×10^5 CFU/mL using sterile MHb, following determination of optical density of this bacterial solution at 600 nm (OD_{600}).

100 μ L of the prepared solution was aliquoted into all wells in row A of a black 96-well microtiter plate, as well as the first well of row H. Into all other wells, 70 μ L of bacterial solution was added. 40 μ L of sample peptide was then added to all wells of row A, allowing two-fold serial dilutions of the experimental mixture from row A to row G.

40 μ L of a control peptide known to exhibit activity against the sample organism was added to the first well of row H, and a two-fold serial dilution was carried out horizontally from well one to well seven, creating a positive control. The remaining wells of row H were left free of antimicrobial agent, allowing them to act as growth controls. An example of plate layout is provided in **Figure 2.3**.



For assays using non-luminescent bacteria, the microtiter plate was covered with adhesive sealing film and incubated for 4 hours (37°C). Following this, 17.5 µL of 500 µmol/L resazurin in sterile dH₂O was added to each well, after which the plate was incubated at 37°C for a further 2 hours.

Resazurin is a blue dye that is reduced by oxidoreductases in metabolically active cells. The product of this metabolism is a pink compound that exhibits a higher fluorescence, allowing this colour change to be measured as an indicator of cell viability.

If the screening was carried out against a luminescent bacterial strain, diluted bacterial solutions were instead prepared using a solution of 20 mmol/L filter-sterilised glucose (Merck Life Science, UK) dissolved in 100 mmol/L sterile Tris(Hydroxymethyl) (Merck Life Science, UK) to reach the desired concentration of 5×10^5 CFU/mL following initial overnight culture in MHb. Additionally, luminescent assays were conducted using opaque white, flat-bottom 96-well microtiter plates. Resazurin dye was not required for this protocol, as bacterial viability could instead be determined by measuring luminescence of the organism.

Following 6 hour incubation, the fluorescence or luminescence of each well was measured using a Tecan Infinite F200 Pro microplate reader (Tecan, Switzerland); where resazurin was present, fluorescence was measured using an excitation/emission spectra of 535/590 nm. Readings were analysed using MATLAB® Toolbox SciXMiner software, an extension of MATLAB®, which would present a relative IC₇₅ (relIC₇₅) - comparative to an in-house control (sequence: KRRWRIWLV) – as well as a score of antimicrobial activity that enabled peptide categorisation (188). Based on the scoring, a peptide was deemed “more active than control” (antimicrobial score ≤ 0.5), “similar activity to control” ($0.5 \leq 2$), “weaker than control” ($2 \leq 20$) and inactive (>20).

Additionally, a control plate was prepared to allow concentration of the bacterial solution to be assessed. The 5×10^5 CFU/mL bacterial solution was subjected to 4 ten-fold serial dilutions; 5 µL aliquots of each dilution were then spotted onto an MH agar plate. The plate was then incubated at 37°C for 24 hours. Following incubation, CFU/mL of the bacterial solution could be calculated from the observed number of control plate colonies using the following equation:

$$\text{Mean CFU per } 5 \mu\text{L} \times 200 \times \text{Dilution Factor}$$

If the concentration was not within the accepted 10^5 to 10^6 CFU/mL range, or if contamination was seen on the plate, the experiment was deemed invalid.

2.9 Antimicrobial Screening of Synthesised Peptides in 10% Serum Solution

A bacterial solution cultured overnight was diluted 1:25 in MHb (400 µL in 10 mL) and incubated at 37°C with shaking (225 rpm) for 2 hours. The culture was then used to prepare two

solutions, with the first solution consisting of 3.6×10^5 CFU/mL bacterial solution and 14% serum (v/v), and the second solution consisting of 3×10^5 CFU/mL bacterial solution and 10% serum (v/v), following an OD₆₀₀ reading.

Plate layout was as described in **Figure 2.3**. 130 µL of the 14% serum solution was aliquoted into all wells in row A of a black 96-well microtiter plate, as well as the first well of row H. Into all other wells, 90 µL of bacterial solution was added. 50 µL of sample peptide was then added to all wells of row A, allowing two-fold serial dilutions of the experimental mixture from row A to row G.

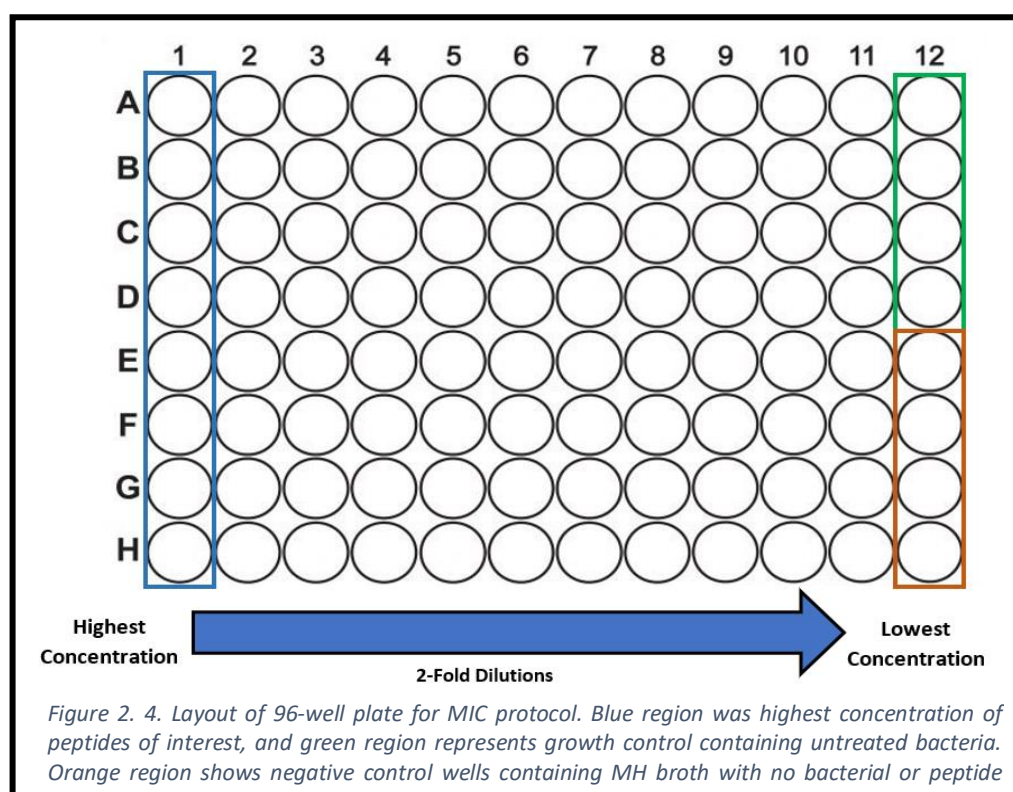
50 µL of a control peptide known to exhibit activity against the sample organism was added to the first well of row H, and a two-fold serial dilution was carried out horizontally, creating a positive control. The remaining wells of row H were used to prepare negative controls, consisting of MHb only, serum only, and 10% serum solution with no antimicrobial agent. A control plate was also prepared as described above (see **2.8**).

2.10 Determining Minimum Inhibitory Concentration (MIC) of Antimicrobial Agents

Optical density readings at 600 nm (OD₆₀₀) of 1:10 dilutions of prepared bacterial solutions (see **2.6**) were obtained using an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, England), allowing the preparation of 1mL bacterial solutions with a concentration of 6×10^5 CFU/mL.

12.5 µL of 1 mg/mL peptide solution was added to all wells of the first column of a polypropylene 96-well microtiter plate, followed by 87.5 µL of sterile MHb. To all remaining wells of the plate, 50 µL of MH broth was added; this allowed the antimicrobial solutions to be subjected to a series of 2-fold dilutions (column 1 to column 11) in order to establish a decreasing concentration gradient for the agents investigated.

50 µL of the prepared 6×10^5 CFU/mL bacterial solution was added to each well containing an antimicrobial solution. Additionally, 50 µL of bacterial solution was added to the first half of column 12 of the plate, allowing it to serve as a growth control. The remainder of column 12 was left clear of bacteria and antimicrobial agent and served as an indicator of media contamination. An example of plate layout is provided in **Figure 2.4**.



Where serum was required to determine antimicrobial efficacy in the presence of serum binding proteins, bacterial solutions of greater concentration would be prepared following determination of optical density, such that their final concentration upon addition of serum would be 6×10^5 CFU/mL. Similarly, serum was added to these bacterial solutions such that the serum concentration would be double that of the concentration required; 50 μ L of this serum-bacteria mixture would be added to the 50 μ L peptide solution mixture already present in the plate wells, halving its concentration.

All plates included an in-house control peptide of known activity against the tested strains. Microtiter plates were covered and incubated at 37°C for 16 – 20 hours in a NuAire air-jacketed incubator (NuAire, Wales). MIC of applied antimicrobial agents could then be determined via visual inspection of bacterial growth in each well. An antimicrobial concentration that inhibited visible bacterial growth was deemed an inhibitory concentration. The lowest concentration that inhibited visible bacterial growth was therefore the MIC of the antimicrobial agent with regards to the sampled bacterial strain.

A control plate was prepared in parallel, involving 3 ten-fold serial dilutions of the prepared 6×10^5 CFU/mL bacterial solution in MHb. Five 5 μ L aliquots of each resultant concentration, as well as the original concentration, were spotted onto an MH agar plate and incubated at 37°C for 16 – 20 hours. Actual CFU/mL of the prepared bacterial solution could then be calculated as previously described (see [2.8](#)).

2.11 Quantification of Antimicrobial Solution Concentration

Antimicrobial solution concentrations were determined via spectrophotometry. A 2 μ L aliquot of the solution of interest was spotted onto the lens of a ThermoScientific Nanodrop Microvolume spectrophotometer (ThermoFisher, USA), allowing absorbance readings at a wavelength of 280 nm to be obtained. Readings were obtained against a dH₂O blank. Antimicrobial solution concentration could then be calculated using the extinction coefficient of the antimicrobial agent (ExPASy Bioinformatics), in accordance with the Lambert-Beer law.

2.12 Quantification of Bacterial Growth via Absorbance Assay

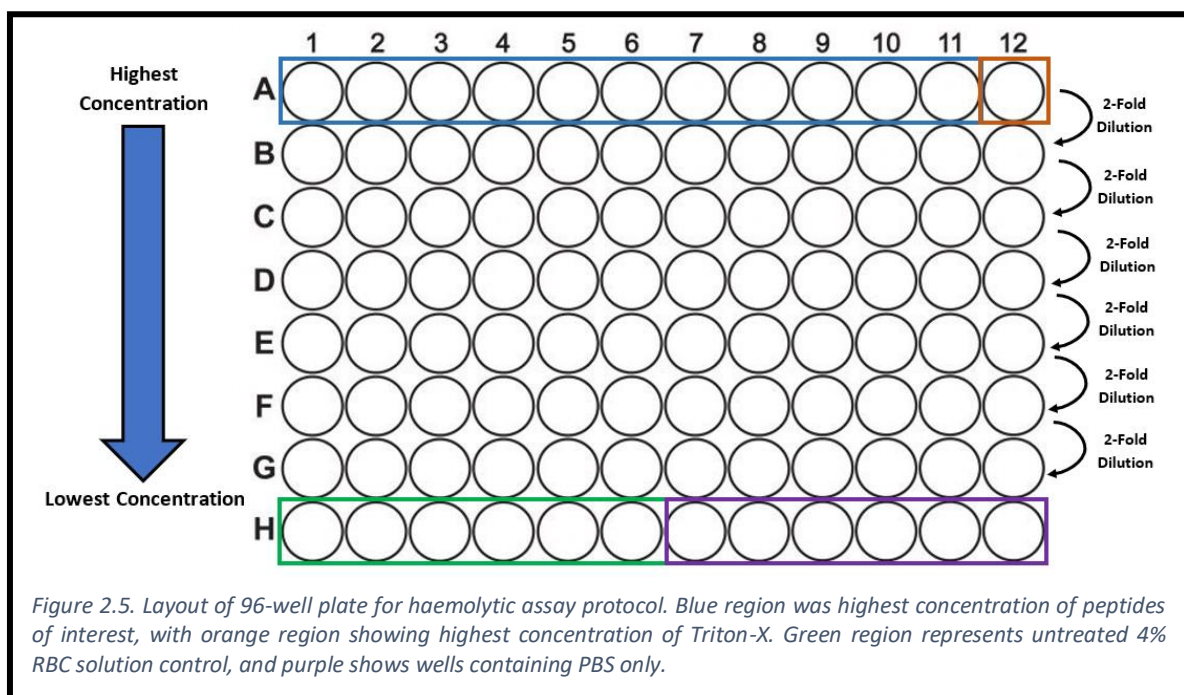
Prepared bacterial solutions were transferred into the wells of a clear, flat-bottom 96-well plate (Corning, USA) as described in [2.10](#) and incubated at 37°C in a Tecan Infinite F200 Pro microplate reader (Tecan, Switzerland); absorbance of the contents of the well (600 nm) was then measured every hour. Also included on the plate were control wells containing identical samples of the growth medium included in the assay, but no bacteria. Bacterial growth could then be quantified by subtracting absorbance of the control well from the well containing the bacterial population of interest.

2.13 Establishing Haemolytic Activity

Fresh blood samples were taken from the author by a trained phlebotomist at the Blood Testing Department of St Georges Hospital and transported in vacutainers containing citrate or Ethylenediaminetetraacetic acid (EDTA) anticoagulants. No samples were stored.

1.5 mL blood samples were transferred to a 2 mL microfuge and centrifuged (1000 RCF, 5 min). The supernatant was removed, and the remaining red blood cell (RBC) pellet was carefully resuspended in an equal volume of 1X phosphate-buffered saline (PBS, Sigma, USA). This process was repeated thrice for the purpose of washing.

A 4% RBC solution was prepared using PBS (v/v). 90 μ L of this solution was added to all but the top row of a 96-well plate (Corning, USA). Additionally, a 5.5% RBC solution was prepared and 130 μ L of this solution was added to the wells of the top row of the plate, such that its final concentration upon addition of 50 μ L peptide solution would also equal 4%. Upon addition of peptide solution, the assay mixture was subjected to two-fold serial dilution down the plate to row G. A series of controls were also included, consisting of untreated 4 % RBC solution, or PBS. A final positive control was prepared, consisting of 50 μ L 3.6% (v/v) Triton-X in PBS, such that its addition to 5.5% RBC solution would result in 1% Triton-X (Alfa Aesar, USA) and 4% RBC. The plate was then incubated at 37°C for 1 hour. An example of plate layout is provided in [Figure 2.5](#).



The plate was centrifuged (1000 RCF, 4°C, 5 min) in a Sigma 3 – 16PK centrifuge (Sigma, USA), and 70 µL of supernatant was transferred from each well to a clear, flat-bottom 96-well plate (Corning, USA). The plates were read using a Tecan Infinite Pro 2000 spectrophotometer (Tecan, Switzerland) at an absorbance of 450 nm. Percentage haemolysis for each well could then be calculated using the following formula:

$$\frac{A - N}{X - N} \times 100$$

Where A = Tecan reading of individual well supernatant at 450 nm

X = Tecan reading of topmost positive control supernatant (1% Triton-X) at 450 nm

N = Tecan reading of untreated 4% RBC solution supernatant at 450 nm

Following haemolytic assay, HC₅₀ could be determined via the generation of a nonlinear regression model of the percentage haemolysis data, facilitated by GraphPad Prism software (GraphPad Software, USA).

2.14 Qualification of Peptide Pharmacodynamics vs *E. coli* via Time-Kill Analysis

A bacterial solution of *E. coli* (strain 15) was diluted 1:25 in MHb (400 µL in 10 mL) and incubated at 37°C with shaking (225 rpm) for 2 hours. The culture was then diluted to a concentration of 1x10⁶ CFU/mL using sterile MHb, following an OD₆₀₀ reading. Peptide solutions of interest were added to 1 mL of this bacterial solution in a 2 mL microfuge tube (STARLAB) such that peptide concentration was triple that of the observed MIC vs *E. coli* strain 15; tubes were then placed in a shaking incubator (225 RPM, 37°C). Samples were taken

from these tubes at predetermined timepoints: 0, 10, 20, 40, 60, 120, 240, and 1440 minutes. These samples underwent 10-fold serial dilution sterile-filtered tris buffer (pH 7.3); 5 selections of 5 μ L aliquots of each dilution (Dilution factors 0 to 10^5) were then spotted onto an MH agar plate and incubated at 37°C for 20 hours. Controls were also included, consisting of a 2 mL microfuge tube containing 1 mL of 1×10^6 CFU/mL *E. coli* solution and no peptide, as well as a tube containing 1 mL of only Mb. Samples of these controls were collected and plated alongside the experimental samples at timepoints 0, 240, and 1440 minutes. Following incubation, colonies present on MH agar plates were counted in order to determine bacterial concentration at the time of sample extraction, as described in [2.8](#).

2.15 Bioinformatic Analysis of Peptide Sequences

Identified AMP sequences were subjected to alignment analysis via BLASTp software provided by the National Centre for Biotechnology Information (NCBI) (212). The following databases were used to confirm the novelty of identified sequences: Database of Antimicrobial Activity and Structure of Peptides (DBAASP), Yet Another AMP Database (YADAMP), Collection of Antimicrobial Peptides (CAMP), and the Antimicrobial Peptide Database (APD3).

2.16 Maintenance and Harvest of HEK293 Cells

1 mL of HEK293 human embryonic kidney (HEK) cell solution stored at -196 °C was removed from cold storage and warmed to 37.5 °C in a water bath. A growth medium was prepared, consisting of 89% (v/v) High-Glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma, USA) containing L-glutamine, 10% (v/v) Foetal Bovine Serum (FBS, Sigma, USA), and 1% Penicillin-Streptomycin (10,000 IU/mL, Sigma, USA). 5 mL growth medium was added to the HEK solution and centrifuged (4 min, 800 rpm). The supernatant was removed and replaced with an additional 5 mL growth medium.

Medium containing HEK293 cell solution was then transferred to a 75cm² flask (Thermo Fisher, USA) and incubated (37°C, 5% CO₂) until required. Adherence and growth of cells were observed via light microscopy.

HEK293 cells were harvested at approximately 90% confluence for continued cultivation as well as experimental purposes, except for calcium release imaging where confluence of sample cells could not exceed 70%. Adherent cells were released from the flask by washing with PBS (Sigma, USA) followed by exposure to warm porcine Trypsin-EDTA solution (Sigma, USA) for 2 min. Cells in suspension were then transferred to fresh flasks/growth medium at a concentration suitable for either experimentation or cell line maintenance and incubated (37°C in humidified environmental air + 5% CO₂) until required.

2.17 Determining Peptide Influence on HEK 293 Calcium Release

Approximately 1×10^4 HEK293 cells were placed into the wells of an 8-well by 4-well section of a clear, flat-bottom 96-well plate (Corning, USA) treated with poly-d-lysine (Sigma, USA). Cells were incubated (37°C , humidified environmental air +5% CO_2) in 150 μL of an assay medium consisting of 90% (v/v) High-Glucose DMEM (Sigma, USA) containing L-glutamine, and 10% (v/v) FBS (Sigma, USA) for 16 hours.

Peptide-mediated calcium release was determined using a ThermoFisher Fluo-4 Calcium Imaging Kit (ThermoFisher, USA). A reaction mixture was prepared consisting of 50% (v/v) assay medium and 50% Fluo-4 reagent solution. The wells of interest were emptied of medium and 40 μL of this mix was added. The plate was then wrapped in reflective foil and placed in an incubator (as above) for 45 min.

The plate was removed from the incubator and left for 15 min to allow cell acclimatisation to room temperature. To experimental wells, 5 μL of peptide solution was added and resultant fluorescence was measured (excitation 494 nm/emission 506 nm) every 60 seconds for 30 minutes in a GloMax Multi Detection Plate Reader. 5 μL of sterile dH_2O was added to control wells in the place of peptide solution.

After 30 minutes, 5 μL thapsigargin (Bio-Techne, USA) was added to wells of the plate with the exception of selected control wells, and fluorescent measurements continued for a further 30 minutes

2.18 Determining Peptide Cytotoxicity via Resazurin Metabolism

Approximately 1×10^4 HEK293 cells were placed into the wells of a clear, flat-bottom 96-well plate (Corning, USA). Cells were incubated (37°C , humidified environmental air +5% CO_2) in 150 μL of an assay medium consisting of 90% (v/v) High-Glucose DMEM (Sigma, USA) containing L-glutamine, and 10% (v/v) FBS (Sigma, USA) for 16 hours.

Following incubation, medium was removed and replaced with 100 μL fresh assay medium. 100 μL solutions of the peptides of interest were then added, prepared in a series of 2-fold serial dilutions. Controls were also included, consisting of wells with 100 μL of water instead of peptide solution to confirm expected cell growth and establish background levels of fluorescence, and wells with 100 μL of cytotoxic Triton-X (0.1% v/v, Sigma, USA) to indicate fluorescence at maximum cytotoxicity. The cells were then incubated (as above) for 2 hours. A solution of resazurin was prepared, consisting of 6 mg of resazurin powder (Aldrich, USA) dissolved in 50 mL of demineralised water; this solution was then passed through a sterile 0.22 μm filter (Sigma, USA). 40 μL of sterile resazurin was added to each well, and the cells were incubated (as above) overnight. Following incubation, the fluorescence of each well was measured using a Tecan Infinite F200 Pro microplate reader (Tecan, Switzerland) with

excitation/emission spectra of 535/590 nm. Resultant values could then be used to calculate percentage viability using the following formula:

$$\frac{\text{Peptide treated fluorescence} - \text{Background fluorescence}}{\text{Maximum toxicity fluorescence} - \text{Background fluorescence}} \times 100$$

2.19 Determining Peptide Cytotoxicity via Lactate Dehydrogenase (LDH) Release

Approximately 1×10^4 cells of a HEK293 cell culture were placed into the wells of a clear, flat-bottom 96-well plate (Corning, USA). Cells were incubated (37°C, humidified environmental air +5% CO₂) in 150 µL of an assay medium consisting of 90% (v/v) High-Glucose DMEM (Sigma, USA) containing L-glutamine, and 10% (v/v) FBS (Sigma, USA) for 16 hours.

Quantification of extracellular LDH was facilitated by a Thermo Scientific Pierce LDH Cytotoxicity Assay Kit (ThermoFisher, USA). Following incubation, assay medium was replaced with 90 µL fresh assay medium. 10 µL solutions of the peptides of interest were then added, prepared in a series of 2-fold serial dilutions. Wells with 10 µL of ultrapure water instead of peptide solution were used as controls for expected cell growth, and maximum LDH release wells were prepared with 10 µL of a 10X lysis buffer provided by the kit. A Reaction Mix was prepared consisting of 11.4 mL of 5% (v/v) Substrate Mix – a lyophilizate provided by the kit – in ultrapure water and 600 µL of a provided Assay Buffer.

Treated cells were incubated (as above) for 1 hour, after which 50 µL of sample medium was taken from each well and transferred to a clear, flat-bottom plate (Corning, USA) followed by 50 µL of prepared Reaction Mix. The solutions were then incubated at room temperature for 30 min, after which 50 µL of a Stop Solution was added to each well.

Absorbance of each well was measured at 450 nm and 600 nm using a Tecan Infinite F200 Pro microplate reader (Tecan, Switzerland). Extracellular LDH could then be determined by subtracting absorbance of a well at 600 nm with that of its absorbance at 450 nm. Resultant values could then be used to calculate percentage toxicity using the following formula:

$$\frac{\text{Peptide treated LDH release} - \text{Spontaneous LDH release}}{\text{Maximum LDH release} - \text{Spontaneous LDH release}} \times 100$$

2.20 Determining Peptide Effect on HEK293 Cell Growth Characteristics

Approximately 5×10^3 HEK293 cells were placed into the wells of a clear, flat-bottom 96-well plate (Corning, USA). Cells were incubated (37°C, humidified environmental air +5% CO₂) in 100 µL of an assay medium consisting of 90% (v/v) High-Glucose DMEM (Sigma, USA) containing L-glutamine, and 10% (v/v) FBS (Sigma, USA) for 4 hours. Following incubation, medium was removed and replaced with 135 µL fresh assay medium. 15 µL solutions of the peptides of interest were then added, prepared in a series of 10-fold serial dilutions. Controls

were also included, consisting of wells with 100 μ L of water instead of peptide solution as a control for expected cell growth, and wells with 15 μ L of cytotoxic dimethyl sulfoxide (DMSO, Sigma, USA).

The cells were incubated (as above) for 72 hours and cell growth was recorded using Ptychographic Quantitative Phase Imaging (QPI), facilitated by a Phasefocus LiveCyte Kinetic Cytometer. Imaging output was analysed by Livecyte 2 analysis software.

2.21 In Silico Statistical Analysis

Statistical analysis of generated data was performed using GraphPad Prism version 8.02 for Windows (GraphPad Software, USA).

2.22 Purchase and Storage of *Galleria mellonella* larvae

Galleria mellonella larvae in late instar stage were purchased from Livefoods UK. The larvae were kept in 4°C cold storage, remaining in their packaging consisting of wood shavings until required. The larvae were stored for a maximum of 14 days before use.

2.23 Determining Peptide Toxicity in *G. mellonella* larvae

Peptides of interest were dissolved in PBS (Sigma, USA), such that their final concentration was 2.5 mg/mL. Ten *G. mellonella* larvae were injected with 20 μ L of a peptide solution, administered to the last left proleg using a 0.3 mL insulin syringe (Terumo, Japan). Control populations were included, consisting of a selection of larvae injected with 20 μ L PBS and a separate group with no injections at all. Injected larvae were placed in petri dishes containing a small amount of wood shavings and incubated at 37°C, then inspected every 24 hours for five days. Larvae were determined to be dead if they failed to respond to physical stimuli.

2.24 Determining Pathogenicity of *E. coli* in *G. mellonella* larvae

1 mL of a bacterial suspension was made from an overnight culture of *E. coli* (strain 15) and centrifuged (13,000 g, 2 min) in a Sci Spin mini centrifuge. The supernatant was discarded, and the pellet was resuspended in PBS (Sigma, USA); the concentration of this bacterial suspension was then determined via spectrophotometry, as previously described (see [2.7](#)). The bacterial solution was then used to prepare a series of bacterial concentrations ranging from 1×10^2 to 1×10^7 CFU/20 μ L.

20 μ L samples of each bacterial suspension concentration were injected into the last left proleg of separate groups of ten *G. mellonella* larvae. Control populations were included, consisting of a selection of larvae injected with 20 μ L PBS, a separate group with no injections at all, and a final group injected with 10^5 CFU *P. aeruginosa* strain PAO1 as this was a dose known to be lethal within 24 hours. Injected larvae were placed in petri dishes containing a small amount of

wood shavings and incubated at 37°C, then inspected every 24 hours for five days. Larvae were determined to be dead if they failed to respond to physical stimuli.

Control plates were prepared in parallel, involving three ten-fold serial dilutions of the prepared bacterial solutions. Five 5 µL aliquots of each resultant concentration, as well as the original concentration, were spotted onto an MH agar plate and incubated at 37°C for 16 – 20 hours. Species contamination and actual CFU/mL of the prepared bacterial solution could then be determined by observing and counting the number of colonies following incubation. If the concentration was not within the expected range, or if contamination was seen on the plate, the experiment was deemed invalid.

2.25 Determining Peptide Efficacy in *G. mellonella* larvae

A 1×10^4 CFU/20 µL solution of *E. coli* (strain 15) was prepared as previously described (see [2.24](#)). 20 µL samples of this bacterial solution were injected into the last left proleg of *G. mellonella* larvae, separated into groups of ten. After 30 minutes, the *G. mellonella* groups were injected with 20 µL of a chosen peptide solution of interest at a known concentration. Injected larvae were placed in petri dishes containing a small amount of wood shavings and incubated at 37°C, then inspected every 24 hours for five days. Larvae were determined to be dead if they failed to respond to physical stimuli.

Control populations were also included consisting of a selection of larvae injected with two 20 µL sterile PBS doses (Sigma, USA) at 30 minute intervals, a separate group with no injections at all, and a final group injected with 10^4 CFU of *E. coli* strain 15, followed by 20 µL of sterile PBS 30 minutes later. Control plates were prepared in parallel with the assay, involving three ten-fold serial dilutions of the 1×10^4 CFU/20 µL bacterial solution. Five 5 µL aliquots of each resultant concentration, as well as the original concentration, were spotted onto an MH agar plate and incubated at 37°C for 16 – 20 hours. Species contamination and actual CFU/mL of the prepared bacterial solution could then be determined by observing and counting the number of colonies following incubation, as described in [2.8](#). If the concentration was not within the expected range, or if contamination was seen on the plate, the experiment was deemed invalid.

3. Isolation & Evaluation of Peptides Sourced from the Human Lung Peptidome

3.1 Introduction

As previously mentioned, the human lung has potential as a source of bioactive peptides. Particular consideration should be given to airway surface liquid (ASL), the fluid that lines the luminal surface of the airways, as it contains thousands of proteins and peptides critical for defence against inhaled pathogens and foreign particles (213). In addition, it is reasoned that peptides occurring naturally in humans are less likely to have toxic interactions with human cells than those of non-human origin. The environment within the lung can be altered by the interactions between the host and any foreign particles that enter the airways (214,215). In the case of bacteria, both the pathogen and the host's immune cells will produce peptides and proteins. Among these products will be proteases that can cleave proteins in the ASL, potentially adding to the peptidome even further. It is for this reason that a section of this project investigated peptides isolated from patients with and without cystic fibrosis, a condition known to predispose its sufferers to chronic bacterial infection (216), as well as patients with *P. aeruginosa* infection. The pathology of cystic fibrosis involves the dehydration of the ASL and mucostasis of the affected, forming a glucose-rich layer of mucus on the airway surfaces of the affected; this provides a favourable environment for bacterial infection, with the predominant pathogen involved in these infections being *P. aeruginosa* (103).

The discovery and development of AMPs for therapeutic purposes has seen several promising candidates remain in clinical development for decades due to cytotoxicity and susceptibility to proteolysis or protein binding; it is not uncommon for AMPs with encouraging bioactivity *in vitro* to perform poorly in living models or be restricted to topical administration for these reasons (217–219). The components of serum – namely the proteolytic enzymes and binding proteins found therein – have previously been implicated in the *in vivo* neutralisation of AMP activity (217,220). Therefore, the capacity of an antimicrobial peptide to tolerate the presence of serum must be investigated when determining its therapeutic viability.

Attention was also given to the effects of these peptides on calcium signalling as a measure of bioactivity. Eukaryotic cells contain internal calcium stores that can be released intracellularly in response to various physiological challenges (221). Specifically, calcium signalling has been implicated in multiple facets of the human inflammatory response, including lymphocyte activation and differentiation, lymphocyte mediated cytotoxicity, and the cytoskeletal rearrangements required for phagocytosis (222). Similarly, while the importance of calcium signalling in prokaryotes is less understood, it has been demonstrated that calcium ions are involved in numerous prokaryotic physiological processes, including motility and cell structure

(223). Thus, AMPs that induce changes to these levels could be said to have therapeutic potential as an antimicrobial or anti-inflammatory agent.

Study Design

Isolation of peptides was carried out by a separate team led by Dr Robert Tarran at the University of North Carolina (UNC); sputum samples were taken from 8 patients with cystic fibrosis (CF), as well as a non-CF group of 4. These peptides were size fractionated and analysed via liquid chromatography and tandem mass spectrometry in order to isolate the sequences of peptides with a mass not exceeding 10 kDa. While antimicrobial efficacy of these peptides was determined on-site, the UNC team independently carried out a series of assays investigating their effects on calcium release, as well as stimulation and inhibition of cell growth; the data gained from these investigations would later inform the selection of a secondary set of peptide candidates, comprising a number of peptides of this population as well as additional peptides from the same peptidome. Also included for evaluation were numerous peptides of known antimicrobial activity whose sequences were taken from online AMP databases.

Direct antimicrobial activity was determined *in vitro* using the described screening protocol (see [2.8](#)) against epidemic MRSA-15 and a luminescent H174 strain of *P. aeruginosa*. Selected candidates were purified and subjected to MIC assay against three bacteria: *E. coli* (strain K12), epidemic MRSA (strain 15, as above), and *P. aeruginosa* (strain PAO1). This provided a combination of both gram-positive and gram-negative bacteria, as well as a spectrum of expected resistance from *E. coli* K12 - which is typically relatively susceptible to the mechanisms of our tested AMPs - to *P. aeruginosa* PAO1, which is characteristically tolerant. The strategy for evaluating AMP efficacy against a bacterial strain was a two-step process. An antimicrobial screening would be carried out at multiple concentrations, allowing the IC₇₅ - the concentration at which 75% growth inhibition had been achieved - of each candidate to be determined. These IC₇₅ values would then be compared to a control peptide of known activity against the tested strain; this comparison minimised the effect of plate-to-plate variation when assessing peptide activity. Comparisons would generate a relIC₇₅ that could be used to categorise candidates in order of efficacy.

Where possible, data were investigated for statistical significance using analysis of variance (ANOVA) tests followed by post-hoc multiple comparisons testing; this involved either Dunnett's multiple comparisons testing - which compares the mean of each sample group with the mean of a designated control - or Games-Howell's testing - which compares the mean of each group with the mean of every other group - where appropriate. This approach was selected in preference to statistical analyses with greater power - such as the unpaired t-test - as the

post-hoc test mitigates the greater risk of Type I error associated with multiple statistical comparisons by using multiplicity adjusted P values (224,225).

To investigate potential efficacy in an *in vivo* model, candidates derived from the lung peptidome that possessed antimicrobial activity were then re-assessed in the presence of human serum to test their resistance to serum components. Maximum concentration of peptide solutions used for MIC assay typically did not exceed 64 µg/mL; this maximum was chosen as it provided an acceptable degree of sensitivity for the identification of antimicrobial activity while excluding peptides that, while active, possessed efficacy that would be too impractical for further drug development.

Subsequent assays were carried out using HEK293 human embryonic kidney cells due to the ease and reliability of their cultivation. Calcium signalling assays investigated the effects of selected peptides on basal intracellular calcium levels, as well as the rise in intracellular calcium due to phospholipase C (PLC) and store-operated calcium entry (SOCE) pathways (226). The mechanisms of SOCE have been implicated in an array of cellular processes; of particular relevance is the role of calcium signalling in the inflammatory response (221,227,228). To elaborate, calcium signalling involves the storage, release, and external influx of calcium ions into the intracellular space. These events are assisted by the well-characterised activation of the PLC enzyme reaction cascade, as well as the interaction of the two membrane proteins STIM and Orai that facilitate a tightly regulated influx of calcium ions into a cell following a release of calcium from the endoplasmic reticulum (229,230). The calcium assay protocol involves the introduction of an agent that – once bound to calcium - fluoresces upon exposure to a known excitation wavelength; this agent is allowed to permeate the HEK cells and all free fluorophore is subsequently washed away. Thus, detected fluorescence is due to interactions between the fluorescent agent and any available unbound calcium ions in the cytosol. Additionally, cell capacity to sequester calcium is measured through the use of thapsigargin; a compound able to prevent calcium re-uptake into the endoplasmic reticulum through inhibition of sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), a calcium ion pump (231). The inhibition of SERCA via thapsigargin leads to a brief yet characteristic upsurge in observable fluorescence when it is administered.

Following investigation of peptide-mediated calcium release, peptide cytotoxicity was determined using both resazurin metabolism and release of the lactate dehydrogenase (LDH) enzyme. While the measurement of resazurin fluorescence facilitates a more accessible assay, the method lacks the sensitivity provided by quantification of LDH release; it is for this reason that the former assay was utilised for the complete peptide set while LDH release was only measured in select candidates of suspected toxicity.

Finally, effects of peptide on HEK cell proliferation were measured using quantitative phase imaging on live cells; this had the benefit of allowing cell count and subsequent proliferation rates to be accurately measured in real-time without the potential interference that may arise from the presence of fluorescent dyes.

Hypothesis, Aims, and Objectives

Hypothesis: The human lung peptidome is a viable source of bioactive peptides.

Aim: Evaluate the therapeutic capacity of peptides isolated from human lungs.

Objectives:

- Analyse antimicrobial efficacy of peptides sourced from lung samples via MIC against a selection of gram-negative and gram-positive bacteria.
- Investigate effects of peptide candidates on calcium-mediated cell signalling.
- Confirm that observed changes to calcium release are due to effects on cell signalling and not cytotoxic interactions through evaluation of resazurin metabolism and LDH release.
- Characterise effect of peptide candidates on cell proliferation via phase microscopy.

3.2 Results

Confirming Activity of the Antimicrobial Control Peptide

In order to elucidate the antimicrobial activity of the control peptide against the bacteria selected for screening, it was subjected to MIC assays prior to the project ([3.1](#)). These assays were carried out in triplicate against epidemic Methicillin-resistant *Staphylococcus aureus* (MRSA) strain 15 and *P. aeruginosa* strain PAO1, as well as once against *E. coli* strain K12.

The in-house control was determined to be highly efficacious against the strains of interest; candidates of interest would therefore be selected if they presented similar potency following an antimicrobial screen.

Table 3.1. Modal MIC values of in-house control peptide against *E. coli* K12, MRSA E15, and *P. aeruginosa* PAO1 strains, expressed as modal concentrations.

Peptide	Sequence	<i>E. coli</i> (µg/mL)	MRSA (µg/mL)	<i>P. aeruginosa</i> (µg/mL)
Control	KRRWRIWL	4	8	16

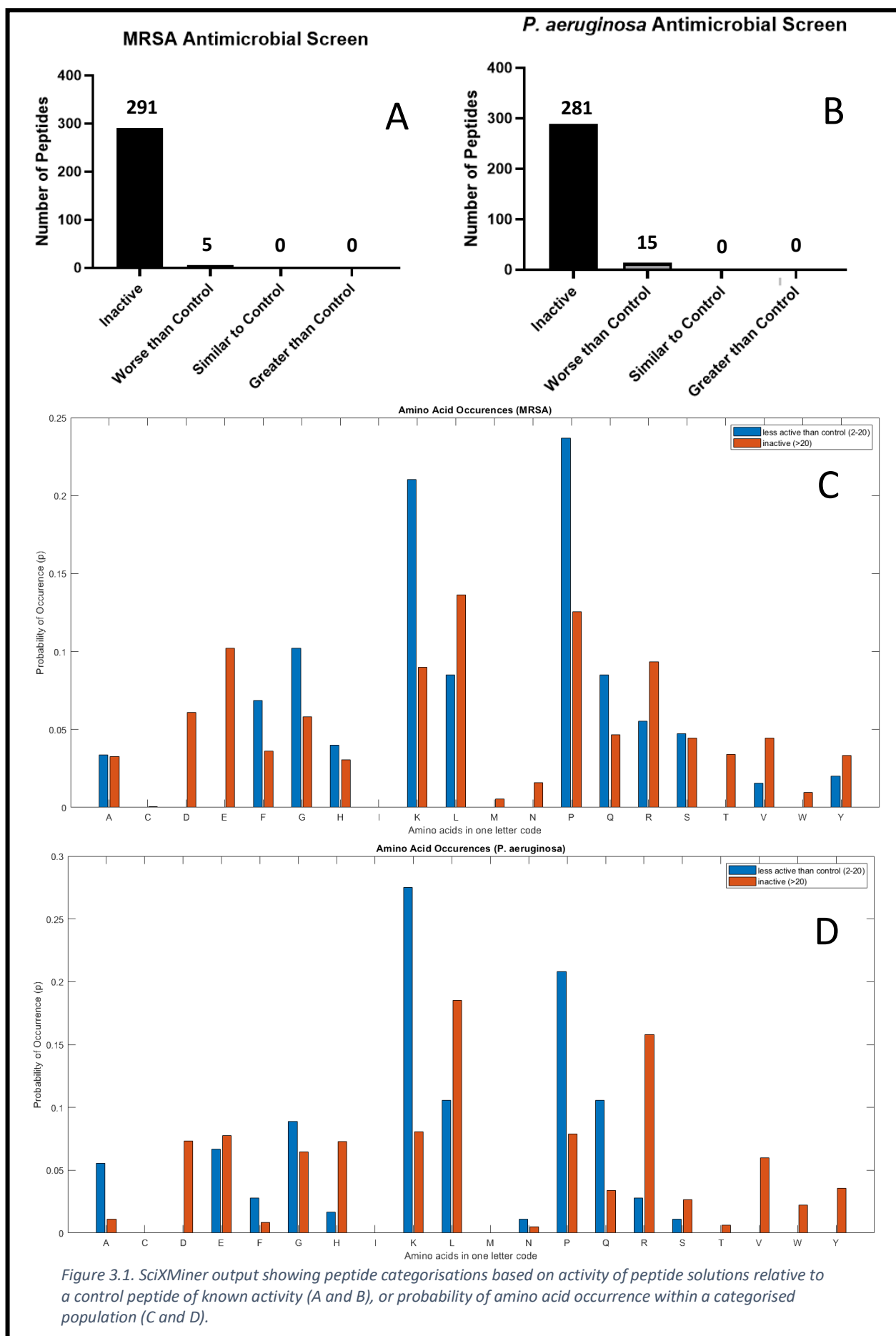
Identifying Antimicrobial Efficacy of Primary Peptidome Group

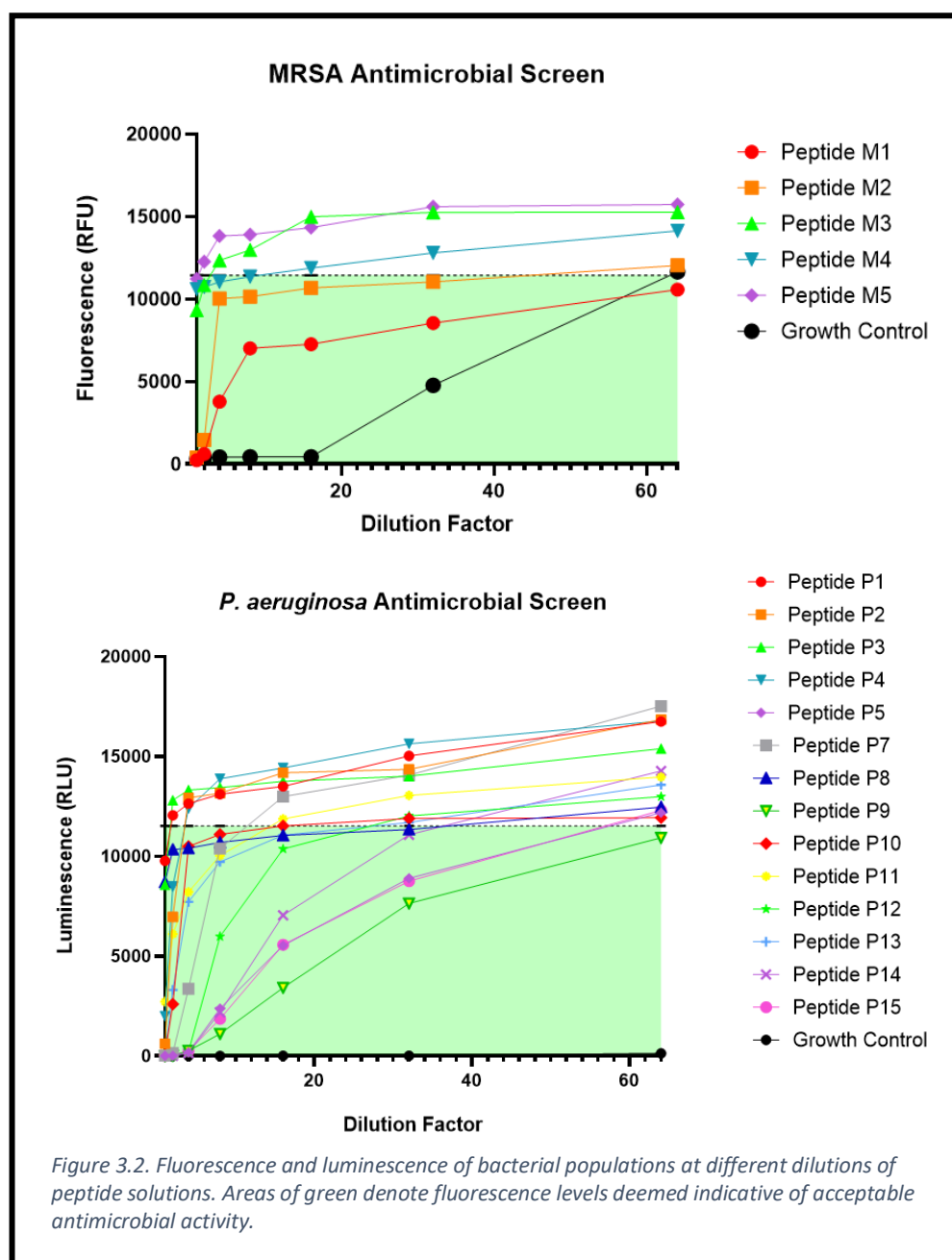
Analysis of the lung fluid samples by the Tarran group identified 140 peptide sequences that were found solely in the non-fibrotic group, and 55 peptides that were identified in at least 75% of the CF samples but not found in the non-CF group. A further 101 peptides of bacterial origin were identified and attributed to *Pseudomonas* species. These 296 peptides ([Appendix B, Table 1](#)) were synthesised via SPOT in duplicate - with the remaining synthesis spots being in-house control peptide - and screened for antimicrobial activity against epidemic MRSA strain 15 and luminescent *P. aeruginosa* strain H174. Screened peptides were categorised in accordance with their relIC_{75} , as calculated via SciXMiner software. Of the 296 peptides screened, 5 displayed antimicrobial activity against MRSA and 15 showed activity against *P. aeruginosa*, although all exhibited weaker activity than the in-house control ([Figure 3.1A, 3.1B](#)). The remaining peptides did not show activity – determined through reduction in fluorescence or luminescence when introduced to the chosen bacteria - and thus were categorised as “inactive” by SciXMiner software. Peptide sequences were also analysed for associations between the presence of specific amino acid residues and the observed activity of the peptide ([Figure 3.1C, 3.1D](#)). Across the 296 peptides tested against MRSA, the amino acid with the greatest probability of being present in the sequence of a peptide designated less active than control – which was the most active category identified – was proline (P), followed by lysine (K). This is distinct from the population of peptides that were inactive against MRSA, where the amino acid with the greatest probability of occurrence was leucine (L), followed by proline. Several amino acids –

cysteine (C), aspartic acid (D), glutamic acid (E), methionine (M), asparagine (N), threonine (T), and tryptophan (W) – were found in sequences that were inactive against MRSA, but not in those that displayed activity against the same organism.

Against *P. aeruginosa*, the amino acid with the highest probability of occurring in a sequence that showed activity was lysine, followed by proline. This differs from the peptides that showed no activity against *P. aeruginosa*, where the amino acid with the highest probability of occurrence was leucine, followed by arginine (R). The amino acids aspartic acid, threonine, valine (V), tryptophan, and tyrosine (Y) were found in sequences that were inactive against *P. aeruginosa*, but not those that showed activity.

Activity of the candidates designated less active than the control was further investigated by comparing relationships between dilution of peptide solution and observed reduction in fluorescence or luminescence (**Figure 3.2**); a peptide that reduced luminescence or fluorescence to less than 80% of what was shown by the bacterial growth control was deemed antimicrobial. While all peptides were able to sufficiently reduce fluorescence of MRSA at the highest concentration (Dilution Factor 1), capacity to reduce fluorescence decreased as peptides were diluted, with only two peptides able to achieve sufficient fluorescence reduction at the lowest tested concentration (Dilution Factor 64). Similarly, examining the luminescence of *P. aeruginosa* following incubation with the selected peptides identified a single peptide that was able to achieve sufficient reduction in luminescence at the lowest tested concentration.





Further Characterisation of Antimicrobial Activity

Peptide candidates were selected for further characterisation based on their observed activity, determined by the greatest dilution at which they could reduce bacterial growth to a sufficient degree. Peptides M1 and M2 were selected from the MRSA screen, and peptides P8, P9, P10, P12, and P13 were chosen from the screen against *P. aeruginosa*. Peptide candidates were synthesised and purified off-site by Genscript Biotech (New Jersey, USA, [Appendix C – Preliminary Lung Peptide Set](#)). The efficacy of pure peptides could then be investigated by determining MIC against the chosen bacterial strains, both separately and in combined solutions to identify synergistic effects ([Table 3.2A - C](#)).

Peptide candidates were tested against *E. coli* strain K12, epidemic MRSA strain 15, and *P. aeruginosa* strain PAO1. Despite efficacy having been observed in the initial efficacy screens with crude peptides, MIC of all purified candidates could not be determined as they exceeded the highest tested concentration of 512 µg/mL.

Table 3.2A - C. Sequences of selected peptide candidates (A), alongside modal MIC values of the selected lung peptidome candidates individually (B) and following combination of peptide solutions (C). All assays were carried out in triplicate.

Peptide Name	Sequence
M1	KPSKGLK
M2	KRHHGYKRKF
P8	KLKLKF
P9	KRGKPK
P10	KRHYKL
P12	KLHEEELQEL
P13	LDEERQGPPL

A

Peptide	<i>E. coli</i> MIC (µg/mL)	MRSA MIC (µg/mL)	<i>P. Aeruginosa</i> MIC (µg/mL)
M1	>512	>512	>512
M2	>512	>512	>512
P8	>512	>512	>512
P9	>512	>512	>512
P10	>512	>512	>512
P12	>512	>512	>512
P13	>512	>512	>512

B

Solution	Peptides Included	<i>E. coli</i> MIC (µg/mL)	MRSA MIC (µg/mL)	<i>P. Aeruginosa</i> MIC (µg/mL)
Solution 1	M1 + M2	>512	>512	>512
Solution 2	P8 + P9 + P10 + P11 + P12	>512	>512	>512
Solution 3	M1 + M2 + P8 + P9 + P10 + P11 + P12	>512	>512	>512

C

Investigating Effects of Peptides on Intracellular Calcium Release

Following evaluation of direct antimicrobial activity, capacity of selected candidates to affect intracellular calcium levels was investigated to identify alternative bioactivity. Purified peptide solutions were introduced to HEK293 human embryonic kidney cells and incubated for 30 min, after which point a thapsigargin solution was added that is known to inhibit calcium re-uptake, resulting in a characteristic rise in intracellular calcium (**Figure 3.3**).

It was observed that while no candidates raised basal intracellular calcium – measured as fluorescence - independently, HEK cells treated with 6 of the 7 peptides (Peptides M1, M2, P8, P9, P10, and P12) showed a reduced rise in mean fluorescence upon addition of thapsigargin.

Dunnett's multiple comparisons test following a two-way ANOVA identified that this reduction was statistically significant ($P < .05$, $n=3$) when comparing fluorescence of peptide M1 to the control from time-point 39 minutes to time-point 42 minutes (9 to 12 minutes post addition of thapsigargin, respectively).

For all peptides where a reduction in the thapsigargin response was seen, calcium fluorescence assays were repeated using three separate concentrations to identify dose-response relationships (**Figure 3.4**). Of all peptides tested, only Peptide M1 showed a consistent increase in magnitude of response – suppression of the thapsigargin-mediated rise in intracellular calcium – as peptide concentration increased. As before, Dunnett's multiple comparisons test was employed following an ANOVA, but no statistically significant difference ($P < .05$, $n=3$) between the fluorescence induced by any two peptide candidates could be found. Games-Howell's multiple comparisons testing identified significance between fluorescence of cells treated with peptide P8 at 100 $\mu\text{g/mL}$ and the control population ($P = .0102$, $n=3$). Additionally, significance was identified in the differences between cells treated with different concentrations of peptides M2 and P10 ($P = .0128$ and $.0215$ respectively, $n=3$), but the concentrations at which this significance occurred could not be found.

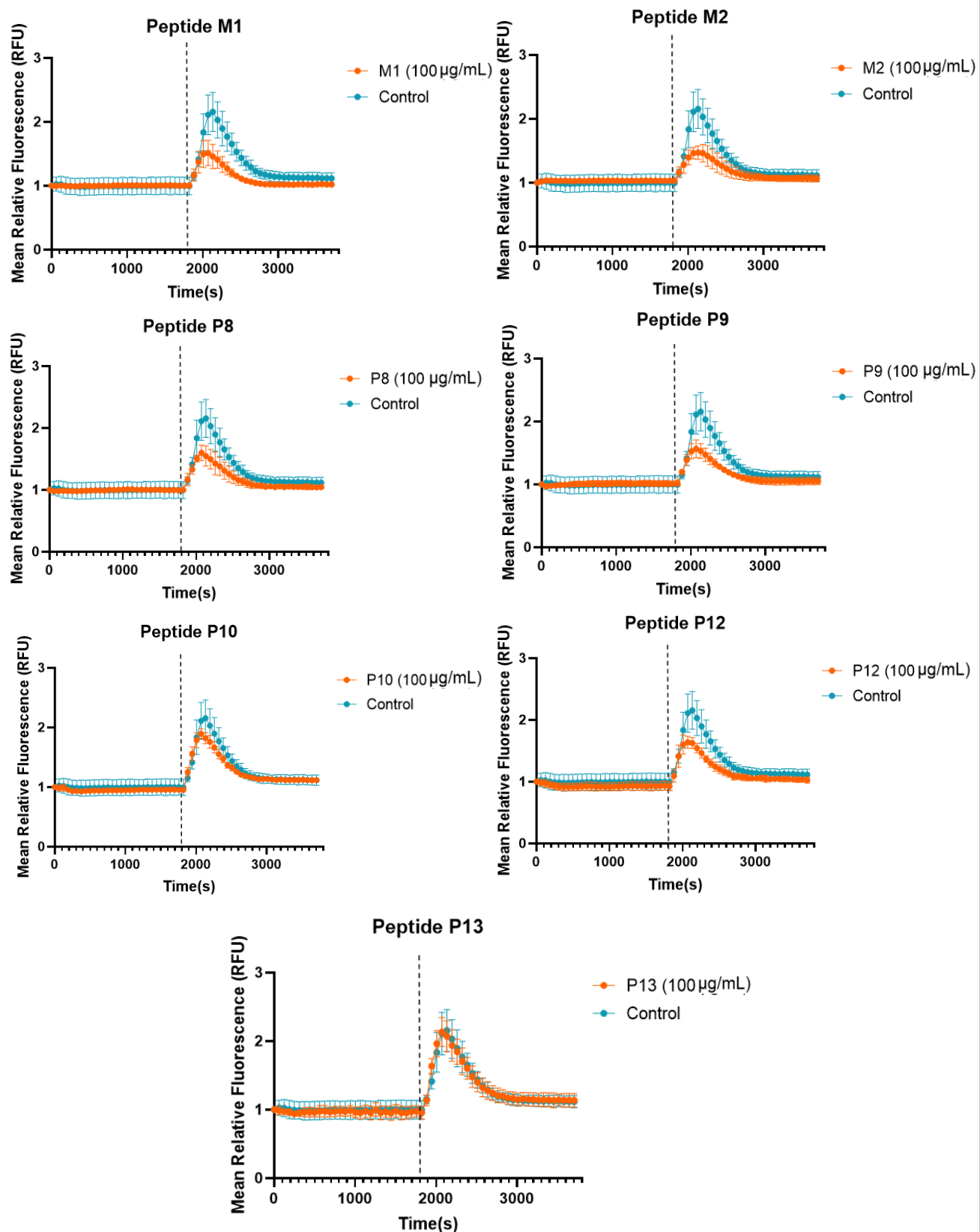
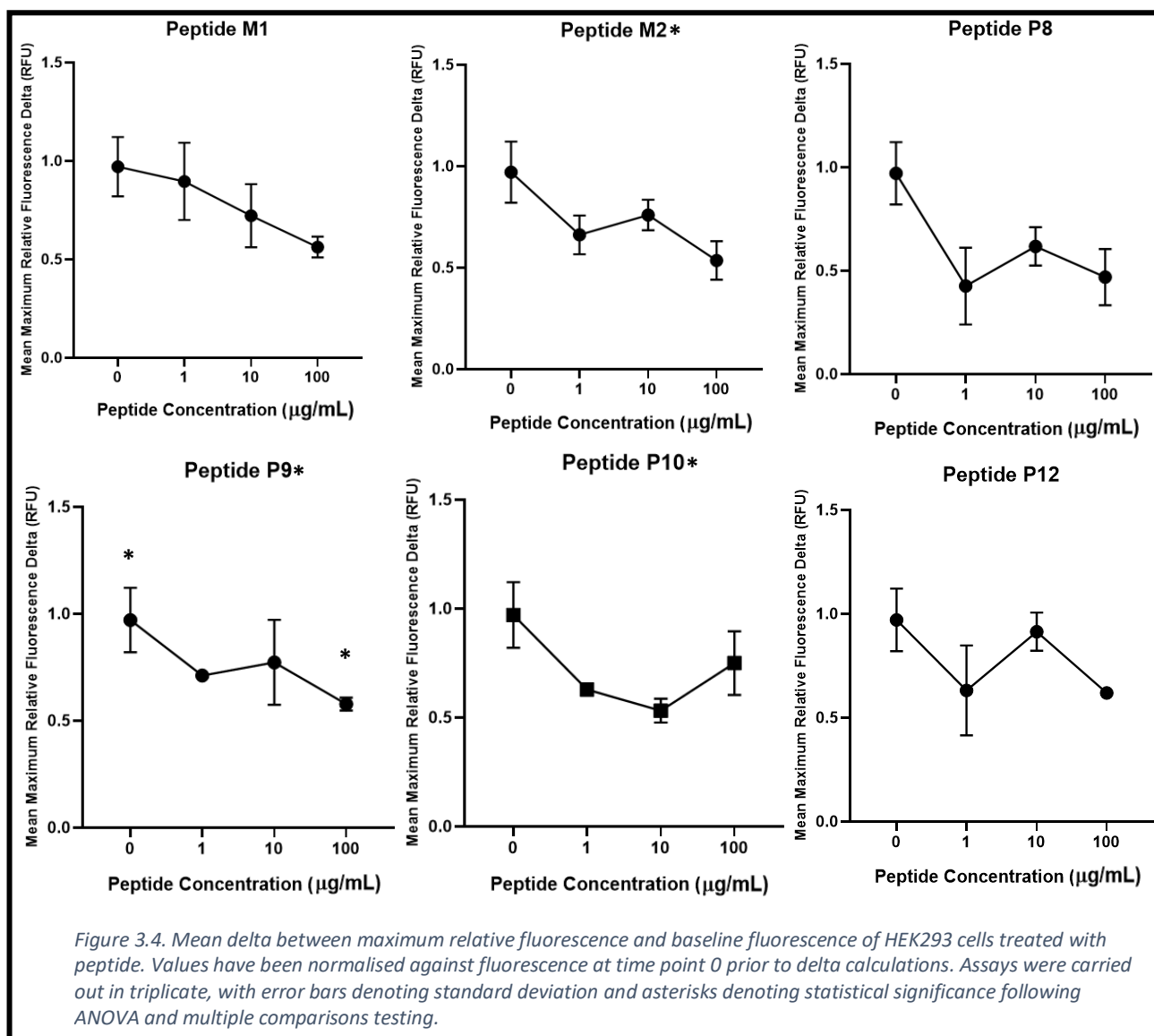


Figure 3.3. Changes in intracellular calcium of HEK293 cells following exposure to selected peptides and thapsigargin, measured as mean fluorescence. Values have been normalised against cell fluorescence at time-point 0s. Assays were carried out in triplicate, and error bars showing standard deviation are included. Time point at which thapsigargin was added is denoted by a dotted vertical line.



Identifying Antimicrobial Efficacy of Secondary Peptidome Group

The Tarran group received a collection of peptides with sequences isolated from the lung peptidome or identified on AMP databases; this collection included the 296 isolated peptides of the primary set. All peptides were independently assayed by the Tarran team for inhibition or promotion of HEK cell proliferation, as well as suppression or stimulation of intracellular calcium release. A subsequent selection of peptides was made based on the data gained from this investigation; a total of 54 peptides of interest ([Appendix B, Table 2](#)) were synthesised and purified by Synpeptide (Shanghai, China), and given designations based on their observed properties ([Table 3.3](#)). Following synthesis and purification, these peptides were delivered for validation of experimental data and determination of antimicrobial efficacy.

Table 3.3. Designations of secondary peptide set.

Prefix-	Meaning	-Suffix	Meaning
AMP	Peptide sequence was identified within AMP database	9J	Antimicrobial activity of peptide was experimentally validated by colleague "J"
LU	Peptide was isolated from lung peptidome	AGO	Peptide is labelled "calcium agonist" by UNC team
KA	Antimicrobial activity of peptide was experimentally validated by colleague "KA"	ANT	Peptide is labelled "calcium antagonist" by UNC team
DEBY	-experimentally validated by colleague "DEBY"	BAC	Peptide is a derivative of the AMP "Bactenicin"
-	-	CAN	Peptide sequence was identified within the genomic data of <i>cannabis sativa</i>
-	-	GRI	Peptide is labelled "inhibitor of cell growth" by UNC team
-	-	GRP/S	Peptide is labelled "promotor/stimulator of cell growth" by UNC team
-	-	NK	Peptide is a negative control for antimicrobial activity
-	-	RRP	Peptide is labelled "promotor/stimulator of cell growth" by UNC team

This secondary peptide set contained several peptides selected from AMP databases that were known to possess antimicrobial activity; the purpose of including these peptides was twofold, as it provided an internal control of expected antimicrobial activity, as well as an opportunity to investigate indirect bioactivity of these peptides via the analysis of their effects on intracellular calcium and cell growth. As before, MIC assays were carried out against *E. coli* strain K12,

epidemic MRSA strain 15, and *P. aeruginosa* strain PAO1; of the 54 peptides assayed, 12 exhibited antimicrobial activity at the highest tested concentration of 64 µg/mL (**Table 3.4**). Against *E. coli*, 3 of the 12 active peptides (Peptides KABAC7, KACAN3, and KACAN4) exhibited an MIC of 4 µg/mL, with an additional 5 peptides (AMPAGO4, AMPGRS2, AMPNK1, KACAN1, and KACAN5) displaying an MIC of 8 µg/mL. All observed activity against *E. coli* occurred at peptide concentrations below 64 µg/mL, the highest concentration tested. The lowest MIC against *P. aeruginosa* was 2 µg/mL – exhibited solely by KACAN4 –, with a further 5 peptides (AMPAGO4, AMPNK1, and AMPNK2, KACAN1, and KACAN3) presenting an MIC of 8 µg/mL. Of the 12 peptides with identified activity, only peptide AMP9J1 presented an MIC at the highest concentration of 64 µg/mL against *P. aeruginosa*, with a further 2 peptides (KABAC7 and KACAN5) presenting MICs greater than the highest tested concentration. The tested MRSA strain was associated with higher MICs: while 4 of the active peptides (AMPAGO2, AMPAGO4, AMPNK2, and KACAN1) presented the lowest MIC of 8 µg/mL, the remaining 8 peptides had an MIC equal to or greater than 64 µg/mL, the highest tested concentration. Of the peptides tested, AMPAGO4 and KACAN1 presented relatively low MICs against all three tested strains.

Table 3.4. Modal MIC values of antimicrobial controls from the secondary peptidome set against selected bacterial strains. All assays were carried out in triplicate.

Peptide	Sequence	<i>E. coli</i> MIC (µg/mL)	MRSA MIC (µg/mL)	<i>P. aeruginosa</i> MIC (µg/mL)
AMP9J1	KRRVRWIW	16	64	16
AMP9J2	RVKWWIRVR	32	>64	64
AMPAGO2	FWGALIKGAALKLIPSVVGLFKKKQ	32	8	16
AMPAGO4	RIGVLLARLPKLFSLFKLMGKKV	8	8	8
AMPGRS2	KRRWRIWLV	8	>64	16
AMPNK1	VNWKILGKIIKVVK	8	>64	8
AMPNK2	FIGAIARLLSKIFGKR	32	8	8
KABAC7	RWRRPIRRRPIRPPFWR	4	64	>64
KACAN1	IFIFKFFKKLIKQYKR	8	8	8
KACAN3	GQWRWLGIWKKRRRL	4	>64	8
KACAN4	RKFFKKIKKQYIRLLF	4	>64	2
KACAN5	IKKLIKKNIKFFLMLH	8	>64	>64

The efficacy of these candidates was then re-tested against 2 of the 3 bacterial strains used previously, incubated in MHb containing 25% human serum (**Table 3.5**). Ultimately, antimicrobial activity against the tested strains could not be determined as MICs in the presence of serum exceeded the highest tested concentration of 64 µg/mL.

Table 3.5. Modal MIC values of antimicrobial controls from the secondary peptidome set against selected bacterial strains in the presence of serum. All assays were carried out in triplicate.

Peptide	Sequence	MRSA MIC (µg/mL)	<i>P. aeruginosa</i> MIC (µg/mL)
AMP9J1	KRRVRWIIW	>64	>64
AMP9J2	RVKWWIRVR	>64	>64
AMPAGO2	FWGALIKGAALKLIPSVVGLFKKKQ	>64	>64
AMPAGO4	RIGVLLARLPKLFSLFKLMGKKV	>64	>64
AMPGRS2	KRRRWRIWLV	>64	>64
AMPNK1	VNWKKILGKIIKVVK	>64	>64
AMPNK2	FIGAIARLLSKIFGKR	>64	>64
KABAC7	RWRRPIRRRPIRPPFWR	>64	>64
KACAN1	IFIFKFFKKLIKQYKR	>64	>64
KACAN3	GQWRWLGWIWKKRRRL	>64	>64
KACAN4	RKFFKKIKKQYIRLLF	>64	>64
KACAN5	IKKLIKKNIKFFLMLH	>64	>64

Investigating Effects of Secondary Peptidome Group Peptides on Intracellular Calcium Release

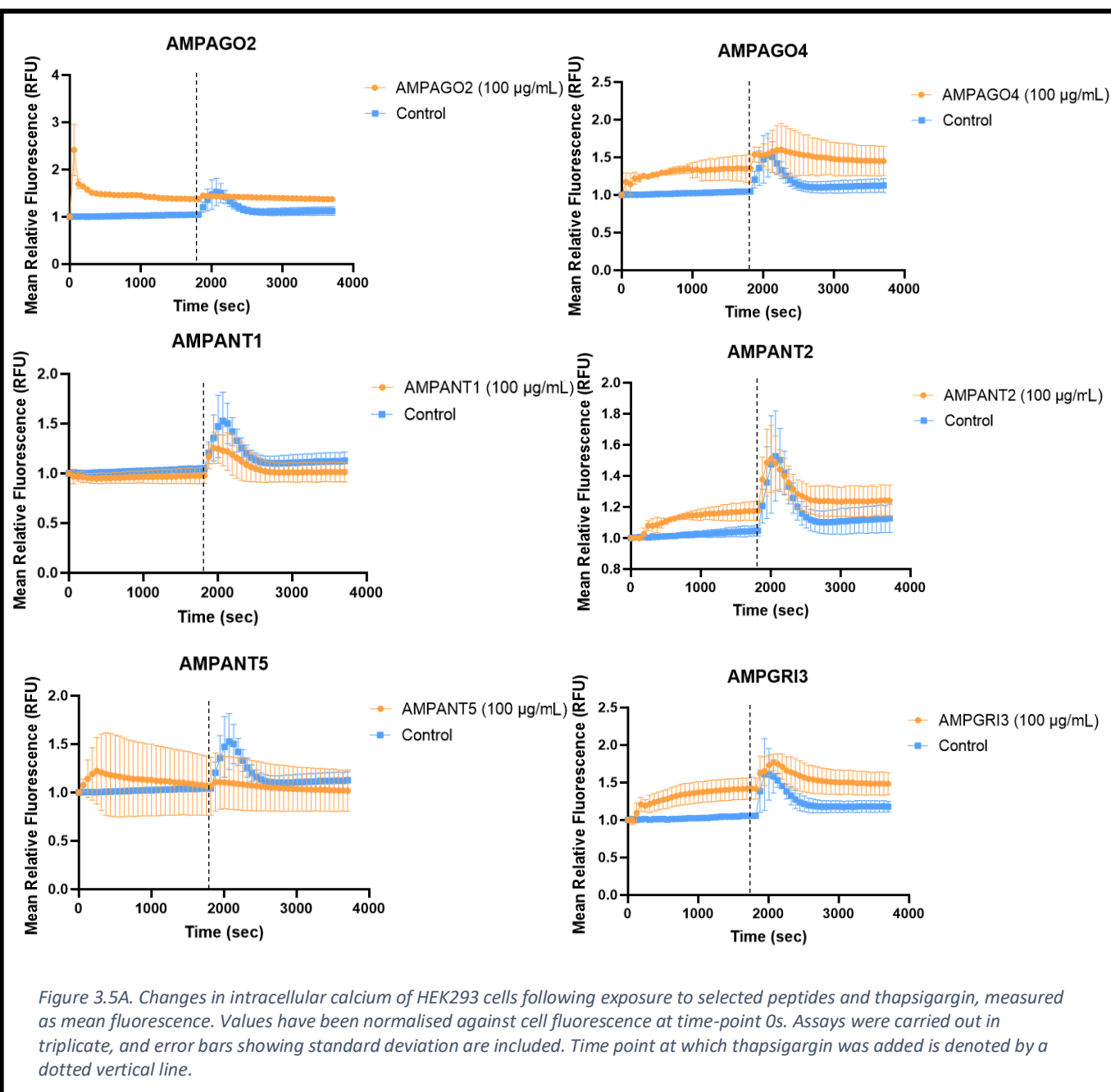
All peptides of interest from the secondary peptidome set were assessed for influences on HEK cell calcium release (**Figure 3.5A, 3.5B**). Of the 54 peptides evaluated, 11 were associated with changes in calcium fluorescence when compared to a non-peptide treated control. Notably, HEK cells treated with any 1 of 8 peptides (AMPAGO2, AMPAGO4, AMPANT2, AMPANT5, AMPGRI3, AMPNK1, AMPNK2, and KACAN3) exhibited an increase in mean relative fluorescence prior to the addition of thapsigargin; this increased fluorescence persisted to the end of the assay after the effects of thapsigargin had been introduced and resolved. HEK cells treated with AMPNK1, AMPNK2, or KACAN3 also displayed a greater increase in mean fluorescence upon addition of thapsigargin than those treated with other peptides or when compared to the untreated control.

Contrarily, cells treated with AMPANT1, AMPANT5, or KACAN1 showed a suppressed increase in mean fluorescence upon addition of thapsigargin when compared to the control; this ranges from a noticeable reduction of the thapsigargin-associated fluorescence increase – as is seen with AMPANT1 – to an immediate reduction in fluorescence to levels below baseline, as is the case with KACAN3. Mean relative fluorescence of HEK cells treated with KACAN1 did not return to baseline levels at any point of the assay following exposure. Finally, cells treated

with AMPGRS1 display a delayed response to introduction of thapsigargin before returning to fluorescence levels comparable to the control once the thapsigargin effect had been resolved.

Dunnett's multiple comparisons tests following two-way ANOVA identified statistically significant differences in mean relative fluorescence ($P < .05$, $n=3$) between untreated control cells and cells treated with AMPAGO2 from minute 4 to minute 30 of incubation following treatment. This significance ends upon addition of thapsigargin - at which point the fluorescence of AMPAGO2-treated cells does not exceed those of untreated cells to a significant degree - before resuming at minute 40 until minute 45. Significant difference was also seen between untreated cells and cells treated with AMPAGO4 from minute 5 to minute 8 of incubation following treatment, as well as between cells treated with AMPANT2 and the untreated cells from minute 8 to minute 11, and between cells treated with AMPNK2 and untreated cells from minute 2 to minute 7.

Finally, statistically significant difference was found at three intervals between the untreated cell population and cells treated with AMPNK1: from minute 5 to minute 7, minute 38 to 41, and from minute 43 until the end of the assay.



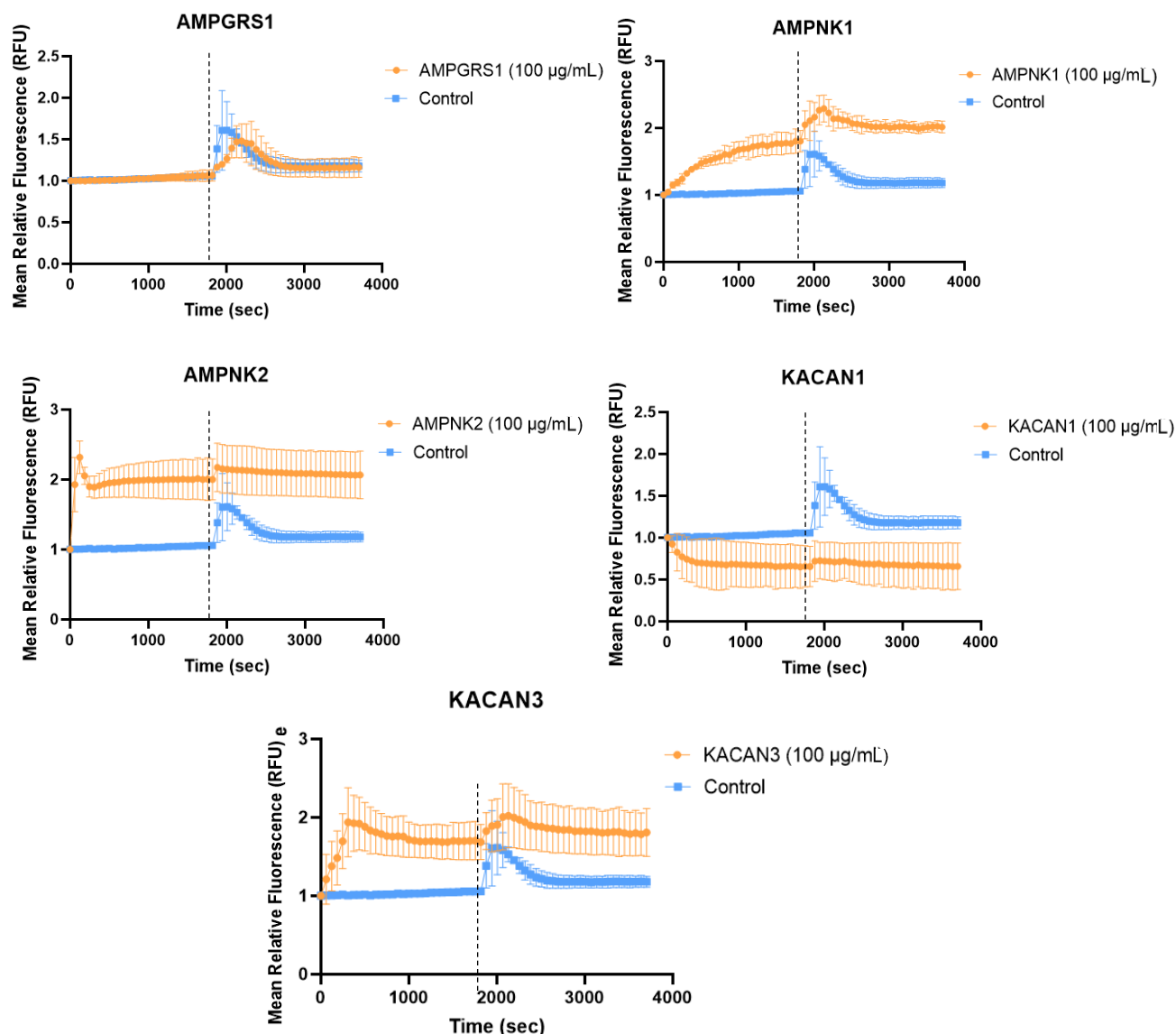


Figure 3.5B. Changes in intracellular calcium of HEK293 cells following exposure to selected peptides and thapsigargin, measured as mean fluorescence. Values have been normalised against cell fluorescence at time-point 0s. Assays were carried out in triplicate, and error bars showing standard deviation are included. Time point at which thapsigargin was added is denoted by a dotted vertical line.

As before, calcium-mediated fluorescence assays were then repeated with selected peptides of interest to determine dose-response relationships influencing the observed changes in relative fluorescence. Baseline fluorescence of HEK cells was recorded at time-point 0s, immediately following addition of peptide solution; this value was then subtracted from the highest relative fluorescence shown by HEK293 cells in the 30 minutes prior to (**Figure 3.6**) or following (**Figure 3.7**) addition of thapsigargin. Calculated fluorescence deltas were then subjected to one-way ANOVA, after which multiple comparisons tests were conducted between delta values exhibited by peptide-treated cells and those of the untreated control population at the same point in time, as well as those shown by cells treated with the same peptide at the 3 varying concentrations.

Intensity of independent changes to HEK cell fluorescence – changes that were seen prior to the addition of thapsigargin – appeared to consistently increase as peptide concentration increased for cell populations treated with either AMPAGO2, AMPNK1, or KACAN3. In addition, cells treated with peptides AMPAGO4, AMPANT2, and AMPGRI3 all display considerable increase in mean fluorescence delta at the highest tested concentration of 100 µg/mL when compared to the 1 µg/mL and 0 µg/mL populations; a similar trend is shown by cells treated with AMPNK2, although this increase occurs between 1 µg/mL and 10 µg/mL solution concentrations, with the mean delta remaining fairly constant when the concentration is raised to 100 µg/mL from 10 µg/mL. Statistical analysis of the differences between responses of cell populations treated with any peptide candidates did not identify significance ($P < .05$, $n=3$), but significance was found in the difference between responses for cells treated at 0, 1, 10, or 100 µg/mL for peptides AMPAGO2 ($P=.0179$, $n=3$) AMPAGO4 ($P=.0161$, $n=3$), or AMPNK1 ($P=.0144$, $n=3$). However, the source of this significance could not be identified following multiple comparisons testing.

Consistent trends in mean fluorescence delta of cells after the addition of thapsigargin were observed for AMPNK2-treated cells, where mean delta increased as peptide concentration increased, as well as for peptides AMPANT2 and KACAN1, where mean fluorescence delta decreased as peptide concentration decreased. Statistical analysis did not identify significance in the difference between cell responses following individual peptide treatments, nor due to changes concentration of peptide used.

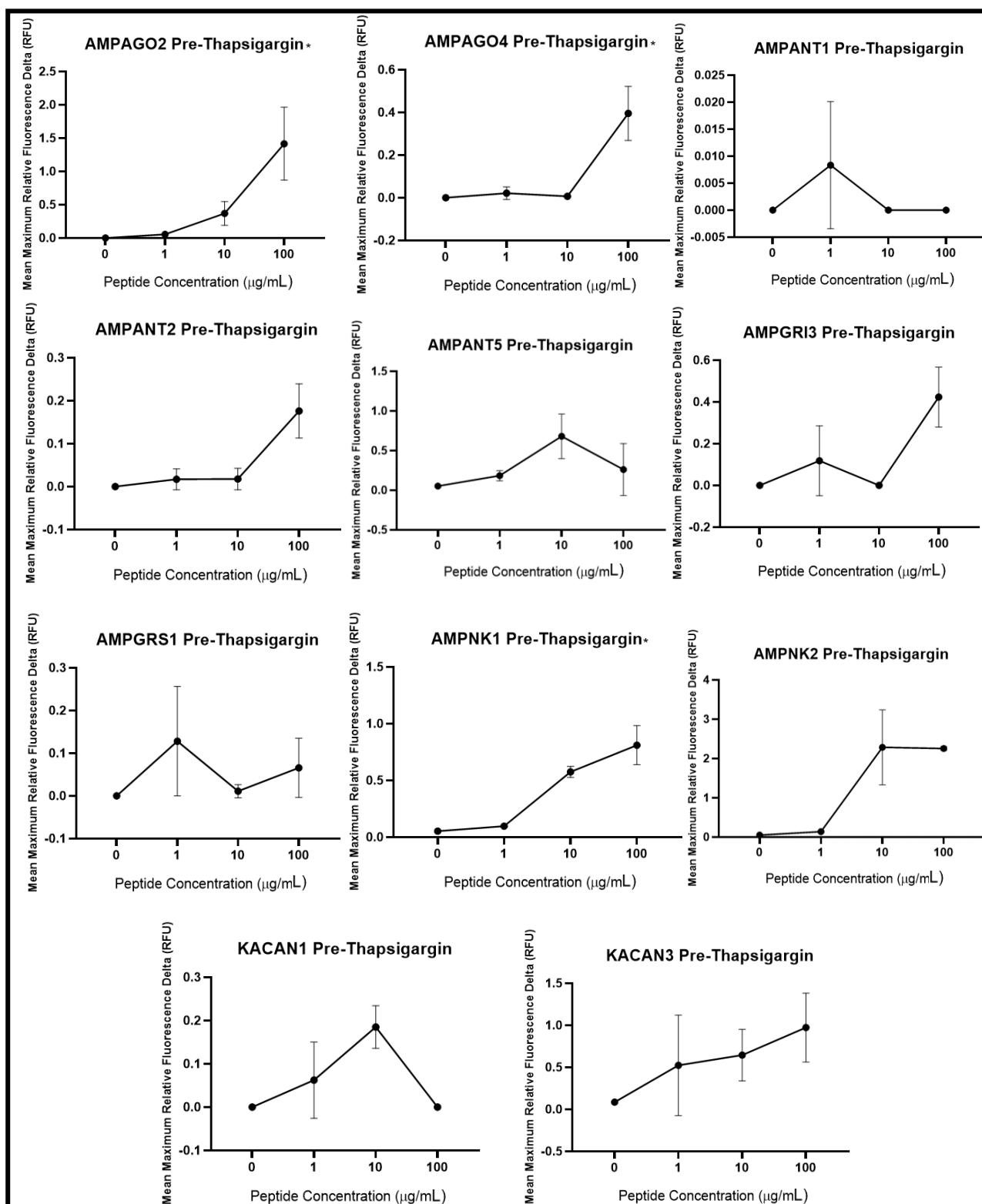
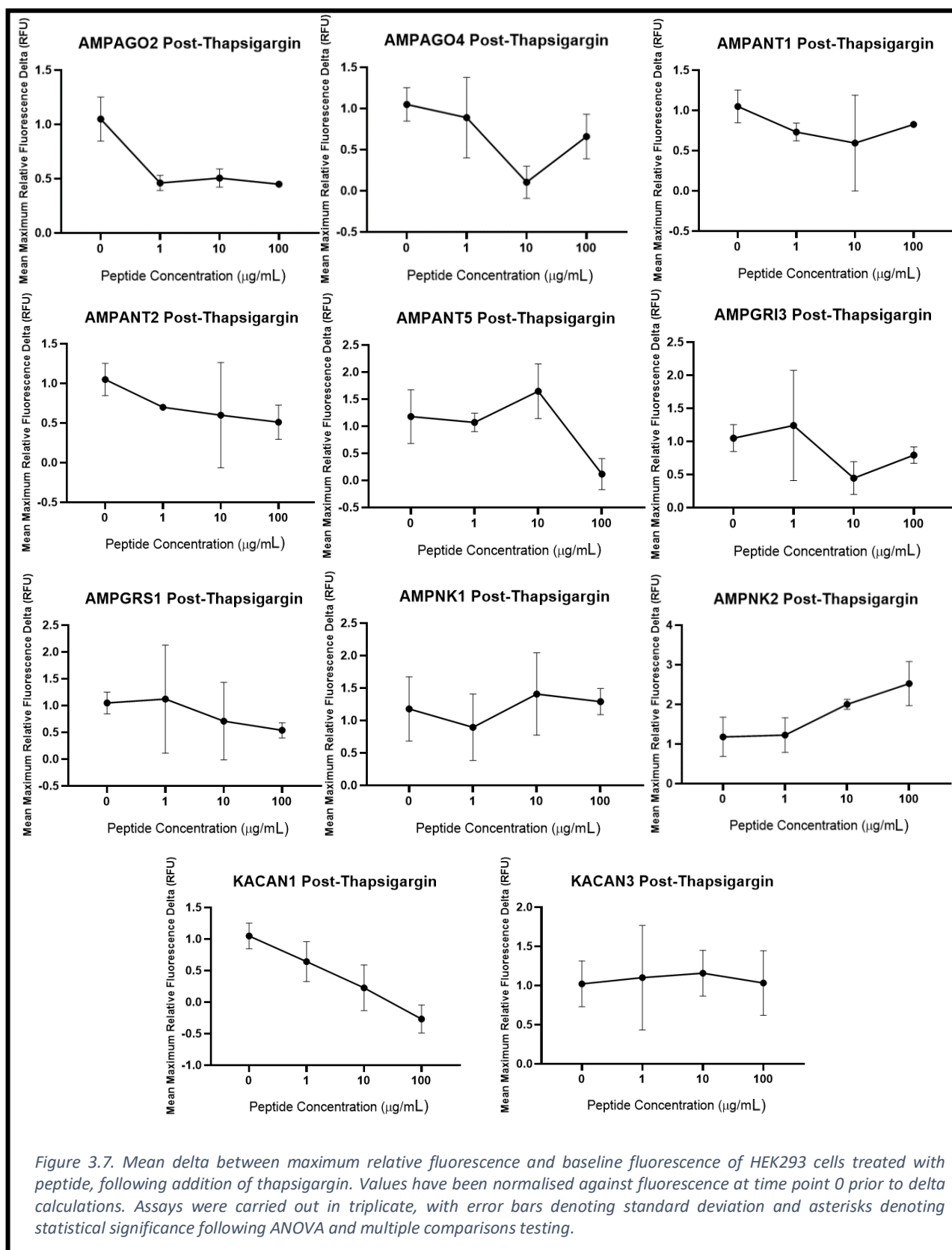
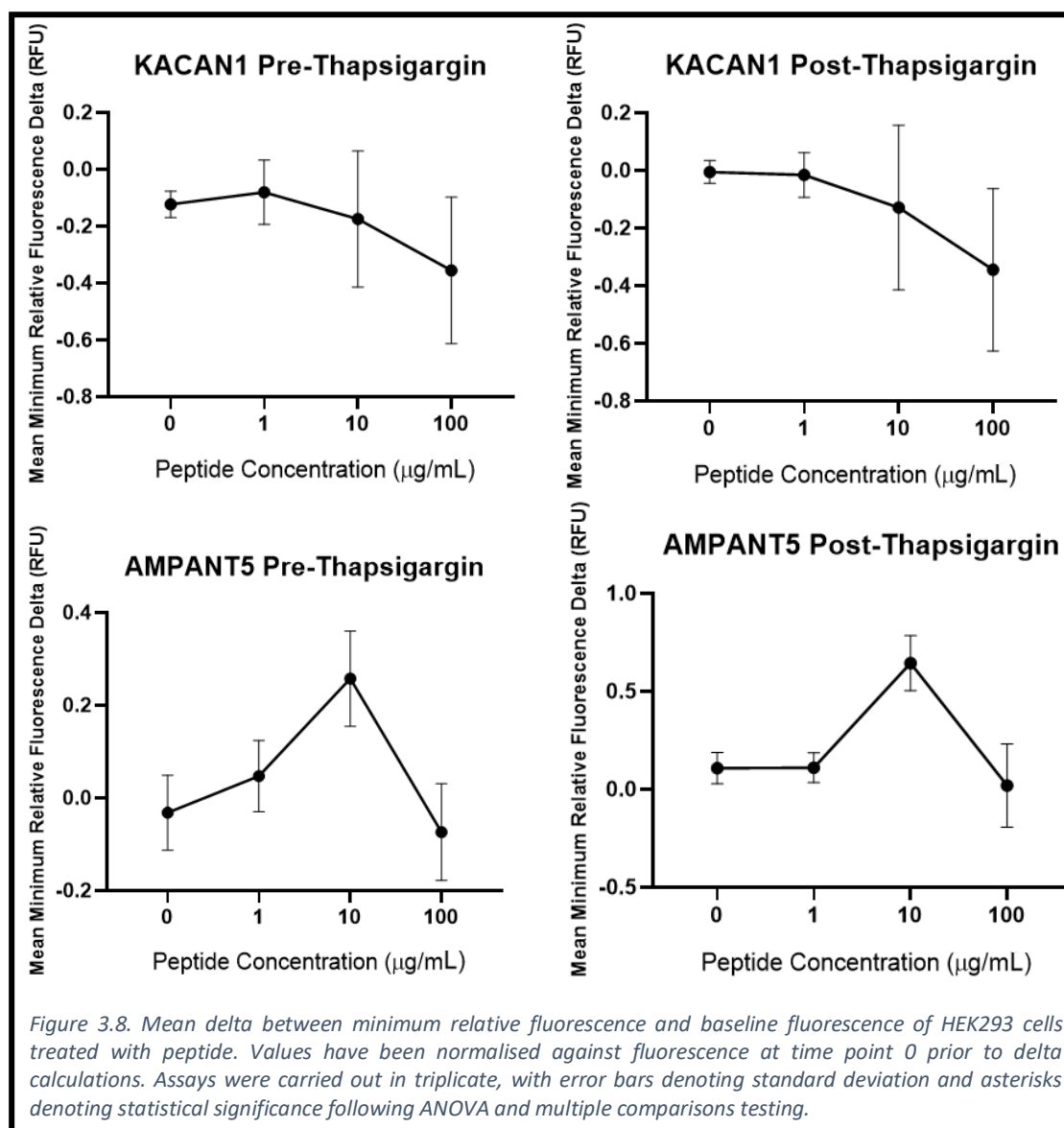


Figure 3.6. Mean delta between maximum relative fluorescence and baseline fluorescence of HEK293 cells treated with peptide, prior to addition of thapsigargin. Values have been normalised against fluorescence at time point 0 prior to delta calculations. Assays were carried out in triplicate, with error bars denoting standard deviation and asterisks denoting statistical significance following ANOVA and multiple comparisons testing.



For peptide-treated cells where mean relative fluorescence was reduced to below baseline – as was observed for cells treated with KACAN1 and AMPANT5 – an additional analysis was carried out where baseline fluorescence was instead subtracted from the lowest relative

fluorescence shown in either 30 minute time period (**Figure 3.8**). Mean fluorescence delta of cells treated with KACAN1 decreased as concentration of peptide increased both before and after addition of thapsigargin, although the pre-thapsigargin mean fluorescence of cells treated with a 1 $\mu\text{g/mL}$ solution was greater than the untreated 0 $\mu\text{g/mL}$ population. Additionally, cells treated with AMPANT5 showed considerable divergence in fluorescence delta when treated at 10 $\mu\text{g/mL}$ compared the other tested concentrations. However, no statistical significance was found following analysis of differences between fluorescence deltas of the two peptides, nor of either peptide tested at the 3 differing concentrations.



Investigating Secondary Peptidome Group Cell Cytotoxicity

Analysis of maximum fluorescence prompted investigation into cytotoxic capacity of the peptides of interest; it was hypothesised that observed increases in calcium-mediated fluorescence – specifically increases that were independent of thapsigargin action - could be due to calcium release as a result of cell lysis as opposed to any peptide interactions with intracellular calcium stores. HEK293 cells were once again treated with one of 54 peptides and incubated; resultant viability of treated cells was quantified via the addition of resazurin, and fluorescence was read at 1 and 2 hours post-treatment (**Figure 3.9**). Percentage of cell viability was normalised against an untreated control population. Of the 5 established peptides of interest (AMPAGO2, AMPANT5, AMPNK1, AMPNK2, and KACAN3), AMPAGO2 and AMPNK2 were associated with reduction in HEK cell viability following treatment, with AMPAGO2-treated cells exhibiting 0% viability after both 1 and 2 hours incubation and AMPNK2-treated cells exhibiting 33.9% viability after 1 hour before decreasing further to 21.1% after 2 hours. Reduction in cell viability was not seen following treatment with AMPANT5, AMPNK1 or KACAN3, with viability instead exceeding that of the untreated population after 1 hour and remaining above 100% after 2 hours incubation.

Of the remaining 49 peptides – excluding the established peptides of interest – cells treated with any 1 of 10 peptides (AMPANT1, AMPANT4, AMPGRI2, AMPGRS2, DEBY1, DEBY2, KABAC7, KACAN1, KACAN2, and KACAN5) displayed an increase in cell viability greater than 20% when compared to the untreated control population after 1 hour of incubation. Of these populations, cells treated with DEBY1 exhibited greatest viability with a calculated percentage viability of 153.0%. Cells treated with any 1 of 5 peptides within this group of 10 (AMPANT4, DEBY1, DEBY2, KACAN2, and KACAN5) maintained a 20% or greater relative increase in cell viability after 2 hours of incubation; this was also seen for cells treated with any 1 of an additional 8 peptides (AMPANT3, AMPGRI1, LUAGO4, LUAGO5, LUGRI3, LUGRP4, LURRP5, and LURRP8) for a total of 13. Within this group of 13, the greatest increase in cell viability was exhibited by cells treated with KACAN5 – with a viability of 154.1% - while the viability of cells treated with DEBY1 had dropped to 146.3%.

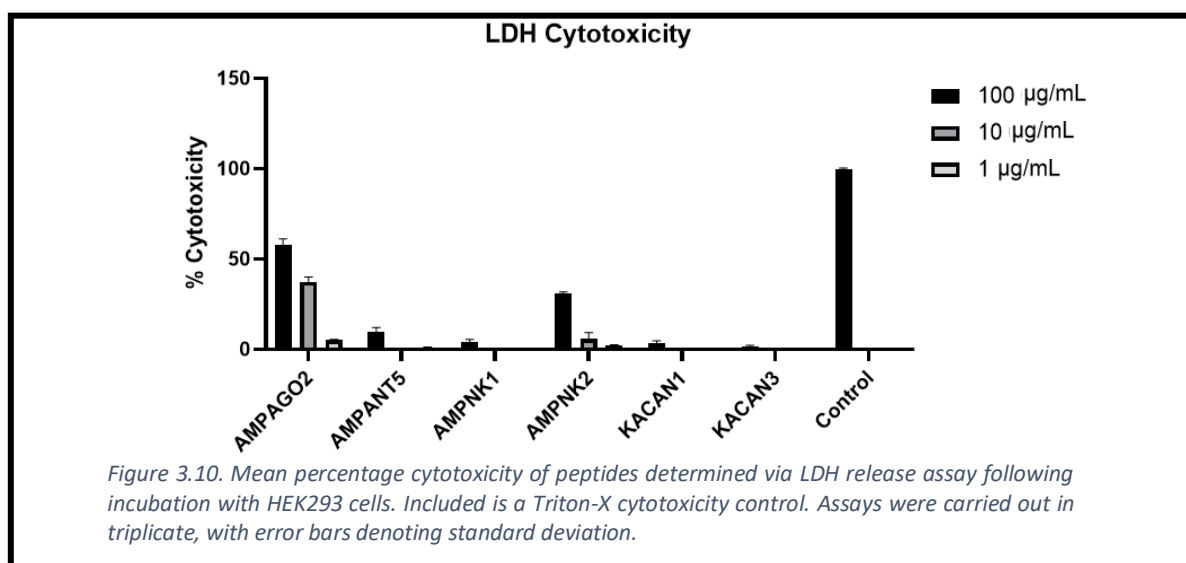
Contrarily, cells treated with 9 of these 49 peptides (Peptides LUAGO7, LUANT3, LUGRI1, LUGRI4, LUGRP2, LURRP6, LURRP9, LURRP10, and LURRP11) displayed a reduction in relative viability after 1 hour incubation, with the greatest reduction in viability – being reduced to 54.3% - observed in cells treated with LUGRI4. Cells treated with 6 of these 9 peptides (LUAGO7, LUANT3, LUGRI1, LURRP6, LURRP9, and LURRP11) were able to reach a relative viability of 80% or higher by 2 hours of incubation, with LUGRI1-treated cells showing the greatest increase in relative viability between these two timepoints (+28.2%). Of the total population of 54 peptides, no other peptide had a viability as low as those treated with AMPAGO2, with AMPNK2-treated cells achieving the second-highest reduction in viability at

Figure 3.9 displays five bar charts showing the mean percentage viability of various peptide solutions (100 µg/ml) determined via resazurin-mediated fluorescence following 1hr and 2hr incubation with HEK293 cells. The y-axis represents Viability (%) from 0 to 200. The x-axis lists the peptides. Each peptide has two bars: a black bar for 1 hour and a grey bar for 2 hours. Error bars represent standard deviation.

The peptides are grouped into five rows:

- Row 1:** AMP9J1, AMP9J2, AMPAGO1, AMPAGO2, AMPAGO3, AMPAGO4, AMPANT1, AMPANT2, AMPANT3, AMPANT4, AMPANT5, AMPGR11.
- Row 2:** AMPGR12, AMPGR13, AMPGRS1, AMPGRS2, AMPNK1, AMPNK2, DEBY1, DEBY2, KABAC7, KACAN1, KACAN2, KACAN3.
- Row 3:** KACAN4, KACAN5, LUAGO1, LUAGO2, LUAGO3, LUAGO4, LUAGO5, LUAGO6, LUAGO7, LUANT1, LUANT2, LUANT3.
- Row 4:** LUANT4, LUANT5, LUGR1, LUGR2, LUGR3, LUGR4, LUGR5, LUGRP1, LUGRP2, LUGRP3, LUGRP4, LURRP5.
- Row 5:** LURRP6, LURRP7, LURRP8, LURRP9, LURRP10, LURRP11.

Potential cytotoxicity of established peptides of interest (AMPAGO2, AMPANT5, AMPNK1, AMPNK2, and KACAN3) as well as the peptide most strongly associated with reduction of calcium-mediated fluorescence (KACAN1) was further investigated via evaluation of LDH release following peptide treatment (**Figure 3.10**). Of these 6 peptides, AMPAGO2 and AMPNK2 displayed cytotoxicity above 10%: 57.7%, and 31.0% respectively at the highest tested concentration of 100 $\mu\text{g/mL}$. When tested at 10 $\mu\text{g/mL}$, only AMPAGO2 exhibited cytotoxicity above 10% - 37.4% - and no cytotoxicity exceeding 10% was seen of any peptide when tested at the lowest concentration.

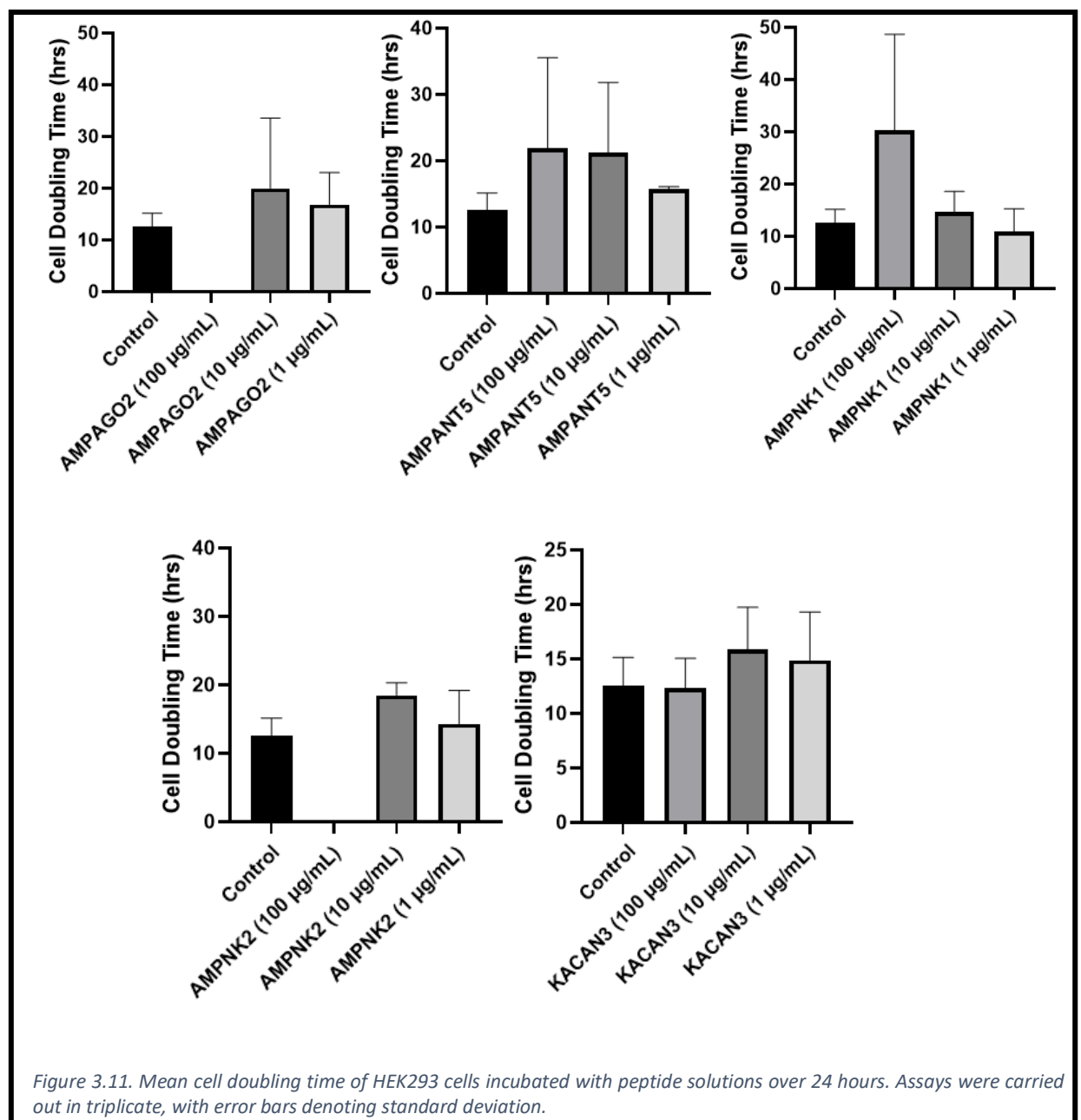


Investigating Effects of Secondary Peptidome Group on HEK Cell Proliferation

Following indications of increased HEK cell viability, effects of peptides within the secondary peptidome group were monitored via phase microscopy to determine whether stimulatory or inhibitory effects on HEK cell proliferation could be directly observed (**Figure 3.11**). Of the established peptides of interest (AMPAGO2, AMPANT5, AMPNK1, AMPNK2, and KACAN3), all but KACAN3 displayed increases in mean cell doubling time at 100 $\mu\text{g/mL}$ when compared to the control; in the case of AMPAGO2 and AMPNK2, this was to the extent that cells did not replicate following exposure to the peptide or at all over 24 hours. Similarly, all peptides of interest increased mean cell doubling time at 10 $\mu\text{g/mL}$ with the greatest increase being seen in cells treated with AMPANT5 (+8.7 hours), followed by AMPAGO2 (+7.5 hours). At 1 $\mu\text{g/mL}$, all peptides of interest continued to increase mean cell doubling time when compared to the control, with the exception of AMPNK1 which instead exhibited a reduction of 1.6 hours.

Of the remaining 49 peptides tested, 19 (LUAGO5 - 7, LUANT1 - 4, LUGRI2 - 5, LUGRP1 & 3, and LURRP6 - 10) reduced mean cell doubling time by 5 hours or more when compared to

the untreated control population (**Figure 3.12A, 3.12B**). Cells treated with 100 $\mu\text{g/mL}$ LUGRI3 exhibited the greatest reduction in cell doubling time of these peptides – and therefore of all peptides tested –, with a mean time 11.3 hours lower than that of the untreated control population. Dose-dependent nature of these effects varied between peptides, with solutions of LUAGO7 displaying more potent reduction as the concentration decreased; contrarily, LUGRI2, LUGRI3, LUGRI5, LUGRP1, LUGRP3, and LURRP8 all showed diminishing effects as concentration of peptide solution decreased. Peptides LUAGO6, LUANT1, LUANT2, LUGRI1, LUGRI4, LURRP6, and LURRP7 all displayed greater reductions in mean cell doubling time at 10 $\mu\text{g/mL}$ than at either of the other concentrations tested; a notable inverse to this trend was observed in cells treated with LUANT4, which reduced mean cell doubling time at 100 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ but completely halted cell division at 10 $\mu\text{g/mL}$.



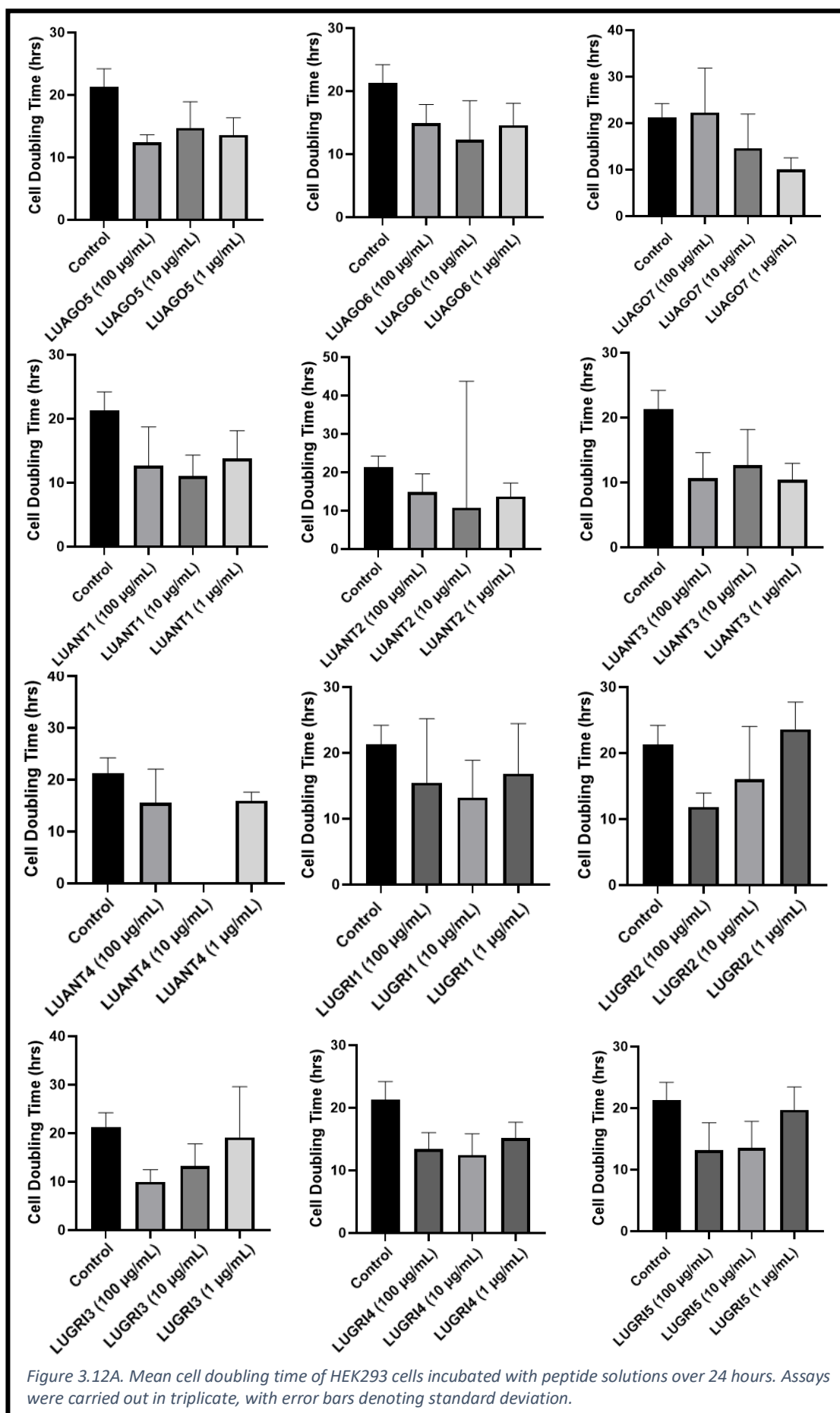


Figure 3.12A. Mean cell doubling time of HEK293 cells incubated with peptide solutions over 24 hours. Assays were carried out in triplicate, with error bars denoting standard deviation.

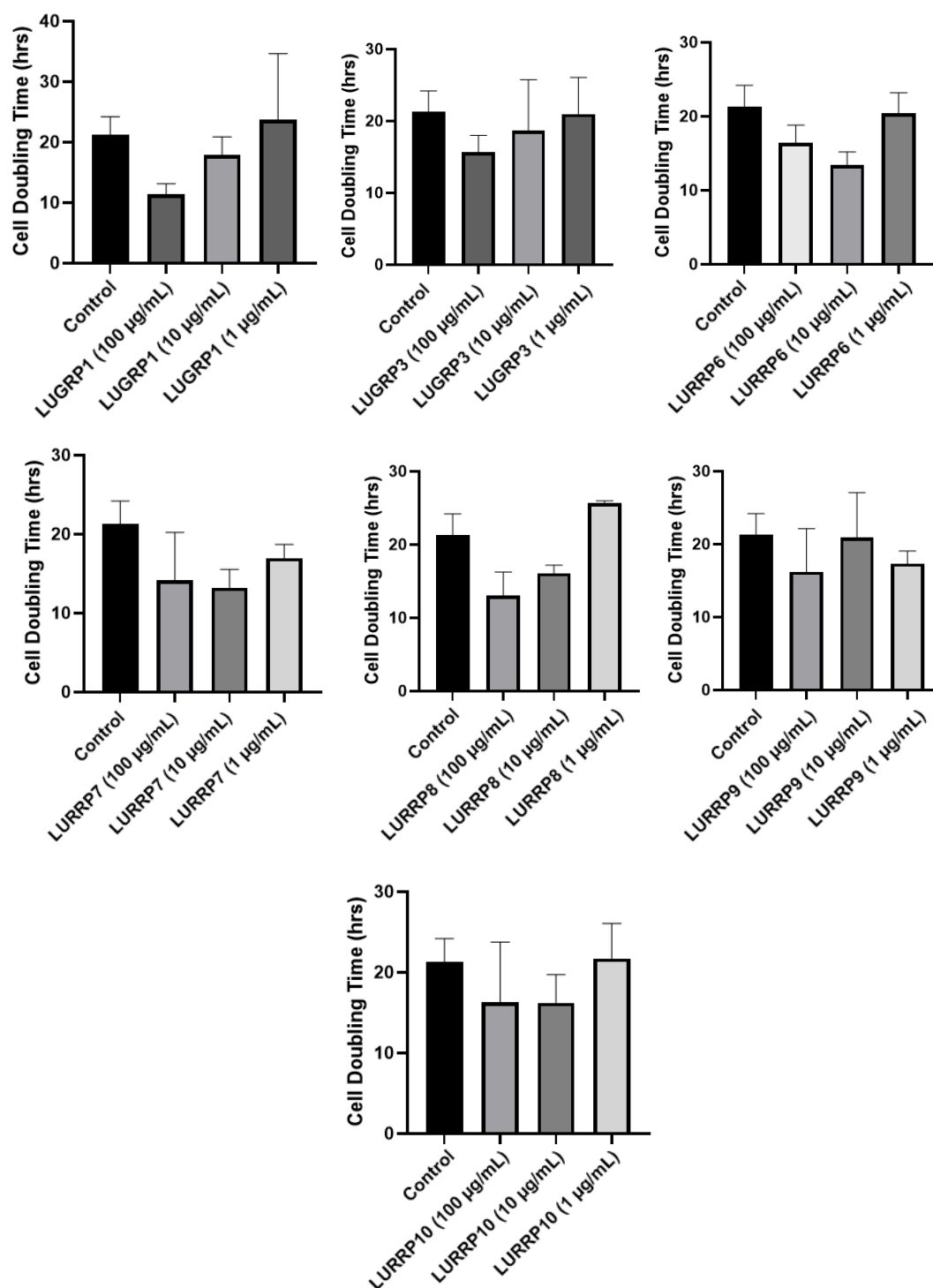


Figure 3.12B. Mean cell doubling time of HEK293 cells incubated with peptide solutions over 24 hours. Assays were carried out in triplicate, with error bars denoting standard deviation.

3.3 Discussion

Summary of Findings

An initial selection of 296 peptides were isolated from lung fluid samples taken from both healthy donors and patients with cystic fibrosis by a group of researchers at the University of North Carolina. Following SPOT synthesis, crude variants of these peptides were screened for antimicrobial activity against multidrug resistant strains of *S. aureus* and *P. aeruginosa* and compared against an experimentally validated in-house control, with potential candidates being purified for further elucidation of efficacy. Following evaluation of purified candidate variants, no antimicrobial activity could be identified for the candidates on their own, nor in combination with other selected candidate peptides.

Further investigation of peptide bioactivity identified a number of correlations between peptide incubation and resultant calcium release within human embryonic kidney cells; however, changes in intracellular calcium following peptide treatment were determined to be statistically insignificant.

A secondary selection of candidates was independently assessed by the North Carolina group for influences on calcium signalling and promotion of cell proliferation. Candidates selected from these assays were screened for antimicrobial activity which yielded 12 peptides with known MICs against the tested multidrug resistant strains. Efficacy of these peptides was discovered to be sensitive to the presence of serum, such that MIC following incubation in 25% serum solution exceeded the highest concentration tested. This secondary set was then tested for influences on human embryonic kidney cell calcium signalling, identifying five candidates that were associated with increases in calcium-dependent fluorescence, or reduced response to the inhibitory action of thapsigargin. Statistically significant differences were found between a number of candidates within this set compared to untreated populations when investigating changes in intracellular calcium release, changes to the cellular response to thapsigargin, as well as changes to strength of response depending on peptide concentration. However, the sources of observed significance have yet to be fully elucidated.

Cytotoxic capacity of these candidates was also investigated, and it was found that 2 of the 5 peptides identified via calcium-fluorescence assay were associated with reduced cell viability; cells treated with the remaining peptides instead exhibited greater cell viability following 1 and 2 hours incubation. Furthermore, peptides within the secondary candidate group were also associated with increased viability of treated cells, and so were further investigated using phase microscopy. Microscopy identified several peptides associated with reduced cell doubling time – and thus increased cell replication – although this did not appear to correlate with the increases in cell viability previously observed.

Interpretations of Findings

The MIC assay of the in-house control peptide identified that it has potent activity against the tested *E. coli*, *P. aeruginosa*, and MRSA strains. This has significant implications due to the methodology of the antimicrobial assays, which use this control peptide as a reference for categorisation of antimicrobial activity. While this approach serves to identify peptides of similarly or greater activity, it should be noted that AMPs less potent than the control – such as those categorised as ‘less active’ - may still possess therapeutic viability, particularly following optimisation (232,233).

This project’s interest in the lung peptidome as a viable source of AMPs arose partially from the hypothesis that the susceptibility of cystic fibrosis patients to recurrent pathogenic infection would be reflected in the peptidome, yielding more antimicrobial compounds than would otherwise be found. The inclusion of the preliminary antimicrobial screen following SPOT synthesis of the primary peptidome group was to provide a relatively high-throughput means of identifying antimicrobial peptides through their observed impact on fluorescence or luminescence associated with living bacterial cells. However, while preliminary screening of crude peptides isolated from the lung peptidome presented active candidates, no antimicrobial activity could be determined following purification and re-evaluation.

There are several potential explanations for this occurrence. It should be noted that the sequences of the crude peptides synthesised via SPOT will differ from their purified variants synthesised on resin, in that the SPOT peptides will possess a glycine residue on their C-terminal; this is due to the requirement of a spacer molecule between the peptide chain and the cellulose membrane during the SPOT coupling process. Glycine was chosen as a suitable spacer due to its relatively high stability (234), and its position at the end of the peptide chains mitigates the risk of structural alteration due to the inclusion of an additional amino acid, despite its flexibility. Regardless, the possibility remains that the activity exhibited by the crude peptide variants was a result of the presence of the additional glycine residue – and the subsequent chemical or structural alterations this may have induced – which would therefore not be observed by the pure variants.

One must also consider that while the preliminary screen was conducted against *P. aeruginosa* strain H174 – a genetically modified variant of strain H1001 (235) -, the subsequent MICs were instead against strain PAO1; while strain H174 was initially included to take advantage of the greater sensitivity gained from the luminescence assay, strain PAO1 has the advantage of greater characterisation in literature due to its more common use in laboratory settings (236,237). However, this alteration does affect the direct comparability of the preliminary screening and subsequent MIC assay data. While this could potentially explain the discrepancy seen between the anti-*Pseudomonas* activity data, it does not account for the reduction in

activity seen against MRSA, for which the same strain was used throughout. That said, of the 7 peptide candidates tested against MRSA following the preliminary screen of the primary peptidome group, only 2 of them (Peptides M1 and M2) were selected for anti-*Staphylococcus* activity; it is therefore unlikely that this is the sole cause of the discrepancies seen.

Upon consideration of the candidates' sequences and resultant properties, it is notable that the purified candidates identified in the primary set yielded no activity, despite each peptide sequence incorporating a number of hydrophobic residues, and all but 2 of them (peptides P12 and P13) possessing a net positive charge. As previously stated, the presence of these characteristics is associated with antimicrobial activity, as they can facilitate greater attraction to and interaction with the bacterial membrane; it is reported that hydrophobicity contributes greatly to a peptide's capacity to insert into the target membrane (238,239). However, it should be noted that peptide activity remains difficult to predict, due to the multitude of factors that confer and influence antimicrobial properties (239). It is possible that one or more of these factors is insufficient or unsuitable for these candidates, hindering their antimicrobial potential.

MIC assays of the purified secondary peptide group identified candidates with activity against both *E. coli* and *P. aeruginosa*, with the lowest MIC exhibited against both bacteria being 4 µg/mL. However, MICs against the tested MRSA strain were considerably higher; this has particular significance upon consideration that *S. aureus* is the only gram-positive bacterium of the three organisms tested. As previously mentioned, selective action of AMPs is often dependent on the electrostatic attraction that occurs between the cationic peptide and the anionic pathogen (217). Both gram-negative and gram-positive bacteria possess a membrane that is rich in negatively charged phospholipids, but this property is further compounded by negatively charged teichoic acids in the cell wall of gram-positive bacteria as well as negative lipopolysaccharides in the outer membrane of gram-negative bacteria (240); that is to say that the properties contributing to the selective mechanisms of AMPs vary between gram-positive and gram-negative bacteria, which may manifest as observable differences in efficacy between the two bacterial categories.

This occurrence may also explain why active peptides that were excluded from entry into the primary peptidome group following preliminary antimicrobial screening were later identified as efficacious following independent selection by the Tarran group. 1 of the 2 pathogens utilised in the preliminary antimicrobial screen was E-MRSA strain 15; the same strain that all but a few peptides proved to be relatively ineffective against during the MIC assays. They therefore would have likely been eliminated as candidates due to the relatively poor antimicrobial activity score given to them by the SciXMiner software.

All candidates displayed considerable reduction of efficacy in the presence of serum; this is an arguably expected outcome upon consideration of the components within serum, such as its

binding proteins and proteolytic enzymes. While the peptide candidates do possess cleavage sites for certain proteases found in humans – trypsin and chymotrypsin, and plasmin – these would not reasonably be active in serum due to the presence of various serine protease inhibitors (241); it is thus more likely that the observed reduction in efficacy can be attributed to binding proteins. Regardless, antimicrobial evaluation identified a number of efficacious peptides including 2 peptides (AMPAGO4 and KACAN1) that exhibited broad-spectrum activity against all three tested strains *in vitro*. This provides a promising foundation for the development of a novel therapeutic.

All but 1 of the 7 peptides tested in the primary peptidome group reduced the rise in intracellular calcium in response to thapsigargin. As mentioned previously, thapsigargin acts on the SERCA ion pump to inhibit re-uptake into the endoplasmic reticulum, causing a rapid build-up of calcium within the cytosol (231). In the presence of a calcium-specific fluorophore, this results in an increase in fluorescence. A reduction of this fluorescence increase therefore suggests a reduction in cytosolic calcium; this can be due to peptide-facilitated efflux of ions out of the cell or influx of ions into the endoplasmic reticulum, although the fact that no changes in calcium are seen prior to the addition of thapsigargin lends credence to the latter hypothesis. These changes are therapeutically relevant when one considers the implication of calcium signalling in inflammation; it has been reported in literature that a number of inflammatory mediators – such as histamine and bradykinin – increase intracellular calcium levels (242,243). Thus, the observed reductions in calcium-mediated fluorescence suggest potential anti-inflammatory properties of the peptides associated with them.

However, interpretation of these data is tempered by the fact that no statistically significant differences between treated and untreated cells were found following ANOVA and multiple comparison tests of maximum fluorescence ($P < .05$, $n=3$). This assessment is despite the apparent trends exhibited by the calcium fluorescence curves, most likely due to the large standard deviation of the averaged data points which in turn suggest considerable variability in the observable effects of the peptide at the tested concentrations. This variability was observed across a number of the assays conducted but was conspicuously absent in the untreated control populations, suggesting that there are indeed peptides within the tested group able to affect HEK cell growth and/or calcium signalling, but that these effects are inconsistent. Thus, while the null hypothesis must be accepted based on the data presented, it is not necessarily accurate to state that these peptides are inactive.

Peptides associated with increased or decreased calcium-mediated fluorescence were also identified within the secondary peptidome group; several of these peptides exhibited effects with statistically significant difference to the untreated control. Following assessment of cytotoxicity, 3 of these peptides were not only deemed non-toxic but they also displayed potential for promotion of HEK cell proliferation as they were also associated with increased

cell viability; among these peptides was KACAN3 - the only peptide that maintained statistical significance when comparing increases in maximum fluorescence to the untreated control across all tested concentrations – and AMPNK1, which maintained statistically significant differences at the two highest tested concentrations. However, neither of these peptides reduced cell doubling time following introduction to HEK cell culture and were instead associated with increased doubling time at multiple concentrations.

Regardless, it is notable that treatment of HEK cells with these peptides induced an increase in cytosolic calcium that was independent of thapsigargin action; this suggests either induction of calcium release from the endoplasmic reticulum, upregulation of calcium influx from the environment, or a peptide-mediated mechanism of re-uptake inhibition similar to that of thapsigargin itself. It is also important to note that fluorescence increase upon addition of thapsigargin in cells treated with these peptides exceeded that of cells treated with thapsigargin alone, suggesting a mechanism of cytosolic calcium increase that is independent of SERCA inhibition. If cell calcium signalling is established in the role of inflammation, it stands to reason that induction of calcium release is potentially indicative of inflammatory action; this hypothesis is supported by both the characterised effects of calcium signalling on immune cell activity as well as the immunomodulatory capacities displayed by other AMPs in literature (217,244,245).

In contrast, the peptide AMPANT5 displayed considerable capacity to suppress thapsigargin-associated fluorescence at 100 µg/mL, although no reduction in fluorescence is seen before thapsigargin is added; this reduction in maximum fluorescence was to a statistically significant degree, highlighting an encouraging candidate for potential anti-inflammatory action. Curiously, cells treated with the same peptide at 10 µg/mL displayed the opposite effect. While not as common as a direct dose-response relationship, peptides that exhibit structural and functional changes at different concentrations have been reported (246–248); further investigation into this property may therefore unearth considerable therapeutic applications due to the flexibility of its effects.

Unfortunately, HEK cell viability studies following incubation with peptide solution identified that 2 of the 5 promising candidates – peptides AMPAGO2 and AMPNK2 – severely reduced cell viability at concentrations associated with greater calcium fluorescence; it is thus more likely that the observed fluorescence was due to cell lysis and subsequent calcium release than any induced cell signalling response, which renders them unsuitable for therapeutic application. This hypothesis was further supported by the microscopy assays, which identified no growth following treatment of these peptides at the highest tested concentration. Contrarily, cells treated with the remaining 3 peptides – AMPANT5, AMPNK1, and KACAN3 – exhibited considerably greater viability than the untreated control. This has interesting implications due to the discovery that while cells treated with AMPNK1 or KACAN3 displayed increased calcium

fluorescence, the opposite was seen from cells treated with AMPANT5 at the concentrations used for the assessment of cytotoxicity. Further investigation into the trends uncovered through these assays therefore has the potential to elucidate multiple calcium-mediated pathways of promotion of cell proliferation.

Ultimately, the data gained from the microscopy growth assays casts doubt on the correlation between cell viability observed during the cytotoxicity assays and actual HEK cell proliferation, as of the 18 peptides associated with increased cell viability over either 1 or 2 hours of incubation, only 2 of those peptides produced an observable reduction in cell doubling time. However, there does appear to be a link between low cell viability and increased cell doubling time – as well as peptide cytotoxicity as determined via LDH assay and increased cell doubling time - which is most strongly exhibited by peptides AMPAGO2 and AMPNK2 which displayed both lowest viability and highest toxicity.

Intracellular calcium levels have long been implicated in regulation of the cell cycle (249). However, this study has identified peptides with no statistically significant effects on intracellular calcium that regardless showed an effect on cell growth; this could suggest either that the peptides employ a calcium-independent mechanism of cell cycle regulation, or that the effect is so variable at the tested concentrations that significance is difficult to determine. It therefore stands to reason that there is value in investigating the validity of the latter hypothesis, such that it can be confirmed or refuted. A combination of assays carried out at higher concentrations – that would facilitate more consistent peptide effects – and a separate assay with a greater threshold for reporting calcium activity may shed light on the matter, allowing the therapeutic potential of these peptides to be more adequately determined.

As previously mentioned, peptides within the secondary peptidome set were given designations based on properties reported by the UNC team. While this investigation identified peptides associated with increases and decreases in calcium release, the total number is far fewer than the total population of -ANT and -AGO labelled peptides reported to inhibit or promote calcium release respectively. Similarly, of the 19 peptides associated with decreased cell doubling time following microscopy, 7 of them had been previously associated with promotion of cell growth compared to the 9 that had reportedly exhibited cell growth inhibition. Regardless, the data of this project identify several low-toxicity peptides isolated from the lung peptidome with the capacity to induce or inhibit cellular calcium release and/or cell proliferation.

Limitations

While critical for the large-scale synthesis of potential peptide candidates, the nature of SPOT synthesis introduces limitations that must be considered when interpreting the output of this study. To begin, due to the large number of peptides being synthesised in parallel, directly monitoring success and quality of amino acid coupling is not feasible; this means that side

products – unwanted variations of the desired amino acid sequence – may have arisen over the course of this synthesis and facilitated the discrepancy in efficacy seen between the crude and pure antimicrobial assays. Although this is mitigated somewhat by the inclusion of quality control peptide sequences, the presence of side products may have contributed to the antimicrobial activity initially observed by acting in place of or in combination with the peptide of interest; it has been observed in literature that peptides have the capacity to work in synergy with other components of immunity to induce a greater antimicrobial effect. This has been seen, for example, in the case of the AMP LL-37 (250,251).

An additional drawback of this synthesis method is that the total quantity of the completed peptide is difficult to measure, and so resultant solutions made with said peptide are of unknown concentration; it is for this reason that preliminary screening activity is presented in terms of dilution factor and not concentration of solution. What this means is that while concentration of peptide solution was known for the subsequent MIC assays, concentration of any one peptide solution may have varied between assays which could contribute to the discrepancies observed between the two investigations. Yield of synthesis will also vary from peptide to peptide on the SPOT cellulose membrane, which must be considered when attempting to make direct comparisons of efficacy between products of synthesis.

Finally, while serving as practical and accessible determinants of antimicrobial screening, neither the preliminary screen nor the subsequent MIC protocols attempt to emulate the environment of the respiratory tract where a number of these peptides of interest were sourced. It is known that environmental factors such as pH and salt concentration can influence AMP activity, as can the presence or absence of components such as binding proteins and proteolytic enzymes (252,253). Thus, repeats of the assay incorporating a more suitable medium – such as minimal medium or glucose solution, for example – may serve to facilitate greater activity among the peptide set.

The employed calcium fluorescence assay has the advantage of considerable sensitivity; even slight modifications to calcium-mediated fluorescence can be detected and attributed to peptide activity. However, the assay is limited in its ability to characterise those modifications once they have been detected and thus it is unclear, for example, how these changes in calcium fluorescence arise and therefore if they can indeed be linked to pro- or anti-inflammatory activity. However, the dose-dependent nature of these calcium changes does implicate the peptides in their manifestation at the very least.

A secondary purpose of the resazurin cytotoxicity assay – besides quantifying peptide toxicity – was identifying whether said toxicity was confounding the calcium fluorescence data; as the calcium assays took place over an hour, resazurin-mediated fluorescence was measured after an hour of incubation for comparison. However, cell viability assay protocols involving resazurin

recommend between 1 and 4 hours for the dye to fully permeate (254); it is for this reason that measurements were also carried out after 2 hours. Thus, while the fluorescence recorded after 1 hour provides a minimum value for cell viability, the true capacity of the peptides at this time point may not have been seen. This is a factor that must be taken into consideration when interpreting the data from this assay, as well as when comparing the supposed toxicity of the different peptides involved.

The imaging strategy employed to observe growth of peptide-treated HEK cells – ptychographic quantitative phase imaging – has the benefits of being unaffected by lens-induced visual aberrations as well as the capacity to image transparent cells very clearly (255). However, the computational nature of the process introduces susceptibility to improper identification of cells due to the presence of physical debris; this mislabelling would affect the calculated cell count, and therefore the derived doubling time of the cell population. It should be noted, however, that the margin of this error diminishes considerably if a suitably large population of cells is used for imaging, so long as they are adequately prepared.

4. Isolation & Evaluation of Peptides Identified Using *In Silico* Metagenomic Analysis

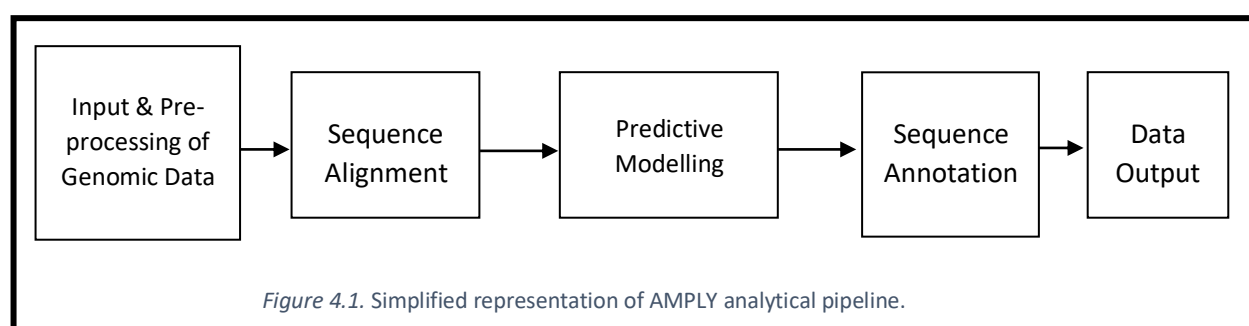
4.1 Introduction

The selection of individual peptidomes for AMP discovery can be a meticulous process, such that many naturally occurring peptides undoubtedly remain undiscovered to this day. There has been a recent shift to computational methods of AMP identification, using informationally trained pattern-recognition software able to predict the sequences and chemical properties of antimicrobial peptides found in the reading frames of genomic data (256,257).

Software of this nature is trained via machine learning to identify genomic regions that produce AMPs when expressed. The resultant ‘neural network’ is typically built using a library of empirical data – formed from both established AMP databases as well as experimentally validated data - allowing outcome probabilities to be calculated from the observed trends (258–260). The length and predicted hydrophobicity of an AMP sequence, for example, are qualities known to influence antimicrobial activity and cell selectivity of the resultant peptide (163,261), and so are prime candidates for *in silico* analysis. While AMPs have been identified in every known form of life, analysis of genomic data remains a relatively untapped source of peptide sequences, despite almost 100,000 living organisms having been fully sequenced to date (204,212). With the majority of this genomic data being readily available to the public, this method of AMP identification is a powerful tool for candidate selection.

One instance of software developed for *in silico* prediction of AMP sequences is AMPLY, which attempts to predict and characterise therapeutic candidates as they are generated.

AMPLY is the name of a metagenomic analysis tool, developed by Dr Benjamin Thomas and Prof Chris Creevey of Queen’s University (211). Its primary purpose is to carry out a series of characterisation steps on genomic data, identifying regions within a given genome that encode AMPs and predicting their efficacy on gram-positive and gram-negative bacteria, as well as other notable properties such as haemolytic activity. This is achieved through a series of pairwise alignments against established and experimentally generated AMP data repositories, as well as rigorous predictive modelling algorithms (**Figure 4.1**).



Study Design

The AMPLY analytical pipeline is able to – among other things – assign a score of predicted bioactivity to generated peptide sequences using its CORE-T data repository. CORE-T is a compilation of hundreds of peptide MIC data points gathered by both the original software developers and the data generated by the Hilpert group. The software was used to analyse the genomes of 20 different organisms (**Table 4.1**), yielding over 4500 peptide sequences from which 2000 sequences (**Appendix B, Table 3**) were selected based on their bioactivity scores and anomaly indexes. These peptides were synthesised via SPOT synthesis and subjected to antimicrobial screening vs *E. coli* strain 15. Previous investigations highlighted the considerable inhibitory effect that the presence of serum can have on AMP activity; for this reason, all preliminary screens were carried out using a bacterial growth medium consisting of 90% MH broth and 10% human serum, such that peptides rendered ineffective by serum components would be eliminated from consideration.

Therapeutic candidates were then chosen (**Appendix B, Table 4**) based on observed antimicrobial activity and purified for further investigation regarding spectrum of activity, haemolytic and cytotoxic activity, and resistance to the action of serum binding proteins. Like other mammalian cells, human erythrocytes carry a net neutral charge due to the components of their membranes; this facilitates selective interaction between the typically cationic AMPs and their negatively charged pathogenic targets (217). However, haemolytic and cytotoxic interactions have been displayed by several natural and synthetically altered AMPs despite the differences in membrane composition, with associations being made between peptide helicity and hydrophobicity – two additional factors reported to influence peptide-target interactions -, and haemolytic capacity (262). While investigation of haemolysis can be used as a rapid indicator of peptide toxicity, the preparation of erythrocyte samples can render them more vulnerable to lysis, confounding the resultant data. For this reason, cytotoxic assays on HEK293 cells were also carried out as in the previous study under the hypothesis that this would allow a more accurate evaluation of peptide cytotoxicity.

Finally, remaining candidates were screened for activity against the ESKAPE pathogens to determine peptide MICs against a highly clinically relevant group of bacteria. As previously, maximum concentration of peptide solutions used for MIC assay did not exceed 64 µg/mL. MIC assays to determine activity against the ESKAPE pathogen group were carried out against the following strains: *E. faecium* strain AGR 15, *S. aureus* strain 15, *P. aeruginosa* strain PAO1, and clinical isolates of *A. baumannii*, *K. pneumoniae*, and *E. cloacae*. *E. cloacae* was chosen to represent the Enterobacter species because it is recognised across all known Enterobacteriaceae as the third most prevalent bacterium exhibiting resistance to third-generation cephalosporins, following after *K. pneumoniae* and enteric *E. coli* (119). In addition, *E. cloacae* is known to

produce a number of ESBLs and has exhibited expression of the QnrA protein, conferring resistance to fluoroquinolones (119,120).

Table 4.1. Organisms chosen as sources for AMPLY analysis and sequence derivation.

Organism Name	Common Name	Number of Sequences Identified via AMPLY	Number of Peptides Selected
<i>Bacillus anthracis</i>	<i>Bacillus anthracis</i>	17	17
<i>Burkholderia pseudomallei</i>	<i>Burkholderia pseudomallei</i>	143	100
<i>Canis lupus familiaris</i>	Domesticated dog (boxer)	226	100
<i>Canis lupus lupus</i>	Eurasian wolf	62	60
<i>Crocodylus porosus</i>	Saltwater crocodile	343	100
<i>Danaus plexippus plexippus</i>	Monarch butterfly	193	100
<i>Falco peregrinus</i>	Peregrine falcon	219	100
<i>Galdieria sulphuraria</i>	Algae	20	18
<i>Gallus gallus</i>	Red junglefowl	474	241
<i>Ganoderma lucidum</i>	Reishi mushroom	198	100
<i>Helianthus annuus</i>	Sunflower	288	124
<i>Mesobuthus martensii</i>	Chinese scorpion	328	123
<i>Metallosphaera sedula</i>	Archaea	72	51
<i>Ophiophagus hannah</i>	King cobra	178	100
<i>Orcinus orca</i>	Killer whale	290	100
<i>Panthera tigris altaica</i>	Siberian tiger	354	130
<i>Plasmodium falciparum</i>	<i>Plasmodium falciparum</i>	229	100
<i>Rhincodon typus</i>	Whale shark	603	136
<i>Takifugu rubripes</i>	Japanese pufferfish	262	100
<i>Talitrus saltator</i>	Sandhopper amphipod	100	100

Hypothesis, Aims, and Objectives

Hypothesis: Bioactive peptides can be identified within genomic data using AMPLY analytical software.

Aim: Evaluate the therapeutic capacity of peptides identified via *in silico* metagenomic analysis.

Objectives:

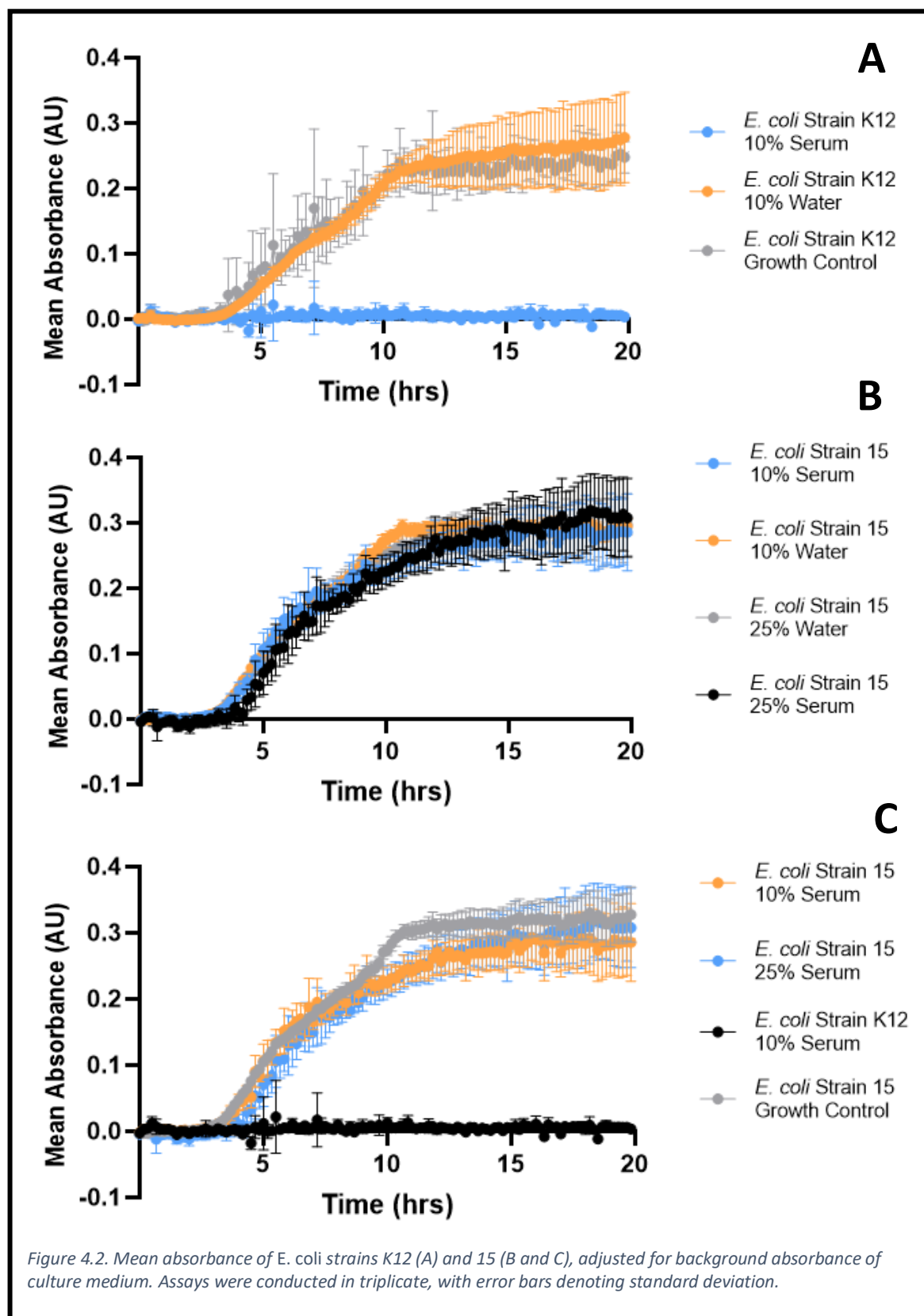
- Identify an *E. coli* strain that is tolerant of the presence of serum.
- Identify and eliminate candidates sensitive to the presence of serum during antimicrobial screen against serum-tolerant *E. coli* strain.
- Establish antimicrobial activity of chosen candidates in the absence of serum against the *E. coli* strain by determining MIC.
- Determine therapeutic viability of selected peptide candidates by investigating haemolytic and cytotoxic activity.
- Carry out MIC assays against bacteria from the ESKAPE pathogen group to establish breadth of activity and ability to treat multidrug resistant bacteria.

4.2 Results

Preliminary Screening of AMPLY Peptide Set for Antimicrobial Activity and Serum Tolerance

Peptides selected by AMPLY software were to undergo antimicrobial screening in the presence of serum to eliminate candidates that would be neutralised by serum binding proteins and other obstructive mechanisms; this required the use of a bacterial strain that is unaffected by serum, such that any observed inhibition of bacterial growth could be reasonably attributed to the action of the peptide. Additionally, a bacterial strain with susceptibility to serum action was also required to act as a suitable control. Finally, investigations were also carried out to determine whether the dilution of MH broth – as a result of the inclusion of serum – would itself have an effect on bacterial growth. Samples of *E. coli* strain K12 incubated in an MH broth solution containing 10% human serum exhibited severely reduced absorbance when compared to the growth control containing no serum; this effect was not seen with *E. coli* incubated using a 10% water solution (**Figure 4.2A**). Dunnett's multiple comparisons test following one-way ANOVA found no statistically significant difference ($P < .05$, $n=3$) in the absorbance of cells incubated in 10% water solution when compared to the growth control at any timepoint throughout the 20-hour assay, while significance was seen between *E. coli* K12 in 10% serum solution and all other included populations.

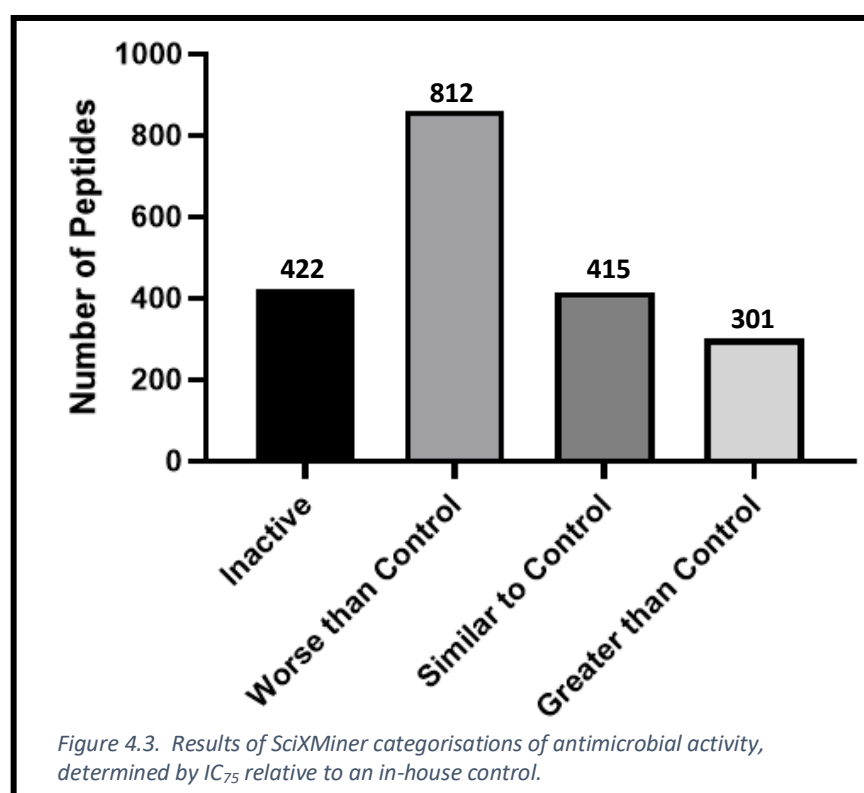
E. coli strain 15 displayed no reductions in growth compared to the growth control following incubation in either 10% serum solution, 25% serum solution, 10% water solution, or 25% water solution (**Figure 4.2B**). Additionally, no statistically significant differences between *E. coli* population conditions were found following one-way ANOVA and multiple comparisons testing ($P < .05$, $n=3$). Comparison of these data (**Figure 4.2C**) shows that reduced growth is seen in both populations exposed to serum between 10 and 11.5 hours of incubation when compared to the growth control. However, there were no statistically significant differences found following ANOVA.



Following identification of a serum-tolerant bacterial strain, 2000 peptides were synthesised via SPOT synthesis, utilising amino acid sequences identified by AMPLY ([Appendix B, Table 3](#)). These crude peptides were then screened for antimicrobial activity against *E. coli* strain 15 in an MH broth solution containing 10% human serum and categorised into categories of activity relative to the in-house peptide control via SciXMiner software ([Table 4.2, Figure 4.3](#)). Of the 2000 peptides screened, 422 of them presented no activity against the bacterial strain at the highest concentration tested; of the remaining 1528 peptides, 716 of them exhibited activity similar or greater to that of the in-house control with the remaining 812 peptides displaying antimicrobial activity in the presence of serum, but to a lesser degree than that of the control.

Table 4.2. MIC values of in-house control peptide against *E. coli* strain 15 in a 10% serum/MH broth solution expressed as a modal concentration. Assays were carried out in triplicate.

Peptide	Sequence	<i>E. coli</i> 10% Serum MIC ($\mu\text{g/mL}$)
Control	KRRWRIWLV	16



Following SciXMiner analysis, activity profiles of the selected peptides could be compiled and grouped according to genome of origin ([Table 4.3](#)). Observations could also be made regarding correlations between the presence of specific amino acid residues and the resultant activity of the peptide; these were also grouped by source genome to determine variations in this correlation across organisms, should they be present ([Figure 4.4A-C](#)). Numerically speaking,

the greatest contribution across all organisms to the 301 peptides that were categorised “greater than control” was made by peptides sourced from the red junglefowl (*Gallus gallus*) genome with 48 peptides, followed by the those of the whale shark (*Rhincodon typus*) genome with 28, and then the Chinese scorpion (*Mesobuthus martensii*) genome with 26. The lowest contribution was made by the red algae (*Galdieria sulphuraria*) peptide group, of which only 1 was more active than the control. The genome of *G. sulphuraria* also provided the fewest number of “inactive” peptides across all organisms – with 4 out of a total 422 -, while the greatest contribution was provided by peptides sourced from the monarch butterfly (*Danaus plexippus plexippus*) with 38.

However, when expressed in terms of peptide population percentage per organism, the greatest contribution to the peptides categorised “greater than control” was instead made by the peptides derived from the sandhopper (*Talitrus saltator*) genome, with 25% of its 100 peptides falling into this category. This is then followed by the peptide group identified within the *M. martensii* genome – with 21.1% of its 123 peptides being placed into this category -, and then that of the *R. typus* with 20.6% of its 136 peptides. The lowest contribution to this group was provided by peptides identified from the genomes of the peregrine falcon (*Falco peregrinus*) and the bacterium *Burkholderia pseudomallei*, both of which had 2% of 100 peptides fall into this category. The genome of *D. plexippus plexippus* remains the greatest contributor to the inactive group, with 38% of its 100 peptides showing no activity against the tested *E. coli* strain in the presence of serum. Peptides sourced from the sunflower (*Helianthus annuus*) genome had the lowest population percentage of inactive peptides, with 7.26% of 124 peptides showing no activity.

Across all peptide sequences synthesised the 5 most common amino acids present within a sequence were lysine (K), leucine (L), arginine (R), phenylalanine (F), and alanine (A), in descending order of occurrence. Peptide sequences with lysine were most commonly categorised as more active than the control (“fully active”), as well as similar in activity to the control (“similar to control”). This is also true of sequences with leucine but is not the case with arginine; peptides with this amino acid were instead most commonly categorised as less active than the control (“worse than control”). Contrarily, peptides identified within the genomes of *B. pseudomallei* and the reishi mushroom (*Ganoderma lucidum*) had relatively few lysine residues in their sequences, with both presenting a majority of arginine residues instead. Similarly, while lysine was the most commonly incorporated amino acid for peptide sequences found within the genomes of *H. annuus*, the domesticated dog (*Canis lupus familiaris*) and the bacterium *Bacillus anthracis*, sequences from these genomes containing this amino acid were more frequently categorised as “inactive”; this is true when compared to categorisations of peptides from these genomes that did not contain lysine, as well as frequency of lysine-containing peptides from these genomes that were given any of the other available categorisations.

Table 4.3. SciXMiner categorisations of AMPLY-predicted peptide candidates, grouped by organism.

Source Organism	SciXMiner Categorisation			
	Greater Than Control	Siimilar To Control	Less Active Than Control	Inactive
<i>Bacillus anthracis</i>	2	3	7	5
<i>Burkholderia pseudomallei</i>	2	3	59	36
<i>Canis lupus familiaris</i>	21	21	37	21
<i>Canis lupus lupus</i>	4	9	30	17
<i>Crocodylus porosus</i>	9	23	36	32
<i>Danaus plexippus plexippus</i>	7	13	42	38
<i>Falco peregrinus</i>	2	16	47	35
<i>Galdieria sulphuraria</i>	1	5	8	4
<i>Gallus gallus</i>	48	65	93	35
<i>Ganoderma lucidum</i>	11	22	47	20
<i>Helianthus anuus</i>	22	47	46	9
<i>Mesobuthus martensii</i>	26	31	52	14
<i>Metallosphaera sedula</i>	2	5	37	7
<i>Ophiophagus hannah</i>	5	14	51	30
<i>Orcinus orca</i>	13	19	42	26
<i>Panthera tigris altaica</i>	22	28	58	22
<i>Plasmodium falciparum</i>	13	17	56	14
<i>Rhincodon typus</i>	28	38	56	14
<i>Takifugu rubripes</i>	15	19	45	21
<i>Talitrus saltator</i>	25	23	40	12

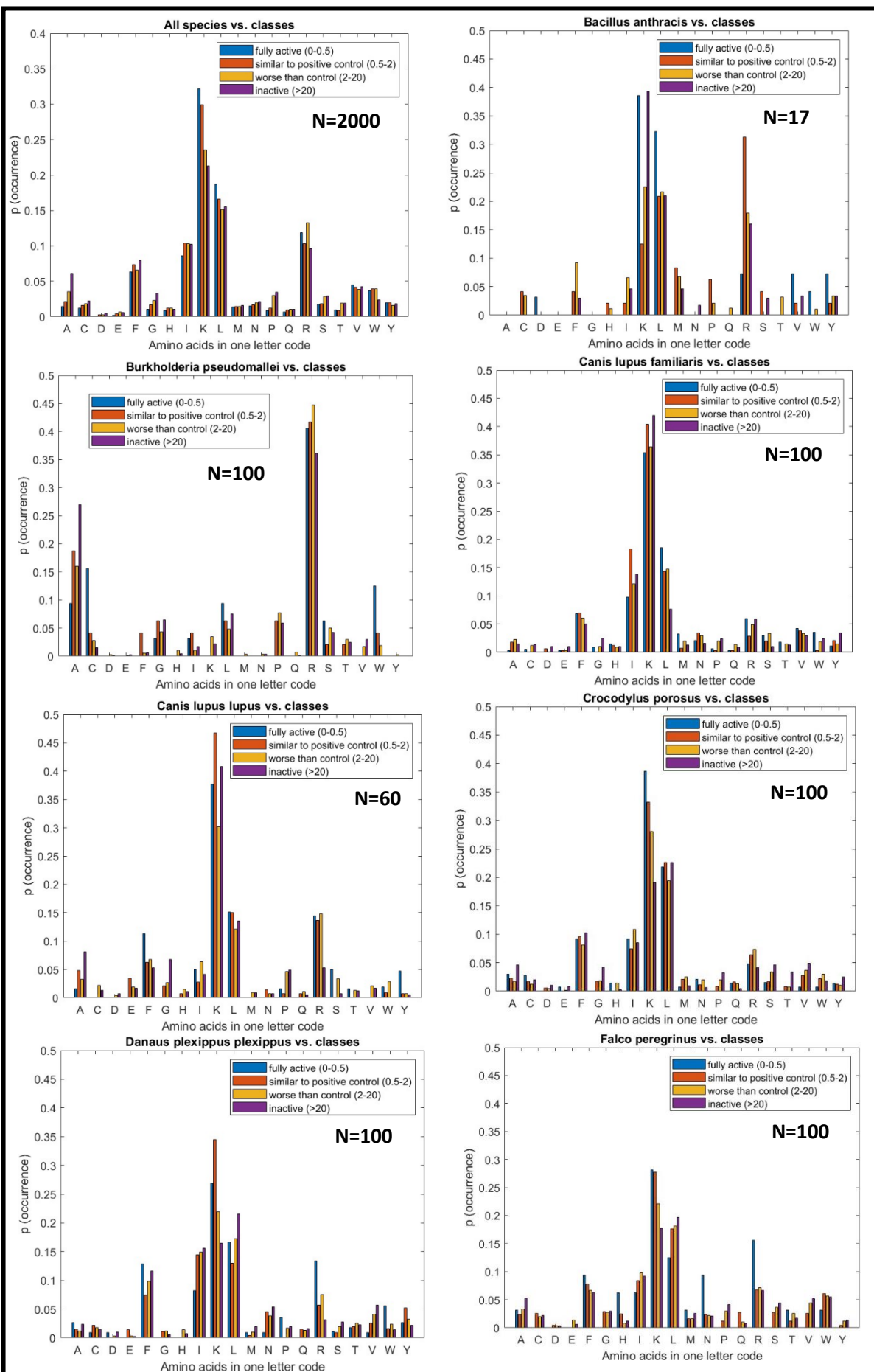


Figure 4.4A. SciXMiner output showing probability of amino acid occurrence within peptide reIC_{75} categorisations, grouped by organism. Number of peptides sourced from each organism (N) is shown.

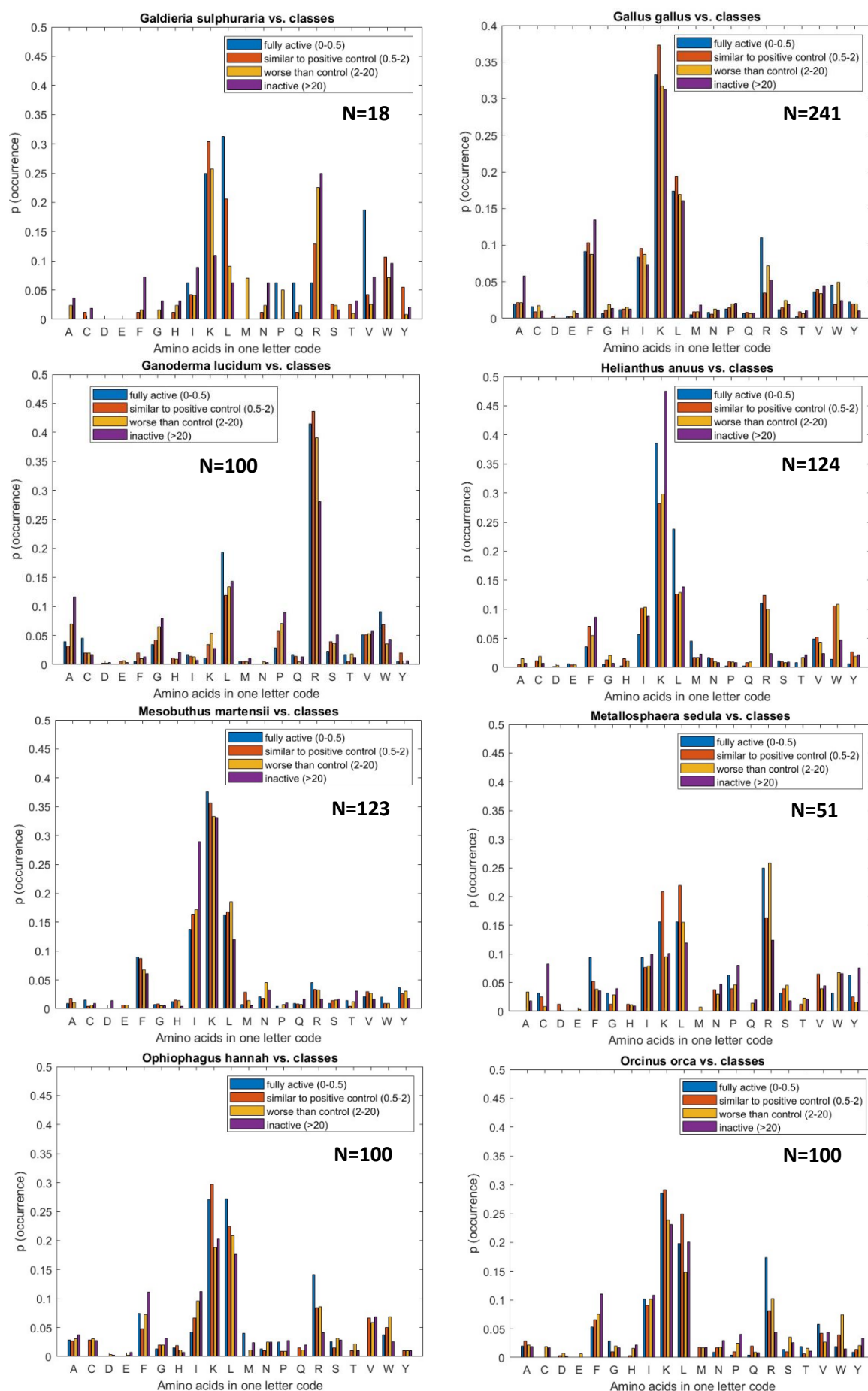


Figure 4.4B. SciXMiner output showing probability of amino acid occurrence within peptide *reIC₇₅* categorisations, grouped by organism. Number of peptides sourced from each organism (N) is shown.

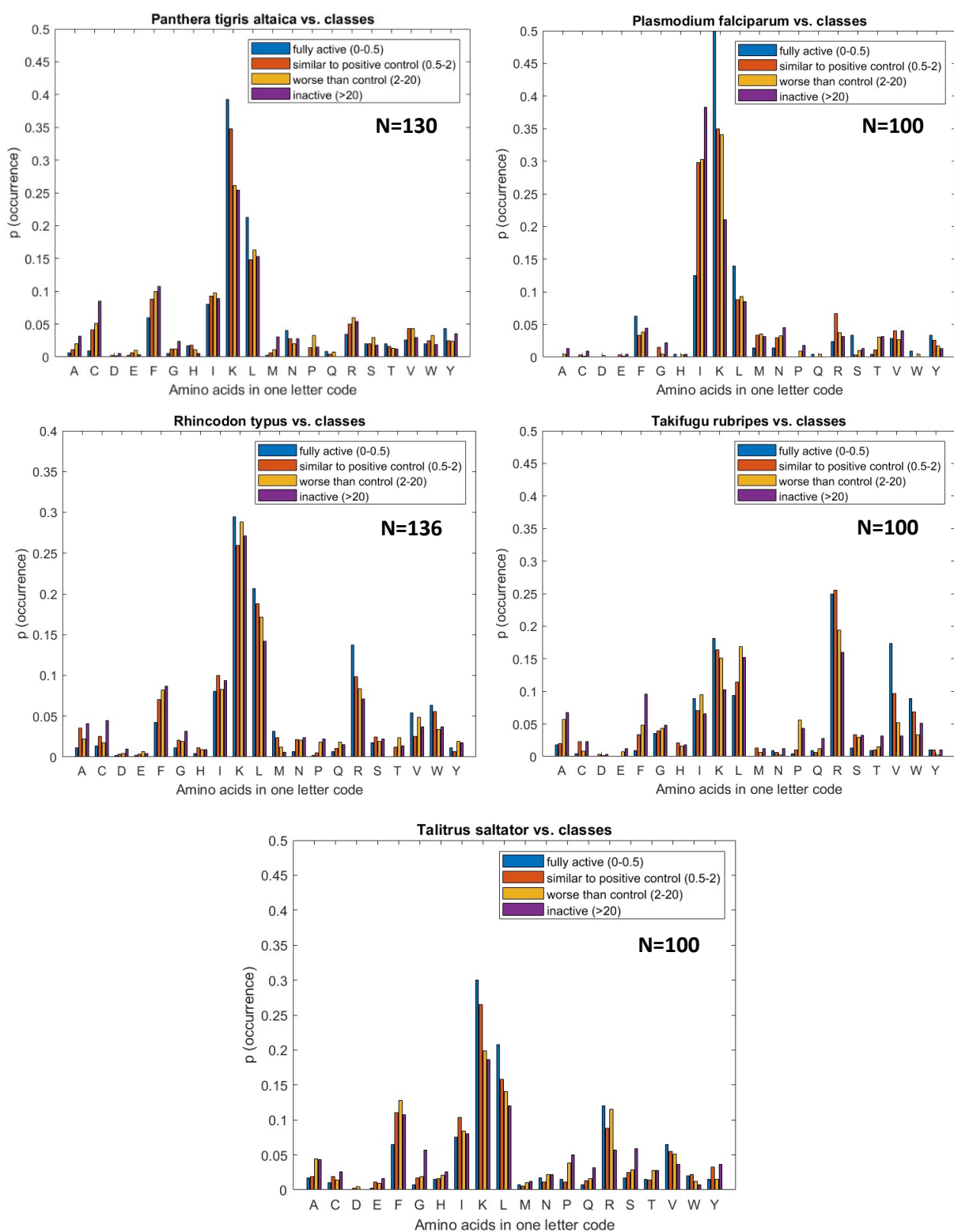


Figure 4.4C. SciXMiner output showing probability of amino acid occurrence within peptide *reIC₇₅* categorisations, grouped by organism. Number of peptides sourced from each organism (N) is shown.

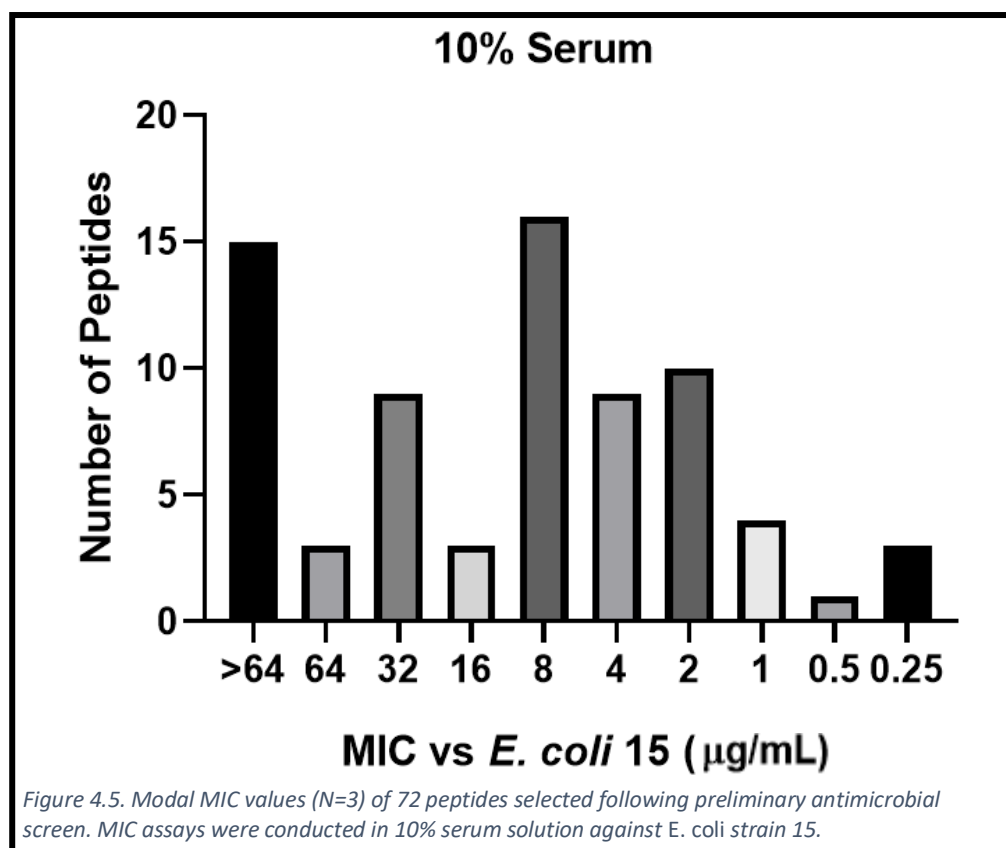
Candidate Selection and Further Evaluation of Serum Tolerance

From the population of 2000, 72 peptides were selected for further characterisation; 3 candidates showing the lowest relIC₇₅ were chosen from each of the included organism genomes, followed by the 12 peptides of the lowest relIC₇₅ that had yet to be selected (**Appendix B, Table 4**). Candidates were then given designations based on the organism from which they were sourced (**Table 4.4**), followed by a numerical ranking based on their relIC₇₅ in descending order.

Table 4.4. Designations of selected peptides within the AMPLY peptide set.

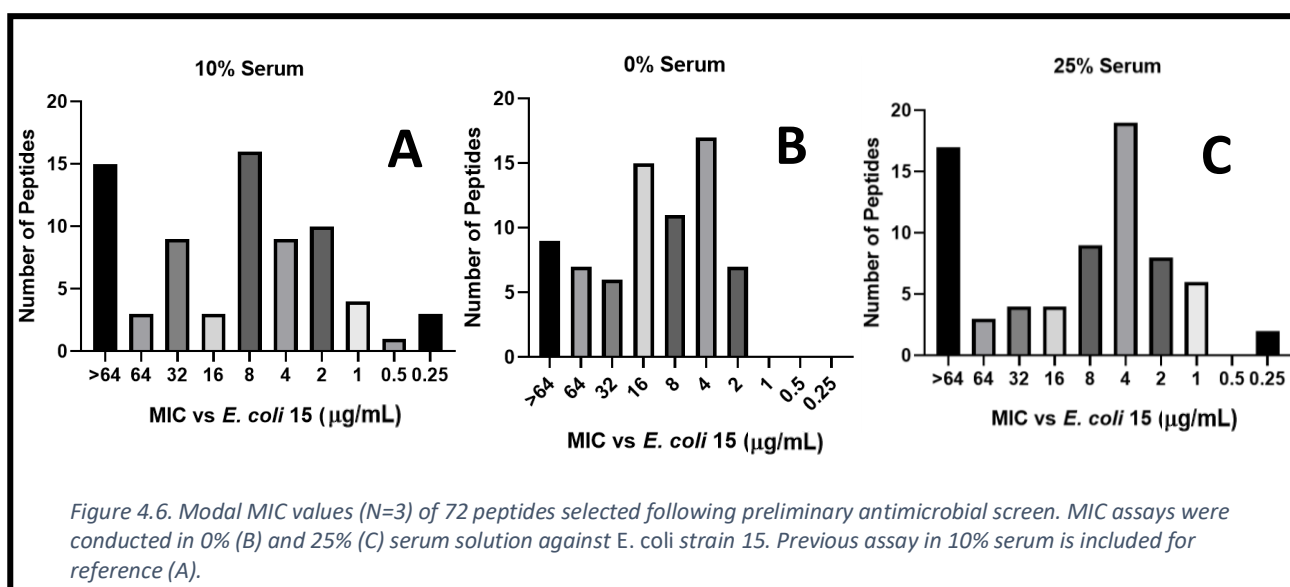
AMPLY-Peptide Designation	Organism Name	Common Name
BACAN	<i>Bacillus anthracis</i>	<i>Bacillus anthracis</i>
BURPS	<i>Burkholderia pseudomallei</i>	<i>Burkholderia pseudomallei</i>
CANFA	<i>Canis lupus familiaris</i>	Domesticated dog (boxer)
CANLU	<i>Canis lupus lupus</i>	Eurasian wolf
CROPO	<i>Crocodylus porosus</i>	Saltwater crocodile
DANPL	<i>Danaus plexippus plexippus</i>	Monarch butterfly
FALPE	<i>Falco peregrinus</i>	Peregrine falcon
GALGA	<i>Gallus gallus</i>	Red junglefowl
GALSU	<i>Galdieria sulphuraria</i>	Algae
GANLU	<i>Ganoderma lucidum</i>	Reishi mushroom
HELAN	<i>Helianthus annuus</i>	Sunflower
MESMA	<i>Mesobuthus martensii</i>	Chinese scorpion
METSE	<i>Metallosphaera sedula</i>	Archaea
OPHHA	<i>Ophiophagus hannah</i>	King cobra
ORCOR	<i>Orcinus orca</i>	Killer whale
PANAL	<i>Panthera tigris altaica</i>	Siberian tiger
PLAFA	<i>Plasmodium falciparum</i>	<i>Plasmodium falciparum</i>
RHITY	<i>Rhincodon typus</i>	Whale shark
TAKRU	<i>Takifugu rubripes</i>	Japanese pufferfish
TALSA	<i>Talitrus saltator</i>	Sandhopper amphipod

Pure variants of these 72 peptides were acquired from Genscript (New Jersey, USA), and antimicrobial activity against *E. coli* strain 15 in 10% serum solution was re-evaluated in the absence of side products associated with crude SPOT synthesis (**Figure 4.5**). Of the 72 peptides tested, the MICs of 15 could not be determined as they exceeded the highest tested concentration. Of the 57 remaining, 54 peptides exhibited modal MICs lower than the highest tested concentration, with the most common modal MIC of the total peptide set – shown by 16 of 72 – being 8 $\mu\text{g/mL}$. Contrarily, the lowest observed modal MIC was 0.25 $\mu\text{g/mL}$; this activity was exhibited by 3 peptides within the peptide set of 72.



Further MIC assays were conducted to determine both activity of the peptide candidates in the absence of serum and subsequent activity in 25% serum solution (**Figure 4.6**); these could then be compared with the data from the 10% serum solution assays to determine whether the effect of serum on these peptides was concentration dependent. It was seen that the total number of peptides with an MIC exceeding the highest tested concentration increased as serum concentration increased, rising from 9 of 72 peptides in the absence of serum to 17 of 72 when tested in 25% serum solution, as well as the 15 peptides of 72 observed previously. The lowest MIC displayed by the peptide set when tested in 0% serum solution was 2 $\mu\text{g/mL}$; this MIC was displayed by 7 of the 72 peptides tested. Contrarily, as was seen during the 10% serum solution tests the lowest MIC observed following incubation in 25% serum solution was 0.25 $\mu\text{g/mL}$; this activity was displayed by 2 peptides within the set. While the most common MIC when

tested in 10% serum was 8 $\mu\text{g/mL}$, this decreased in the absence of serum with 17 of the 72 peptides showing an MIC of 4 $\mu\text{g/mL}$; this was followed by 15 peptides exhibiting an MIC of 16 $\mu\text{g/mL}$ and then a further 11 peptides with an MIC of 8 $\mu\text{g/mL}$. In 25% serum solution the most commonly observed MIC remained 4 $\mu\text{g/mL}$ – shown by 19 of 72 peptides –, followed by 9 peptides with an MIC of 8 $\mu\text{g/mL}$ and 8 peptides with an MIC of 2 $\mu\text{g/mL}$.



Haemolytic capacity of the AMPLY peptide set was then evaluated; this would allow therapeutic viability of all peptides to be more thoroughly evaluated and compared (**Table 4.5A-C**), such that the pool of candidates selected for further study could be reduced. Of all peptides assessed, AMPLY-GALGA7 showed the greatest tolerance to red blood cells with a mean HC_{50} of 2660 $\mu\text{g/mL}$. The lowest HC_{50} shown by any peptide within the set was 11.28 $\mu\text{g/mL}$, shown by AMPLY-DANPL2; this peptide exhibited a modal MIC of 16 $\mu\text{g/mL}$ against *E. coli* strain 15, which dropped to 4 $\mu\text{g/mL}$ when incubated in both 10% and 25% serum solutions. Across the population of 72 peptides, 42 of them presented a mean HC_{50} that exceeded 256 $\mu\text{g/mL}$, a concentration 4 times greater than the highest concentration tested during the MIC assays; 25 of these 42 peptides exhibited a mean HC_{50} exceeding 10 times the highest concentration tested. Within the remaining 30 peptides, 10 of them showed mean HC_{50} values lower than 64 $\mu\text{g/mL}$.

These data were also grouped by genome of origin, allowing correlations between the source of the candidates and their observed activity, toxicity, and serum tolerance to be identified (**Table 4.6**). Across all organisms where there was a most commonly observed modal MIC in the absence of serum (12 of 20 organism groups), the lowest was shown by the group of peptides derived from the *B. anthracis* genome (AMPLY-BACAN) whose most frequently observed modal MIC was 2 $\mu\text{g/mL}$. This was followed jointly by the 4 organism groups – AMPLY-GALGA, AMPLY-MESMA, AMPLY-RHITY, and AMPLY-TAKRU – whose most commonly

observed modal MIC was 4 µg/mL, the second lowest value seen. The most commonly observed modal MICs of these 5 groups did not increase when tested in the presence of serum. The lowest most commonly observed modal MIC value remained 2 µg/mL when the candidates were tested in serum; in addition to the AMPLY-BACAN group, this value was most frequently shown by the groups AMPLY-GANLU, AMPLY-HELAN, and AMPLY-OPHHA. It should be noted that of these 3 groups, only AMPLY-OPHHA did not present an additional modal MIC with the same frequency as it did 2 µg/mL.

In the absence of serum, there were 3 organism groups where the most commonly observed modal MIC exceeded that of the highest tested concentration: AMPLY-BURPS, AMPLY-CANLU, and AMPLY-PANAL. These groups continue to present exceedingly high MICs when tested in serum, and are joined by groups AMPLY-CANFA, AMPLY-FALPE, and AMPLY-GANLU. As noted previously, the AMPLY-GANLU group also presented the lowest most commonly observed modal MIC when tested in serum; this occurrence of this MIC within this group was identical to that of the MICs that were too high to be determined.

Across all organism groups the highest mean HC₅₀ was 922.2 µg/mL, shown by peptides derived from the *B. pseudomallei* genome (AMPLY-BURPS); this group also presented the highest most commonly observed MICs both with and without the presence of serum. With the exception of this group, the highest mean HC₅₀ was shown by the AMPLY-GANLU group, followed by the AMPLY-MESMA group. The lowest mean HC₅₀ observed across all groups was 98.46 µg/mL, shown by the AMPLY-BACAN group; this group also presented the lowest most commonly observed MICs with and without the presence of serum.

A selection of candidates was made based on the observed antimicrobial and haemolytic properties of this peptide population; of the 72 peptides investigated a single candidate was chosen from each organism, followed by the 4 peptides within this population that had not yet been selected (**Table 4.5A-D**).

Table 4.5A. Modal MIC and mean HC₅₀ of peptide candidate groups AMPLY-BACAN to AMPLY-FALPE. Candidates selected for further characterisation are highlighted in green.

Peptide	<i>E. coli</i> MIC (µg/mL)			HC ₅₀ (µg/mL)
	0% Serum	10% Serum	25% Serum	
AMPLY-BACAN1	2	2	2	93.7
AMPLY-BACAN2	8	32	4	35.09
AMPLY-BACAN3	2	0.5	0.25	166.6
AMPLY-BURPS1	>64	>64	>64	441.3
AMPLY-BURPS2	>64	>64	>64	804.3
AMPLY-BURPS3	32	32	64	1521
AMPLY-CANFA1	64	8	8	27.56
AMPLY-CANFA2	>64	16	8	167.7
AMPLY-CANFA3	16	256	>64	627.5
AMPLY-CANFA4	16	32	>64	907.3
AMPLY-CANLU1	>64	>512	>64	109.4
AMPLY-CANLU2	8	32	8	566
AMPLY-CANLU3	>64	512	>64	30.01
AMPLY-CROPO1	32	>64	16	808
AMPLY-CROPO2	8	8	8	866.5
AMPLY-CROPO3	8	8	8	38.29
AMPLY-DANPL1	16	4	4	26.7
AMPLY-DANPL2	32	4	8	320.2
AMPLY-DANPL3	16	8	8	412.4
AMPLY-FALPE1	4	8	4	59.85
AMPLY-FALPE2	64	>64	>64	387.7
AMPLY-FALPE3	16	8	16	1967

Table 4.5B. Modal MIC and mean HC₅₀ of peptide candidate groups AMPLY-GALGA to AMPLY-METSE. Candidates selected for further characterisation are highlighted in green.

Peptide	<i>E. coli</i> MIC (µg/mL)			HC ₅₀ (µg/mL)
	0% Serum	10% Serum	25% Serum	
AMPLY-GALGA1	4	2	1	18.99
AMPLY-GALGA2	4	4	2	395.7
AMPLY-GALGA3	2	1	1	207.9
AMPLY-GALGA4	4	1	1	1559
AMPLY-GALGA5	64	>64	>64	544.8
AMPLY-GALGA6	4	8	4	252.7
AMPLY-GALGA7	>64	>64	>64	1216
AMPLY-GALSU1	8	8	4	990.1
AMPLY-GALSU2	4	4	4	215.7
AMPLY-GALSU3	32	32	32	328
AMPLY-GANLU1	8	>64	>64	307.7
AMPLY-GANLU2	2	2	4	410.7
AMPLY-GANLU3	32	32	16	1905
AMPLY-HELAN1	>64	16	>64	339.9
AMPLY-HELAN2	2	2	2	52.13
AMPLY-HELAN3	4	4	4	1706
AMPLY-MESMA1	64	64	32	2970
AMPLY-MESMA2	4	4	4	807.8
AMPLY-MESMA3	32	>64	>64	218.4
AMPLY-MESMA4	16	32	32	851.9
AMPLY-MESMA5	8	2	4	1260
AMPLY-MESMA6	4	4	8	147.9
AMPLY-MESMA7	4	0.25	0.25	10.21
AMPLY-MESMA8	16	64	4	1268
AMPLY-MESMA9	16	32	64	57.03
AMPLY-METSE1	8	16	4	65.18
AMPLY-METSE2	64	>64	>64	265.1
AMPLY-METSE3	16	64	>64	294.8

Table 4.5C. Modal MIC and mean HC₅₀ of peptide candidate groups AMPLY-OPHHA to AMPLY-TALSA. Candidates selected for further characterisation are highlighted in green.

Peptide	<i>E. coli</i> MIC (µg/mL)			HC ₅₀ (µg/mL)
	0% Serum	10% Serum	25% Serum	
AMPLY-OPHHA1	2	1	8	147.5
AMPLY-OPHHA2	8	32	64	1108
AMPLY-OPHHA3	16	0.25	2	670.8
AMPLY-ORCOR1	8	8	8	806.5
AMPLY-ORCOR2	4	4	8	572.7
AMPLY-ORCOR3	64	>64	>64	421.6
AMPLY-PANAL1	>64	8	4	148
AMPLY-PANAL2	8	8	2	49.02
AMPLY-PANAL3	32	512	>64	270.0
AMPLY-PANAL4	>64	>64	>64	136
AMPLY-PLAFA1	16	8	4	99.04
AMPLY-PLAFA2	4	2	2	883.3
AMPLY-PLAFA3	64	8	32	821.4
AMPLY-RHITY1	4	8	16	68.69
AMPLY-RHITY2	4	2	2	433.6
AMPLY-RHITY3	16	4	8	210.1
AMPLY-TAKRU1	4	2	2	667.3
AMPLY-TAKRU2	8	0.25	1	70.33
AMPLY-TAKRU3	4	4	4	15.55
AMPLY-TALSA1	16	8	4	243
AMPLY-TALSA2	16	8	4	978.3
AMPLY-TALSA3	2	0.125	1	83.11

Table 4.5D. Amino acid sequences and organism sources of selected AMPLY candidates.

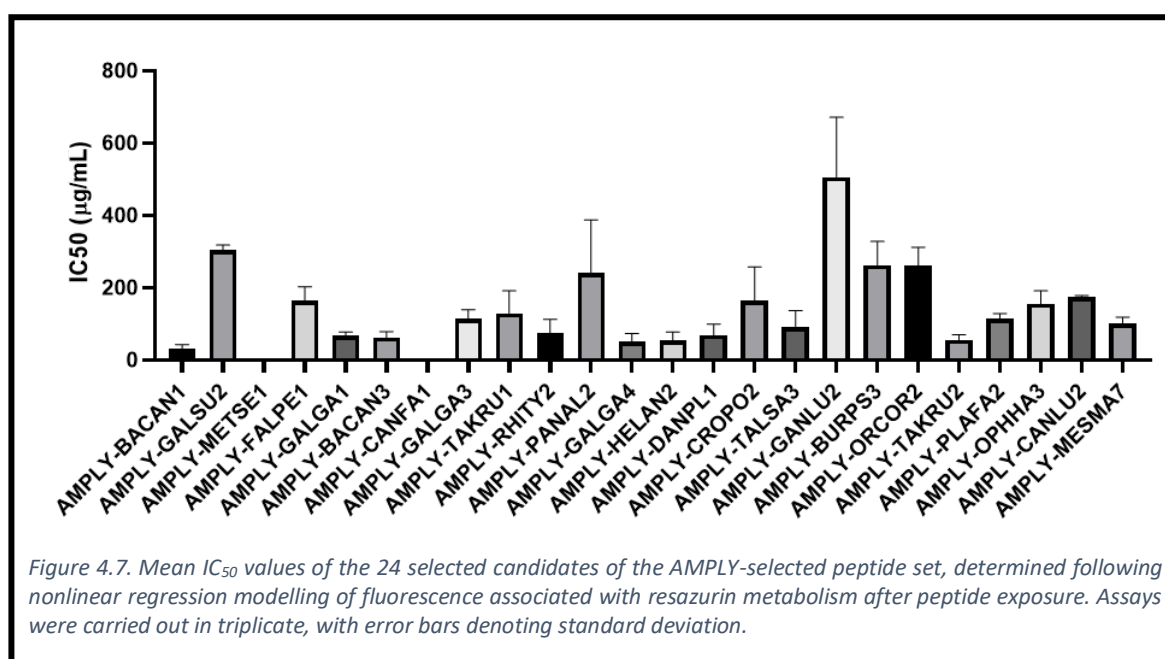
Peptide	Sequence	Source
AMPLY-BACAN1	KRWVKLLYKLLK	<i>Bacillus anthracis</i>
AMPLY-BACAN3	LKKLKKKLKRYLKDLV	<i>Bacillus anthracis</i>
AMPLY-BURPS3	RRWWRCISRLGWRRWR	<i>Burkholderia pseudomallei</i>
AMPLY-CANFA1	LKKLLKHKSIVKLIKI	<i>Canis lupus familiaris</i>
AMPLY-CANLU2	YFFFFFKRRRRRRTP	<i>Canis lupus lupus</i>
AMPLY-CROPO2	RCNRKFELLKILKKKF	<i>Crocodylus porosus</i>
AMPLY-DANPL1	RALFFVKKIKKIKRL	<i>Danaus plexippus plexippus</i>
AMPLY-FALPE1	KVPFKCEYKKWRFIWK	<i>Falco peregrinus</i>
AMPLY-GALGA1	LFLLIYKKKKKKKKKR	<i>Gallus gallus</i>
AMPLY-GALGA3	LKRKKKKVVAHLFLLV	<i>Gallus gallus</i>
AMPLY-GALGA4	LSRKISWKKIHKIKL	<i>Gallus gallus</i>
AMPLY-GALSU2	KKQRVLLKLLVVIPL	<i>Galdieria sulphuraria</i>
AMPLY-GANLU2	RRVRVRVQLLRRARA	<i>Ganoderma lucidum</i>
AMPLY-HELAN2	MKIMKMMKKIMKMMKK	<i>Helianthus annuus</i>
AMPLY-MESMA7	KKKKKKKNILKLYKVFFL	<i>Mesobuthus martensii</i>
AMPLY-METSE1	KLRCRIKILPKRFIPK	<i>Metallosphaera sedula</i>
AMPLY-OPHHA3	WKLPWKRLKLKRLK	<i>Ophiophagus hannah</i>
AMPLY-ORCOR2	RVRFRGWGRFRVRVRV	<i>Orcinus orca</i>
AMPLY-PANAL2	LSLSKINKLLKKYKKI	<i>Panthera tigris altaica</i>
AMPLY-PLAFA2	SILTSLVIKKKKKKK	<i>Plasmodium falciparum</i>
AMPLY-RHITY2	LSHYKRKLLKSLLKKL	<i>Rhinocodon typus</i>
AMPLY-TAKRU1	LLKKKIILLRKAIK	<i>Takifugu rubripes</i>
AMPLY-TAKRU2	RLKKIWRVFKASWKAA	<i>Takifugu rubripes</i>
AMPLY-TALSA3	RPKVRVKIWWKARVKV	<i>Talitrus saltator</i>

Table 4.6. Most frequently observed modal MIC of AMPLY-predicted peptide candidates against *E. coli* and mean HC_{50} , grouped by genome of origin.

AMPLY-Peptide Designation	Organism Common Name	Number of Peptides	0% Serum Most Frequent Modal MIC ($\mu\text{g/mL}$)	0 – 25% Serum Most Frequent Modal MIC ($\mu\text{g/mL}$)	Mean HC_{50} ($\mu\text{g/mL}$)
BACAN	<i>Bacillus anthracis</i>	3	2	2	98.46333
BURPS	<i>Burkholderia pseudomallei</i>	3	>64	>64	922.2
CANFA	Domesticated dog (boxer)	4	16	>64 and 8	274.2533
CANLU	Eurasian wolf	3	>64	>64	235.1367
CROPO	Saltwater crocodile	3	32 and 8	8	570.93
DANPL	Monarch butterfly	3	16	8 and 4	253.1
FALPE	Peregrine falcon	3	N/A	>64 and 8	804.85
GALGA	Red junglefowl	7	4	4	599.2986
GALSU	Algae	3	N/A	4	511.2667
GANLU	Reishi mushroom	3	N/A	>64, 32, and 2	874.4667
HELAN	Sunflower	3	N/A	4 and 2	699.3433
MESMA	Chinese scorpion	9	4	4	843.4711
METSE	Archaea	3	N/A	>64	208.36
OPHHA	King cobra	3	N/A	2	642.1
ORCOR	Killer whale	3	N/A	8	600.2667
PANAL	Siberian tiger	4	>64	>64	150.755
PLAFA	<i>Plasmodium falciparum</i>	3	N/A	8	601.2467
RHITY	Whale shark	3	4	4	237.4633
TAKRU	Japanese pufferfish	3	4	4	251.06
TALSA	Sandhopper amphipod	3	16	16, 8, and 4	434.8033

Investigating Cytotoxicity and Antimicrobial Efficacy Against the ESKAPE Pathogens

24 peptides (**Table 4.7**) were taken forward for additional characterisation of their therapeutic viability. Having established the haemolytic capacity of these candidates, efforts were made to investigate the toxicity of the chosen peptides on additional cell types, facilitated by measuring resazurin metabolism of HEK293 cells following incubation in the presence of peptide (**Figure 4.7**). Data from these assays were subjected to nonlinear regression analysis (four parameters), allowing IC_{50} values to be extrapolated for all peptides tested with the exception of AMPLY-METSE1 and AMPLY-CANFA1, whose cytotoxicity could not be quantified because it was not observed during the study. Of the remaining 22 peptides, the highest IC_{50} – a mean value of 506.53 $\mu\text{g/mL}$ – was shown by AMPLY-GANLU2; this peptide also presented the greatest mean HC_{50} of the 24 peptides chosen for further characterisation. The lowest mean IC_{50} value of 36.63 $\mu\text{g/mL}$ was shown by AMPLY-BACAN1; similarly, this peptide presented the lowest mean HC_{50} of the 24 peptides chosen. Within this population, 4 peptides – AMPLY-GALSU2, AMPLY-GANLU2, AMPLY-BURPS3, and AMPLY ORCOR2 – exhibited a mean IC_{50} exceeding 256 $\mu\text{g/mL}$, with a further 6 peptides – AMPLY-FALPE1, AMPLY-TAKRU1, AMPLY-PANAL2, AMPLY- CROPO2, AMPLY-OPHHA3, and AMPLY-CANLU2 – presenting a mean IC_{50} exceeding 128 $\mu\text{g/mL}$, double that of the highest concentration tested during MIC assays.



The therapeutic potential of the chosen peptide candidates was then further investigated, with activity against the ESKAPE pathogens being determined via MIC assay in the absence of serum (**Figures 4.8A-G, Table 4.7**). Assays were conducted using peptide solutions with concentrations ranging from 64 $\mu\text{g/mL}$ to 0.0625 $\mu\text{g/mL}$. Across all assays conducted against all

bacterial strains, the most common modal MIC observed was 4 µg/mL, with a mean frequency of 5.83, followed by 8 µg/mL with a mean frequency of 5.33. Contrarily, the least frequent modal MIC shown by this group of candidates was 1 µg/mL with a mean frequency of 0.33, followed by 64 µg/mL with a mean frequency of 0.67. 1 µg/mL was also the lowest modal MIC observed across all assays despite not being the lowest concentration tested. Finally, the mean frequency of peptides whose MIC could not be determined – as it exceeded the highest tested concentration – was 4.5, with considerable deviation due to the tolerance exhibited by the selected VRE strain.

The lowest modal MIC seen against *E. cloacae* was 1 µg/mL, displayed solely by AMPLY-BACAN1. However, a further 5 peptides exhibited modal MICs of 2 µg/mL, the second lowest concentration seen. The most common MIC against *E. cloacae* was 8 µg/mL – shown by 8 of 24 peptides – although the number of peptides with lower MICs against the same organism – 10 of 24 – is moderately greater. The greatest observed MIC of all peptides tested against was 16 µg/mL. However, the MIC could not be determined for a single peptide – AMPLY-CANFA1 – as it exceeded the highest concentration tested.

Against MRSA the peptide set presented 2 µg/mL as the lowest modal MIC, with the most common MIC being 4 µg/mL; this value was exhibited by 9 of 24 peptides tested, compared to the 4 of 24 peptides that were able to inhibit MRSA growth at a lower concentration. The highest MIC observed against this pathogen – 64 µg/mL – was shown solely by AMPLY-PANAL2; similarly, AMPLY-CANFA1 was the only peptide whose activity could not be determined. This was also the case for the peptide when tested against *K. pneumoniae*, whereas all other peptides tested displayed observable activity. As with MRSA, the lowest observed modal MIC against *K. pneumoniae* was 2 µg/mL, shown by 4 of 24 peptides tested; this was also the third most common modal MIC exhibited against this pathogen, exceeded by 32 µg/mL which was shown by 5 peptides, as well as 4 µg/mL which was shown by 7 of 24 peptides tested, making it the most common modal MIC shown against the pathogen.

A. baumannii is the first of the 6 pathogens tested to have known MICs for all peptides assessed, although this was also seen when the peptide candidates were assessed against *P. aeruginosa*. Against *A. baumannii*, the most common modal MIC was once again 4 µg/mL – exhibited by 11 of 24 peptides, one of which being the previously unresponsive AMPLY-CANFA1 – followed by 2 µg/mL, which was shown by 6 of the 24. *A. baumannii* is the only tested pathogen against which the highest MIC observed is not 64 µg/mL; instead, 2 peptides – AMPLY-FALPE1 and AMPLY-BURPS3 – exhibited modal MICs of 32 µg/mL and were outperformed by all other peptides. *A. baumannii* is also one of two bacteria where the lowest observed MIC is 1 µg/mL; this activity was shown by a single peptide, AMPLY-GALGA4. Against *P. aeruginosa*, the tested peptides most commonly display a modal MIC of 8 µg/mL – shown by 11 of 24 peptides tested –, with the second most common MIC being 16 µg/mL, shown by a further 6 peptides.

The lowest MIC observed against this pathogen was 2 µg/mL; this was shown by the peptide AMPLY-BACAN1 and no others, marking the second time that the lowest MIC can be attributed solely to this peptide.

Finally, attempts to characterise activity against VRE were inconclusive, as no activity could be observed against the pathogen for any of the 24 peptide candidates at any of the tested concentrations.

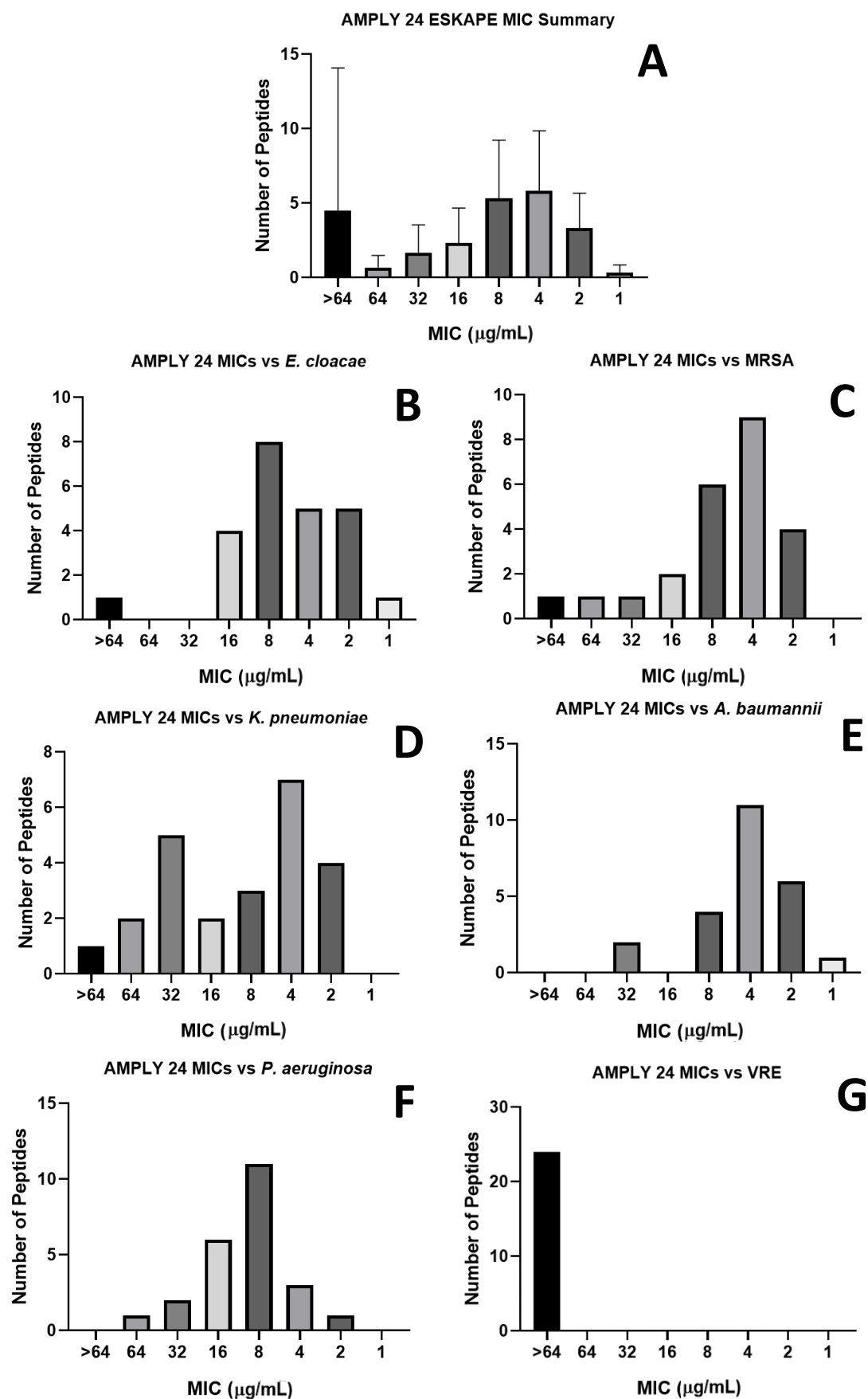


Figure 4.8. Modal MIC values of selected peptide candidates against strains of the ESKAPE pathogen group (B - G), and mean frequency of all modal MIC values across all strains tested (A), with error bars denoting standard deviation. Assays were conducted in triplicate.

Table 4.7. Summary of observed properties of selected peptide candidates, including modal MICs against ESKAPE group pathogens and mean IC₅₀ and HC₅₀ extrapolated from nonlinear regression models (four parameters, n=3).

Peptide	MIC (µg/mL)						Toxicity (µg/mL)	
	<i>E. cloacae</i>	MRSA	<i>K. pneumoniae</i>	<i>A. baumannii</i>	<i>P. aeruginosa</i>	VRE	RBC HC ₅₀	HEK IC ₅₀
AMPLY-BACAN1	1	2	2	4	2	>64	35.09	30.63
AMPLY-BACAN3	8	8	2	2	4	>64	808	62.41
AMPLY-BURPS3	16	16	32	32	64	>64	65.18	253.4
AMPLY-CANFA1	>64	>64	>64	4	32	>64	866.5	984
AMPLY-CANLU2	16	4	32	8	16	>64	136	152.6
AMPLY-CROPO2	8	8	8	8	8	>64	218.4	151.3
AMPLY-DANPL1	8	4	64	4	8	>64	807.8	66.21
AMPLY-FALPE1	8	8	32	32	32	>64	167.7	162.9
AMPLY-GALGA1	16	4	64	8	4	>64	627.5	68.19
AMPLY-GALGA3	4	4	4	4	8	>64	412.4	111.4
AMPLY-GALGA4	4	16	4	1	4	>64	252.7	49.75
AMPLY-GALSU2	4	32	8	4	16	>64	804.3	279.6
AMPLY-GANLU2	2	4	2	4	8	>64	1268	480.2
AMPLY-HELAN2	8	4	8	2	8	>64	307.7	50.76
AMPLY-MESMA7	2	2	4	4	16	>64	243	97.36
AMPLY-METSE1	16	8	16	8	16	>64	1521	959.8
AMPLY-OPHHA3	2	2	32	4	16	>64	270	131.5
AMPLY-ORCOR2	2	4	4	4	8	>64	265.1	190.8
AMPLY-PANAL2	8	64	4	4	16	>64	544.8	245.8
AMPLY-PLAFA2	2	4	4	2	8	>64	1108	113.1
AMPLY-RHITY2	4	8	2	2	8	>64	207.9	72.76
AMPLY-TAKRU1	8	4	32	2	8	>64	387.7	133.1
AMPLY-TAKRU2	8	8	16	2	8	>64	294.8	53.45
AMPLY-TALSA3	4	2	4	4	8	>64	147.9	85.58

4.3 Discussion

Summary of Findings

This project aimed to evaluate the therapeutic viability of peptide sequences identified by the AMPLY software. To this end, the genomes of 20 organisms were processed through the software's analytical pipeline and 2000 peptide sequences were selected from the output, based on predicted antimicrobial activity. After identifying a suitable bacterial strain for preliminary screening, these peptides were synthesised via SPOT and the crude products were screened for antimicrobial activity against a strain of *E. coli* in the presence of an MH broth solution containing 10% human serum. The antimicrobial screen identified 1528 peptides with activity against the chosen bacterial strain in the presence of serum; the organism with the greatest proportion of most active peptides was identified to be *G. gallus* in terms of numerical contribution, or *T. saltator* when considering proportion of peptides contributed within a genome. These data facilitated the selection of 72 peptides exhibiting antimicrobial activity despite the actions of serum binding proteins or proteolytic enzymes.

Pure variants of the 72 candidates of interest were synthesised off-site and the conditions of the preliminary screen were repeated using known concentrations of peptide solutions, such that efficacy of these peptides in the absence of side products could be determined. 15 of the 72 candidates were eliminated following this investigation, as their MICs exceeded that of the highest tested concentration. The remaining peptides presented a range of modal MICs against the bacterial strain from 64 µg/mL to 0.25 µg/mL, with the most common value observed being 8 µg/mL. Further investigations were carried out using the same peptide set in 0% and 25% serum solution, to establish baseline efficacy and degree of serum tolerance, respectively. Activity and serum tolerance of peptides was noted per organism of origin, with organism groups with the most serum-tolerant peptides being identified.

While the number of peptides with MICs exceeding the highest tested concentration increased as serum concentration increased, the relationship between serum concentration and inhibition of peptides that retained activity in the presence of serum was not so linear; the addition of serum was associated with a shift towards lower modal MICs, with the peptide set only displaying MICs lower than 2 µg/mL in the serum-containing solutions. Additionally, the addition of serum was associated with a considerable decrease of peptides with MICs equal to or greater than 8 µg/mL.

Investigating haemolytic capacity of this peptide set identified tolerance of red blood cells across a number of peptide candidates, with only 10 of the 72 peptides exhibiting mean HC₅₀ values lower than the highest concentration utilised during the antimicrobial activity studies. Additionally, more than half of this peptide set presented mean HC₅₀ values 4 times or more greater than this concentration. The organism group with the lowest haemolytic activity was

revealed to be the group with the least activity; the inverse was also observed, with the organism group with highest activity also being the most haemolytic.

The population of candidates was then further reduced, with the resultant 24 peptides being taken further for further characterisation of therapeutic viability; this took the form of cytotoxicity assays quantifying metabolism of resazurin, and MIC assays against strains of the ESKAPE pathogen group to establish spectrum of activity in the context of a clinically relevant selection of bacteria. Investigation of cytotoxicity identified several peptides with low toxicity – including 2 peptides where toxicity was not observed at all – with mean IC₅₀ values ranging from 36.63 µg/mL to 506.53 µg/mL where they could be determined.

MIC assays against the ESKAPE pathogens confirmed activity of the peptide set against 5 of the 6 tested bacterial strains; MICs could not be determined against the chosen strain of VRE. All tested peptides exhibited activity against *A. baumannii* and *P. aeruginosa*, with only a single peptide of the set of 24 showing a modal MIC above the highest tested concentration. Most common MIC values presented range from 4 µg/mL to 8 µg/mL where activity is seen, with greatest mean frequency of modal MIC being 4 µg/mL across all strains and tested candidates.

Interpretation of Findings

Identification of a suitable organism for studying serum tolerance not only required investigation of bacterial growth in the presence of serum but also consideration of any effects that would arise from diluting the MH broth to reach the required serum concentration; it is for this reason that bacterial solutions were included where MH broth was diluted using a proportion of water equal to those used for the serum solutions. Comparing mean absorbance of *E. coli* strain 15 in 10% and 25% water diluted solutions revealed no observable or statistically significant differences ($P < .05$, $n=3$) between the two populations; it was concluded from this that any differences in absorbance observed between serum- and water-containing solutions of *E. coli* strain 15 could thus be attributed to the presence of serum. The absence of these differences established that the growth of *E. coli* strain 15 was not adversely affected by serum, and so could be used to test peptide activity in the required conditions. Similarly, no differences were observed between *E. coli* strain K12 in 10% water solution and the 0% serum growth control. This cannot be said when comparing absorbances between strain K12 in 10% serum and 10% water solutions, as the presence of serum was associated with significant reduction in K12 absorbance; this property allowed the strain to be used as a control for the inhibitory effect of serum when evaluating the suitability of strain 15.

All 2000 peptides chosen for SPOT synthesis were selected based on the potential bioactivity scores given to them by the AMPLY neural network; these scores were based on experimentally validated MIC data used to establish trends between amino acid residues and motifs, and the resultant efficacy of the peptide (211). Despite activity being predicted for all peptides chosen

the preliminary screen identified 422 that presented no activity against the bacterial strain tested, suggesting a failure rate of roughly 20% for the software's predictive algorithms. While a margin of error is to be expected for any *in silico* selection method, there is an additional explanation for the events seen here: the inclusion of serum, the effects of which AMPLY is not currently equipped to account for. Hypothetically, some or all of the peptides within this population of 422 may indeed possess antimicrobial ability that is negated by concentrations of serum as low as 10% as opposed to improper selection by the software. Regardless, the exclusion of these peptides is an intended outcome of the experiment's design and facilitated the selection of several suitable candidates for further study.

The observed correlation between occurrence of arginine, lysine, or leucine and greater antimicrobial activity is unsurprising on consideration of what is known about AMP properties; the positive charges of lysine and arginine are believed to contribute to AMP activity through the electrostatic interactions previously described, and leucine-rich sequences have long been associated with a broad and potent showcase of AMP activity (263–265). Conspicuously absent is a presence of serine-rich sequences, as a number of the earliest peptide and protein structures with antimicrobial properties – particularly against organisms such as *S. aureus* – as well as those used to this day are serine derivatives (266).

The comparatively high proportion of “greater than control” peptides found within the genomes of *G. gallus* and *T. saltator* is difficult to comment on, as little context is provided by established literature. As an organism, interest in *T. saltator* arises primarily from its sensitivity and resultant behavioural responses to various environmental conditions, and there is yet to be a published study regarding the antimicrobial potential of its peptidome (267–269). However, studies have already identified the junglefowl as a viable source of AMPs, with candidates showing efficacy *in vitro* being derived from *G. gallus* haemoglobin (270).

Once pure variants of the 72 chosen candidates were acquired, assessing antimicrobial activity in 0%, 10%, and 25% serum solutions revealed unexpected trends against *E. coli* strain 15. While the previous project – and many instances in literature (217,220) – showcased the typically inhibitory effects that serum has on AMP activity, this selection of peptides as a whole has several examples of increases in activity in the presence of serum; while the most common modal MIC is 4 µg/mL for both 0% and 25% serum assays, the number of peptides showing this MIC is greater in the 25% serum assays, suggesting a greater frequency of peptides active at lower concentrations in higher percentages of serum. Frequency of modal MICs above 8 µg/mL decrease considerably when serum is included in the assays, and MICs below 2 µg/mL were only observed when bacteria and peptides were incubated in the presence of serum, showing values as low as 0.25 µg/mL for both 10% and 25% serum solutions. Reports of increasing peptide activity in the presence of serum are far less common, although possible explanations do exist. As previously mentioned, there is the possibility of greater environmental stress placed on

the bacterial population by the dilution of MH broth, although this hypothesis is somewhat less likely in light of the preliminary screening data regarding 10% and 25% water solutions where no discernible changes in growth were seen.

There also lies the possibility that the selected candidates are simply resistant to the effects of serum associated with reduced peptide activity: namely, conjugation to binding proteins and degradation by proteolytic enzymes (271,272). However, while a reduction in activity would not be observed if this were the case, it would be unexpected to see an increase in activity either; that this increase is shown by the peptide set suggests that there are additional factors affecting their performance. One final hypothesis arises on consideration of one of the components of serum: the complement proteins that contribute to innate immunity. It is known that certain AMPs are able to influence the immune system, exhibiting modulatory effects to indirectly clear pathogenic infection; multiple hypotheses have also been published regarding mechanisms of collaboration between AMPs and the complement system (273,274). It is therefore possible that the increased efficacy displayed in the presence of serum is the result of synergistic action between the peptides and the antimicrobial properties of the serum itself.

Evaluating haemolytic capacity of this peptide set proved encouraging, with the lowest HC_{50} observed still being exceeded by the MIC of the same peptide: AMPLY-DANPL2. Similarly, the 24 peptides taken forward for further testing showed promising tolerance to HEK cells, with the majority of this population exhibiting IC_{50} values in considerable excess of the maximum concentration used for MIC assays and the lowest IC_{50} seen exceeding the most common modal MIC shown by the group in any tested conditions by a factor of 4. These data combined suggest relatively low toxicity across this peptide set, despite the considerable antimicrobial activity displayed. Analysis of these peptides in groups of their source organism identified a positive correlation between antimicrobial activity and haemolytic activity; this is arguably unsurprising, as the contribution of AMP amphipathicity and hydrophobicity to both activity and toxicity is commonly reported in literature, if not yet fully understood (217,262,275,276).

The peptide set showed considerable efficacy against the ESKAPE pathogen group with the notable exception of VRE, against which no observable activity was displayed. All peptides tested displayed activity against *A. baumannii* and *P. aeruginosa*, with only a single peptide – AMPLY-CANFA1 – showing no activity at the tested concentrations against *E. cloacae*, *K. pneumoniae*, and MRSA; this shows a considerably broad spectrum of activity against pathogens with numerous and varying mechanisms of resistance, as well as both gram-negative and gram-positive organisms.

The resistance shown by VRE is notable; the varying profiles of performance shown by the AMPLY peptide set suggest that differing mechanisms of action are employed by the candidates. However, the universal tolerance observed by this organism hints at a possible

commonality in mechanism across the selected candidates, or a considerably broad spectrum of resistance from the pathogen itself.

Additionally, comparing the MIC data for the ESKAPE pathogens with the aforementioned toxicity studies suggest sizeable therapeutic windows for the treatment of this group; AMPLY-BACAN1 – the peptide with the lowest HC_{50} value of 36.71 $\mu\text{g/mL}$ – exhibited modal MICs ranging from 1 to 4 $\mu\text{g/mL}$ against the strains, with the exception of VRE. Similarly, AMPLY-BURPS3 – the only other peptide with a HC_{50} less than 128 $\mu\text{g/mL}$ – presented modal MICs ranging from 16 $\mu\text{g/mL}$ to 64 $\mu\text{g/mL}$. When considering the IC_{50} values of these peptides against HEK293 cells, the number of peptides with a toxic dose below 64 $\mu\text{g/mL}$ rises from 2 to 5. Of these peptides, only one shows a modal MIC greater than 16 $\mu\text{g/mL}$ against all tested ESKAPE pathogen strains – again, with the exception of VRE –, with AMPLY-DANPL1 exhibiting a mean IC_{50} of 66.21 $\mu\text{g/mL}$ and an MIC of 64 $\mu\text{g/mL}$ against *K. pneumoniae*. However, it should be noted that the modal MICs of this same peptide range from 4 $\mu\text{g/mL}$ to 8 $\mu\text{g/mL}$ against the remaining ESKAPE pathogens tested. With these points in mind, the tolerance to host cells and antimicrobial efficacy shown by this candidate group provide strong evidence that these peptides have therapeutic potential warranting further study.

Limitations

While steps were taken to ensure that a serum-tolerant bacterial strain was used for the serum tolerance studies, selection of a suitable strain was made with the assumption that an organism susceptible to serum would present inhibited growth. Serum is known to contain factors - such as the components of the complement system and various inflammatory mediators - that directly contribute to the clearance of bacterial cells (277) and so it was hypothesised that a bacterial population unaffected by the presence of these factors was therefore suitable for use. However, it remains possible that the presence of serum components acted as stressors for the bacteria which would alter the resultant activity of the peptides tested; this was not a factor that was investigated during the assay. Similarly, while the possibility of nutrient deficiency due to dilution of MH broth solution was investigated prior to preliminary screening, this depletion of available resources may have acted as an additional stressor, impacting bacterial competence without observably affecting growth.

However, the previously described serum studies involving peptides isolated from the human lung peptidome highlighted the potent inhibitory effect that serum can have on peptide activity; it can be reasoned that the inhibitory effect that serum may have on the bacterial population is overshadowed by the considerable effect that serum would have on peptide activity if they were susceptible to its presence. In this instance, peptide sensitivity to serum would still be determinable even though accurate peptide activity would be difficult to quantify.

The preliminary antimicrobial screen used to select candidates from a population of 2000 peptides relied on a single organism – a gram-negative pathogen - to act as the determinant of activity. While this approach was considered an acceptable compromise between utility and practicality, it is limited by the narrow scope used to measure peptide activity as a result; the resistance mechanisms employed by the chosen organism may just happen to be more or less resistant to the actions of the peptide set, leading to a skewed representation of antimicrobial capacity with no other data to compare. This is compounded by the fact that these peptides would not be assessed for activity against a gram-positive pathogen until considerably further on in the project. It is therefore possible that peptides with promising activity against gram-positive bacteria were excluded from the study before being investigated for this property.

This limitation also manifests during the MIC assays against the ESKAPE pathogen group; each member of the group was represented by a single strain, once again for the sake of practicality although a number of the chosen strains had the additional benefit of being well-characterised due to their common use in laboratory settings. Regardless, this limited selection introduces the possibility of efficacious candidates being discarded due to a perceived lack of activity. This is particularly relevant in light of the investigation into activity against VRE, where no MICs could be determined; there lies the possibility that the chosen strain of *E. faecium* was unexpectedly resistant to the mechanisms of the peptide set when other members of its species may not have been so. This is not a hypothesis that was extensively explored by this project.

5. Further Characterisation of Therapeutic Candidates

Identified Using *In Silico* Metagenomic Analysis

5.1 Introduction

The mechanisms of action shown by AMPs are numerous and varied, particularly when considering the distinction between AMPs with immunomodulatory activity and those that act directly on pathogens. In addition, AMPs with bacteriostatic properties have been reported in literature, exhibiting the ability to inhibit growth of a bacterial population without directly causing bacterial cell death (278,279). Elucidation of a novel AMP's mode of action has benefits beyond the academic as greater understanding of these mechanisms allows for peptide optimisation – improvements to activity, stability, cytotoxicity – while minimising impacts to peptide efficacy.

A critical hurdle when developing AMPs is *in vivo* performance. Despite tens of thousands of AMPs being reported in literature, only a select few have successfully progressed through clinical trials with a scant 36 AMPs undergoing clinical or pre-clinical trials as of 2019 (280). As previously mentioned, performance of AMPs *in vivo* is often greatly diminished when compared to *in vitro* data which suggests detrimental pharmacokinetics that impact peptide efficacy; it is for this reason that commercially available peptides are often administered topically or – less commonly – intravenously (281). This trend places particular significance on the inclusion of living models when assessing AMP candidates for therapeutic viability, but the inclusion of mammalian models is prohibitively costly – in terms of both money and time – and often necessitates ethical considerations that complicate experimental design.

It is perhaps for these reasons that many studies now report the use of *Galleria mellonella* greater wax moth larvae when investigating how a peptide interacts with the physiology of a living organism (282,283). These organisms have many advantages when compared to traditionally-used mammalian models as they are considerably cheaper to acquire and maintain, requiring no specialised equipment for their storage. These larvae can therefore serve as an additional screening measure, reducing the number of candidates selected for the more demanding mammalian trials. Additionally, despite evolutionarily diverging from vertebrates millennia ago, similarities remain between the mechanisms of insect immunity and those of the innate immunity seen in mammals (284); this property has allowed *G. mellonella* to be used to study virulence of an array of both gram-negative and gram-positive bacteria, including all members of the ESKAPE pathogen group and *E. coli* (285).

Similarly, this organism has properties that make it suitable for efficacy modelling; in addition to topical applications, known doses of both bacteria and antimicrobial agents can be injected into the haemolymph - a substance analogous to mammalian blood – of wax moth larvae,

allowing an approximation of intravenous administration. Progression of infection can then be monitored, with the wax worm larvae showing several distinct signs of health or distress such as skin melanisation or the formation of cocoons (282).

Study Design

The previous study established activity of the selected peptide candidates against the ESKAPE pathogens in the absence of serum; for this project, attention was turned to evaluating activity of these peptides in the presence of serum as an indicator of potential *in vivo* efficacy. As before, serum tolerance of the chosen bacterial strains was investigated prior to any efficacy assays, with absorbance of light being measured in solutions of MH broth containing either 25% serum or 25% water to evaluate the influence of broth dilution as well as serum action on bacterial cell growth. *E. coli* strain K12 was once again included for this study, acting as an indicator for the inhibitive effects of serum. Following this, peptide activity in the presence of serum was investigated via MIC determination to allow comparison between these assays and those for 0% serum activity, with the maximum concentration tested once again being 64 µg/mL. Upon consideration of the resistance shown by the chosen *E. faecium* strain, this bacterium was not included in the serum assays for the ESKAPE group. For the assays, 25% serum solutions were prepared as activity of this peptide set in the presence of a high serum concentration had been observed previously.

The results of these experiments were used to further refine the selection of candidates to facilitate greater depth of characterisation. While cytotoxicity of the larger set of peptides had been investigated through the measurement of resazurin metabolism, with the number of candidates reduced it became feasible to investigate LDH release following exposure to the peptides, providing a more sensitive quantification of HEK cell toxicity. Additionally, while MIC assays had adequately confirmed the presence of activity against the ESKAPE pathogen group, the magnitude of this activity was only loosely defined at this stage; as inhibition of bacterial growth was determined visually for these assays, the degree of inhibition could not be compared between peptides once the bacterial population could not be seen by the naked eye, nor could comparisons be made between peptides presenting identical MICs. For this reason, efforts were made to achieve precise quantification of activity by measuring the absorbance of bacterial populations following incubation in the presence of the remaining peptide candidates. The bactericidal or bacteriostatic nature of said activity was investigated through the generation of time-kill curves, providing a representation of the effects of peptide-bacteria interaction over 24 hours.

Attention was then turned to *in vivo* performance of the remaining peptide candidates, by this stage narrowed down to 4. *G. mellonella* larvae were first used to determine progression of *E. coli* strain 15 infection in the absence of peptide, as well as peptide toxicity. Prior to evaluation

of efficacy in the wax moth larvae models, an antibiotic known to be active against the chosen *E. coli* strain – meropenem, a β -lactam - was evaluated for serum tolerance to confirm that it would be suitable for use as an *in vivo* activity control. Once this had been established, the peptide candidates were introduced to infected larvae; progression of infection in these treated larvae was monitored and could be compared to a non-peptide-treated population to evaluate therapeutic effects associated with the treatment.

Hypothesis, Aims, and Objectives

Hypothesis: The selected peptides are suitable candidates for therapeutic application.

Aim: Determine the bactericidal or bacteriostatic nature of the peptides' activity, as well as their and *in vivo* suitability.

Objectives:

- Determine tolerance of selected ESKAPE pathogen strains to the presence of 25% human serum.
- Evaluate activity of peptide candidates against members of the ESKAPE pathogen group in the presence of 25% human serum.
- Select final therapeutic candidates for further characterisation following analysis of cytotoxicity via LDH release, and anti-ESKAPE activity in the presence of serum.
- Carry out detailed quantification of peptide activity in the absence of serum via measurement of bacterial absorbance of light (600 nm).
- Elucidate modes of action exhibited by this peptide set via generation of time-kill curves.
- Establish model of *E. coli* strain 15 infection progression in *G. mellonella* wax moth larvae.
- Determine suitability of meropenem for use as an activity control against *E. coli* strain 15.
- Determine toxicity of peptide candidates and meropenem in *G. mellonella* wax worm model.
- Investigate *in vivo* efficacy of peptides using *G. mellonella* wax worm model.

5.2 Results

Determining Activity of Peptide Candidates Against ESKAPE Pathogens in the Presence of Serum

The chosen strains from the ESKAPE pathogen group were incubated for 20 hours in MH broth containing 25% serum or 25% water to determine the effect that both serum and broth dilution would have on bacterial growth (**Figure 5.1**); statistically significant differences ($P < .05$, $n=3$) in mean absorbance when compared to the 0% serum population were then determined using two-way ANOVA and Dunnett's multiple comparisons testing. *E. cloacae* showed a greater rate of absorbance increase when incubated in serum than when incubated in MH broth alone; this difference was statistically significant from time-point 5.17 hours and continued to be so for the remainder of the time measured. Contrarily, *E. cloacae* incubated in a 25% water solution showed a reduced rate of absorbance increase when compared to the 0% serum population, with this difference first reaching statistical significance at time-point 13.7 hours.

MRSA showed a similar trend, with mean absorbance rising more quickly in the presence of serum and less quickly in the 25% water solution when compared to the 0% serum populations. The mean absorbance of 25% serum and 0% MRSA populations reached similar values in the final hours measured; this is supported by the multiple comparisons test, which identifies statistical significance in mean absorbance differences from time-point 9.7 hours to time-point 16.5 hours but does not do so for the remainder of the time measured. This was not seen when comparing the 25% water and 0% serum MRSA populations, where statistically significant differences were exhibited from time-point 10.2 hours until the assays were concluded.

K. pneumoniae showed slower rates of mean absorbance increase in both 25% serum and 25% water solutions when compared to the 0% serum populations. These differences were statistically significant between 0% and 25% serum populations from time-point 3.7 hours to point 4.7 hours, and again between time-points 9.3 hours and 19 hours; statistical significance of this difference did not persist until the final hour. The *K. pneumoniae* populations incubated in 25% water solution exhibited the lowest absorbance increase by the final time measured. When compared to the 0% serum populations, the difference in absorbance is statistically significant between time-points 4.5 hours and 6.2 hours, as well as from time-point 8.5 hours until the final time measured. Additionally, differences between mean absorbance of *K. pneumoniae* in 25% water and 25% serum solutions were themselves statistically significant from time-point 11.7 hours until the final time measured.

A. baumannii displayed very similar mean absorbance values when incubated in 0% serum and 25% water solutions; no statistically significant differences were observed between these mean absorbances across all measured time-points. When compared to the 0% serum population, *A. baumannii* incubated in 25% serum presented an initially reduced rate of absorbance increase

between 5 and approximately 15 hours of incubation, but mean absorbance of this population would go on to exceed that of the 0% serum population by the final time measured. The observed trends are supported by the subsequent statistical analysis, which identified statistically significant difference between these two populations from time-point 5.3 hours to 12.7 hours, as well as from time-point 17.7 hours until the final time measured.

P. aeruginosa exhibited considerably different growth curves in the 3 solutions tested, with the population incubated in 25% water solution once again showing the slowest increase in mean absorbance from approximately 15 hours; the mean absorbance of this population begins to taper off at this time. A similar trend is shown by the population incubated in 0% serum solution, although the absorbance increases at a considerably faster rate before approaching a plateau – reaching a higher mean absorbance overall - when compared to the 25% water solution population. For approximately the first 10 hours of incubation, the *P.aeruginosa* population incubated in 25% serum solution presented a similar rate of mean absorbance increase to that of the 25% water population; after this point, mean absorbance continued to increase without a plateau, reaching the highest mean absorbance of the 3 populations by the final time measured.

Two-way ANOVA and multiple comparisons testing following the *P. aeruginosa* absorbance assays identified statistically significant differences between mean absorbance of populations incubated in 0% serum and 25% serum solutions between time-points 5.5 hours and 15.5 hours, and again from time-point 17.8 hours until the final time measured. Differences in mean absorbance between 0% serum solution and 25% water solution populations were statistically significant from 6.3 hours of incubation; this significance was maintained until the final time measured. Lastly, comparing absorbance curves of *P. aeruginosa* incubated in 25% serum and 25% water solutions identified statistically significant differences from time-point 5.8 hours to time-point 10 hours, and again from time point 13.7 hours until the final time measured.

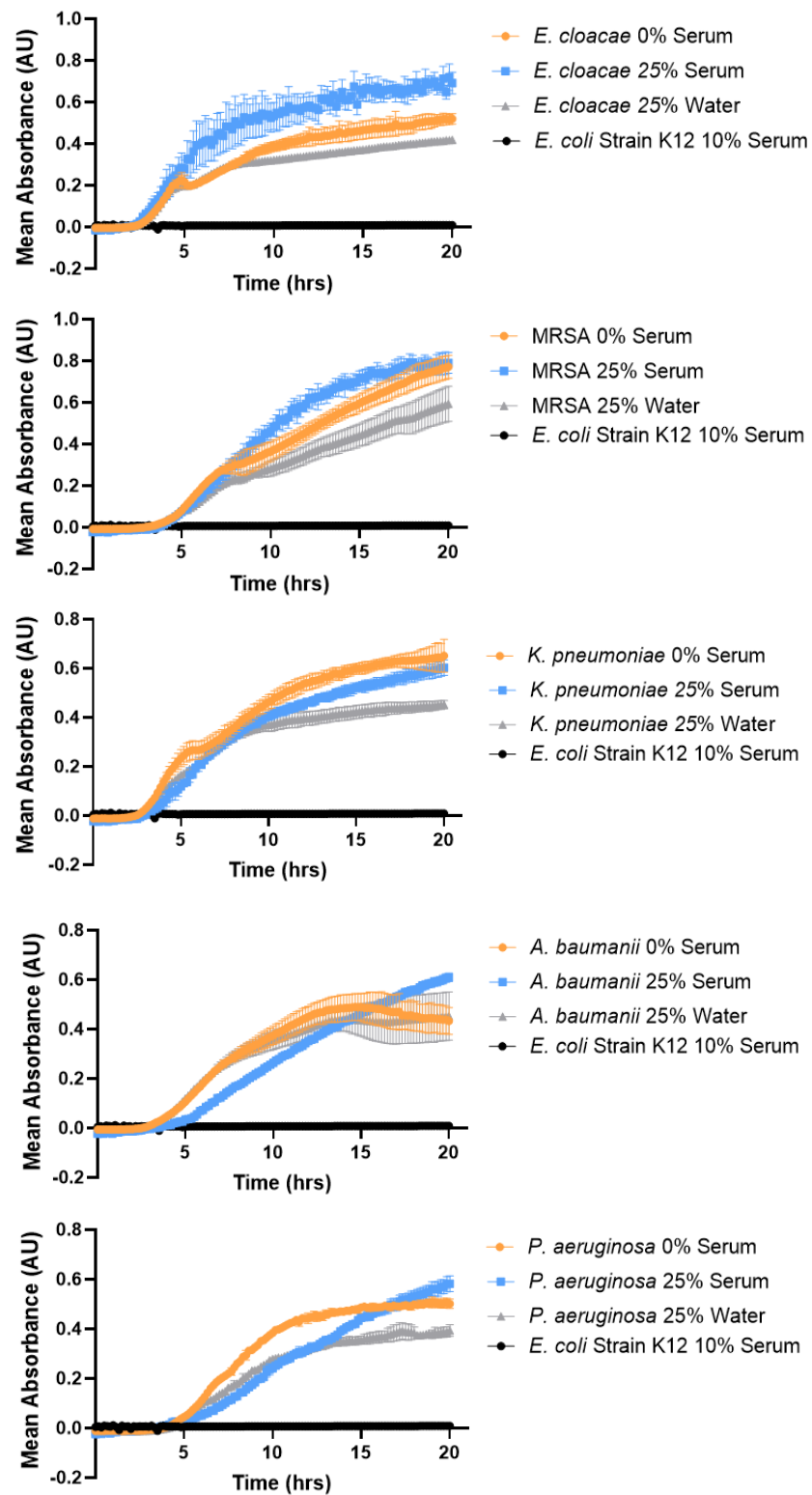


Figure 5.1. Mean absorbance of ESKAPE pathogens, adjusted for background absorbance of culture medium. Assays were conducted in triplicate, with error bars denoting standard deviation.

Following characterisation of the serum tolerance of the chosen bacterial strains, the 24 peptide candidates were assessed for activity against these ESKAPE pathogens in the presence of serum (**Figure 5.2, Table 5.1**). Across all peptide candidates tested against all bacterial strains in these conditions, the most common modal MIC observed was 64 µg/mL, with a mean frequency of 5 against any given strain; this value, however, was exceeded by the number of peptides for which an MIC could not be determined – a mean frequency of 7.6 - due to it being greater than the highest concentration tested. Contrarily, the least common modal MIC across the peptide set was 0.25 µg/mL - which had a mean frequency of 0.2 - followed by concentrations 0.125 µg/mL, 2.5 µg/mL, and 1 µg/mL, which all had a mean frequency of 0.4.

When tested against *E. cloacae* in the presence of serum, the most common modal MIC value observed was 8 µg/mL, exhibited by 7 of 24 peptides; this value is exceeded only by the number of peptides that were unable to display activity at the highest tested concentration in these conditions, of which there were 8. 2 of 24 peptides displayed MICs lower than this most common value, and these peptides – AMPLY-GALGA4 and AMPLY-OPHHA3 – also exhibited the lowest modal MIC seen against this bacterial strain in the presence of serum. The greatest modal MIC observed against *E. cloacae* in the presence of serum was 64 µg/mL, with a frequency of 4 of 24 peptides tested.

MICs could not be determined for the majority of peptides tested against MRSA in the presence of serum, with 18 of 24 peptides tested showing no observable activity at the highest concentration tested. Of the remaining 6 peptides, 5 of them presented a modal MIC of 64 µg/mL; peptide AMPLY-BACAN1 was the exception, exhibiting a modal MIC of 16 µg/mL. Against *K. pneumoniae*, MIC could not be determined for 1 peptide within the set: AMPLY-CANLU2. Across the remaining 23 peptides, the greatest modal MIC observed was 64 µg/mL. This concentration is also one of two most common modal MICs exhibited against this bacterial strain in these conditions; a peptide frequency of 5 was seen for both 64 µg/mL and 4 µg/mL, followed by 32 µg/mL and 8 µg/mL which both had a peptide frequency of 4. The lowest modal MIC observed against this bacterial strain in the presence of serum was 0.5 µg/mL, which was exhibited by AMPLY-BACAN3 and no other candidates.

A. baumannii is the only tested pathogen for which MICs in the presence of serum could be determined for all peptide candidates. The highest observed MIC against this bacterial strain in serum was 64 µg/mL; this activity was shown by 2 of 24 peptides tested, AMPLY-METSE1 and AMPLY-CROPO2. 4 of 24 peptides presented modal MIC values of either 32 µg/mL, 16 µg/mL, or 8 µg/mL, with this being the highest peptide frequency observed against this organism throughout the assays, followed by the modal MIC of 2 µg/mL which was exhibited by 3 of the peptides tested. The lowest modal MIC observed against all strains tested was 0.125 µg/mL; this activity was observed entirely against *A. baumannii* and was attributed to peptides AMPLY-GALGA3 and AMPLY-MESMA7.

Lastly, MICs of 11 peptides could not be determined when investigating activity against *P. aeruginosa* in the presence of serum. Of the remaining 13 peptides, 9 of them displayed a modal MIC of 64 µg/mL with a further 3 showing a modal MIC of 32 µg/mL. The lowest modal MIC shown by the peptide set against this organism in the presence of serum was 8 µg/mL; this activity was shown by AMPLY-BACAN3 and no other candidates.

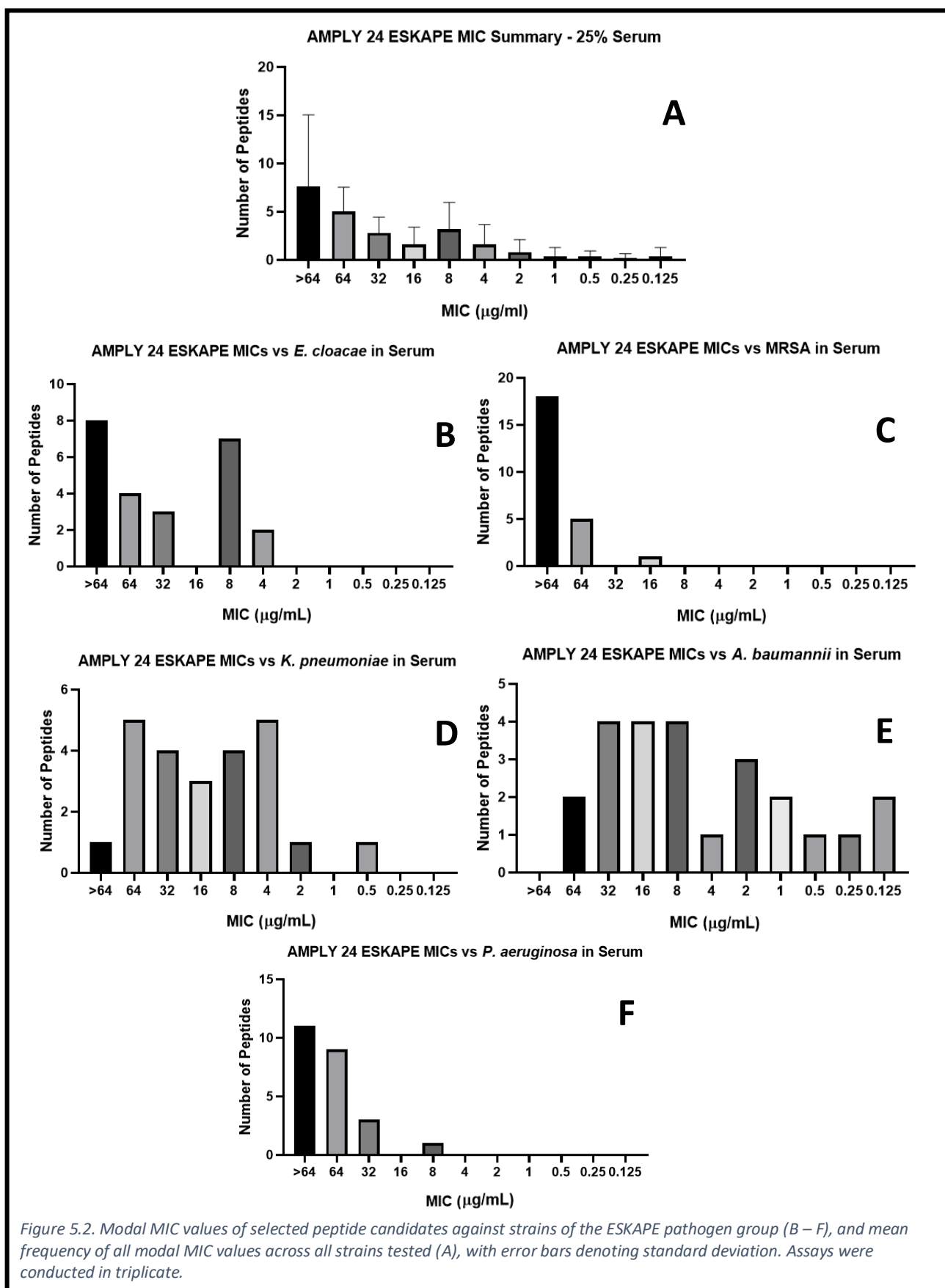


Figure 5.2. Modal MIC values of selected peptide candidates against strains of the ESKAPE pathogen group (B – F), and mean frequency of all modal MIC values across all strains tested (A), with error bars denoting standard deviation. Assays were conducted in triplicate.

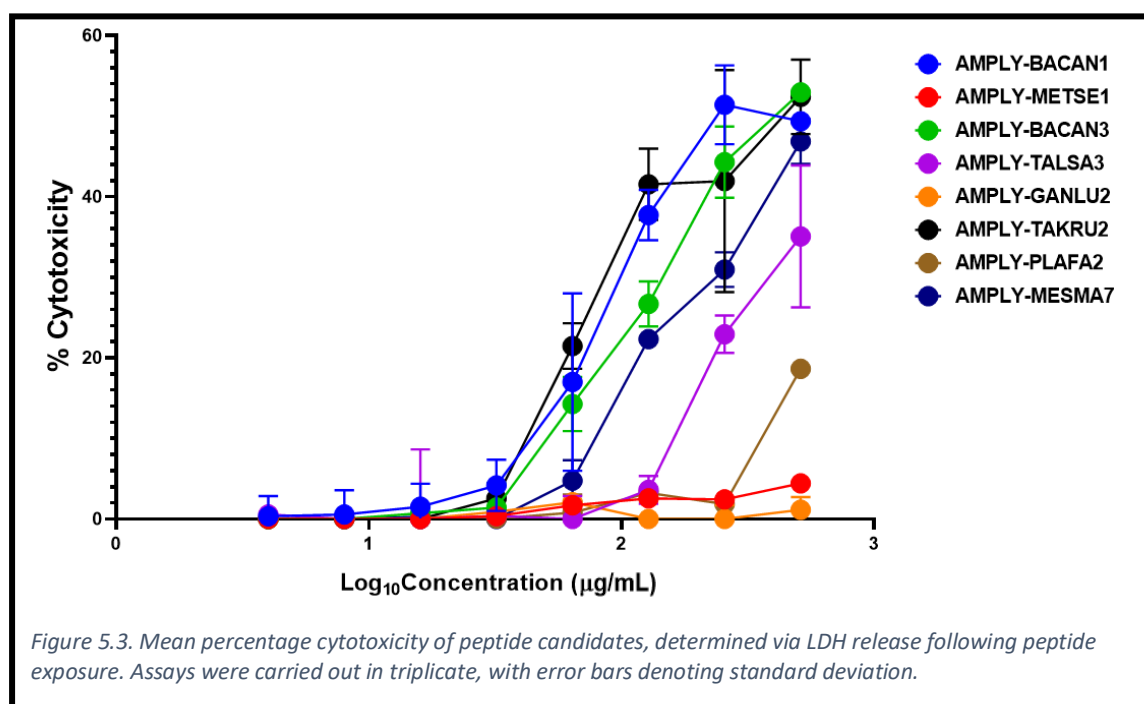
Table 5.1. Mean HC_{50} , mean IC_{50} , and modal MICs of selected peptide candidates against strains of the ESKAPE pathogen group in 25% serum solution. Assays were carried out in triplicate. In parentheses are the MICs of peptide candidates against the pathogen in the absence of serum. Peptides selected for further characterisation are highlighted in green.

Peptide	25% Serum MIC ($\mu\text{g/mL}$)					Toxicity ($\mu\text{g/mL}$)	
	<i>E. cloacae</i>	MRSA	<i>K. pneumoniae</i>	<i>A. baumannii</i>	<i>P. aeruginosa</i>	RBC HC_{50}	HEK IC_{50}
AMPLY-BACAN1	(1) 8	(2) 16	(2) 8	(4) 2	(2) 32	35.09	30.63
AMPLY-BACAN3	(8) 8	(8) >64	(2) 0.5	(2) 1	(4) 8	808	62.41
AMPLY-BURPS3	(16) 64	(16) 64	(32) 32	(32) 8	(64) >64	65.18	253.4
AMPLY-CANFA1	(>64) 32	(>64) >64	(>64) 16	(4) 32	(32) 64	866.5	984
AMPLY-CANLU2	(16) >64	(4) 64	(32) >64	(8) 32	(16) >64	136	152.6
AMPLY-CROPO2	(8) >64	(8) >64	(8) 32	(8) 64	(8) >64	218.4	151.3
AMPLY-DANPL1	(8) 8	(4) >64	(64) 8	(4) 8	(8) >64	807.8	66.21
AMPLY-FALPE1	(8) 64	(8) >64	(32) 64	(32) 32	(32) >64	167.7	162.9
AMPLY-GALGA1	(16) >64	(4) 64	(64) 64	(8) 16	(4) 64	627.5	68.19
AMPLY-GALGA3	(4) 32	(4) 64	(4) 32	(4) 0.125	(8) 64	412.4	111.4
AMPLY-GALGA4	(4) 4	(16) >64	(4) 4	(1) 2	(4) 64	252.7	49.75
AMPLY-GALSU2	(4) >64	(32) >64	(8) 64	(4) 0.5	(16) >64	804.3	279.6
AMPLY-GANLU2	(2) 64	(4) >64	(2) 64	(4) 32	(8) >64	1268	480.2
AMPLY-HELAN2	(8) >64	(4) >64	(8) 16	(2) 16	(8) >64	307.7	50.76
AMPLY-MESMA7	(2) 64	(2) >64	(4) 2	(4) 0.125	(16) 64	243	97.36
AMPLY-METSE1	(16) >64	(8) >64	(16) 64	(8) 64	(16) >64	1521	959.8
AMPLY-OPHHA3	(2) 4	(2) >64	(32) 8	(4) 8	(16) >64	270	131.5
AMPLY-ORCOR2	(2) >64	(4) 64	(4) 32	(4) 16	(8) >64	265.1	190.8
AMPLY-PANAL2	(8) 8	(64) >64	(4) 4	(4) 16	(16) 64	544.8	245.8
AMPLY-PLAFA2	(2) 32	(4) >64	(4) 16	(2) 0.25	(8) 64	1108	113.1
AMPLY-RHITY2	(4) >64	(8) >64	(2) 4	(2) 4	(8) 64	207.9	72.76
AMPLY-TAKRU1	(8) 8	(4) >64	(32) 4	(2) 8	(8) 64	387.7	133.1
AMPLY-TAKRU2	(8) 8	(8) >64	(16) 4	(2) 2	(8) 32	294.8	53.45
AMPLY-TALSA3	(4) 8	(2) 8	(4) 1	(4) 32	(8) 32	147.9	85.58

Further Characterisation of AMP Candidate Efficacy

A further selection of candidates was made based on the observed tolerance to serum when tested against bacteria of the ESKAPE pathogen group; peptides were prioritised based on exhibited breadth of activity followed by degree of relative efficacy, and finally cytotoxicity where it had been previously established via resazurin assay ([Table 5.1](#)). The reduction in candidate number made it practical to further investigate cytotoxicity by measuring the release of LDH following HEK cell incubation in the presence of peptide ([Figure 5.3](#)). The assays were carried out at a maximum concentration of 512 $\mu\text{g/mL}$ and serially diluted 2-fold to a minimum concentration of 4 $\mu\text{g/mL}$. At the highest concentration tested, all but two of the peptide candidates exhibited percentage cytotoxicity lower than 50%, with AMPLY-BACAN3 presenting the highest mean cytotoxicity observed at this concentration - 53.0% - and AMPLY-TAKRU2 showing a mean toxicity of 52.4%; mean percentage cytotoxicity did not exceed 50% across the peptide set at lower concentrations with the exception of AMPLY-BACAN1, which displayed a mean cytotoxicity of 51.4% when administered at 256 $\mu\text{g/mL}$. Additionally, when tested at 512 $\mu\text{g/mL}$, 3 of 8 peptides displayed mean percentage cytotoxicity below 20%, with 2 of those peptides – AMPLY-METSE1 and AMPLY-GANLU2 – showing mean cytotoxicity below 5%.

Across the peptide set, mean percentage cytotoxicity did not exceed 5% until peptide solutions were administered at a concentration of 64 $\mu\text{g/mL}$; it was at this point that mean cytotoxicity of AMPLY-BACAN1 rose to 17.0%, AMPLY-BACAN3 rose to 14.3%, and AMPLY-TAKRU2 rose to 21.5%. Mean percentage cytotoxicity did not exceed 0.5% for any peptide solutions when tested at the lowest concentration of 4 $\mu\text{g/mL}$.



The implementation of a more sensitive cytotoxicity assay allowed a final reduction of the peptide candidate population; of the 8 remaining peptides of interest, 4 were chosen for further investigations in light of their exhibited cytotoxicity during the LDH release assays as well as the established breadth and efficacy of their antimicrobial activity in both the presence and absence of serum (**Table 5.2**). The aforementioned activity of these peptides against the ESKAPE pathogens was then further characterised through measurement of bacterial absorbance following 20 hours of incubation in the presence of peptide (**Figure 5.4**); while previous MIC assays provided valuable discrete data regarding antibacterial efficacy, it was hypothesised that determining absorbance would provide continuous data to more precisely quantify peptide activity, as well as allow candidate activity at a given concentration to be compared with other peptides that had previously presented the same MIC.

The four remaining candidates were incubated with the ESKAPE bacteria at concentrations shown to be efficacious during previous MIC assays. As before, statistical significance was determined via one-way ANOVA and post-hoc Dunnett's multiple comparisons tests ($P < .05$, $n=3$). Statistically significant reductions in mean *E. cloacae* absorbance were shown by 3 of 4 peptide candidates – AMPLY-BACAN3, AMPLY-PLAFA2, and AMPLY-MESMA7 – when compared to the control, with multiple comparisons testing showing P values below .0001 for AMPLY-BACAN3 and AMPLY-PLAFA2, and a P value of .0003 for AMPLY-MESMA7. Statistically significant changes to mean *E. cloacae* absorbance following incubation in the presence of AMPLY-GANLU2 were not found when compared to the control at the established MIC. Additionally, no statistical significance was seen when comparing reductions in mean absorbance between the 3 peptides active against the organism.

When tested against MRSA, no statistically significant reductions in mean absorbance were identified across any of the peptides tested at their established MICs. Mean absorbance of MRSA increased following incubation in the presence of AMPLY-GANLU2; this increase was determined to be statistically significant following analysis.

Of the 4 peptides, only AMPLY-BACAN3 failed to significantly reduce mean absorbance when tested against *K. pneumoniae* at the established MIC, presenting a P value of .2311 following statistical analysis. Decreases in mean absorbance when compared to the control were deemed statistically significant following incubation in the presence of peptides AMPLY-GANLU2, AMPLY-PLAFA2, and AMPLY-MESMA7 with P values of .0036, .0053, and .0187, respectively. Additionally, statistical significance was not found when comparing inhibitory effects between AMPLY-BACAN3 and AMPLY-GANLU2 – both of which were tested at 2 µg/mL – nor between AMPLY-PLAFA2 and AMPLY-MESMA7 which were both tested at 4 µg/mL.

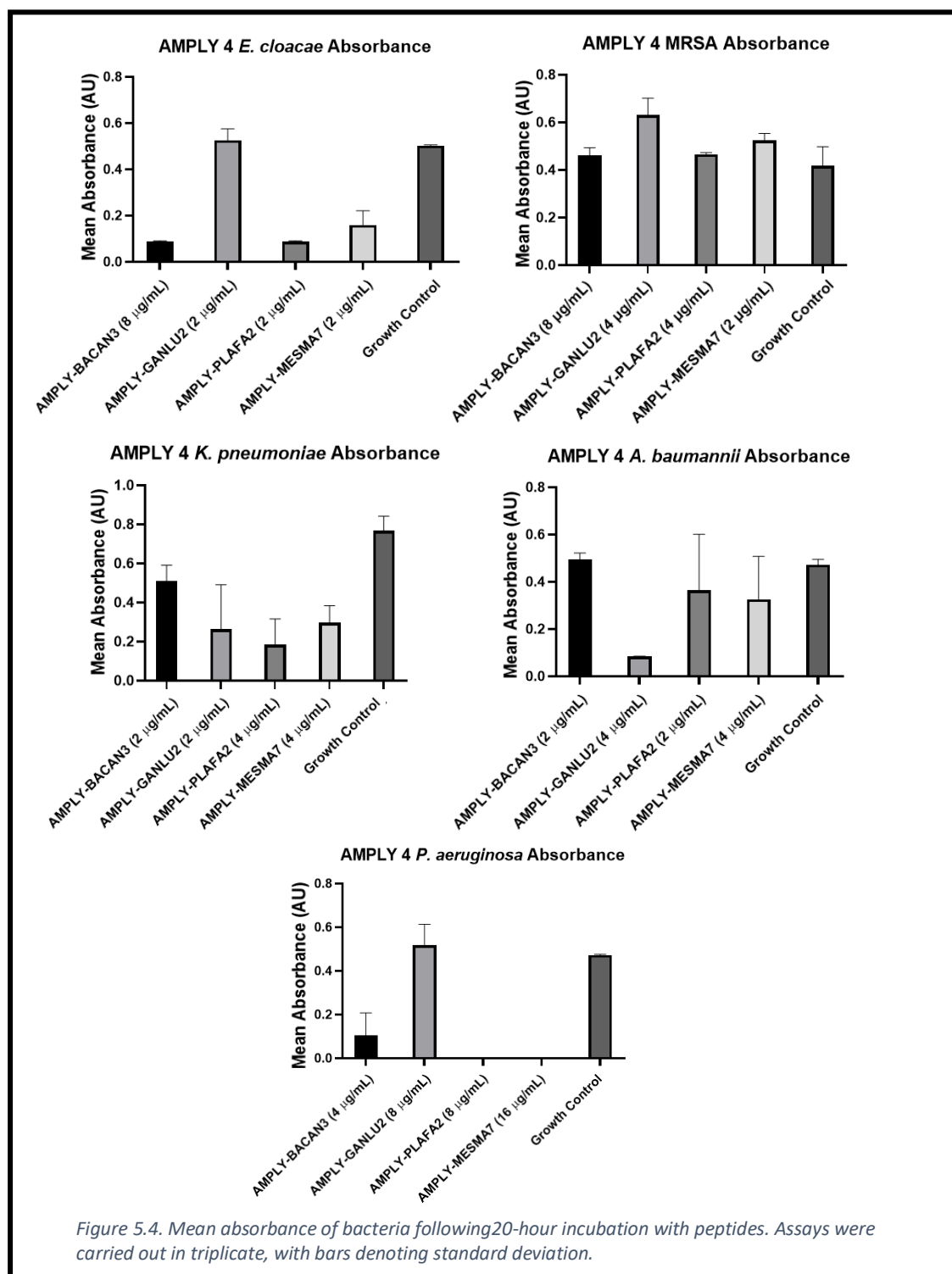
Only AMPLY-GANLU2 reduced mean *A. baumannii* absorbance when tested against it at the established MIC, with a P value of .0124 when compared to the growth control. Changes in mean absorbance following 20-hour incubation of *A. baumannii* in the presence of the remaining 3 peptides were not found to be statistically significant. Additionally, while significance was found when comparing changes in mean absorbance between AMPLY-GANLU2 and AMPLY-MESMA7 – with both peptides being tested at their established MIC of 4 µg/mL – no significance was found when comparing the remaining peptide candidates with each other, both of which had an established MIC of 2 µg/mL against this *A. baumannii* strain.

Finally, 3 of 4 peptide candidates at their established MIC significantly reduced mean absorbance of *P. aeruginosa* after 20 hours of incubation when compared to the growth control; changes in mean absorbance in the presence of peptides AMPLY-BACAN1, AMPLY-PLAFA2, and AMPLY-MESMA7 all presented P values less than .0001 following statistical analysis. Incubation in the presence of AMPLY-GANLU2 at its established MIC against *P. aeruginosa* did not reduce mean absorbance to a statistically significant degree. As a result, differences between the two peptides tested at the same concentration – AMPLY-GANLU2 and

Table 5.2. Summary of observed properties of final peptide candidates, including modal MICs against all tested pathogens in 25% serum and mean IC₅₀ and HC₅₀ extrapolated from nonlinear regression models (four parameters, n=3). In parentheses are the MICs of peptide candidates against the pathogen in the absence of serum.

Peptide	25% Serum MIC (µg/mL)						Toxicity (µg/mL)		
	<i>E. coli</i>	<i>E. cloacae</i>	MRSA	<i>K. pneumoniae</i>	<i>A. baumannii</i>	<i>P. aeruginosa</i>	RBC HC ₅₀	HEK IC ₅₀ (Resazurin)	HEK IC ₅₀ (LDH)
AMPLY-BACAN3	(2) 0.25	(8) 8	(8) >64	(2) 0.5	(2) 1	(4) 8	808	62.41	132
AMPLY-GANLU2	(2) 4	(2) 64	(4) >64	(2) 64	(4) 32	(8) >64	1268	480.2	249.3
AMPLY-MESMA7	(4) 0.25	(2) 64	(2) >64	(4) 2	(4) 0.125	(16) 64	243	97.36	186.1
AMPLY-PLAFA2	(4) 2	(2) 32	(4) >64	(4) 16	(2) 0.25	(8) 64	1108	113.1	221.5

AMPLY-PLAFA2 – were statistically significant, with a P value less than .0001.



Absorbance assays were carried out against the ESKAPE group bacteria at multiple concentrations, allowing mean IC_{50} to be determined for each peptide candidate against the selected strains (**Figure 5.5, Figure 5.6**).

Against *E. cloacae* the lowest mean IC_{50} was shown by AMPLY-BACAN3 with an inhibitory concentration of 1.51 μ g/mL; this was the only peptide with an IC_{50} below 2 μ g/mL against this organism, while AMPLY-GANLU2, AMPLY-MESMA7, and AMPLY-PLAFA2 presented

mean IC₅₀s of 3.56 µg/mL, 4.00 µg/mL, and 4.64 µg/mL respectively. Statistical analysis of the differences between these concentrations identified statistical significance (P=.032, n=3), the source of which could not be identified following Games-Howell's multiple comparisons testing.

Contrarily, AMPLY-BACAN3 presented the highest mean IC₅₀ against MRSA with a value of 13.10 µg/mL; the lowest mean IC₅₀ was instead shown by AMPLY-MESMA7 with a value of 5.24 µg/mL. AMPLY-MESMA7 and AMPLY-GANLU2 were the only candidates to exhibit a mean IC₅₀ less than 10 µg/mL against MRSA, with AMPLY-GANLU2 presenting a value 9.22 µg/mL. Despite this, no statistically significant difference was found between any of the observed IC₅₀s following ANOVA and post-hoc comparison.

AMPLY-BACAN3 once again presented the lowest mean IC₅₀ when the candidates were tested against *K. pneumoniae* and was the only candidate with a mean IC₅₀ lower than 2 µg/mL (1.83 µg/mL), while all candidates exhibited mean IC₅₀s below 5 µg/mL. Contrasting with its activity against MRSA, AMPLY-MESMA7 presented the highest IC₅₀ – 3.78 µg/mL – against *K. pneumoniae*, with AMPLY-GANLU2 and AMPLY-PLAFA2 presenting mean IC₅₀ values of 2.78 µg/mL and 2.98 µg/mL respectively. Statistical significance was not found when comparing IC₅₀s for the peptide candidates against this organism.

As was the case when tested against *E. cloacae* and *K. pneumoniae*, all candidates presented a mean IC₅₀ lower than 5 µg/mL against *A. baumannii*. The lowest IC₅₀ of 2.26 µg/mL was shown by AMPLY-GANLU2; this is the only organism tested against which AMPLY-GANLU2 exhibits the lowest mean IC₅₀ of the candidate group. AMPLY-BACAN3, AMPLY-PLAFA2, and AMPLY-MESMA7 displayed mean IC₅₀s of 3.04 µg/mL, 3.88 µg/mL, and 4.56 µg/mL respectively. It should also be noted that AMPLY-PLAFA2 and AMPLY-MESMA7 exhibited considerable variability in efficacy against *A. baumannii*, with standard deviation equalling or exceeding 80% of the mean values themselves. Analysis of variance identified no statistically significant differences in the IC₅₀s of the candidates against this organism.

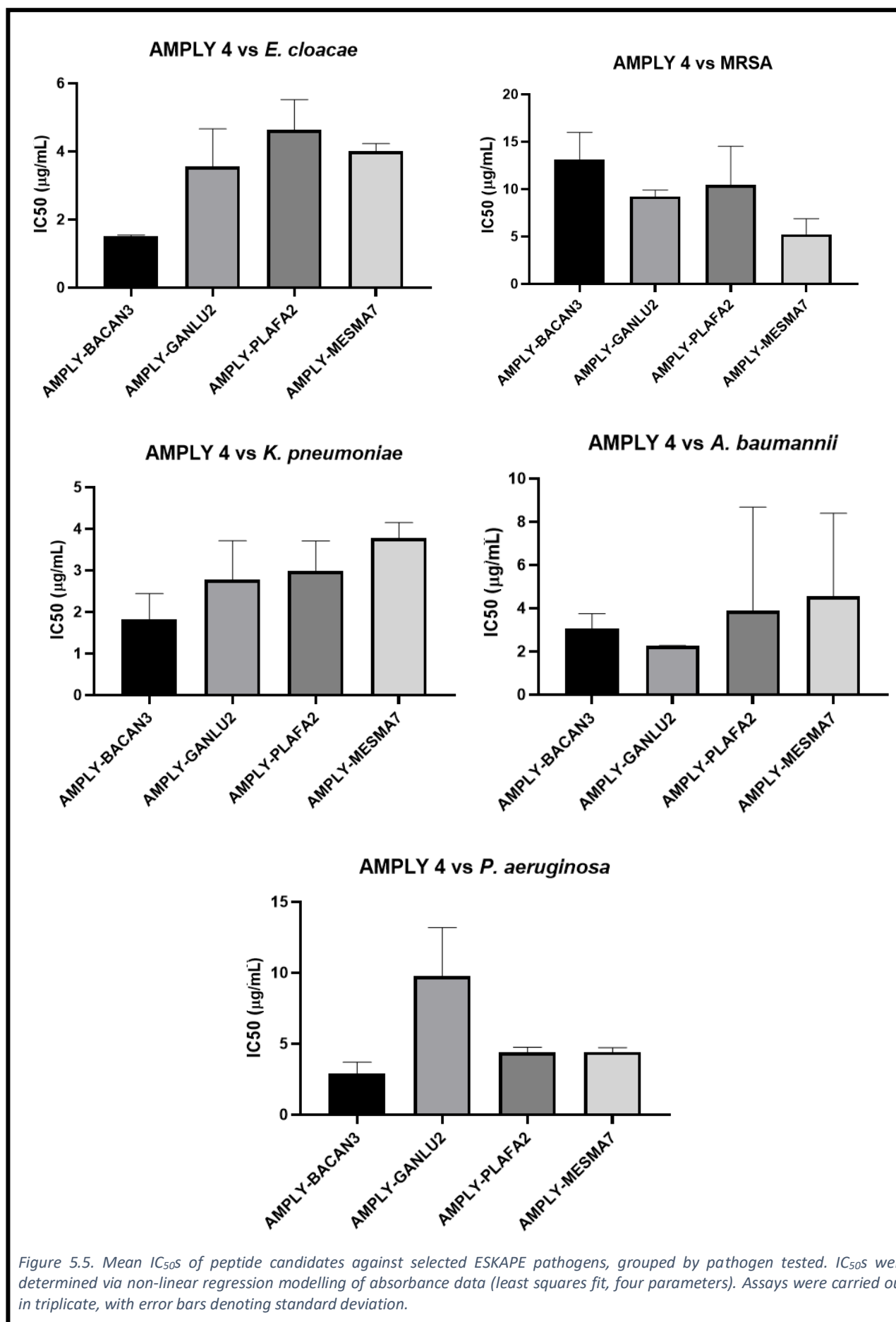
AMPLY-GANLU2 was the only candidate to present a mean IC₅₀ exceeding 5 µg/mL when tested against *P. aeruginosa*, with a value of 9.78 µg/mL. The lowest mean IC₅₀ against this organism was shown by AMPLY-BACAN3 – with a mean value of 2.89 µg/mL – followed by AMPLY-PLAFA2 and AMPLY-MESMA7, which presented mean IC₅₀s of 4.40 µg/mL and 4.42 µg/mL respectively. Statistical analysis via ANOVA identified no statistically significant differences between these values.

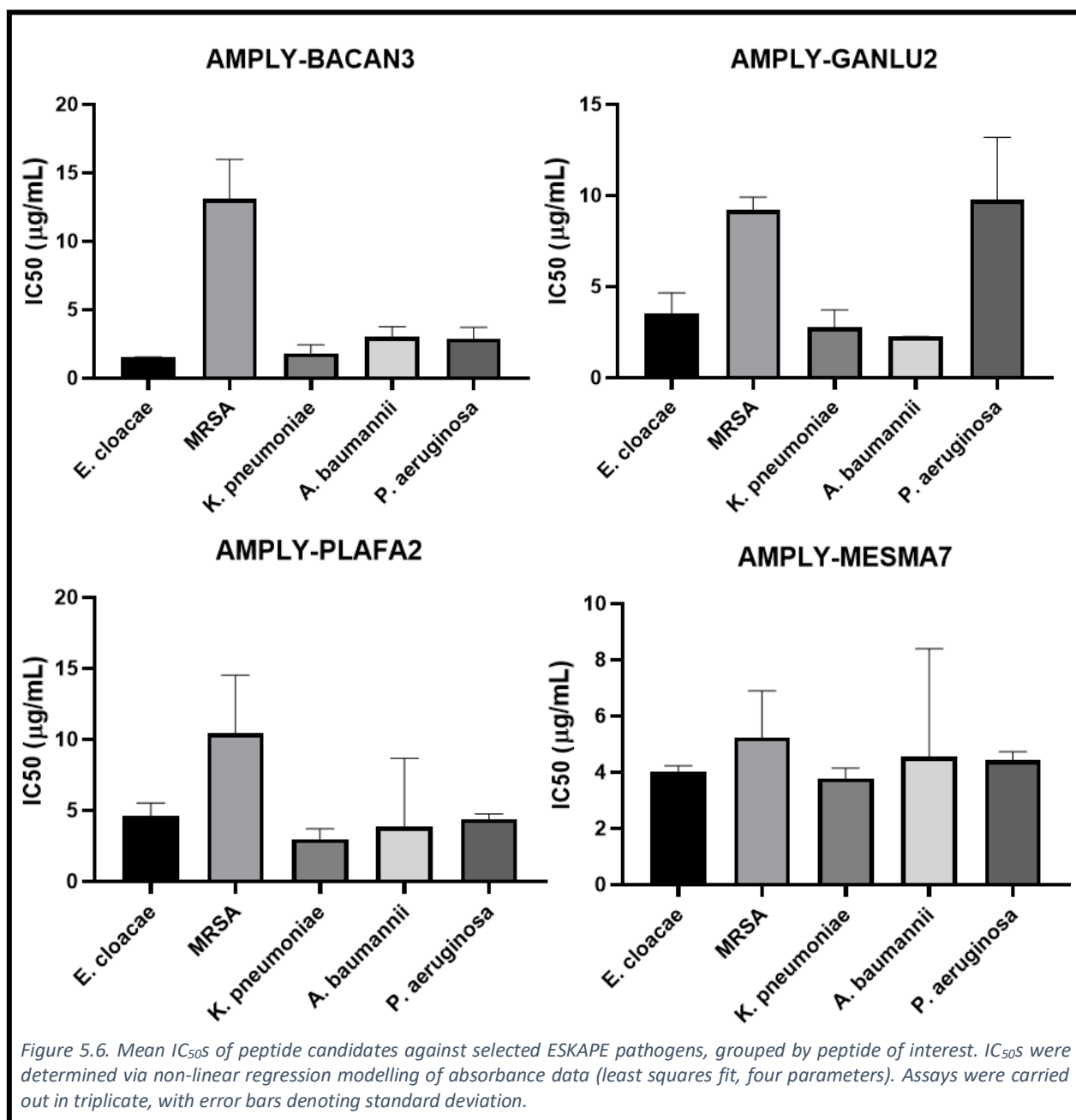
Across the 5 pathogens tested, AMPLY-BACAN3 presented its lowest IC₅₀ value against *E. cloacae*; AMPLY-BACAN3 is the only peptide of the set for which this is the case, and its inhibitory concentration, as previously noted, was the lowest of all candidates against this organism. Similarly, the highest IC₅₀ exhibited by this peptide was against MRSA, for which its

inhibitory concentration was the highest of all peptides tested. Statistical analysis of the differences between the inhibitory concentrations of this peptide identified significance ($P=.0096$, $n=3$), the source of which could not be identified following Games-Howell's multiple comparisons testing.

AMPLY-GANLU2 presented its lowest mean IC_{50} when tested against *A. baumannii* and was the only peptide to do so; it is also 1 of 2 peptide candidates that presented mean IC_{50} values lower than 10 $\mu\text{g/mL}$ against all of the tested bacteria, with the other being AMPLY-MESMA7. Additionally, it is the only peptide for which its highest mean IC_{50} was observed against *P. aeruginosa*, although it should be noted that its mean IC_{50} against MRSA – the pathogen against which all other candidates showed their highest mean IC_{50} s – is comparable to the other candidates of the set. Statistical analysis of the differences between the IC_{50} s of this peptide confirmed significance ($P=.029$, $n=3$), the source of which could not be identified.

Both AMPLY-PLAFA2 and AMPLY-MESMA7 presented their lowest mean IC_{50} when tested against *K. pneumoniae*, although neither of these were the lowest value shown by the peptide set against this organism. Like all other candidates with the exception of AMPLY-GANLU2, the highest IC_{50} shown by these peptides was against MRSA. No statistically significant differences were identified between the IC_{50} s of these peptides for any of the pathogens tested.



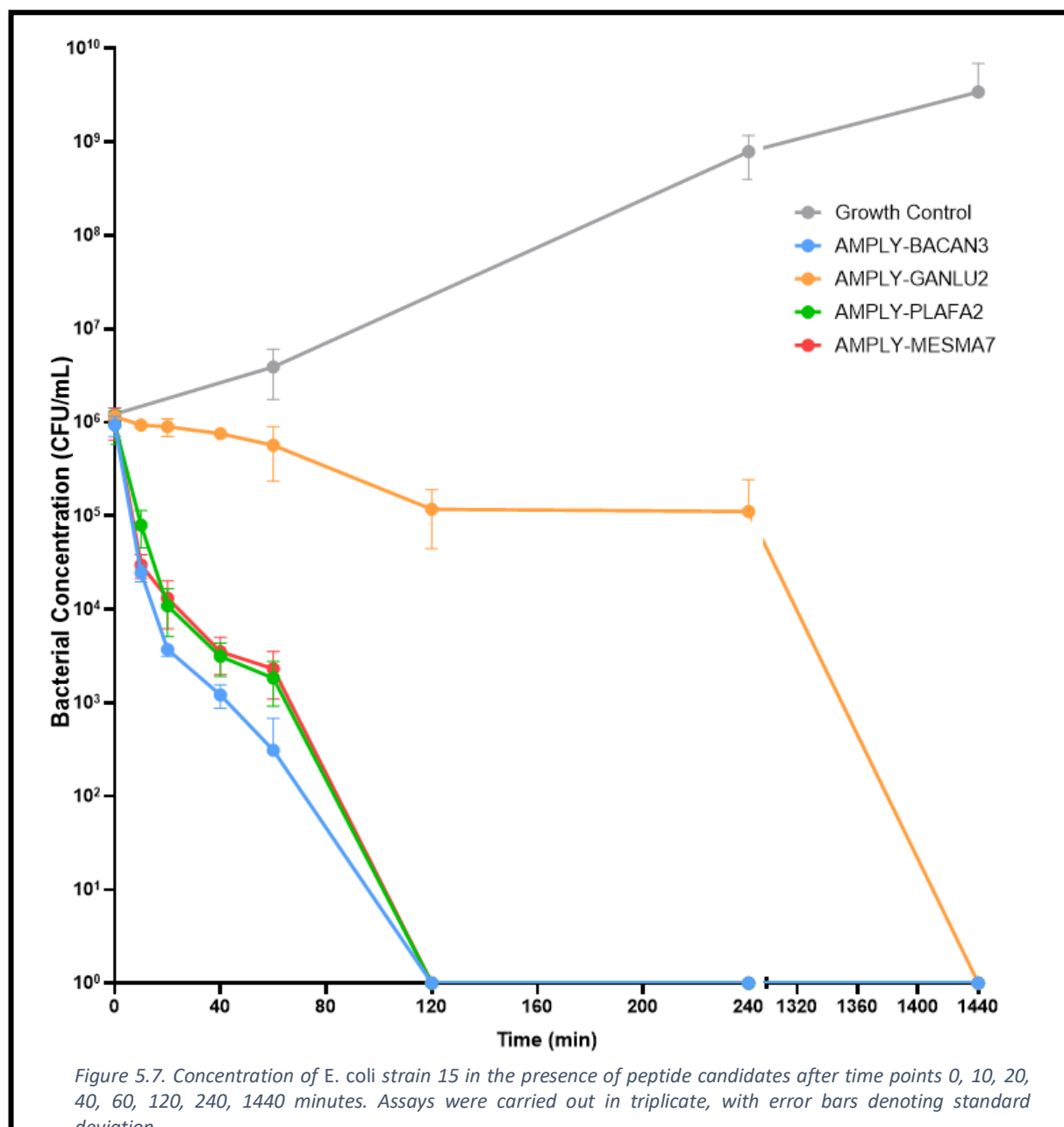


Time-Kill Assays of AMP Candidates

Time-kill kinetics assays were carried out to determine whether the activity of the peptide candidates was bacteriostatic or bactericidal; solutions of *E. coli* strain 15 were incubated in the presence of each candidate, with samples being extracted at selected intervals to measure effects of the peptides on bacterial concentration over 24 hours (**Figure 5.7**). A decrease in mean bacterial concentration was observed for all candidates following incubation of 10 minutes or more. This decrease was identified as statistically significant when compared to the mean concentration of the growth control from 60 minutes of incubation: the first time-point at which growth control concentration was determined following time-point 0. Statistical significance persisted at all subsequent time-points, as determined by two-way ANOVA and Dunnett's multiple comparisons testing ($p < .05$, $n=3$).

Incubation of bacteria in the presence of AMPLY-BACAN3, AMPLY-PLAFA2, or AMPLY-MESMA7 resulted in a reduction of mean bacterial concentration to 0 CFU/mL by 120 mins in all assays and this reduction persisted until the conclusion of the assays 22 hours later. Statistical significance between activity of these three peptides was not identified for any time-points of the assay.

E. coli incubated in the presence of AMPLY-GANLU2 also showed a decrease in mean bacterial concentration within 10 minutes of incubation, with statistically significant difference being identified between this population and the growth control from the 60 minute time-point to the conclusion of the assays. Significance was also observed when comparing activity of this peptide to any one of the other 3 candidates between time-points 10 and 240 minutes, where the reduction of mean bacterial concentration due to AMPLY-GANLU2 was considerably less than that of AMPLY-BACAN3, AMPLY-PLAFA2, or AMPLY-MESMA7 to the point that reduction of mean bacterial concentration was not observed between time-points 120 minutes and 240 minutes. Mean bacterial concentration was reduced to 0 CFU/mL by the conclusion of the assays after 24 hours of incubation but the time-point at which this occurred was not observed.

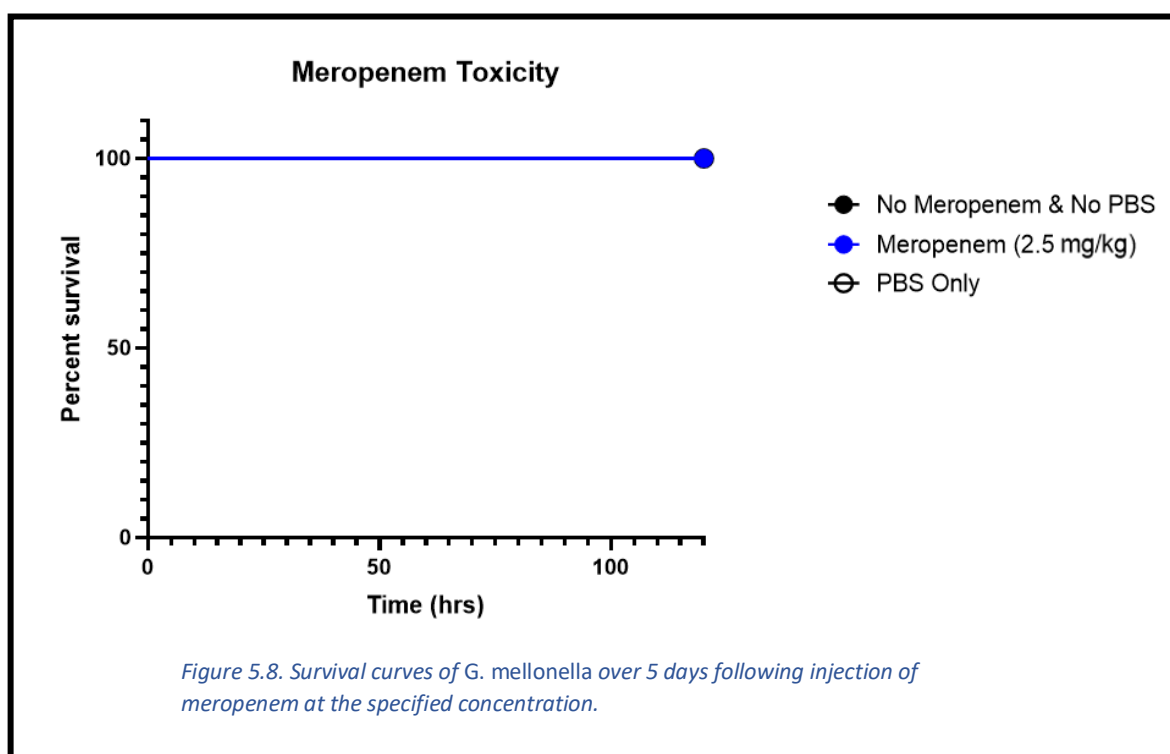


In vivo* Studies of AMP Candidate Activity Using *G. mellonella

Prior to *in vivo* investigations, an antimicrobial agent was selected to act as a positive control of efficacy. This agent – meropenem – was chosen due to its known activity against *E. coli* strain 15 and was subsequently assessed for activity against this organism in the presence of serum (**Table 5.3**) as well as toxicity in *G. mellonella* (**Figure 5.8**). Modal MICs against the bacterial strain without serum were very low for meropenem, approximately 1000 times lower than all those shown by the peptide candidates identified by the AMPLY software. Meropenem modal MIC did increase in the presence of 25% serum solution but remained in ng/mL ranges. Additionally, *G. mellonella* larvae injected with meropenem showed 100% survival after 5 days incubation.

Table 5.3. MIC values of meropenem against *E. coli* strain 15, expressed as modal concentration. Assays were carried out in triplicate.

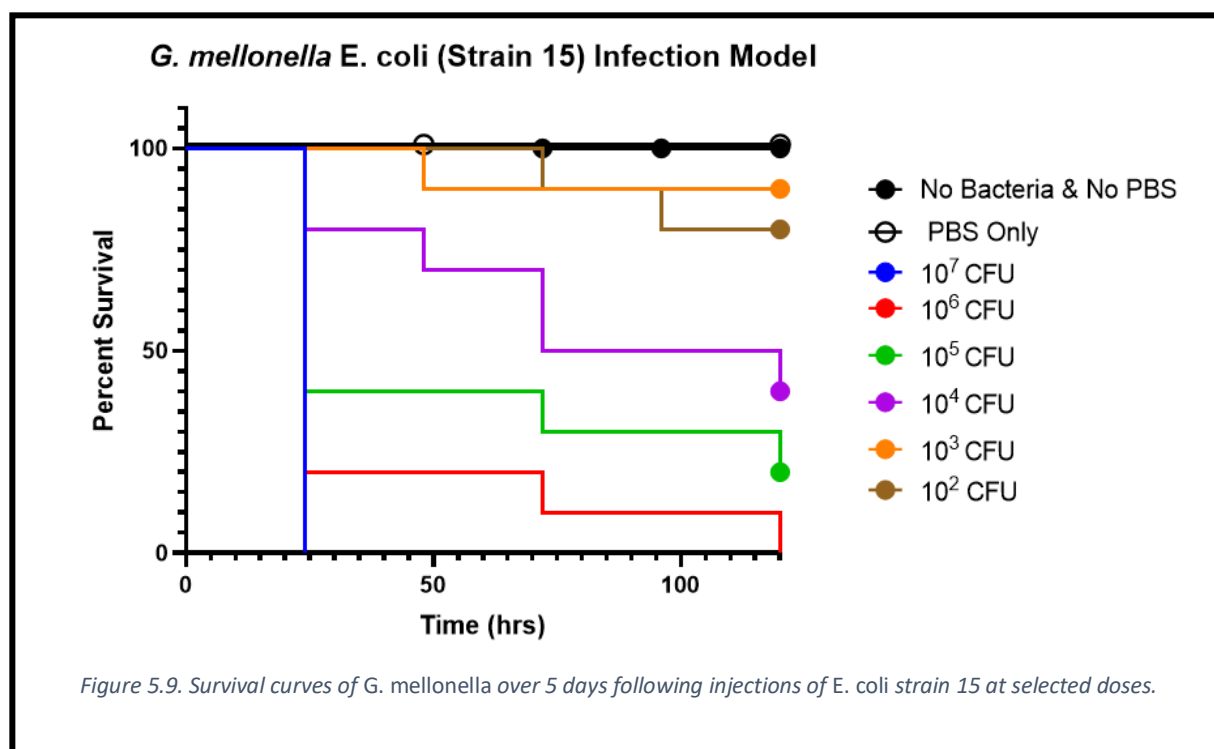
Antibiotic	<i>E. coli</i> 0% Serum MIC (ng/mL)	<i>E. coli</i> 25% Serum MIC (ng/mL)
Meropenem	31.25	62.50



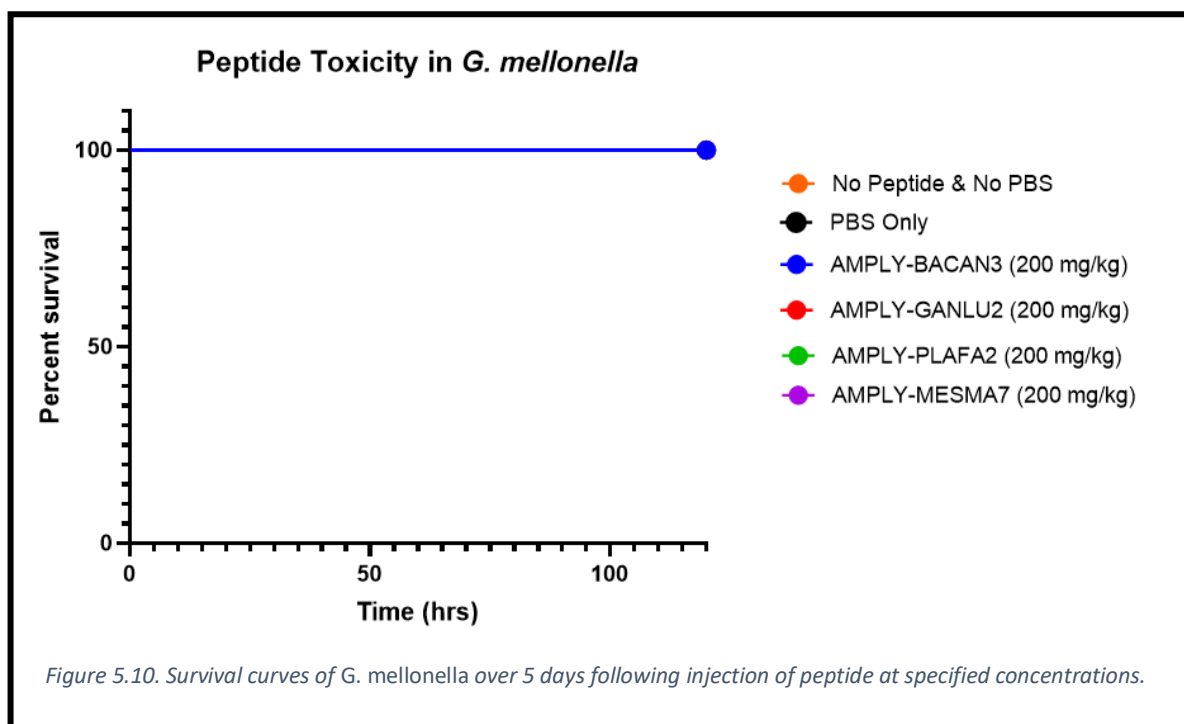
Lethality of the chosen *E. coli* strain was then investigated so that an appropriate bacterial concentration could be used to assess peptide efficacy *in vivo*. To this end, *G. mellonella* populations were injected with the pathogen at selected concentrations and the progression of their survival was monitored over 5 days (**Figure 5.9**). Percentage survival consistently

decreased as initial bacterial concentration increased, with larvae injected with 10^7 CFU/mL *E. coli* exhibiting 100% reduction in population within the first 24 hours. An exception to this trend was the *G. mellonella* population injected with 10^2 CFU/mL *E. coli* – the lowest concentration tested – which presented 80% survival after 120 hours of infection; this was a reduction in survival compared to the population injected with 10^3 CFU/mL *E. coli*, a dose 10 times more concentrated. The only other larvae population to reach 0% survival over the course of the assay had been injected with 10^6 CFU/mL *E. coli*, with 80% of this population dying within the first 24 hours and the remaining deaths being recorded after 72 and 120 hours post-infection.

The greatest increase in tolerance was observed when comparing larvae populations injected with 10^4 CFU/mL and 10^3 CFU/mL *E. coli* after 120 hours, with the sample population showing a 40% increase in survival (50% to 90% survival after 5 days) after this 10-fold reduction in bacterial concentration.



The peptide candidates were screened for toxic interactions with *G. mellonella* larvae before *in vivo* efficacy was assessed; to this end, highly concentrated doses of peptide solutions were injected into populations of wax moth larvae and their subsequent survival was monitored over 5 days (**Figure 5.10**). No reductions in survival were observed in the control or treated populations by the end of the assay.



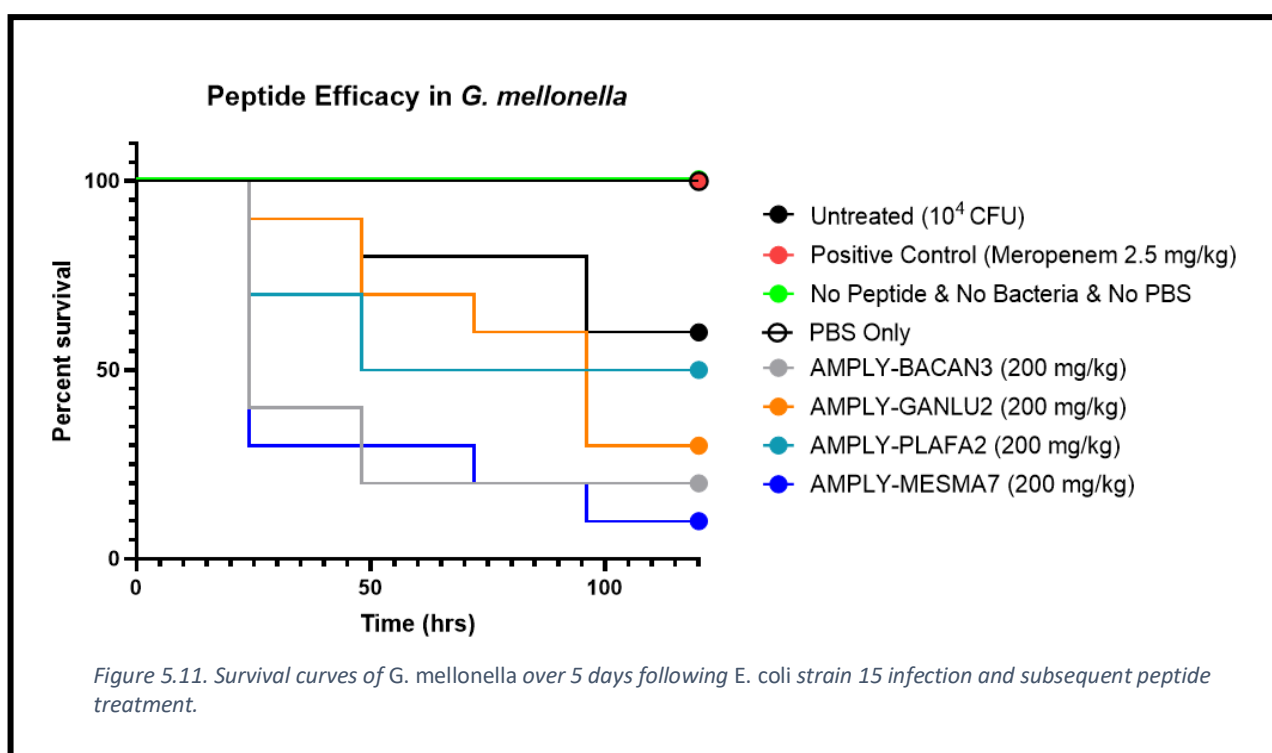
Peptide candidates could then be assessed for activity against *E. coli* strain 15 in the chosen model, with *G. mellonella* larvae being injected with 10^4 CFU of bacterial solution. As before, survival of treated populations was then monitored over 5 days (**Figure 5.11**). Larvae treated with meropenem were the only group to show no reduction in survival, excluding PBS and completely non-injected controls. Larvae treated with a 10^4 CFU bacterial dose and no subsequent peptide exhibited 60% survival by the end of the assay, with the first reduction – from 100% to 90% - occurring within the first 24 hours, and a further 10% reduction occurring within 48 hours. The resultant population numbers were maintained until at least 96 hours had passed, with a further 20% of the population being lost between this time-point and the conclusion of the assay.

All peptide treatments resulted in worse survival than the untreated 10^4 CFU population, with the injection of 2 of 4 candidates – AMPLY-MESMA7 and AMPLY-PLAFA2 – causing a population reduction greater than 50% within the first 24 hours. Survival within the first 24 hours was inferior to the untreated population for all peptide-treated larvae with the exception of those injected with AMPLY-GANLU2, although survival within this group would be surpassed by the untreated population within 48 hours after dropping to 70% survival compared to the 80% survival of the untreated group. Survival of these two groups were equal after 96 hours of infection with both reaching 60% survival; the groups would diverge by the conclusion of the assay with survival of the population treated with AMPLY-GANLU2 dropping a further 20% in the final 24 hours.

Of the peptide-treated populations, the lowest overall reduction in survival was shown by those treated with AMPLY-PLAFA2; survival of this population dropped by 30% within the first 24 hours, and by a further 20% in the following 24 hours. However, the remaining population persisted until the assay's conclusion with the group ultimately presenting 50% survival following infection and treatment.

Wax moth larvae treated with AMPLY-MESMA7 exhibited a 70% reduction in population within the first 24 hours following infection and peptide injection; a reduction in survival 7-fold greater than the untreated control population and the largest population reduction observed within the first 24 hours of the assay. This same population also displayed the lowest overall survival, with only 10% of the treated larvae remaining after the assay's conclusion.

Similarly, the *G. mellonella* population treated with AMPLY-BACAN3 presented the second-lowest reduction in population within the first 24 hours following infection and treatment with a percentage survival of 40% at this time-point. Wax moth larvae treated with this peptide following infection also showed the second-lowest percentage survival after 5 days, with 20% of the original population still remaining.



5.3 Discussion

Summary of Findings

The remaining 24 peptide candidates identified by AMPLY software were investigated for antimicrobial activity against selected members of the ESKAPE pathogen group in the presence of serum to determine whether efficacy would decrease when compared to their MICs exhibited in the absence of serum, gained from the previous project. Prior to this, the bacteria of interest were themselves screened for growth abnormalities in the presence of serum. Statistically significant changes to growth rate – determined via measurements of absorbance - in the presence of serum were identified across all strains, with the population matching or exceeding that of the 0% serum group by time-point 20 hours where the assays concluded. With these findings under consideration, the bacteria were taken forwards for peptide MIC evaluation.

MICs across the candidate set were higher when tested in the presence of serum, with a shift in most commonly observed modal MIC to 64 µg/mL and a larger proportion of the set presenting MICs that exceeded the highest concentration tested. MICs below 1 µg/mL were still observed, but at considerably lower frequency.

A refinement of the candidate pool was carried out based on the data gathered, and the remaining 8 peptides were subjected to HEK293 cell LDH release assays for a more sensitive measure of cytotoxicity. These data were encouraging, with approximately 50% of HEK cell populations tolerating the presence of the peptides at the highest concentration tested; this concentration far exceeded the highest MICs at which activity was observed for the selected candidates against any of the tested strains. HEK cell viability also increased as peptide concentration decreased, identifying the dose-dependent nature of the toxic activity where it was observed.

A final selection was made in light of the cytotoxicity data – along with all relevant data gathered previously – with 4 peptides being taken forward for further analysis of efficacy against the ESKAPE pathogen group. Measuring absorbance of bacterial populations in the presence of these peptides allowed IC₅₀ values to be generated; these data provided a contrast to the MIC values acquired under similar conditions, which itself proved an inconsistent indication of bacterial growth inhibition during the absorbance assays. Analysis of IC₅₀s identified little statistically significant difference between the activity of the candidates against the ESKAPE strains.

Time-kill kinetics assays were then carried out to characterise the mechanisms of candidate activity, using *E. coli* as the sample organism. Candidates were revealed to be primarily bactericidal in nature, with the majority eradicating the bacterial population within 2 hours.

AMPLY-GANLU2 displayed a less rapid effect, but also cleared the bacterial population within 24 hours.

Efforts then turned to investigating *in vivo* peptide activity, following validation of the efficacy of meropenem – the positive activity control – against the chosen bacterial strain. Both meropenem and the peptide candidate group exhibited no toxic interactions with the wax moth larvae at the tested concentrations, and so were used to treat larvae populations following infection with an *E. coli* dose deemed appropriate. Ultimately, treating the model with peptide candidates conferred no observable protection from the activity of *E. coli*, with treated populations displaying considerably reduced survival when compared to the non-treated infection control.

Interpretation of Data

While it is true that none of the selected bacterial strains displayed inhibited growth when incubated in 25% serum solution it must also be noted that changes in growth were indeed observed, with some bacterial strains showing an increase in absorbance compared to the control by the final time-point. While it has been established both in literature and throughout this study – seen in the case of *E. coli* strain K12 -, that the presence of serum can certainly have inhibitory effects on bacterial growth due to serum components like complement proteins and albumin (277), serum is also rich in various hormones and electrolytes that may contribute to bacterial proliferation (272,286). Thus, it can be hypothesised that the presence of these components is what allowed bacteria in those conditions to maintain and exceed growth rates of the control populations.

Upon analysis of the in-serum MIC data, it was observed that the number of peptides neutralised in the presence of serum – with MIC exceeding that of the highest tested concentration – would differ depending on which pathogen the peptides were being tested against, with some peptides displaying activity comparable to their non-serum activity against one strain while not showing activity at all against others. The implication of this phenomenon is notable as it suggests that the observed decrease in activity is not due solely to the presence of serum; were this the case, it would be expected to present as a consistent reduction in the activity of any affected peptide against all strains. Instead, the nature of the chosen pathogen may be a factor in peptide efficacy just as it is in the absence of serum, with resultant changes in activity being caused by a combination of serum components, bacterial resistance factors, and impediments to peptide mechanism of action. This in turn suggests that further elucidation of peptide mechanisms may allow understanding of the nature of these impediments, such that optimisations can be carried out to increase in-serum activity against a greater number of strains.

Regardless of the explanation, this phenomenon does confound the analysis of the following absorbance assays somewhat; while a reduction in activity is seen across the candidate set of 24 peptides it remains unknown to what extent this is due to inhibitory effects of serum on the peptides versus stimulatory effects of serum on bacterial growth. However, these data do serve as an indicator of peptide activity in the presence of serum and therefore of activity in the circulation of *in vivo* models and living hosts. Considering the data generated by these assays, many of the peptide candidates would not currently be suitable for therapeutic application in spite of their activity *in vitro* and would require modification to achieve greater serum tolerance.

This is ultimately exemplified by the investigations incorporating *G. mellonella* as living models; while the peptides tested had been selected for their high efficacy and low toxicity, they were unable to increase survival of larvae infected with *E. coli*. While the most apparent hypothesis for this outcome is peptide neutralisation due to components of the *G. mellonella* haemolymph, it must be noted that populations treated with peptide candidates consistently displayed decreased survival when compared to the non-treated infection control population. Upon consideration that no toxic interactions were observed between peptide candidates and wax moth larvae at the tested concentration, these data instead suggest that the reduced survival is due to the presence of both *E. coli* and peptide, implicating the result of interactions between the two. It was previously hypothesised that the peptide candidates may recruit complement proteins present in serum when acting to inhibit bacterial growth; this was originally posited to explain the increase in activity observed against this same *E. coli* strain in serum when compared to MIC assays in MH broth alone. It can thus be reasoned that any immunomodulatory properties the peptides possess may stimulate the immune response of the *G. mellonella* subjects – including synergistic action with the host's own AMPs -, with the observed deaths being an adverse reaction to the resultant inflammation.

Additionally, the lipopolysaccharides found in the outer membrane of gram-negative bacteria such as *E. coli* are known to act as endotoxins that – in humans – are contributors to the pathophysiology of septic shock due to their potent induction of the inflammatory response (287). These endotoxins are released upon lysis of bacterial cells; in light of what is known about AMP membrane interaction as well as the observed bactericidal activity of the peptide candidates, it can be hypothesised that their interactions with the chosen pathogen may also lead to the release of these inflammatory endotoxins, once again presenting as a greater loss of *G. mellonella* larvae when the peptides were administered.

The absorbance assays themselves identified many discrepancies between expected peptide activity – informed by the MIC data – and what was ultimately observed and quantified in the form of IC₅₀s; this highlights an oversight in the approach to peptide candidate selection, as many of those decisions were heavily influenced by MIC data. A known aspect of the MIC assay protocol is that growth inhibition is determined by visually inspecting wells containing

bacterial and peptide solutions, with an absence of turbidity or visible colonies suggesting peptide activity (288). While both these assays and those measuring absorbance are used to identify peptide inhibition of growth – as opposed to explicitly bactericidal activity - the nature of calculated IC₅₀ values as continuous data-points allows for greater validation through statistical analysis when compared to the categorical values of an MIC assay; this confers a greater degree of reliability to the quantification of peptide efficacy.

These studies also further highlighted positive aspects of the candidates' therapeutic potential, with the absorbance assay, LDH release assay, and time-kill kinetics assay data providing promising depictions of the peptides' efficacy and host cell tolerance *in vitro*. All peptide candidates presented IC₅₀ values in a range of 1 – 15 µg/mL, although values were consistently higher when peptides were tested against MRSA, the only gram-positive pathogen of the bacteria of interest. The peptide candidates all exhibited considerable broad-spectrum activity, with 2 of 4 peptides exhibiting no significant differences when considering effects on absorbance of the 5 tested ESKAPE pathogens.

The data gained from the LDH release assays present an encouraging portrayal of the candidates' toxicity, with all peptides presenting approximate LD₅₀s far in excess of their presented IC₅₀s and MIC values. Notably, 3 of the 8 peptides tested – 2 of which would be taken forward as part of the final candidate selection – showed less than 20% cytotoxicity at the highest tested concentration, with the remaining peptide of the final set not exceeding 50% cytotoxicity until this concentration. In addition, these peptides all exhibited complete bacterial clearance during the time-kill kinetics assays, with 3 of 4 peptides fully inhibiting growth within 2 hours. These data combined suggest considerable therapeutic potential should these candidates be used as the foundation for further development.

Limitations

This project involved a number of factors that obscure the conclusions that can be drawn from its data. Preliminary screening of the ESKAPE pathogen strains in serum – as previously mentioned – identified statistically significant changes in growth compared to the populations in non-serum-containing growth media. While the resultant in-serum efficacy data can be analysed with these growth changes under consideration, this is an arguably imprecise and subjective approach to peptide characterisation and also impacts the degree to which the following in-serum MIC data can be compared to previous assays carried out in non-serum-containing medium. However, it can also be argued that the presence of serum is itself a confounding factor regardless of whether changes to growth are seen; as was mentioned earlier, there is no guarantee that an absence of observed changes in absorbance confirms complete serum tolerance. This “obscuration of conclusions” is therefore a necessary concession made to carry out tolerance studies at all, though the argument could be made that the absence of further steps

to investigate the impact of serum as an environmental stressor is a greater limitation of the study.

Nonetheless, it should also be noted that the inclusion of serum in the preliminary and subsequent assays serves to test the peptides in an environment more closely approaching what they would encounter *in vivo*; thus, the concession of confounded data may be regarded as a justified exchange between consistency of data and insight into peptide-bacteria interactions in a living model.

The absence of IC₅₀s in the presence of serum is a noticeable one, particularly in light of the identified inconsistencies between MIC assay and absorbance assay depictions of efficacy. However, it should be noted that the purpose of the in-serum MIC assays was merely to determine the existence of activity in those conditions, as opposed to the objective comparisons that IC₅₀ values would allow. The utility of data gained from in-serum absorbance studies would arguably be limited due to the effects of serum on bacterial growth discussed previously which - while acceptable for MIC assays to confirm absence or presence of activity - would obstruct any attempts to compare peptide-bacteria interactions to a meaningful degree.

Lastly, while this does not detract from the observed performance of the peptides during the *G. mellonella* studies, it should be noted that this modelling system is not without its drawbacks, foremost among them being the relative lack of characterisation of the organism due to its novelty. To date, the *G. mellonella* genome has yet to be sequenced fully and there are no reported RNA interference mutant libraries (285). In addition, *G. mellonella* larvae are commonly purchased from various independent pet food suppliers; the lack of established stock centres for the organism means that the acquisition of specific genotypes is currently unsupported and conditions under which the samples are maintained may not necessarily be standardised. This is particularly relevant due to reports that the immune response of *G. mellonella* is affected by factors such as temperature and diet (289,290). Regardless, while this model may not supplant established mammalian models it currently provides a cost-effective and technically straightforward means of collecting preliminary *in vivo* data.

6. Final Discussion

Implications and Applications

The investigation of peptides from the human lung peptidome did not identify peptides within the ASL that displayed direct antimicrobial activity, but several candidates were associated with changes to intracellular calcium release and proliferation rates in HEK293 cells.

The significance of this relationship is difficult to quantify, as there is relatively little focus on the manipulation of intracellular calcium release in the context of antimicrobial activity.

However, links between bacterial function and intracellular calcium levels have been established, to the point that the release of calcium into the cytosol of *S. aureus* has been reported to activate efflux pumps involved in its multidrug resistance (291); this not only implicates calcium signalling in antimicrobial resistance, it also highlights the utility of an agent that could potentially inhibit this response - such as KACAN1 or AMPANT5 – by employing a relatively uncharacterised pathway. Alternatively, as previously mentioned, it is known that calcium signalling has a role in inflammatory mediation in eukaryotes (228). Therefore, the observed influences of these peptides on calcium release may be the initial steps to greater understanding of the immunomodulatory abilities that many AMPs possess.

The applications of an agent that can stimulate or inhibit cell proliferation are more clearly defined in literature, with much attention already having been given to compounds with wound healing or anticancer properties (292,293). AMPs have already been investigated for their anticancer activity, with several peptides being able to selectively trigger human cancer cell cytotoxicity due to the negatively charged phospholipids expressed on their membranes (294), while their applications regarding the promotion of tissue repair remain mostly unexplored. The peptides identified in the lung peptidome study exhibited properties relevant to these applications, albeit inconsistently; these candidates may therefore require further modification to become suitable for medicinal use.

The AMPLY software identified an array of sequences with predicted activity; 72 of those peptides now have confirmed activity following purification and MIC evaluation. The data gained from these studies therefore further validate the use of AMPLY as an additional strategy in the relatively novel field of computational AMP sequence prediction, and prove that this approach can be feasibly applied to a large cohort of genomic data. The successful application of this software to genomic data – much of which had not previously been investigated as a source of AMP sequences - highlights the underutilised potential of these databases, which have been experimentally validated and can be publicly accessed with considerable ease and no cost. Additionally, the observed properties of the peptides tested – particularly regarding activity and

cytotoxicity – can be used to further improve AMPLY’s predictive algorithms, refining the means by which therapeutically viable candidates can be identified.

The AMPLY peptides identified remain as promising therapeutic candidates, with the investigations carried out providing the initial information required for further development. Much is now known about the candidates’ antimicrobial activity against several MDR bacteria while critical areas requiring improvement – such as the deleterious interactions seen between the peptides and the *in vivo* model – have been identified.

Future Work

While the necessary preliminary steps have been taken to establish the potential of these candidates, a considerable amount remains unknown about both their discovered properties and the limitations to their application.

While the calcium release studies identified that certain peptides isolated from the human lung peptidome are able to affect intracellular calcium levels in human cells, whether these peptides could be developed as anti-inflammatory agents is still a matter of speculation. Interactions between these peptides and cells involved in inflammation have yet to be investigated and could be carried out using protocols for the culture of leukocytes that are well-established (295,296); this would reasonably be the next relevant area of interest if the anti-inflammatory capacity of these peptides is to be elucidated. Should peptide-dependent calcium responses be observed in these studies, these data could be cross-referenced with the original HEK293 cell experiments to identify consistencies in active peptides and the effects they have on the two cell types; this would also shed greater light on whether the variability of the effects observed in the initial studies was due to the peptides themselves or an intrinsic quality of the HEK293 cells. Additionally, should the peptide effects be as variable as was observed in the HEK cell calcium release investigation, assays would be repeated to allow more informed identification of outliers as well as the application of more powerful statistical analysis.

A number of peptides within this set displayed effects on HEK293 cell proliferation, but this is a very preliminary determination of wound healing capacity. A time- and cost-effective study that could be carried out following the growth assays would incorporate scratch assays to investigate whether the bioactive peptides induce controlled cell proliferation and migration or simply increase the rate of cell doubling. Additionally, these assays could feasibly be carried out on multiple relevant cell types; the keratinocytes that comprise the majority of the epidermis and the epithelial cells that line numerous internal surfaces in the body are potential subjects for this study that could result in very meaningful implications for the applications of the peptides tested.

The informational yield of these studies would be greatly increased by utilising the same quantitative phase imaging (QPI) that facilitated the growth assays described previously. QPI

allows for characterisation of cell properties beyond visual assessment - such as in-depth morphology, confluence, and mass – that can facilitate greater elucidation of the effects of peptide-cell interaction (255). In this way, a greater understanding of the effects of the peptide candidates on cell health and cell behaviour – in addition to cell growth and motility – could be gained.

Immediate efforts regarding the 4 remaining AMPLY-predicted peptides would focus on further characterisation of their modes of action, as this would inform attempts to modify and optimise the peptides of interest later on. These efforts would investigate the nature of interaction between the candidates and affected bacteria; this could be achieved through electron microscopy to directly visualise physical changes following exposure, as well as flow cytometry to acquire quantitative data regarding both physical and physiological changes of the bacterial cells. These findings could be compounded by the inclusion of small-angle X-ray scattering (SAXS) techniques, such as those used to characterise the interactions between AMPs and *E. coli* or MRSA (297,298). Additionally, RNA sequencing would give insight to changes in bacterial protein expression upon exposure to peptides of interest which could in turn generate hypotheses regarding peptide mode of action. These investigations would go on to incorporate not only the tested *E. coli* strain but bacteria of the ESKAPE pathogen group as well.

The results from the *G. mellonella* larvae studies are somewhat inconclusive, as it is currently unknown whether the reduced survival seen in the peptide efficacy models was due to peptide inactivity, peptide toxicity, or factors that have yet to be considered. Toxicity and efficacy assays against *E. coli* would first be repeated with the peptides of interest so that consistent trends - should any exist - could be identified. Should a reduction in survival of *G. mellonella* larvae repeatedly be observed when peptides are administered in the presence of pathogen, attention would turn to the causes of this response so that the presence or absence of peptide activity in the living model could definitively be determined. Protocols for the extraction and incubation of *G. mellonella* haemolymph are reported in literature (299); haemolymph extracted from wax moth larvae given bacterial and peptide doses could be cultured in order to quantify bacterial CFU. These data would clarify whether low survival is due to a failure to clear the infection, or because of unforeseen interactions between bacteria, host, and peptide activity.

There are many avenues of AMP modification; optimisations strategies could be applied not only to the most promising peptides but to various candidates that had been previously excluded due to undesirable levels of toxicity or insufficient activity. AMP sequences can be structurally cyclised or conjugated to lipids or sugars to induce changes in activity, toxicity, and stability (148,300). Alternatively, amino acid residues within a sequence can be substituted with either naturally occurring L-amino acids or D-amino acid isomers, conferring different chemical properties – such as hydrophobicity, amphipathicity and net charge – that will once again result in changes to the factors governing a peptide's antimicrobial capacity (301,302). These factors

include sensitivity to enzymatic degradation, an aspect that has yet to be investigated within this study but remains a critical component of peptide pharmacokinetics (303). It should be noted, however, that the effects of peptide modification are dependent on the properties of the subject peptide and thus are not fully predictable. While optimisation is a very necessary step in peptide development, it is expected to require many permutations of these candidates to be synthesised and tested before they are deemed suitable for clinical trials.

Similarly, adjustments to peptide formulation could be considered to improve pharmacokinetics; combining AMPs with lipid or metal nanoparticles has been associated with greater bioavailability, increasing peptide half-life and decreasing the amount of peptide required to have the desired effect (304–306). These strategies in particular would be implemented at a much later stage of the peptides' development, and thus are primarily speculative at this juncture.

Conclusion

The development of novel therapeutic agents is a justifiably lengthy process. The investigations described here are the first necessary steps towards expanding the options available for the treatment of multidrug resistant infection. These studies demonstrated that humans as well as other relatively overlooked organisms are viable sources of antimicrobial – or otherwise bioactive – peptides that possess both efficacy and low toxicity *in vitro*, and that these sources can be explored in an economical and timely manner using efficient AMP-prediction software.

These studies also highlight that more work is required to further develop the promising candidates that have been identified thus far. With the preliminary assays completed there is now the possibility for extensive optimisation and characterisation, such that potent analogues of the peptides identified here can be used to treat a variety of MDR infections.

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Appendices

Appendix A – Amino Acids

Table 1. Fmoc-protected amino acids used for synthesis, as well as their protecting group, where applicable.

Amino Acid Derivative	Protecting Group
Fmoc-Ala-OH.H ₂ O	-
Fmoc-Arg(Pbf)-OH	2,2,4,6,7-Pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl
Fmoc-Asn(Trt)-OH	Trityl
Fmoc-Asp(OtBu)-OH	<i>tert</i> -Butyl ester
Fmoc-Cys(Trt)-OH	Trityl
Fmoc-Gln(Trt)-OH	Trityl
Fmoc-Glu(OtBu)-OH.H ₂ O	<i>tert</i> -Butyl ester
Fmoc-Gly-OH	-
Fmoc-His(1-Trt)-OH	Trityl
Fmoc-Ile-OH	-
Fmoc-Leu-OH	-
Fmoc-Lys(Boc)-OH	<i>tert</i> -Butyloxycarbonyl
Fmoc-Met-OH	-
Fmoc-Phe-OH	-
Fmoc-Pro-OH	-
Fmoc-Ser(tBu)-OH	<i>tert</i> -Butyl
Fmoc-Thr(tBu)-OH	<i>tert</i> -Butyl
Fmoc-Trp(Boc)-OH	<i>tert</i> -Butyloxycarbonyl
Fmoc-Tyr(tBu)-OH	<i>tert</i> -Butyl
Fmoc-Val-OH	-

Appendix B – Sequence Tables

Table 1. Sequences of primary lung peptidome set.

Peptide Number	Sequence
1	EHKRF
2	EPDWR
3	KDRRH
4	KEFYI
5	KHEFR
6	KLYYL
7	KYFKR
8	KYYRL
9	LDYYY
10	LEERY
11	PRYFR

12	RFAYR
13	SNRYY
14	WHEFP
15	YKFKR
16	YKKRF
17	YKRKF
18	YNRYF
19	AERELV
20	AFHQLP
21	DDPEVR
22	DEFLKL
23	DEPLLK
24	DEQLRL
25	DFEELK
26	DFQGKL
27	DHERLH
28	DHLVLL
29	DLRQPL
30	DVDRLK
31	EALRPL
32	EDHVKL
33	EDYDHE
34	EHDLEV
35	ELFHAL
36	ELRQEL
37	ELVRDL
38	ERDHEL
39	EVDCLK
40	EVFERL
41	EVFQKL
42	FLLNKV
43	FPPKPK
44	FQPLLH
45	GQLLKF
46	GTFLRK
47	GVLQRH
48	GYRSNY
49	HLETTF
50	HRPELL
51	HSELPQ
52	HSLEQP
53	HSRGYH
54	KAELEL
55	KARLVL
56	KDAELF

57	KDELKY
58	KDLQPL
59	KDLVKL
60	KEDLLK
61	KEFLSE
62	KFDEPL
63	KGDYDL
64	KGEFDL
65	KGERRL
66	KGNVEY
67	KGYRPV
68	KLDDLL
69	KLKLKF
70	KLRQQF
71	KLVDKK
72	KMNLQL
73	KQDVWF
74	KRGKPK
75	KRHYKL
76	KSLELK
77	KTEPLE
78	KTVELF
79	KYDLNL
80	LARRLP
81	LDDFQK
82	LDKLLL
83	LDLNRL
84	LDPKLF
85	LDRELF
86	LEGRQL
87	LENLRF
88	LGRFGY
89	LLDVYH
90	LLKPWA
91	LLRDLL
92	LPQKPR
93	LRGQRL
94	LRGRGF
95	LRKLPF
96	LSQTLR
97	LSRKVH
98	LTDWMQ
99	LTEEKL
100	LTFRRL
101	LTYKSR

102	MERLSV
103	NHPRPP
104	PDLREL
105	PDPRPR
106	PRFPSV
107	PRNEAL
108	PRTTSR
109	PRWPQP
110	PYFDLP
111	QEPLYP
112	QKRVLL
113	QLLEKL
114	QLSLPR
115	RAFDQL
116	RLGRFG
117	RLRGFG
118	RNLLDD
119	RPRALP
120	RRDRAP
121	RTLLRP
122	RVLDPF
123	SDGFRE
124	SHRGYR
125	SSLLKY
126	SYRWPR
127	TAEERL
128	TDLERT
129	TELLPK
130	TERDTL
131	TFRKAL
132	TLRFEP
133	TPEEKL
134	TRRLPW
135	TVRNEL
136	TVRWPA
137	VLEYLK
138	VSEDLR
139	WRRLLV
140	YQPVPE
141	YRPRAP
142	YVELMK
143	ALPRRAL
144	APEEHPV
145	APPLSQR
146	DDDFEKL

147	DELFQDR
148	DETYVPK
149	DVTTEVK
150	EDPEVKF
151	EGEEERL
152	ELQAEAK
153	EVLLTPK
154	EVPWEDK
155	EVPWEDR
156	EVPWENR
157	FLDEERQ
158	GEATLTH
159	GPPRPPQ
160	GRLGRLV
161	GRPPRPA
162	HLTPEEK
163	KEEEEDR
164	KEPPPKP
165	KGLDGLY
166	KGSPEGR
167	KGVAQLL
168	KPERPPP
169	KPSKGLK
170	KSETLVL
171	KYKKTLL
172	KYVPPPP
173	LELLKDL
174	LNPGVAR
175	LSGHEVP
176	LTGRSKL
177	LTPLQVP
178	NDLFERL
179	PPGPPPR
180	PRAPTFR
181	PRFPSVL
182	QRLVREL
183	RGLLTKA
184	RPPSRPQ
185	RPSRPPQ
186	SDGLRLL
187	SDLLVPK
188	SEEEFLR
189	SNYLYDN
190	STVRPVK
191	TEQGHEL

192	TLGMMMLL
193	VGRLEAV
194	YDKLEKT
195	YQLSVNK
196	YRPRAPL
197	YTKKVPQ
198	AEFAEVSK
199	APPAGKPQ
200	DSWDVKLG
201	DVVLGPLQ
202	ESSLTKDA
203	GDSWDVKL
204	GPPKPGPP
205	GRPSRPPQ
206	KGPPPPPP
207	KRKFHEKH
208	LAAEALKL
209	NWDDMEKL
210	PPPPGRPQ
211	PPPPGRQP
212	PPQGGRPS
213	QGPPPRPG
214	QPVPEQPL
215	SGNEDEFR
216	TSVQKRAL
217	TVGTLTEL
218	WSPKEEDR
219	WSPQEEDR
220	YQPVPEQP
221	DDHDPVDKL
222	DDHDSVDKL
223	DETEQWEKF
224	DHPPLPQTS
225	DSSEEKFLR
226	EDLDTNADK
227	GTGASGSFK
228	HHDEEKDVL
229	KFHEKHSH
230	LLSGKPEGR
231	SHEDPEVQF
232	SQSPPGKPQ
233	STVLTSKYR
234	TDDDKQDHL
235	DDEDGLPEKL
236	EEAEEEAQRA

237	GPPPQGGKPQ
238	GPPPQGGKPQ
239	KLHEEELQEL
240	KRHHGYKRKF
241	LDEERQGPPL
242	NNSRLKLGLK
243	PATGGVKKPH
244	SPQSPPGKPQ
245	SPRSPPGKPQ
246	VDFNSEEDAK
247	YEKEDSDEVH
248	AAENEEDEHSV
249	DVGSYQEKVDV
250	DVSHEDPEVKF
251	EDPQGDAQKT
252	FHEKHHSRGY
253	GDGSDEEVDGK
254	GPPAQGGKSQ
255	GPPPPPPGKPQ
256	GPPPQGDNKS
257	GPPPQGGNKPQ
258	GPSPQGGNKPQ
259	KFHEKHHSRG
260	KNNQLTNNQRL
261	NETSQHHDDLE
262	QPLPPAGKPQ
263	TYTEHAKRKT
264	VGGLKEDTEH
265	DSHAKRHHGYK
266	GPGRLLLLPP
267	GPPPQGDKSRP
268	GPPQGGHPRPP
269	KAAPSVKPK
270	KPAPPKPEPK
271	LFAEEKAVADTR
272	QQGPPPPQGPQ
273	RHPDYSVLLR
274	SHRPPPPGKPE
275	SPPGKPQGGPPQ
276	AQSGPVRSGPKF
277	EDPQGDAQKTD
278	GPGRLLLLPPY
279	GPPPQGDKSRSPQ
280	GPPPQGDNKSRS
281	GTGASGSFKNKK

282	GTGSETESPRNPS
283	NKPQGPPPPGKPQ
284	SEHPGLSLGDTAK
285	DSGSSEEQGGSSRA
286	GPPPPGKPQGPPQ
287	GRPQGPPQQGGHQQ
288	RQGPPLGGQSQPS
289	GNQPQGPPPPGKPQ
290	KAKAAKPKKAPKSPA
291	KATKSPAKPKAVKPK
292	PEPAKSAPAPKKGSK
293	PQQGGHPPPPQGRPQ
294	SVDSGSSEEQGGSSR
295	TEESPASDEAGEKEAK
296	SPPGKPQGPPPPQGGNQPQ

Table 2. Sequences of secondary lung peptidome set.

Peptide	Sequence
AMP9J1	KRRVRWIIW
AMP9J2	RVKWWIRVR
AMPAGO1	EGPVGLADPDGPASAPLGAP
AMPAGO2	FWGALIKGAALKIPSVVGLFKKKQ
AMPAGO3	GLKEIFKAGLSLVKGIAAHVAS
AMPAGO4	RIGVLLARLPKLSLFLMGKKV
AMPANT1	NWKKGKEVLKAL
AMPANT2	IWSAIWSGIKGLL
AMPANT3	IPPFIKKVLTTVF
AMPANT4	VLPLVGNLLNDLL
AMPANT5	VRRFPWWWPFLRR
AMPGR11	AVPAVRKTNETLD
AMPGR12	GLWQKIKDKASELVSGIVEGVK
AMPGR13	IGGIISFFKRLF
AMPGRS1	AVKPKTAKPKTAKPKTA
AMPGRS2	KRRWRIWLV
AMPNK1	VNWKKILGKIIKVVK
AMPNK2	FIGAIARLLSKIFGKR
DEBY1	PQQGGHPRPP
DEBY2	PQQGGHPPPP
KABAC7	RWRRPIRRRPIRPPFWR
KACAN1	IFIFKFFKKLIKQYKR
KACAN2	WWWRTWKWGWRRRRWR
KACAN3	GQWRWLGWIWKKRRRL
KACAN4	RKFFKKIKKQYIRLLF
KACAN5	IKKLIKKNIKFFLMLH

LUAGO1	VNEYKKL
LUAGO2	LDRELF
LUAGO3	KLYYL
LUAGO4	RAFDQLR
LUAGO5	GDPLPKSPF
LUAGO6	GNDLLAAAK
LUAGO7	VLGEKGKNL
LUANT1	KEEPPV
LUANT2	LHEGLET
LUANT3	KQDVWF
LUANT4	MERLSV
LUANT5	RRDRAP
LUGRI1	YKKRF
LUGRI2	DHERLH
LUGRI3	SNRYY
LUGRI4	KQDVWF
LUGRI5	HSRGYH
LUGRP1	SVDSGSSEEQGGSSR
LUGRP2	PQQGGHPPPPQGRPQ
LUGRP3	SEHPGLSLGDTAK
LUGRP4	KNLHPL
LURRP10	NKPQGPPPPGKPQ
LURRP11	TDLERT
LURRP5	PEPAKSAPAPKKGSK
LURRP6	SPPGKPQGPPPQ
LURRP7	GPPPPGKPQGPPPQ
LURRP8	APEEHPV
LURRP9	VSEDLR

Table 3. Sequences of peptide sequences identified by AMPLY.

Sequence	Source
AARIVAARARRA	<i>Burkholderia pseudomallei</i>
AARRVARHLLYS	<i>Burkholderia pseudomallei</i>
AIPWRKKKWIWR	<i>Crocodylus porosus</i>
AKKLKAKAKEML	<i>Ophiophagus hannah</i>
ALHVRLARRARA	<i>Burkholderia pseudomallei</i>
ALKKILRMLKKL	<i>Rhincodon typus</i>
ALSMVLRPLLRP	<i>Crocodylus porosus</i>
ALWSALWRMLCW	<i>Falco peregrinus</i>
AMPNYFCIVFKK	<i>Ophiophagus hannah</i>
AREARAVRRAAL	<i>Burkholderia pseudomallei</i>
ARLLRALRLRAG	<i>Burkholderia pseudomallei</i>
ARRIAPLKRSR	<i>Burkholderia pseudomallei</i>
ARRRAALARRAG	<i>Burkholderia pseudomallei</i>

ASKVICLWRNAK	<i>Ophiophagus hannah</i>
CVVALHLKMGKI	<i>Ganoderma lucidum</i>
FFFFFFPRRKK	<i>Gallus gallus</i>
FFKKRKLVLKFY	<i>Rhincodon typus</i>
FFRKFFKCLRKR	<i>Bacillus anthracis</i>
FFYLRLRYFSRF	<i>Panthera tigris altaica</i>
FINFFLIKALKT	<i>Danaus plexippus plexippus</i>
FIRRLHFIHRF	<i>Crocodylus porosus</i>
FIRVFRKFLKFK	<i>Talitrus saltator</i>
FKHLAKLFSKIQ	<i>Orcinus orca</i>
FKKFQTKFIKIF	<i>Talitrus saltator</i>
FKKKIKKIKKKL	<i>Helianthus annuus</i>
FKKKKKKPWFFF	<i>Canis lupus lupus</i>
FKKRKIIKKFIK	<i>Canis lupus familiaris</i>
FKLFNQILLKFL	<i>Crocodylus porosus</i>
FKLKKLKKKKLK	<i>Danaus plexippus plexippus</i>
FKNLILLYQKYR	<i>Danaus plexippus plexippus</i>
FKRIIKILLRNL	<i>Mesobuthus martensii</i>
FKRLKRRKKKIF	<i>Gallus gallus</i>
FKTIKKRKIFKK	<i>Mesobuthus martensii</i>
FKVFVKVTKVIF	<i>Rhincodon typus</i>
FLCKLFTIFKK	<i>Crocodylus porosus</i>
FLFKKYIYFLQL	<i>Danaus plexippus plexippus</i>
FLFRLIAKLGKV	<i>Falco peregrinus</i>
FLGILGKVLGKV	<i>Ophiophagus hannah</i>
FLKKKKKKKAIF	<i>Gallus gallus</i>
FLKLFKKISQRI	<i>Danaus plexippus plexippus</i>
FLPILKPILKFL	<i>Gallus gallus</i>
FLPLILSLRKIV	<i>Ophiophagus hannah</i>
FLSILKKIYATV	<i>Falco peregrinus</i>
FLTIFKKVLKFL	<i>Ophiophagus hannah</i>
FMKLKKIKKLKF	<i>Ophiophagus hannah</i>
FPKIKKKKIKSV	<i>Orcinus orca</i>
FSSLLFTVAIKR	<i>Crocodylus porosus</i>
FTALIKSLLRRC	<i>Danaus plexippus plexippus</i>
GFFKFVRRLKQ	<i>Talitrus saltator</i>
GFLVPVRVRCKV	<i>Crocodylus porosus</i>
GILKLLKIFAA	<i>Gallus gallus</i>
GKFPWSKILGKL	<i>Gallus gallus</i>
IAFLLVVRILYF	<i>Galdieria sulphuraria</i>
IFKKLLRSLKFF	<i>Talitrus saltator</i>
IFRIIFRIYVF	<i>Danaus plexippus plexippus</i>
IIFCFCFRRKKK	<i>Gallus gallus</i>
IIIMIAILKNLK	<i>Panthera tigris altaica</i>
IIKKKKKKNLFF	<i>Gallus gallus</i>

IIKVHLKRFKKL	<i>Metallosphaera sedula</i>
IILIITKLLNSL	<i>Danaus plexippus plexippus</i>
IILKKILLWKL	<i>Ophiophagus hannah</i>
IISHLKLYKKLL	<i>Mesobuthus martensii</i>
IKIKIKIKIK	<i>Takifugu rubripes</i>
IKILLKHWWKIL	<i>Gallus gallus</i>
IKISGKKIFYLL	<i>Gallus gallus</i>
IKKKLKKLLKIL	<i>Mesobuthus martensii</i>
ILFLKKKKKKNF	<i>Canis lupus familiaris</i>
ILITAICKLIKK	<i>Falco peregrinus</i>
ILKKLLKIRLKI	<i>Rhincodon typus</i>
ILMSTLKKTLAK	<i>Ophiophagus hannah</i>
ILQTVLLRLLK	<i>Talitrus saltator</i>
ILWMKILKKYLK	<i>Gallus gallus</i>
IQKLIIFIKKKC	<i>Rhincodon typus</i>
IRARIRVKVMVK	<i>Canis lupus familiaris</i>
IVKKLFKKKKIK	<i>Canis lupus familiaris</i>
IVSKLKKKKKKL	<i>Helianthus anuus</i>
IWFRIRIRIRIR	<i>Takifugu rubripes</i>
KAVQWRWLQLSL	<i>Takifugu rubripes</i>
KCKLRCCRYRIC	<i>Metallosphaera sedula</i>
KFFKAYCFFKFF	<i>Panthera tigris altaica</i>
KFFLKPRILGVS	<i>Falco peregrinus</i>
KFKKKTFLRIYR	<i>Rhincodon typus</i>
KFNKKKKVFGII	<i>Danaus plexippus plexippus</i>
KHIRKKIKFKKI	<i>Canis lupus familiaris</i>
KIIRKIDIIRK	<i>Canis lupus familiaris</i>
KIKKKKLFKRIS	<i>Gallus gallus</i>
KIKKKKPFGIFF	<i>Panthera tigris altaica</i>
KILFKNLIKYLK	<i>Gallus gallus</i>
KILKILKKILKY	<i>Takifugu rubripes</i>
KIVSSPVVKTL	<i>Crocodylus porosus</i>
KIYWKLLKKLLV	<i>Galdieria sulphuraria</i>
KKILISFLGKI	<i>Metallosphaera sedula</i>
KKINRVLSWILP	<i>Orcinus orca</i>
KKKIKKKKLLLF	<i>Falco peregrinus</i>
KKKIKKGKIFKK	<i>Orcinus orca</i>
KKKKKEIFHLWL	<i>Gallus gallus</i>
KKKKKKKKVLMF	<i>Gallus gallus</i>
KKKKKKKKVYFL	<i>Helianthus anuus</i>
KKKKNLLRFPWW	<i>Falco peregrinus</i>
KKKKRLKKAICK	<i>Canis lupus lupus</i>
KKKRIVKIKKWL	<i>Canis lupus familiaris</i>
KKKTIALKKKK	<i>Canis lupus lupus</i>
KKLKKKLKRYLK	<i>Bacillus anthracis</i>

KKLTRYCYCPIR	<i>Metallosphaera sedula</i>
KKVAKKAAAPAK	<i>Burkholderia pseudomallei</i>
KKVLKILKKLWK	<i>Ophiophagus hannah</i>
KKVLPKKKKKKK	<i>Canis lupus lupus</i>
KLFSKIQNKAUVW	<i>Ophiophagus hannah</i>
KLGKLIKNFWKI	<i>Danaus plexippus plexippus</i>
KLIPILASKFTK	<i>Talitrus saltator</i>
KLLLKNLKKFSL	<i>Mesobuthus martensii</i>
KLVQKKKKEKLG	<i>Canis lupus lupus</i>
KLWKKLYRRLKL	<i>Falco peregrinus</i>
KMCIFYKKKKKR	<i>Gallus gallus</i>
KRIFCKKIIKII	<i>Canis lupus lupus</i>
KRWVKLLYKLLK	<i>Bacillus anthracis</i>
KTIFQIIKKIII	<i>Mesobuthus martensii</i>
KTKFFKKIKFLL	<i>Danaus plexippus plexippus</i>
KWISFMKFISFK	<i>Talitrus saltator</i>
KWKILIKKLFKL	<i>Rhincodon typus</i>
KWKKLFLKLRLK	<i>Takifugu rubripes</i>
KWKMICCKITKF	<i>Ophiophagus hannah</i>
KWLRLMWLKKLLK	<i>Helianthus annuus</i>
LAFKELFRAWKR	<i>Gallus gallus</i>
LAFSACKIAKQC	<i>Ophiophagus hannah</i>
LAHRVARVPLLS	<i>Ganoderma lucidum</i>
LCLCKKKKKKKK	<i>Canis lupus lupus</i>
LDKIFACLIFKK	<i>Danaus plexippus plexippus</i>
LFFRKYYKKIYK	<i>Mesobuthus martensii</i>
LFKKKIIKKKLD	<i>Crocodylus porosus</i>
LFLTRLRLLLVS	<i>Crocodylus porosus</i>
VKKIIIIKIKKK	<i>Canis lupus familiaris</i>
VWLWWLWRKLRKG	<i>Ophiophagus hannah</i>
WARIWRLFRKLLR	<i>Gallus gallus</i>
WKGRYVLWAWWWVW	<i>Falco peregrinus</i>
WLRVRRRIIRAWK	<i>Metallosphaera sedula</i>
WRWLGRHRWLWLW	<i>Takifugu rubripes</i>
WRWLRLNIWLWTMR	<i>Takifugu rubripes</i>
WWWWRRRRRRRWC	<i>Galdieria sulphuraria</i>
YIGRKCAFgyfCR	<i>Crocodylus porosus</i>
YIKILLKKKKTKK	<i>Crocodylus porosus</i>
YKLRKFFKKFLKK	<i>Mesobuthus martensii</i>
AIKWLSIWKLVRK	<i>Falco peregrinus</i>
ALKVALKVLKVSL	<i>Ophiophagus hannah</i>
ALLCKVYKLNFKK	<i>Panthera tigris altaica</i>
CQFFICKYVKKKK	<i>Gallus gallus</i>
DFVIHLIKKILSL	<i>Danaus plexippus plexippus</i>
FFFVLLKKKKKKI	<i>Takifugu rubripes</i>

FFKIILRKAQKIM	<i>Talitrus saltator</i>
FFKKAIIIDKKI	<i>Rhincodon typus</i>
FFKLLARLSRRGL	<i>Metallosphaera sedula</i>
FFLIKKIIKKIAR	<i>Ophiophagus hannah</i>
FIHFKKFIKLFKR	<i>Orcinus orca</i>
FISIFLLKKFCRF	<i>Orcinus orca</i>
FKIIKKKLKKLRK	<i>Mesobuthus martensii</i>
FKIKLLKYYSKKI	<i>Orcinus orca</i>
FKKKIIHFLKRFV	<i>Mesobuthus martensii</i>
FKKPRICGVSLSF	<i>Orcinus orca</i>
FKKVVYLKKLFKK	<i>Crocodylus porosus</i>
FKSLKKLWRIFKK	<i>Gallus gallus</i>
FLGGLIKLVKKAK	<i>Takifugu rubripes</i>
FLIFFRTYRRKKC	<i>Helianthus anuus</i>
FLIIRVIKLGNL	<i>Ophiophagus hannah</i>
FLIWYKKKKKKKK	<i>Mesobuthus martensii</i>
FLKKKKKKILFIY	<i>Canis lupus familiaris</i>
FLLGVIPKCINKK	<i>Ophiophagus hannah</i>
FLPVIKRLKTIL	<i>Falco peregrinus</i>
FLRFLKSLWLIYR	<i>Orcinus orca</i>
FMLILVNMNMKIL	<i>Danaus plexippus plexippus</i>
FPPFHVHYFPFRR	<i>Gallus gallus</i>
FRHKFKKPFKILS	<i>Danaus plexippus plexippus</i>
FRIRIRIVKWAIR	<i>Helianthus anuus</i>
FVIFFLIKRRRKK	<i>Gallus gallus</i>
FYKKKKKFLKFF	<i>Canis lupus familiaris</i>
GFLKLLKTFLKKL	<i>Rhincodon typus</i>
GIIQKIFKKIVKR	<i>Gallus gallus</i>
GKIIKLKVLKLL	<i>Mesobuthus martensii</i>
GLKKLKLKLYKL	<i>Crocodylus porosus</i>
GLKLLKLKLLKV	<i>Rhincodon typus</i>
GLLKLLKALTIL	<i>Ophiophagus hannah</i>
GRPPCPPYRIVCR	<i>Canis lupus familiaris</i>
GRRCWIFRVHCIR	<i>Falco peregrinus</i>
GRRRARVRRRRLY	<i>Metallosphaera sedula</i>
GRVKIKIKIYLIK	<i>Ophiophagus hannah</i>
HLKVVKNLKALS	<i>Canis lupus familiaris</i>
HLVWNFIRLLMRK	<i>Danaus plexippus plexippus</i>
HTCFIRIRPIFRK	<i>Bacillus anthracis</i>
ICKILKKLKLWTI	<i>Mesobuthus martensii</i>
IFFNKLKKNLNII	<i>Danaus plexippus plexippus</i>
IIKIIIMKILKNI	<i>Mesobuthus martensii</i>
IIIQIIKPMIIK	<i>Danaus plexippus plexippus</i>
IIKIQIKIKINIK	<i>Mesobuthus martensii</i>
IKKRAKKTLLKPR	<i>Falco peregrinus</i>

IKWKKIFKNVMSL	<i>Panthera tigris altaica</i>
ILRRARRSRWRPR	<i>Metallosphaera sedula</i>
INVKKKLKIKKK	<i>Panthera tigris altaica</i>
IPWLWIWKTNFL	<i>Canis lupus familiaris</i>
IQPWKWPYILCWK	<i>Metallosphaera sedula</i>
IRKFPFFILFFK	<i>Ophiophagus hannah</i>
IRLTCRFRCPAQI	<i>Takifugu rubripes</i>
IYKKKKKKRERL	<i>Canis lupus familiaris</i>
KALKSILKLLKI	<i>Mesobuthus martensii</i>
KCALWKILIKRVL	<i>Ophiophagus hannah</i>
KCLLLFFFLKRCW	<i>Orcinus orca</i>
KFIKFFKKIKTIY	<i>Rhincodon typus</i>
KFWKRLLKKFLRK	<i>Rhincodon typus</i>
KIFIKKRKIKKI	<i>Canis lupus familiaris</i>
KIFILALKKALF	<i>Rhincodon typus</i>
KIISIIKILPKN	<i>Mesobuthus martensii</i>
KIPKGIIKKRIK	<i>Falco peregrinus</i>
KIVSLFKKKKIYH	<i>Ophiophagus hannah</i>
KIWQKLKRIKQIH	<i>Canis lupus familiaris</i>
KKDKKVLLAHFLF	<i>Falco peregrinus</i>
KKFFKKLFQYKII	<i>Rhincodon typus</i>
KKKKAIRHFLFFF	<i>Ophiophagus hannah</i>
KKKKIFLAKLFSF	<i>Canis lupus familiaris</i>
KKKKILHLPLCLFF	<i>Takifugu rubripes</i>
KKKKKKKKLVYFF	<i>Helianthus anuus</i>
KKKLEAWWWYFKK	<i>Panthera tigris altaica</i>
KKKLFFKKIKKKN	<i>Helianthus anuus</i>
KKKLKKFIKALSK	<i>Crocodylus porosus</i>
KKKQLLPFFIFF	<i>Ophiophagus hannah</i>
KKLKKLLKIYIKG	<i>Helianthus anuus</i>
KKLLQLKKLKKLL	<i>Ophiophagus hannah</i>
KLALLKMLIKLLK	<i>Orcinus orca</i>
KLFKRIKKQIIFL	<i>Gallus gallus</i>
KLIILNLKLMK	<i>Rhincodon typus</i>
KLLLKLVCCKLL	<i>Ophiophagus hannah</i>
KLVLKQLPFLIFV	<i>Danaus plexippus plexippus</i>
KMLKKLNKLNKLL	<i>Ophiophagus hannah</i>
KRKLLKKIFSKLW	<i>Canis lupus lupus</i>
KTSLIIPRHLKLL	<i>Falco peregrinus</i>
KVFRKILKYHLAK	<i>Danaus plexippus plexippus</i>
KVGLKAFRRILKK	<i>Takifugu rubripes</i>
KVKKKKVFKKHKI	<i>Gallus gallus</i>
KWHSWCCLRKLLK	<i>Ophiophagus hannah</i>
LFGQALGLLKKVL	<i>Ophiophagus hannah</i>
LFKNMLKMLKNLL	<i>Canis lupus familiaris</i>

LFLLRIWIRYVSV	<i>Crocodylus porosus</i>
LFVFLFVKIAKKN	<i>Orcinus orca</i>
LGWKWPWNKLGYS	<i>Metallosphaera sedula</i>
LIINKLWKLLKI	<i>Orcinus orca</i>
LIINLKIYKLLK	<i>Mesobuthus martensii</i>
LIKKCLKKLKKLL	<i>Panthera tigris altaica</i>
LIKMIYKTYLILF	<i>Crocodylus porosus</i>
LIKNLMKKLKNLL	<i>Mesobuthus martensii</i>
LIWRLVRRNLRR	<i>Gallus gallus</i>
LKHLKKLKKLHLK	<i>Mesobuthus martensii</i>
LKKDLKKLKKLVK	<i>Crocodylus porosus</i>
LKMLKKAACKTILA	<i>Gallus gallus</i>
LKRFLKKLLKRCW	<i>Mesobuthus martensii</i>
LKSCWIRIVKRIK	<i>Gallus gallus</i>
LLIILLRRRKWAK	<i>Orcinus orca</i>
LLIYKIYKKILLY	<i>Orcinus orca</i>
LLKALKKSLKYLK	<i>Ophiophagus hannah</i>
LLKILIFKLWQKL	<i>Canis lupus familiaris</i>
LLLIKDLMLKKLL	<i>Helianthus annuus</i>
LLNKRIFKTILKY	<i>Gallus gallus</i>
LPLLIKILIKKLLK	<i>Helianthus annuus</i>
LRALVLSWRLLLR	<i>Falco peregrinus</i>
LRMIFLFVKIYIR	<i>Danaus plexippus plexippus</i>
LSFLSKVLPNVKL	<i>Danaus plexippus plexippus</i>
LSLNPLKLLKFLK	<i>Talitrus saltator</i>
LVKKKKKKKKKVQF	<i>Gallus gallus</i>
LVLGRRSGWFKLF	<i>Crocodylus porosus</i>
LWLRARRRRACWLR	<i>Ophiophagus hannah</i>
LWRAVWVKVLKKLK	<i>Orcinus orca</i>
LYIKKKKKKKRSL	<i>Canis lupus familiaris</i>
LYLITLFSLVIKL	<i>Orcinus orca</i>
MFKLKKAVLLIVL	<i>Danaus plexippus plexippus</i>
MFLKKKKKKKKKT	<i>Canis lupus familiaris</i>
MFLKKLLKISTKV	<i>Ophiophagus hannah</i>
MGLWSLVLCISK	<i>Falco peregrinus</i>
MKIKGKIIRKIK	<i>Crocodylus porosus</i>
MKKLYIKKKKKKK	<i>Canis lupus familiaris</i>
MLGKIKCIKTC	<i>Panthera tigris altaica</i>
MLKKILKIKNVF	<i>Helianthus annuus</i>
MLLKLKIPKNLL	<i>Orcinus orca</i>
MLPAWKKLLKILK	<i>Helianthus annuus</i>
MSLLKHIAIPVLK	<i>Falco peregrinus</i>
MWKTLLKHILKSA	<i>Crocodylus porosus</i>
NWSSIFLKVAKLV	<i>Ophiophagus hannah</i>
PALVYGCFLPRKR	<i>Ophiophagus hannah</i>

PFKIKKKKFIFSL	<i>Helianthus anuus</i>
QLKMLKLLWKMLK	<i>Helianthus anuus</i>
RIILLRFVTNILF	<i>Danaus plexippus plexippus</i>
RKINLLKIKFKKK	<i>Crocodylus porosus</i>
MASILFCILLRKC	<i>Orcinus orca</i>
MGIIIVIVKKIVIK	<i>Orcinus orca</i>
MKKIKKFLKKRLKK	<i>Orcinus orca</i>
MKKKLFFKKKKKKF	<i>Helianthus anuus</i>
MLRLLIFYLFFFK	<i>Falco peregrinus</i>
MLSLLKKKKKKKYL	<i>Crocodylus porosus</i>
MWRRKLRPIILKKL	<i>Bacillus anthracis</i>
NKLLKFLKAFCIFF	<i>Orcinus orca</i>
NLLKRLFKKLLFFL	<i>Ophiophagus hannah</i>
NYVKFKKKKKKMLL	<i>Gallus gallus</i>
PFFLKLCFLIFKK	<i>Crocodylus porosus</i>
PWLWPFKKKKIPKK	<i>Crocodylus porosus</i>
RALLRLLMRGFKRL	<i>Takifugu rubripes</i>
RGRRWWWWWCLWRW	<i>Helianthus anuus</i>
RITLVRVFPQCWK	<i>Ophiophagus hannah</i>
RIVHRIKKIKKKL	<i>Crocodylus porosus</i>
RKKKIFFFFLKKGT	<i>Gallus gallus</i>
RKKLRRLKYSRPCL	<i>Danaus plexippus plexippus</i>
RNLWWLLRSLIVK	<i>Crocodylus porosus</i>
RRLGSRRRRRSWR	<i>Metallosphaera sedula</i>
RRRRWQRWKRWARR	<i>Burkholderia pseudomallei</i>
RRVWSWKRRRLRR	<i>Ganoderma lucidum</i>
RVFIKASIKLPVA	<i>Danaus plexippus plexippus</i>
RWLRRILSVRRWLR	<i>Ganoderma lucidum</i>
RWYFWVCRRGCFWK	<i>Panthera tigris altaica</i>
VFKIICKKKKKLKK	<i>Canis lupus familiaris</i>
VKKRIMHIFKIPFV	<i>Orcinus orca</i>
VKVINKISYVWIGL	<i>Danaus plexippus plexippus</i>
VRRWLRRFLAVVRR	<i>Metallosphaera sedula</i>
VWGKRRCWWRWWWR	<i>Helianthus anuus</i>
WDLTRFKKAIKN	<i>Danaus plexippus plexippus</i>
WFRWWLLRFRLWWW	<i>Helianthus anuus</i>
WLKLLKKMLCGLLV	<i>Falco peregrinus</i>
WLKWWRRWQKLERR	<i>Rhincodon typus</i>
WQKLFFKKTNILKK	<i>Falco peregrinus</i>
WRKKKKKKMLLTYV	<i>Gallus gallus</i>
WSRIWRVVFYWRWR	<i>Falco peregrinus</i>
WVFFSFFFFRKRK	<i>Gallus gallus</i>
WWLWLWLKMWWWMKW	<i>Ganoderma lucidum</i>
WYFVKKKKKKKKKK	<i>Canis lupus familiaris</i>
YKRIFKKLIFVLIK	<i>Orcinus orca</i>

YRKKKKKKKNYHLL	<i>Orcinus orca</i>
YVALLKKLKLIFH	<i>Gallus gallus</i>
AKILMKNNGCRNWWCL	<i>Ophiophagus hannah</i>
CWEKKKKKKIVLFIL	<i>Rhincodon typus</i>
FFFAAVKKKKKKKKK	<i>Gallus gallus</i>
FFLKKKKIKKIKKEY	<i>Canis lupus familiaris</i>
FFSKYFIVGKILFSL	<i>Crocodylus porosus</i>
FHHLRKKKKRRLPWW	<i>Orcinus orca</i>
FIFSKISLQILKIFN	<i>Danaus plexippus plexippus</i>
FKKLRRKKMKLSIRK	<i>Bacillus anthracis</i>
FKKRKKVMIKYYYFF	<i>Gallus gallus</i>
FKSFFKKIFRIFKKT	<i>Mesobuthus martensii</i>
FLFFLFFLFIFYFF	<i>Orcinus orca</i>
FLGLLKKVLKRVLK	<i>Gallus gallus</i>
FLIIKKKKLKKI	<i>Takifugu rubripes</i>
FLKKKKKVVKIFML	<i>Gallus gallus</i>
FLTILKKVLCPLQKK	<i>Ophiophagus hannah</i>
FLVFFFFLRRKKKKK	<i>Gallus gallus</i>
FMLKKKKKKKNLKL	<i>Canis lupus lupus</i>
FQLILNKILKKILKK	<i>Orcinus orca</i>
FYAKKKKKKKKIGFL	<i>Helianthus anuus</i>
GFACLILTRKYRII	<i>Ophiophagus hannah</i>
ICFFFLKKKKKKALK	<i>Gallus gallus</i>
IFKLFGRLFKNLII	<i>Helianthus anuus</i>
IFYKKKKKKKERKKG	<i>Canis lupus familiaris</i>
IIFLICCRQRIFFKR	<i>Ophiophagus hannah</i>
IIIIIIINKIIKIN	<i>Plasmodium falciparum</i>
IIIIKTMIKLTKKLC	<i>Helianthus anuus</i>
IIKKFKIKFYIIK	<i>Plasmodium falciparum</i>
IIKLLMKWKMKRKMK	<i>Crocodylus porosus</i>
IILLIKIKKIIMNL	<i>Plasmodium falciparum</i>
IYKRKFAKKIFKKL	<i>Mesobuthus martensii</i>
IKIYFKKLFFKKILK	<i>Mesobuthus martensii</i>
IKKEIKIKKIYFFR	<i>Gallus gallus</i>
IKKINIKKKIKKKEI	<i>Panthera tigris altaica</i>
IKKKMWLKPMMKLLK	<i>Helianthus anuus</i>
IKNIAKTFFISLRAI	<i>Metallosphaera sedula</i>
IKNKFIKKIKIKKI	<i>Canis lupus familiaris</i>
ILKKNLSYLKKLLKL	<i>Canis lupus familiaris</i>
ILLAKKKKKKKKKLF	<i>Gallus gallus</i>
ITKKLFKIQKKLLKI	<i>Gallus gallus</i>
ITLFKIKNKKILKNN	<i>Ophiophagus hannah</i>
KCKFLKLLKLGRKLF	<i>Rhincodon typus</i>
KHLKLLKALKWLLK	<i>Panthera tigris altaica</i>
KIIKVFKKIFKNTIF	<i>Panthera tigris altaica</i>

KIKKKKVQLLKTLLK	<i>Canis lupus familiaris</i>
KIYFKKKKKIKLFFR	<i>Orcinus orca</i>
KKFKHNRIWKFIVKA	<i>Gallus gallus</i>
KKHFKMFKNLFKKFS	<i>Falco peregrinus</i>
KKKKIFLKVFFFFFK	<i>Gallus gallus</i>
KKKKIYFKKACFPLW	<i>Gallus gallus</i>
KKLKIKFFKKAKNIG	<i>Panthera tigris altaica</i>
KKLSKIVLLLFLFFY	<i>Gallus gallus</i>
KKLTKMIKKKKIFKK	<i>Orcinus orca</i>
KKRKIIQLYFKKTKI	<i>Danaus plexippus plexippus</i>
KKWLHVPKKKVFFVI	<i>Gallus gallus</i>
KKYIKWKYKKKTLK	<i>Orcinus orca</i>
KLIPGIICKILKKNL	<i>Mesobuthus martensii</i>
KLLKVNKKLLKKVIK	<i>Rhincodon typus</i>
KLLLFKRLKLFNLK	<i>Danaus plexippus plexippus</i>
KLLLKMKLLKKRDF	<i>Rhincodon typus</i>
KLRKKKNLVLPTIYY	<i>Falco peregrinus</i>
KPKQVVFCLRRKCQR	<i>Falco peregrinus</i>
KSLKKHVKKHLKMIF	<i>Canis lupus familiaris</i>
KTIFKSKTIGIRIP	<i>Canis lupus familiaris</i>
KTIKKLIKFLFMLH	<i>Gallus gallus</i>
KVLKKNLKKIKIKIK	<i>Canis lupus familiaris</i>
KWKKLMKKLVKESKK	<i>Helianthus anuus</i>
KWKKLMKKVIKGSRK	<i>Helianthus anuus</i>
LAGLFSKILLMRRC	<i>Takifugu rubripes</i>
LFIFKKKKKKIKKAF	<i>Gallus gallus</i>
LFNKKIAFSFLHFWK	<i>Ophiophagus hannah</i>
LFSILKMMKILKKNM	<i>Ophiophagus hannah</i>
LFVVFVIKKNFFKK	<i>Ophiophagus hannah</i>
LHQIICKILKKLKLW	<i>Mesobuthus martensii</i>
LIVLKKKKKILKCTL	<i>Gallus gallus</i>
LKKGKKRIKKWYRK	<i>Helianthus anuus</i>
LKKIKENLKKIKLL	<i>Helianthus anuus</i>
LKLKLLKIKRAKKA	<i>Orcinus orca</i>
LKLLKKRKKIKSLLK	<i>Canis lupus familiaris</i>
LKNKINVKKKFKKKK	<i>Panthera tigris altaica</i>
LLIKAWLNMLEKLLK	<i>Falco peregrinus</i>
LLIVISKITIKIKI	<i>Orcinus orca</i>
LLKIRRKILKIWKKL	<i>Mesobuthus martensii</i>
LLKKPLRFLKLLKI	<i>Takifugu rubripes</i>
LLKLKYWKKIKKKIV	<i>Gallus gallus</i>
LLLLLLLYKPFLKK	<i>Panthera tigris altaica</i>
LLPHVVLKKKKKKK	<i>Gallus gallus</i>
LLVKKKKKKIVLGFL	<i>Crocodylus porosus</i>
LPIIKILSGKRKNL	<i>Falco peregrinus</i>

LPWKKQKKLPRLWR	<i>Galdieria sulphuraria</i>
LRCHKRCWRCRIR	<i>Panthera tigris altaica</i>
LRKFRKIIKFQKLLK	<i>Falco peregrinus</i>
LWKIWRLLKKLKKR	<i>Orcinus orca</i>
LWRLLVFPLWRFLRR	<i>Ophiophagus hannah</i>
LWRRALWRRTWGSWW	<i>Gallus gallus</i>
LYRKKKKKKRFYAFR	<i>Mesobuthus martensii</i>
LYWRRRRSWIRWTVL	<i>Galdieria sulphuraria</i>
MIFVEIKKKKIFCLM	<i>Orcinus orca</i>
MRVFLRRILVINNKI	<i>Danaus plexippus plexippus</i>
MVILIKKIKNFYNKL	<i>Metallosphaera sedula</i>
NFKKLLKLLYSRYKV	<i>Panthera tigris altaica</i>
NIKYVLLKKKKKRKI	<i>Danaus plexippus plexippus</i>
NLLKLQKKNRWLCFV	<i>Rhincodon typus</i>
PLFISLVVCKQLKI	<i>Ophiophagus hannah</i>
QAVKIWLKHCRRMKW	<i>Gallus gallus</i>
RCFIKIRFVRQCRNL	<i>Rhincodon typus</i>
RILFLKKKKKKILLS	<i>Rhincodon typus</i>
RKALKKLLKKLLG	<i>Ophiophagus hannah</i>
RLKRLFHLRKLKLL	<i>Orcinus orca</i>
RLWFRWFRMKRFFWF	<i>Gallus gallus</i>
RRRLVTPWRRQARRR	<i>Falco peregrinus</i>
RRVINRWWRWRWRWR	<i>Orcinus orca</i>
RWQKWRRMRWIWRLV	<i>Rhincodon typus</i>
RWRWQIFRYRWRRQR	<i>Ganoderma lucidum</i>
FKYLKTVIRFFLTLLF	<i>Crocodylus porosus</i>
FLALVKKNVLPNIFKK	<i>Danaus plexippus plexippus</i>
FLFPFLKKKKKKKERK	<i>Gallus gallus</i>
FLGKYCHLIFCKIFRK	<i>Panthera tigris altaica</i>
FLILIPKISNLVNKII	<i>Orcinus orca</i>
FLKHYILSPRKKKKKK	<i>Plasmodium falciparum</i>
FLKKKKKKKKKAVVMF	<i>Gallus gallus</i>
FLKKKKKKRKILGFRL	<i>Canis lupus lupus</i>
FLKLKFKRIIVKKFII	<i>Crocodylus porosus</i>
FLKVHFRRFIFKVLII	<i>Rhincodon typus</i>
FLLKKKKKSLDLWLFL	<i>Orcinus orca</i>
FLLKRFFILEKKRFCV	<i>Talitrus saltator</i>
FLLLIKILVTVKKKKK	<i>Orcinus orca</i>
FLLPFKKKKKKLKKII	<i>Plasmodium falciparum</i>
FLLRLRRRQRLPGIGK	<i>Ganoderma lucidum</i>
FLLVYCLVLVLAIRRC	<i>Panthera tigris altaica</i>
FLSIVKKILNKIYCTL	<i>Falco peregrinus</i>
FLSLKIDKLKKKIIKW	<i>Panthera tigris altaica</i>
FLVTSLLFLLSLKLGK	<i>Crocodylus porosus</i>
FLWRRKKIILKRKRKK	<i>Orcinus orca</i>

FPPRIIHMPFLFKTILS	<i>Falco peregrinus</i>
FQIFKKLKKILTNNKI	<i>Plasmodium falciparum</i>
FQKKKKKKKKKNPLLI	<i>Rhincodon typus</i>
FQKKTLLLLLILTTLTK	<i>Danaus plexippus plexippus</i>
FQKLKKKKIKWVKKL	<i>Mesobuthus martensii</i>
FQVKLLLKLLKIKKKP	<i>Panthera tigris altaica</i>
FRFAIKKKLKKLKKK	<i>Helianthus annuus</i>
FRPPIYPFRPPIYPFR	<i>Orcinus orca</i>
FRRKRSLLRWRTPLPL	<i>Metallosphaera sedula</i>
FRRLCEVRWRWPGRWK	<i>Ganoderma lucidum</i>
AAACAANRAPLRPPAA	<i>Burkholderia pseudomallei</i>
AAAPKLISPFKHSLL	<i>Falco peregrinus</i>
AAFLPRGVRPRRARLA	<i>Burkholderia pseudomallei</i>
AAGHGRTSRRRPLHSR	<i>Ganoderma lucidum</i>
AAICQPFWPWRRWLG	<i>Gallus gallus</i>
AAPRCRWRRSSRALT	<i>Talitrus saltator</i>
AASAKASRAVPAIAG	<i>Burkholderia pseudomallei</i>
AASRRAPRRRPRRSAA	<i>Burkholderia pseudomallei</i>
ADVPLLKLLPLGVCRR	<i>Ganoderma lucidum</i>
AERAAAARRATRCPRS	<i>Ganoderma lucidum</i>
AEWRGWKALWSWLKRK	<i>Orcinus orca</i>
AFLHLLLRAPARGKP	<i>Takifugu rubripes</i>
AFLSPFPIPLFAFSA	<i>Falco peregrinus</i>
AFSLFISSLRRVFKKF	<i>Talitrus saltator</i>
AGGRGGSCYSQCQHYH	<i>Talitrus saltator</i>
AGLRRHRRWAGLRRRR	<i>Rhincodon typus</i>
AGPRPPPGRHCVRPI	<i>Takifugu rubripes</i>
AGSRRAAAARGRRGAR	<i>Burkholderia pseudomallei</i>
AIAFLNKLILKIKKKL	<i>Rhincodon typus</i>
AIIILVNTMKLKKGSL	<i>Plasmodium falciparum</i>
AIKRLMKNSVKFLQSL	<i>Crocodylus porosus</i>
AIKYIFFFKKKHGVIF	<i>Mesobuthus martensii</i>
AKKKKKKNLLPIYFL	<i>Orcinus orca</i>
AKKWAKHLKKKRVVNT	<i>Talitrus saltator</i>
ALLFTIVLKVLTRKIK	<i>Panthera tigris altaica</i>
ALPLRALLRPPPRGLP	<i>Ganoderma lucidum</i>
ALQARRGAARLLPHLL	<i>Takifugu rubripes</i>
ALRAARPRGLPPRRAS	<i>Canis lupus familiaris</i>
AMRPGWRIRRAASSRR	<i>Burkholderia pseudomallei</i>
AMYVIFYKKLKKKEK	<i>Gallus gallus</i>
ANPKPLSYTNLYPKPY	<i>Orcinus orca</i>
APARPARPTRGSR CIR	<i>Burkholderia pseudomallei</i>
APLFNTRKKDRRAEKK	<i>Crocodylus porosus</i>
APPKAEAKAKALKAKK	<i>Canis lupus lupus</i>
APRRPRLRRRSRRPSA	<i>Burkholderia pseudomallei</i>

ARAI RRP RRR RRR ARK	<i>Burkholderia pseudomallei</i>
ARARA APLRPTLLARA	<i>Burkholderia pseudomallei</i>
ARARGRRRGVSRRRAG	<i>Burkholderia pseudomallei</i>
ARARRRRSRTASAARR	<i>Burkholderia pseudomallei</i>
ARFSFISKIKKCVFAF	<i>Rhincodon typus</i>
ARGRRRRRLPGRDRSA	<i>Burkholderia pseudomallei</i>
ARHNGNFKITINFVVG	<i>Galdieria sulphuraria</i>
ARKRFLRRPIRRRRFR	<i>Takifugu rubripes</i>
ARPPSLFLLRPPRHG	<i>Rhincodon typus</i>
ARRARRAPARAARANG	<i>Burkholderia pseudomallei</i>
ARRCWRWWCWCWRWWL	<i>Helianthus anuus</i>
ARRRRRMKRTRRTT	<i>Burkholderia pseudomallei</i>
ARRRRRRRALQRAARG	<i>Burkholderia pseudomallei</i>
ARSARRRRRAACRAGRL	<i>Burkholderia pseudomallei</i>
ARSRRRRRRRRRAIR	<i>Burkholderia pseudomallei</i>
ARTAARRPAARRRARA	<i>Burkholderia pseudomallei</i>
ARVGPPRPLALPCPAL	<i>Gallus gallus</i>
ASRLFLLRHLPGCARR	<i>Takifugu rubripes</i>
ASRRSGDRRGARRRAR	<i>Burkholderia pseudomallei</i>
ASRTVPRTARLRAARR	<i>Burkholderia pseudomallei</i>
ASVSSGVRVRRRRRR	<i>Ganoderma lucidum</i>
ATCRRAGRRRTRRPAA	<i>Burkholderia pseudomallei</i>
AVAGSRRQPPRARRAA	<i>Burkholderia pseudomallei</i>
AVAKFNGKAAIGGLVK	<i>Rhincodon typus</i>
AVHAAARRTVSSGRGA	<i>Burkholderia pseudomallei</i>
AWGWKKPCRSLLWWLLR	<i>Gallus gallus</i>
AWLFKKKKKKKKVLLL	<i>Gallus gallus</i>
AWPWRCRVRGWRLWTS	<i>Danaus plexippus plexippus</i>
AYPRGPLLALRPSRPR	<i>Ganoderma lucidum</i>
CARIRRAPRNGRRGGR	<i>Burkholderia pseudomallei</i>
CCSTHKRIWRYIYICI	<i>Panthera tigris altaica</i>
CDKGLLPKIYKKILKL	<i>Panthera tigris altaica</i>
CFLWFKKKKKKKKSL	<i>Gallus gallus</i>
CFPIWWQLFLKKKKKK	<i>Gallus gallus</i>
CFRFLVCFTLTRNFVL	<i>Plasmodium falciparum</i>
CHKFIKDIKNLKKKL	<i>Mesobuthus martensii</i>
CLKNKLKKHLKKLKKK	<i>Panthera tigris altaica</i>
CPCPSRWKWLWVWKRI	<i>Rhincodon typus</i>
CPQRPIIFKKKFKFLV	<i>Rhincodon typus</i>
CRGRWCCSCCCCYRGR	<i>Rhincodon typus</i>
CRKRRKCMACRCICL	<i>Panthera tigris altaica</i>
CRNFLRAWSVGWWWWW	<i>Ganoderma lucidum</i>
CRRRRRRRRCRRHHLRA	<i>Takifugu rubripes</i>
CYMGICYRPFCSFIIR	<i>Falco peregrinus</i>
EAYLSARRLWCAFRK	<i>Panthera tigris altaica</i>

EMKKLLKLEFLKLLKK	<i>Falco peregrinus</i>
FCKKKKKKKIASFWAP	<i>Crocodylus porosus</i>
FFFFFFLLKVKCKMSG	<i>Panthera tigris altaica</i>
FFFFFREAAECRRKK	<i>Takifugu rubripes</i>
FFFFKKKFYKKKKKKA	<i>Rhincodon typus</i>
FFFFKKKKKKKILLAI	<i>Gallus gallus</i>
FFFIFFIFILKFLFI	<i>Canis lupus lupus</i>
FFFLFFYFFKNRLSHF	<i>Takifugu rubripes</i>
FFFVLVFNIIKKKKKKK	<i>Plasmodium falciparum</i>
FFKFLRLKLVWLVLFL	<i>Takifugu rubripes</i>
FFPLLGSFLFFFKKKK	<i>Rhincodon typus</i>
FFPSLLKKKKKKKKSF	<i>Crocodylus porosus</i>
FFRLKKKKKKKPLFIV	<i>Canis lupus familiaris</i>
FFYIINLKKKKKKKKS	<i>Plasmodium falciparum</i>
FGKILRSLIFKILMKN	<i>Orcinus orca</i>
FGTQQGLIGKRLKIAK	<i>Talitrus saltator</i>
FIFIRIIKILMSKLRA	<i>Takifugu rubripes</i>
FIIHKKKKKKKKKIPL	<i>Plasmodium falciparum</i>
FIKLLKKKSLFILFFL	<i>Falco peregrinus</i>
FIKLRRFPCISSLLKV	<i>Panthera tigris altaica</i>
FILNKPFLHLFFKK	<i>Gallus gallus</i>
FIYKKYKKKLFDISLK	<i>Rhincodon typus</i>
FKAIHKIKKKKKIKK	<i>Canis lupus familiaris</i>
FKAWQRLRTVLTRYKK	<i>Panthera tigris altaica</i>
FKFAFYFIFYLFRFKK	<i>Panthera tigris altaica</i>
FKFYKIKKKKKKKKKL	<i>Mesobuthus martensii</i>
FKFYMVCKKLLKPLMR	<i>Panthera tigris altaica</i>
FKILMKALKLGPLRLI	<i>Gallus gallus</i>
FKKAWGRWEVRRRVLK	<i>Falco peregrinus</i>
FKKGVTKLFPVLTLR	<i>Talitrus saltator</i>
FKKIIIFKIYKKIMIK	<i>Helianthus anuus</i>
FKKILLHLLKLLNLF	<i>Danaus plexippus plexippus</i>
FKKIVKAFLKKTFIKI	<i>Rhincodon typus</i>
FKKKKKKLCLCFFFL	<i>Panthera tigris altaica</i>
FKKLKIIIIKKKSRGW	<i>Falco peregrinus</i>
FKKLKKKYTKVKKKK	<i>Helianthus anuus</i>
FKKMYKKIKKYQNKKI	<i>Helianthus anuus</i>
FKLLLLKLNKLFKSC	<i>Rhincodon typus</i>
FKNIHFKKKKKKKFS	<i>Rhincodon typus</i>
FKNVFVFLIKKKKKKL	<i>Gallus gallus</i>
FKRFKKKKKILKLYL	<i>Mesobuthus martensii</i>
FKRLRKIWNGFKAIFK	<i>Rhincodon typus</i>
FKSDVSLCKKFFKLL	<i>Orcinus orca</i>
FKSVKKTVKKPLFGFF	<i>Takifugu rubripes</i>
FKTFLITKLLILLVRK	<i>Rhincodon typus</i>

LIRKFKKLFKVI	<i>Falco peregrinus</i>
LIRYMIYKYFLF	<i>Canis lupus familiaris</i>
LIVKKKLLKKIKK	<i>Mesobuthus martensii</i>
LIVKKMLGKLLK	<i>Takifugu rubripes</i>
LKALKKEKKIKK	<i>Falco peregrinus</i>
LKALKKKKNLKK	<i>Falco peregrinus</i>
LKALYKLLKIL	<i>Mesobuthus martensii</i>
LKIYLKKKIKIK	<i>Canis lupus lupus</i>
LKKKKKKKILFW	<i>Crocodylus porosus</i>
LKKLLKIKNAIF	<i>Mesobuthus martensii</i>
LKLKLFELIKLF	<i>Danaus plexippus plexippus</i>
LKLKLFRRKKK	<i>Gallus gallus</i>
LKLKLLKMFQL	<i>Rhincodon typus</i>
LKLLLLQLKKK	<i>Rhincodon typus</i>
LKMLCKKLLKIL	<i>Crocodylus porosus</i>
LKRIKKLPKLLK	<i>Helianthus annuus</i>
LKSGVIFLITKK	<i>Ophiophagus hannah</i>
LKSKKKKIKKL	<i>Gallus gallus</i>
LKSLWRLRRLLR	<i>Ganoderma lucidum</i>
LKVS KMFKRIWN	<i>Orcinus orca</i>
LLAKYKKKKIFK	<i>Canis lupus lupus</i>
LLFLDFVLFYFF	<i>Danaus plexippus plexippus</i>
LLKMLKKKLLKG	<i>Falco peregrinus</i>
LLKMLRLKFLKL	<i>Bacillus anthracis</i>
LLKRKLKKLLKI	<i>Mesobuthus martensii</i>
LLKVLLRAWKKL	<i>Ophiophagus hannah</i>
LLLKEALKMLKK	<i>Gallus gallus</i>
LLLLKKKKKKKR	<i>Canis lupus lupus</i>
LLLLFLKKKKK	<i>Canis lupus lupus</i>
LLRLKKLLKI	<i>Rhincodon typus</i>
LLLSKLICFNT	<i>Falco peregrinus</i>
LLWLKLLKLLK	<i>Ophiophagus hannah</i>
LLNNLWKYLLRA	<i>Falco peregrinus</i>
LLQIRIWLFTI	<i>Danaus plexippus plexippus</i>
LLRLRLRLRLC	<i>Ganoderma lucidum</i>
LLRQCWVRLYLL	<i>Takifugu rubripes</i>
LLRRVHRLRR	<i>Burkholderia pseudomallei</i>
LLRRQVWLKLL	<i>Crocodylus porosus</i>
LLSIVKKTVGLF	<i>Gallus gallus</i>
LMILKILLWKKL	<i>Falco peregrinus</i>
LMLLSLLKSLWK	<i>Gallus gallus</i>
LMWIVHRARWIL	<i>Gallus gallus</i>
LPFLKKFQKIL	<i>Ophiophagus hannah</i>
LRAQVLALLPA	<i>Ganoderma lucidum</i>
LRARLRRARRV	<i>Burkholderia pseudomallei</i>

LRLPLRLHHLCL	<i>Ganoderma lucidum</i>
LRPLRLLRLLRL	<i>Burkholderia pseudomallei</i>
LTRKLKKKFKKI	<i>Canis lupus familiaris</i>
LVILKLLKLLKK	<i>Panthera tigris altaica</i>
LVLCKILICYRK	<i>Ophiophagus hannah</i>
LVLQLLKIKLWI	<i>Rhincodon typus</i>
LVLRKHFKMLVK	<i>Ophiophagus hannah</i>
LWKKNLKKLKKW	<i>Mesobuthus martensii</i>
LWLIFKKKKKKF	<i>Helianthus anuus</i>
LYNKILKKLLKI	<i>Helianthus anuus</i>
MALIKKIAMIW	<i>Danaus plexippus plexippus</i>
MGCRWVARWKCK	<i>Falco peregrinus</i>
MKALKLLKMLKK	<i>Orcinus orca</i>
MKKKKWKKMLK	<i>Canis lupus lupus</i>
MLKKIKKRERK	<i>Canis lupus lupus</i>
MLKKLLRVNWKS	<i>Ophiophagus hannah</i>
MLKPWWFFKKS	<i>Canis lupus familiaris</i>
MLLLLKRKMKKL	<i>Bacillus anthracis</i>
MSFKTLLKHILK	<i>Orcinus orca</i>
MWKKILHLLKLF	<i>Panthera tigris altaica</i>
NKAHLIKLLKKK	<i>Canis lupus lupus</i>
NWRKLRKNWKKL	<i>Metallosphaera sedula</i>
PFNKKKKIFVLL	<i>Crocodylus porosus</i>
PLLQKIVQKLLK	<i>Danaus plexippus plexippus</i>
QKIIIVIKILNK	<i>Mesobuthus martensii</i>
RARARLLARRAR	<i>Burkholderia pseudomallei</i>
RAVAIAAARRAA	<i>Burkholderia pseudomallei</i>
RAVLRPRAARAR	<i>Ganoderma lucidum</i>
RCLRCLRCLRCL	<i>Burkholderia pseudomallei</i>
RCRCRGGSGSGS	<i>Burkholderia pseudomallei</i>
RFLPLVLLRPRL	<i>Ganoderma lucidum</i>
RIFKRIFKRIFK	<i>Crocodylus porosus</i>
RKAFRKKLKKLI	<i>Crocodylus porosus</i>
RLRKFLRRWKFR	<i>Canis lupus lupus</i>
RMKLYRKIRKKK	<i>Bacillus anthracis</i>
RPISWWWLGWSL	<i>Takifugu rubripes</i>
RRALCLQRCWIR	<i>Canis lupus familiaris</i>
RRKTRHAFARAA	<i>Burkholderia pseudomallei</i>
RRLAPRRARLAF	<i>Burkholderia pseudomallei</i>
RRLLVWLTWGRK	<i>Ganoderma lucidum</i>
RRLPGLRPLLRF	<i>Ganoderma lucidum</i>
RRLPRILRLFTR	<i>Metallosphaera sedula</i>
RSIKKYLKMKKL	<i>Crocodylus porosus</i>
RVHLIKCLMKNL	<i>Ophiophagus hannah</i>
RYKCLRKIQYRM	<i>Bacillus anthracis</i>

SLKLFKEILIKI	<i>Danaus plexippus plexippus</i>
STVQKLVKWLK	<i>Falco peregrinus</i>
VKKKKKLLCIF	<i>Gallus gallus</i>
VKKQLKLLQKLL	<i>Takifugu rubripes</i>
VKMLKKLLNIIN	<i>Mesobuthus martensii</i>
VNRILLRRLVR	<i>Bacillus anthracis</i>
VNYRWKKIIAKI	<i>Falco peregrinus</i>
VRLRALRLRRAA	<i>Burkholderia pseudomallei</i>
VTLRRWPIKWWK	<i>Galdieria sulphuraria</i>
VVVFKLWKRTSC	<i>Gallus gallus</i>
VWCCPLWMWLRW	<i>Falco peregrinus</i>
WCSLLKKLKKLL	<i>Ophiophagus hannah</i>
WILLRKCPRLFF	<i>Crocodylus porosus</i>
WIWKKKKKKFFK	<i>Rhincodon typus</i>
WKKILIKNILKV	<i>Mesobuthus martensii</i>
WKKVFQKVSLLI	<i>Ophiophagus hannah</i>
WKLFAKILITKF	<i>Ophiophagus hannah</i>
WKLFFKGGWAML	<i>Ophiophagus hannah</i>
WKPKWRIWWQWW	<i>Orcinus orca</i>
WKSFLKTASFL	<i>Falco peregrinus</i>
WKWLLWRWAERR	<i>Falco peregrinus</i>
WRLDRIWHWKAL	<i>Ophiophagus hannah</i>
WRRCWPARRWPPW	<i>Gallus gallus</i>
WRWLGRLWCAGW	<i>Falco peregrinus</i>
WRWRCRLWHPAW	<i>Danaus plexippus plexippus</i>
WTKVWRLLRKLK	<i>Canis lupus familiaris</i>
WWVLQHWRFLLR	<i>Ophiophagus hannah</i>
YCKSKKICVFLK	<i>Panthera tigris altaica</i>
YFKKFVPMFRKF	<i>Talitrus saltator</i>
YIKLKILLLLLK	<i>Gallus gallus</i>
YKKKKKKLILLF	<i>Helianthus annuus</i>
YLKNMLKKLFNI	<i>Mesobuthus martensii</i>
YRKKFKKIMRLF	<i>Crocodylus porosus</i>
YSKIKKKKLFNI	<i>Mesobuthus martensii</i>
FSEKMLKKLSKKLRL	<i>Mesobuthus martensii</i>
FSFHFLRRKRKIKKKK	<i>Gallus gallus</i>
FVCDRFRKKRFFFRFL	<i>Talitrus saltator</i>
FVFPPFFYKKKLLLLL	<i>Panthera tigris altaica</i>
FVHFIIICVRFRRKKK	<i>Danaus plexippus plexippus</i>
FVIFYFIIIFKRFYFK	<i>Panthera tigris altaica</i>
FVKTFIKNFIHIFK	<i>Danaus plexippus plexippus</i>
FVLCFTALCRYRGGFF	<i>Canis lupus lupus</i>
FVNKLFCALHSADKK	<i>Crocodylus porosus</i>
FWDLKILPKFAKKVKK	<i>Rhincodon typus</i>
FYKKKKSATRNYWIRI	<i>Rhincodon typus</i>

FYMYRKKKKKKIFFLI	<i>Plasmodium falciparum</i>
FYYGNLLMVKKKKKKK	<i>Talitrus saltator</i>
GAAPRARTRCRGRPK	<i>Burkholderia pseudomallei</i>
GAGVRVRVRSIAGAG	<i>Takifugu rubripes</i>
GALSRSSRRRRWWAR	<i>Ganoderma lucidum</i>
GAPRRRRRRRSARRKR	<i>Ganoderma lucidum</i>
GARLASRRRHRRRGS	<i>Ganoderma lucidum</i>
GAVRRTRRRRSGARGR	<i>Ganoderma lucidum</i>
GCHRIRRMWRIWRWYK	<i>Rhincodon typus</i>
GCKNPGALKLGFCKK	<i>Crocodylus porosus</i>
GCMRVRARCVRWRRGW	<i>Rhincodon typus</i>
GCPPKLGCFRCCALKF	<i>Panthera tigris altaica</i>
GFKKIFKKLTIKFLKK	<i>Rhincodon typus</i>
GGARQWRWRWRWRWRW	<i>Burkholderia pseudomallei</i>
GGAVGAARLCARRRRR	<i>Burkholderia pseudomallei</i>
GGKAPRKQLATKAARK	<i>Takifugu rubripes</i>
GGKKKTTKTSRSTKAG	<i>Takifugu rubripes</i>
GGRAGVPGRPRRVCAW	<i>Falco peregrinus</i>
GGRRGRVLPPARVRRR	<i>Ganoderma lucidum</i>
GGTAPTIPHLLRGGLH	<i>Canis lupus lupus</i>
GHIGHLVTRAAFNFGK	<i>Canis lupus familiaris</i>
GHILCKIKRSFLLFAK	<i>Talitrus saltator</i>
GIAKFFRLRKKGVVRK	<i>Orcinus orca</i>
GIFVLYLKLFLWRK	<i>Gallus gallus</i>
GIIKKIAATIIIIIR	<i>Plasmodium falciparum</i>
GILKKFYIILISLPH	<i>Orcinus orca</i>
GKEKKKKKKKKRRIVL	<i>Gallus gallus</i>
GKKEKKWGKKKKFFHL	<i>Gallus gallus</i>
GKKKNLLISYKLTIG	<i>Gallus gallus</i>
GKRCLHVHIKKFKIVV	<i>Talitrus saltator</i>
GKVAFLKTISLPKKN	<i>Talitrus saltator</i>
GLRAARGRRARRTRRA	<i>Burkholderia pseudomallei</i>
GPGRALPAGPARPRAG	<i>Canis lupus lupus</i>
GPRRPRVRLRVAVGI	<i>Ganoderma lucidum</i>
GRAARRVRRARLYVL	<i>Burkholderia pseudomallei</i>
GRASRRRPRRPRVIRR	<i>Burkholderia pseudomallei</i>
GRHSYYPASCCPHLPR	<i>Talitrus saltator</i>
GRIGRLVTRAAFTSKK	<i>Takifugu rubripes</i>
GRIKTLKVLKKMIFK	<i>Falco peregrinus</i>
GRLRRRLGRLARALS	<i>Ganoderma lucidum</i>
GRPGSRRGIPRARYQR	<i>Ganoderma lucidum</i>
GRQVGSKILLLLKIKK	<i>Rhincodon typus</i>
GRRGSRRSVRRASRRC	<i>Ganoderma lucidum</i>
GRRLGVRVWRRWMRG	<i>Ganoderma lucidum</i>
GRWRGARRRARRFAKR	<i>Ganoderma lucidum</i>

GSARRRRRRACLGCA	<i>Burkholderia pseudomallei</i>
GTRVGRNAPFSIHRPI	<i>Metallosphaera sedula</i>
GTWTRWKCWIWKQKRC	<i>Takifugu rubripes</i>
GWRFHRWRRRCVIVWW	<i>Helianthus anuus</i>
HGRSQRKRRCRFCVCR	<i>Burkholderia pseudomallei</i>
HGSCRWRWWRRWRRRW	<i>Gallus gallus</i>
HIKKLRLHDKFFHLLK	<i>Canis lupus familiaris</i>
HIKRLKKALKKALKKL	<i>Mesobuthus martensii</i>
HILKILLKKLKNVVNL	<i>Gallus gallus</i>
HILYAFFKKKKKKKKK	<i>Rhincodon typus</i>
HKIVDFFRKKKKLNVK	<i>Canis lupus lupus</i>
HKTfNKFFLRMKKKTL	<i>Talitrus saltator</i>
HLLLLLLLLKKKKLFL	<i>Gallus gallus</i>
IADAKCRRMMRWIRRL	<i>Rhincodon typus</i>
IAKILFRKKVNVIPVI	<i>Falco peregrinus</i>
IAVMWKREIWWLKRRL	<i>Panthera tigris altaica</i>
ICWPACRRRFRNPVRK	<i>Rhincodon typus</i>
IEQLIRKKKIYFLLKK	<i>Mesobuthus martensii</i>
IFFFFKRQKMKSMLKK	<i>Takifugu rubripes</i>
IFFKVHKKISEVLRII	<i>Talitrus saltator</i>
IFKFLFRKDVIIKLA	<i>Danaus plexippus plexippus</i>
IFKINKLTKKIIRKK	<i>Rhincodon typus</i>
IFLLKKIALLFKKNC	<i>Mesobuthus martensii</i>
IFPCNFRYKVKKNIIN	<i>Danaus plexippus plexippus</i>
IFRFFQNYVLRAREKR	<i>Talitrus saltator</i>
IFRKIKKLIKDLELL	<i>Takifugu rubripes</i>
IFRLHKVYKIFKQKLL	<i>Talitrus saltator</i>
IGGIIFIKLLFKTFI	<i>Crocodylus porosus</i>
IGRPLSAGASAGAAL	<i>Burkholderia pseudomallei</i>
IHLERKRCVCVCVRAR	<i>Panthera tigris altaica</i>
IIALKINKKKIFHYIK	<i>Mesobuthus martensii</i>
IIFRILLRFLKKHLRS	<i>Gallus gallus</i>
IIIIIVVRTKKILI	<i>Plasmodium falciparum</i>
IIIIIKNIITIIII	<i>Plasmodium falciparum</i>
IIIIKGIYMRNKRRK	<i>Plasmodium falciparum</i>
IIIIILMRMLKMNLLK	<i>Plasmodium falciparum</i>
IIIIKKIIKKIIKK	<i>Takifugu rubripes</i>
IIKKIIIIINKNNKK	<i>Gallus gallus</i>
IIKILKTKDKLKKIG	<i>Canis lupus familiaris</i>
IIKMIIIIKINITKI	<i>Plasmodium falciparum</i>
IIILNKLRTVLKKNLK	<i>Mesobuthus martensii</i>
IIINMITILMMMKKKK	<i>Plasmodium falciparum</i>
IIKIINIGIIKINIG	<i>Plasmodium falciparum</i>
IIKILSKQMKKPAYVA	<i>Canis lupus familiaris</i>
IIKKISTIIVKILLNN	<i>Mesobuthus martensii</i>

IIKKYWFLIKRIVKKV	<i>Helianthus anuus</i>
IIKLIVIKINVLKVLK	<i>Gallus gallus</i>
IIKMIVKRIVKRIVKR	<i>Plasmodium falciparum</i>
IIKMLLKIKLKLNLKI	<i>Mesobuthus martensii</i>
IIKNMIIKNMIIKKVK	<i>Plasmodium falciparum</i>
IILKIMIILKIIITLK	<i>Plasmodium falciparum</i>
IIMIMMMMIIIIIKK	<i>Falco peregrinus</i>
IINKLKIKKKIKKKIL	<i>Canis lupus familiaris</i>
IIRIIKMKVITIGMK	<i>Plasmodium falciparum</i>
IIRWRLRWVPRKAVRR	<i>Helianthus anuus</i>
IISRLTIRFPIRSKLL	<i>Metallosphaera sedula</i>
IIVILLYFFMSSKVYK	<i>Plasmodium falciparum</i>
IIVRIMGKCLNTFKKK	<i>Panthera tigris altaica</i>
IKALKVQLKYRCQCQK	<i>Rhincodon typus</i>
IKAVKSRLFRKWKCV	<i>Panthera tigris altaica</i>
IKCFKKRKKKLLLLL	<i>Gallus gallus</i>
IKDIIIIIIITLKK	<i>Mesobuthus martensii</i>
IKIFFLFFWRRMKRRK	<i>Crocodylus porosus</i>
IKIFFLNRKKKKKKIY	<i>Plasmodium falciparum</i>
IKILFKNGRGRVTRAR	<i>Orcinus orca</i>
IKKDIIKKKIIKKK	<i>Plasmodium falciparum</i>
IKKIIIIKKIIKKYLL	<i>Plasmodium falciparum</i>
RLCLFCKCCPRVR	<i>Panthera tigris altaica</i>
RLCYIRYYVCVAR	<i>Danaus plexippus plexippus</i>
RLPFQYAIRRRRR	<i>Metallosphaera sedula</i>
RRLWRLIRSLLL	<i>Crocodylus porosus</i>
RRWSRIHRVPRR	<i>Metallosphaera sedula</i>
SFLKLIKFLKKW	<i>Danaus plexippus plexippus</i>
SKVIPKLFCILNK	<i>Ophiophagus hannah</i>
VFWKKIIKKGKII	<i>Falco peregrinus</i>
VGKFLKLKLLKI	<i>Mesobuthus martensii</i>
VGVLWLRLWRKLW	<i>Ophiophagus hannah</i>
VIWKKIIKIIFLN	<i>Danaus plexippus plexippus</i>
IKKITHFKKKKKFFLY	<i>Talitrus saltator</i>
IKKKKIIKKNIYIL	<i>Plasmodium falciparum</i>
IKKKKKKKVFPLPHL	<i>Gallus gallus</i>
IKKLKNIKFRYKKLKF	<i>Canis lupus familiaris</i>
IKKNKTKLKNTITKVK	<i>Orcinus orca</i>
IKKVMLLLLKRKMKKL	<i>Bacillus anthracis</i>
IKLIFIWTIKVNGYYF	<i>Danaus plexippus plexippus</i>
IKMIIKMIIKMTIKMI	<i>Plasmodium falciparum</i>
IKMLKPCCLCCQIRWR	<i>Rhincodon typus</i>
IKMVIKTIKTVIIKK	<i>Plasmodium falciparum</i>
IKNFISWNLKFKAIR	<i>Rhincodon typus</i>
IKNLLCFAKCLVFKQG	<i>Panthera tigris altaica</i>

IKRNFIKRRPIFKRLV	<i>Metallosphaera sedula</i>
IKVKNILGKIFKTINK	<i>Danaus plexippus plexippus</i>
ILFCKLLIHRLLKKVLF	<i>Rhincodon typus</i>
ILFIFIMIQUIKKKKII	<i>Plasmodium falciparum</i>
ILFKKKKKKKKKKKKK	<i>Plasmodium falciparum</i>
ILIKNLKKILNIRKNI	<i>Canis lupus familiaris</i>
ILILFFFKLLKKNVKL	<i>Crocodylus porosus</i>
ILKIIKLRFIKSLKK	<i>Plasmodium falciparum</i>
ILKIKKKIFKMIKKCC	<i>Crocodylus porosus</i>
ILKKKKKKKIFSILLLL	<i>Rhincodon typus</i>
ILKKVLKPWLLFLKYI	<i>Gallus gallus</i>
ILKLLPLILLLRVIKV	<i>Takifugu rubripes</i>
ILKNKELKKLMMMLLK	<i>Falco peregrinus</i>
ILKQLFITFKINKQK	<i>Danaus plexippus plexippus</i>
ILLIKILLKWKNNLLKK	<i>Gallus gallus</i>
ILLKIIKKFAKLLSKV	<i>Mesobuthus martensii</i>
ILLKTLKKLLKLELQS	<i>Mesobuthus martensii</i>
ILNFKKKKKNIATITI	<i>Plasmodium falciparum</i>
ILNIVKKIKVKKIAIK	<i>Canis lupus familiaris</i>
ILRKRFFKKKKKKFFK	<i>Mesobuthus martensii</i>
ILRKRIKKKNVIKLFK	<i>Gallus gallus</i>
IMGTKKKMKMVRKLLK	<i>Canis lupus familiaris</i>
IMIMNLKKKKKKIFLV	<i>Mesobuthus martensii</i>
INIKKKKKQKKFKKK	<i>Panthera tigris altaica</i>
IPLKEGKRKKLVPPFL	<i>Plasmodium falciparum</i>
IQRRRSAAVAAARAARR	<i>Burkholderia pseudomallei</i>
IRKARKILFKFKKNL	<i>Canis lupus familiaris</i>
IRKLIKKGFKNITYYL	<i>Danaus plexippus plexippus</i>
IRKLKKYRGTLKNIFK	<i>Orcinus orca</i>
IRNIIKITLIIIRKR	<i>Plasmodium falciparum</i>
IRRLARRGGVKRISGL	<i>Takifugu rubripes</i>
IRRPLVIAKTRARRRR	<i>Ganoderma lucidum</i>
IRVRVRVRVCVRVWVR	<i>Rhincodon typus</i>
ITILLKRLTNFLLSKL	<i>Plasmodium falciparum</i>
ITQKPKLTLTIHAIKK	<i>Helianthus annuus</i>
IQAQRSIIFITKRGNK	<i>Rhincodon typus</i>
IVFIKIIKKLMINKK	<i>Mesobuthus martensii</i>
IVFIKNLNQIYFLRNL	<i>Metallosphaera sedula</i>
IVPIRALLSFSRFLC	<i>Panthera tigris altaica</i>
IWFQHRLLYKIYRKKK	<i>Panthera tigris altaica</i>
IWKKKIGQSIKRIKRK	<i>Plasmodium falciparum</i>
IWWVGAQVCRKDRQRR	<i>Canis lupus familiaris</i>
IYINPAIFIKLKKKKF	<i>Helianthus annuus</i>
IYPIKFLIKKIIKK	<i>Mesobuthus martensii</i>
KAACKAEAFELRKVVK	<i>Ganoderma lucidum</i>

KAKREIKKKKIYIHKK	<i>Gallus gallus</i>
KALLLRFLLLFKKK	<i>Gallus gallus</i>
KALPVRQPSHLRLPFL	<i>Ganoderma lucidum</i>
KAPALGGSCGAARRRG	<i>Canis lupus lupus</i>
KARRVVAKVELRVLR	<i>Ganoderma lucidum</i>
KASRRPTASSRPTRSK	<i>Burkholderia pseudomallei</i>
KASWGARRRRRKRKRK	<i>Orcinus orca</i>
KCAYKHPNYMKNLKKK	<i>Mesobuthus martensii</i>
KCHRPLFYKVCYCYKA	<i>Panthera tigris altaica</i>
KCYNKLLKKALKHKLK	<i>Rhincodon typus</i>
KDFKIVIIKILNNLKE	<i>Orcinus orca</i>
KDVLNLLKYLYLLKK	<i>Panthera tigris altaica</i>
KFFKKLVILRPFQKKS	<i>Crocodylus porosus</i>
KFIIKKTVKKKKKKK	<i>Canis lupus familiaris</i>
KFIPFLKLTFAKIKKK	<i>Danaus plexippus plexippus</i>
KFKFGLRGKIQFTCKF	<i>Talitrus saltator</i>
KFKFKNTGFPKIYFLK	<i>Danaus plexippus plexippus</i>
KFKKKKKKKKGICGLF	<i>Gallus gallus</i>
KFKKKKKKKKKLYIRK	<i>Plasmodium falciparum</i>
KFKKMFLKSLAALWRK	<i>Rhincodon typus</i>
KFLKIKKFEKKFVKKF	<i>Talitrus saltator</i>
KFLYRVKMIKNLMKIH	<i>Mesobuthus martensii</i>
KFNLISIKFHVRCFKR	<i>Talitrus saltator</i>
KFPIRKMLKKMLKML	<i>Canis lupus familiaris</i>
KFRWGLLGRKLLYSIP	<i>Rhincodon typus</i>
KFSKRFLKKKKKKKY	<i>Crocodylus porosus</i>
KFVYFKIKKKKKKKK	<i>Helianthus annuus</i>
KGAVRRARRSRCIRAG	<i>Ganoderma lucidum</i>
KGIKVLGKKAEEVLRI	<i>Rhincodon typus</i>
KGKFWLWSPKLILIKI	<i>Rhincodon typus</i>
KGKICYRKGVRCMILI	<i>Panthera tigris altaica</i>
KGKKGKKEKKGKKGKK	<i>Canis lupus lupus</i>
KGKKIKILLKKIFKK	<i>Rhincodon typus</i>
KGLQWRKLLKLISGNI	<i>Falco peregrinus</i>
KGPKIRYKDVQKLEIK	<i>Takifugu rubripes</i>
KGRRRLRLRLWLWLP	<i>Ganoderma lucidum</i>
KGWNWKKVRFKLLWKK	<i>Takifugu rubripes</i>
KGYGLRVIRVRVRVK	<i>Takifugu rubripes</i>
KHFTLKDFAKYYLKFK	<i>Talitrus saltator</i>
KHLKILSKLLFLNIFC	<i>Canis lupus familiaris</i>
KHLRHWYIAFLLVRI	<i>Galdieria sulphuraria</i>
KHSIIIVKILEKFLK	<i>Mesobuthus martensii</i>
KICIKIYMKIIKKLNL	<i>Mesobuthus martensii</i>
KICWLRVLLKKKKKKK	<i>Gallus gallus</i>
KIEKRAPFFIAPFQLF	<i>Talitrus saltator</i>

KIFFTKIFKKINKNKN	<i>Panthera tigris altaica</i>
KIFHLAYLALKKYLSS	<i>Gallus gallus</i>
KIFKKFKKFKKKLKIP	<i>Helianthus anuus</i>
KIFKKSRFFSHKIFKK	<i>Canis lupus familiaris</i>
KIFKRICHYHLHLPK	<i>Gallus gallus</i>
KIFRMIKKSKCFKYRK	<i>Panthera tigris altaica</i>
KIGKWRIKHSRLGSGG	<i>Crocodylus porosus</i>
KIIIIKRWLRKLLRNL	<i>Takifugu rubripes</i>
KIIIIIIIIQIKFI	<i>Mesobuthus martensii</i>
KIIKKTIFKKICRKK	<i>Plasmodium falciparum</i>
KIIIKRKALQIRKLFN	<i>Orcinus orca</i>
KIIIVIIIIKELGQK	<i>Crocodylus porosus</i>
KIIKILIKILITKTL	<i>Takifugu rubripes</i>
KIIVKIIIIIVKIIK	<i>Plasmodium falciparum</i>
KIILKKIQIFKILIKI	<i>Mesobuthus martensii</i>
KIINIKKKRVHLIKKK	<i>Panthera tigris altaica</i>
KIIQKLKKIYKKVKFK	<i>Mesobuthus martensii</i>
KIITIKIITIKIITIK	<i>Plasmodium falciparum</i>
KIIVKNLTIITYIIVK	<i>Mesobuthus martensii</i>
KIKIKMKLKIKRKLKL	<i>Rhincodon typus</i>
KIKIKNKKKLKKLKKI	<i>Orcinus orca</i>
KIKIKVLIFHFLKKLE	<i>Mesobuthus martensii</i>
KIKKIIIIKKIFKFSK	<i>Gallus gallus</i>
KIKKKKKDQIFRLVYKK	<i>Crocodylus porosus</i>
KIKKKKKFFFGICLFYF	<i>Mesobuthus martensii</i>
KIKKKKIAHLLLFQKK	<i>Gallus gallus</i>
KIKKKKKYSKSSLEIF	<i>Canis lupus familiaris</i>
KIKKKKLFNLWLIFFF	<i>Gallus gallus</i>
KIKKKKLITAFYLLAI	<i>Mesobuthus martensii</i>
KIKMVNAKKIKLQIQM	<i>Rhincodon typus</i>
KIKRIKNLKVIIKKK	<i>Orcinus orca</i>
KIKRVIMKITIKIKII	<i>Plasmodium falciparum</i>
KIKTIIIIIIIRVIK	<i>Plasmodium falciparum</i>
KIKTNKIFKILLKFHL	<i>Panthera tigris altaica</i>
KILKKNIKKKAGKVIA	<i>Falco peregrinus</i>
KILLAFKRYRRIHRLI	<i>Gallus gallus</i>
KILLVLIKNRSIPKIN	<i>Plasmodium falciparum</i>
KILPEWFIKRALKKFK	<i>Talitrus saltator</i>
KILNRNKKYLFVILVK	<i>Talitrus saltator</i>
KIMIKRRRKIKTLKVI	<i>Plasmodium falciparum</i>
KINFYIIMVSTKII	<i>Plasmodium falciparum</i>
KINLVMQRFKKIIKFL	<i>Orcinus orca</i>
KINMMKMILIIKKIK	<i>Plasmodium falciparum</i>
KIRKKKKKKKKKHIHI	<i>Plasmodium falciparum</i>
KIRPPLLKKKKKKKKK	<i>Plasmodium falciparum</i>

KIVKVIKKIKPKKIF	<i>Mesobuthus martensii</i>
KIVNSLLFKALASGVG	<i>Crocodylus porosus</i>
KIYSKILKKFLKKQLK	<i>Orcinus orca</i>
KKAHAKKHHAKKHAKK	<i>Burkholderia pseudomallei</i>
KKAKIKIKIKKKAKKK	<i>Falco peregrinus</i>
KKAVRIFLKKLKYLIK	<i>Talitrus saltator</i>
KKCFLFCFSFFFFFMK	<i>Panthera tigris altaica</i>
RFGRRALARLKLALAGAL	<i>Canis lupus lupus</i>
RFKKFAIFCRKKLSKS	<i>Rhincodon typus</i>
KKEIIIIILIKKYIIK	<i>Plasmodium falciparum</i>
KKFFFLKQIWPKKKKK	<i>Panthera tigris altaica</i>
KKFFIKIFKLRDKIII	<i>Talitrus saltator</i>
KKFFKVFILWRKKNKV	<i>Canis lupus familiaris</i>
KKFKKKEIKPILLHIK	<i>Panthera tigris altaica</i>
KKFMLSFKLKKKKKKK	<i>Plasmodium falciparum</i>
KKGERLKIKKKKRKKR	<i>Gallus gallus</i>
KKHLKKLKYIPKELK	<i>Panthera tigris altaica</i>
KKIIGFLVLKKILKNK	<i>Plasmodium falciparum</i>
KKIIKKQIFILKYIIK	<i>Gallus gallus</i>
KKIINKLVKIYNLLKI	<i>Mesobuthus martensii</i>
KKIKINKKRKKEKKKR	<i>Gallus gallus</i>
KKIKLKKILIKKVIYF	<i>Panthera tigris altaica</i>
KKIKTYKIKFKKDFVR	<i>Rhincodon typus</i>
KKIMIKRIMIKRIMIK	<i>Plasmodium falciparum</i>
KKITLKIILNIKIKKI	<i>Plasmodium falciparum</i>
KKKGFQNRTRHVNKRL	<i>Talitrus saltator</i>
KKKGHAEVWRGQACWV	<i>Canis lupus lupus</i>
KKKIIIMKKKMILKK	<i>Plasmodium falciparum</i>
KKKKAKKIVIAFLKIL	<i>Gallus gallus</i>
KKKKHLKKLKNLKKK	<i>Panthera tigris altaica</i>
KKKKIFNLHGLVEIKV	<i>Mesobuthus martensii</i>
KKKKILKKWRNYVLGK	<i>Panthera tigris altaica</i>
KKKKKKILKYFFLKF	<i>Mesobuthus martensii</i>
KKKKKKKALFALKLLF	<i>Gallus gallus</i>
KKKKKKKFLFIHLIVL	<i>Gallus gallus</i>
KKKKKKKFPLLSFIYL	<i>Gallus gallus</i>
KKKKKKKIKLLFLFLL	<i>Gallus gallus</i>
KKKKKKKKKKKKLIKY	<i>Plasmodium falciparum</i>
KKKKKKKKKKKLISILF	<i>Plasmodium falciparum</i>
KKKKKKKKKKKNLKKSP	<i>Gallus gallus</i>
KKKKKKKKKKRYIFLLF	<i>Plasmodium falciparum</i>
KKKKKKKKKTVHCLHG	<i>Helianthus annuus</i>
KKKKKKKKLVIPFYEI	<i>Gallus gallus</i>
KKKKKKKKVLKSFLFL	<i>Plasmodium falciparum</i>
KKKKKKKKLYIHKLGVF	<i>Gallus gallus</i>

KKKKKKKNKKKKKKKK	<i>Galdieria sulphuraria</i>
KKKKKKKNRKLIFL	<i>Plasmodium falciparum</i>
KKKKKKKRKIIINVIL	<i>Plasmodium falciparum</i>
KKKKKKKRRLKNKKKP	<i>Galdieria sulphuraria</i>
KKKKKKLGVLFVLF	<i>Gallus gallus</i>
KKKKKKPWFFFFLLSL	<i>Canis lupus lupus</i>
KKKKKKRKKKKRIRKV	<i>Gallus gallus</i>
KKKKKKKYLKKIHL	<i>Mesobuthus martensii</i>
KKKKKVHHIHKIKKK	<i>Gallus gallus</i>
KKKKMFYLLFSVLFFL	<i>Gallus gallus</i>
KKKKNQKKNNKKKKK	<i>Rhincodon typus</i>
KKKKRKKERKKERKKE	<i>Canis lupus lupus</i>
KKKKVFLKKVFCVNR	<i>Gallus gallus</i>
KKKKVLKALKVFGFFF	<i>Gallus gallus</i>
KKKLICPKRHKIVYSI	<i>Gallus gallus</i>
KKKKKKKTkFMKLLKK	<i>Danaus plexippus plexippus</i>
KKKLLPSILLILCPI	<i>Panthera tigris altaica</i>
KKKLNLKLSVPYKKIK	<i>Rhincodon typus</i>
KKKNGWLPQFRINCKR	<i>Talitrus saltator</i>
KKKNTHKYFYKKKKKK	<i>Mesobuthus martensii</i>
KKKQLSTTVVIVYYKK	<i>Helianthus anuus</i>
KKKRLKMLFFFFFFVK	<i>Helianthus anuus</i>
KKKSFVILKRRKKKK	<i>Mesobuthus martensii</i>
KKKVFSLLKKGIMIF	<i>Canis lupus familiaris</i>
KKLAKIIMQKKKKKKK	<i>Helianthus anuus</i>
KKLFKKITSKTLFLKA	<i>Rhincodon typus</i>
KKLITIVKKITIIVTI	<i>Plasmodium falciparum</i>
KKLKKKLKNTWSQKKK	<i>Canis lupus familiaris</i>
KKLKKLKRWLKLRLL	<i>Helianthus anuus</i>
KKLKLRLRCSITKK	<i>Takifugu rubripes</i>
KKLLKYPRALKKTLKK	<i>Gallus gallus</i>
KKLLCLQPKICPFL	<i>Panthera tigris altaica</i>
KKLRRRVRRVFKKLR	<i>Orcinus orca</i>
KKLYLPFLKIIAKLFF	<i>Panthera tigris altaica</i>
KKNFSCFYILTKKKV	<i>Talitrus saltator</i>
KKQRVLLKLLVVIPL	<i>Galdieria sulphuraria</i>
KKRIKKLLKKKKVKR	<i>Falco peregrinus</i>
KKRKRKRRCRARRCLL	<i>Ganoderma lucidum</i>
KKRLAKKIRKKLKEG	<i>Talitrus saltator</i>
KKRRRKRKKGTRRRRA	<i>Ganoderma lucidum</i>
KKRRRRRRRRRRRRRR	<i>Canis lupus lupus</i>
KKRRVIKLLKLLLLK	<i>Helianthus anuus</i>
KKTGIKIRIMIGIRK	<i>Plasmodium falciparum</i>
KKTLLKVLISIAQHKIS	<i>Talitrus saltator</i>
KKVIKKLIKIRIRMII	<i>Talitrus saltator</i>

KKVKSIVKCLQRIQAY	<i>Talitrus saltator</i>
KKVLLLLLLLLFLAK	<i>Crocodylus porosus</i>
KKVLLRKILKFYSLYF	<i>Talitrus saltator</i>
KKVQILYNFKKEIRK	<i>Panthera tigris altaica</i>
KKVRQEVLSKKKKKKK	<i>Canis lupus lupus</i>
KLFKKIYKKLIFHTKK	<i>Mesobuthus martensii</i>
KLFKLGPIVQRILYST	<i>Mesobuthus martensii</i>
KLFKLSKLLKLIKFLR	<i>Talitrus saltator</i>
KLIKILRLLVIRIII	<i>Danaus plexippus plexippus</i>
KLILIKKKKKKKKSVL	<i>Gallus gallus</i>
KLKAMLIAIKKILIKK	<i>Rhincodon typus</i>
KLKKIFFFRKIKSEFK	<i>Danaus plexippus plexippus</i>
KLKKKFIFKKARILI	<i>Rhincodon typus</i>
KLKKKKVFLFLFFHLL	<i>Gallus gallus</i>
KLKKKMNSKHLWYKLS	<i>Falco peregrinus</i>
KLKKKYITACIYLLKK	<i>Panthera tigris altaica</i>
KLKKLLILLRKLPCKK	<i>Danaus plexippus plexippus</i>
KLKNPWKAFCFALKLF	<i>Panthera tigris altaica</i>
KLKRKQLYSLVKIVLL	<i>Talitrus saltator</i>
KLKRMIMMKKWKKIKK	<i>Helianthus annuus</i>
KLKRRTHTTTWWRWRI	<i>Rhincodon typus</i>
KLLKEFFRSIYKLKKI	<i>Danaus plexippus plexippus</i>
KLLFIKLLQLFTKLL	<i>Falco peregrinus</i>
KLLKIPKKYYLSLKKL	<i>Gallus gallus</i>
KLLKKLLKTAKKLLK	<i>Talitrus saltator</i>
KLLKMLNFLKSNLYK	<i>Rhincodon typus</i>
KLLLKLIKNLKLNKL	<i>Canis lupus familiaris</i>
KLLVAFFKKKTCLVKI	<i>Rhincodon typus</i>
KLNVKKKFKYINKCLK	<i>Panthera tigris altaica</i>
KLRCRIKLPKRIFPK	<i>Metallosphaera sedula</i>
KLRKLFNNILSKVKHS	<i>Falco peregrinus</i>
KLVLILVSGTLVDCI	<i>Falco peregrinus</i>
KLVQLPWRAIWKSLKK	<i>Orcinus orca</i>
KLVRKKLLKLLKNGK	<i>Talitrus saltator</i>
KLYFKFLHARKKFGFLF	<i>Talitrus saltator</i>
KMGRQSGGLLELRRL	<i>Takifugu rubripes</i>
KMKKQQRKRKKKKRKH	<i>Galdieria sulphuraria</i>
KMMMMRRRRRRRKM RK	<i>Galdieria sulphuraria</i>
KMWLRRLFIFFIFFFF	<i>Takifugu rubripes</i>
KNFKKVRKKKKKKGLL	<i>Plasmodium falciparum</i>
KNIILMLLLKNLKNIG	<i>Canis lupus familiaris</i>
KNIKKLIKTKWRLL	<i>Mesobuthus martensii</i>
KNILLKFYFLLLRV	<i>Orcinus orca</i>
KNIMCLWYFAHSCRK	<i>Canis lupus lupus</i>
KNKLKKHLKNLKKILK	<i>Panthera tigris altaica</i>

KNKNKKKKLKKIFFLK	<i>Panthera tigris altaica</i>
KNLKKLKPCTFVLSLV	<i>Canis lupus familiaris</i>
KNLLNMLSKLLKSLRK	<i>Takifugu rubripes</i>
KNLYGPKKILLKRIVQ	<i>Talitrus saltator</i>
KNVIKIKLNLITIII	<i>Takifugu rubripes</i>
KPKPKPKPKPKPKPKP	<i>Burkholderia pseudomallei</i>
KPRRTTRTARTRRRRR	<i>Burkholderia pseudomallei</i>
KRARRPRRPRARRRTA	<i>Burkholderia pseudomallei</i>
KRFKIKKKKKKKRKKK	<i>Canis lupus lupus</i>
KRIFLIPWNIKNIIIK	<i>Plasmodium falciparum</i>
KRIQRNLKKNLFWKPK	<i>Canis lupus familiaris</i>
KRIRKLFKLFKIILRL	<i>Gallus gallus</i>
KRKKHLKLLKRFFFL	<i>Panthera tigris altaica</i>
KRKKKKKKERKKERKK	<i>Canis lupus lupus</i>
KRKKKKKRRITRSRHH	<i>Galdieria sulphuraria</i>
KRKKKKQKKKTTFIVK	<i>Panthera tigris altaica</i>
KRKKKRKEEFKKKKKK	<i>Canis lupus lupus</i>
KRKKVHKKIIRKILKK	<i>Helianthus annuus</i>
KRKKYLVPLIKKIII	<i>Gallus gallus</i>
KRKTFIKKKKCFHFKM	<i>Mesobuthus martensii</i>
KRLGNASYKILKLLK	<i>Rhinocodon typus</i>
KRLKKSKKTKCLIWK	<i>Gallus gallus</i>
KRLKLFLKLYHKCLK	<i>Gallus gallus</i>
KRLKRLKSRWKLKR	<i>Helianthus annuus</i>
KRRSTRVFRPGGRQGG	<i>Ganoderma lucidum</i>
KRVVRVRVRVRVRVR	<i>Takifugu rubripes</i>
KRWGRKRWWWWLQWGR	<i>Takifugu rubripes</i>
KSFKKKKKKKKELYIG	<i>Gallus gallus</i>
KSFKKWNIIVKILSMK	<i>Canis lupus familiaris</i>
KSKKTLYIFLFFFKI	<i>Gallus gallus</i>
RWFWRRLLYWRWWWRW	<i>Helianthus annuus</i>
RWIWWCAWRRSRRGRR	<i>Ganoderma lucidum</i>
KSLKKNLKKVIFTKIL	<i>Mesobuthus martensii</i>
KSLFMLKLLKILKM	<i>Orcinus orca</i>
KSLWRTVWRFLKLLKR	<i>Orcinus orca</i>
KSMKKFHLLKKSWKK	<i>Crocodylus porosus</i>
KSSRCRLFWSRWWRLL	<i>Ganoderma lucidum</i>
KTGSVALRRRRWAARR	<i>Takifugu rubripes</i>
KTNFLIKAKKKKKKKK	<i>Gallus gallus</i>
KVFCVISVRLFMCRKK	<i>Panthera tigris altaica</i>
KVIKILFKLILKNLKK	<i>Talitrus saltator</i>
KVIPARAAHRWAAVQL	<i>Gallus gallus</i>
KVLFLLKKKKKSVLLL	<i>Gallus gallus</i>
KVPFKCEYKKWRFIWK	<i>Falco peregrinus</i>
KVRKLKLLKLLKSKK	<i>Helianthus annuus</i>

KVRVKVRVRVRVSISG	<i>Takifugu rubripes</i>
KVTLLATLKKKKKKKK	<i>Panthera tigris altaica</i>
KWKIIKKLYHLKYKKN	<i>Plasmodium falciparum</i>
KWLSKTWKAIRATTRR	<i>Burkholderia pseudomallei</i>
KYFRKIRYFKIFSCKM	<i>Panthera tigris altaica</i>
KYHLIPLRISIIKKII	<i>Orcinus orca</i>
KYIIKIINGIYKGIKG	<i>Canis lupus familiaris</i>
KYKAFKKIEVKVKFF	<i>Talitrus saltator</i>
KYLFAKEKKKIKKIKK	<i>Gallus gallus</i>
KYLLKIPGSKLKLLK	<i>Gallus gallus</i>
KYLLLLLLLMKPINI	<i>Danaus plexippus plexippus</i>
KYRFSNFKKKKKKKKK	<i>Mesobuthus martensii</i>
LAANLLKKKNLFGTKI	<i>Falco peregrinus</i>
LALGICWKIKKIKGL	<i>Talitrus saltator</i>
LALKKKKKKYKQLLL	<i>Crocodylus porosus</i>
LALRGRRRRVLLRLL	<i>Ganoderma lucidum</i>
LAPDLKKKKLLHGLLI	<i>Orcinus orca</i>
LARFLRVLRFLRVLA	<i>Takifugu rubripes</i>
LARRGGVKRISGLIYE	<i>Takifugu rubripes</i>
LASLLSLGKALKLLK	<i>Crocodylus porosus</i>
LCALKWMRMLLTALKK	<i>Panthera tigris altaica</i>
LCAVLTKAVKLLKKKK	<i>Gallus gallus</i>
LCIAPSQLKHLKLLKK	<i>Orcinus orca</i>
LCLLKVKSRPLPKRL	<i>Ganoderma lucidum</i>
LCLSQKINKHLKLLKK	<i>Panthera tigris altaica</i>
LCRGMLFGSHLDSKYR	<i>Falco peregrinus</i>
LCRSLRRMHRLRRMFR	<i>Bacillus anthracis</i>
LCTKLVTHPILLIAKL	<i>Danaus plexippus plexippus</i>
LCVLLKLLRCSLLPK	<i>Panthera tigris altaica</i>
LETSLGVLAVGDLLG	<i>Crocodylus porosus</i>
LFCKFKKKKKKILLVF	<i>Crocodylus porosus</i>
LFDKNLNKNLKLKKKK	<i>Panthera tigris altaica</i>
LFLLFKKSLFKKIFK	<i>Panthera tigris altaica</i>
LFIIRKHCLFLLRIK	<i>Mesobuthus martensii</i>
LFIISTKKKKKVGAFI	<i>Panthera tigris altaica</i>
LFIKVHIFFRKITKKY	<i>Talitrus saltator</i>
LFIYNKKKKKKKKRKK	<i>Plasmodium falciparum</i>
LFKIAHTVKLLLPKFK	<i>Orcinus orca</i>
LFKKFYIKKIIKEKK	<i>Canis lupus familiaris</i>
LFKKKKKKKALFQLNI	<i>Crocodylus porosus</i>
LFKKKKKKKKFRTLKK	<i>Rhincodon typus</i>
LFKLATKIIPSLFRRK	<i>Mesobuthus martensii</i>
LFKLLKILLLLLLLK	<i>Plasmodium falciparum</i>
LFKTAAKKAFWCKLLK	<i>Falco peregrinus</i>
LFLFWKKKKKRQKKKK	<i>Gallus gallus</i>

LFLLIYKKKKKKKKR	<i>Gallus gallus</i>
LFLKFRPAKCSMFG	<i>Gallus gallus</i>
LFLMKKKKKKKLLY	<i>Plasmodium falciparum</i>
LFLQVKWKTLLKHVLK	<i>Orcinus orca</i>
LFQIGIMTRLHCILRR	<i>Metallosphaera sedula</i>
LFQILFRIAKKKKKK	<i>Gallus gallus</i>
LFRLFPIFSVLFPIF	<i>Falco peregrinus</i>
LFRVKRTIPKRLKWK	<i>Talitrus saltator</i>
LFSPQLAGKKIVNILI	<i>Crocodylus porosus</i>
LFSSFKIIQKITIKK	<i>Crocodylus porosus</i>
LFTKKKKKAAFIVRKL	<i>Gallus gallus</i>
LFVFLKHLKFFLKILK	<i>Panthera tigris altaica</i>
LFYLKAKKKKKKKPLL	<i>Crocodylus porosus</i>
LGACAAPLALPRPLGA	<i>Crocodylus porosus</i>
LGKSKIKLWFQLKHK	<i>Panthera tigris altaica</i>
LGMKAWKKKKKKLALL	<i>Gallus gallus</i>
LGTLTGLKWPRRLERL	<i>Talitrus saltator</i>
LGWIRKSKRRTEVIPS	<i>Falco peregrinus</i>
LGWVWVCIVFWFVFF	<i>Falco peregrinus</i>
LIDLVVYKVLKLLK	<i>Canis lupus familiaris</i>
LIFALFKKKKKKKKI	<i>Crocodylus porosus</i>
LIFERKIVFHFIMRK	<i>Talitrus saltator</i>
LIISLRRCWHKKIKI	<i>Canis lupus familiaris</i>
LIITIKHTKRLKELN	<i>Takifugu rubripes</i>
LIKFIWFMMRKILFKI	<i>Plasmodium falciparum</i>
LIKFLVVILLKNIKKH	<i>Mesobuthus martensii</i>
LIKIKRVIVIKKKIIL	<i>Plasmodium falciparum</i>
LIKSWSKIIQWRLKWK	<i>Rhincodon typus</i>
LILKFCIKKKKKEIKK	<i>Danaus plexippus plexippus</i>
LILKFNRLKFLKKLVS	<i>Canis lupus familiaris</i>
LILKKILFYKITKKNI	<i>Plasmodium falciparum</i>
LILKLQVKKLMKLLQI	<i>Mesobuthus martensii</i>
LILKNKFKKLLKIKN	<i>Gallus gallus</i>
LILLLLKKKKKKKKK	<i>Gallus gallus</i>
LILLVKKKKIFLKKKK	<i>Talitrus saltator</i>
LILQMNLKLLKLLK	<i>Rhincodon typus</i>
LILRKIVKYDIKHTLR	<i>Rhincodon typus</i>
LILYFKGIKNINKNVD	<i>Danaus plexippus plexippus</i>
LIMIEIKKILKKILR	<i>Plasmodium falciparum</i>
LIRFTKGLRRRKRRWL	<i>Metallosphaera sedula</i>
LIRQLKLLKLLQWAK	<i>Danaus plexippus plexippus</i>
LIRRRRRRRACRGGCA	<i>Burkholderia pseudomallei</i>
LIRRSWRNWWSGPGRT	<i>Metallosphaera sedula</i>
LIRVKHQGFLGKIVKI	<i>Metallosphaera sedula</i>
LIRVLYPCQLKAKLGK	<i>Metallosphaera sedula</i>

LITILYVVKIIRRIKK	<i>Plasmodium falciparum</i>
LIVTSLFLGKKKKKKKI	<i>Panthera tigris altaica</i>
LIYTYIKKKKKKKKKNI	<i>Danaus plexippus plexippus</i>
LKDKVPYIKKIRRKLL	<i>Crocodylus porosus</i>
LKFKIFFPIIKFLLKK	<i>Danaus plexippus plexippus</i>
LKFRFKLKRIKYEKIL	<i>Gallus gallus</i>
LKGILWKILFKRISFL	<i>Falco peregrinus</i>
LKIKIKIKIKIKIKIK	<i>Helianthus anuus</i>
LKIKKNIIVIIKKYI	<i>Plasmodium falciparum</i>
LKIRKLKIKIEKIII	<i>Talitrus saltator</i>
LKKFFSKLKKKKELNE	<i>Panthera tigris altaica</i>
LKKGLGFIKKNKCCFL	<i>Crocodylus porosus</i>
LKKIFIFMIKKILKKR	<i>Plasmodium falciparum</i>
LKKIFLHTKKKKIIKK	<i>Panthera tigris altaica</i>
LKKIMKIVKLKRMKYL	<i>Mesobuthus martensii</i>
LKKKFFKFLFKKLFKK	<i>Gallus gallus</i>
LKKKKIIKIIIIKKG	<i>Gallus gallus</i>
LKKKKKIFQVVSEKLL	<i>Gallus gallus</i>
LKKKKKKKIRKLFSRA	<i>Takifugu rubripes</i>
LKKKKKKKKKTFLMKG	<i>Rhincodon typus</i>
LKKKKKKKKLPLCLKH	<i>Canis lupus lupus</i>
LKKKKKKKKLRLFIMS	<i>Plasmodium falciparum</i>
LKKKKKKKKRISKLSHS	<i>Gallus gallus</i>
LKKKKKKKLLICFLWF	<i>Gallus gallus</i>
LKKKKKNKKCLFLKV	<i>Danaus plexippus plexippus</i>
LKKKKRKMVKVFLLL	<i>Gallus gallus</i>
LKKKNWNKVILLFFFF	<i>Plasmodium falciparum</i>
LKKRKKKKMLISDFL	<i>Canis lupus lupus</i>
LKKLKKKLKRYLKDLV	<i>Bacillus anthracis</i>
LKKLKKKMLPRFIRLI	<i>Rhincodon typus</i>
LKKLLKHKSLVKLIKI	<i>Canis lupus familiaris</i>
LKKLSILLWIKVPALL	<i>Talitrus saltator</i>
LKKPIKMLKKPNSFLL	<i>Canis lupus familiaris</i>
LKKRKRIKMRIKKI	<i>Plasmodium falciparum</i>
LKKSLWKTHGTIFFKK	<i>Falco peregrinus</i>
LKLKLKLNFKLKLKLK	<i>Talitrus saltator</i>
LKLLFFKKRKKHSL	<i>Takifugu rubripes</i>
LKLLKLKILKFLRWK	<i>Crocodylus porosus</i>
LKLLKLLFKLLFKKKK	<i>Gallus gallus</i>
LKLLKLLQKLLKRL	<i>Helianthus anuus</i>
LKLLPSLFIFLYLPHL	<i>Gallus gallus</i>
LKLNWKRMLIKKLK	<i>Rhincodon typus</i>
LKLWLKKKVKAQCQVF	<i>Talitrus saltator</i>
LKMLKKYLKKKMLKRY	<i>Rhincodon typus</i>
LKNKINVKKKTLLKKK	<i>Panthera tigris altaica</i>

LKNLLRRLKLRRLLK	<i>Helianthus anuus</i>
LKPLKKLMKIKSQLLK	<i>Takifugu rubripes</i>
LKRFRYLKKRLHLPVK	<i>Talitrus saltator</i>
LKRKKKKVVAHLFLLV	<i>Gallus gallus</i>
LKVAKKKKKKKKKKV	<i>Canis lupus familiaris</i>
LKWFKRIRINTKMNFL	<i>Danaus plexippus plexippus</i>
LLCALIFYKKKKKKTLL	<i>Gallus gallus</i>
LLCFLKSIINLLMKNK	<i>Canis lupus familiaris</i>
LLEFFLKKIFKRFKKK	<i>Rhincodon typus</i>
LLHLIFKKLKKYIEKL	<i>Gallus gallus</i>
LLIFLLKYIYLKRLKK	<i>Gallus gallus</i>
LLIILRKRIYLYKYIK	<i>Gallus gallus</i>
LLIRKLPFQRLVREIA	<i>Takifugu rubripes</i>
LLKAIKILKKYFIFTL	<i>Orcinus orca</i>
LLKKAVKKNLLKKNLK	<i>Gallus gallus</i>
LLKKKIIKILLRKAIK	<i>Takifugu rubripes</i>
LLKKKKKKRKKILFSL	<i>Canis lupus lupus</i>
LLKKKKKRKKDPFLVL	<i>Gallus gallus</i>
LLKKRINKKILIIKKI	<i>Plasmodium falciparum</i>
LLKLLRIKRKLIKLIK	<i>Gallus gallus</i>
LLKVPFPIKKKKKKFT	<i>Canis lupus lupus</i>
LLKWLWIWKLNNAPK	<i>Takifugu rubripes</i>
LLLCFAFSLRSIRCR	<i>Panthera tigris altaica</i>
LLLCLLKKKKKIQAII	<i>Gallus gallus</i>
LLLFLCFLFALKKKKK	<i>Gallus gallus</i>
LLLFLKKKKKGLVRFL	<i>Orcinus orca</i>
LLLILFPFLKRRRRRR	<i>Orcinus orca</i>
LLLILPLPLTPRRYH	<i>Ganoderma lucidum</i>
LLKLLKILLGFGKWK	<i>Orcinus orca</i>
LLLNKAHLIKLLKKK	<i>Canis lupus lupus</i>
LLKRGKLLKQLLKK	<i>Gallus gallus</i>
LLLHLKLKLYVLKLL	<i>Gallus gallus</i>
LLLLLLLLKKKKKLKA	<i>Gallus gallus</i>
LLLLLLLLKLEMILQK	<i>Crocodylus porosus</i>
LLLLLLLLSKIEIKS	<i>Crocodylus porosus</i>
LLLLLLWYCWWWSRW	<i>Orcinus orca</i>
LLLPKLKICVIKKKNL	<i>Panthera tigris altaica</i>
LLMLFLKKKKKKTLTK	<i>Gallus gallus</i>
LLPFFKQIHNSIFPKL	<i>Orcinus orca</i>
LLPLFFLKKKKKKKNF	<i>Gallus gallus</i>
LLPNVIKLIKTLKSG	<i>Falco peregrinus</i>
LLQKIYWKLLKLLVL	<i>Galdieria sulphuraria</i>
LLQYKMKSKKKKKKI	<i>Plasmodium falciparum</i>
LLRHEKIYKRIIRHIK	<i>Helianthus anuus</i>
LLRHLSLRIISGGVKL	<i>Takifugu rubripes</i>

LLRILLRKLLPLFPPF	<i>Metallosphaera sedula</i>
LLRISIKEIIRLRRRK	<i>Talitrus saltator</i>
LLRRCLWIGDARHLLP	<i>Orcinus orca</i>
LLRVLFCSLWHRGPIS	<i>Gallus gallus</i>
LLSIKRIKKKKLTLP	<i>Rhincodon typus</i>
LLTLLNNKLVIKKKK	<i>Danaus plexippus plexippus</i>
LLTVARRCPRHLLTVA	<i>Takifugu rubripes</i>
LLVLLNHTFKKLLKLL	<i>Rhincodon typus</i>
LLVYFLNRRKKFYFL	<i>Panthera tigris altaica</i>
LLWRRLLQCWRGLNLR	<i>Rhincodon typus</i>
LLYLSMLYIKKKKKKK	<i>Plasmodium falciparum</i>
LMGKHLDLLKKLLKVL	<i>Falco peregrinus</i>
LMLIKVIIIILLKNLK	<i>Mesobuthus martensii</i>
LMRAVLKWLIIIRSLW	<i>Gallus gallus</i>
LMTIKKKKVYLSIKK	<i>Panthera tigris altaica</i>
LNFFVYLKKKKKKKKK	<i>Plasmodium falciparum</i>
LPFLHKLRRLRLRRL	<i>Talitrus saltator</i>
LPIKKNLAKMLKTLL	<i>Crocodylus porosus</i>
LPKPFLKDLRFLKKKY	<i>Gallus gallus</i>
LPLLRPQSRLLLKRFT	<i>Ganoderma lucidum</i>
LPLRQLRRYRLPRLRR	<i>Ganoderma lucidum</i>
LPRRRRRRWCRGARRA	<i>Ganoderma lucidum</i>
LPTIILLQIKILRLLL	<i>Danaus plexippus plexippus</i>
LQKKKKKKLILKKFIK	<i>Plasmodium falciparum</i>
LQLLGKLLKLQKFFLK	<i>Falco peregrinus</i>
LQLLIKEKLKLLKLLK	<i>Helianthus annuus</i>
LQPRRWRCRCRFCRYT	<i>Ganoderma lucidum</i>
LQRKLRSWLRLKCLPF	<i>Gallus gallus</i>
LQRRWDQIFFKKKKKK	<i>Orcinus orca</i>
LRAGTNIAANAGGSRA	<i>Burkholderia pseudomallei</i>
LRCLWLRFCAQLTR	<i>Crocodylus porosus</i>
LRIIALVFFPIFRFFS	<i>Talitrus saltator</i>
LRKCLMLKKLKLKLLK	<i>Rhincodon typus</i>
LRKGNYGIRGWNLARV	<i>Metallosphaera sedula</i>
LRKKKKKKKKKKKYIY	<i>Plasmodium falciparum</i>
LRKKLAFERPNCKIL	<i>Panthera tigris altaica</i>
LRKKLLRVLILRKLL	<i>Talitrus saltator</i>
LRKNLFIRYMKKMLKS	<i>Talitrus saltator</i>
LRKSYFFRLFRFKAQF	<i>Talitrus saltator</i>
LRKTLRKKVIKVVKKK	<i>Talitrus saltator</i>
LRLGWRWWRVGWRRVR	<i>Rhincodon typus</i>
LRLKMLKKLPIIGLLK	<i>Mesobuthus martensii</i>
LRLLLKKKKKIVFLI	<i>Rhincodon typus</i>
LRLRRPPLRPRVRRGL	<i>Ganoderma lucidum</i>
LRLRRRVWLRLSRRA	<i>Ganoderma lucidum</i>

LRPLLGLFSPDALPAA	<i>Canis lupus lupus</i>
LRPRAGPCRVRPLPL	<i>Ganoderma lucidum</i>
LRRGYGRCRRRRRRIR	<i>Ganoderma lucidum</i>
LRRKGRSREGRWWWWW	<i>Orcinus orca</i>
LRRNMVRWRKPQRASR	<i>Metallosphaera sedula</i>
LRRRPVRRFRRLRVGL	<i>Ganoderma lucidum</i>
LRRSRGRAPACRRTRR	<i>Burkholderia pseudomallei</i>
LRRTWSFCWLYWRRHR	<i>Galdieria sulphuraria</i>
LRSLLRHLPFPRSKI	<i>Metallosphaera sedula</i>
LRSYLWRRGYKFFRRL	<i>Metallosphaera sedula</i>
L RTPWCLLRRLRCSWR	<i>Ganoderma lucidum</i>
LRVLLVKLLRRLLVDK	<i>Metallosphaera sedula</i>
LRVLRLRLHLELL	<i>Talitrus saltator</i>
LSFFLSFFPFSFFKIL	<i>Canis lupus lupus</i>
LSGILNLKALKKLLKL	<i>Crocodylus porosus</i>
LSHYKRKLLKSLLKKL	<i>Rhincodon typus</i>
LSLLKINKHLKKLKKL	<i>Panthera tigris altaica</i>
LSLSKINKLLKYYKKI	<i>Panthera tigris altaica</i>
LSQMKPFFKKKKKKLL	<i>Rhincodon typus</i>
LSRKISWKKIHKIKL	<i>Gallus gallus</i>
LSSRRDLGFWGFFWK	<i>Falco peregrinus</i>
LTAKIKLIIVTSKIKL	<i>Plasmodium falciparum</i>
LTIKKFLKSLKKLPLT	<i>Canis lupus familiaris</i>
LTKKLIVLKKKKKKLL	<i>Helianthus anuus</i>
LTPRPTRARARPRRRR	<i>Burkholderia pseudomallei</i>
LTRYRNRPLRPLLRL	<i>Metallosphaera sedula</i>
LVGVAHLRHHRRRRRR	<i>Takifugu rubripes</i>
LVIKFYRARWRLMRH	<i>Talitrus saltator</i>
LVITLLKRLNRLKDK	<i>Rhincodon typus</i>
LVLVKKKKHKDLLLQS	<i>Orcinus orca</i>
LVNSILQLIKKKKILH	<i>Panthera tigris altaica</i>
LVPFFFFLKKKKKKK	<i>Gallus gallus</i>
LVPLWRRRWRRHRGLR	<i>Ganoderma lucidum</i>
LVPPRLPRRPPRPPAL	<i>Canis lupus lupus</i>
LVRRTTRRWLMRLLR	<i>Ganoderma lucidum</i>
LVSYYKKLKKKKKKNLL	<i>Plasmodium falciparum</i>
LWKIVRRILKKITITI	<i>Orcinus orca</i>
LWKTVWRVLKKLKI	<i>Canis lupus familiaris</i>
LWLLHRLHLVWQRR	<i>Helianthus anuus</i>
LWRRVRGIRRWVRGIR	<i>Ganoderma lucidum</i>
LWRWGRCCRWMMWRRRK	<i>Helianthus anuus</i>
LWWPADRLGPVLRWWK	<i>Ganoderma lucidum</i>
LWWWWWGWGWLEFIRRL	<i>Helianthus anuus</i>
LYAVKKIKKKKKKEKK	<i>Helianthus anuus</i>
LYIKKIKTLLLNWKK	<i>Panthera tigris altaica</i>

LYIKKSLRLHLKLLK	<i>Canis lupus familiaris</i>
LYIPVYHRWHRRMKRL	<i>Helianthus anuus</i>
MFLYLKKKKKLLKIW	<i>Panthera tigris altaica</i>
MIFYKKKKKIIRKIIF	<i>Gallus gallus</i>
MIICFKKKKKKKSML	<i>Crocodylus porosus</i>
MKIMKMMMKKIMKMMKK	<i>Helianthus anuus</i>
MKKKKIFLWHLPTALK	<i>Falco peregrinus</i>
MKKVKKKKKKKNIVFFF	<i>Canis lupus familiaris</i>
MKKYYLWLFIKWLVF	<i>Danaus plexippus plexippus</i>
MLKKLKKIVWEKKLK	<i>Panthera tigris altaica</i>
MLKKKKKKLKKIQTF	<i>Crocodylus porosus</i>
MLKNFKKILKKWKNKK	<i>Helianthus anuus</i>
MLLFFFFPFIGKLS	<i>Falco peregrinus</i>
MRILFLKFLFFIKK	<i>Panthera tigris altaica</i>
MSVFAKKKKKKKKKK	<i>Canis lupus familiaris</i>
MWLKLLKLLKFRLLK	<i>Helianthus anuus</i>
NFKKKKKKKLSALLK	<i>Rhincodon typus</i>
NFMKKVIIIIDIKIKV	<i>Plasmodium falciparum</i>
NGNFKSRSPWGWPKLA	<i>Galdieria sulphuraria</i>
NIINPLIKKLMKWIII	<i>Plasmodium falciparum</i>
NILLASLLTKEVLKVW	<i>Rhincodon typus</i>
NIYIYKKKKKKKKKYI	<i>Danaus plexippus plexippus</i>
NKIKMLYMYVGKQCIK	<i>Gallus gallus</i>
NKKLKIINFYTKHKRY	<i>Metallosphaera sedula</i>
NLKKKFKKRKKFEKL	<i>Panthera tigris altaica</i>
NLWKLRRNFVCMHLR	<i>Talitrus saltator</i>
NVKKKKKKIFFHLLL	<i>Gallus gallus</i>
NVKVKVKVKVKVKVI	<i>Helianthus anuus</i>
NYRQEFKKKKKKFILI	<i>Canis lupus lupus</i>
PARRAPRRRLCLRPLA	<i>Burkholderia pseudomallei</i>
PIIKTHLKYKLLKV	<i>Panthera tigris altaica</i>
PIPFASPVFLSPSHL	<i>Falco peregrinus</i>
PLLAQINKILKKNLKK	<i>Canis lupus familiaris</i>
PLPRRRRRRLQRPGLS	<i>Burkholderia pseudomallei</i>
PLVAPRRARRAAKLA	<i>Ganoderma lucidum</i>
PPKKKKIKLIPVIKI	<i>Gallus gallus</i>
PPPFPPKKKKIFLKK	<i>Panthera tigris altaica</i>
PPPSTALTATNMKAFF	<i>Talitrus saltator</i>
PRGRRRWPLFLLPHRL	<i>Ganoderma lucidum</i>
PRRRRLLCLRSSCGK	<i>Metallosphaera sedula</i>
PRVPAPVPAGRARPRV	<i>Ganoderma lucidum</i>
PSKPPRSRKRPRGRR	<i>Burkholderia pseudomallei</i>
PSLWWKWLVGVFLRKL	<i>Rhincodon typus</i>
PWRCWRHPPRSKDPWG	<i>Canis lupus lupus</i>
QKGLQLLLCHRWWLRR	<i>Falco peregrinus</i>

QKLRSCLLKWKKKLVK	<i>Gallus gallus</i>
QLWWRKPFWKWWLWLR	<i>Crocodylus porosus</i>
QMLLKKRKRLTIKIL	<i>Rhincodon typus</i>
QVMAVKKKKKKKKKIL	<i>Crocodylus porosus</i>
QWWIWKLLIIAKRGRL	<i>Helianthus anuus</i>
RAAPRPAIGRPPRPYG	<i>Takifugu rubripes</i>
RAAPVPRRRRPLPVP	<i>Takifugu rubripes</i>
RAFFGWIRRRARAAAR	<i>Burkholderia pseudomallei</i>
RAIARRGGGTCRAARP	<i>Burkholderia pseudomallei</i>
RALFFVKIKIKIKRL	<i>Danaus plexippus plexippus</i>
RAPGAKKKKKKKKKK	<i>Canis lupus lupus</i>
RARARRRRRSLPLLRP	<i>Ganoderma lucidum</i>
RARCWFRWVRRRFWVR	<i>Gallus gallus</i>
RAVAACSRRRRRRRRI	<i>Canis lupus lupus</i>
RAVRARRARRPSCSRA	<i>Burkholderia pseudomallei</i>
RAWAAAWQRLWWWLWR	<i>Ophiophagus hannah</i>
RAWRRGSSLRPGRIWL	<i>Canis lupus lupus</i>
RCLLRENYKKYRYMK	<i>Danaus plexippus plexippus</i>
RCNRKFELLKILKKKF	<i>Crocodylus porosus</i>
RFARVRRARCRPARR	<i>Burkholderia pseudomallei</i>
RFKKFFIFFERLYKK	<i>Panthera tigris altaica</i>
RFKKYFLKLKNSVIF	<i>Mesobuthus martensii</i>
RFKTIYKFFIKIKKKK	<i>Helianthus anuus</i>
RFPLFFFFLKKIGFL	<i>Gallus gallus</i>
RFRKCWRMMRWIRRLV	<i>Rhincodon typus</i>
RFRVWVCFRIRVRVFI	<i>Gallus gallus</i>
RFSILICWRLWLKKKR	<i>Panthera tigris altaica</i>
RFVVKVHRKEKMKIHK	<i>Talitrus saltator</i>
RFVYVRRWWRLVYVR	<i>Helianthus anuus</i>
RGAAARRSRPPPAR	<i>Burkholderia pseudomallei</i>
RGARRGSRRRRRRRCR	<i>Ganoderma lucidum</i>
RGLRRVMRRLVRKLVG	<i>Helianthus anuus</i>
RGNFKPKKKNRPKKIF	<i>Talitrus saltator</i>
RGNGRTARLLPATRK	<i>Ganoderma lucidum</i>
RGPCQRRRRRRCRSCL	<i>Ganoderma lucidum</i>
RGRFRVLVRVRQFRV	<i>Orcinus orca</i>
RGRRVRRTRVVRGGR	<i>Ganoderma lucidum</i>
RGSLTGPPPCRLPLL	<i>Canis lupus lupus</i>
RGVRSVRPVLRPVAR	<i>Danaus plexippus plexippus</i>
RHSYYPASCCPHLPRI	<i>Talitrus saltator</i>
RIAKCRSKTRCVFCFL	<i>Panthera tigris altaica</i>
RILLRSRLKLVSLFKL	<i>Danaus plexippus plexippus</i>
RIQCFCKVLKRILHHW	<i>Ophiophagus hannah</i>
RIREGRRWWRWWWWRR	<i>Gallus gallus</i>
RIRKKVKLILFKLIKK	<i>Gallus gallus</i>

RIRLKKFKDRMLKYRF	<i>Danaus plexippus plexippus</i>
RISVSKFWKRLKLV	<i>Talitrus saltator</i>
RIVRWLRLVIKSKIFR	<i>Talitrus saltator</i>
RIYLINKVLWRNILVF	<i>Talitrus saltator</i>
RKAKITLIFKNGKKEN	<i>Falco peregrinus</i>
RKCRRMMRWIRRLGVV	<i>Rhincodon typus</i>
RKHFKLKPLINKLIRK	<i>Gallus gallus</i>
RKIFKIFCFITLAIKK	<i>Danaus plexippus plexippus</i>
RKIIKRIFKKPSFVKK	<i>Helianthus anuus</i>
RKISKFHLLAKAGKF	<i>Canis lupus familiaris</i>
RKKERKKERKKRKKK	<i>Canis lupus lupus</i>
RKKTQLQKKKKKTLR	<i>Canis lupus lupus</i>
RKKVKRRRNKLNKK	<i>Metallosphaera sedula</i>
RKLFKILKDVLFKIKI	<i>Rhincodon typus</i>
RKLFKRIWKSIIKCLV	<i>Gallus gallus</i>
RKLKAQHXYFQCCGKK	<i>Orcinus orca</i>
RKLKASLKKKKKIRL	<i>Talitrus saltator</i>
RKLKKLLKKNLWKT	<i>Talitrus saltator</i>
RKLRRKHFFKNIFKRL	<i>Falco peregrinus</i>
RKLYRYRRRRPSVYFL	<i>Takifugu rubripes</i>
RKRFFINKKKKKKKK	<i>Plasmodium falciparum</i>
RLFKIKRLKLLRLLK	<i>Helianthus anuus</i>
RLFKMRLKLLRNLLK	<i>Helianthus anuus</i>
RLFRKKRLKLLRLLK	<i>Helianthus anuus</i>
RLFWRLLRYVTNLIFL	<i>Ophiophagus hannah</i>
RLGRAGCYCPLWSCWL	<i>Rhincodon typus</i>
RLHCRCCRLRRRSL	<i>Gallus gallus</i>
RLIFKKKKKCLLLL	<i>Rhincodon typus</i>
RLKIVLKKSLKVWKKW	<i>Rhincodon typus</i>
RLKKIWRVFKASWCAA	<i>Takifugu rubripes</i>
RLKSRLRIKFLKLL	<i>Rhincodon typus</i>
RLLKLLQILLKKLL	<i>Crocodylus porosus</i>
RLLRGLRRRRRGLLG	<i>Orcinus orca</i>
RLMGIRLRRSWWLRL	<i>Ophiophagus hannah</i>
RLPRPRLAPLLKSAS	<i>Ganoderma lucidum</i>
RLRLHRNAKRRRPRAP	<i>Ganoderma lucidum</i>
RLRLRSWLRLRLRPRL	<i>Ophiophagus hannah</i>
RLRRRRRRRARTRRAR	<i>Ganoderma lucidum</i>
RLRRVRAIRRRSCPRR	<i>Burkholderia pseudomallei</i>
RLSAARACRCRRLRRR	<i>Burkholderia pseudomallei</i>
RLSRSPPLLLLLLRR	<i>Takifugu rubripes</i>
RLTGRLRVRRARGGG	<i>Ganoderma lucidum</i>
RLVYVRWWWRLVDVR	<i>Helianthus anuus</i>
RLVYVRWWWRLVDIR	<i>Helianthus anuus</i>
RLWERDLLRCLWRWRA	<i>Metallosphaera sedula</i>

RLWWQWLWWRWWLRWR	<i>Helianthus anuus</i>
RLYFYIKFKKIIKKLK	<i>Panthera tigris altaica</i>
RPKCWIKLKYFTKFRC	<i>Mesobuthus martensii</i>
RPKVRVKIWVKARVKV	<i>Talitrus saltator</i>
RPLLRRRLQSRRWRRR	<i>Ganoderma lucidum</i>
RPPSFYRRRCRRPLL	<i>Bacillus anthracis</i>
RPRPLPRRRRRRAPK	<i>Burkholderia pseudomallei</i>
RPRRRRVRRVRGGRR	<i>Ganoderma lucidum</i>
RPSARLYPRAPIRGGV	<i>Metallosphaera sedula</i>
RPWWKIWRRSLEWILW	<i>Metallosphaera sedula</i>
RAAMRTTCSRSSRRR	<i>Burkholderia pseudomallei</i>
RAASRGRRAGRRRPR	<i>Burkholderia pseudomallei</i>
RAERARRRRRRGRRR	<i>Burkholderia pseudomallei</i>
RRAPRRAPRRAPRRAP	<i>Takifugu rubripes</i>
RRARAGAASRGWGRVR	<i>Canis lupus lupus</i>
RRARRAIRLRRSSRP	<i>Burkholderia pseudomallei</i>
RRASCRPRRRRRWPRR	<i>Burkholderia pseudomallei</i>
RRATRRRRRLRRRRRA	<i>Burkholderia pseudomallei</i>
RREYKIFLKLILRKL	<i>Rhincodon typus</i>
RRFLAKSRRWRVRRVV	<i>Ganoderma lucidum</i>
RRFPARRPRRPRRSAR	<i>Burkholderia pseudomallei</i>
RRFRRPWWWRYYRWVLW	<i>Danaus plexippus plexippus</i>
RRGAPPRTLGHRLFAR	<i>Burkholderia pseudomallei</i>
RRGKPRRPKPSRALGR	<i>Ganoderma lucidum</i>
RRGRRCPAGFRRRPPR	<i>Takifugu rubripes</i>
RRGVRWGGMRCGLGWI	<i>Helianthus anuus</i>
RRHLKVLSHSIFKSRI	<i>Canis lupus lupus</i>
RRIPKVRPPRRRVPR	<i>Ganoderma lucidum</i>
RRKILRPRVRSCDALV	<i>Talitrus saltator</i>
RRKSFIWGWWERLFKG	<i>Gallus gallus</i>
RRLKAGRRSLRAPSLR	<i>Takifugu rubripes</i>
RRLKIKTFKKIFSLLK	<i>Canis lupus familiaris</i>
RRLPPRRLHLLVLHRP	<i>Ganoderma lucidum</i>
RRLPREPARARRRVA	<i>Ganoderma lucidum</i>
RRLRRSWRRRRWRWWR	<i>Gallus gallus</i>
RRLTPYFATRLSRLQK	<i>Metallosphaera sedula</i>
RRPRLRRRPRQVRGAR	<i>Burkholderia pseudomallei</i>
RRPTPRRSRRRWRRAC	<i>Burkholderia pseudomallei</i>
RRRAARSPRLPARPRA	<i>Burkholderia pseudomallei</i>
RRRAASRRPRRPRPRR	<i>Burkholderia pseudomallei</i>
RRRACRKPRRTRCSRR	<i>Ganoderma lucidum</i>
RRRAHLSRRRPILGS	<i>Ganoderma lucidum</i>
RRRAIISAACAAFRGL	<i>Takifugu rubripes</i>
RRRARADRRRVARRL	<i>Burkholderia pseudomallei</i>
RRFRFRERAFSARLWV	<i>Helianthus anuus</i>

RRRKRWIWWQRWWQRV	<i>Gallus gallus</i>
RRRKRWWWWWCWWWN	<i>Falco peregrinus</i>
RRRPARCRRGRPRRTA	<i>Burkholderia pseudomallei</i>
RRRPKKRRRPTRPAPL	<i>Burkholderia pseudomallei</i>
RRRPPLHSRTPLALRV	<i>Metallosphaera sedula</i>
RRRPRNSRKGACILPR	<i>Ganoderma lucidum</i>
RRRPRRSPQAHHLCKL	<i>Takifugu rubripes</i>
RRRRCRARRARPGGG	<i>Ganoderma lucidum</i>
RRRRKRRRRGTWASRL	<i>Ganoderma lucidum</i>
RRRRRLMRRWKRRRL	<i>Ganoderma lucidum</i>
RRRRRSARSMPPRRRCR	<i>Ganoderma lucidum</i>
RRRSSGCPRRRACGCA	<i>Burkholderia pseudomallei</i>
RRRTRAAHRRARRRAA	<i>Ganoderma lucidum</i>
RRRWPRMVRYRRSWSE	<i>Ganoderma lucidum</i>
RRSPALRARRCRRTAR	<i>Burkholderia pseudomallei</i>
RRSPLRLRRRPRSPRR	<i>Orcinus orca</i>
RRSPRRPCRRRAPAAR	<i>Talitrus saltator</i>
RRSRAWRRKYRRKWRG	<i>Ganoderma lucidum</i>
RRSRRSAGSRAAGRLR	<i>Burkholderia pseudomallei</i>
RRVPAARGGSTAARPR	<i>Burkholderia pseudomallei</i>
RRVRVRVQLLRRARA	<i>Ganoderma lucidum</i>
RRWRKWRRIICWMQRL	<i>Rhincodon typus</i>
RRWRTRRCGIRARRSG	<i>Burkholderia pseudomallei</i>
RRWSRFFKVVCCCCWW	<i>Helianthus annuus</i>
RRWTVKLLIRLILELR	<i>Rhincodon typus</i>
RRWWRCISRLGWRRWR	<i>Burkholderia pseudomallei</i>
RSFRVRRRVRVVVFDR	<i>Ganoderma lucidum</i>
RSLIWLYWKKKKNLLV	<i>Panthera tigris altaica</i>
RSPSSGRPRPTGRKR	<i>Burkholderia pseudomallei</i>
RTPIWRRWYWGRRSTS	<i>Ganoderma lucidum</i>
RVDCCRRVRRRFARIR	<i>Takifugu rubripes</i>
RVINCRGAGRFIVLGL	<i>Ophiophagus hannah</i>
RVKVKIKVRVRIKIL	<i>Rhincodon typus</i>
RVLMFCSILFRIFLPS	<i>Ophiophagus hannah</i>
RVLVSRPLVLAIFYPLF	<i>Metallosphaera sedula</i>
RVRFRGWGRFRVRVRV	<i>Orcinus orca</i>
RVRIRVKVRVRVKVLG	<i>Takifugu rubripes</i>
RVRVGVRVRRVRVRVR	<i>Takifugu rubripes</i>
RVRVKVKVRVRVKIRL	<i>Rhincodon typus</i>
RVRVRRLVGIRVRGR	<i>Takifugu rubripes</i>
RVRVRVRVRVGVRVRV	<i>Takifugu rubripes</i>
RVVVRGRVRVRGRGRG	<i>Takifugu rubripes</i>
RWPHRPLRLPLPLR	<i>Talitrus saltator</i>
RWRKWWRMIRWMQRLV	<i>Rhincodon typus</i>
RWRPAWSAARRAGARR	<i>Burkholderia pseudomallei</i>

RWSFCHWRWWWWRRGK	<i>Rhincodon typus</i>
RWWWRMHRWWWRVHI	<i>Helianthus anuus</i>
RYFSRLLLVLKKKKPL	<i>Gallus gallus</i>
SFFICRFKHFRKKKKK	<i>Takifugu rubripes</i>
SGSKGKLVRVRVRGL	<i>Ganoderma lucidum</i>
SILTSLVIKKKKKKK	<i>Plasmodium falciparum</i>
SKINVKKKKLKKKWLK	<i>Panthera tigris altaica</i>
SKISFLVFKKIIKKL	<i>Gallus gallus</i>
SKKKKKKVFNGALLRK	<i>Gallus gallus</i>
SKNIPGFDIKVKINL	<i>Danaus plexippus plexippus</i>
SKVFKKKKKKKKKKV	<i>Canis lupus familiaris</i>
SLICCKKKKKKKKIV	<i>Helianthus anuus</i>
SLILKAVLKKRRKKKK	<i>Orcinus orca</i>
SLKKKKKKKKKIWLFL	<i>Gallus gallus</i>
SLKKVIIKILKNFFKS	<i>Rhincodon typus</i>
SLKNKKKINMKKMFKK	<i>Panthera tigris altaica</i>
SMLMRRRRRRRKMIIA	<i>Galdieria sulphuraria</i>
SRCVWKSRRQRRLRG	<i>Ophiophagus hannah</i>
SRFRHLKKLKLRLNIL	<i>Crocodylus porosus</i>
SRLKRRRRKRRFGQRR	<i>Ganoderma lucidum</i>
SRLRRLMTKLLKLLK	<i>Helianthus anuus</i>
SRPQPRPRPRPQPKPR	<i>Takifugu rubripes</i>
SRWKWLRVSKWLWAWK	<i>Rhincodon typus</i>
STALVKKIWRWWWIAS	<i>Gallus gallus</i>
SYLSARIKKKKIILKK	<i>Mesobuthus martensii</i>
TCKKNLIFKLLKIWK	<i>Mesobuthus martensii</i>
THWKNIILRKAMKNLKK	<i>Falco peregrinus</i>
TKRRYRAIKNFFRKAL	<i>Danaus plexippus plexippus</i>
TLKLFACKKKKKKKKL	<i>Helianthus anuus</i>
TLSLKNKINIKFKKKK	<i>Panthera tigris altaica</i>
TLVKRAKKIFFFFFKK	<i>Gallus gallus</i>
TPRLKAIYLLKLLKKK	<i>Orcinus orca</i>
TSAKKKKKRFCVHILR	<i>Talitrus saltator</i>
TTKPKVKKKIKKIIK	<i>Takifugu rubripes</i>
TVAGCPWRWCPWRRPR	<i>Gallus gallus</i>
VAGKPAPVAGKPAPFA	<i>Talitrus saltator</i>
VCLWLPLRWKKWLGRK	<i>Talitrus saltator</i>
VCVCVLKINKHLKLLK	<i>Panthera tigris altaica</i>
VFFWRAFLHKMLKKFR	<i>Rhincodon typus</i>
VFFYRWFKLRKFQFVK	<i>Talitrus saltator</i>
VFKCGVSCLLPRRPCK	<i>Panthera tigris altaica</i>
VFKKIKLLKKKKKKR	<i>Ophiophagus hannah</i>
VFKKNLKLKPKLETI	<i>Ophiophagus hannah</i>
VFLKFVSRSLKLLLK	<i>Talitrus saltator</i>
VGLAITLIHLGKNKKA	<i>Ophiophagus hannah</i>

VGWRWRWRWRWRWRWR	<i>Canis lupus familiaris</i>
VIWRKILGKWNISLGS	<i>Gallus gallus</i>
VKKFIGVKKSLLALKK	<i>Talitrus saltator</i>
VKKICAKNAFIKKIHK	<i>Rhincodon typus</i>
VKKIILKKTFTKPIKK	<i>Talitrus saltator</i>
VKKKIILKNIFKIIIL	<i>Mesobuthus martensii</i>
VKKKKKKKKRFFDILL	<i>Gallus gallus</i>
VKRINKIKILIKNLVK	<i>Mesobuthus martensii</i>
VKSLLITKTLKFRYHV	<i>Rhincodon typus</i>
VKVVKVKVKVKVKVKVK	<i>Ophiophagus hannah</i>
VLELLKKWMLLPKKLK	<i>Rhincodon typus</i>
VLEWKKKKKKKKKKIHK	<i>Rhincodon typus</i>
VLFIQKLASKVFKKVL	<i>Crocodylus porosus</i>
VLFLKKKKKKQLMKK	<i>Gallus gallus</i>
VLIVACGRIPPIIKLA	<i>Plasmodium falciparum</i>
VLKLWKKWSALLAVLK	<i>Gallus gallus</i>
VLLSKLLPKIRRRQR	<i>Gallus gallus</i>
VLRWRRVHGRWRRHIR	<i>Takifugu rubripes</i>
VNQLKKCFLLLLKKLK	<i>Helianthus annuus</i>
VPSTILRTVLLCAISK	<i>Falco peregrinus</i>
VRAGRRLRRRSAARA	<i>Burkholderia pseudomallei</i>
VRRRRARRRGVGVSPV	<i>Takifugu rubripes</i>
VRRWRRRTVPEVRRR	<i>Takifugu rubripes</i>
VRWSRRHAACLSLLAR	<i>Rhincodon typus</i>
VVLICSLKIFKKCFI	<i>Panthera tigris altaica</i>
VWVRLRRVRVRIRLL	<i>Takifugu rubripes</i>
VYLCIYPHPLKKKKKK	<i>Panthera tigris altaica</i>
WCPGWRRWRRRIPTRG	<i>Ophiophagus hannah</i>
WFWRIIWRWLRLWIR	<i>Ophiophagus hannah</i>
WGARRWWGKRKIIKH	<i>Takifugu rubripes</i>
WGRVIWWRWWWGRVMR	<i>Helianthus annuus</i>
WHRAGLLAFPSPLHWR	<i>Falco peregrinus</i>
WILLRVRIKGGLLLRL	<i>Panthera tigris altaica</i>
WIRRLVRWYVRMRGIL	<i>Rhincodon typus</i>
WIWIWIWIWVRRRG	<i>Panthera tigris altaica</i>
WKIKVKIKVKIKVIHK	<i>Plasmodium falciparum</i>
WKLPWKRLKLKLRLK	<i>Ophiophagus hannah</i>
WKWIVKWFKLLEKLKK	<i>Rhincodon typus</i>
WKWLFWFLKKISFLQS	<i>Takifugu rubripes</i>
WLMPNPWRLLRRGWIR	<i>Gallus gallus</i>
WLRRMWWASRRWRRVW	<i>Takifugu rubripes</i>
WLVRREKAGKRRRLRK	<i>Ganoderma lucidum</i>
WRKWWRMIHWMRRLVG	<i>Rhincodon typus</i>
WRRRWRCWRQRWRWW	<i>Takifugu rubripes</i>
WRS AFLTFRILNWK	<i>Talitrus saltator</i>

WRTWWRTWWRCSSWWRI	<i>Takifugu rubripes</i>
WRVLQWKRRCWGRRCR	<i>Ganoderma lucidum</i>
WRWKPRMKHWWKRIWR	<i>Gallus gallus</i>
WRWLRLAHRLWTWKK	<i>Panthera tigris altaica</i>
WRWLWFRWRWLWFRWR	<i>Helianthus anuus</i>
WRWRWMRRRDCGSSWG	<i>Ganoderma lucidum</i>
WRWWWWWLRRWCWLEK	<i>Helianthus anuus</i>
WRWWWWWRVVAVLWRW	<i>Helianthus anuus</i>
WSIRWHRLFIFNPNS	<i>Helianthus anuus</i>
WSKSFLKKKVFFLLKK	<i>Canis lupus familiaris</i>
WTGRWPGWIWSPWPRK	<i>Panthera tigris altaica</i>
WVPLFKKKKKKKSKL	<i>Helianthus anuus</i>
WWQWRKLMPWGWWRRI	<i>Gallus gallus</i>
WWRWWWWRRRRARPL	<i>Gallus gallus</i>
WWWWRRLMVVVVRWR	<i>Helianthus anuus</i>
WWWWWWWRWRLRWRRR	<i>Helianthus anuus</i>
WYVRRWRRRRGLRGRG	<i>Ganoderma lucidum</i>
YCVKVCLCIGKKKKKK	<i>Panthera tigris altaica</i>
YFFFFFKRRRRRRTP	<i>Canis lupus lupus</i>
YFIKAQNSVKKRKIFF	<i>Ophiophagus hannah</i>
YFKRFFKLAKKKISRG	<i>Gallus gallus</i>
YIVKIISTAVCKIYIK	<i>Ophiophagus hannah</i>
YKEIKLKKLKKIKK	<i>Canis lupus familiaris</i>
YKKIKFLHLLRIAIIK	<i>Crocodylus porosus</i>
YLLCYKKKKKKKKKN	<i>Gallus gallus</i>
YLMSVFYKKKKKKKKK	<i>Helianthus anuus</i>
YNYKYVKILKKNLMK	<i>Helianthus anuus</i>
YRAVRYIEILKKNLKK	<i>Gallus gallus</i>
YWRKKKKILKLLKIG	<i>Mesobuthus martensii</i>
YYKKKKRLKKAIIKSL	<i>Canis lupus lupus</i>
FKKIHKFLNKLMYKLWK	<i>Mesobuthus martensii</i>
IIIIIIIIIVRIIRKI	<i>Plasmodium falciparum</i>
IIIIIRMIILMIKRIT	<i>Plasmodium falciparum</i>
IILYIIIIKLIKNLII	<i>Plasmodium falciparum</i>
ILIIIIIIIIKIKKN	<i>Plasmodium falciparum</i>
KFRKIYKFLKKARKAKL	<i>Mesobuthus martensii</i>
KIKTIIIIIIIRVIKK	<i>Plasmodium falciparum</i>
KILRIRKIYFIFHIIKK	<i>Mesobuthus martensii</i>
KKISLLIKILVNKIIKK	<i>Mesobuthus martensii</i>
MKKLYIKKKKKKKSCTL	<i>Canis lupus familiaris</i>
MVKKKKKKKKKFFVAIYL	<i>Canis lupus familiaris</i>
FFRFRFRFRWKFRISNRF	<i>Mesobuthus martensii</i>
FVYKFFMKKKKIRKRGKI	<i>Mesobuthus martensii</i>
KIIKFRYKFLKIIKKLI	<i>Mesobuthus martensii</i>
KKKKKKKNILKLYKVFFL	<i>Mesobuthus martensii</i>

LLKKKKKKKKKYQLIFF	<i>Mesobuthus martensii</i>
MLYLKKKKKKKIIFCK	<i>Canis lupus familiaris</i>
RIVFIKLNQIYFLRNLV	<i>Metallosphaera sedula</i>
FKKFFKKIFNIGILSIKFF	<i>Mesobuthus martensii</i>
MAVFKIICKKKKKLKKEVK	<i>Canis lupus familiaris</i>
IIVRKIIRKYKYFLIIFRKI	<i>Mesobuthus martensii</i>
AEKVLFLKILRLMR	<i>Ophiophagus hannah</i>
AKKKKKKNLLFMKW	<i>Orcinus orca</i>
AKKVSKRLQKIQLF	<i>Falco peregrinus</i>
CKKILVNYVRRIN	<i>Danaus plexippus plexippus</i>
CKKKFKTVKKFKNV	<i>Helianthus anuus</i>
CKYFIKKKKKLMK	<i>Orcinus orca</i>
CRLIVSLKGCKIIS	<i>Panthera tigris altaica</i>
CWCTRKLICVPIKH	<i>Danaus plexippus plexippus</i>
FAKIFAKKFAKKEL	<i>Gallus gallus</i>
FASFFVWHLKKIWK	<i>Gallus gallus</i>
FFAMIVKRITAVIK	<i>Ophiophagus hannah</i>
FGAILKALKIVLKK	<i>Rhincodon typus</i>
FHSLPKLISSPLF	<i>Falco peregrinus</i>
FILAWWGKIILYAF	<i>Crocodylus porosus</i>
FILKILKKKKKLF	<i>Gallus gallus</i>
FKIFKRKTFRYKIF	<i>Talitrus saltator</i>
FKKKKKKKKTIAL	<i>Helianthus anuus</i>
FKNITVKLLQMKLK	<i>Danaus plexippus plexippus</i>
FKSLNKTCFHTILK	<i>Ophiophagus hannah</i>
FLFILYWKKKKKK	<i>Gallus gallus</i>
FLKAFRVFKHRFR	<i>Crocodylus porosus</i>
FLKAFNKVLHKMLK	<i>Rhincodon typus</i>
FLKKKKKKKLLILI	<i>Gallus gallus</i>
FLKKVARFKIFPKI	<i>Gallus gallus</i>
FLLFKKKKKKEPLL	<i>Ophiophagus hannah</i>
FLYVKMKKKKKKK	<i>Helianthus anuus</i>
FNKKCCHGRYIILF	<i>Danaus plexippus plexippus</i>
FQWKLLCPYQRNIR	<i>Falco peregrinus</i>
FRFKKHYYKKFKLL	<i>Mesobuthus martensii</i>
FRKLRLMKKIFRN	<i>Rhincodon typus</i>
FRRLRISIIRLKIL	<i>Danaus plexippus plexippus</i>
FRVIKIFRVIFRY	<i>Mesobuthus martensii</i>
GKLWFMYFRRKKKI	<i>Gallus gallus</i>
GMLRLLKLLHRKI	<i>Helianthus anuus</i>
GNLSIFFLLIKRFF	<i>Ophiophagus hannah</i>
GVFKKIIKKIIQK	<i>Helianthus anuus</i>
HILFLRLYIGRKTV	<i>Ophiophagus hannah</i>
HWRWWSWKWAKRHR	<i>Takifugu rubripes</i>
IFNKIKKKKNRIFQ	<i>Mesobuthus martensii</i>

IIIRYKIVIKRITI	<i>Helianthus anuus</i>
IIITIITILLKNSL	<i>Mesobuthus martensii</i>
IIIVKGLMKILPFC	<i>Mesobuthus martensii</i>
IIKPPLLLKKNLKK	<i>Canis lupus familiaris</i>
IIKRKIKKVFKKIF	<i>Helianthus anuus</i>
IIPFIILIKLSLLY	<i>Crocodylus porosus</i>
IKIKVKIKNKKILL	<i>Helianthus anuus</i>
IKKEFKKIFLQLKK	<i>Mesobuthus martensii</i>
IKKFFKKVKKIPLL	<i>Takifugu rubripes</i>
IKKIYKKIKIKKKI	<i>Canis lupus familiaris</i>
IKKKKVFLAFLIF	<i>Gallus gallus</i>
IKKQVKIIVKKIHK	<i>Helianthus anuus</i>
IKLKVSLKLPFKLK	<i>Metallosphaera sedula</i>
ILKLIINKIVKWIK	<i>Ophiophagus hannah</i>
ILLILRKKPSKNI	<i>Rhincodon typus</i>
INQIIKDILIKNI	<i>Ophiophagus hannah</i>
INWKKIHKINLMCT	<i>Crocodylus porosus</i>
IPQLTKFLHLWKSF	<i>Danaus plexippus plexippus</i>
IQLLALTQIRKWR	<i>Canis lupus lupus</i>
ISTHLLLLGKLLKI	<i>Gallus gallus</i>
IVKSIFLLKIKTKI	<i>Ophiophagus hannah</i>
IWIFKWVWIRWILR	<i>Ophiophagus hannah</i>
IYSFKKKKKIKKKK	<i>Canis lupus familiaris</i>
KFVKKYKTKPLLI	<i>Rhincodon typus</i>
KHYFSQLKKLKKLL	<i>Ophiophagus hannah</i>
KIDKKKLFFLLLLL	<i>Mesobuthus martensii</i>
KIFKKIILFKKKK	<i>Canis lupus familiaris</i>
KIFKKRLKKVIKKI	<i>Helianthus anuus</i>
KIFLFYKKKKKIFI	<i>Mesobuthus martensii</i>
KIKKILIIILNTYK	<i>Danaus plexippus plexippus</i>
KILKKIKKILKNKK	<i>Helianthus anuus</i>
KINKCKKKKIFKKP	<i>Panthera tigris altaica</i>
KIVMFLIKKKKKKR	<i>Gallus gallus</i>
KIYKKYMKMMKKRF	<i>Mesobuthus martensii</i>
KKIIIVKKIVFKNI	<i>Canis lupus familiaris</i>
KKIKKLHRVFKKIS	<i>Helianthus anuus</i>
KKKKERKRKKEKKK	<i>Canis lupus lupus</i>
KKKKIFFGIFFGFF	<i>Helianthus anuus</i>
KKKKIRLLATFFFF	<i>Crocodylus porosus</i>
KKKKKICLFLAFLF	<i>Gallus gallus</i>
KKKKKKAAAKCYFA	<i>Gallus gallus</i>
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KKKKKKVFLFFFIF	<i>Gallus gallus</i>
KKKMFLLVKFLFLF	<i>Gallus gallus</i>
KKLAIFIKNFRKVF	<i>Talitrus saltator</i>

KKLKKLKS FHLK	<i>Helianthus anuus</i>
KLLRILIKLLRIIL	<i>Canis lupus lupus</i>
KLRRLKALKKLLKL	<i>Canis lupus familiaris</i>
KMLENPLVKKLLKI	<i>Falco peregrinus</i>
KPFFKKKKIKKKIV	<i>Helianthus anuus</i>
KRRLGWLLAFFFFF	<i>Gallus gallus</i>
KSKKLLQLLFFVFF	<i>Ophiophagus hannah</i>
KVFFKMFFKKKKNS	<i>Crocodylus porosus</i>
KVLKFLIKWNKLKI	<i>Talitrus saltator</i>
KWKKLKKKKLFYHL	<i>Mesobuthus martensii</i>
KYFRWKFKFGIKYF	<i>Helianthus anuus</i>
LFLLRRKIRKTRK	<i>Helianthus anuus</i>
LFKKKKKKAWKLF	<i>Gallus gallus</i>
LFMKKKSLLKKK	<i>Bacillus anthracis</i>
LFMLLRRRIWRIC	<i>Helianthus anuus</i>
LFNLILLKKLGN	<i>Danaus plexippus plexippus</i>
LFRNIFKKNIIF	<i>Danaus plexippus plexippus</i>
LFRPTLKLVGK	<i>Orcinus orca</i>
LFSKLLFSLNKF	<i>Danaus plexippus plexippus</i>
LGAVTTFLWLGRAL	<i>Crocodylus porosus</i>
LIAIENILKKKK	<i>Falco peregrinus</i>
LIAKLIYMFCISK	<i>Panthera tigris altaica</i>
LIFKKKKKNFFI	<i>Canis lupus lupus</i>
LIFLLKVRKRKLK	<i>Orcinus orca</i>
LIKRVKAIKKFFKK	<i>Gallus gallus</i>
LILKKEMLKLIKW	<i>Mesobuthus martensii</i>
LILKKIVKKKKKKI	<i>Canis lupus familiaris</i>
LILKLILKLLLLL	<i>Danaus plexippus plexippus</i>
LISLSKLYCLVLKK	<i>Panthera tigris altaica</i>
LIVLLAIVSWIKRK	<i>Takifugu rubripes</i>
LKAVLRLKKWLKI	<i>Panthera tigris altaica</i>
LKINFFIKKKKKIG	<i>Helianthus anuus</i>
LKKKKKKKADKLFL	<i>Gallus gallus</i>
LKKKVKKLFKNFFK	<i>Helianthus anuus</i>
LKKLKKIWKKDFKK	<i>Ophiophagus hannah</i>
LKKLLKISAVNKLL	<i>Mesobuthus martensii</i>
LKMFKKLLMFLFLL	<i>Crocodylus porosus</i>
LKSFSRVIFQLLKK	<i>Canis lupus familiaris</i>
LKYM CWVFFVFFIA	<i>Danaus plexippus plexippus</i>
LLDLLLFKKKKKKR	<i>Crocodylus porosus</i>
LLEKVLRAAARAVL	<i>Falco peregrinus</i>
LLFKFYHLHLLKKK	<i>Talitrus saltator</i>
LLLLLLLLKIIKR	<i>Gallus gallus</i>
LLKKIIQRFLKYNL	<i>Danaus plexippus plexippus</i>
LLLLSFICLKKKKK	<i>Gallus gallus</i>

LLLRCPVFWFYLF	<i>Orcinus orca</i>
LLPLKFKKHLKKLK	<i>Helianthus anuus</i>
LLPLKRKKLKKKLF	<i>Crocodylus porosus</i>
LLRRLRLILIKILM	<i>Canis lupus familiaris</i>
LLSFFFFFFFRRR	<i>Gallus gallus</i>
LLSKIIIIIIKKK	<i>Gallus gallus</i>
LLTSVFKKLKKLLK	<i>Panthera tigris altaica</i>
LMKKIILKKLLKV	<i>Mesobuthus martensii</i>
LMLTYFTLKKRKKR	<i>Bacillus anthracis</i>
LNMLKKLLKKVQLL	<i>Mesobuthus martensii</i>
LRKFIILKKLLKI	<i>Helianthus anuus</i>
LRRLWTSWLKRATQ	<i>Metallosphaera sedula</i>
LTIGLLKKLKCIRI	<i>Talitrus saltator</i>
LTVLPYVPSLFCKI	<i>Crocodylus porosus</i>
LVFNIPSLLVFVLK	<i>Ophiophagus hannah</i>
LVRLFKKKKKKKKV	<i>Falco peregrinus</i>
LWRSLPFRRTLLR	<i>Burkholderia pseudomallei</i>
LWRTFWISLKLPAWK	<i>Panthera tigris altaica</i>
LYFAKKLFKKIHKI	<i>Gallus gallus</i>
MALKILKKVMAHFK	<i>Orcinus orca</i>
SIKFNIIKKYILK	<i>Danaus plexippus plexippus</i>
SILKTFISLKKKTKL	<i>Danaus plexippus plexippus</i>
SIPKSFLCFLLLRKC	<i>Falco peregrinus</i>
VCFRFRSRVKCRSWC	<i>Ophiophagus hannah</i>
VIAFFKQLFTNAKKI	<i>Falco peregrinus</i>
VKLKLFDKLLRLLLL	<i>Crocodylus porosus</i>
VLKLLKIIFSLKLE	<i>Ophiophagus hannah</i>
VNKKILKNLIKVILK	<i>Helianthus anuus</i>
VNVASKSWNKILRKI	<i>Metallosphaera sedula</i>
VPSFKIFVVKFCIAL	<i>Danaus plexippus plexippus</i>
VRIRIRVRIRVRIV	<i>Takifugu rubripes</i>
VVRPFKKVKLVFK	<i>Gallus gallus</i>
VVRRRWMRKRSPW	<i>Metallosphaera sedula</i>
VVWRRRRRWWWWWRW	<i>Helianthus anuus</i>
VWWWWWRRRWQRHR	<i>Helianthus anuus</i>
WAFTFKSLKKLLKII	<i>Mesobuthus martensii</i>
WGLWWRCVCNWWKWF	<i>Orcinus orca</i>
WKFWRRWRRGWCHGR	<i>Rhincodon typus</i>
WKKKKKKKGHLFWIL	<i>Gallus gallus</i>
WKLKFNIVKFKLKF	<i>Helianthus anuus</i>
WKLKKLKKLSCVKL	<i>Ophiophagus hannah</i>
WLKIKFKCVKCFFFF	<i>Panthera tigris altaica</i>
WNRLHRMVWSRLLWR	<i>Gallus gallus</i>
WQLIKIKTKKKKKL	<i>Gallus gallus</i>
WRFVRVRVRVRKKI	<i>Takifugu rubripes</i>

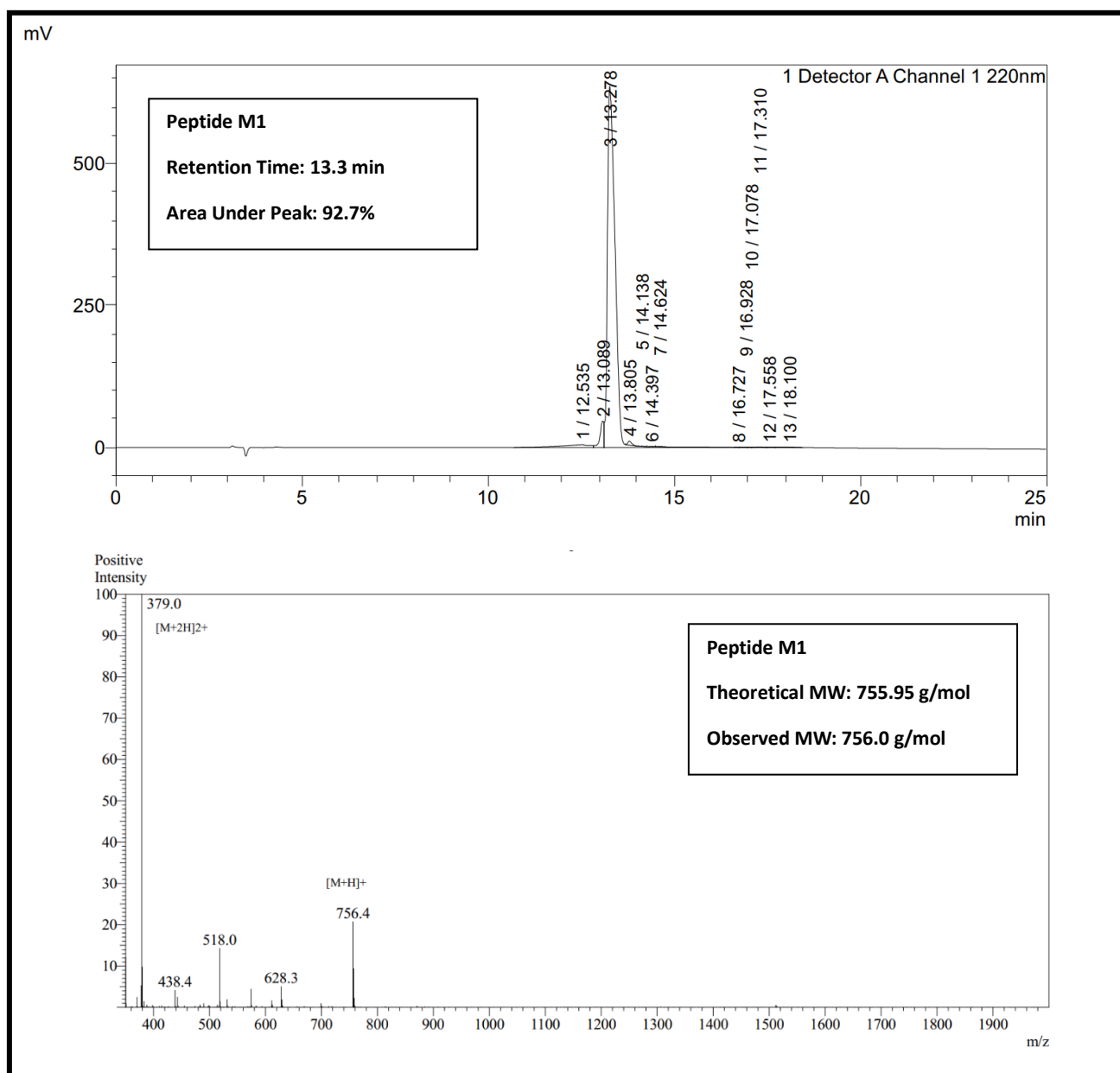
WRKCLWWRWCWWWLW	<i>Metallosphaera sedula</i>
WRLWMLHHWRLLRR	<i>Ophiophagus hannah</i>
WRWAAWWSWLSRWRR	<i>Orcinus orca</i>
WTQVIKILVKNLNTL	<i>Falco peregrinus</i>
YFCFIVPFRVRGGKC	<i>Ophiophagus hannah</i>
KIKKKKNQRCQTQILK	<i>Orcinus orca</i>
ALRVKKDVKALRKLLK	<i>Canis lupus familiaris</i>

Table 4. Sequences of AMPLY peptide candidates selected for purification.

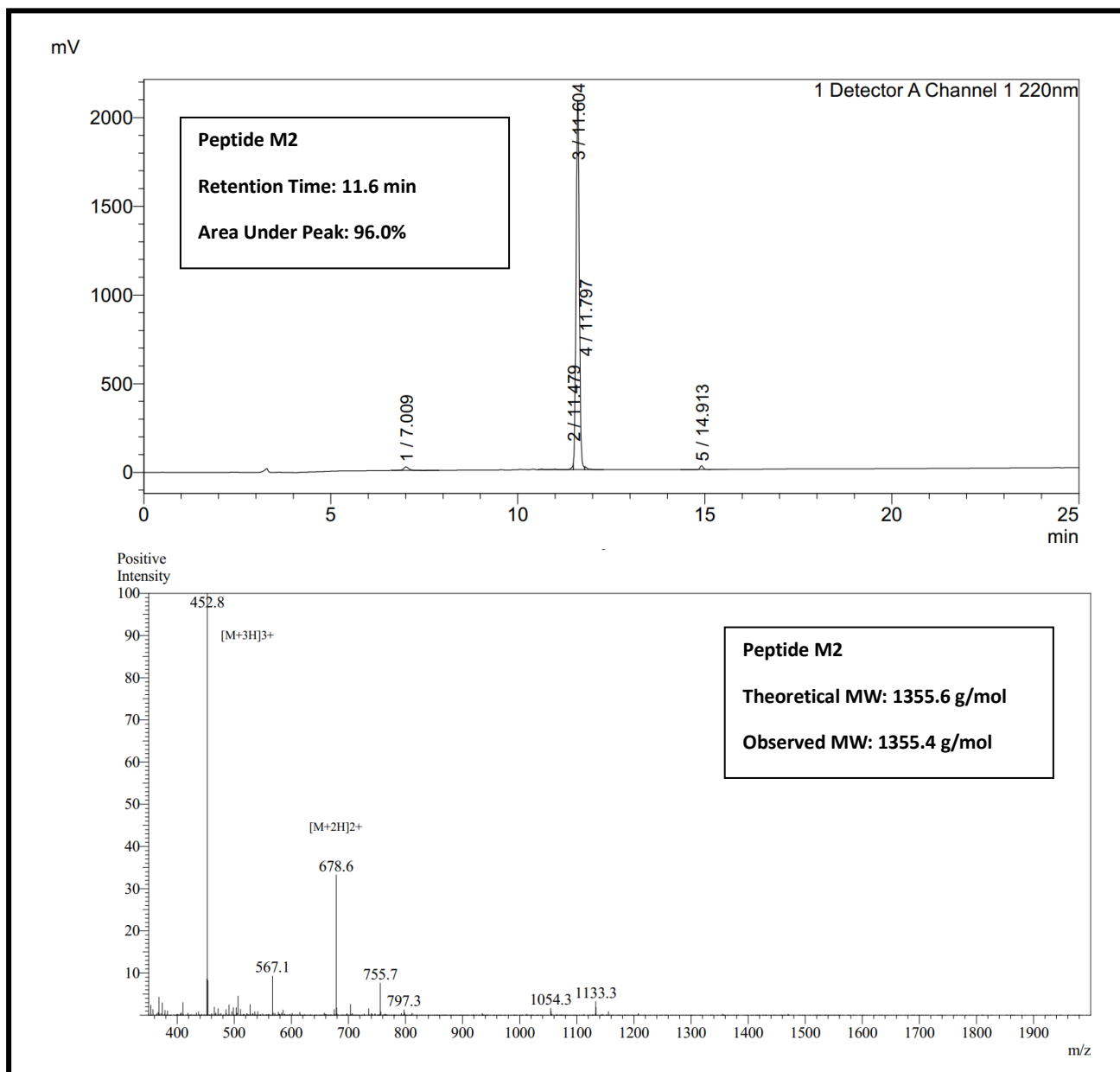
Peptide	Sequence	Source
AMPLY-GALSU1	KIYWKLLKKLLV	<i>Galdieria sulphuraria</i>
AMPLY-BACAN1	KRWVKLLYKLLK	<i>Bacillus anthracis</i>
AMPLY-OPHHA1	KKKKAIRHFLFFF	<i>Ophiophagus hannah</i>
AMPLY-BACAN2	IKKVMLLLLKRKMKKL	<i>Bacillus anthracis</i>
AMPLY-GALSU2	KKQRVLLKKLLVVIPL	<i>Galdieria sulphuraria</i>
AMPLY-METSE1	KLRCRIKILPKRFIPK	<i>Metallosphaera sedula</i>
AMPLY-ORCOR1	KSLWRTVWRFLKKLKR	<i>Orcinus orca</i>
AMPLY-FALPE1	KVPFKCEYKKWRFIWK	<i>Falco peregrinus</i>
AMPLY-GALGA1	LFLIYKKKKKKKKR	<i>Gallus gallus</i>
AMPLY-METSE2	LIRFTKGLRRRKRRL	<i>Metallosphaera sedula</i>
AMPLY-HELAN1	LKIKIKIKIKIKIK	<i>Helianthus annuus</i>
AMPLY-PLAFA1	LKKKKKKKKLRLFIMS	<i>Plasmodium falciparum</i>
AMPLY-GALGA2	LKKKKRKMVKVLLLL	<i>Gallus gallus</i>
AMPLY-BACAN3	LKKLKKKLRKLDLV	<i>Bacillus anthracis</i>
AMPLY-CANFA1	LKKLLKHKSLLVLIKI	<i>Canis lupus familiaris</i>
AMPLY-TALSA1	LKLKLNFKLKLKLK	<i>Talitrus saltator</i>
AMPLY-CROPO1	LKLLKLKILFLFRWK	<i>Crocodylus porosus</i>
AMPLY-TALSA2	LKLWLKKKVKAQCQVF	<i>Talitrus saltator</i>
AMPLY-GALGA3	LKRKKKKVVAHLFLV	<i>Gallus gallus</i>
AMPLY-RHITY1	LLEFFLKIFKRFKKK	<i>Rhincodon typus</i>
AMPLY-TAKRU1	LLKKKIILLRKAIK	<i>Takifugu rubripes</i>
AMPLY-CANLU1	LLKKKKKKRKKILFSL	<i>Canis lupus lupus</i>
AMPLY-GALSU3	LRRTWSFCWLYWRRHR	<i>Galdieria sulphuraria</i>
AMPLY-METSE3	LRSYLWRRGYKFFRRL	<i>Metallosphaera sedula</i>
AMPLY-RHITY2	LSHYKRKLLKSLKKL	<i>Rhincodon typus</i>
AMPLY-PANAL1	LSLLKINKHLKKLKL	<i>Panthera tigris altaica</i>
AMPLY-PANAL2	LSLSKINKLLKYKKI	<i>Panthera tigris altaica</i>
AMPLY-GALGA4	LSRKISWKKIILKL	<i>Gallus gallus</i>
AMPLY-GANLU1	LWRRVRGIRRWVRGIR	<i>Ganoderma lucidum</i>
AMPLY-CANFA2	LYIKKSLRLHLKKLLK	<i>Canis lupus familiaris</i>
AMPLY-PANAL3	MFLYLKKKKKKLLKIW	<i>Panthera tigris altaica</i>
AMPLY-GALGA5	MIFYKKKKKIIRKIIF	<i>Gallus gallus</i>
AMPLY-HELAN2	MKIMKMMKKIMKMMKK	<i>Helianthus annuus</i>
AMPLY-CANFA3	MKKVKKKKKKNIVFFF	<i>Canis lupus familiaris</i>

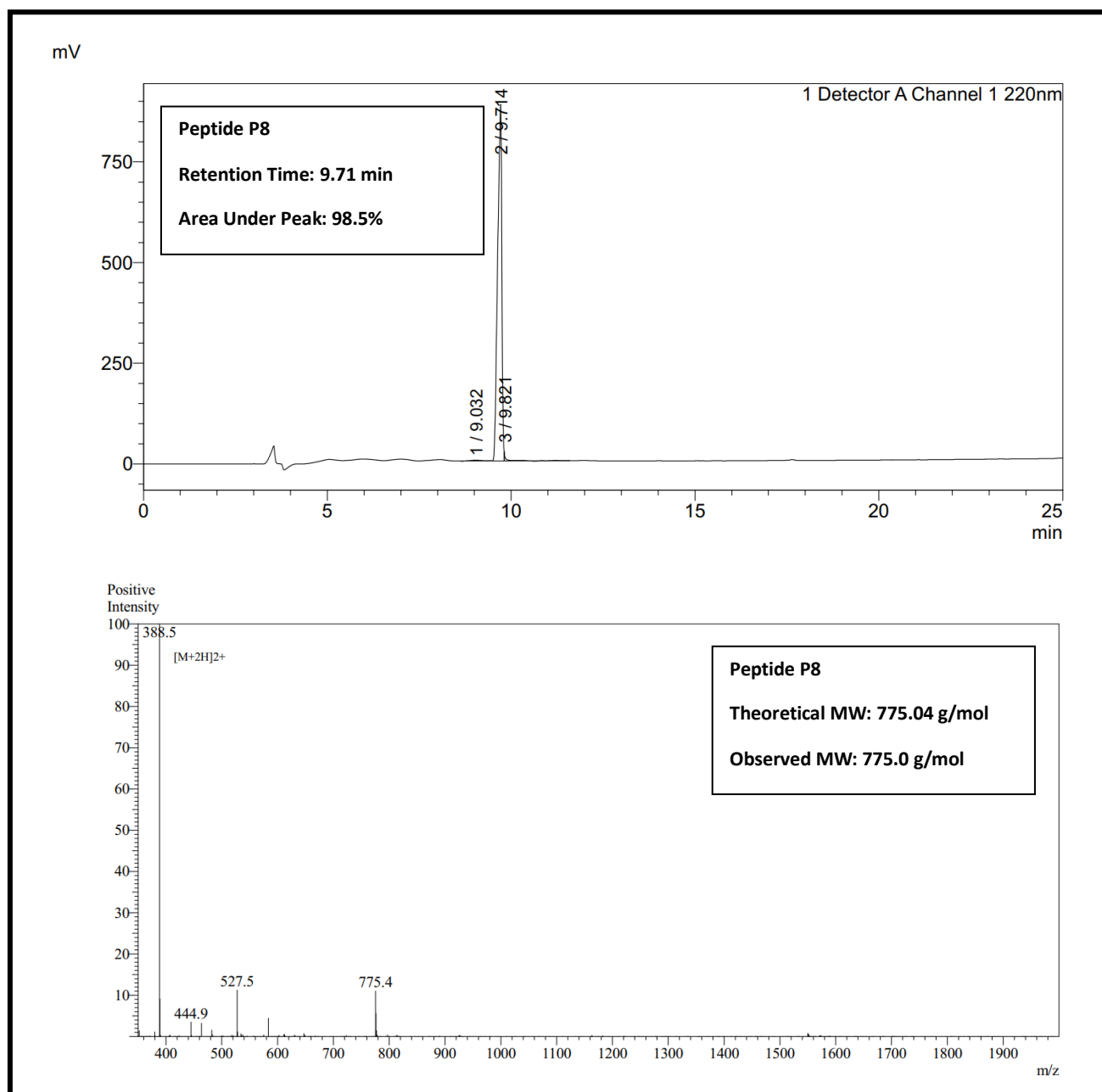
AMPLY-PANAL4	NLKKKFKKRKKFEKL	<i>Panthera tigris altaica</i>
AMPLY-GALGA6	NVKKKKKKIFFHLLL	<i>Gallus gallus</i>
AMPLY-HELAN3	LKNLLRRLKLRRLLK	<i>Helianthus annuus</i>
AMPLY-BURPS1	PARRAPRRRLCLRPLA	<i>Burkholderia pseudomallei</i>
AMPLY-GALGA7	PPKKKKKIKLIPVIKI	<i>Gallus gallus</i>
AMPLY-DANPL1	RALFFVKKKIKKIKRL	<i>Danaus plexippus plexippus</i>
AMPLY-CROPO2	RCNRKFELKILKKKF	<i>Crocodylus porosus</i>
AMPLY-FALPE2	RKLRRKHFFKNIFKRL	<i>Falco peregrinus</i>
AMPLY-BURPS2	RLSAARACRCRRLRRR	<i>Burkholderia pseudomallei</i>
AMPLY-TALSA3	RPKVRVKIWVKARVKV	<i>Talitrus saltator</i>
AMPLY-DANPL2	RRFRPWVWWRYRWVLW	<i>Danaus plexippus plexippus</i>
AMPLY-GANLU2	RRVRVRVQLLRARA	<i>Ganoderma lucidum</i>
AMPLY-RHITY3	RRWRKWRRICWMQRL	<i>Rhincodon typus</i>
AMPLY-BURPS3	RRWWRCISRLGWRRWR	<i>Burkholderia pseudomallei</i>
AMPLY-ORCOR2	RVRFRGWGRFRVRV	<i>Orcinus orca</i>
AMPLY-TAKRU2	RLKKIWRVKASWCAA	<i>Takifugu rubripes</i>
AMPLY-GANLU3	RWVWWCAWRRSRGR	<i>Ganoderma lucidum</i>
AMPLY-PLAFA2	SILTSVKKKKKKK	<i>Plasmodium falciparum</i>
AMPLY-ORCOR3	SLILKAVLKKRRKKK	<i>Orcinus orca</i>
AMPLY-FALPE3	THWKNILRKAMKNLKK	<i>Falco peregrinus</i>
AMPLY-DANPL3	TKRRYRAIKNFFRKAL	<i>Danaus plexippus plexippus</i>
AMPLY-OPHHA2	RLRLRSWLRLRPRL	<i>Ophiophagus hannah</i>
AMPLY-TAKRU3	VWVRLRRVRVIRLL	<i>Takifugu rubripes</i>
AMPLY-PLAFA3	WKIKVKIKVKIKVIK	<i>Plasmodium falciparum</i>
AMPLY-OPHHA3	WKLPWKRLKLKRLK	<i>Ophiophagus hannah</i>
AMPLY-CANLU2	YFFFFKKRRRRRTP	<i>Canis lupus lupus</i>
AMPLY-CROPO3	YKKIKFLHLLRIAIIK	<i>Crocodylus porosus</i>
AMPLY-MESMA1	YWRKKKKILKKLLKIG	<i>Mesobuthus martensii</i>
AMPLY-CANLU3	YYKKKKRLKKAIIKSL	<i>Canis lupus lupus</i>
AMPLY-MESMA2	FKKIHKFLNKLMYKLWK	<i>Mesobuthus martensii</i>
AMPLY-MESMA3	KFRKIYKFLKKARKAKL	<i>Mesobuthus martensii</i>
AMPLY-MESMA4	KILRIRKIYFIFIHIIK	<i>Mesobuthus martensii</i>
AMPLY-CANFA4	MVKKKKKKKKKFVAIYL	<i>Canis lupus familiaris</i>
AMPLY-MESMA5	FFRFRFRWKFRISNRF	<i>Mesobuthus martensii</i>
AMPLY-MESMA6	KIIKFRYKFLKIIKKLI	<i>Mesobuthus martensii</i>
AMPLY-MESMA7	KKKKKKKNILKYVFFL	<i>Mesobuthus martensii</i>
AMPLY-MESMA8	LLKKKKKKKKKYQLIFF	<i>Mesobuthus martensii</i>
AMPLY-MESMA9	IIVRKIIIRKYKFLIFRKI	<i>Mesobuthus martensii</i>

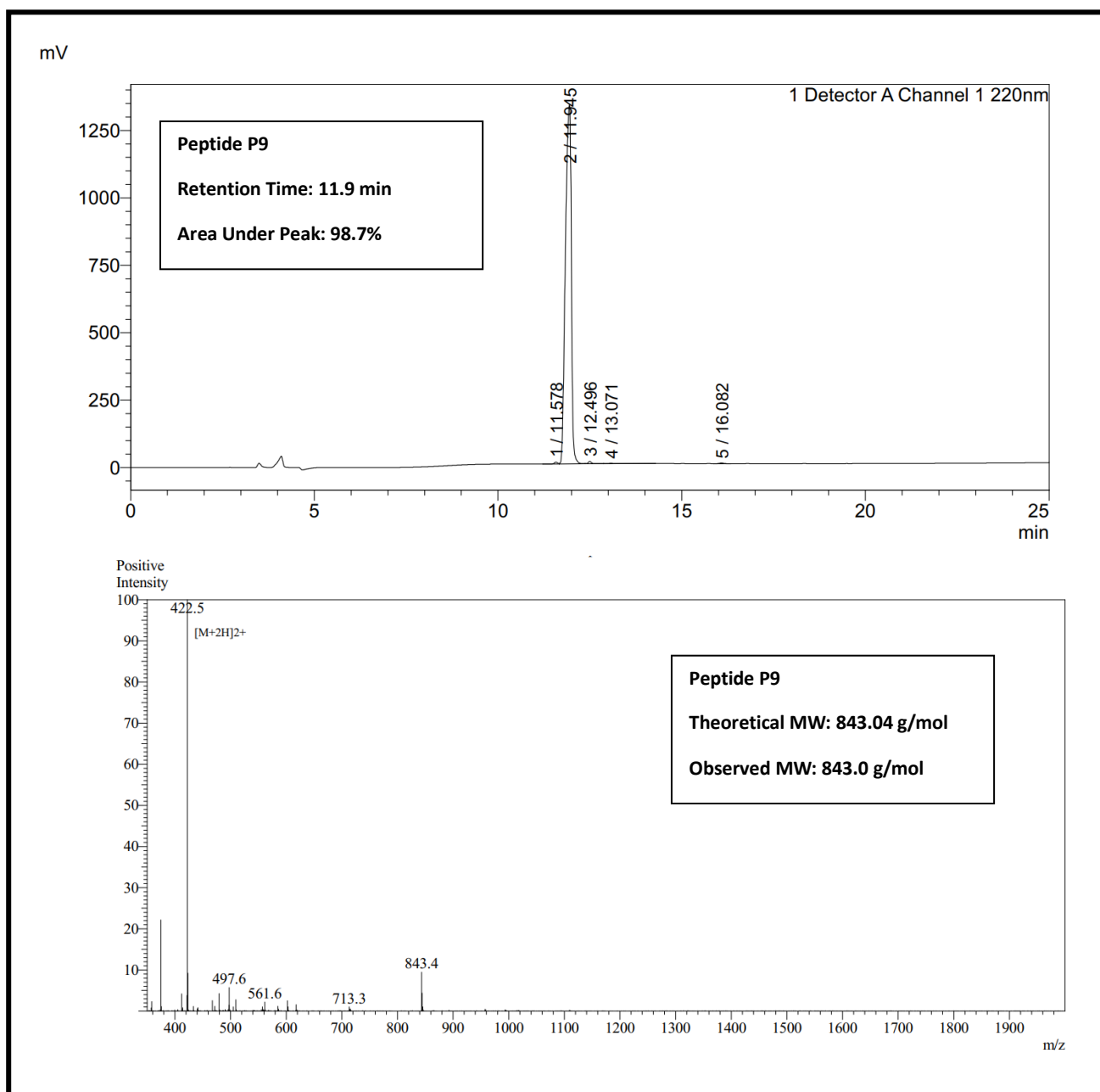
Appendix C – LCMS Data

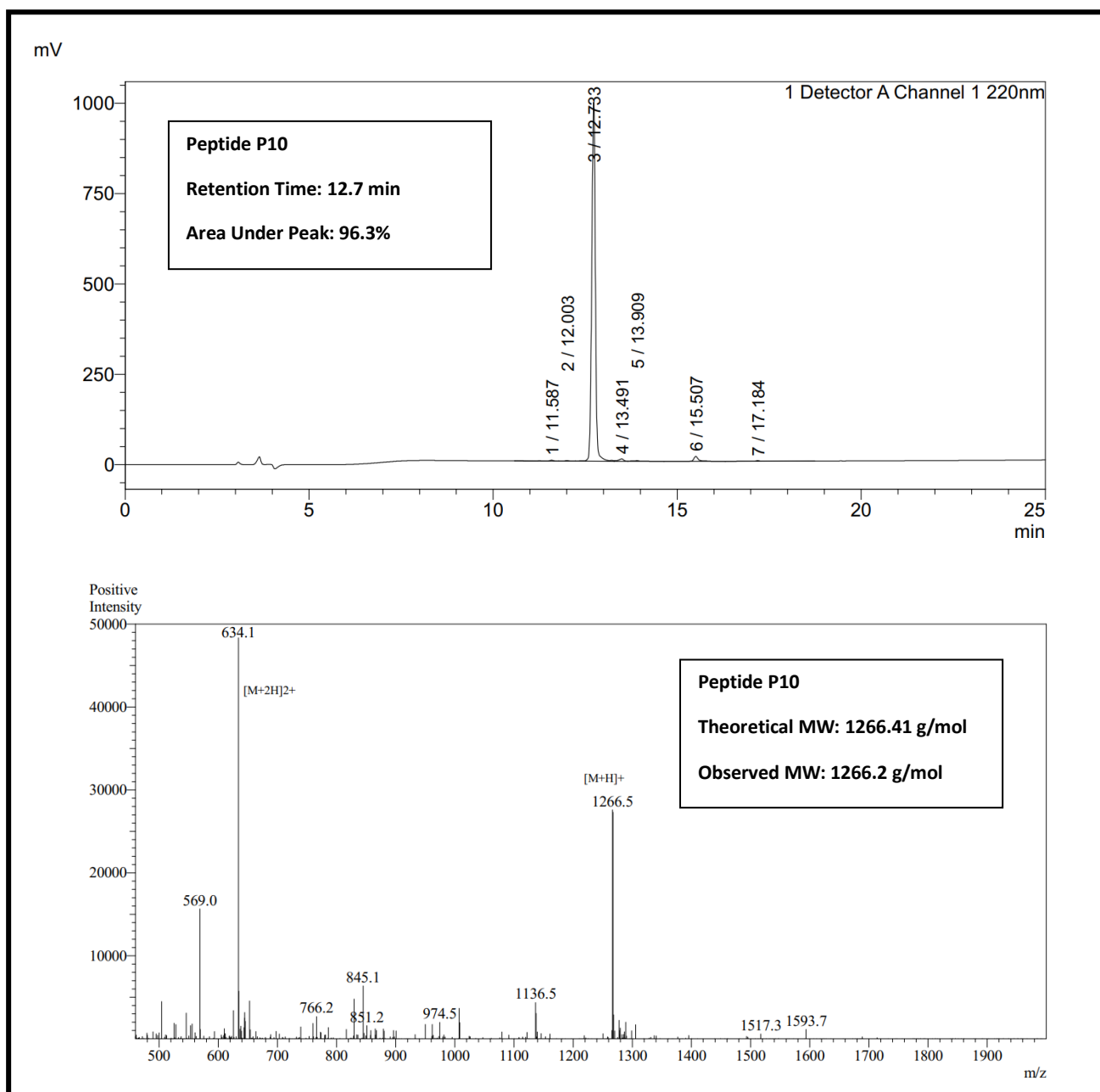


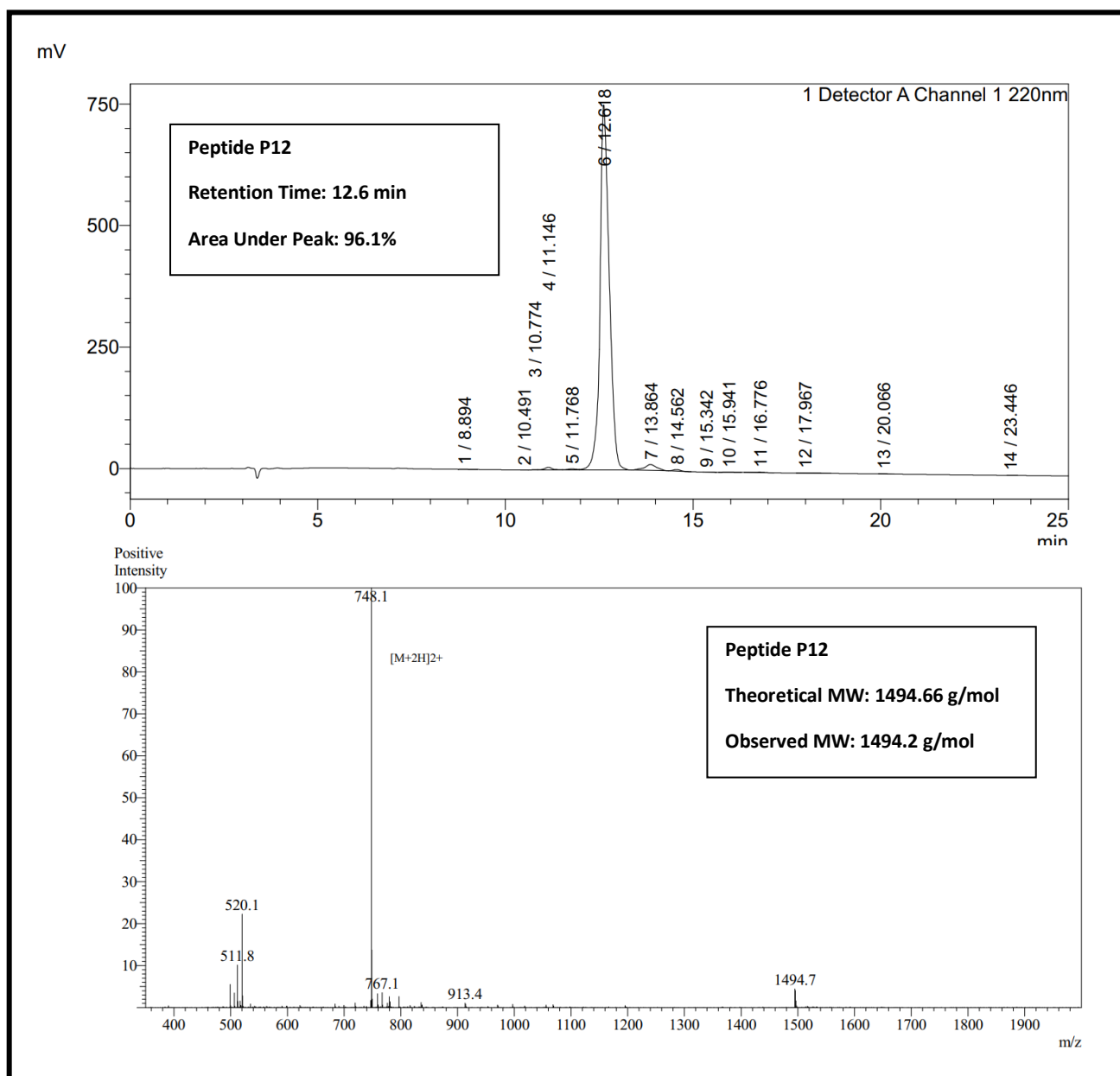
HPLC and LCMS data for preliminary lung peptide set.

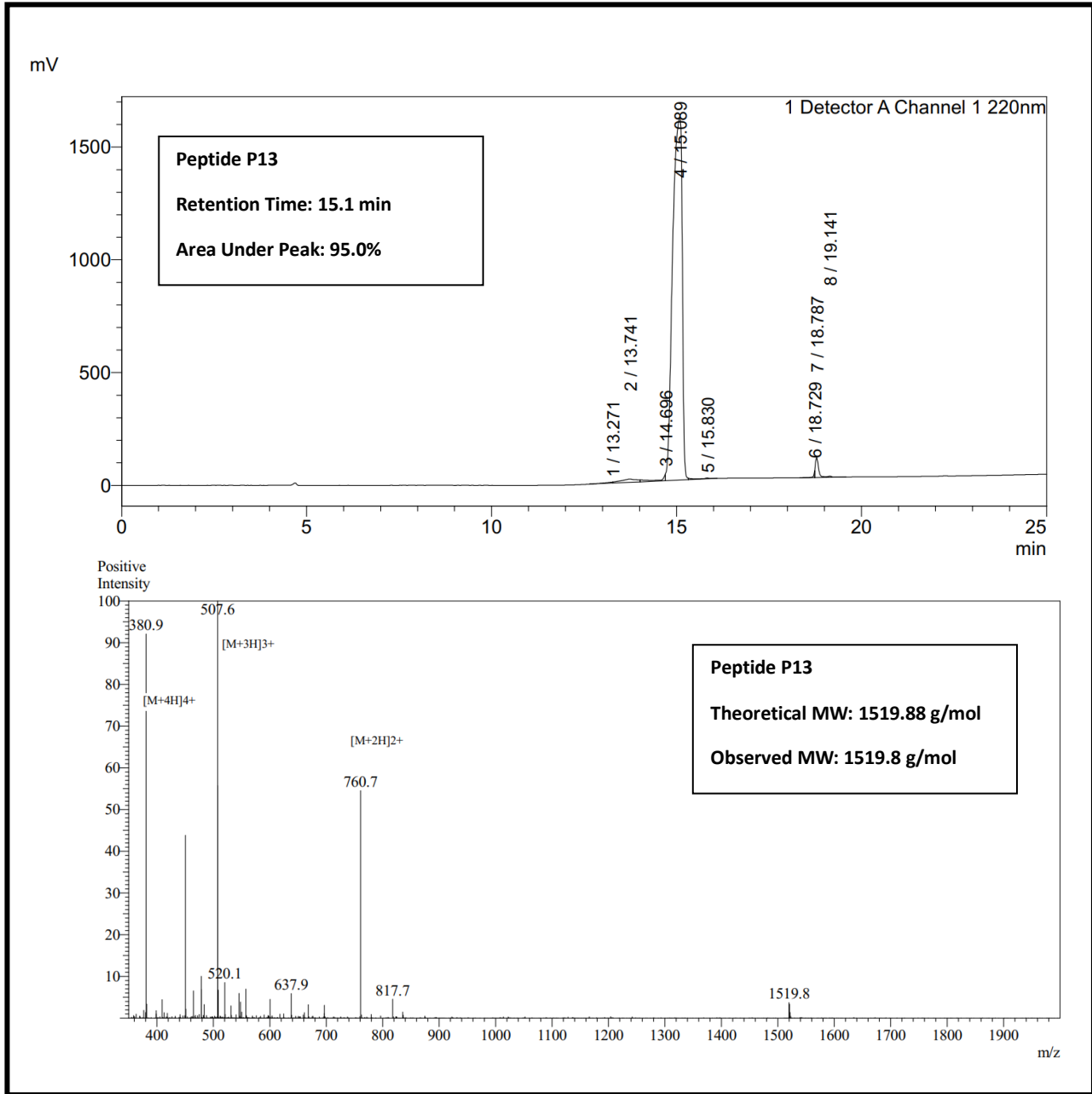


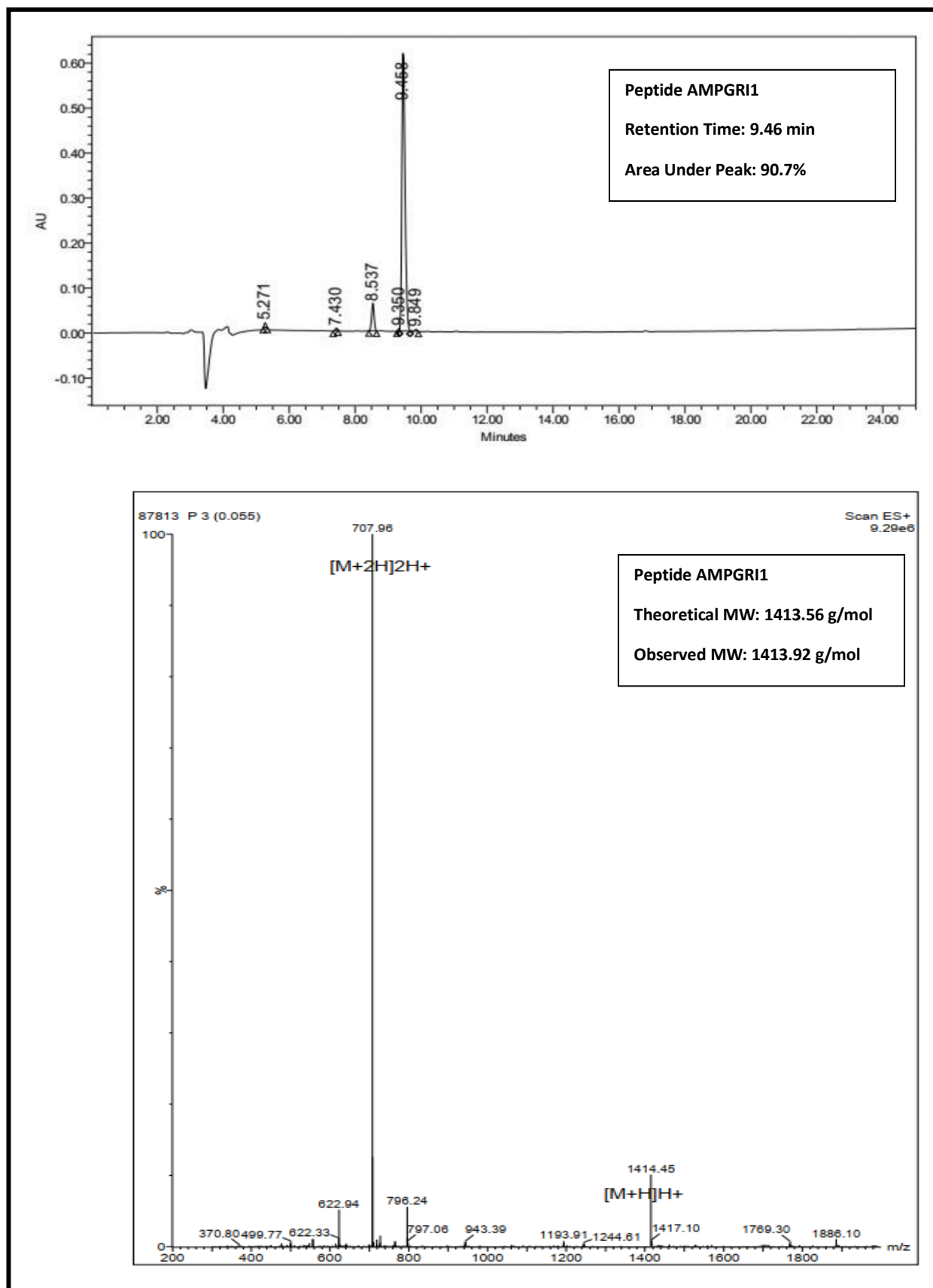


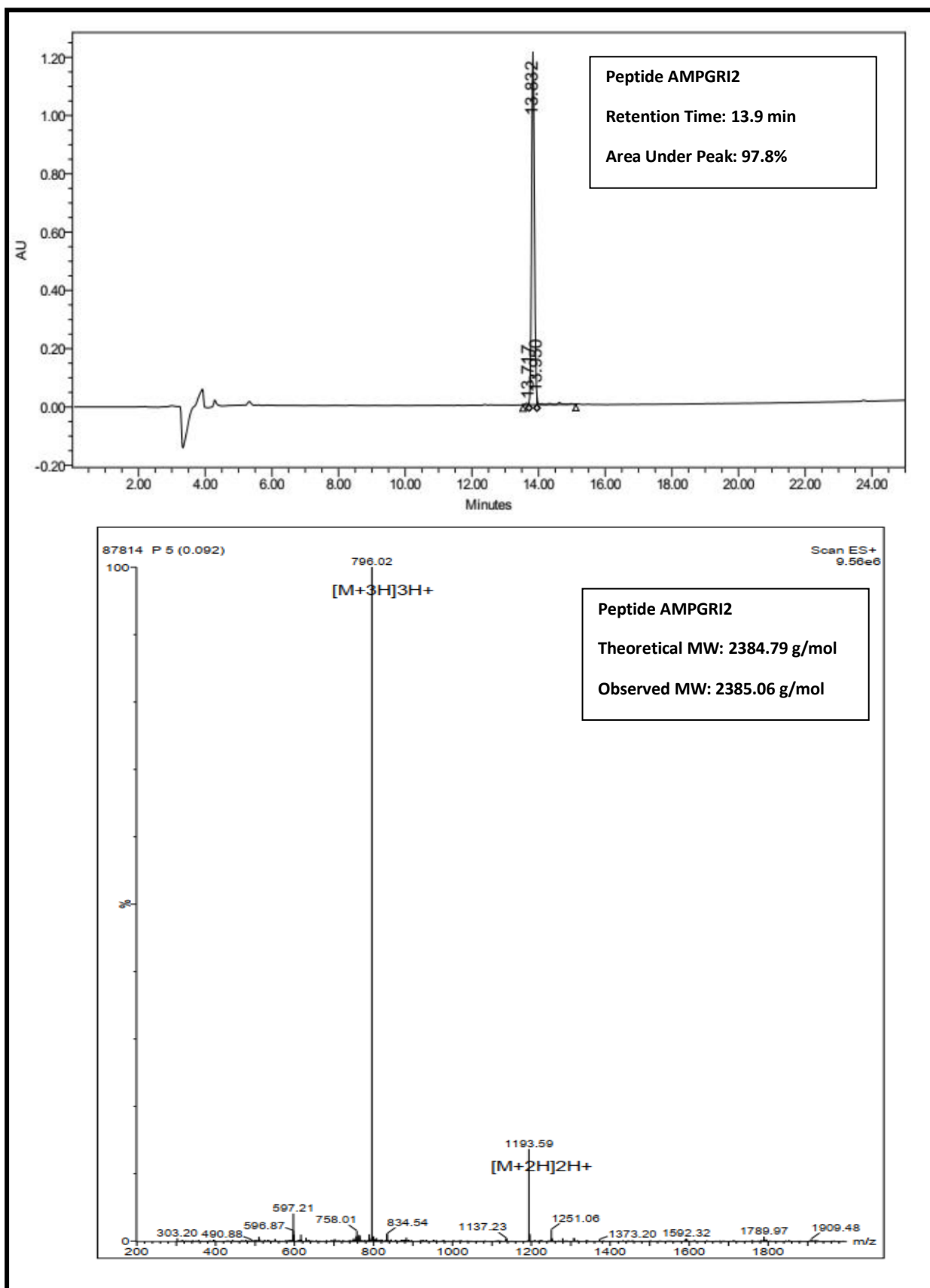


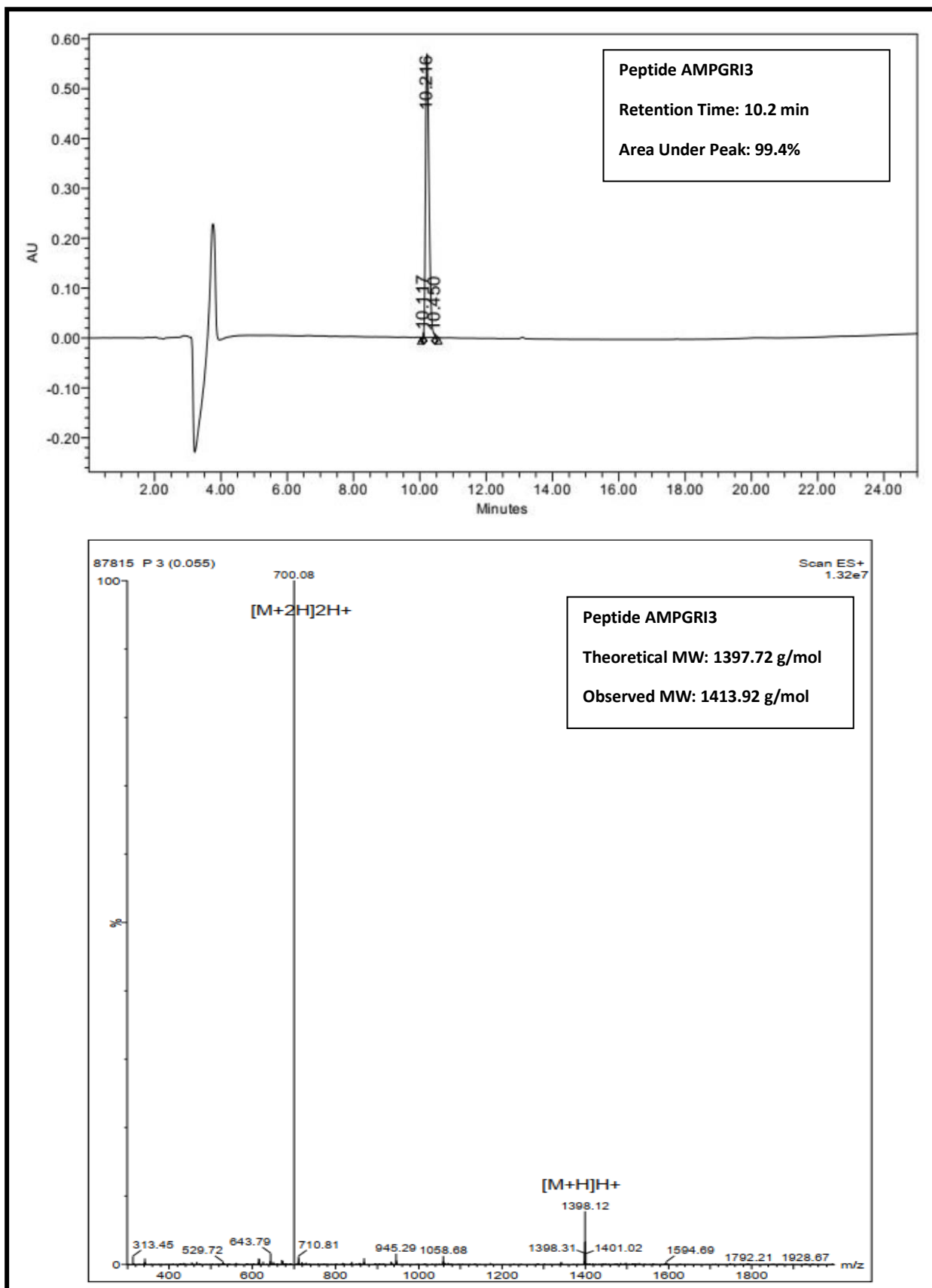


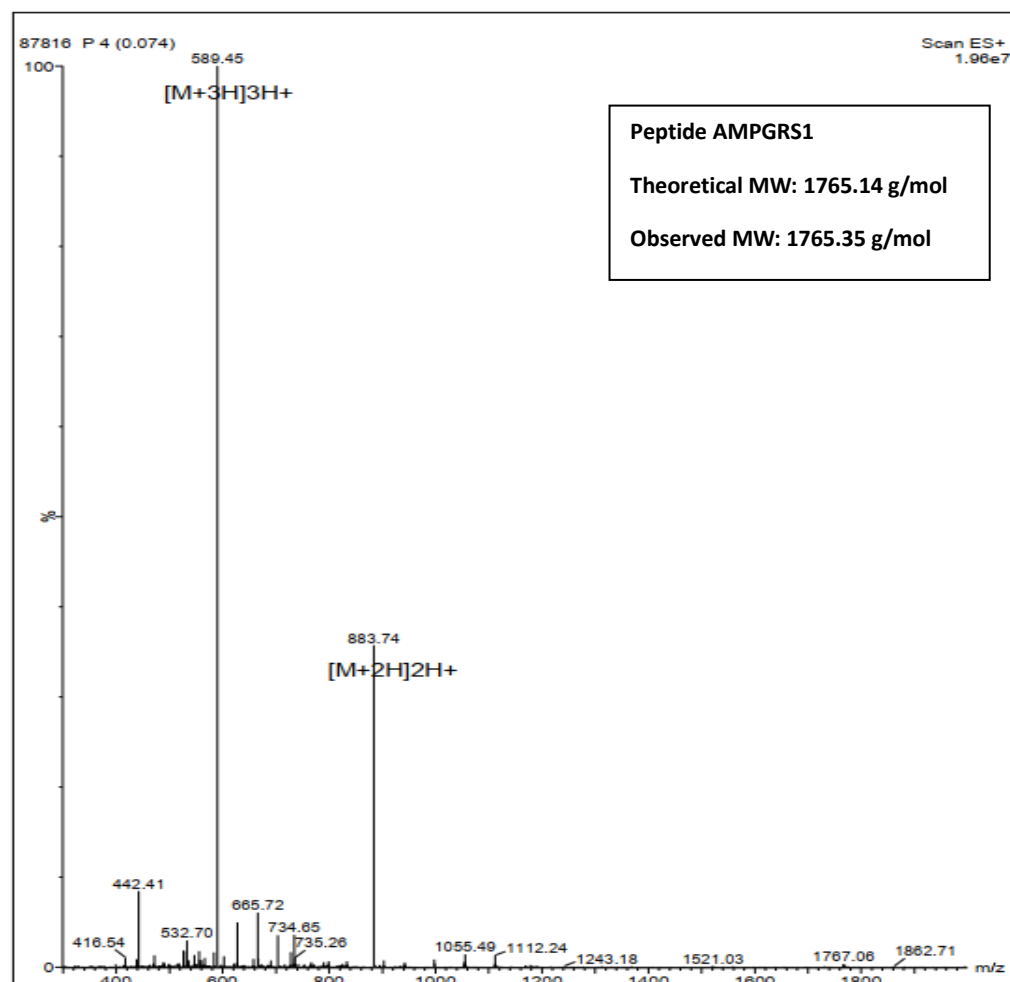
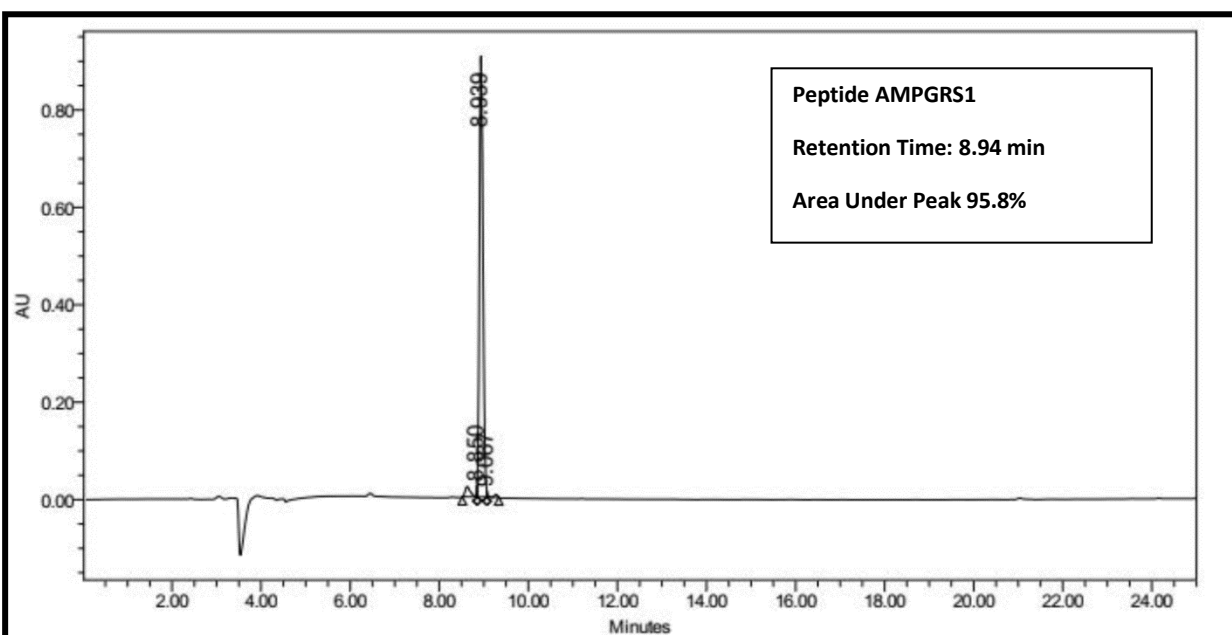


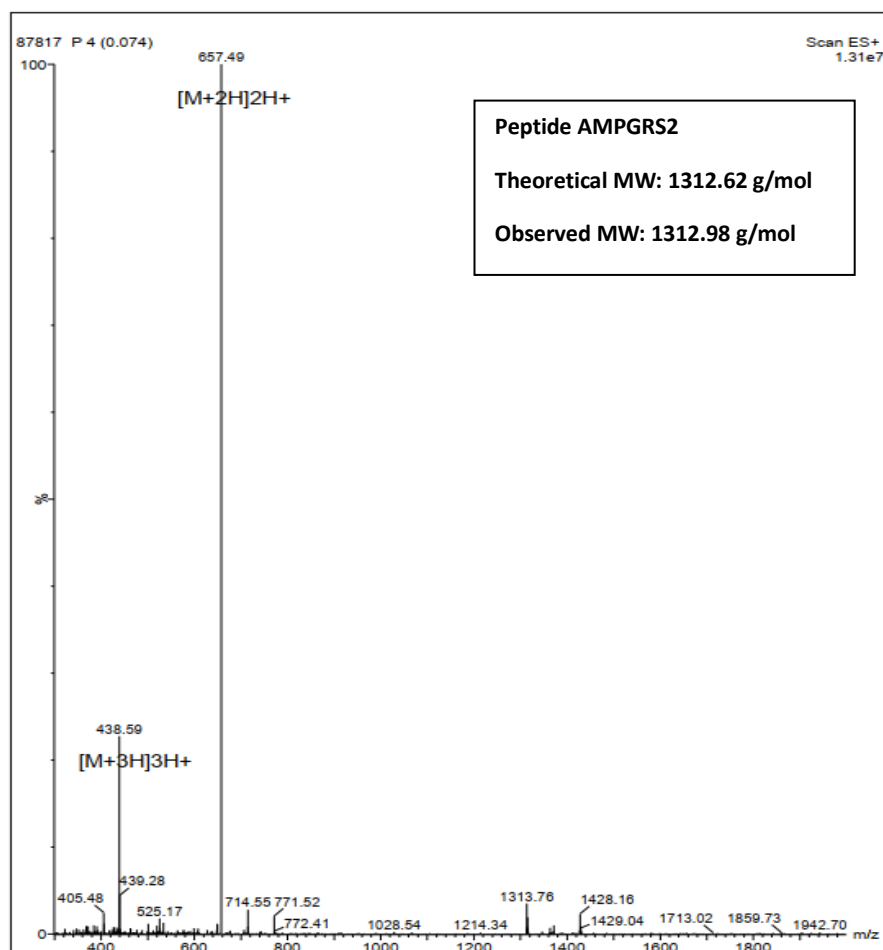
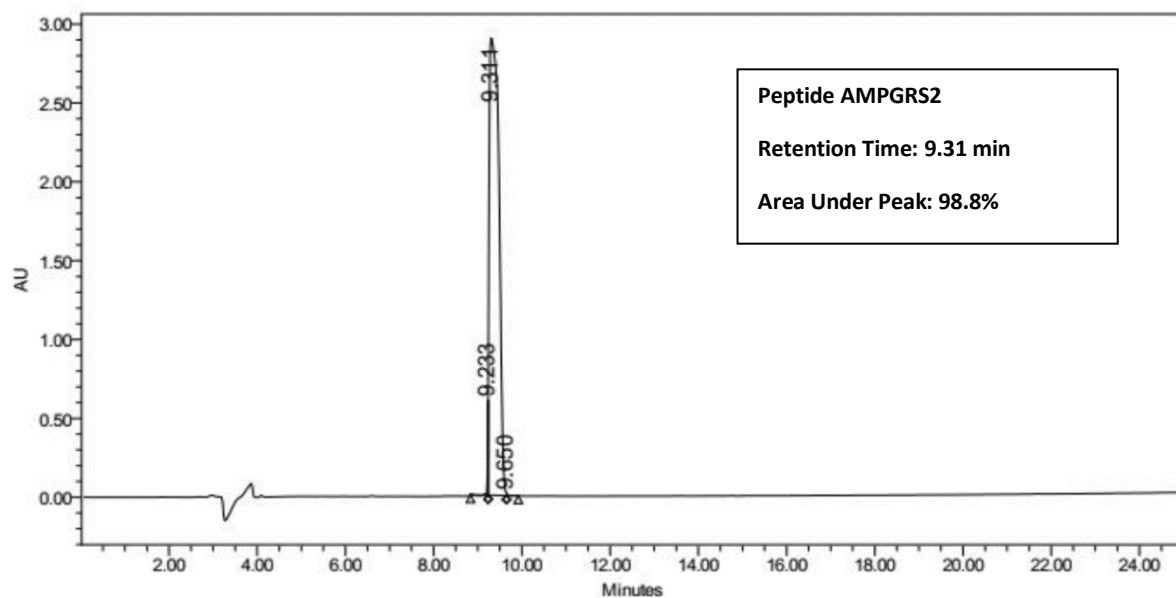


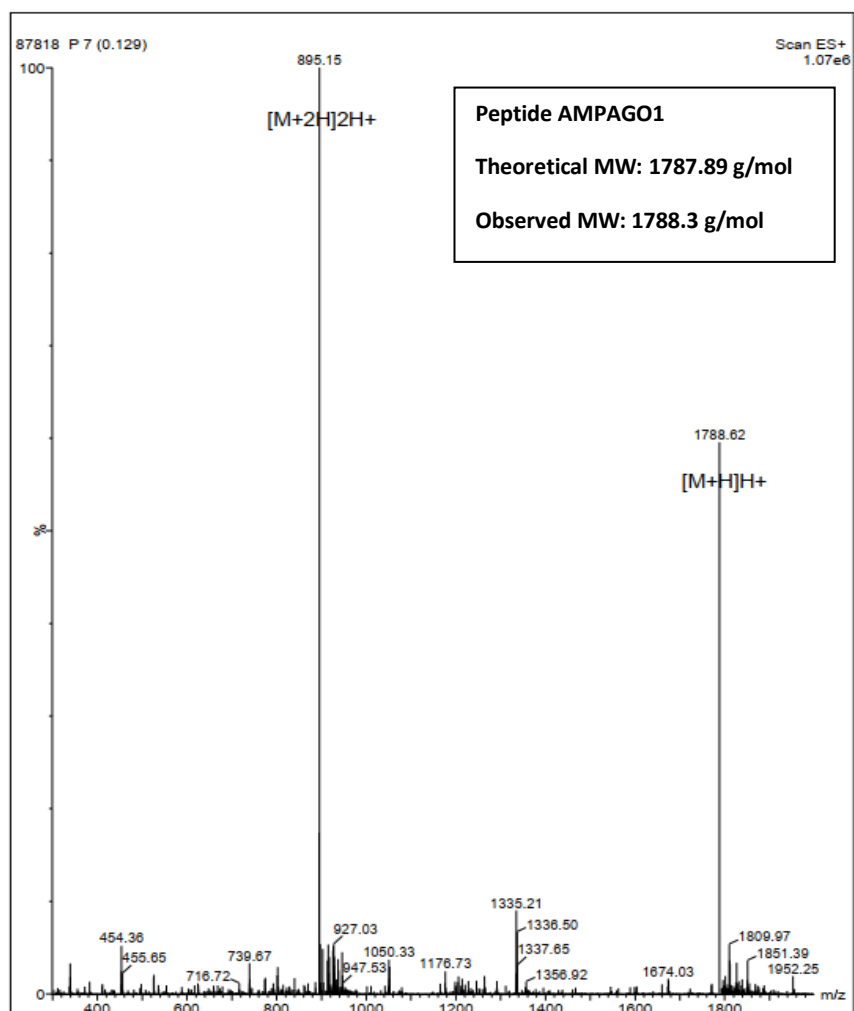
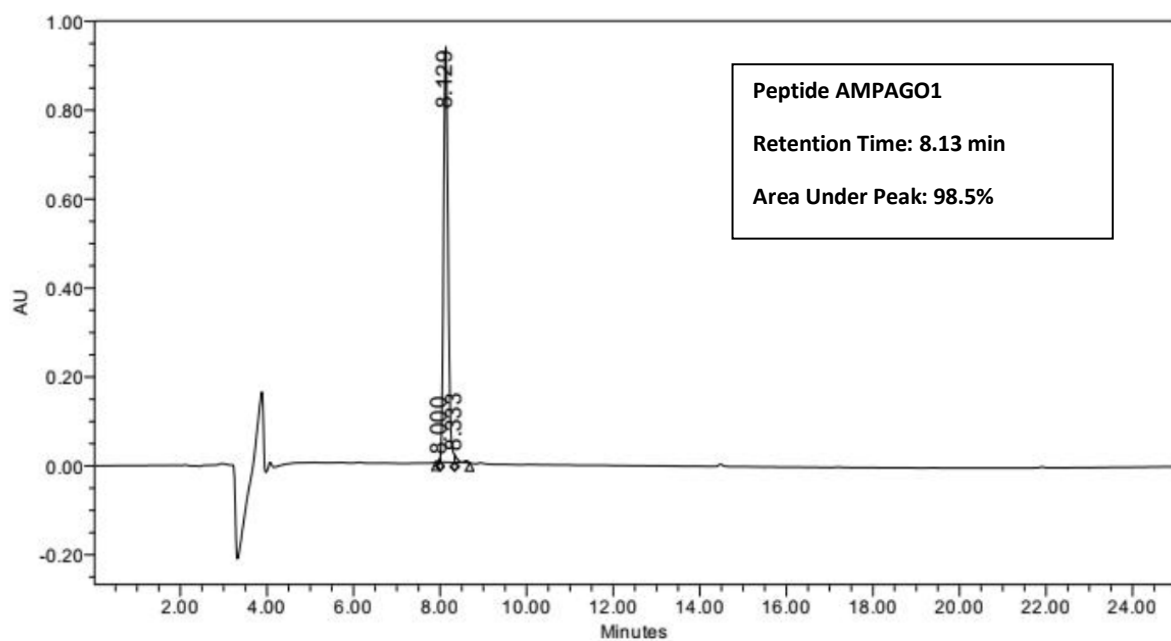


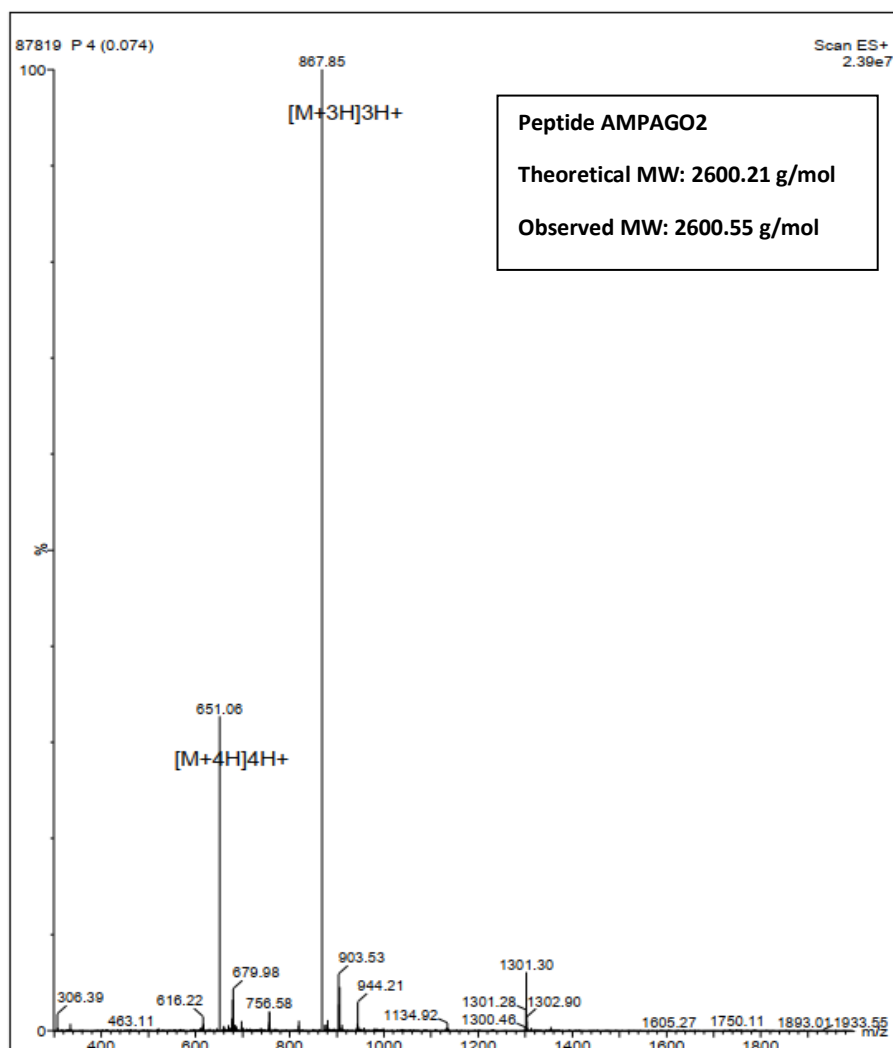
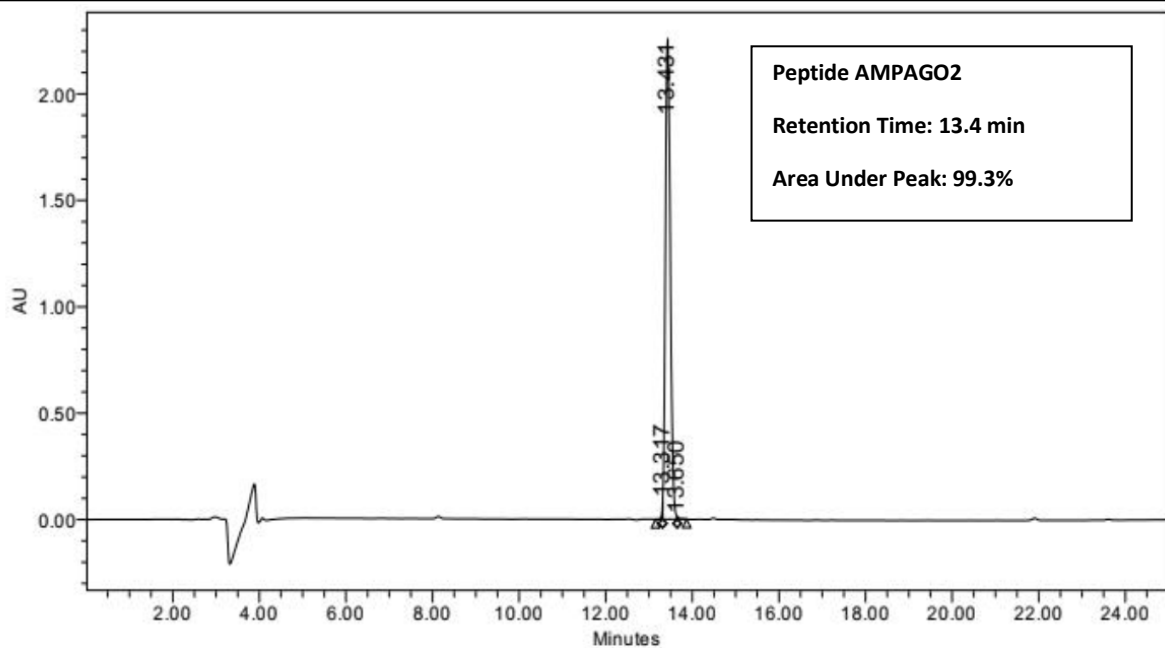


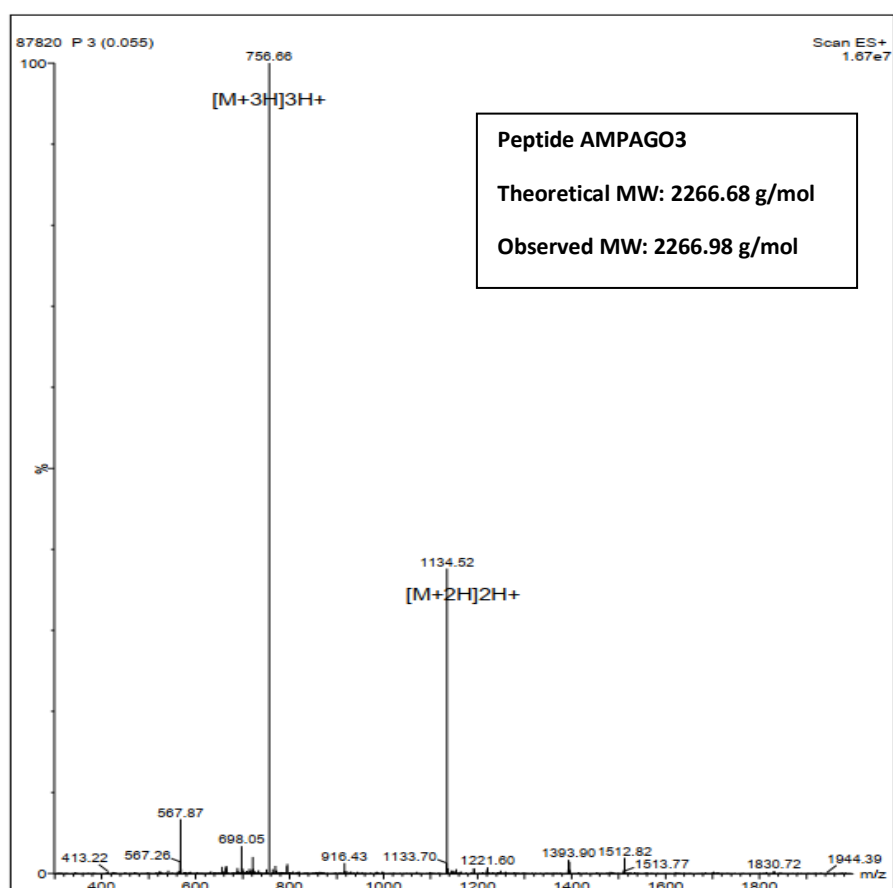
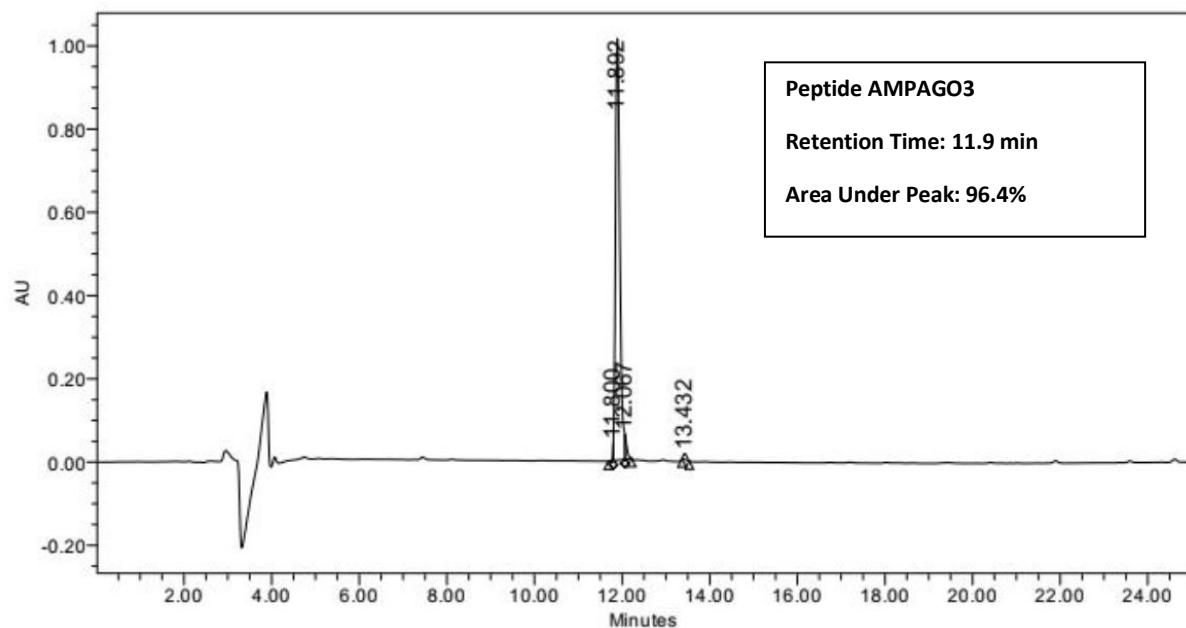


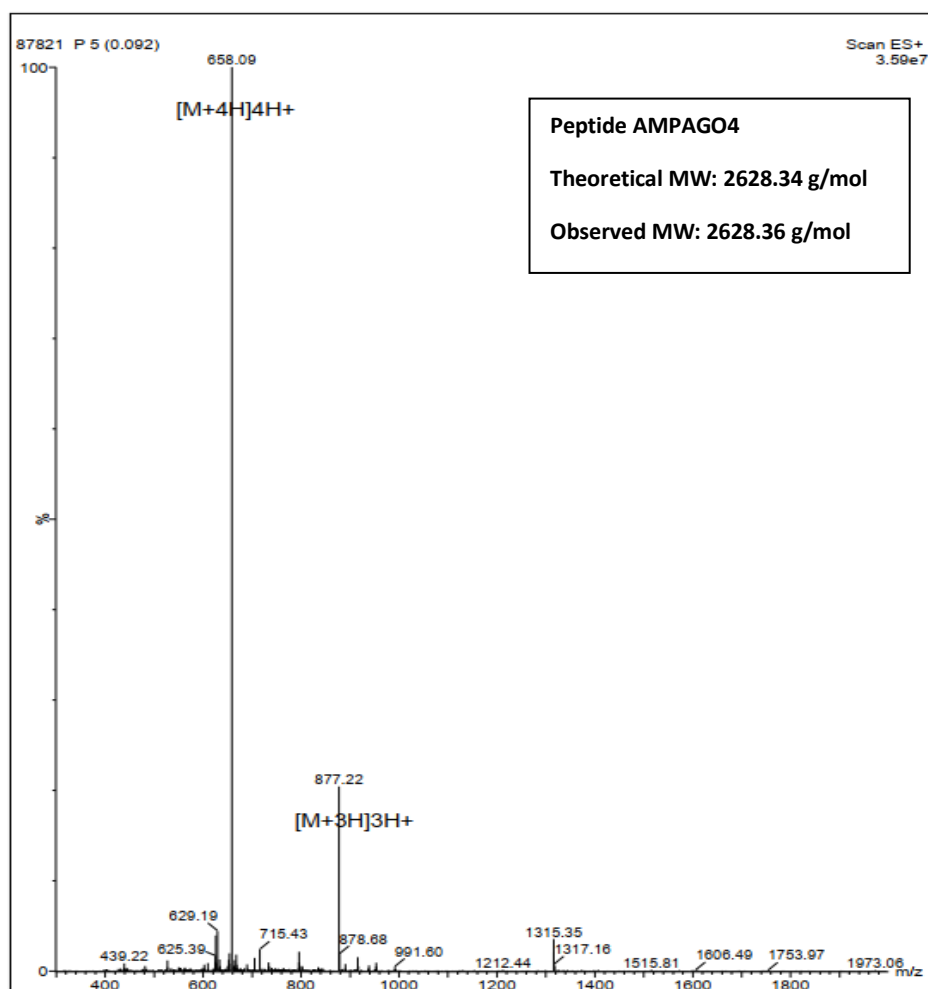
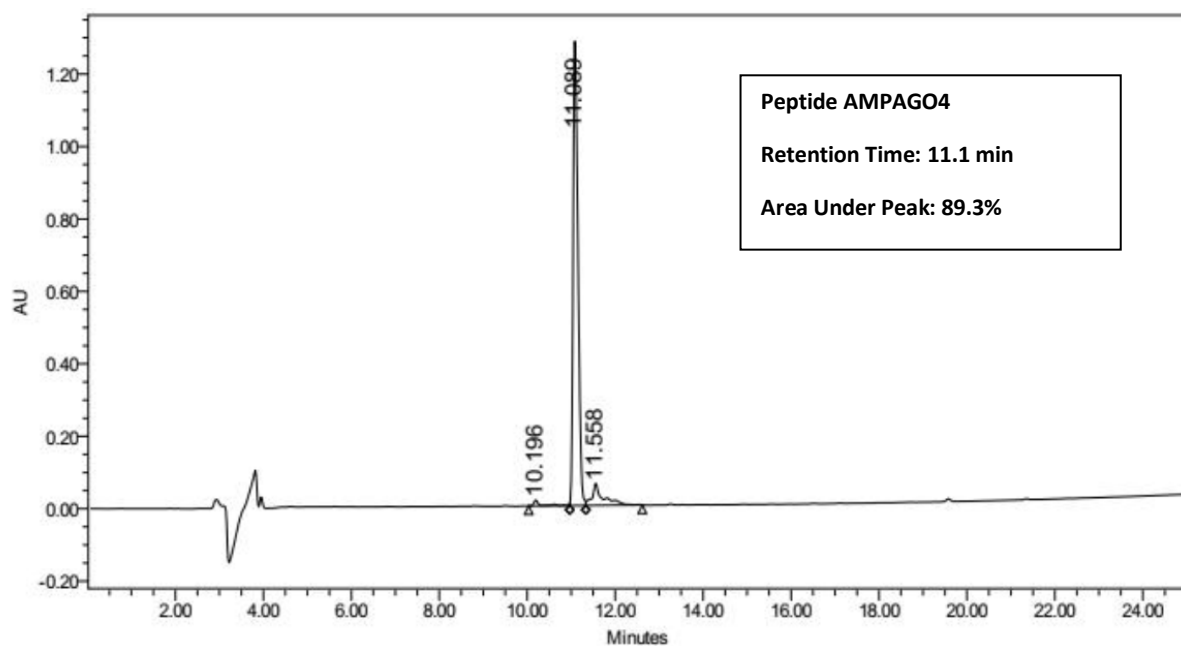


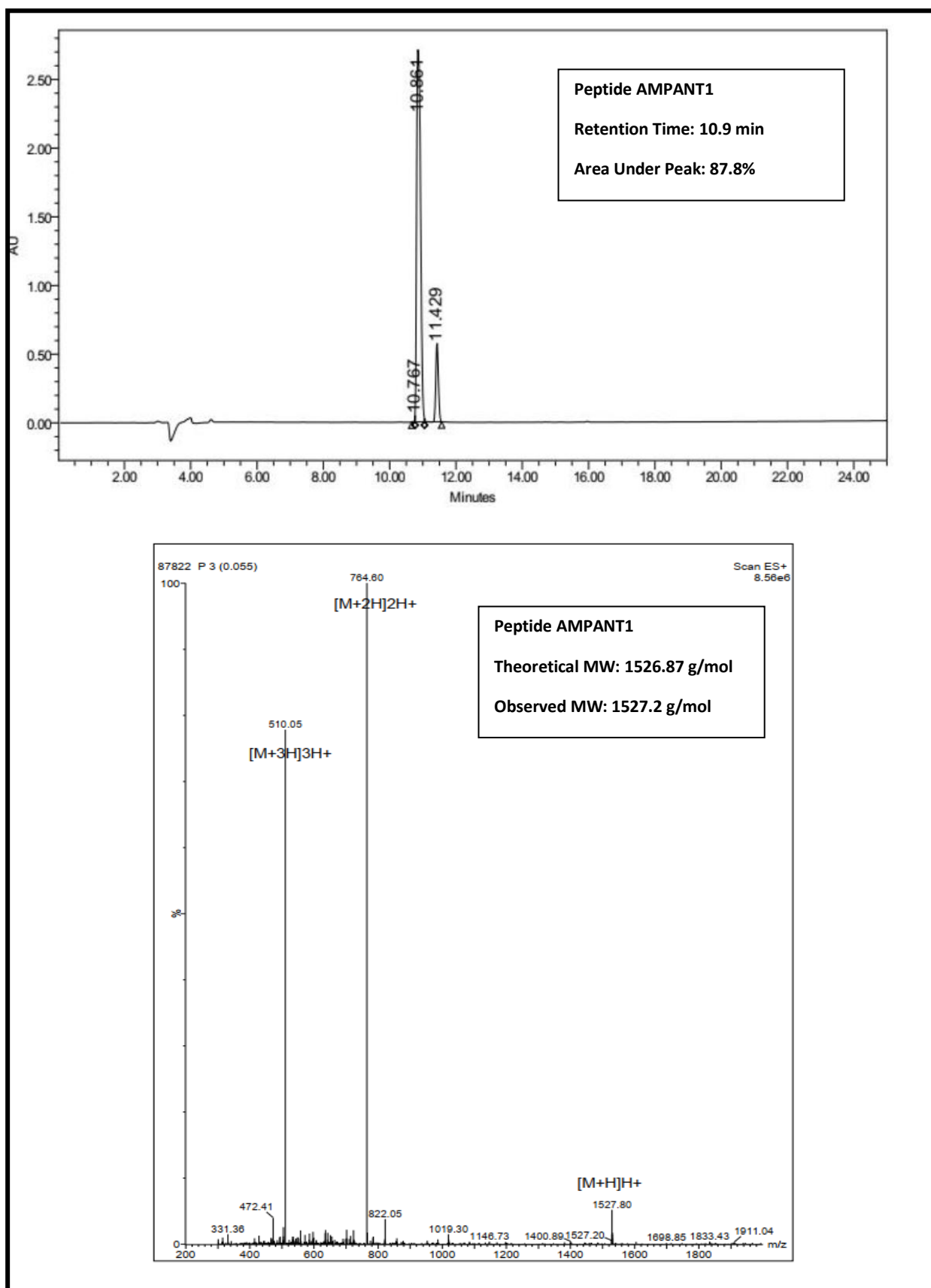


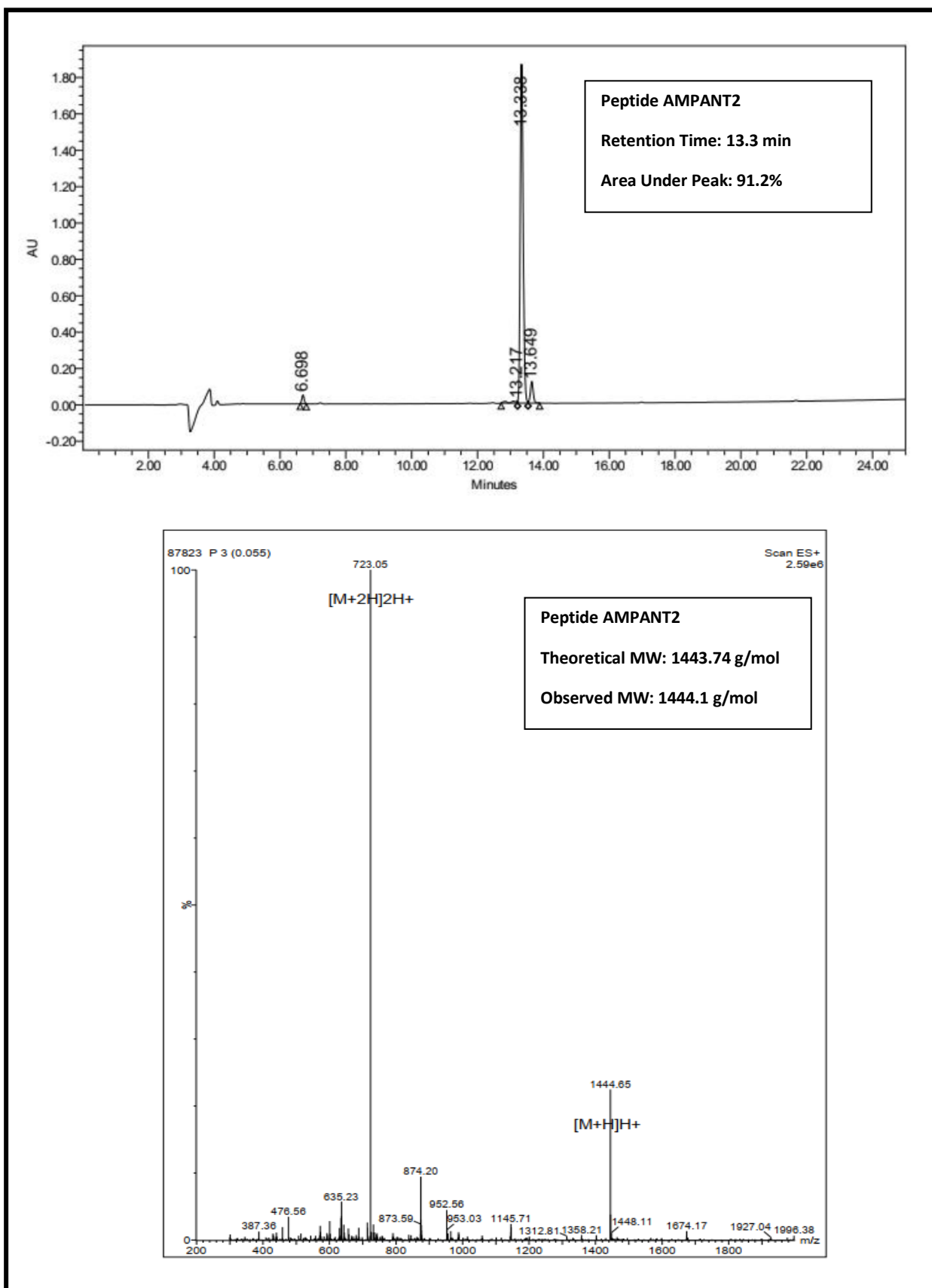


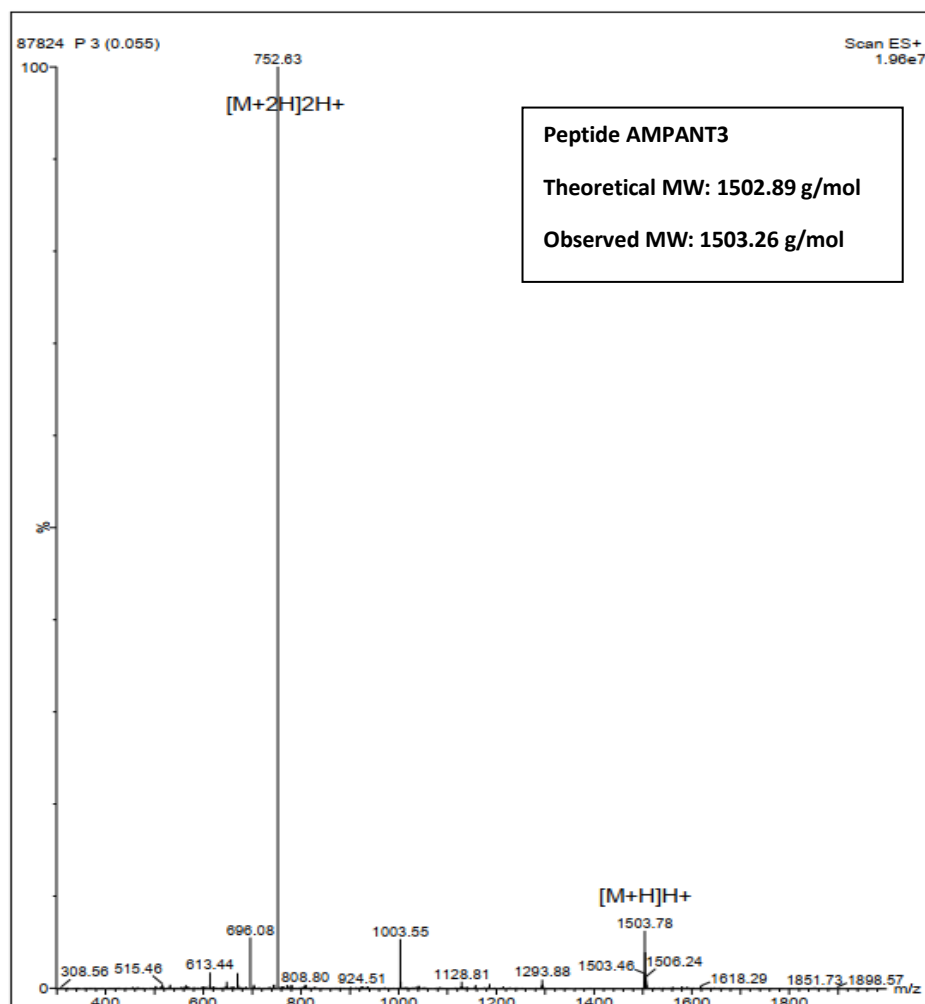
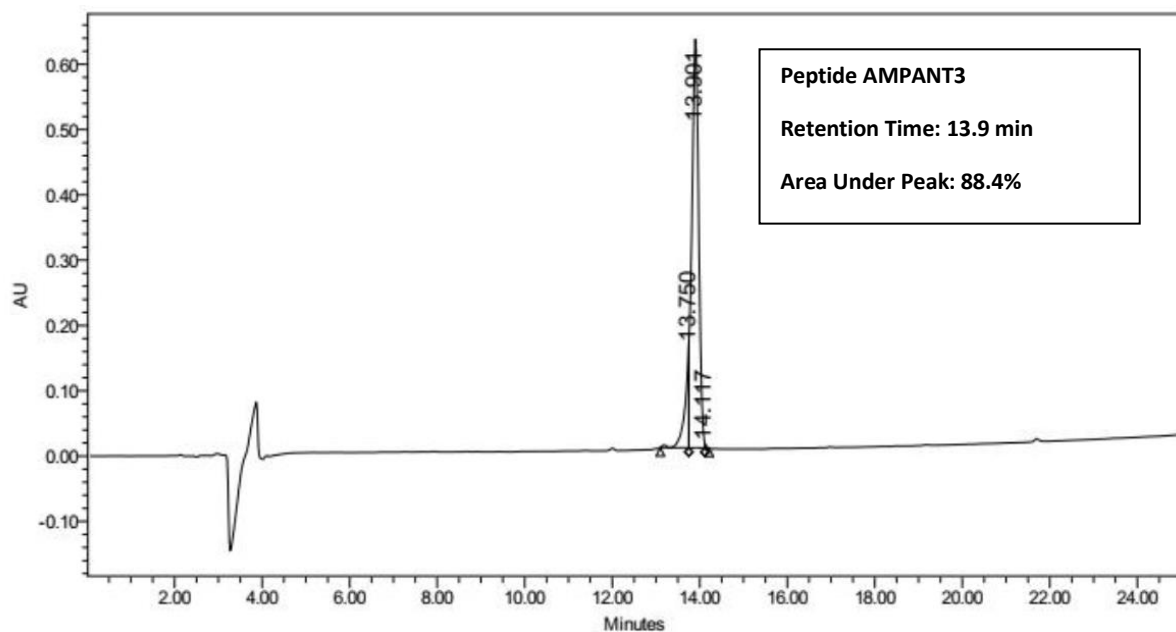


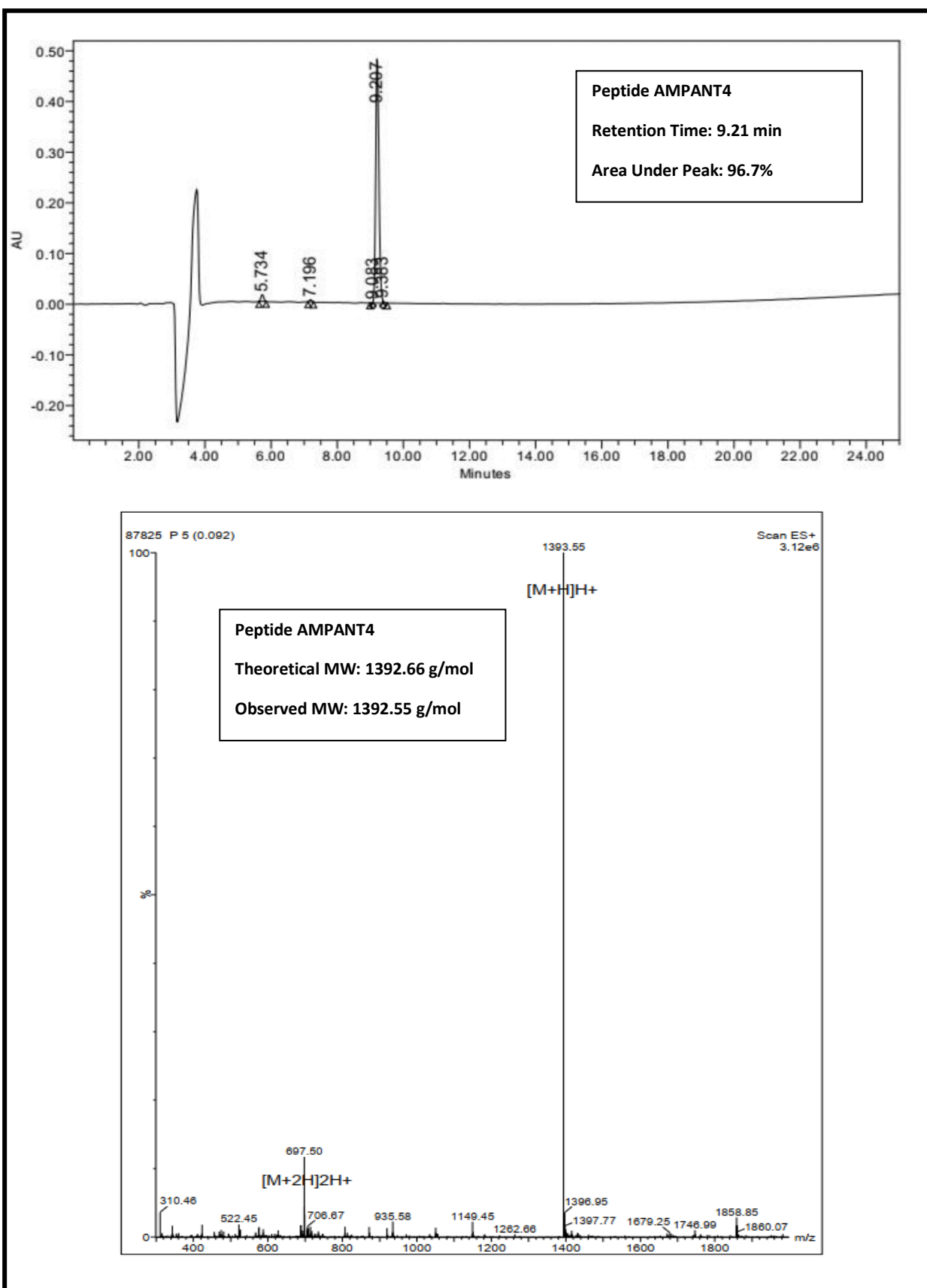


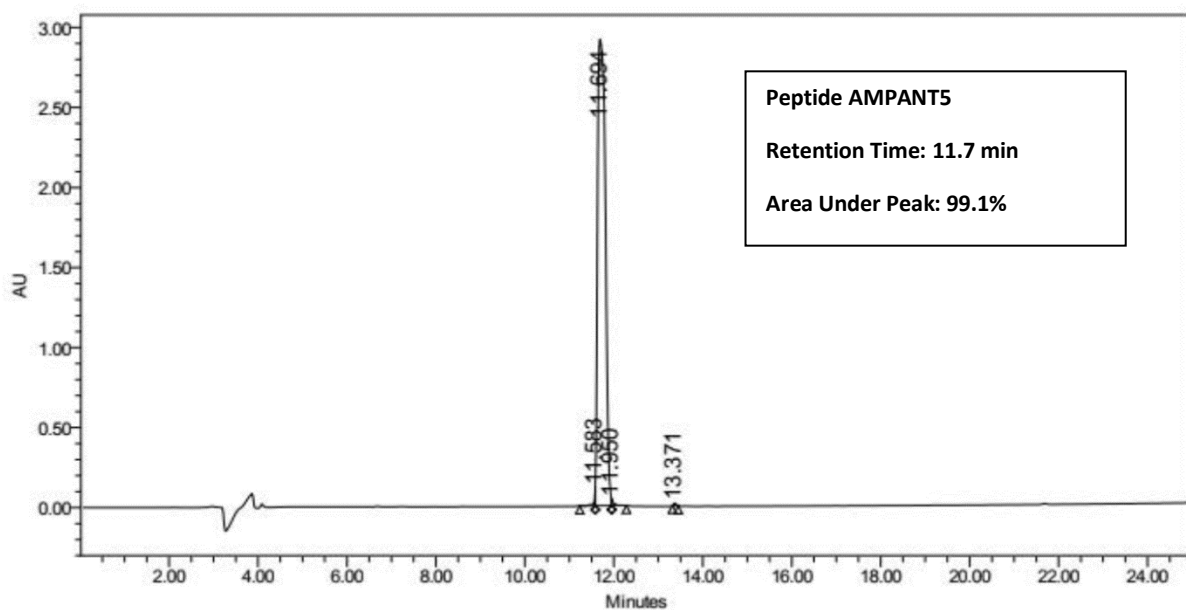








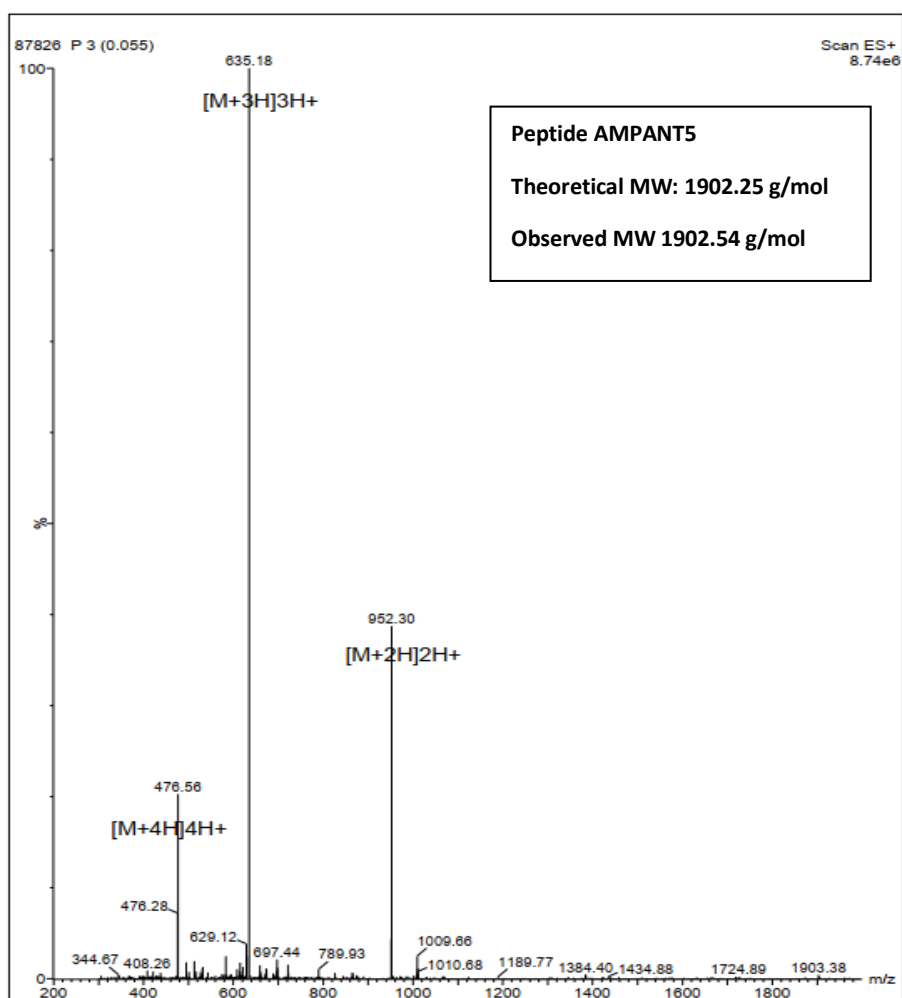




Peptide AMPANT5

Retention Time: 11.7 min

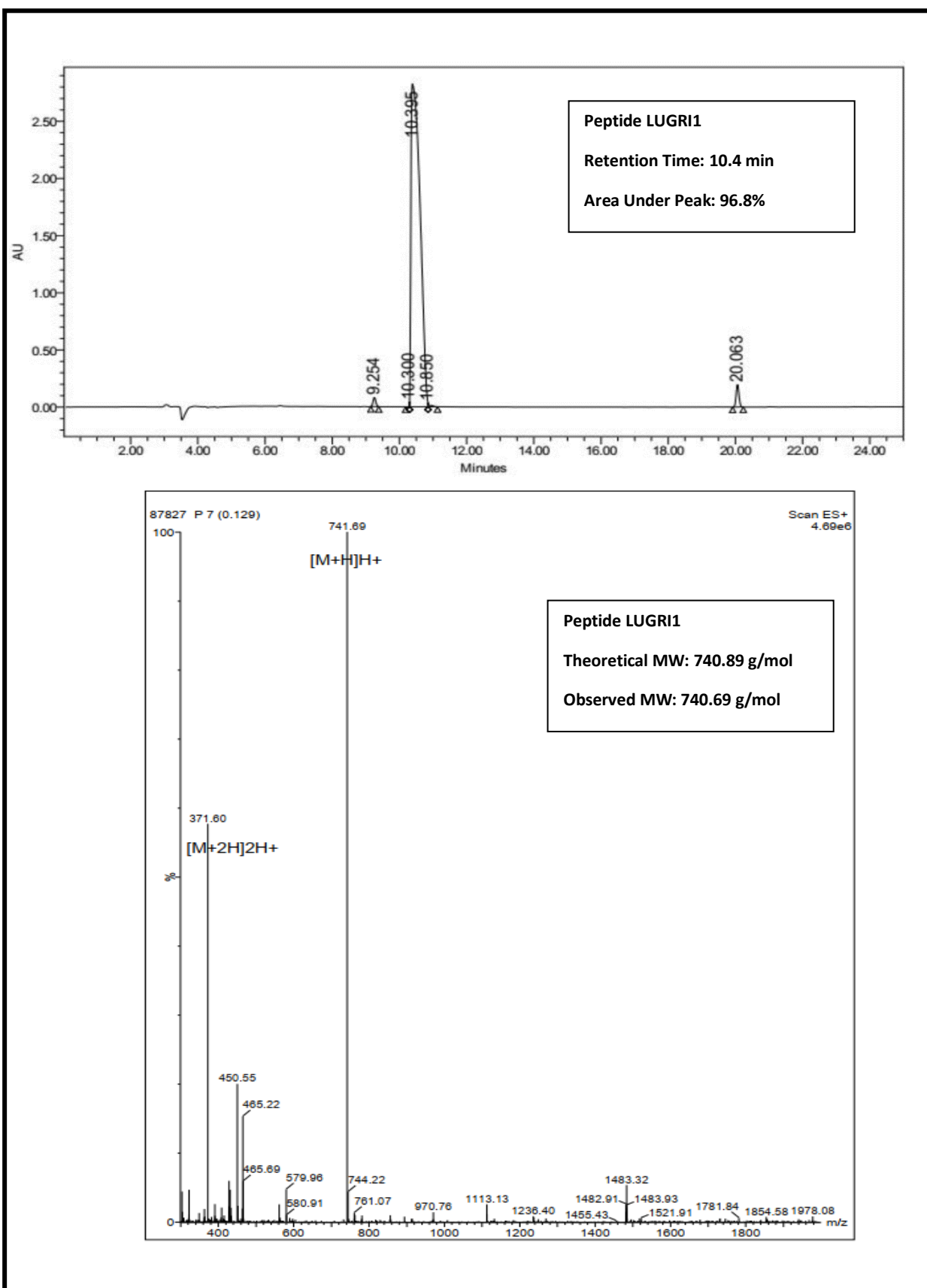
Area Under Peak: 99.1%

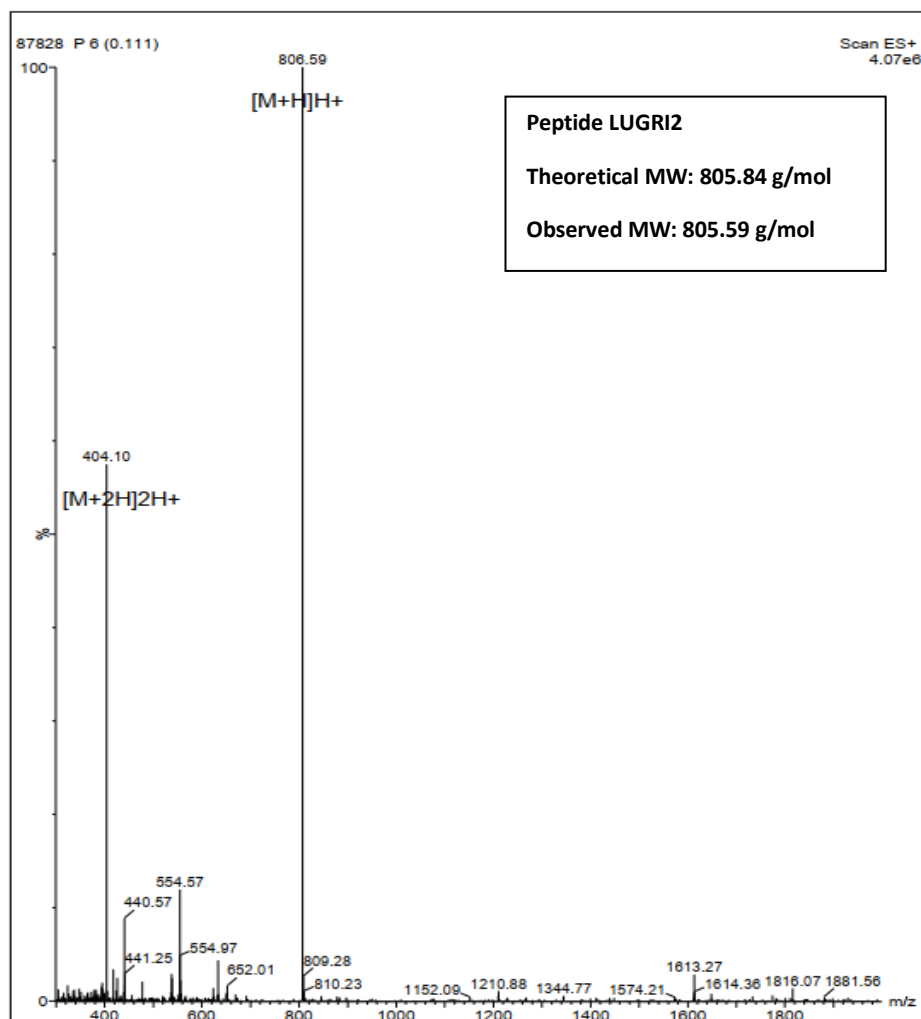
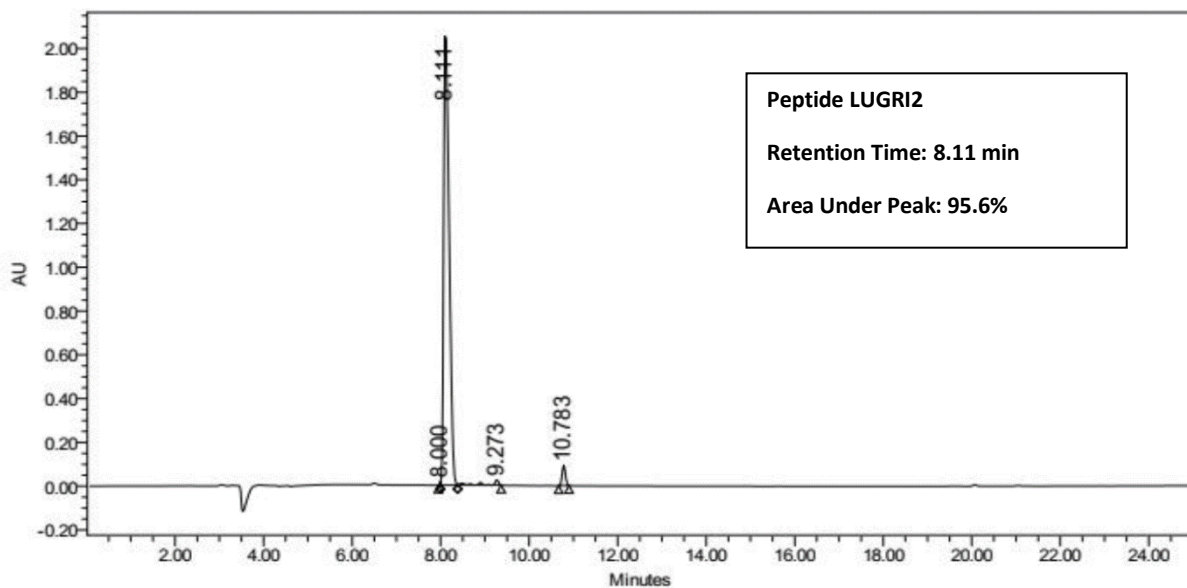


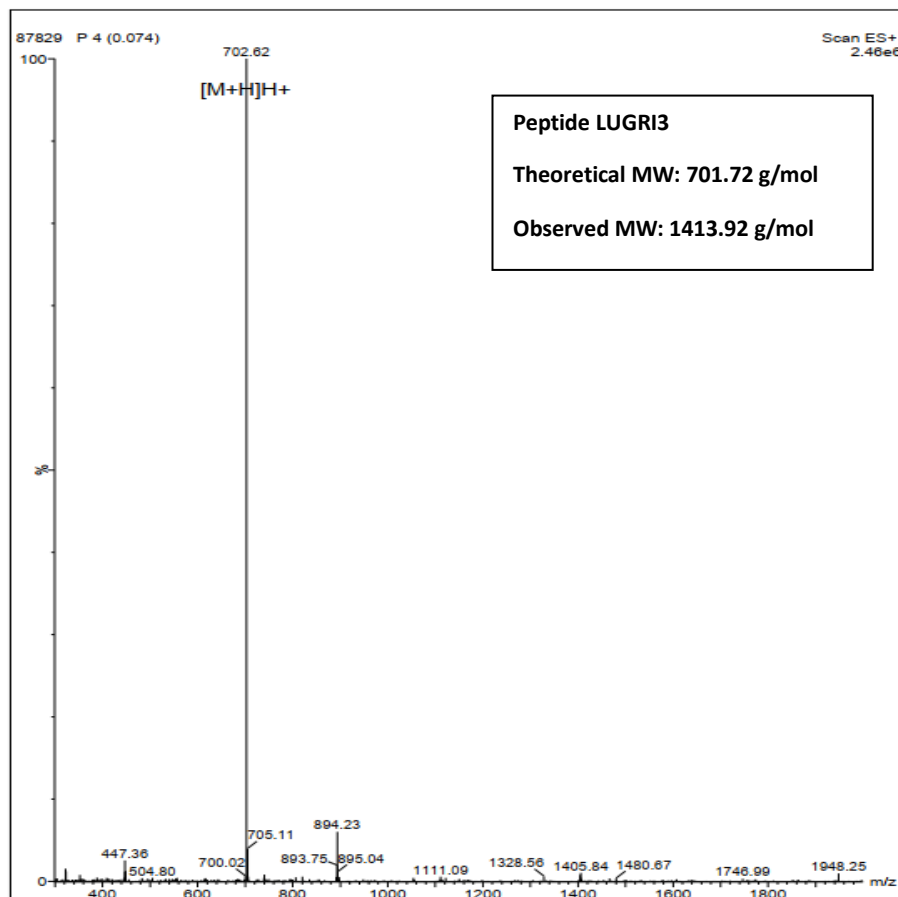
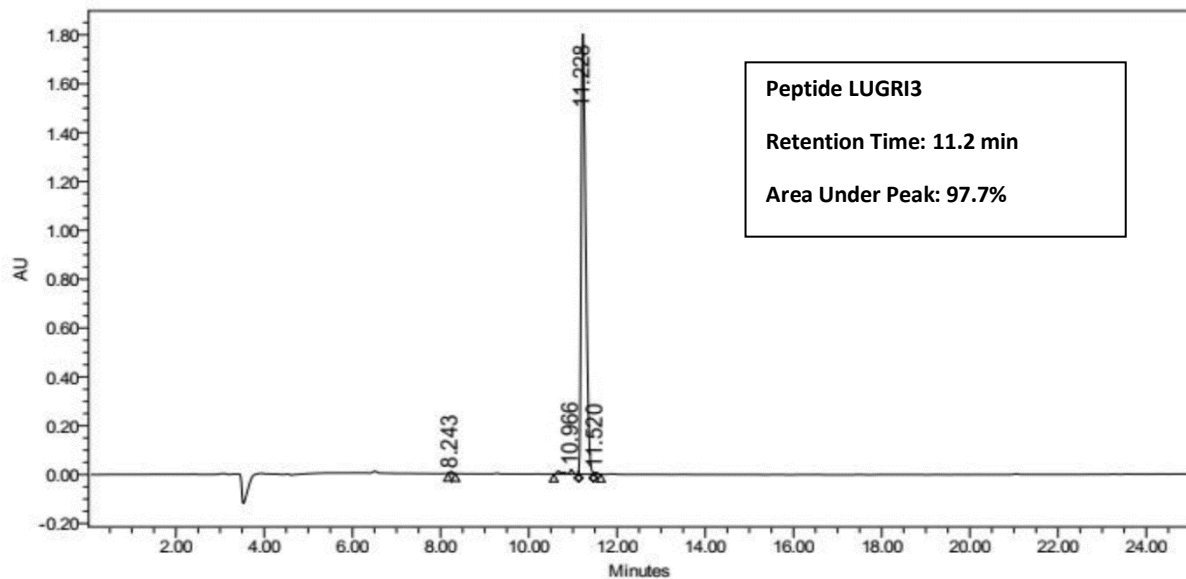
Peptide AMPANT5

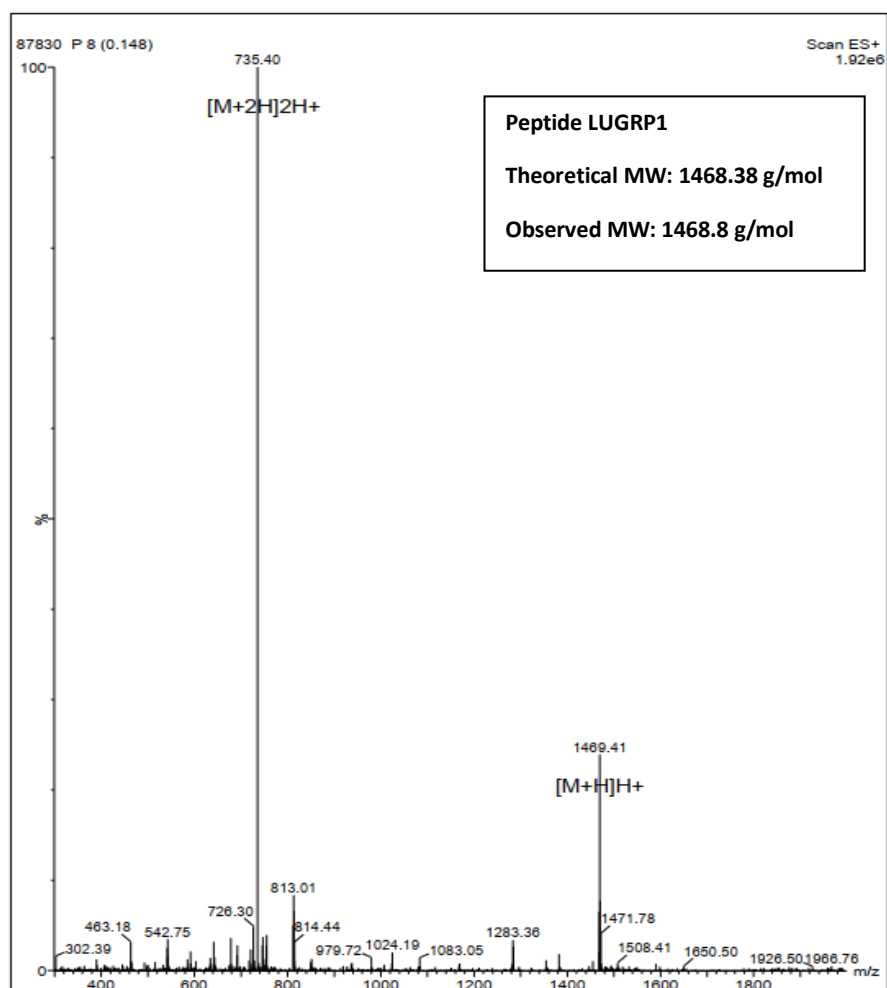
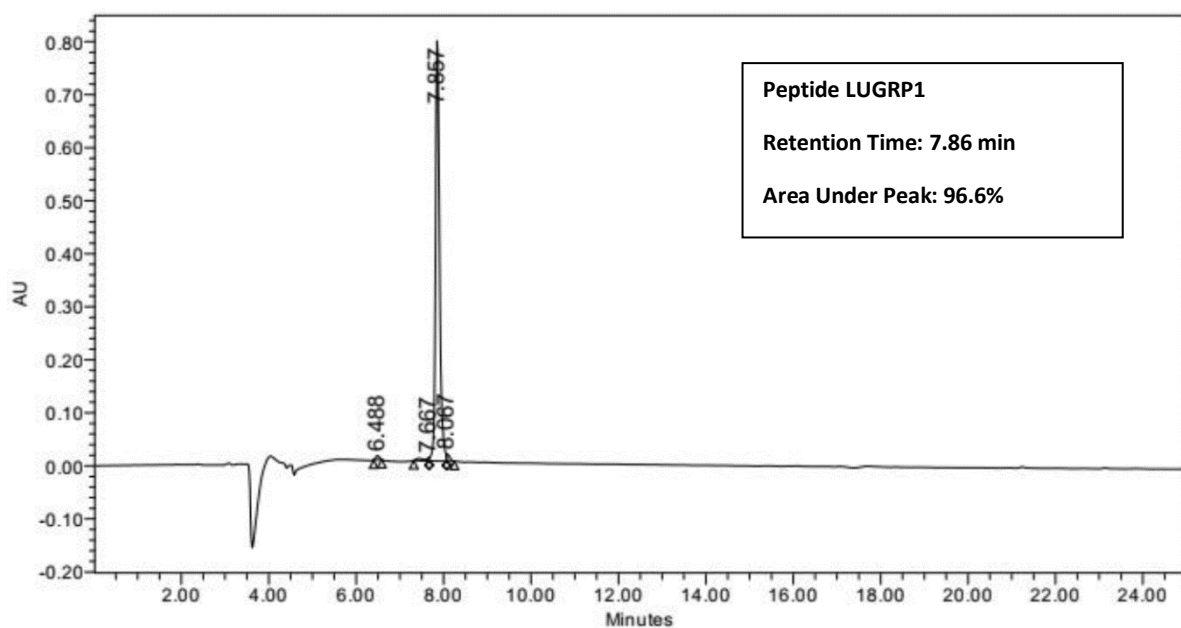
Theoretical MW: 1902.25 g/mol

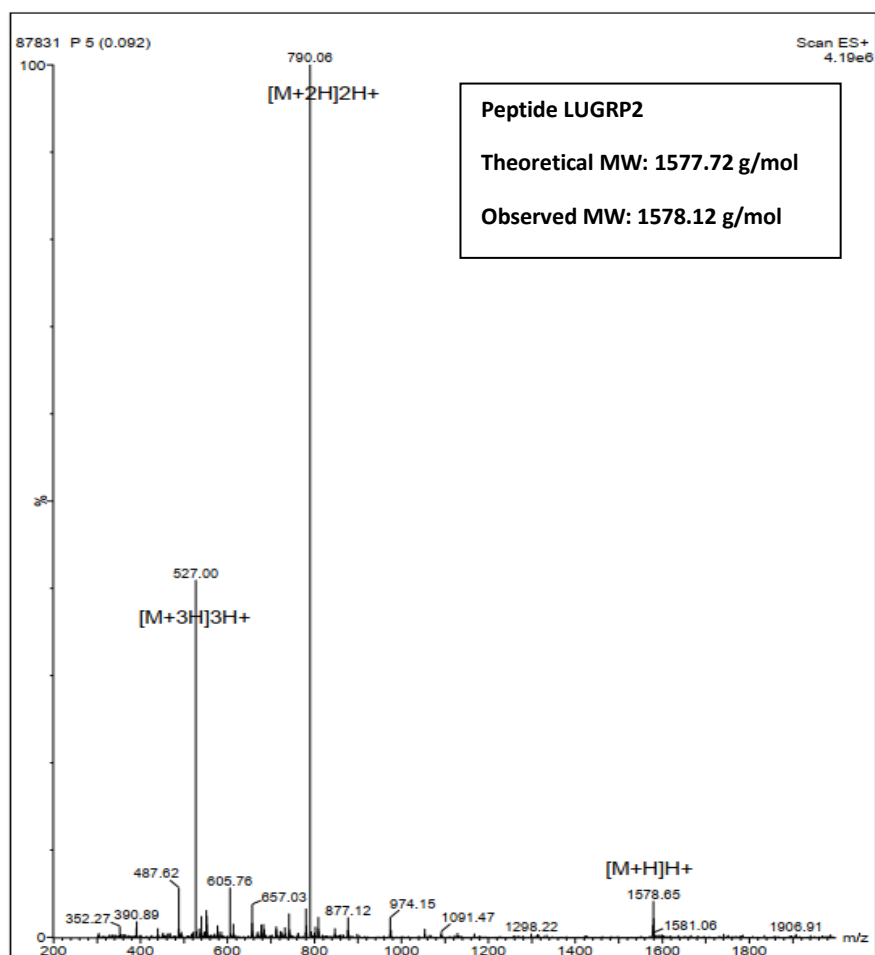
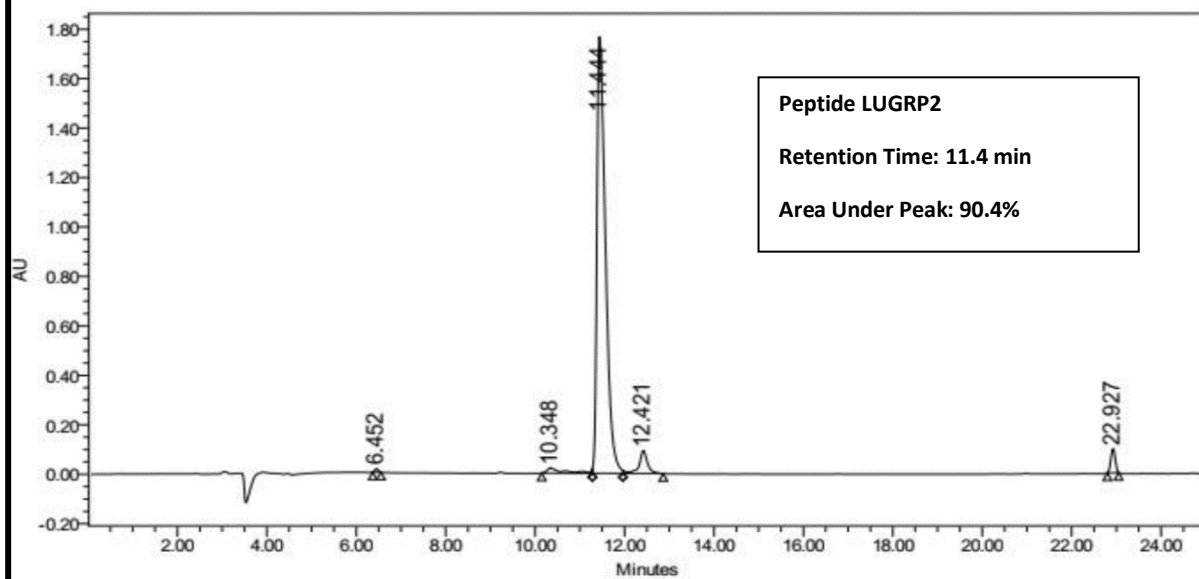
Observed MW 1902.54 g/mol

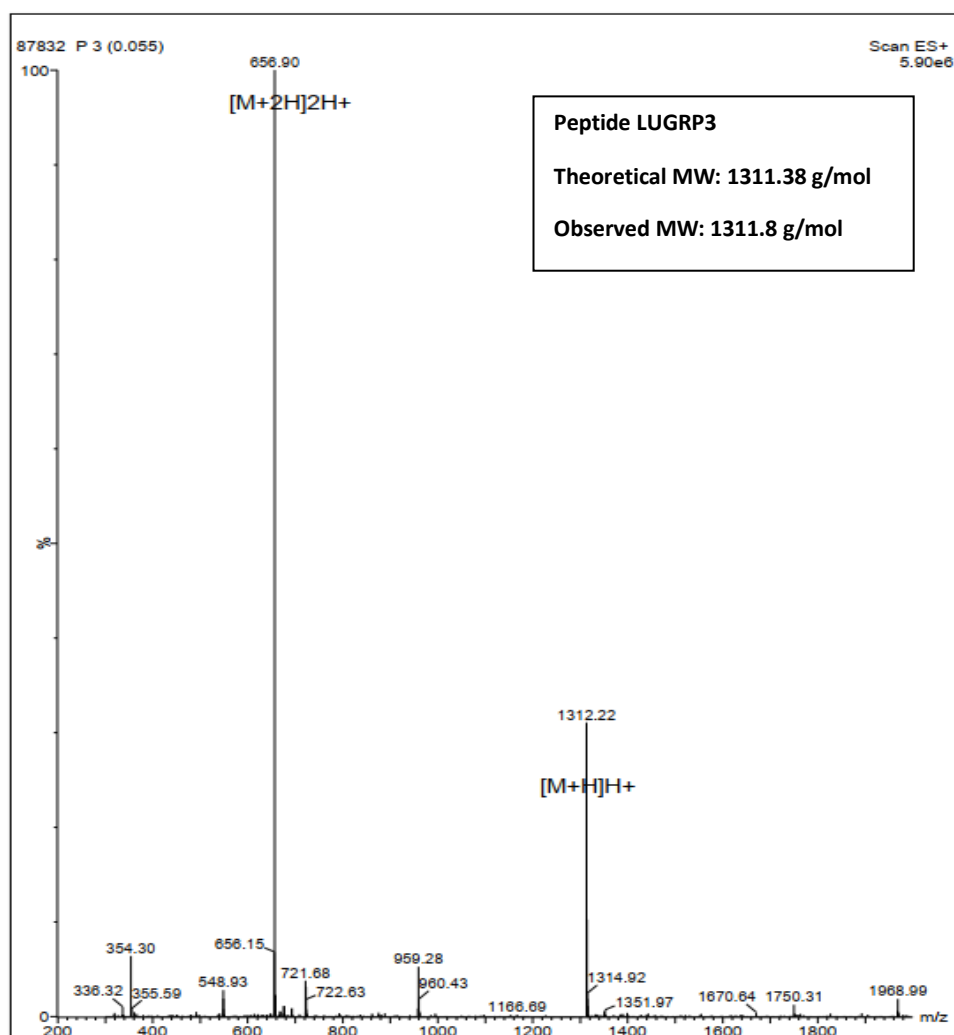
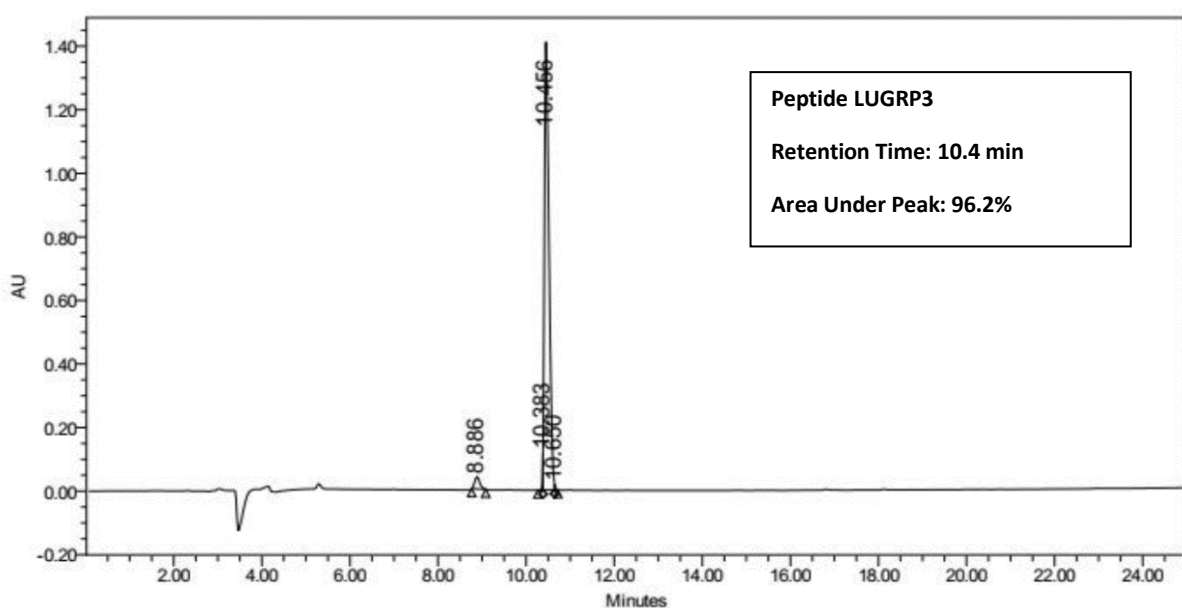


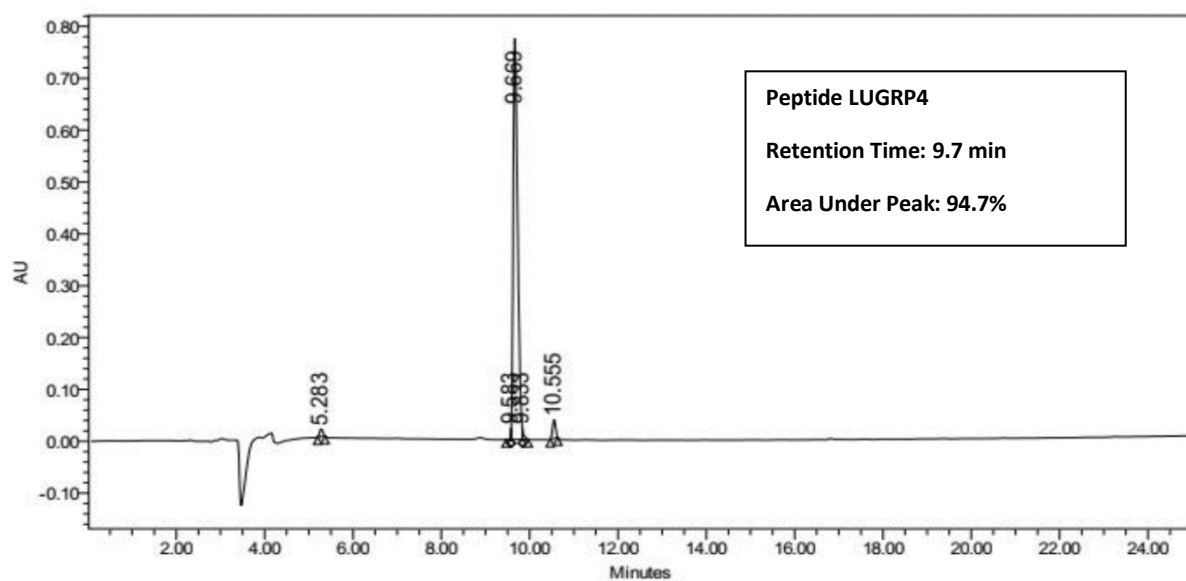








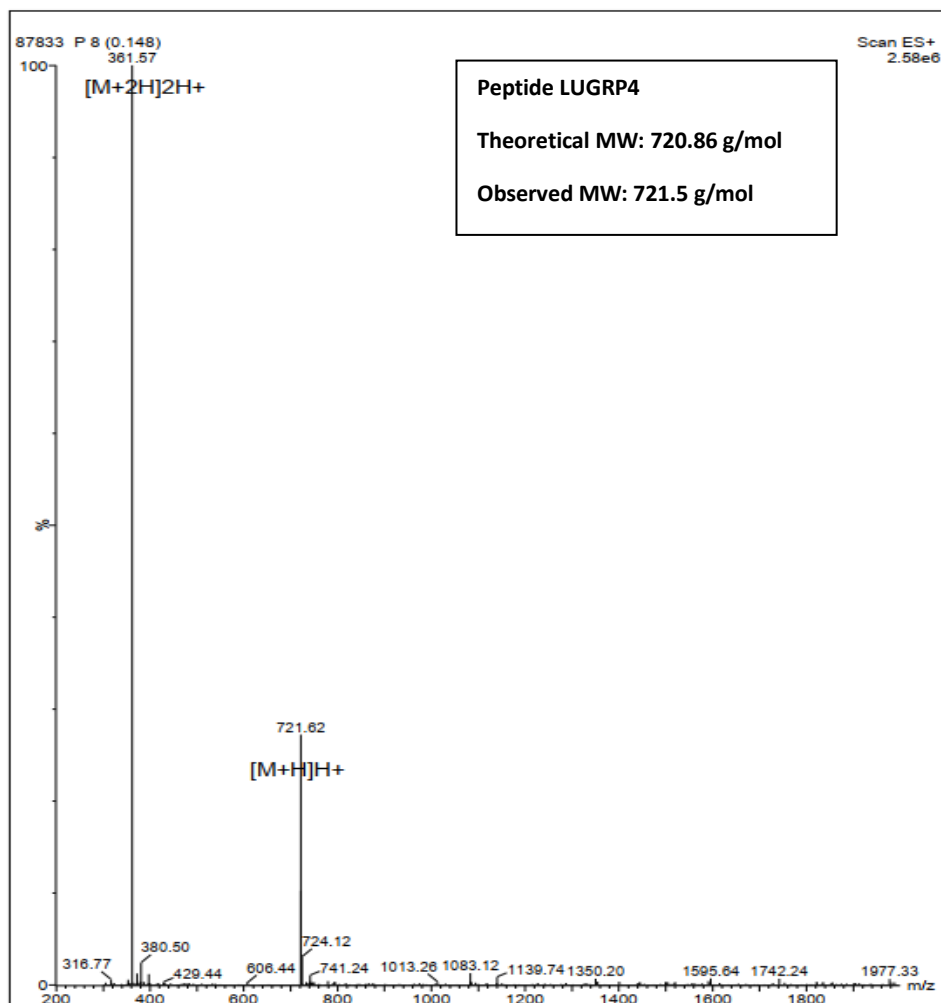




Peptide LUGRP4

Retention Time: 9.7 min

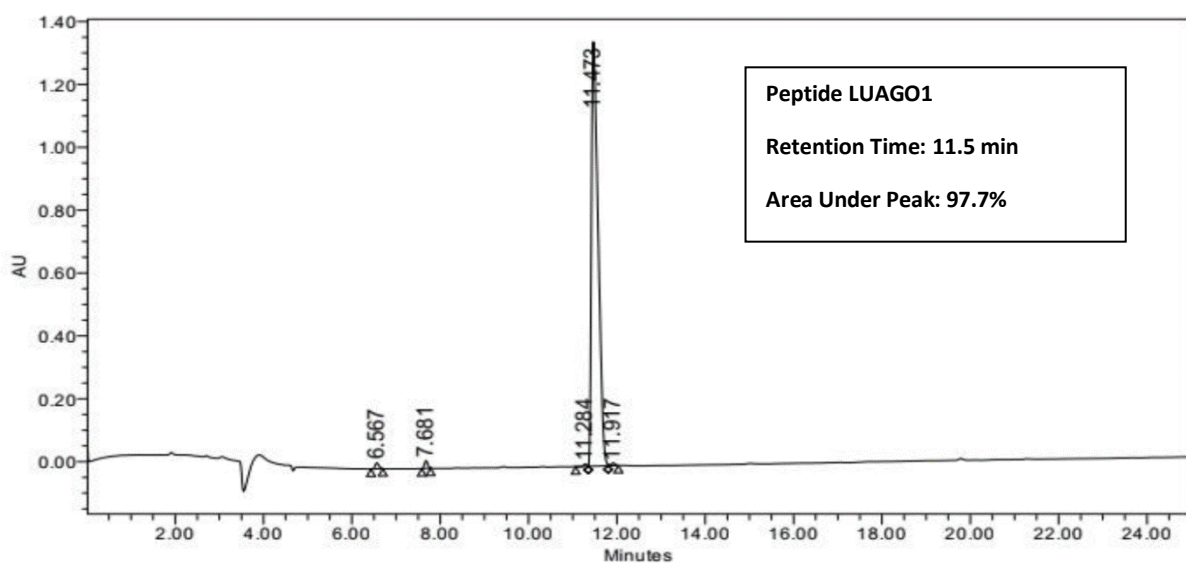
Area Under Peak: 94.7%



Peptide LUGRP4

Theoretical MW: 720.86 g/mol

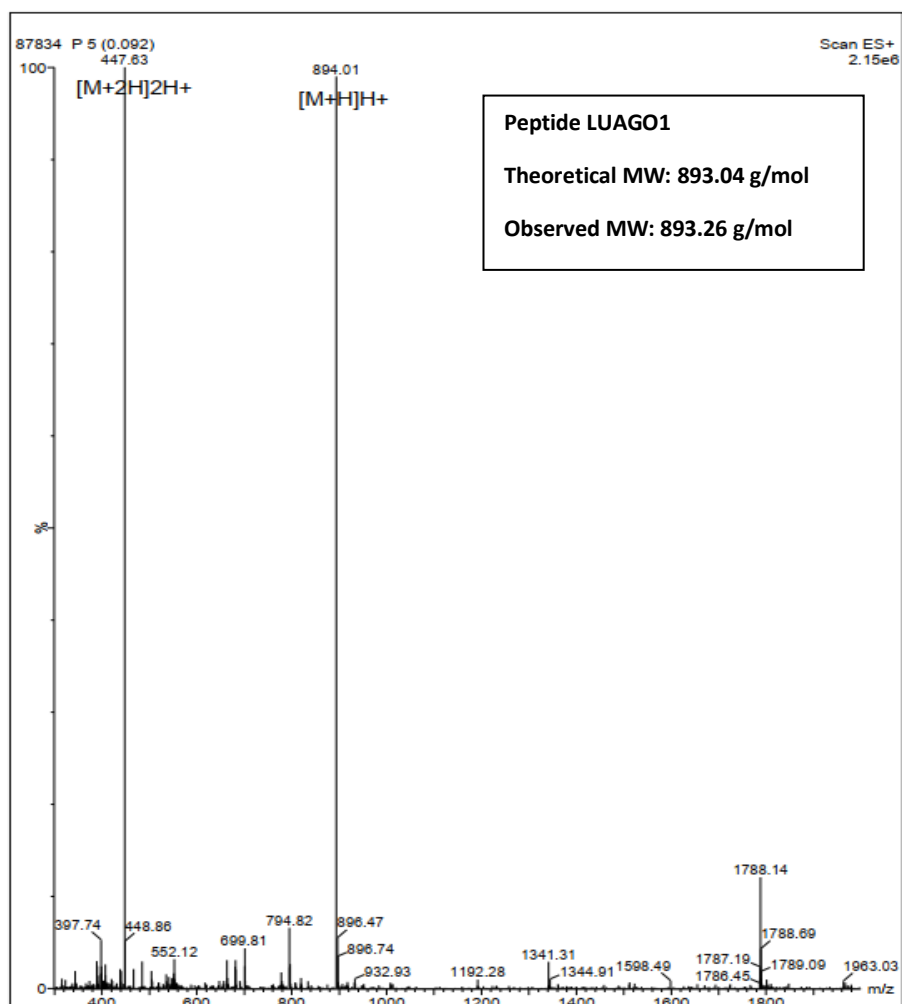
Observed MW: 721.5 g/mol



Peptide LUAGO1

Retention Time: 11.5 min

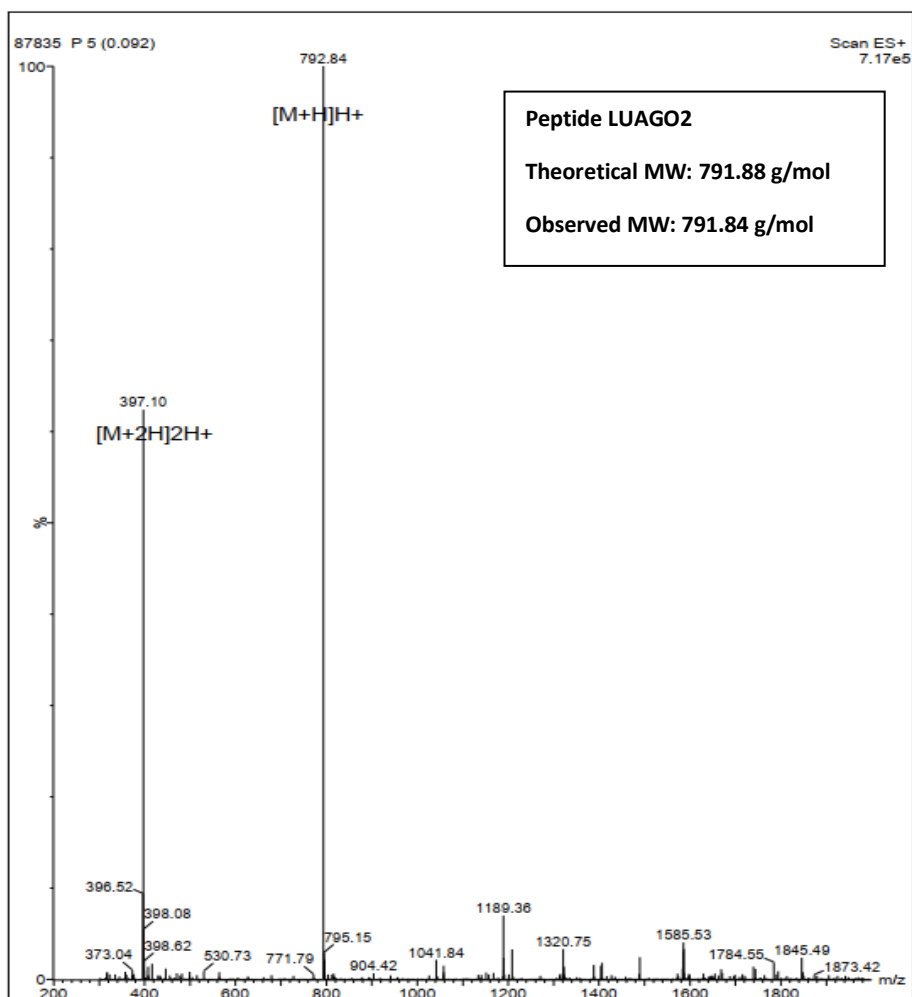
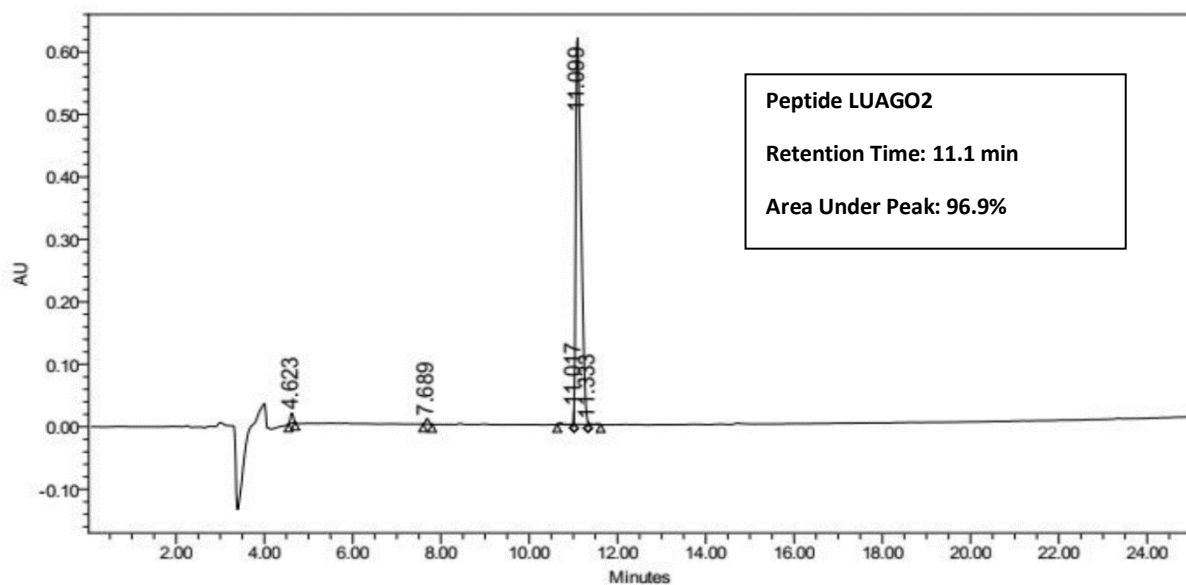
Area Under Peak: 97.7%

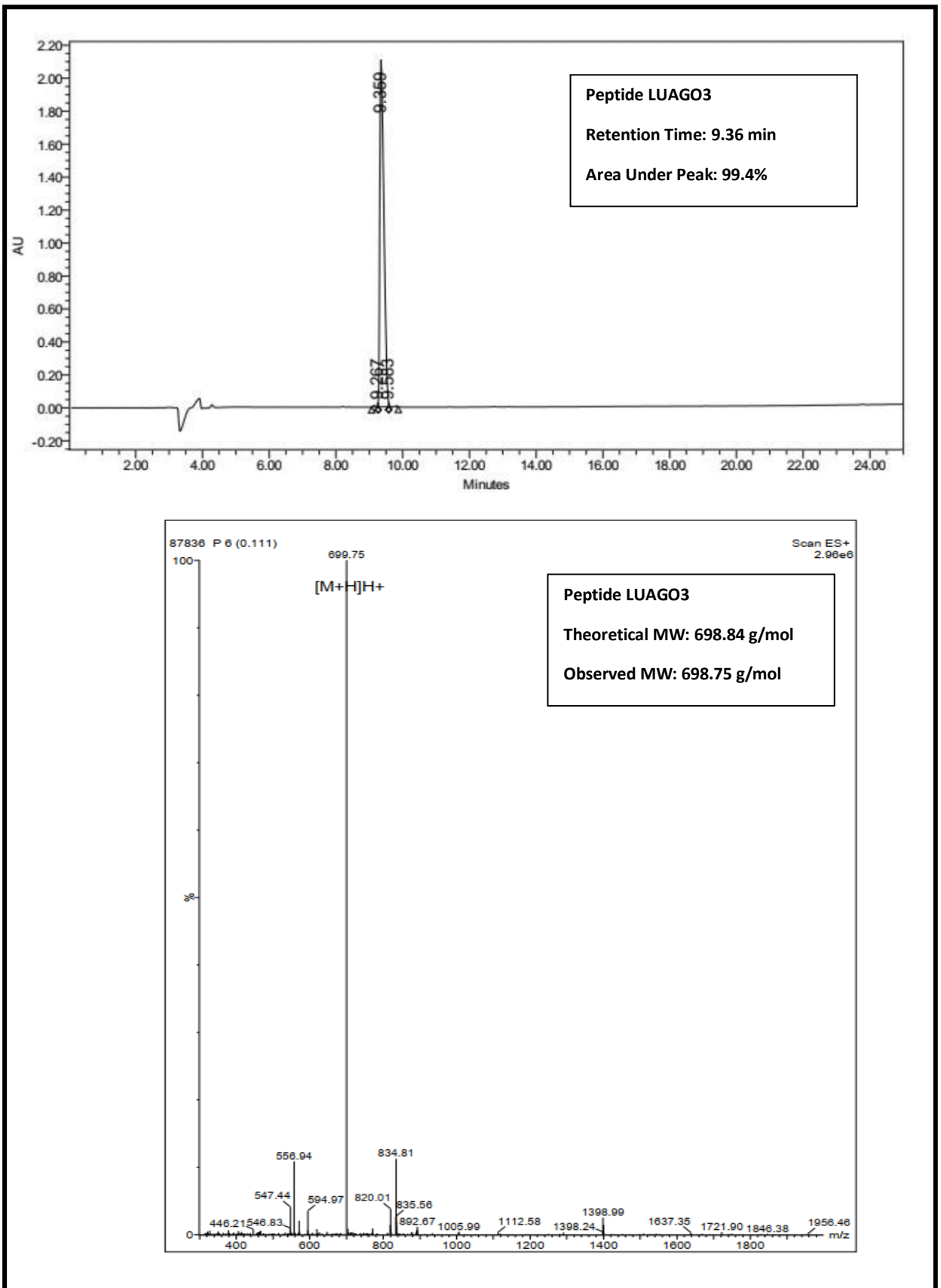


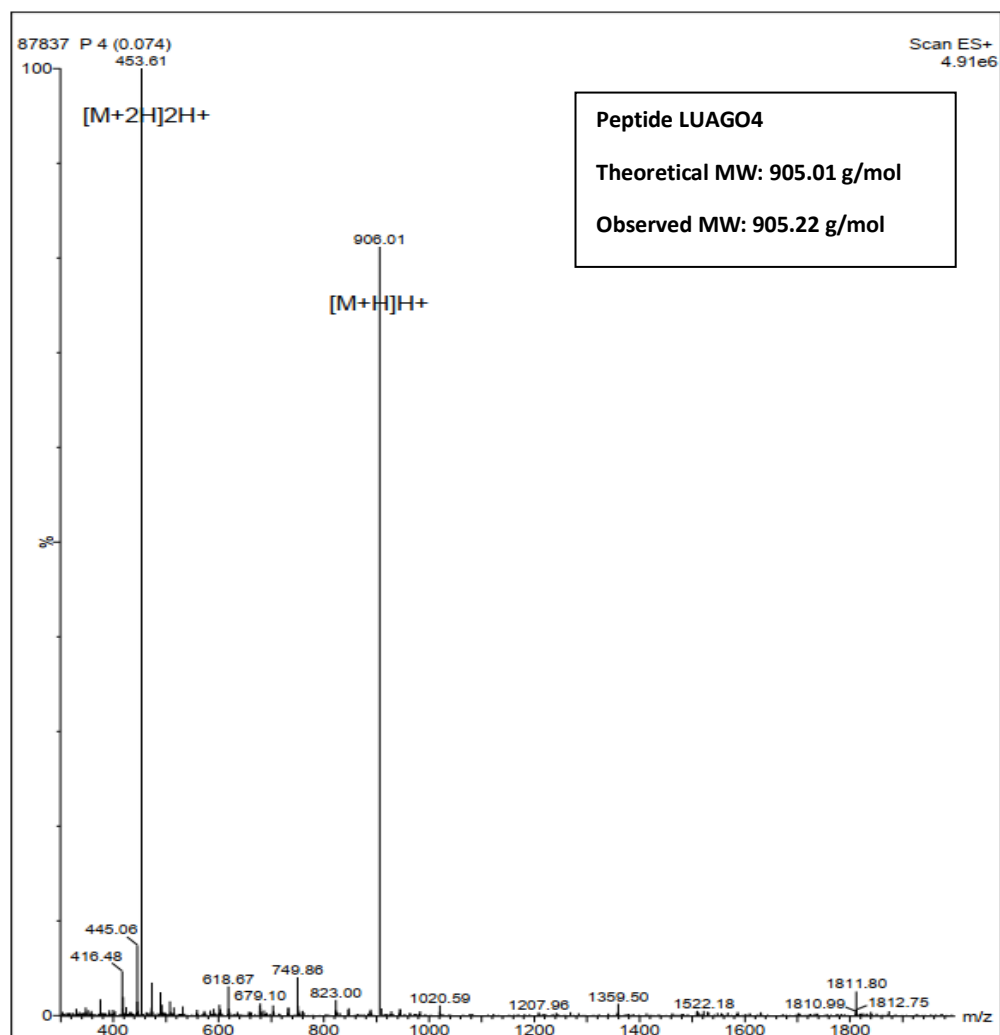
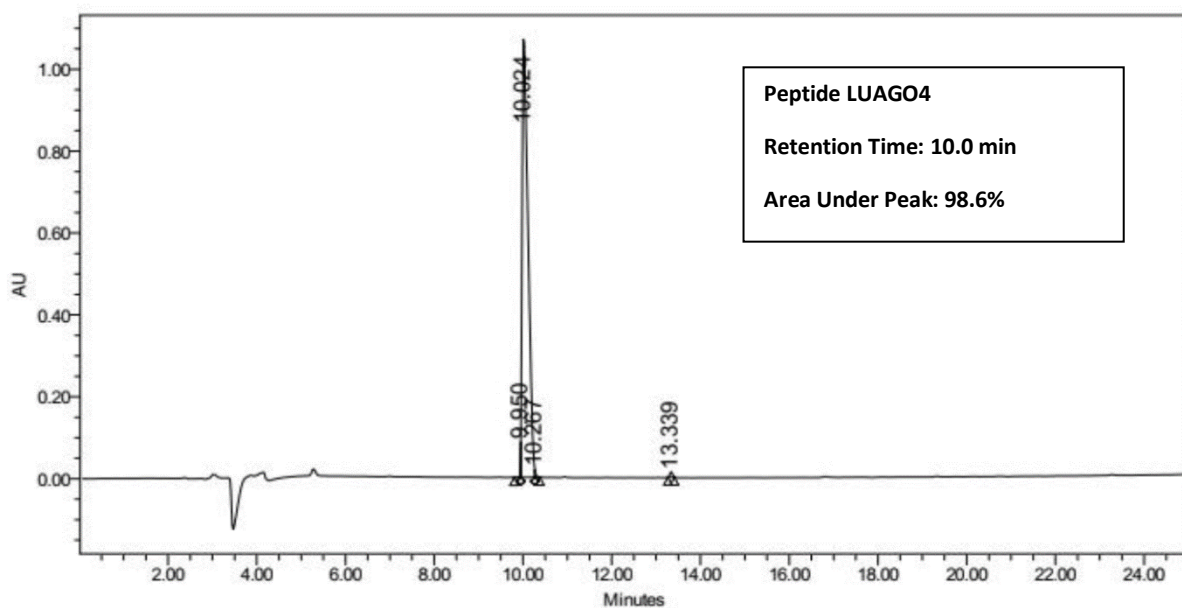
Peptide LUAGO1

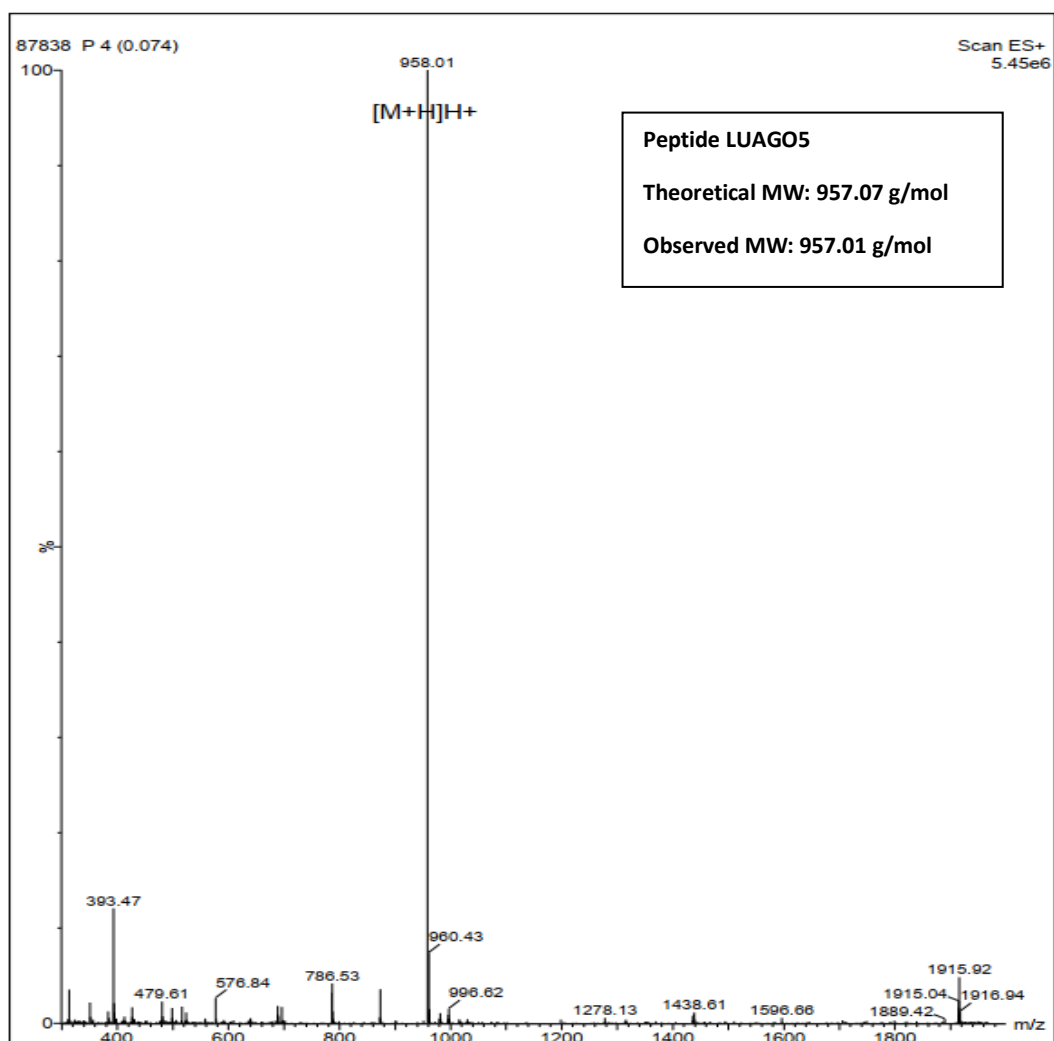
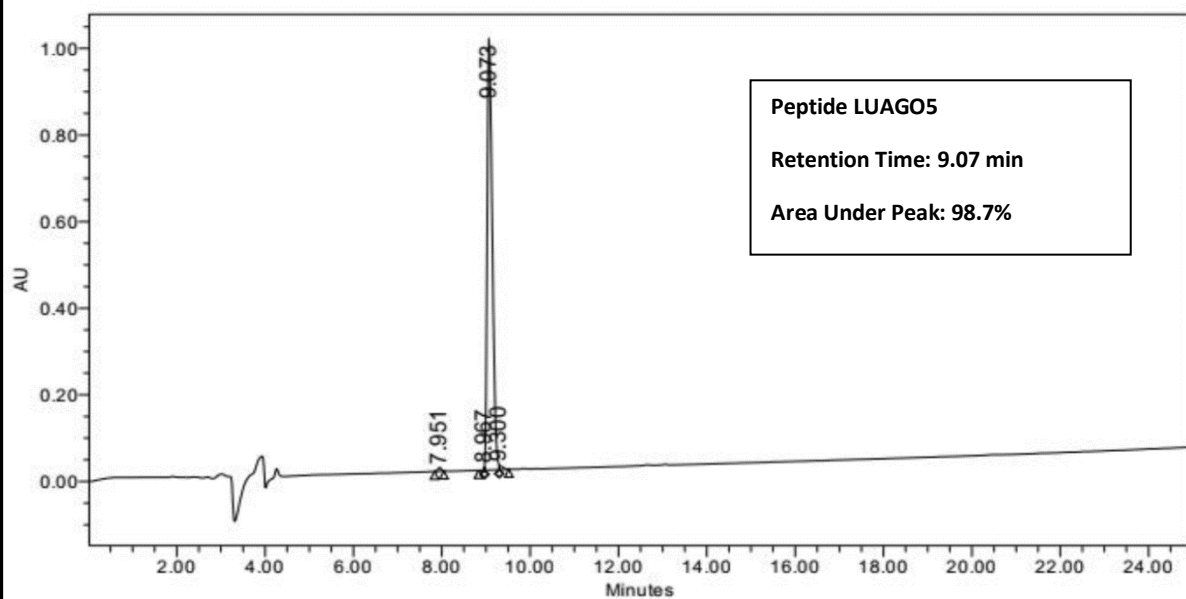
Theoretical MW: 893.04 g/mol

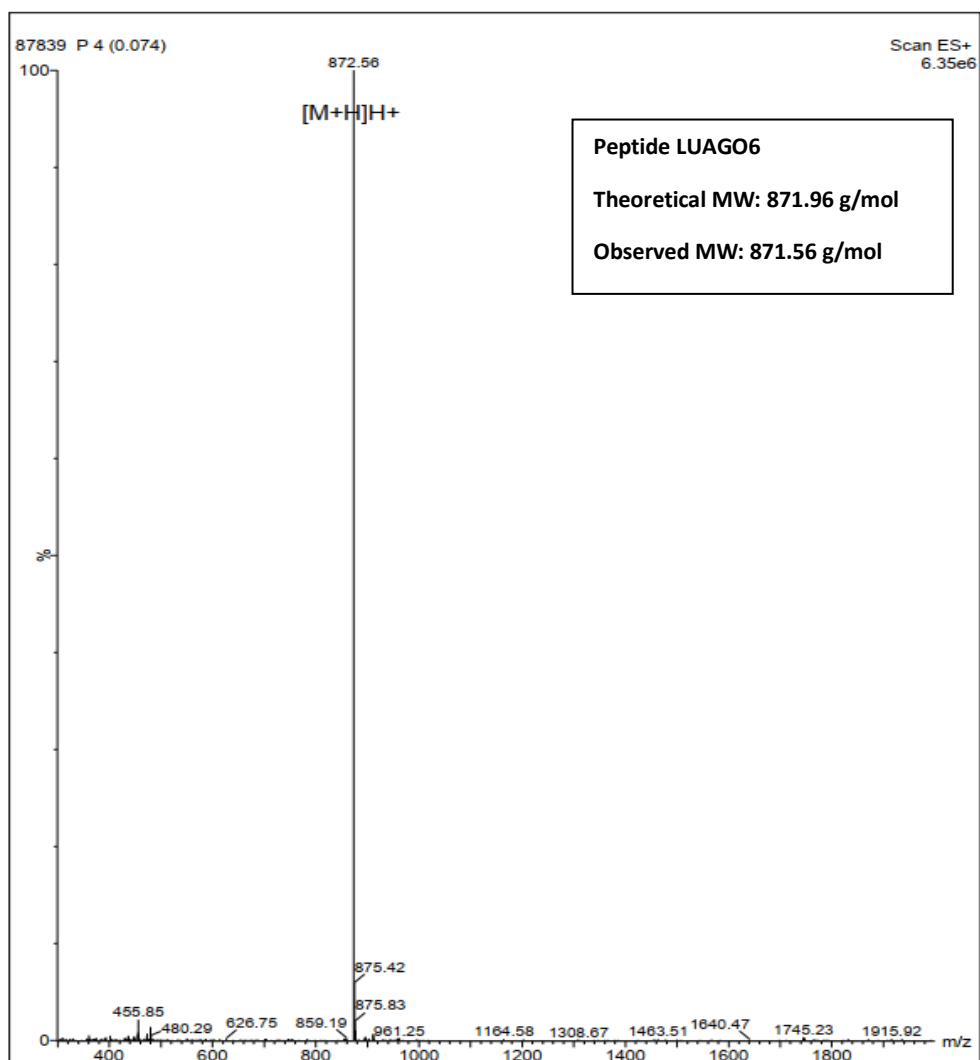
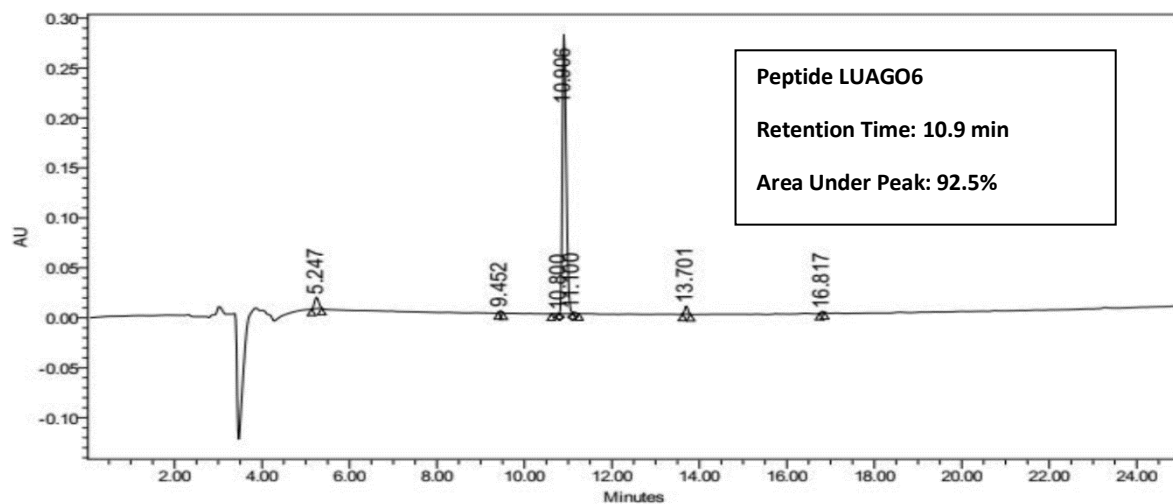
Observed MW: 893.26 g/mol

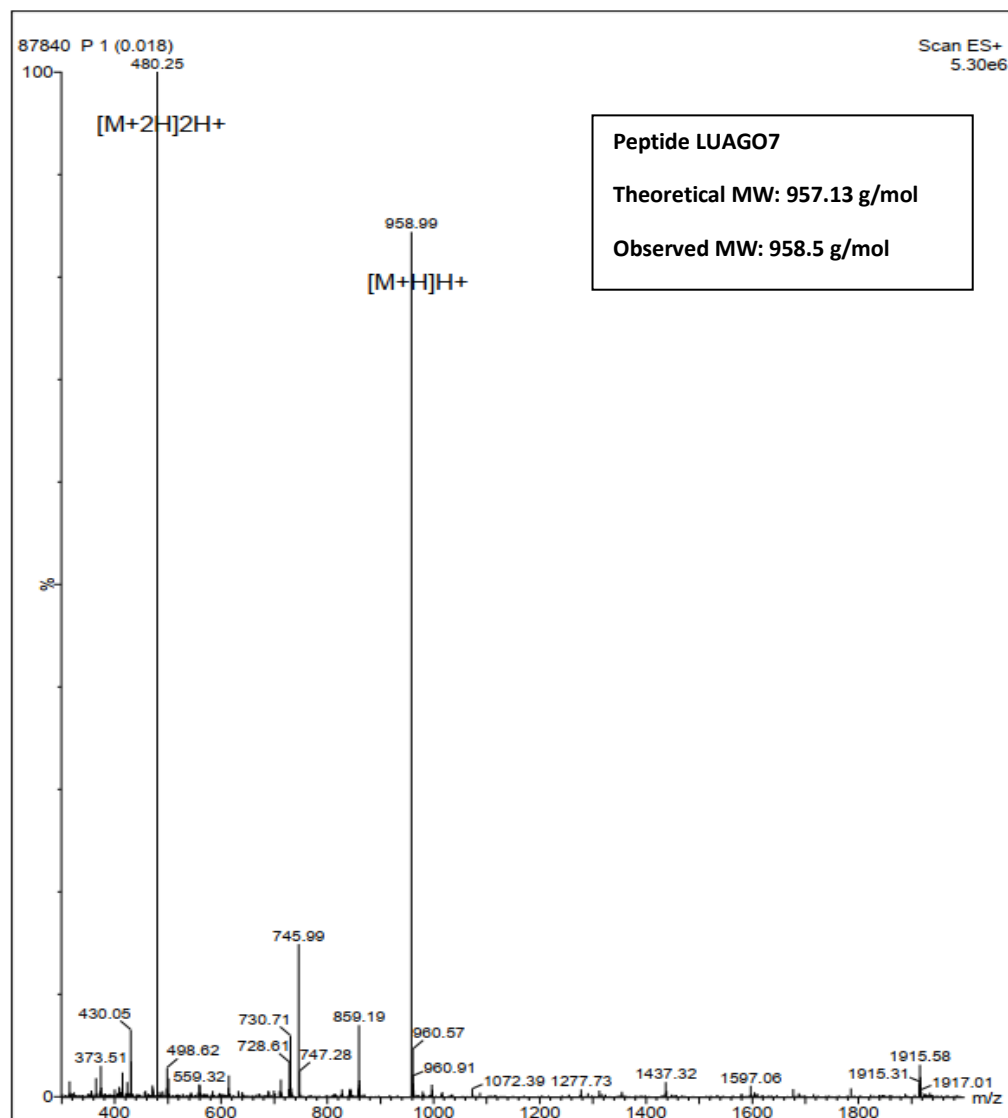
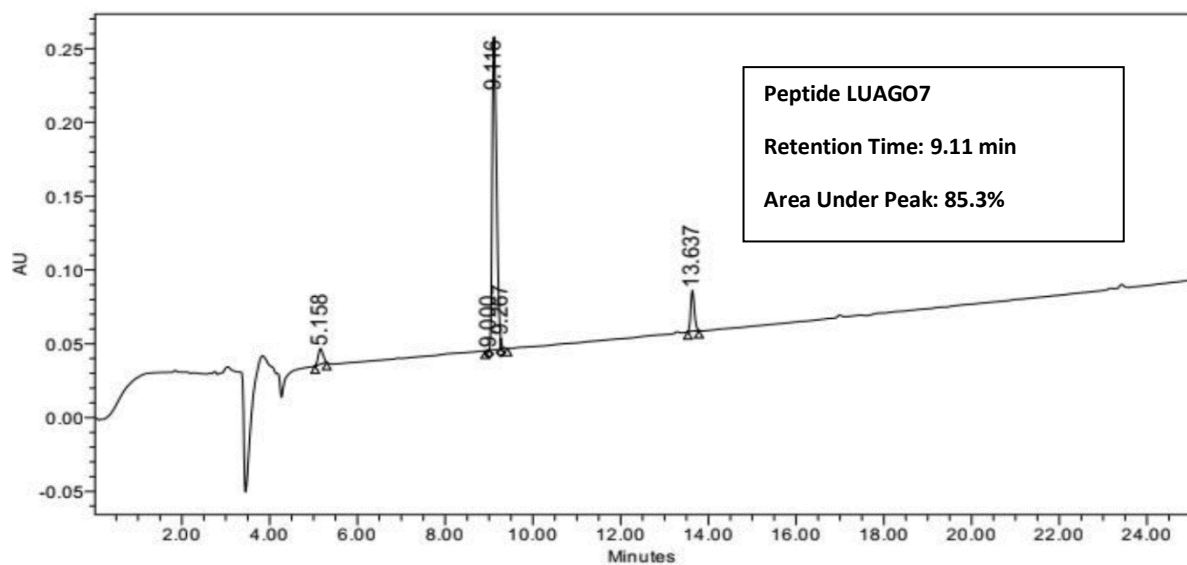


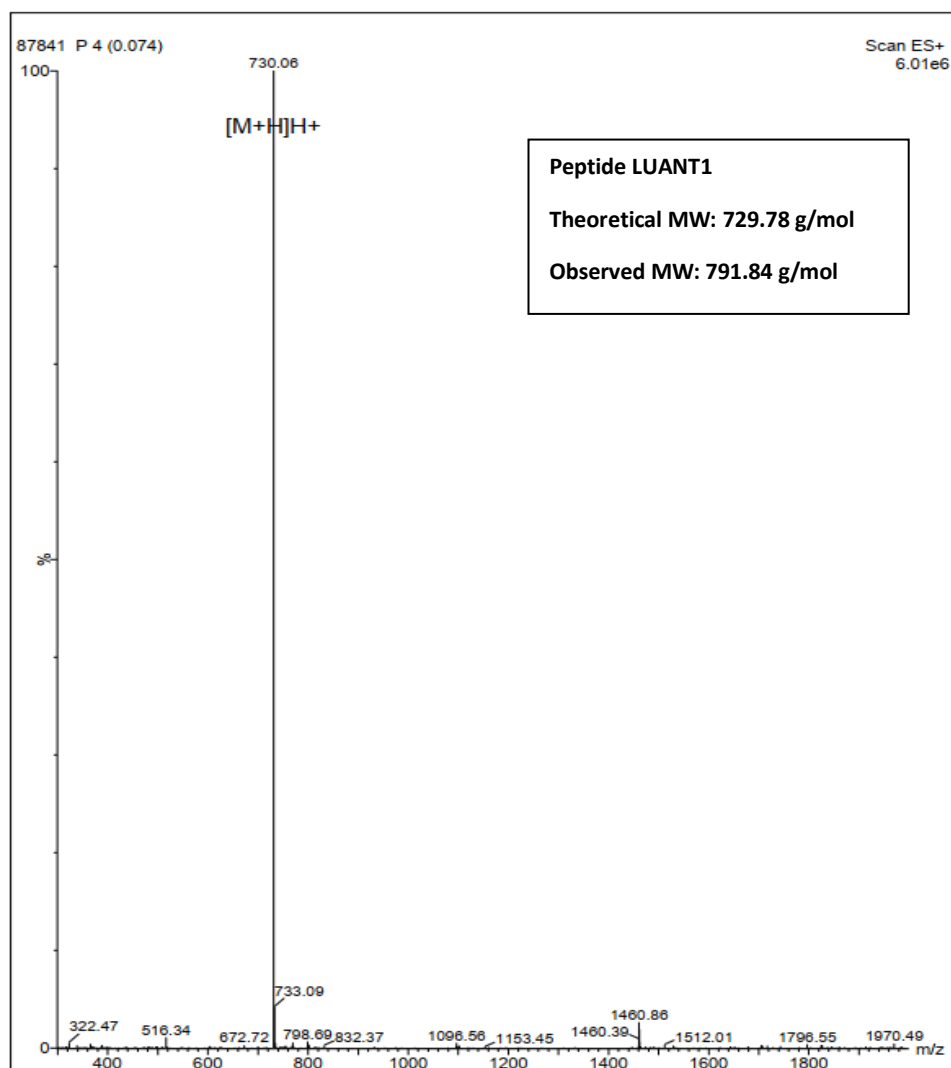
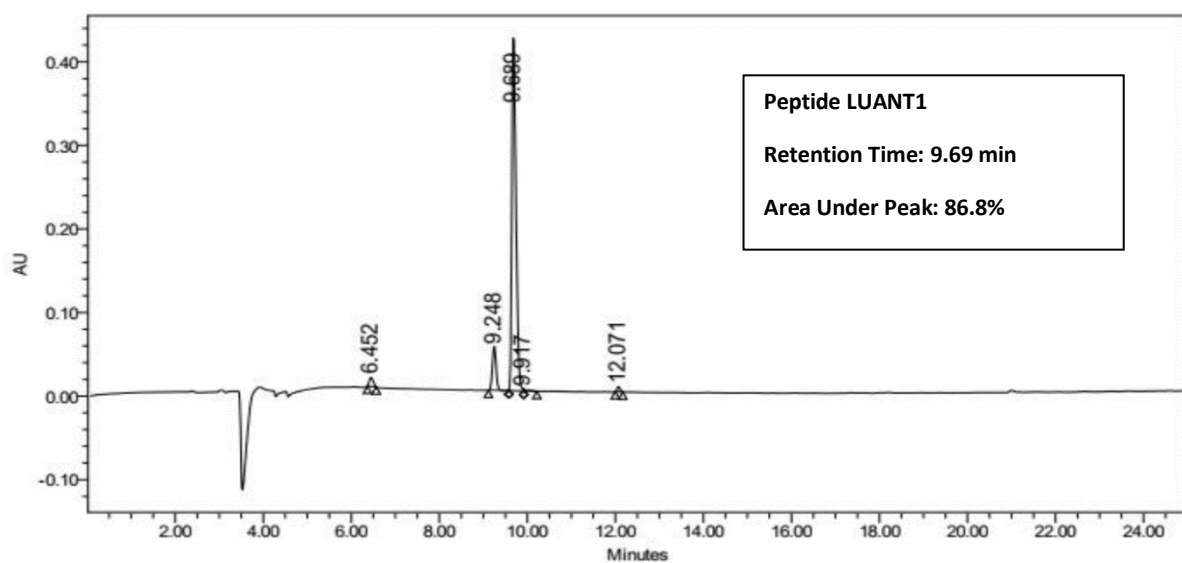


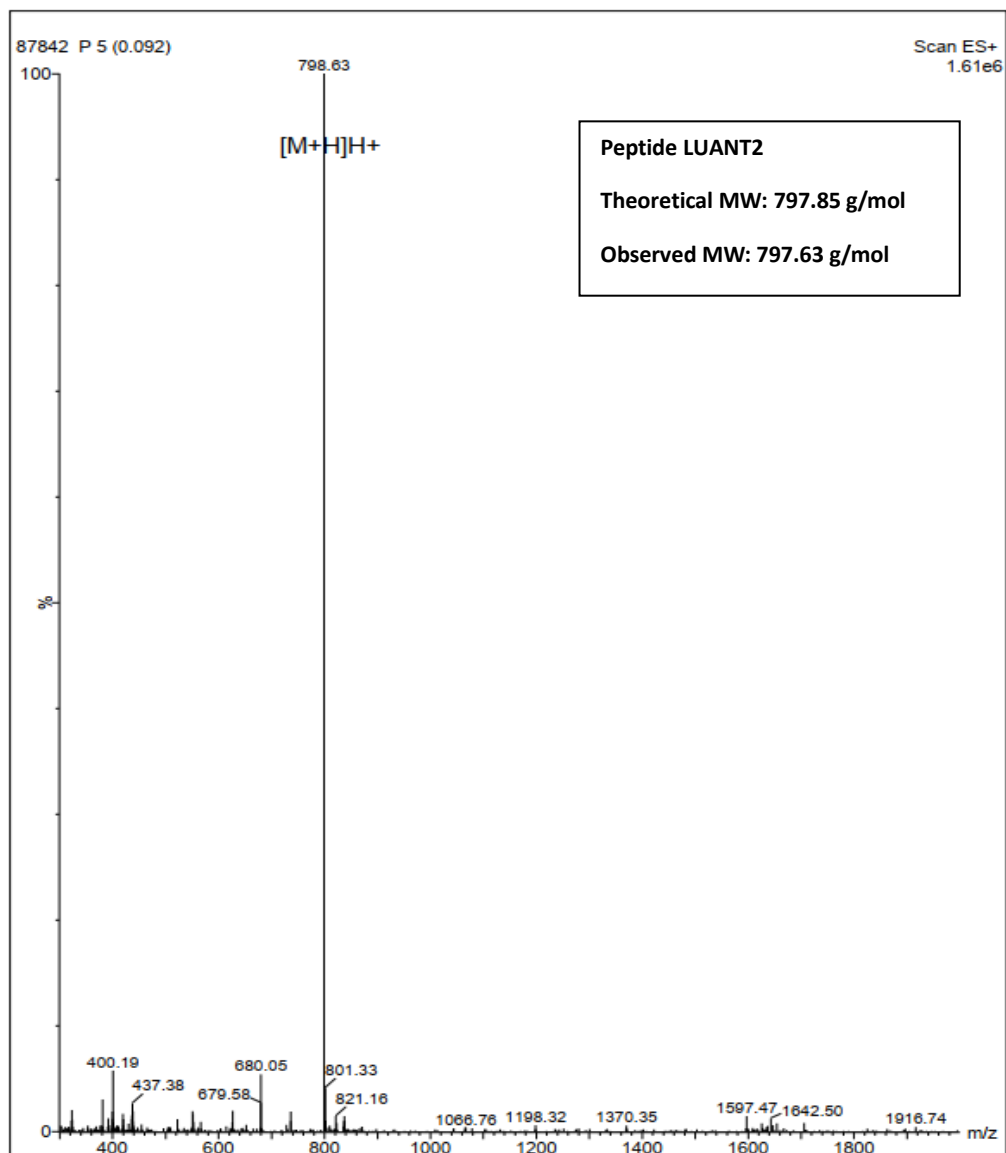
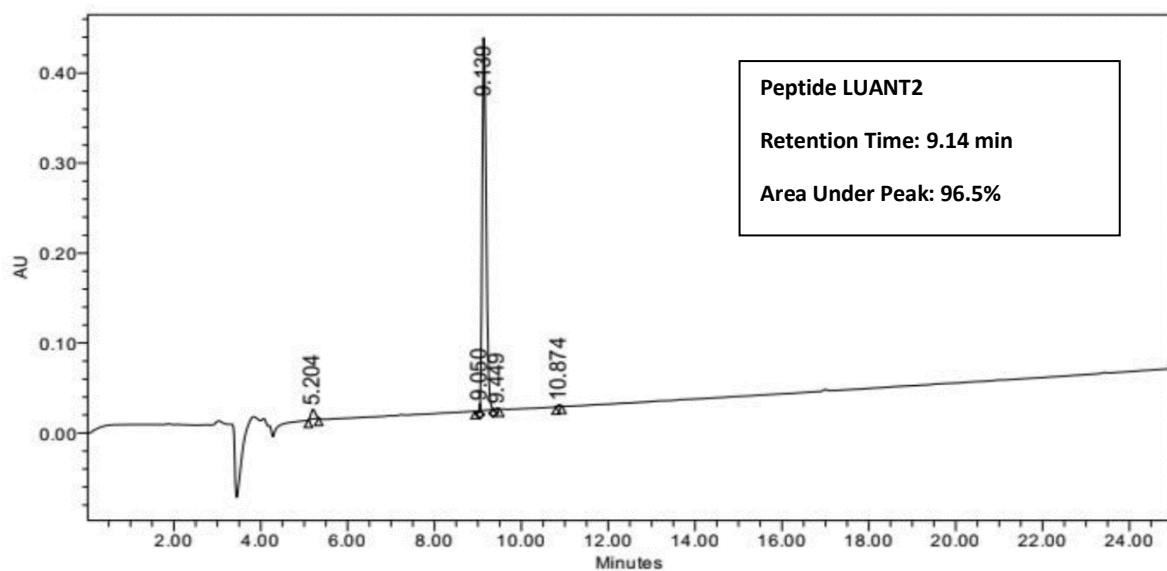


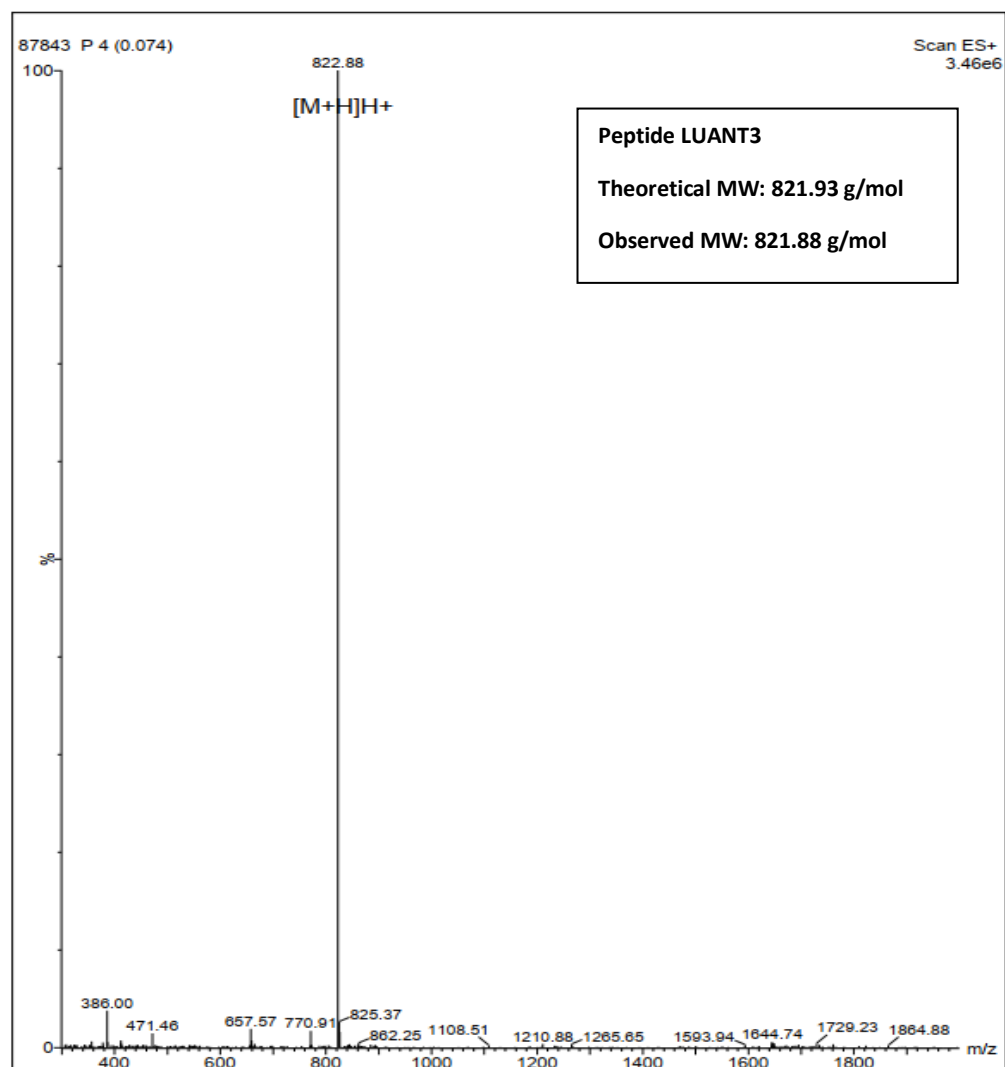
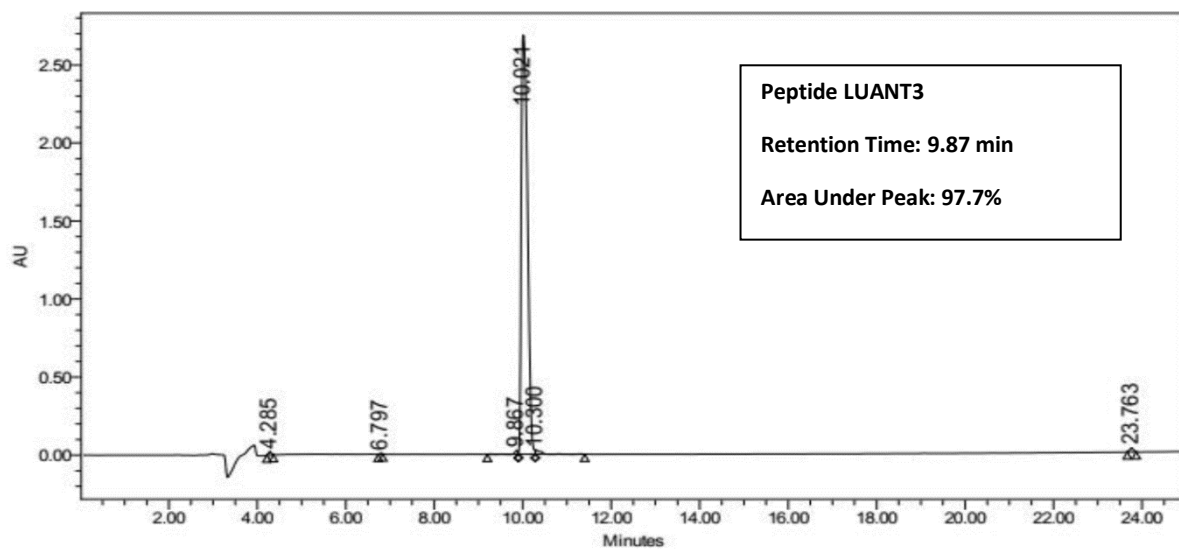


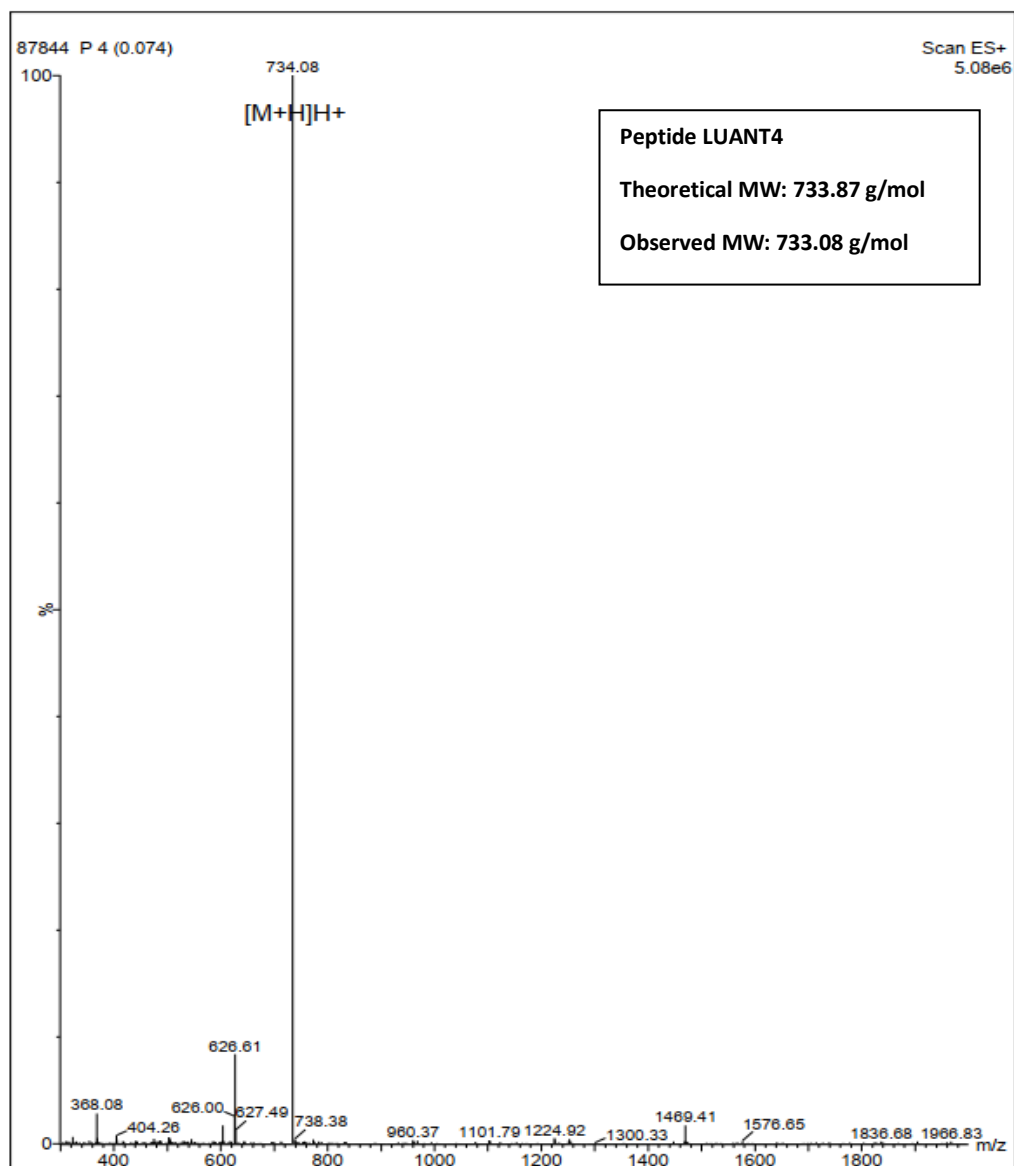
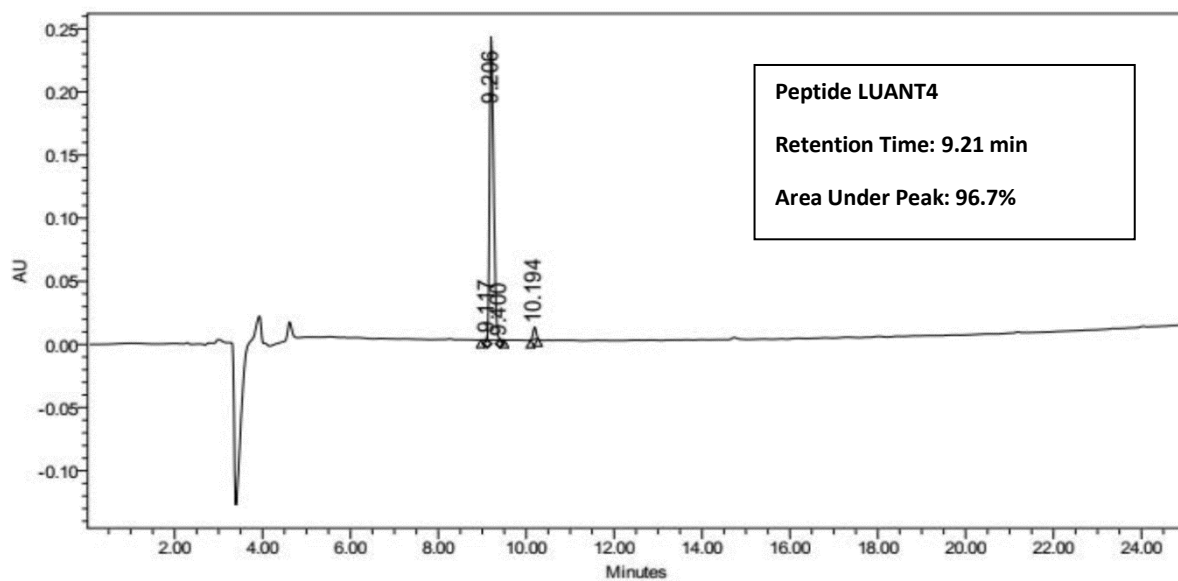


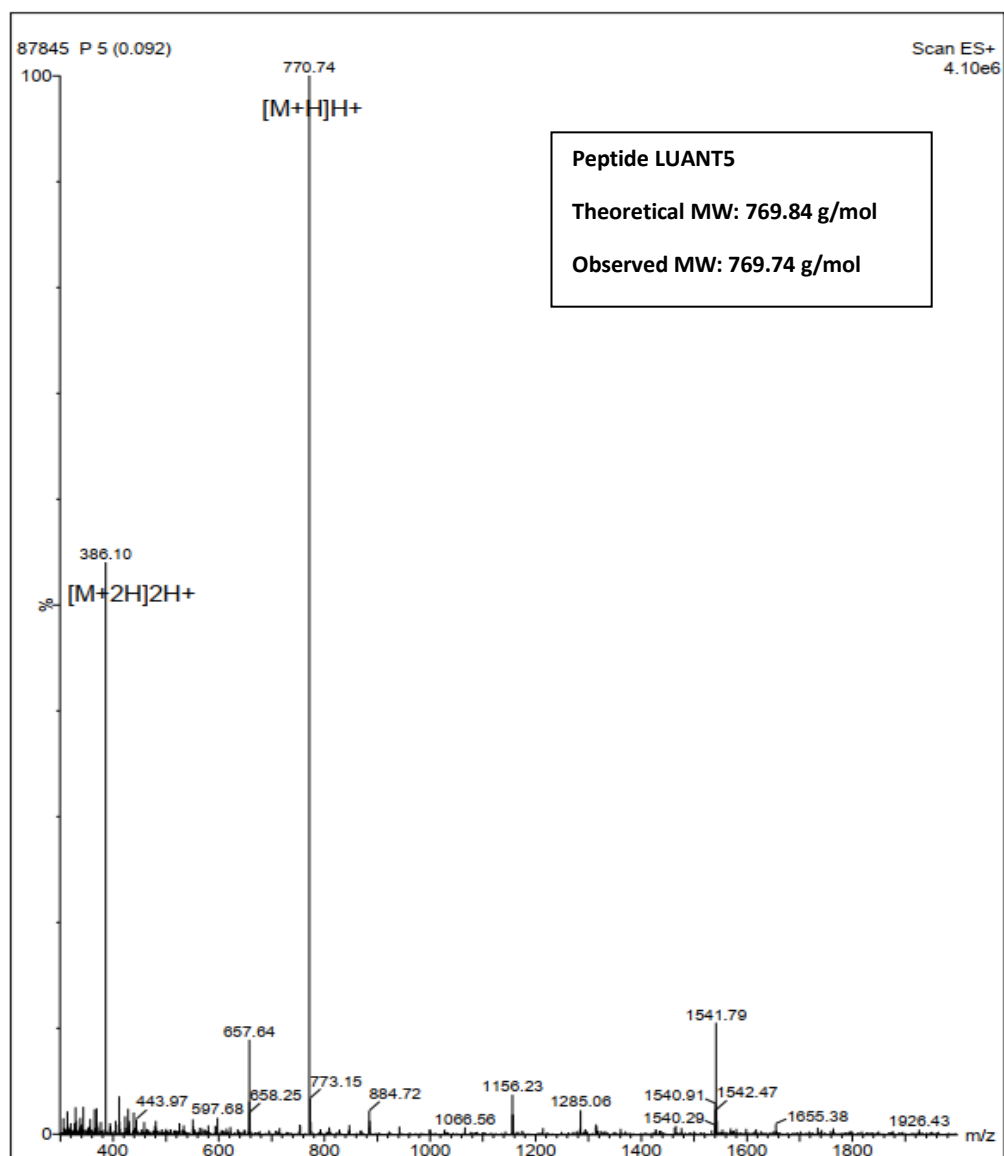
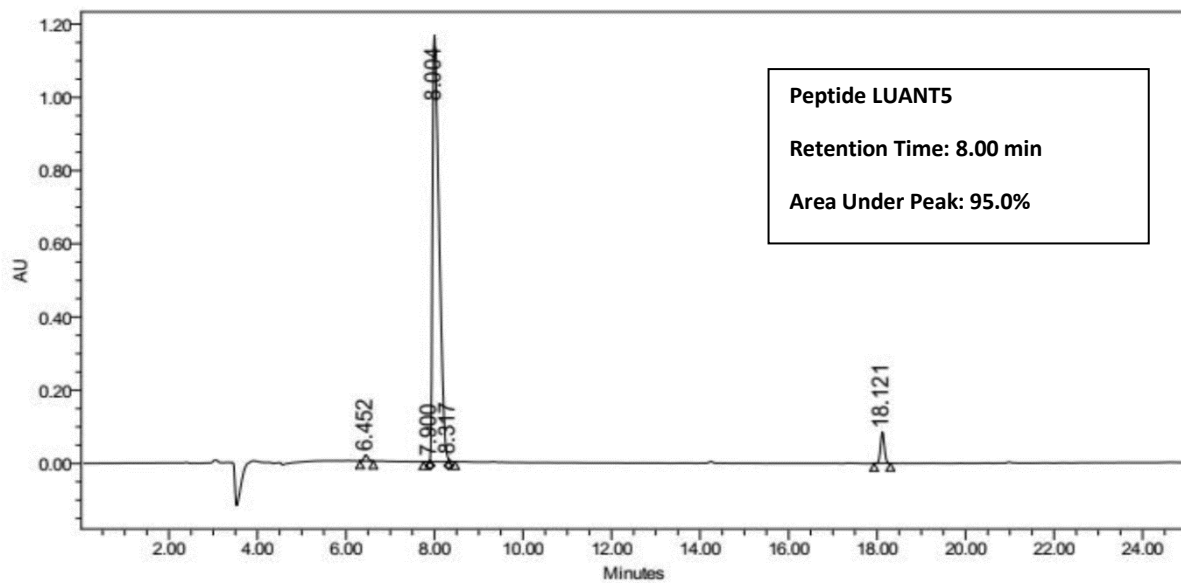


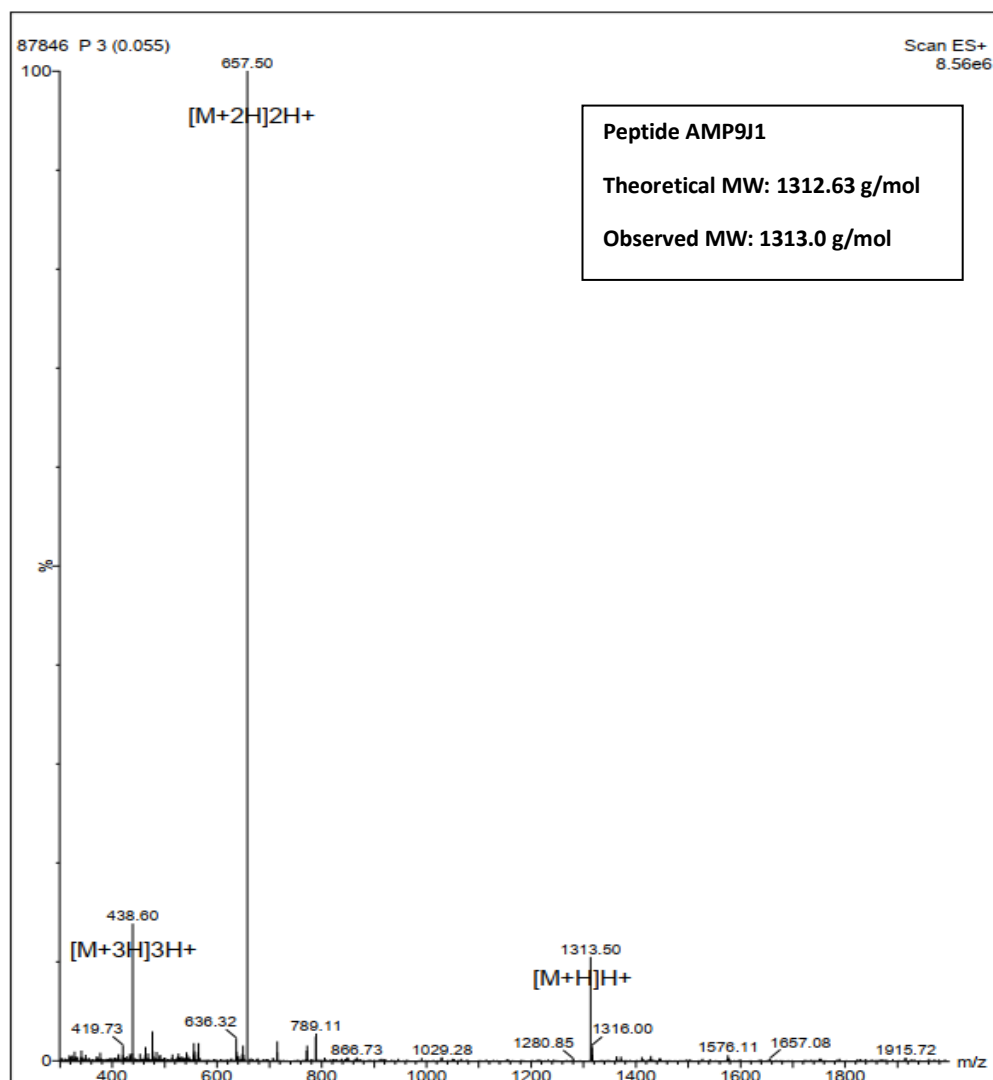
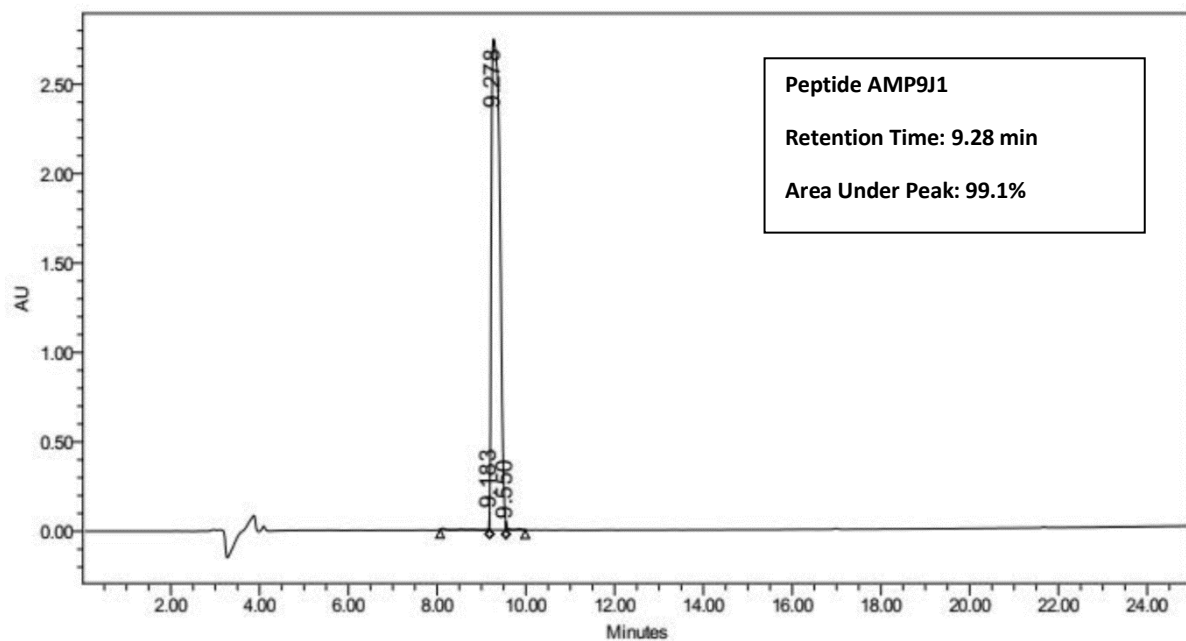


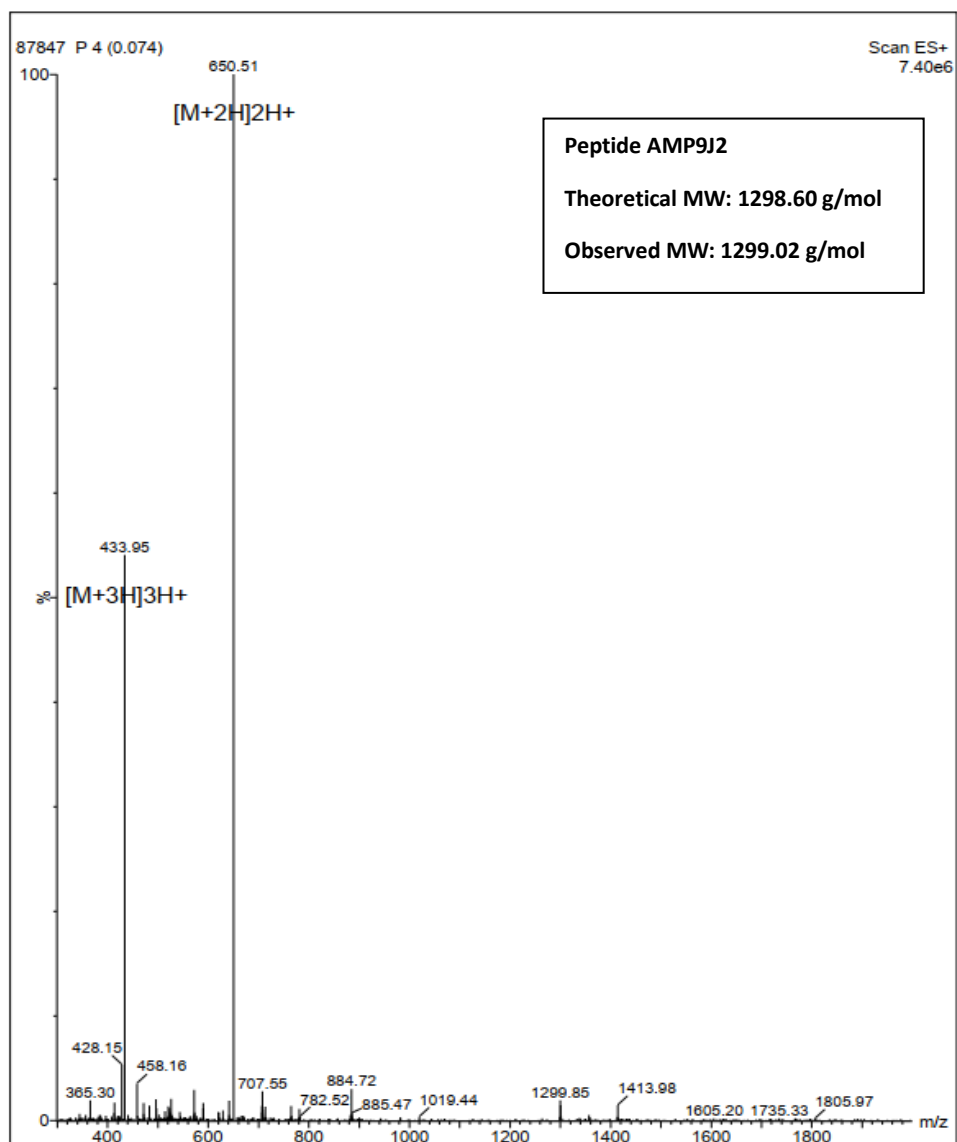
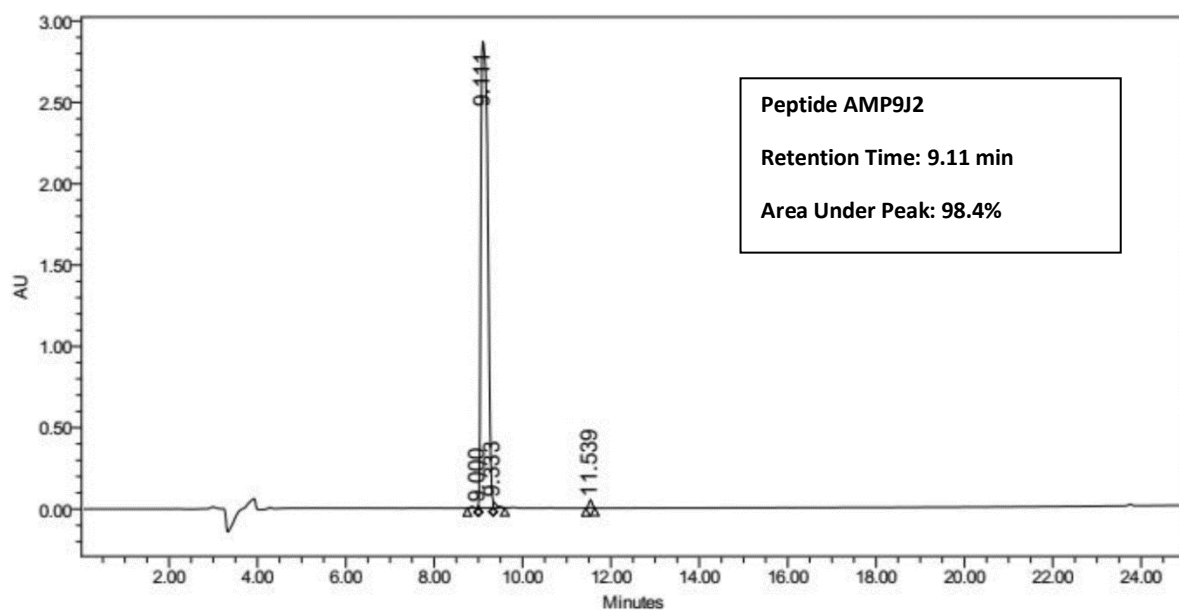


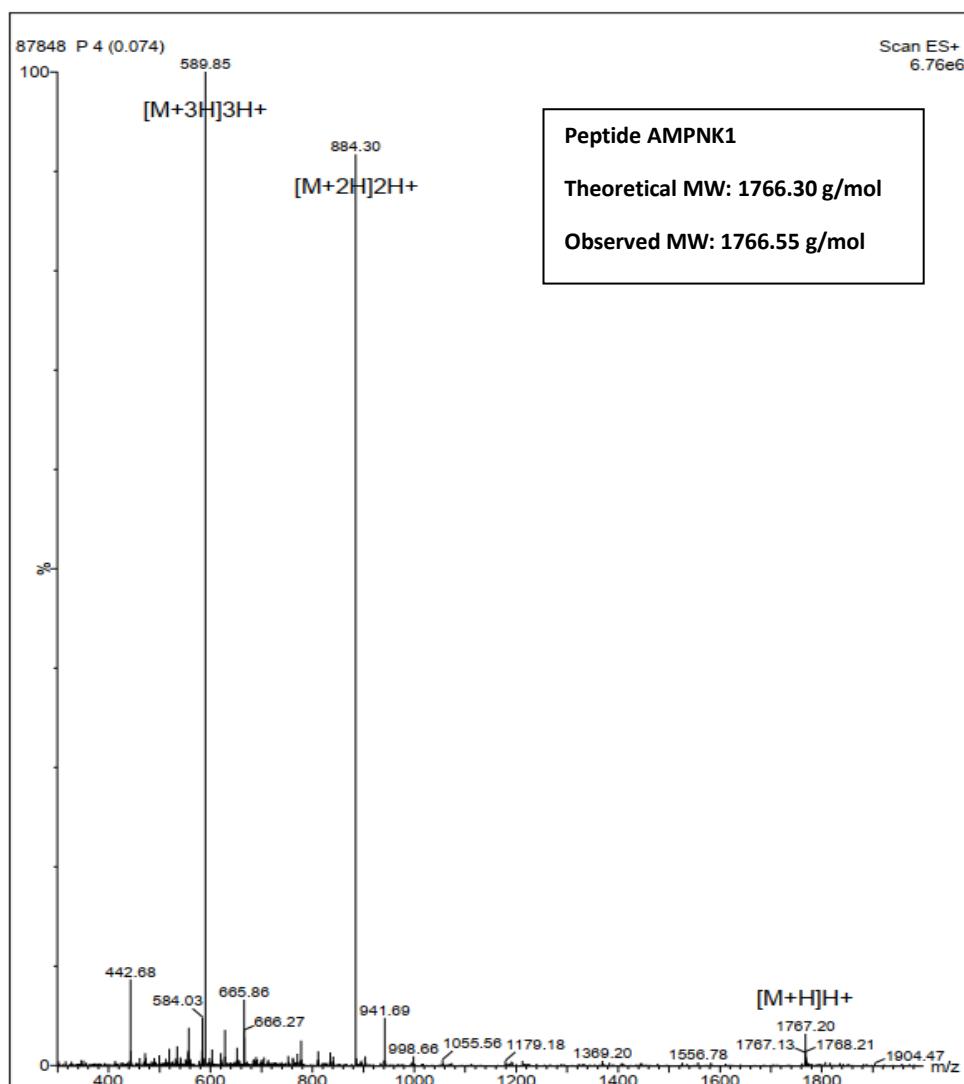
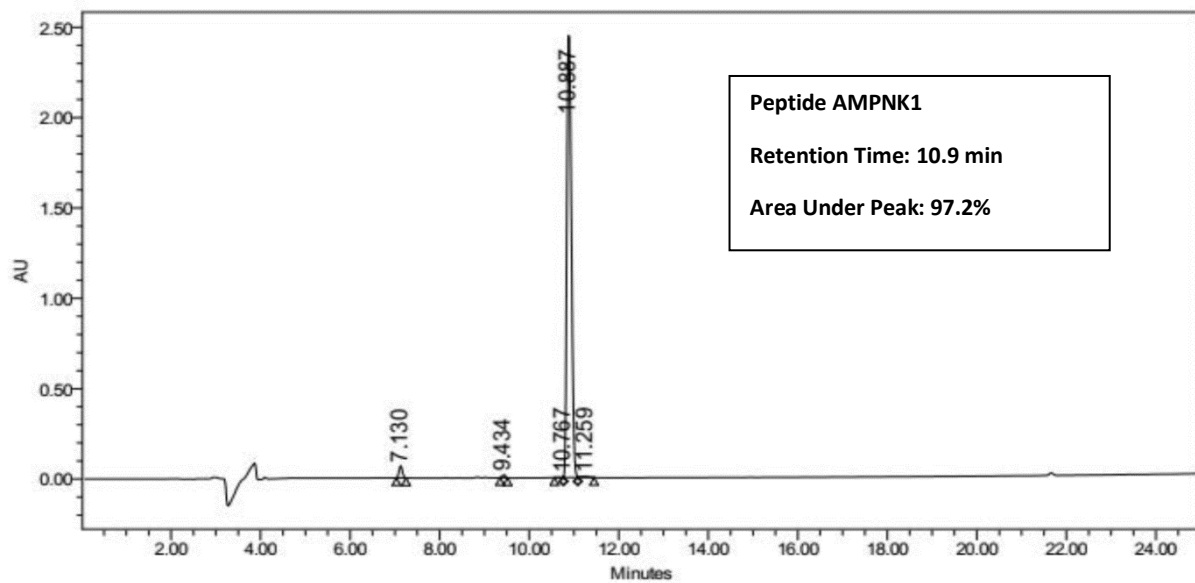


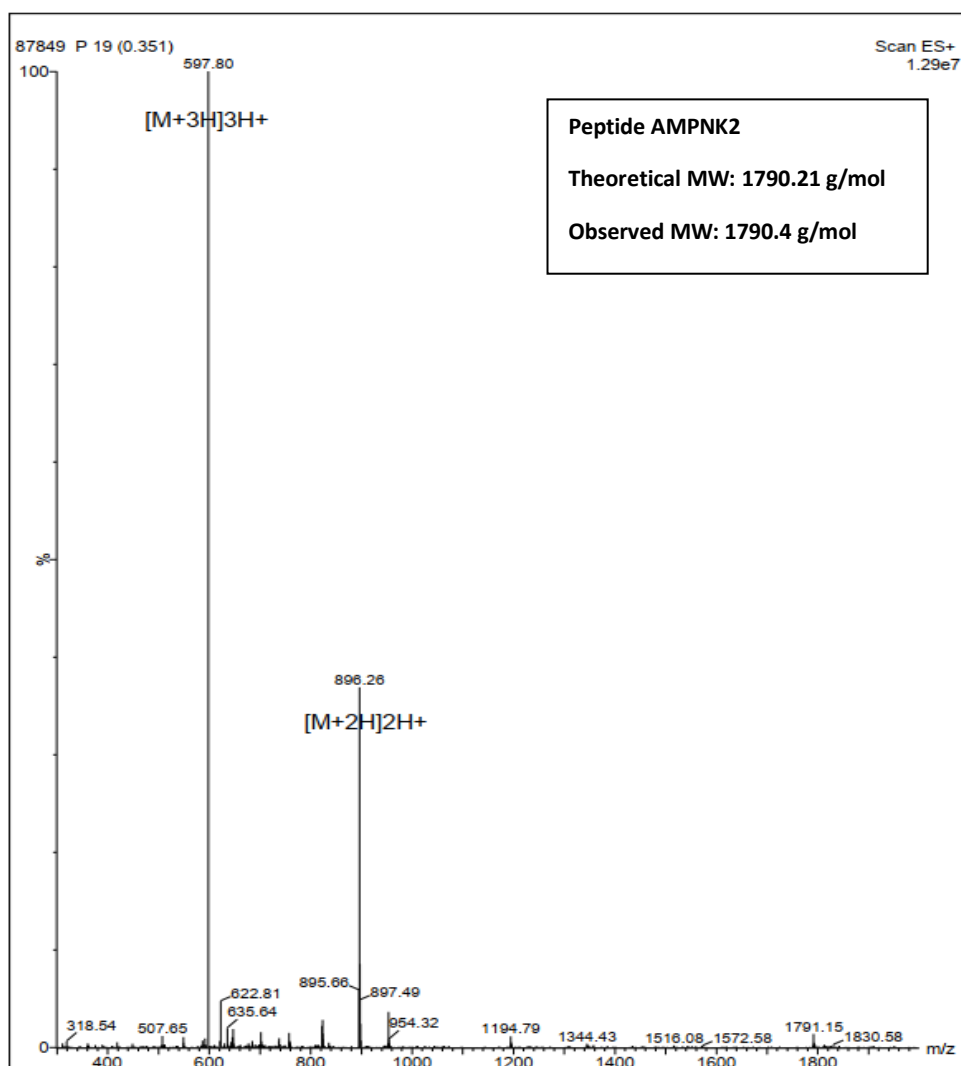
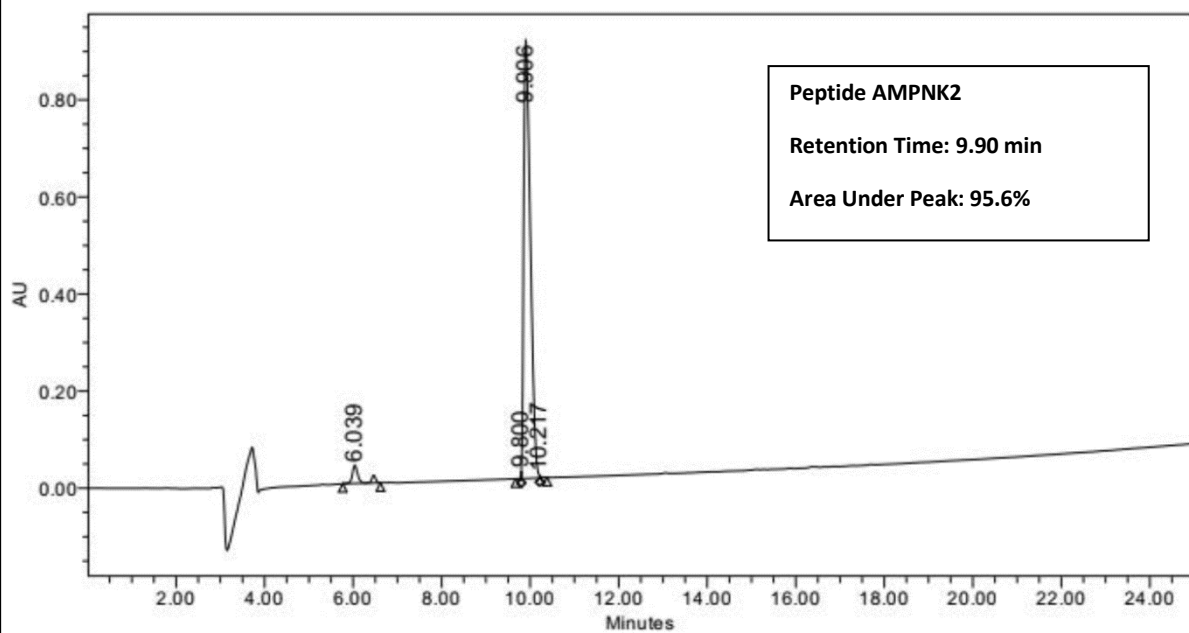


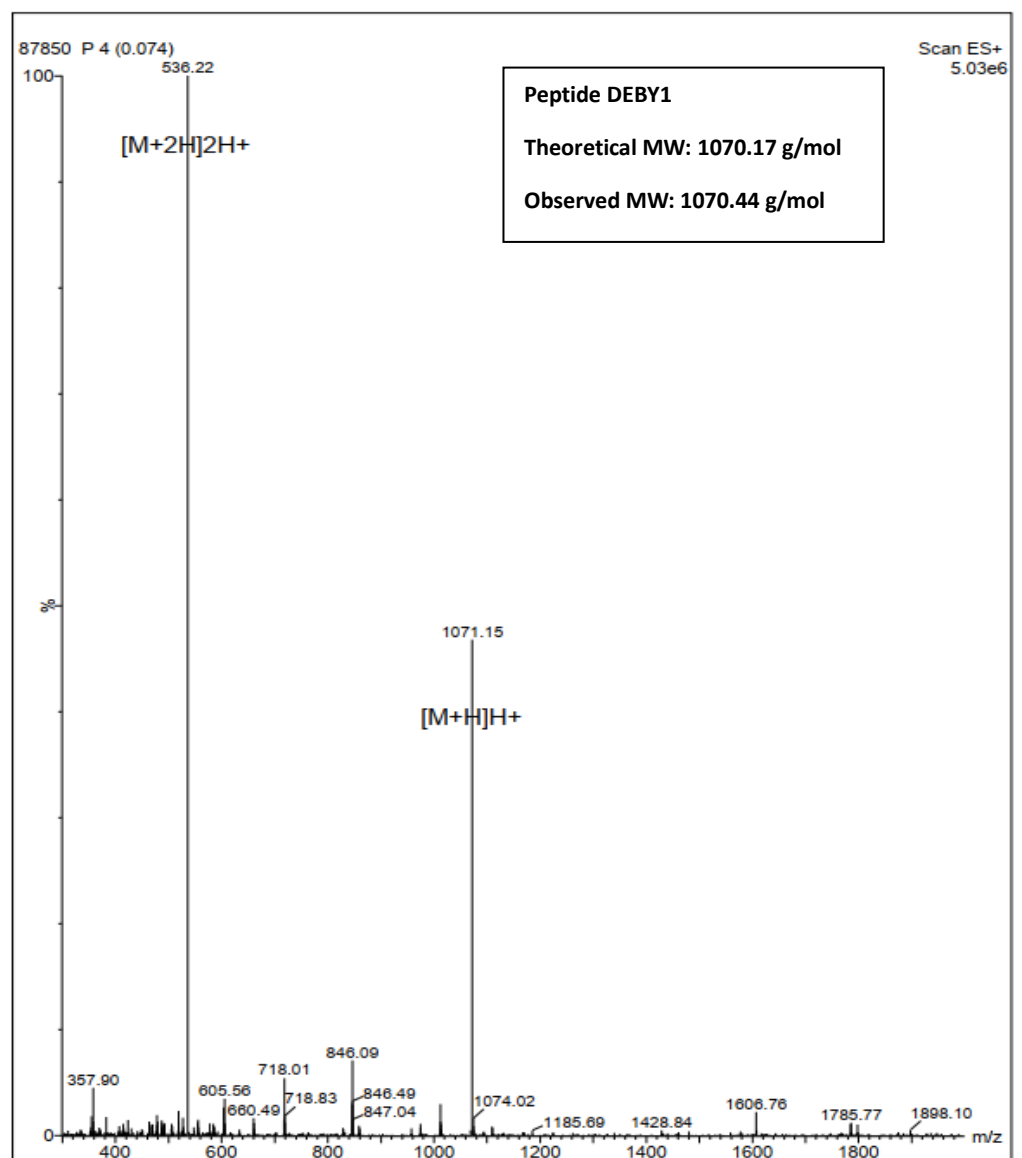
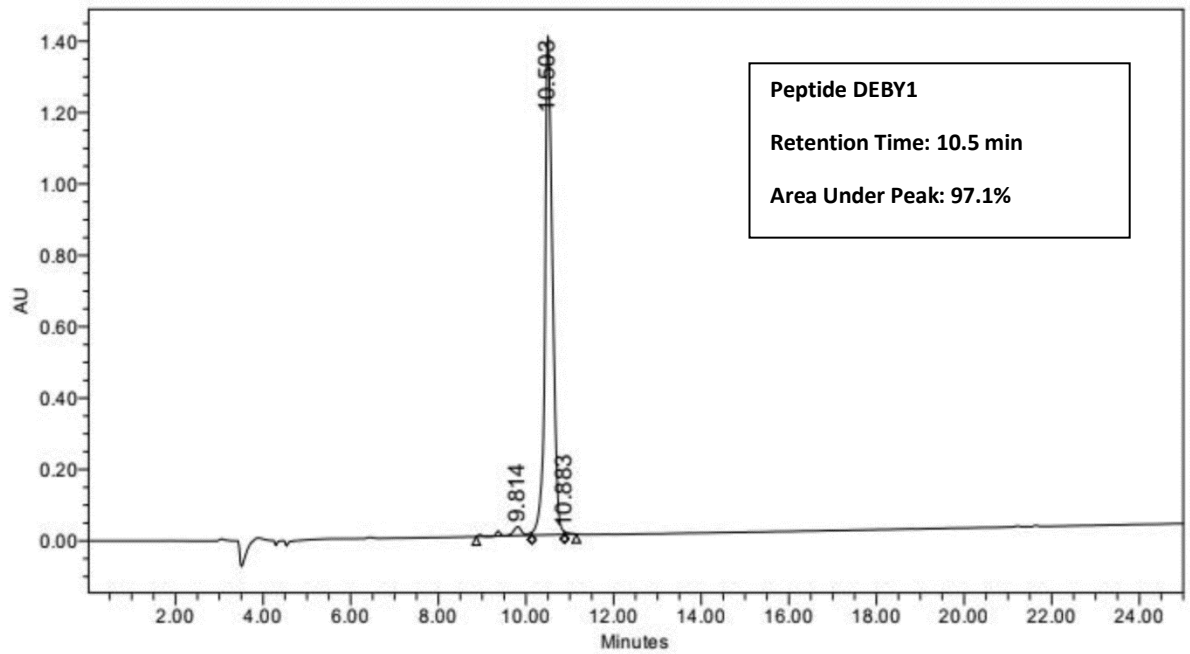


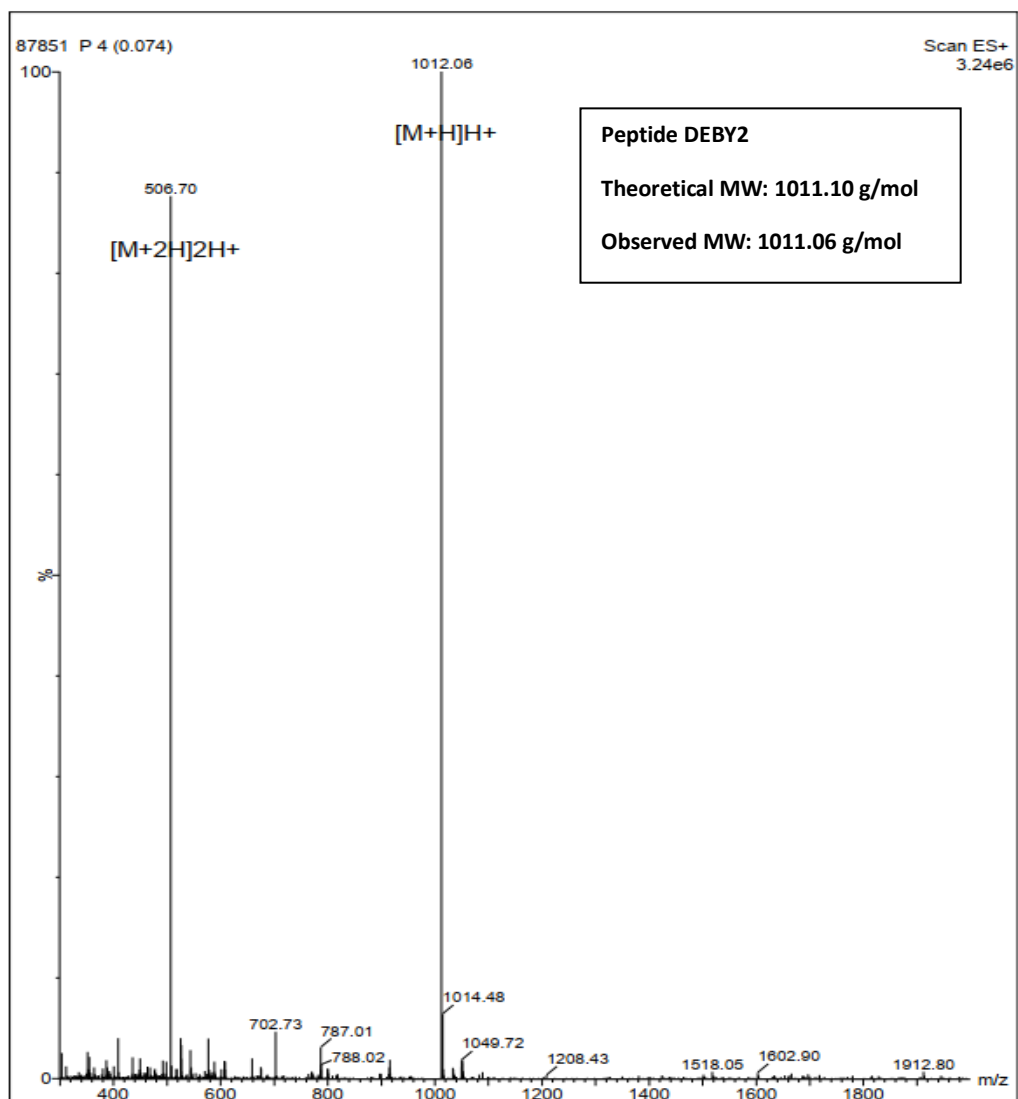
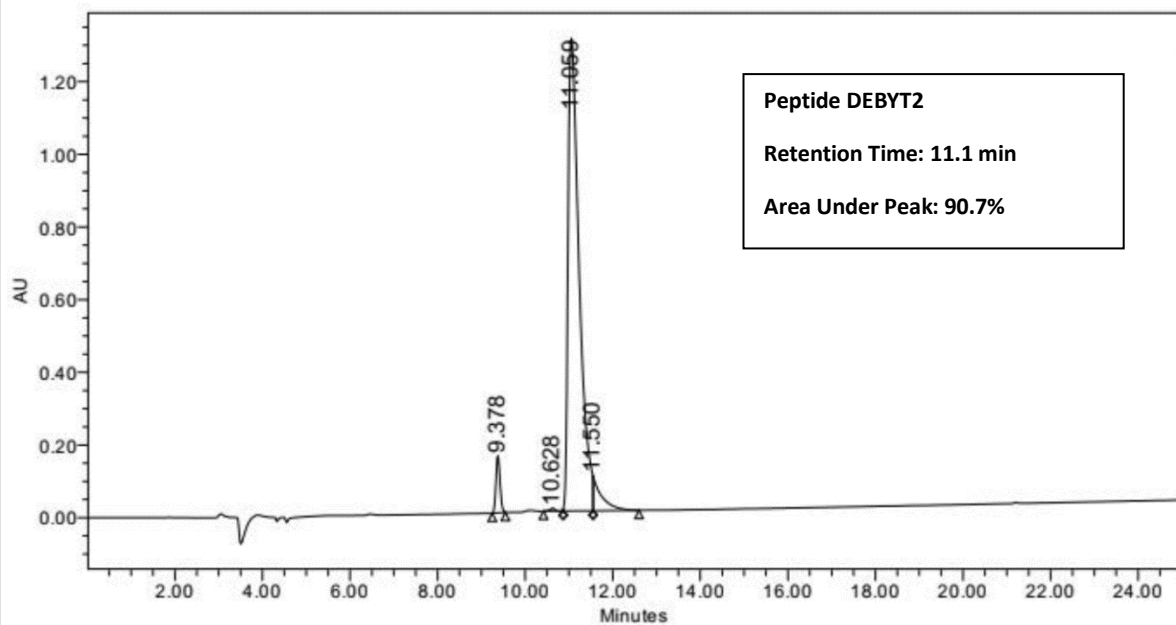


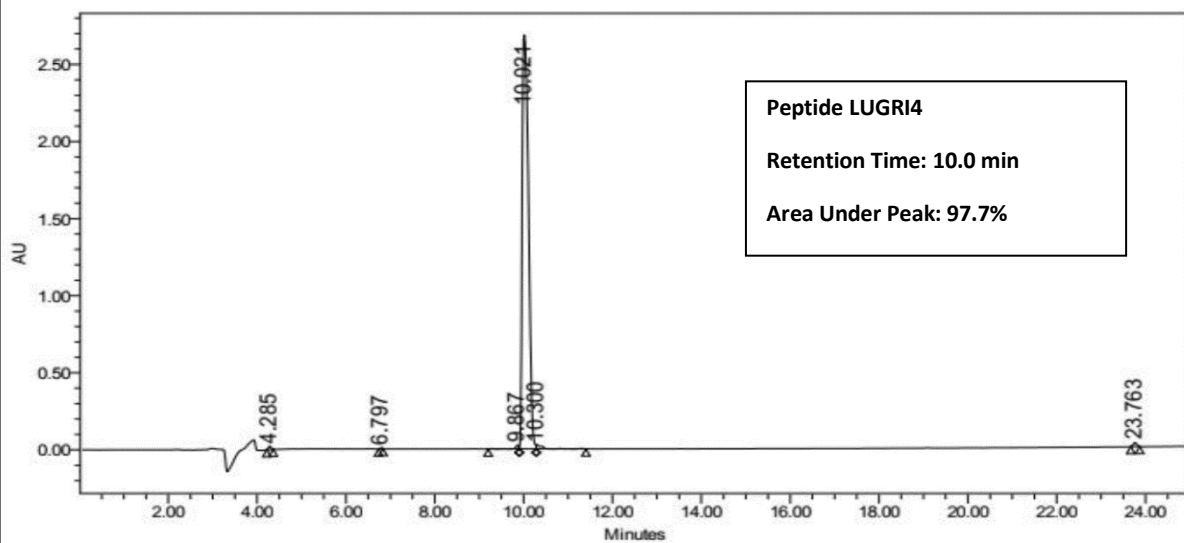








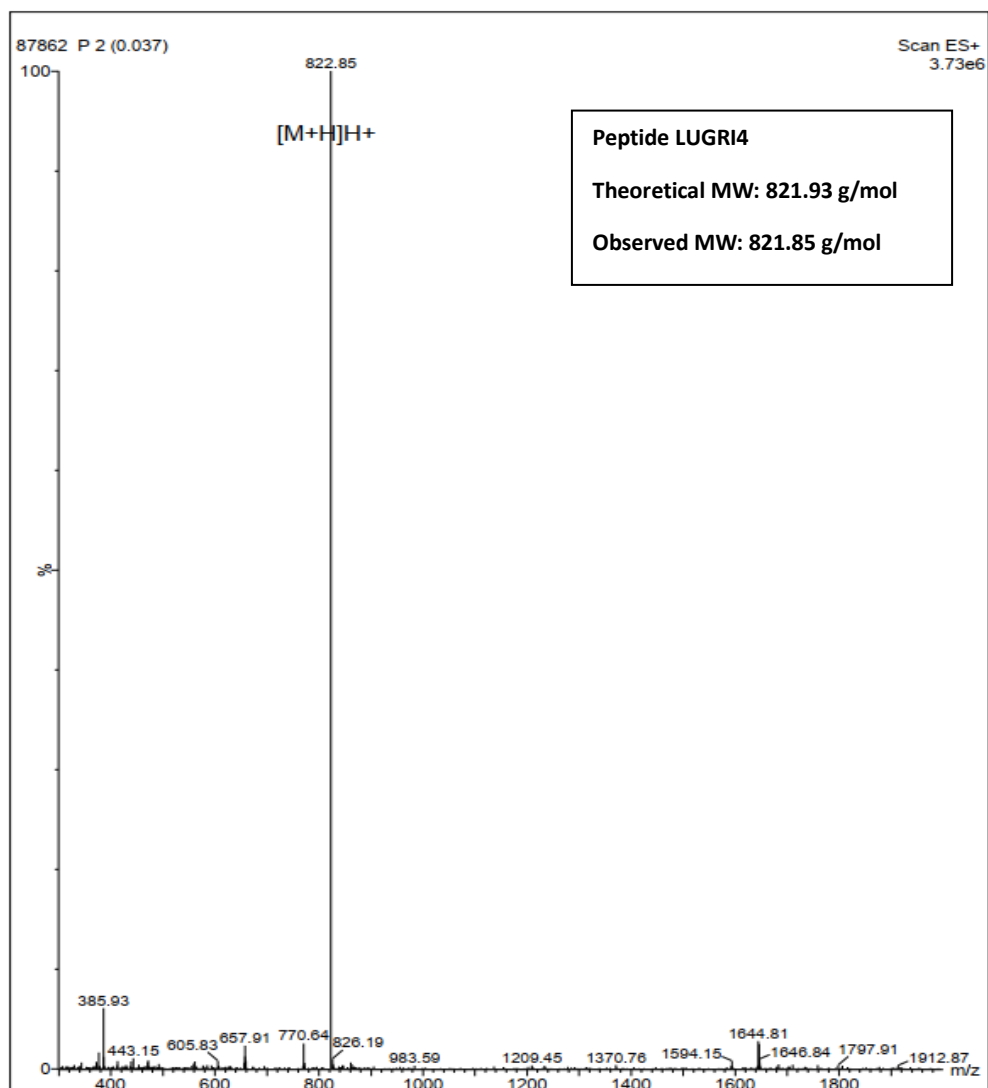




Peptide LUGRI4

Retention Time: 10.0 min

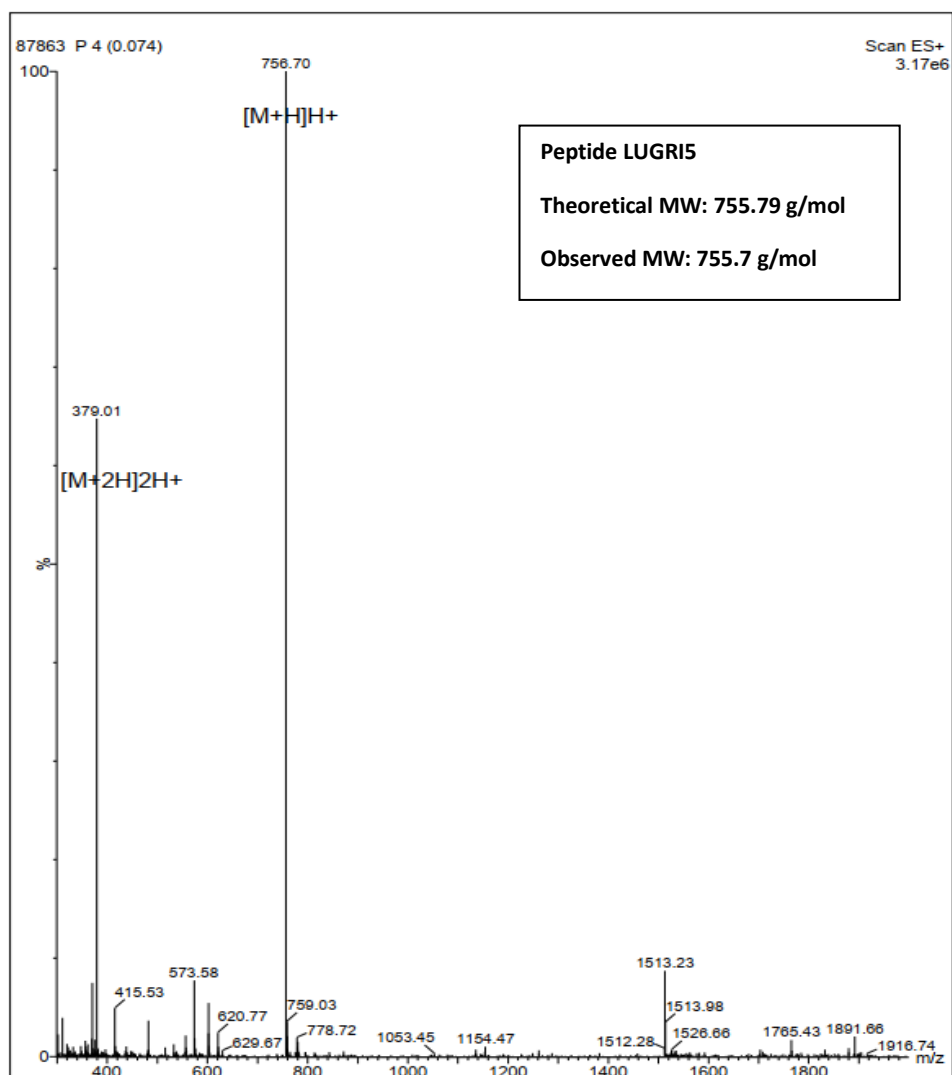
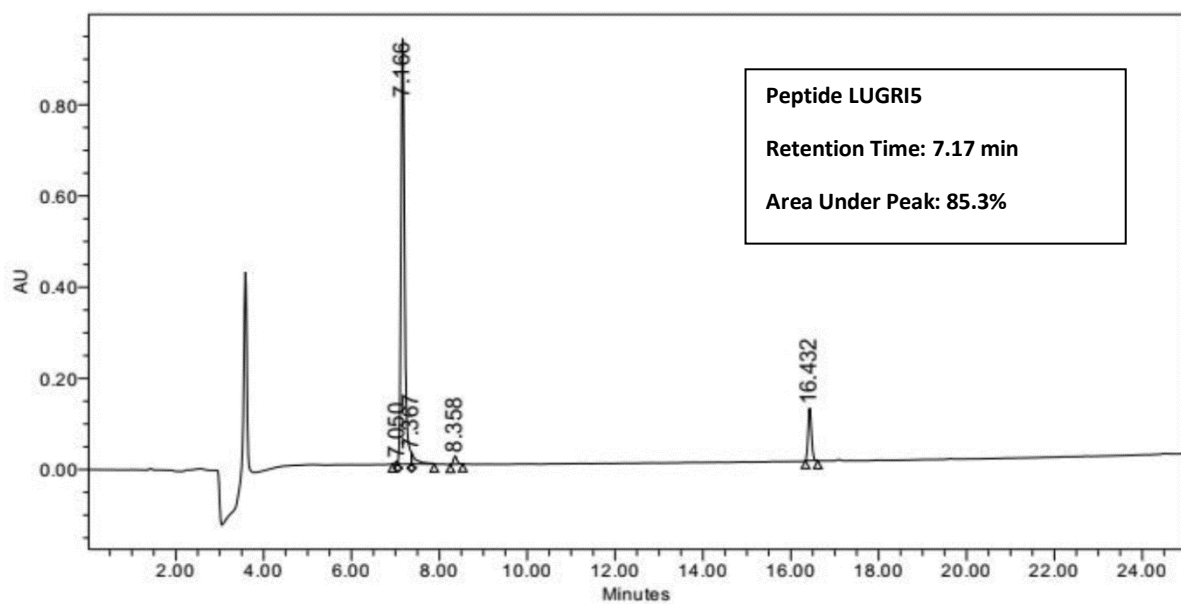
Area Under Peak: 97.7%

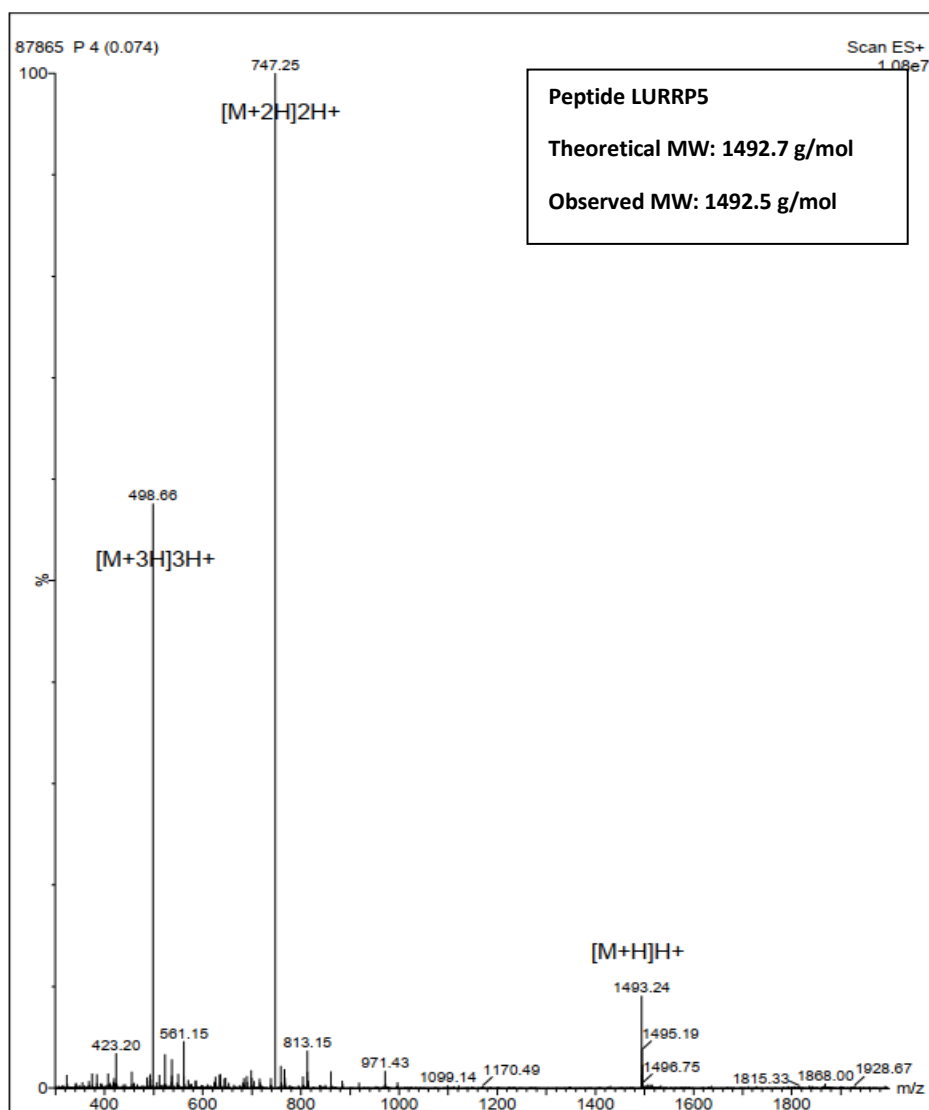
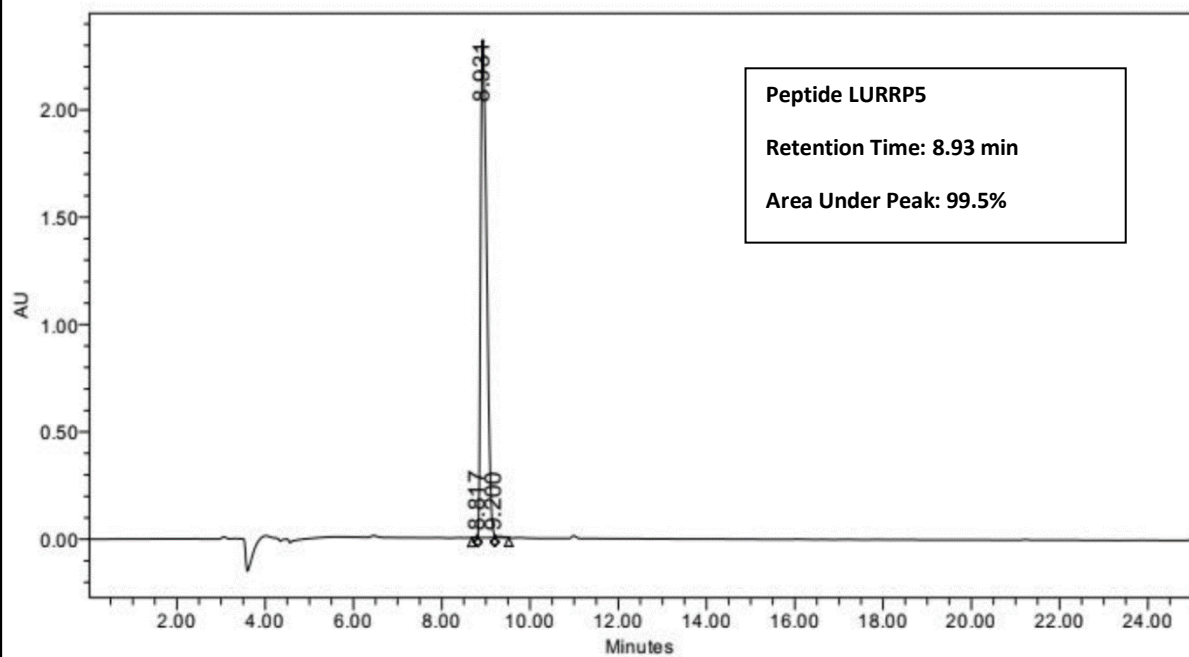


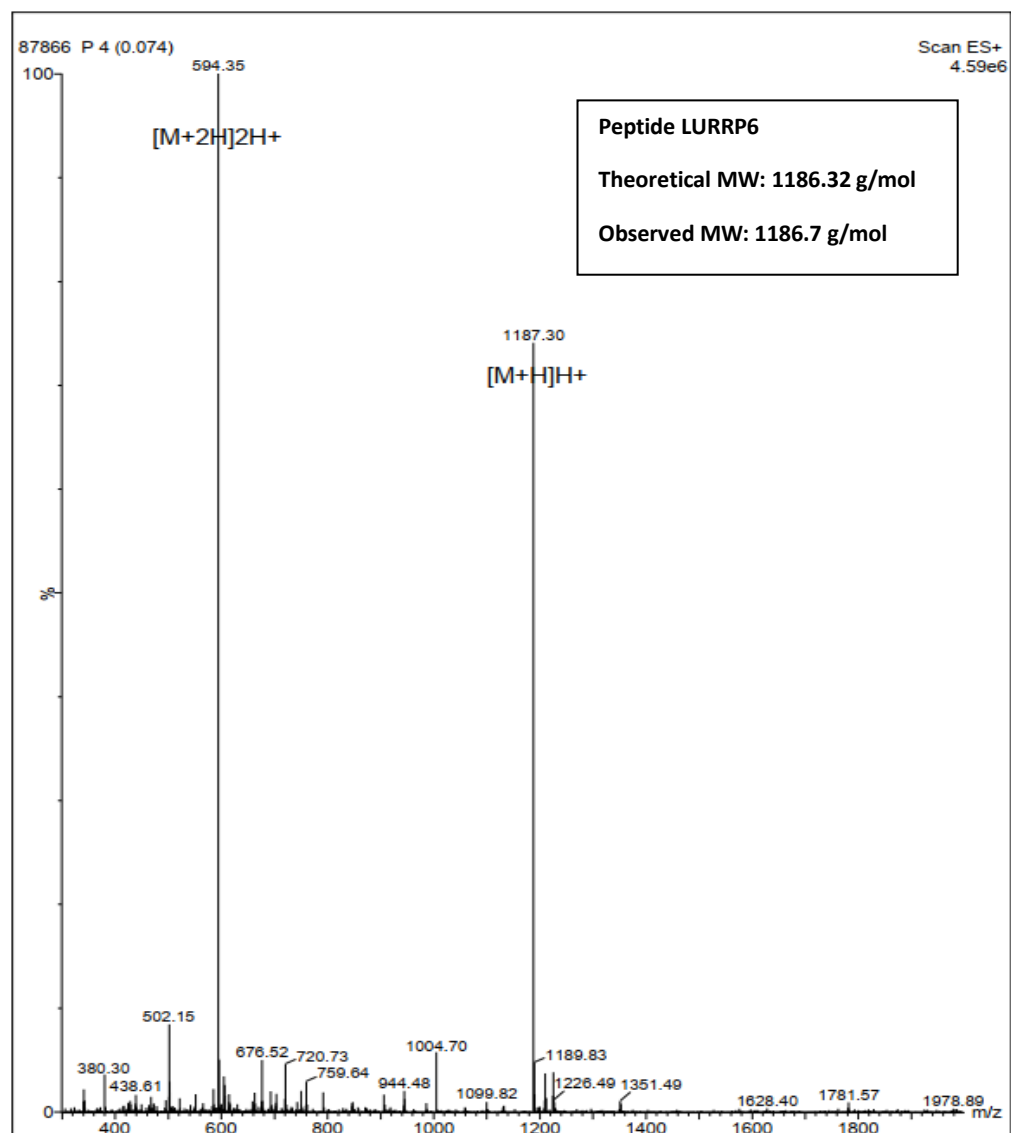
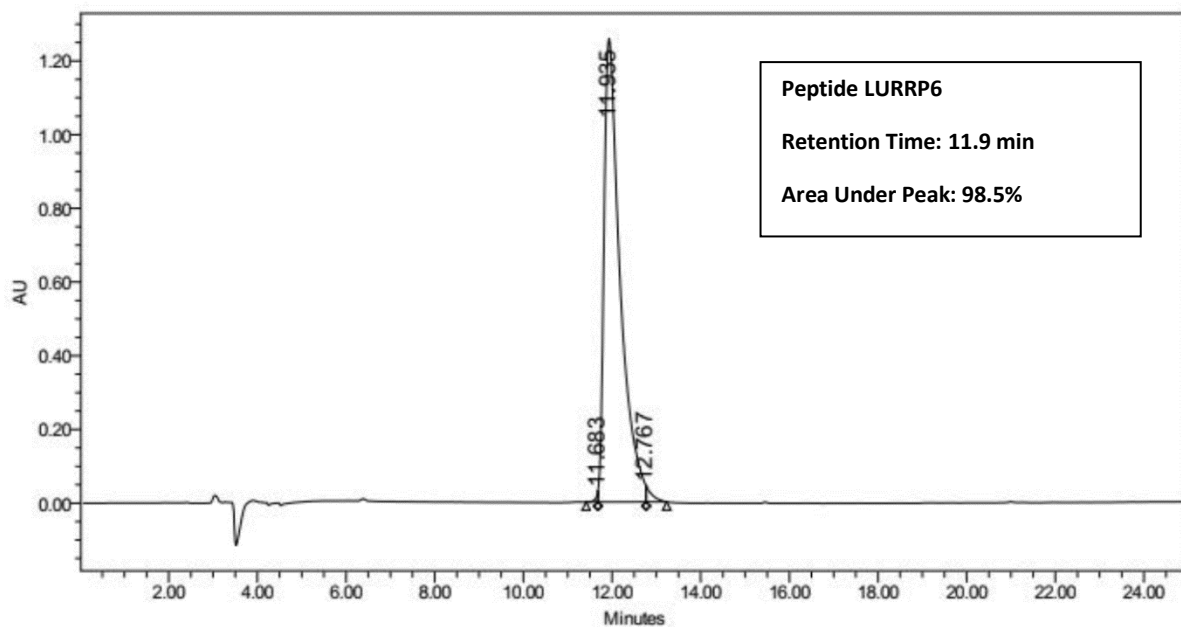
Peptide LUGRI4

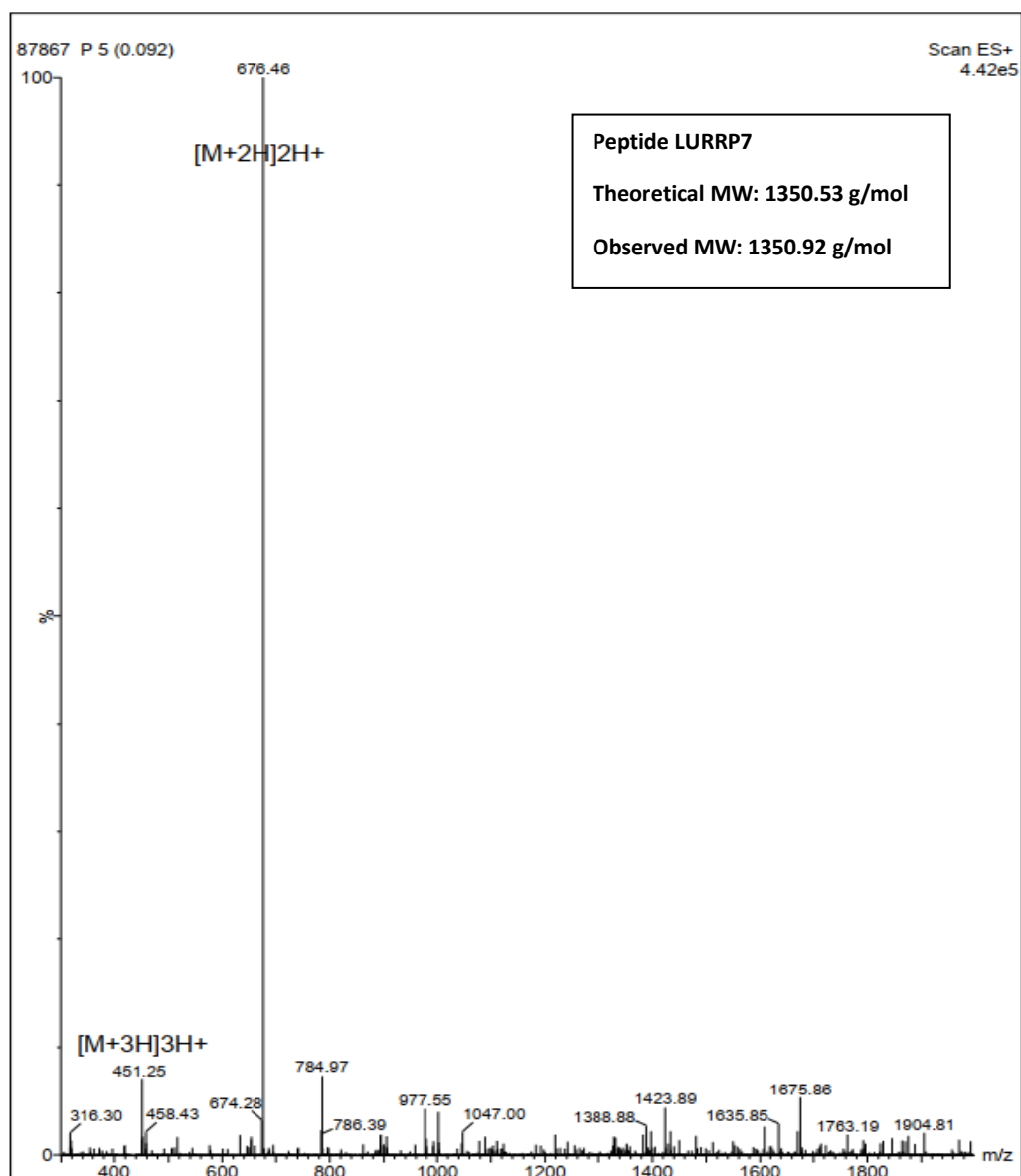
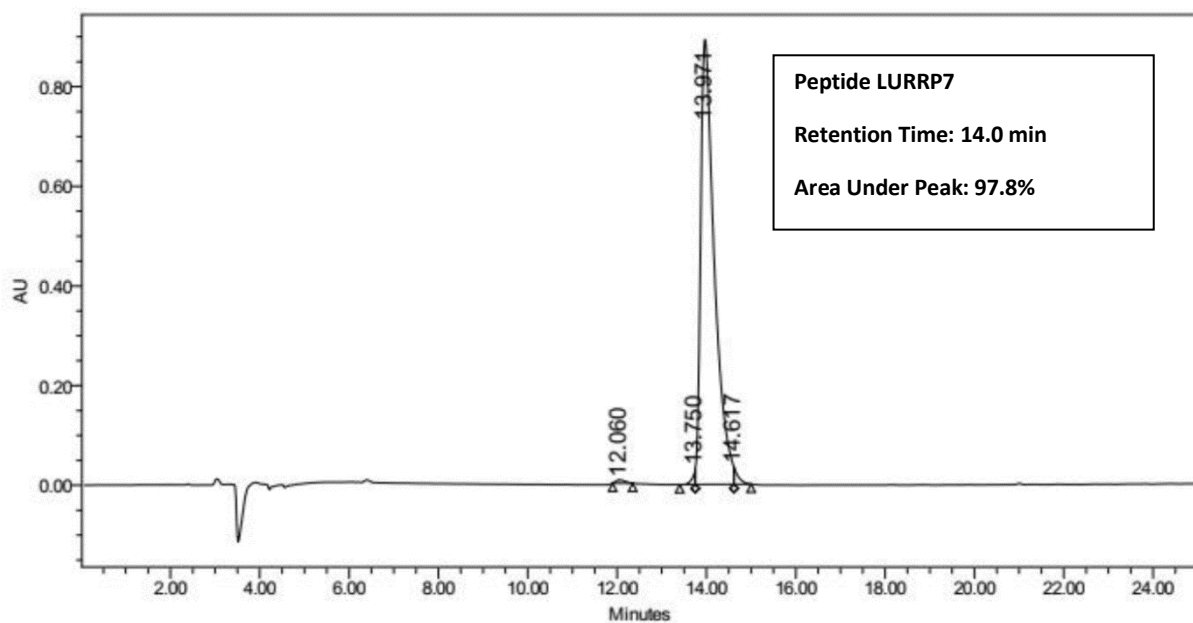
Theoretical MW: 821.93 g/mol

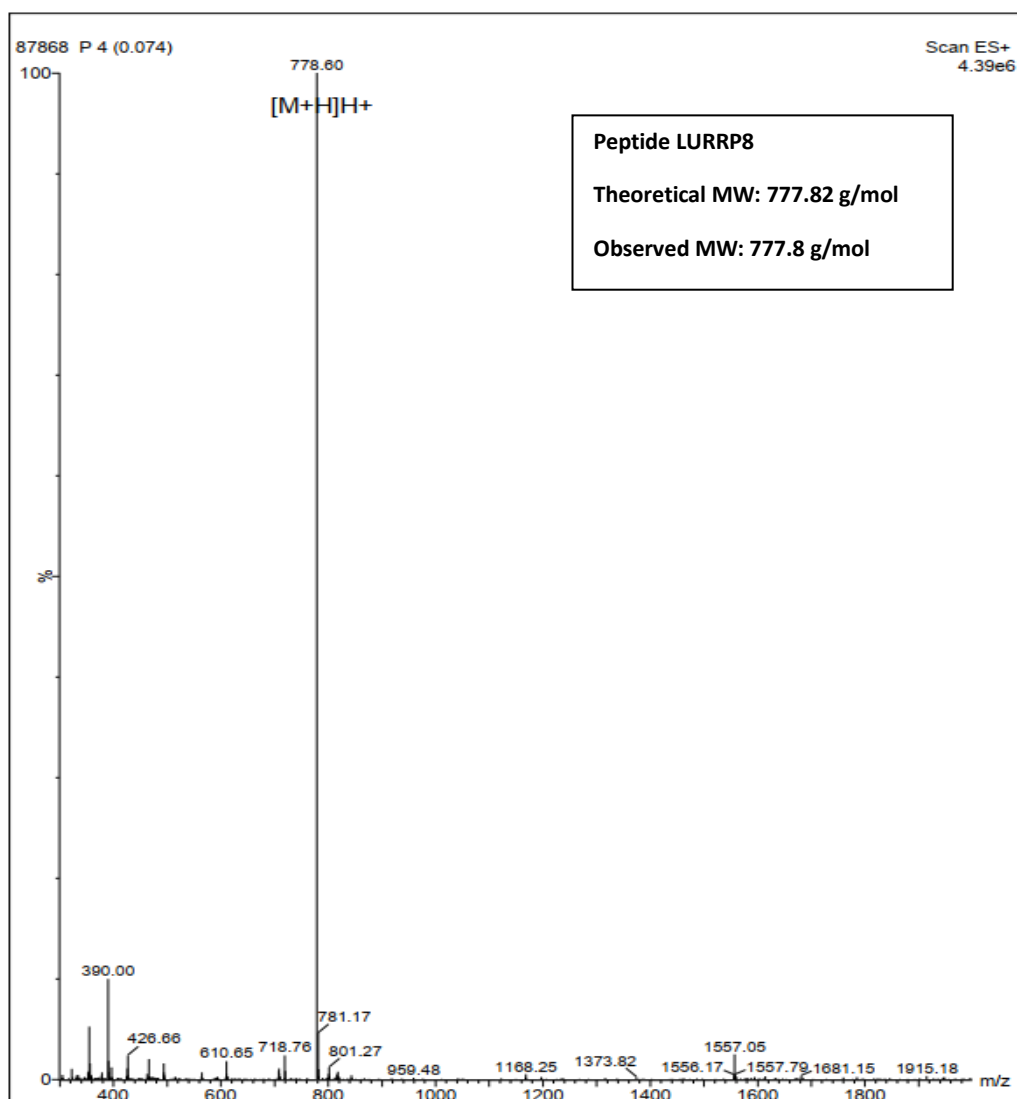
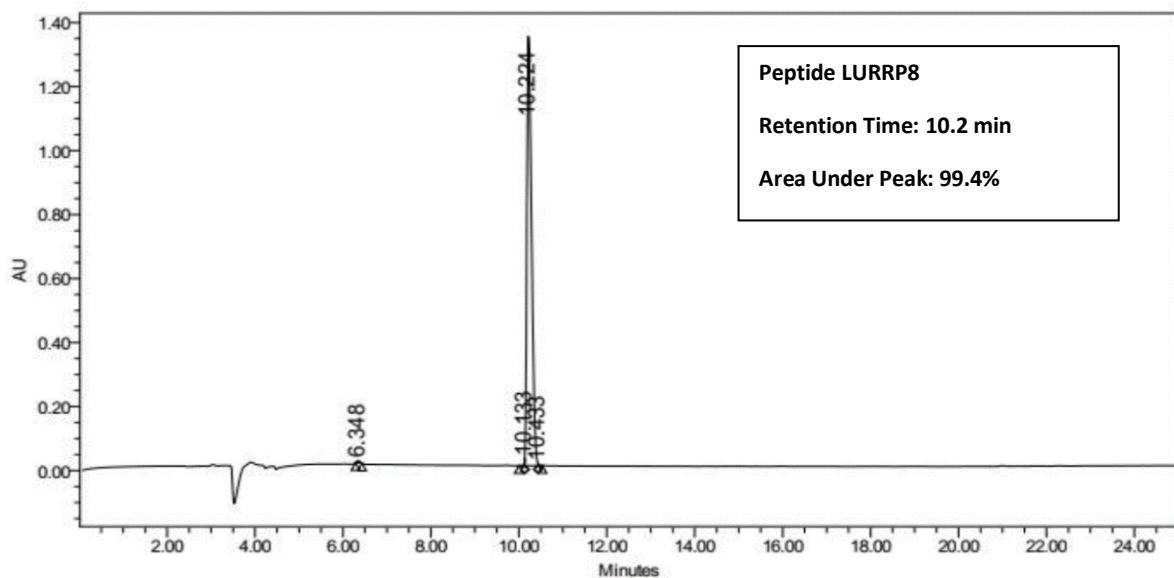
Observed MW: 821.85 g/mol

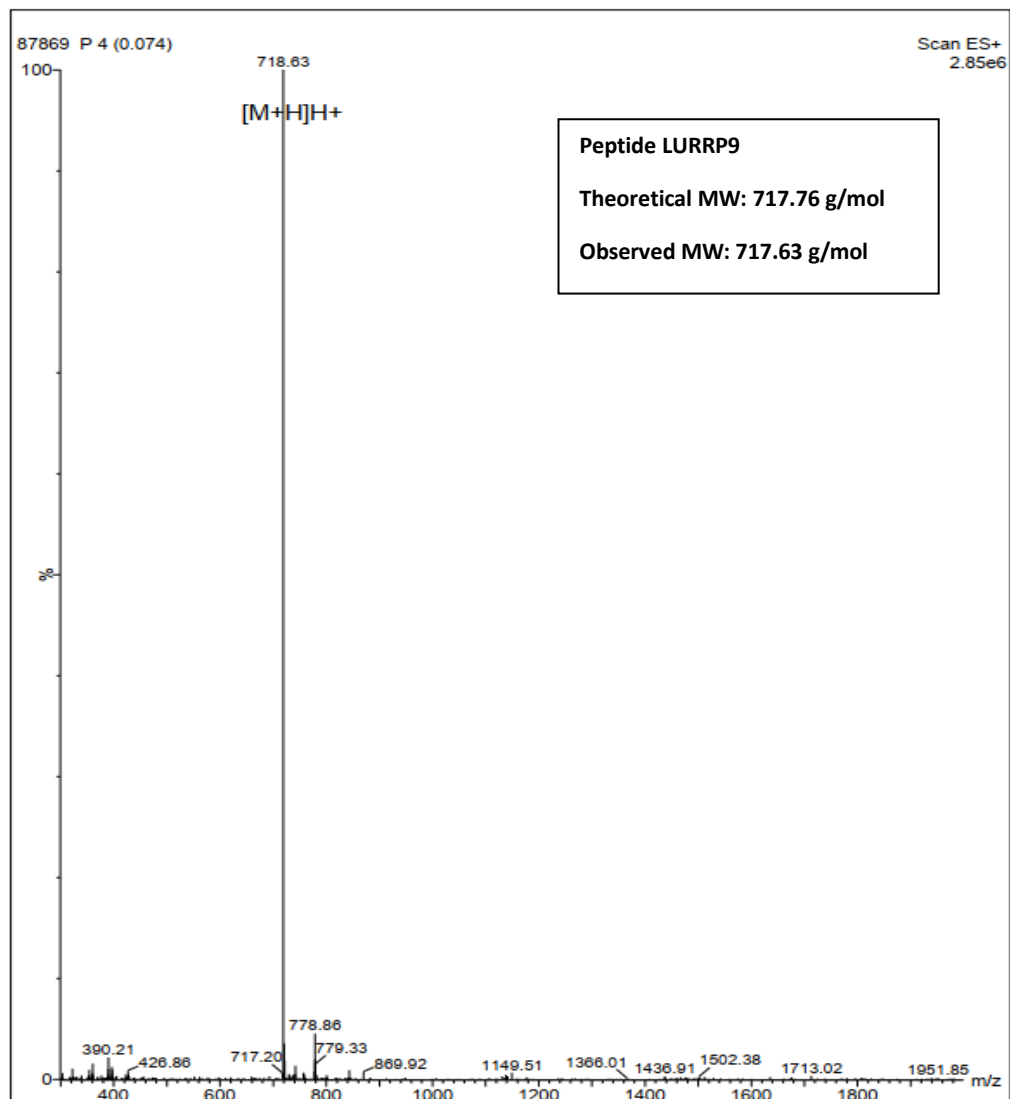
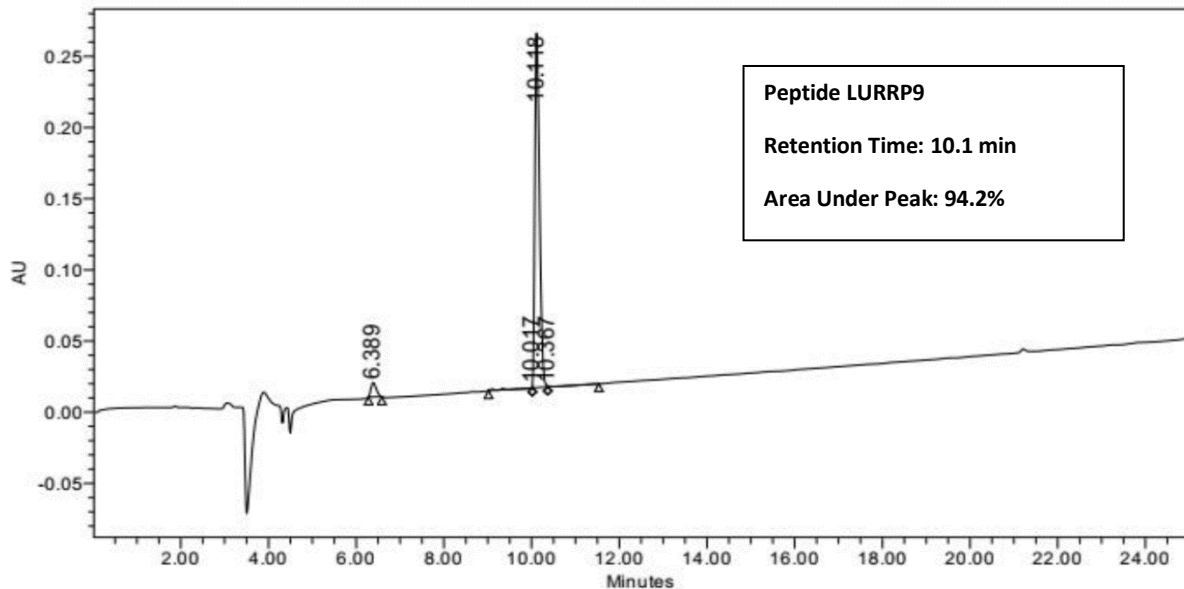


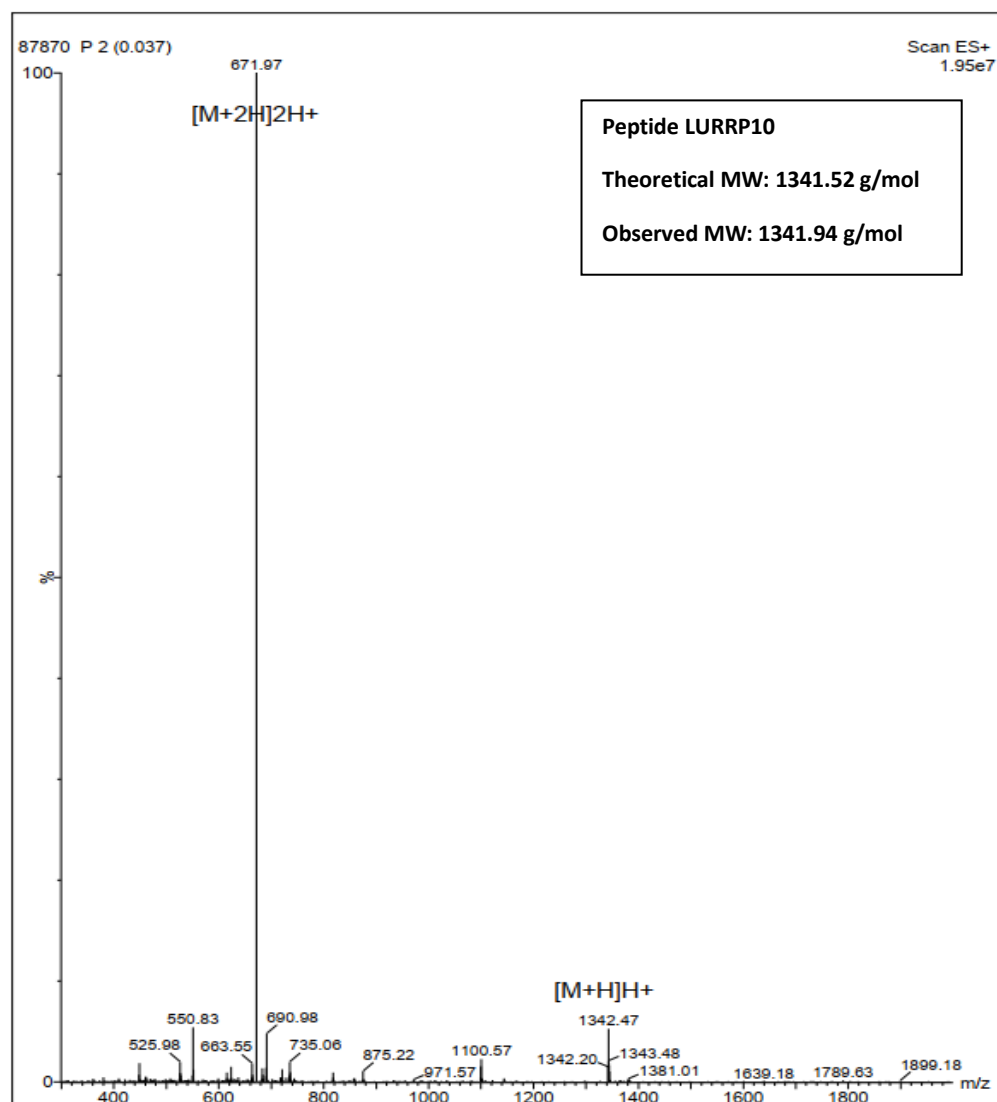
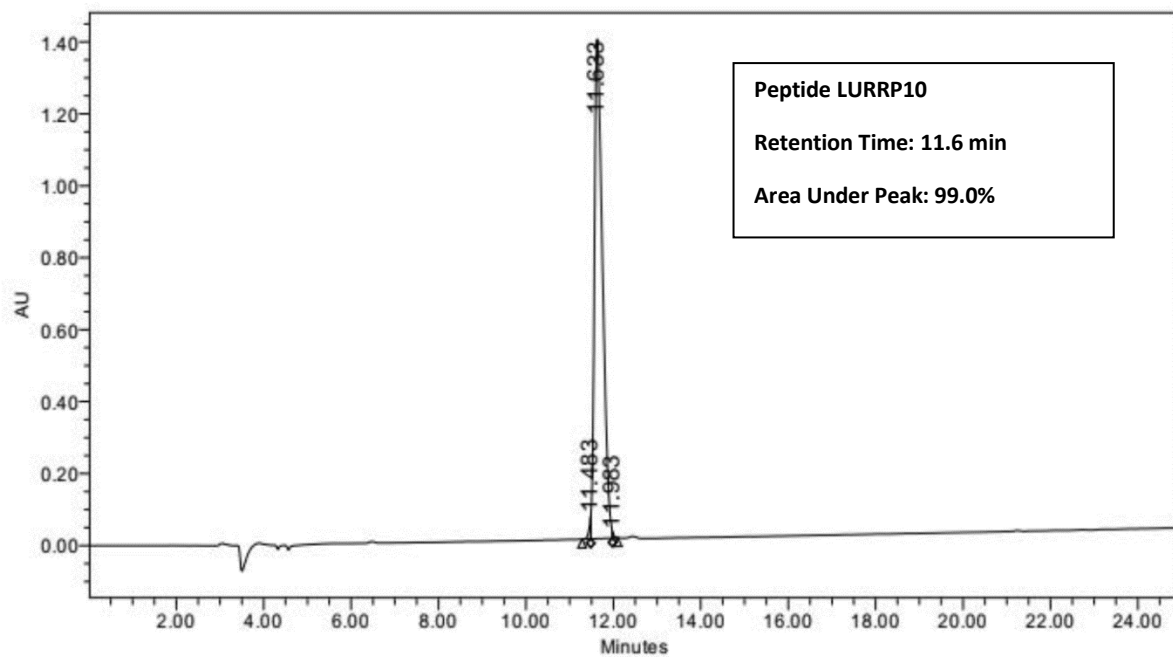


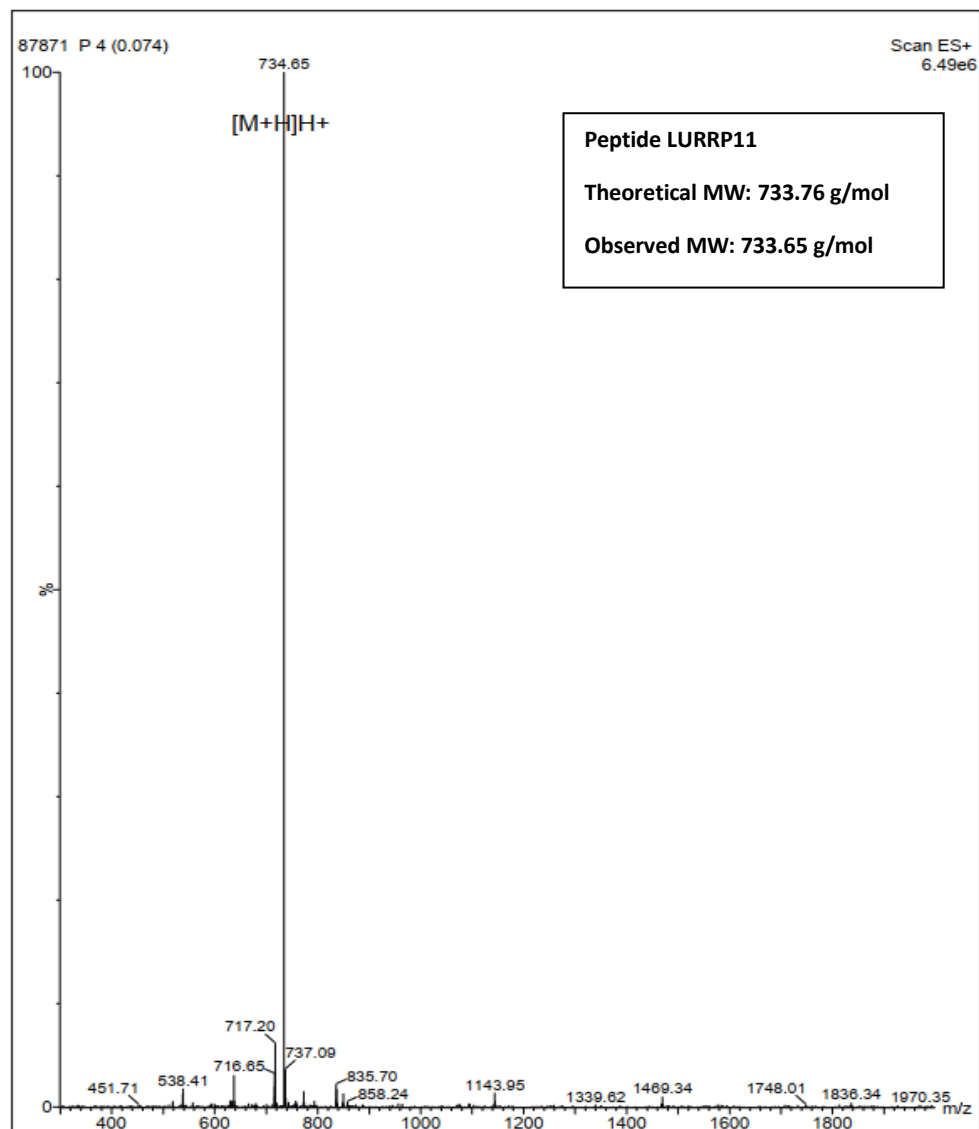
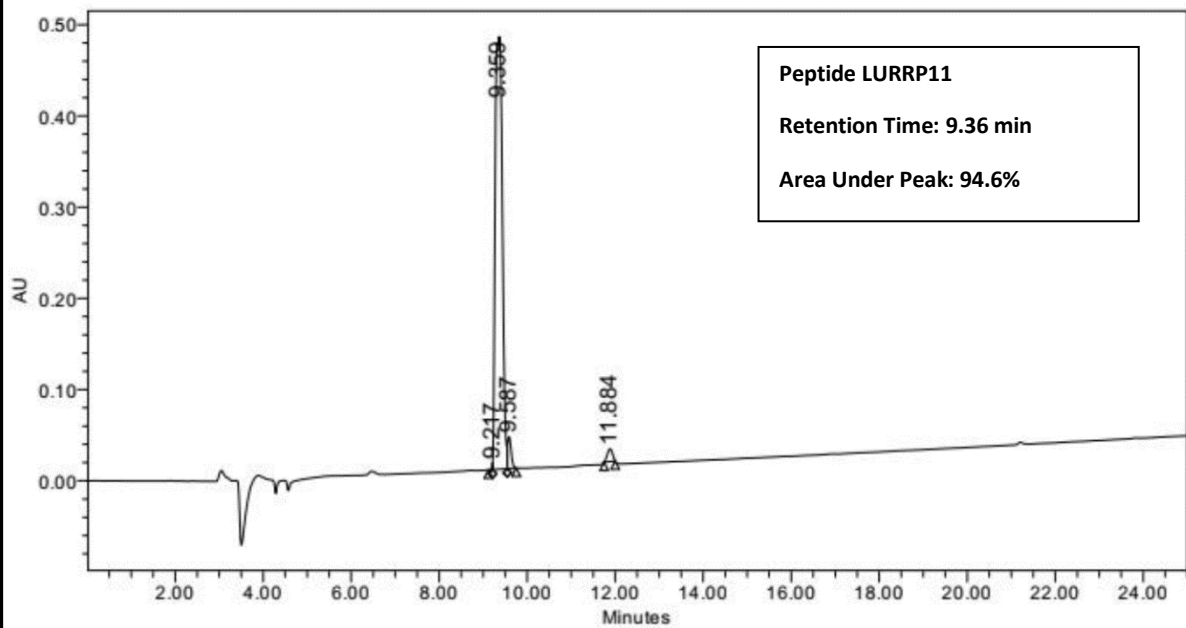


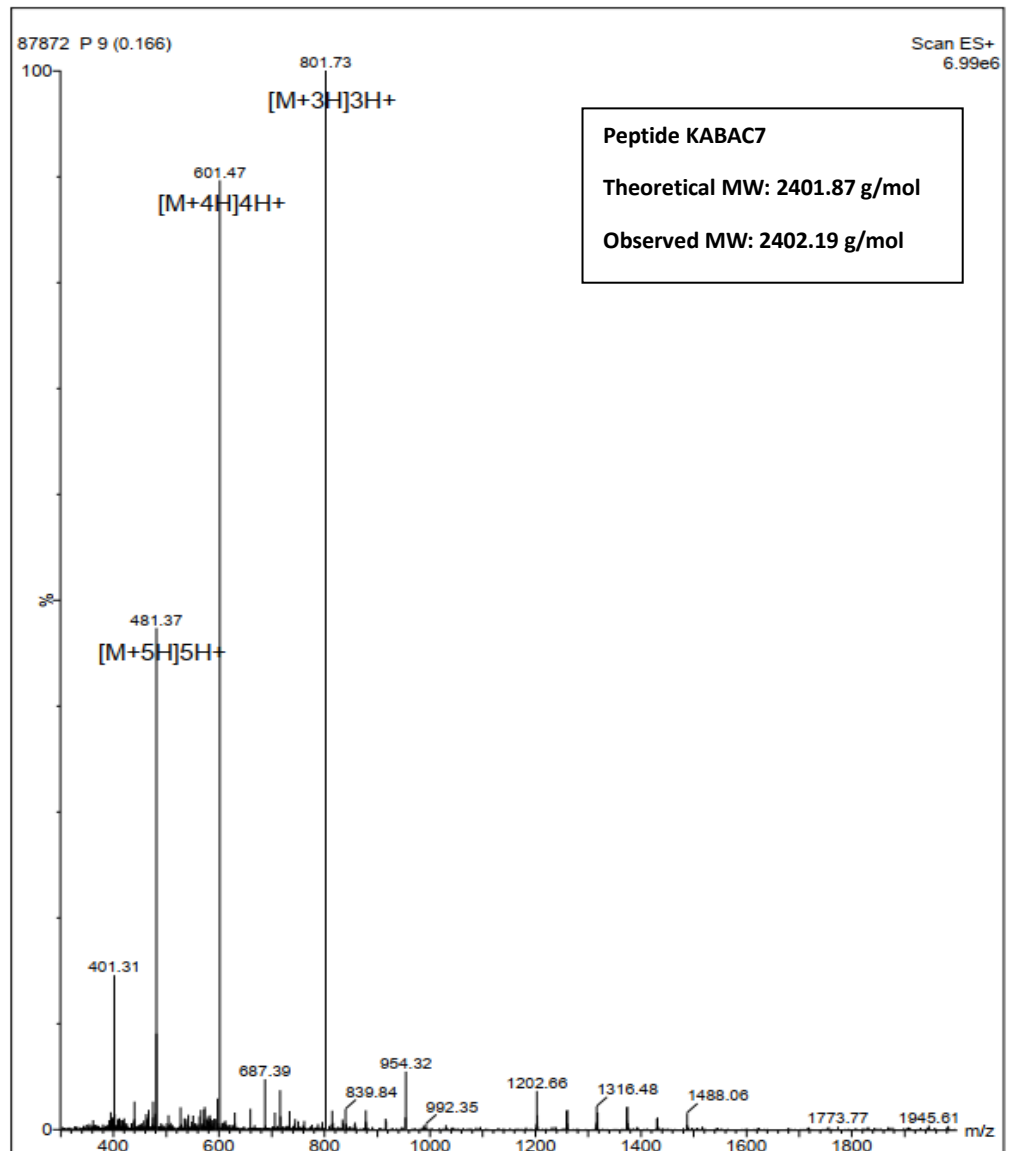
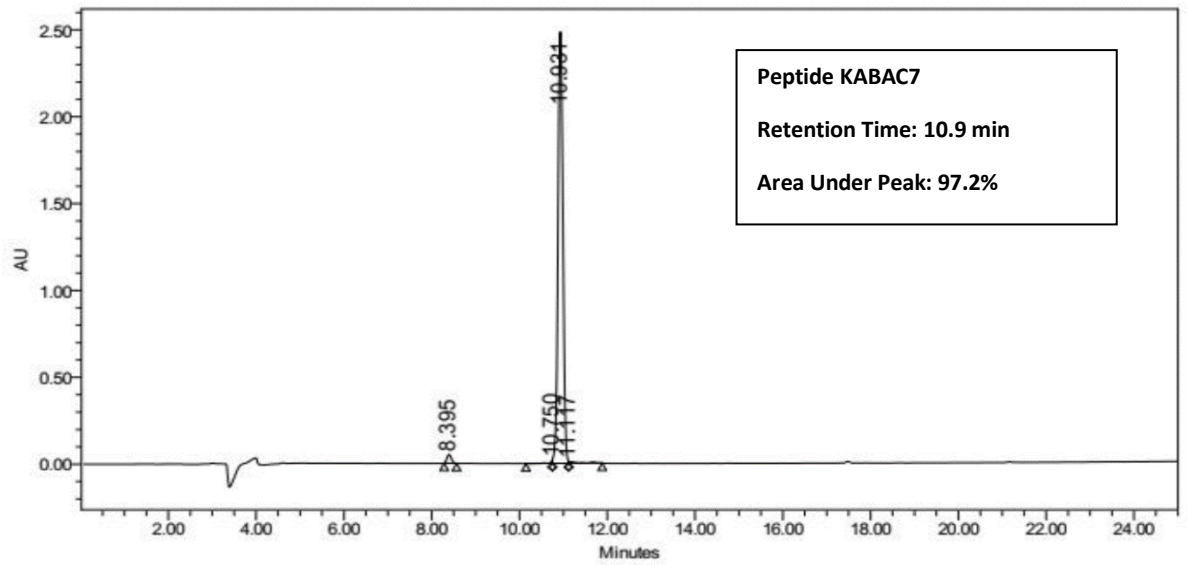


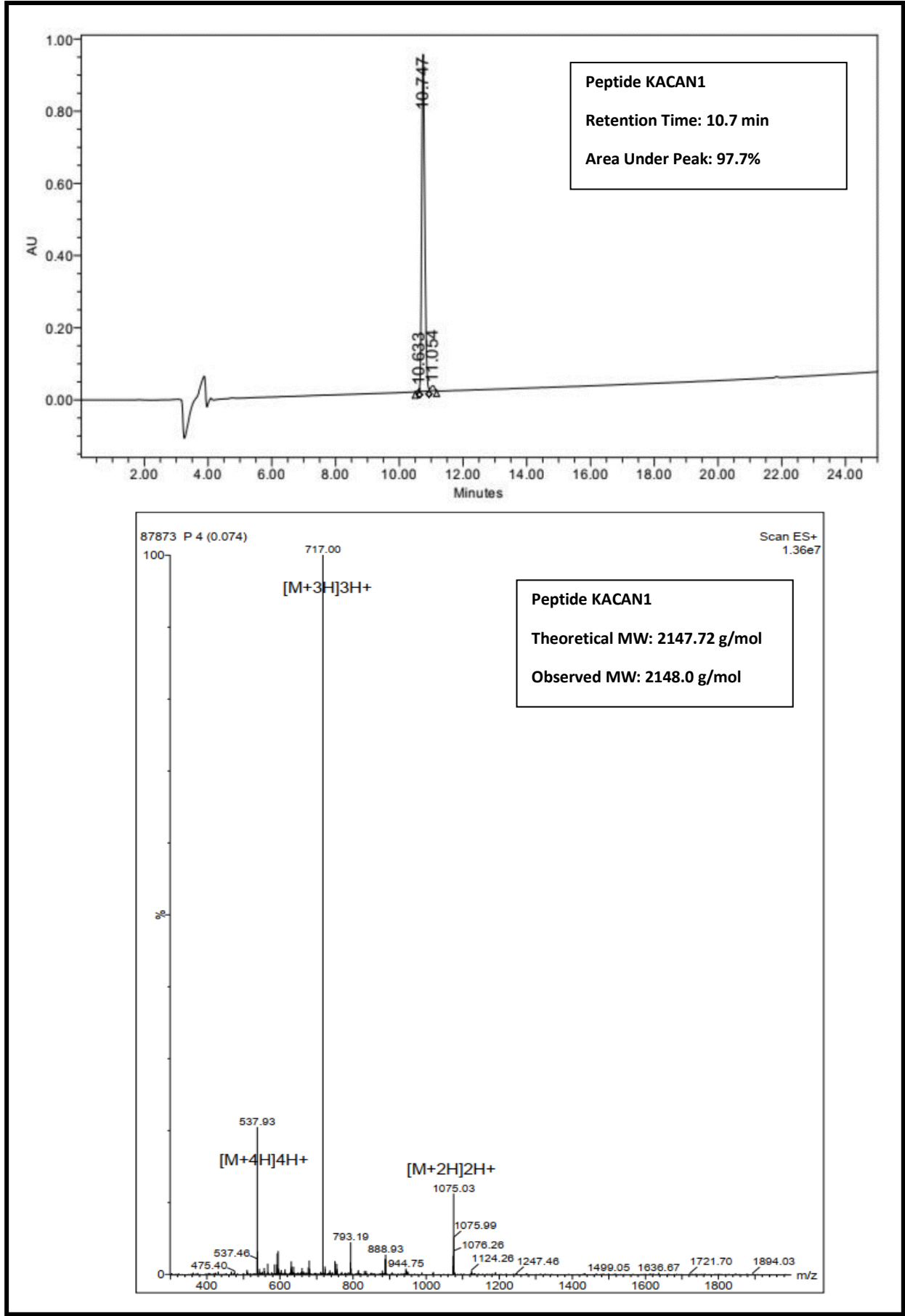


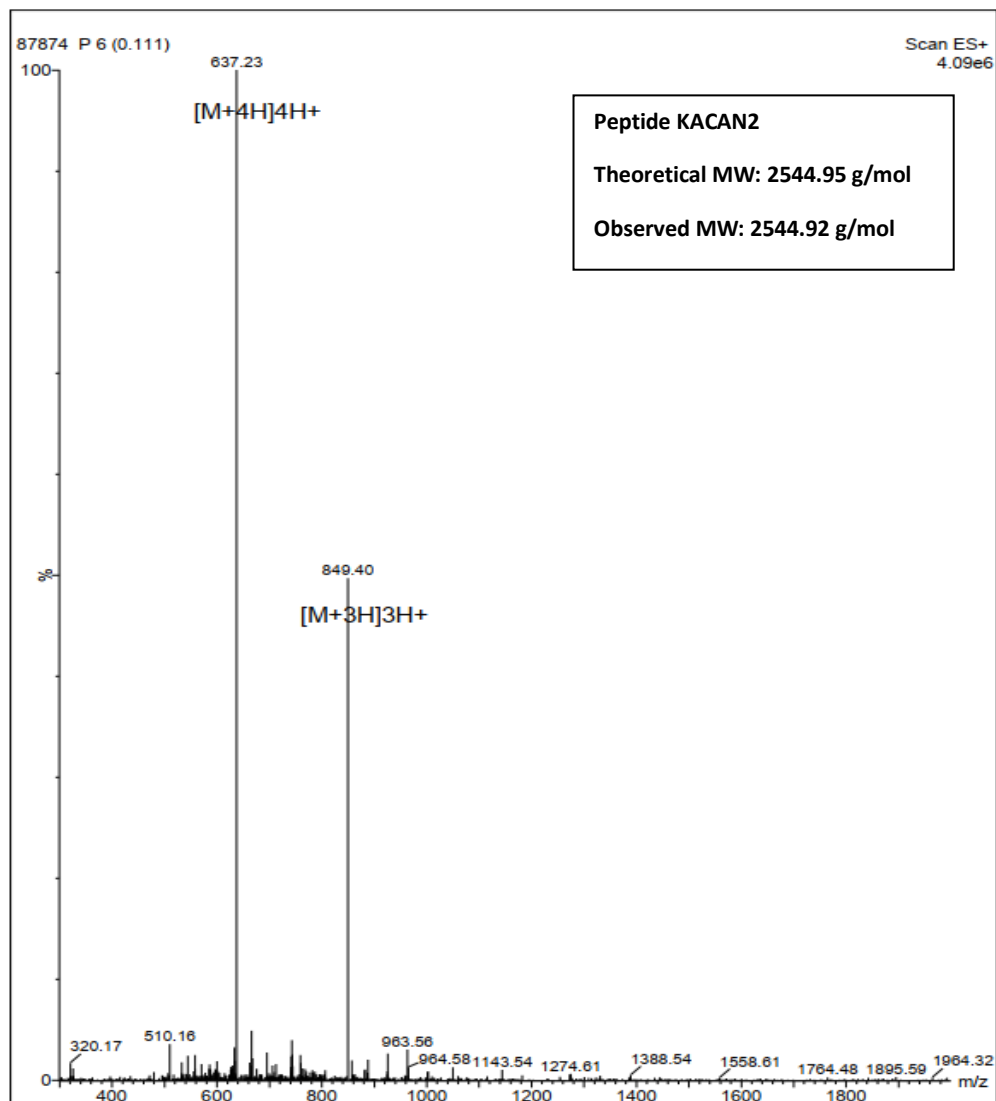
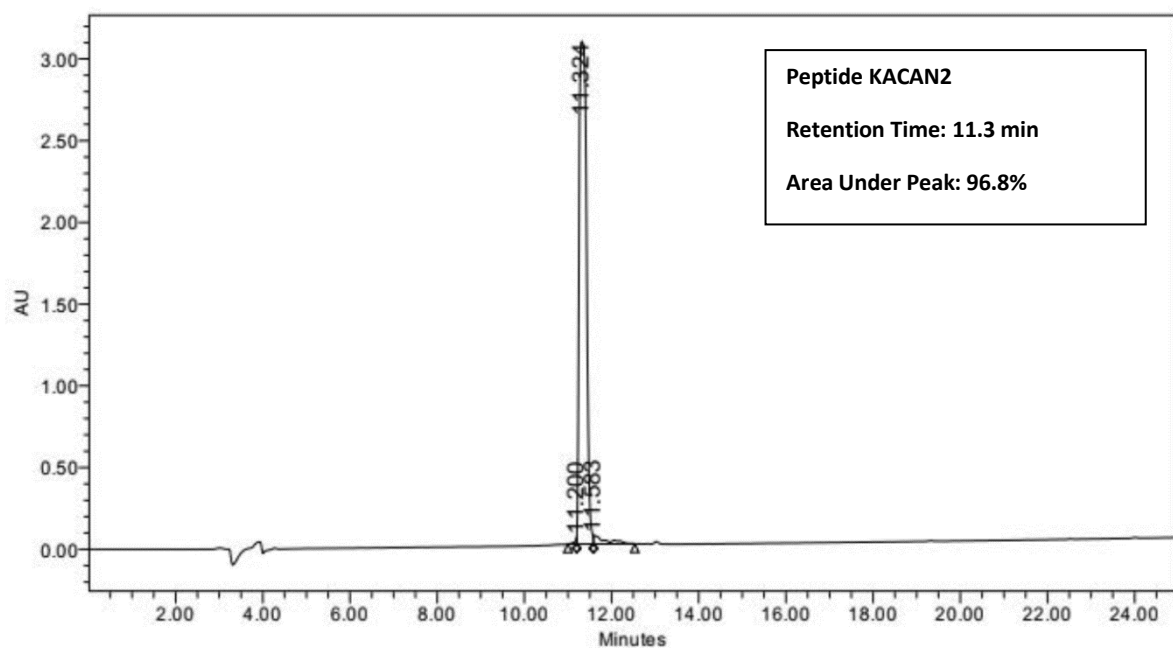


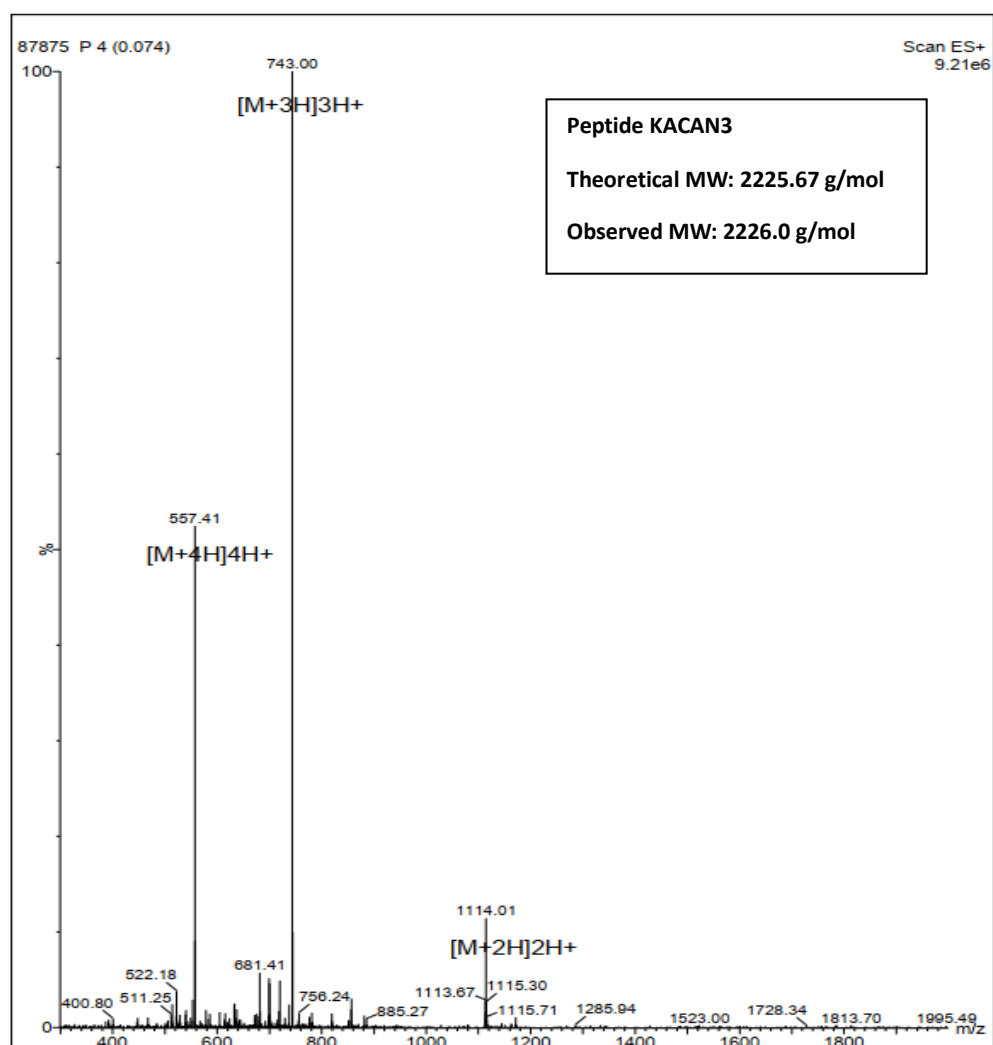
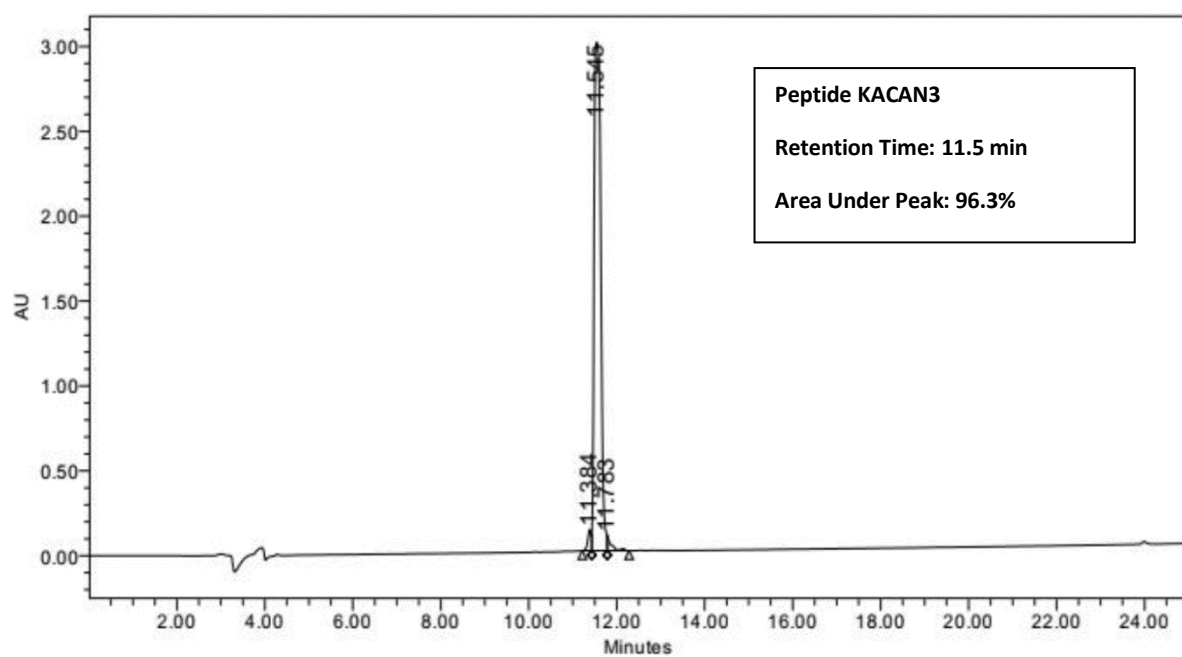


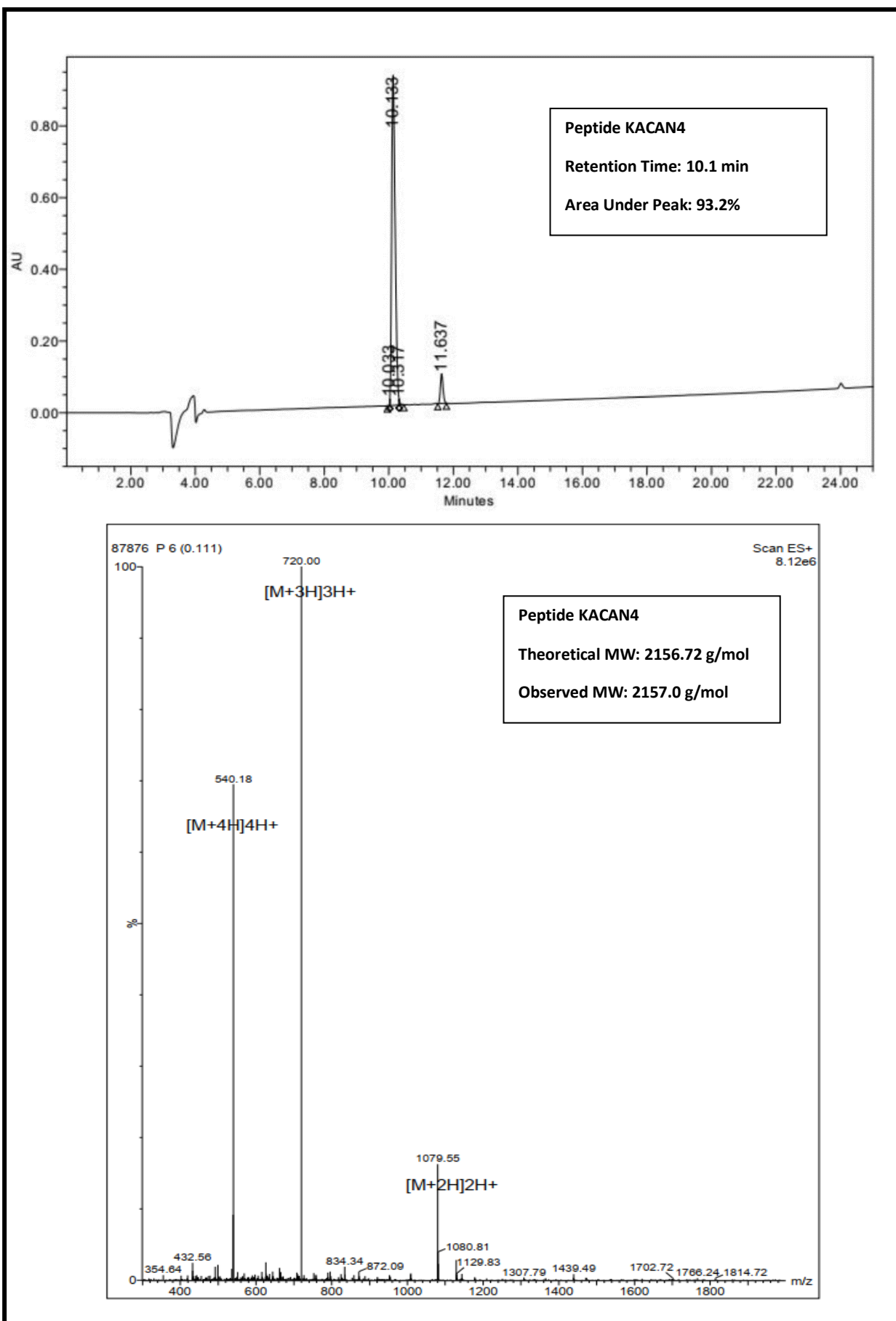


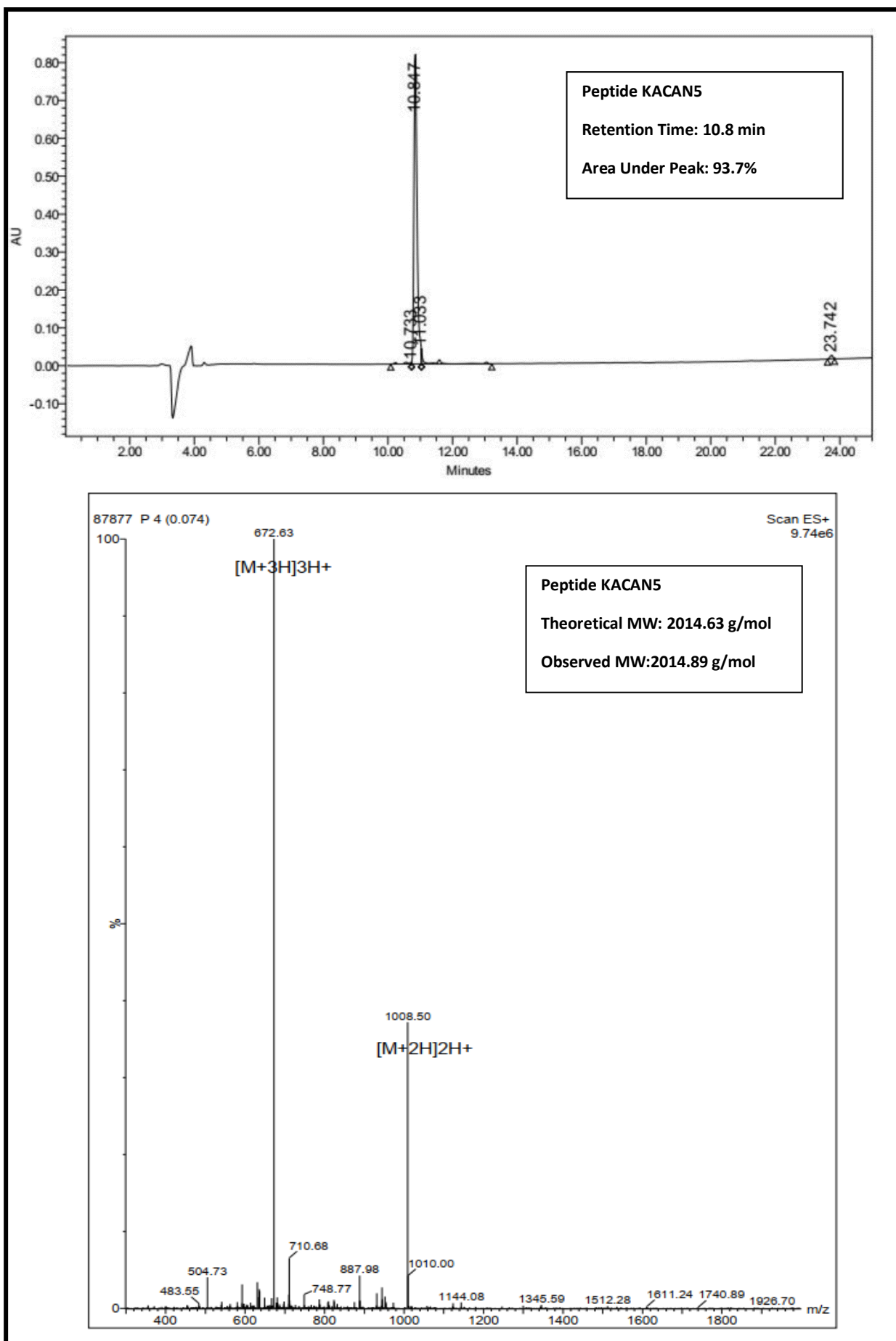


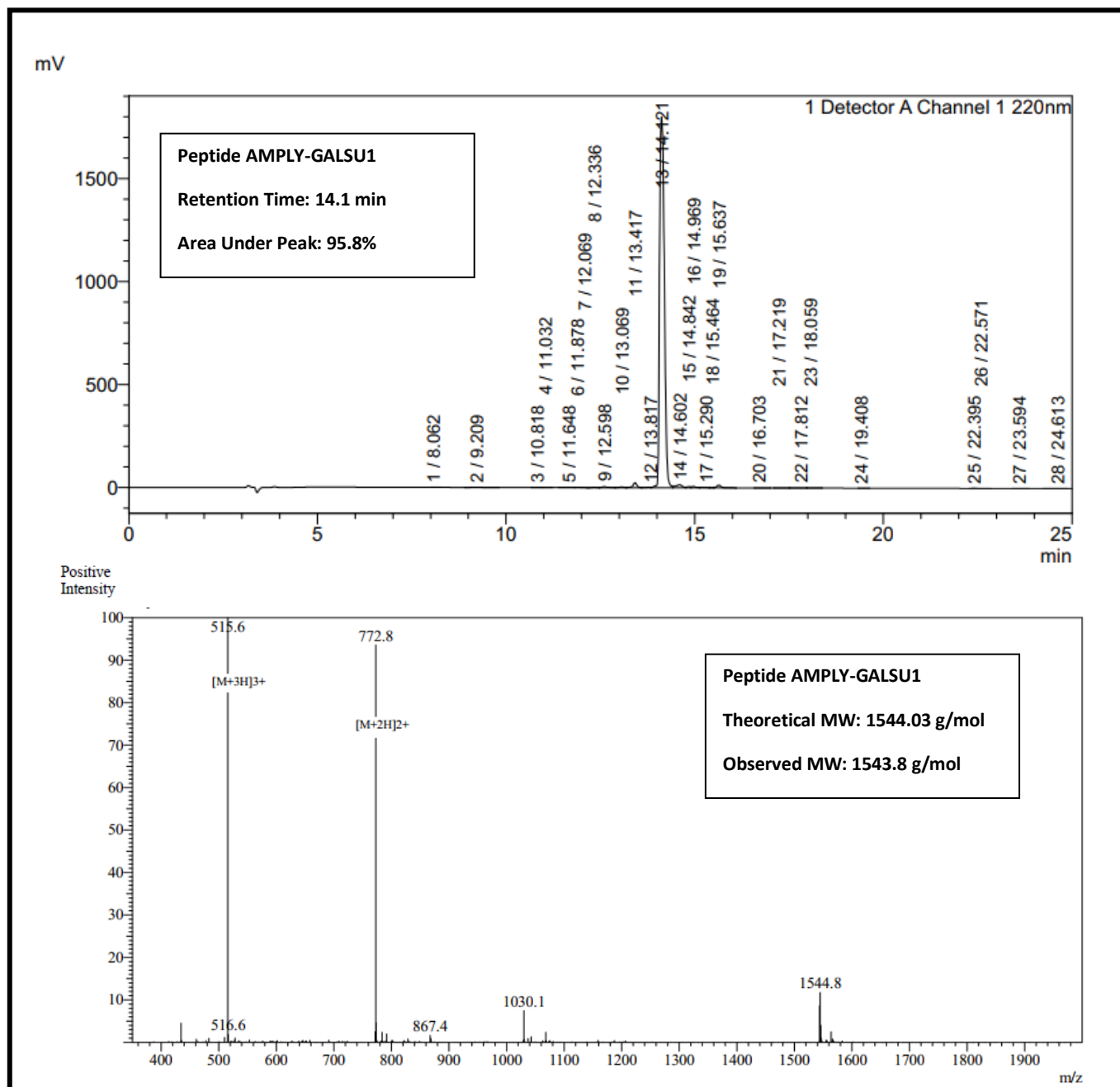


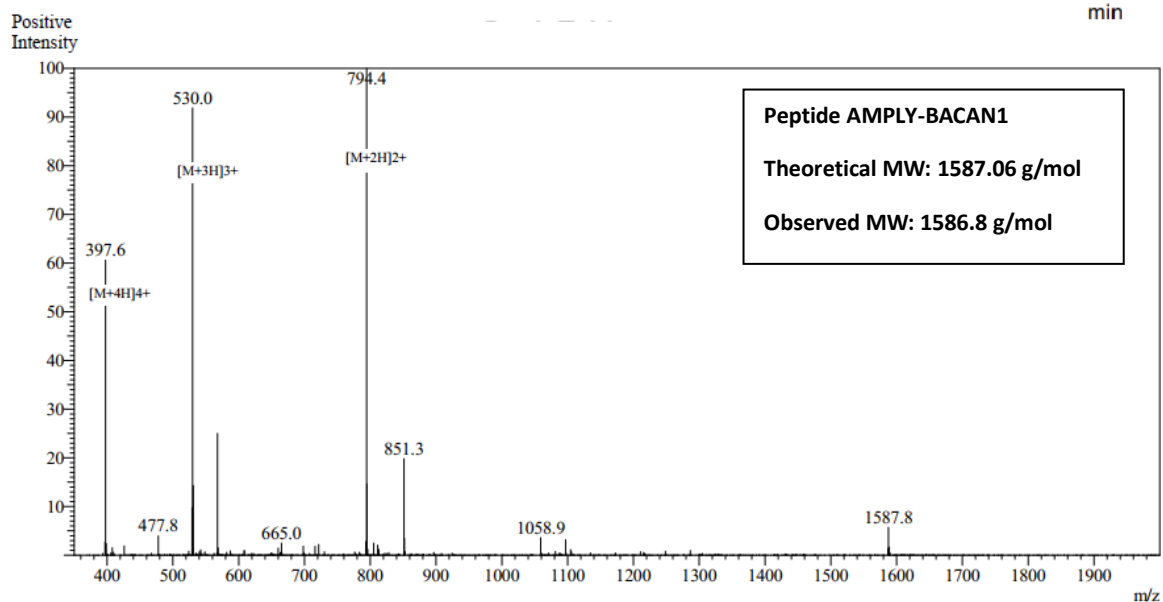
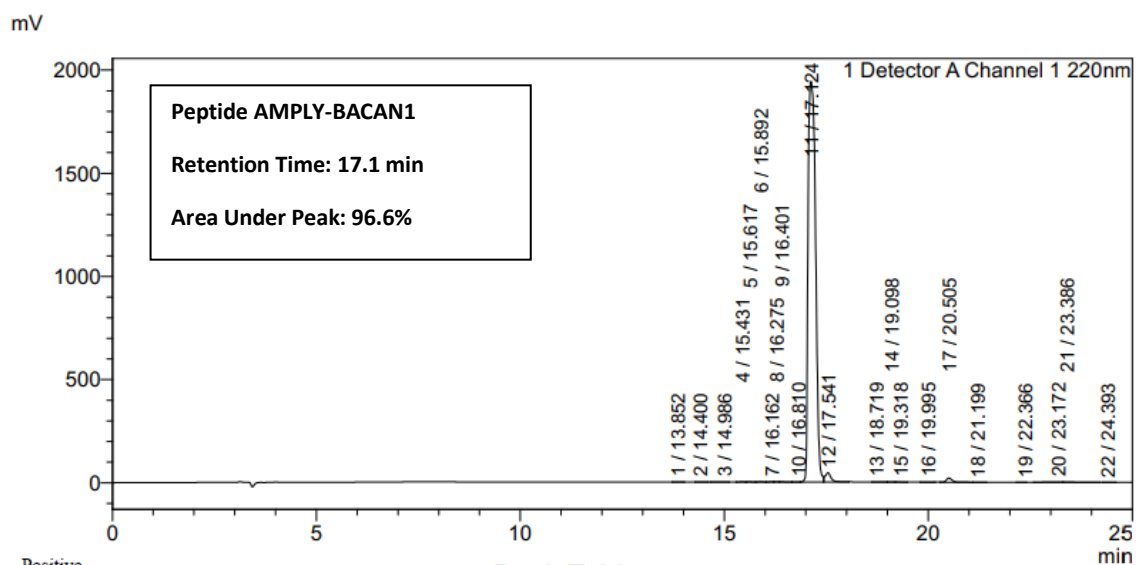


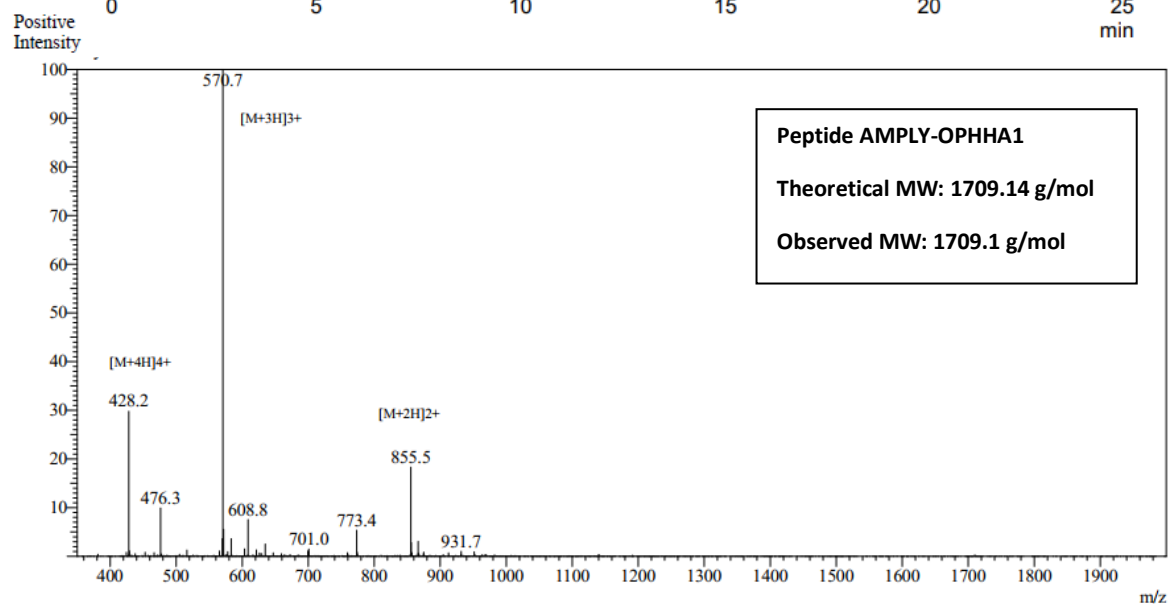
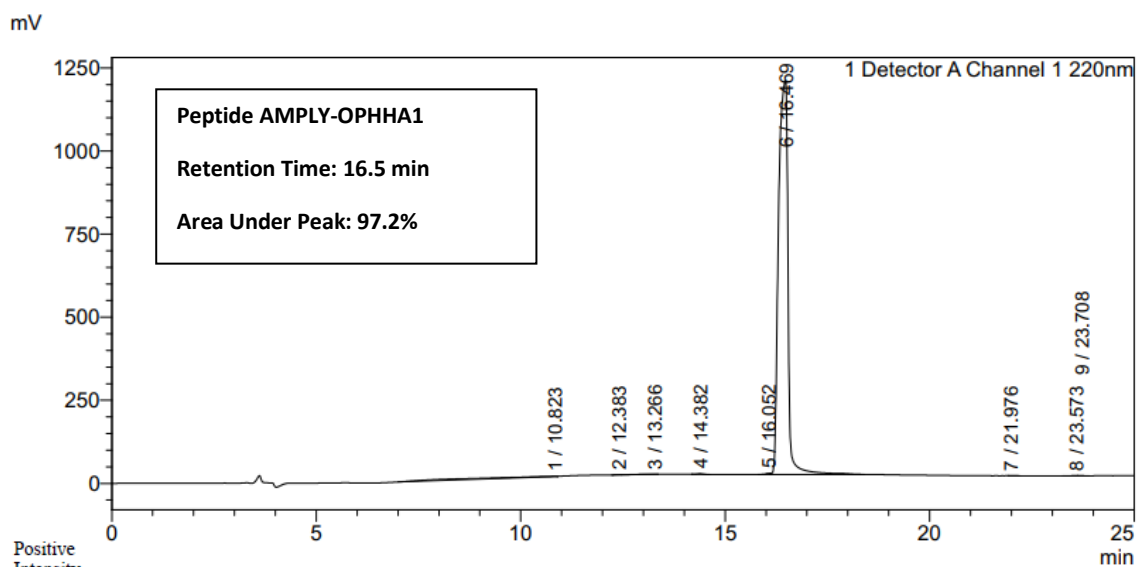




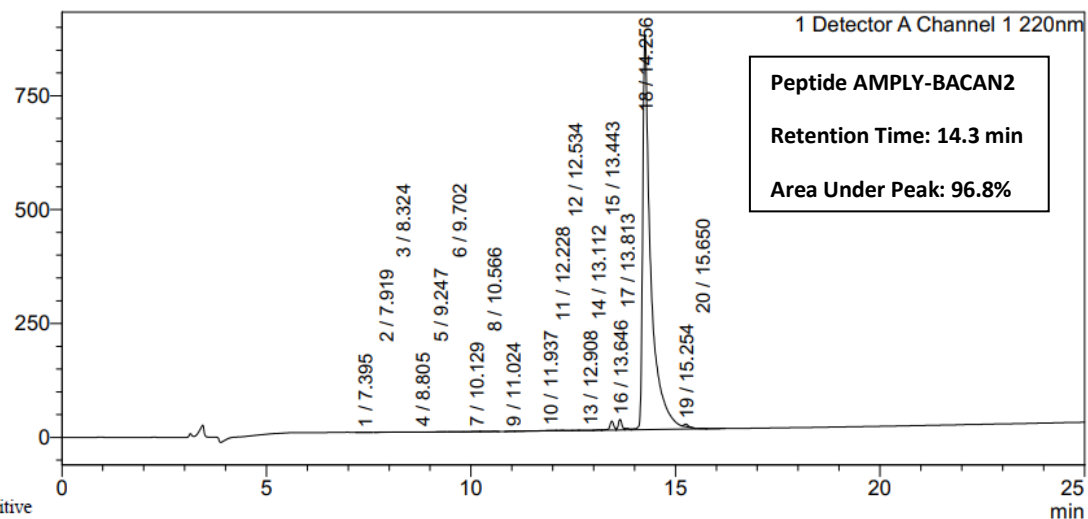




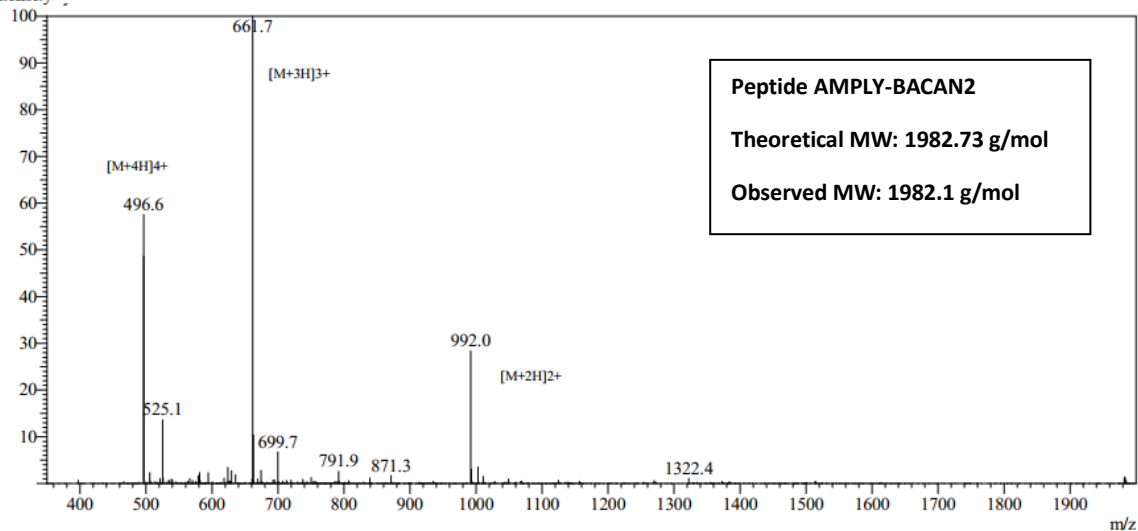




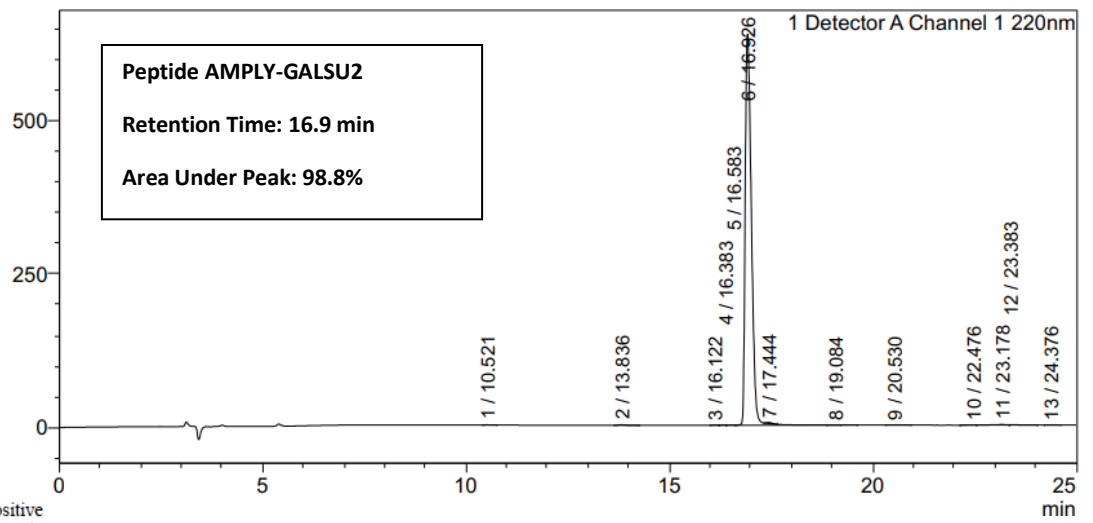
mV



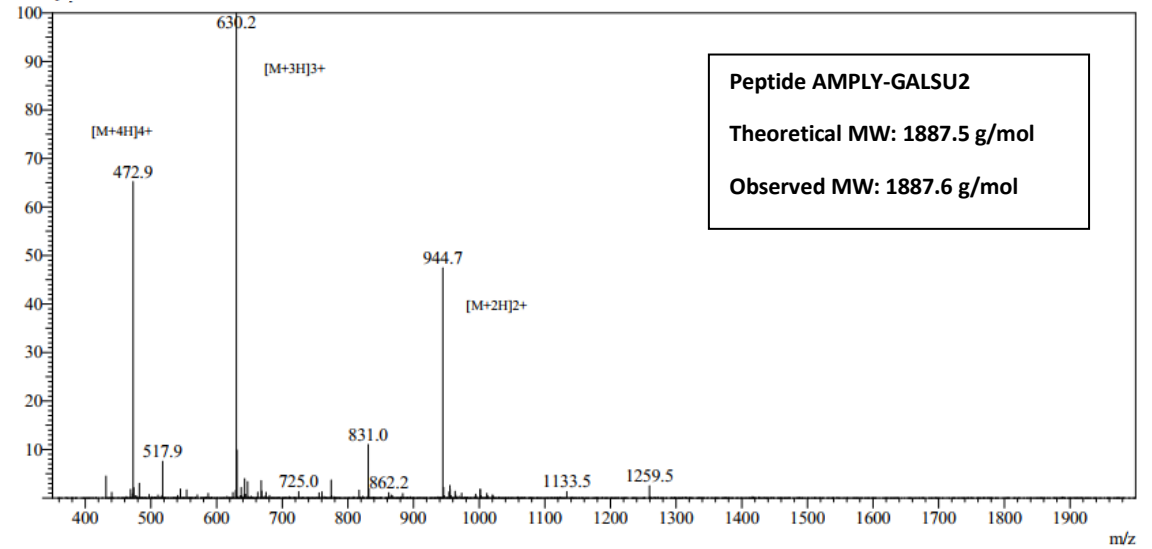
Positive
Intensity



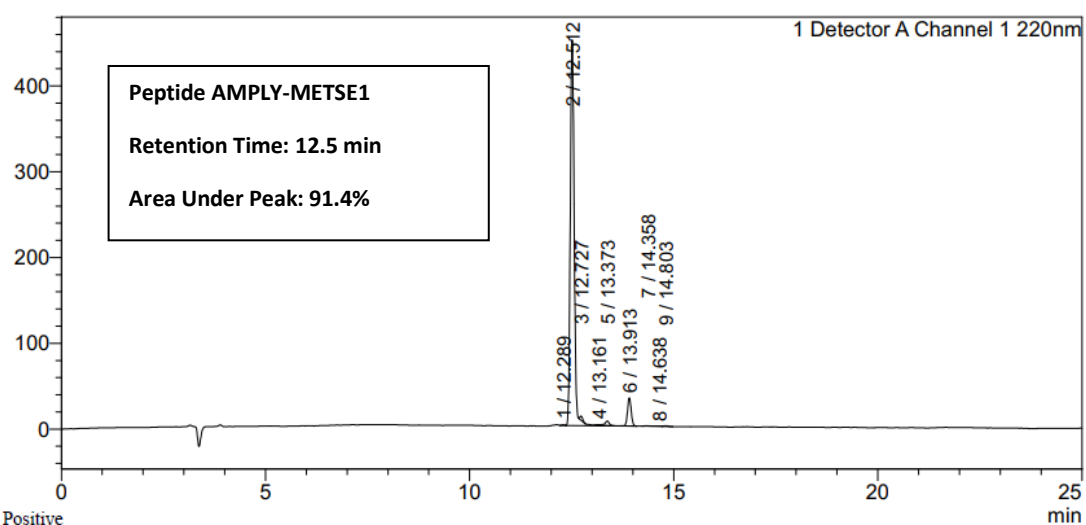
mV



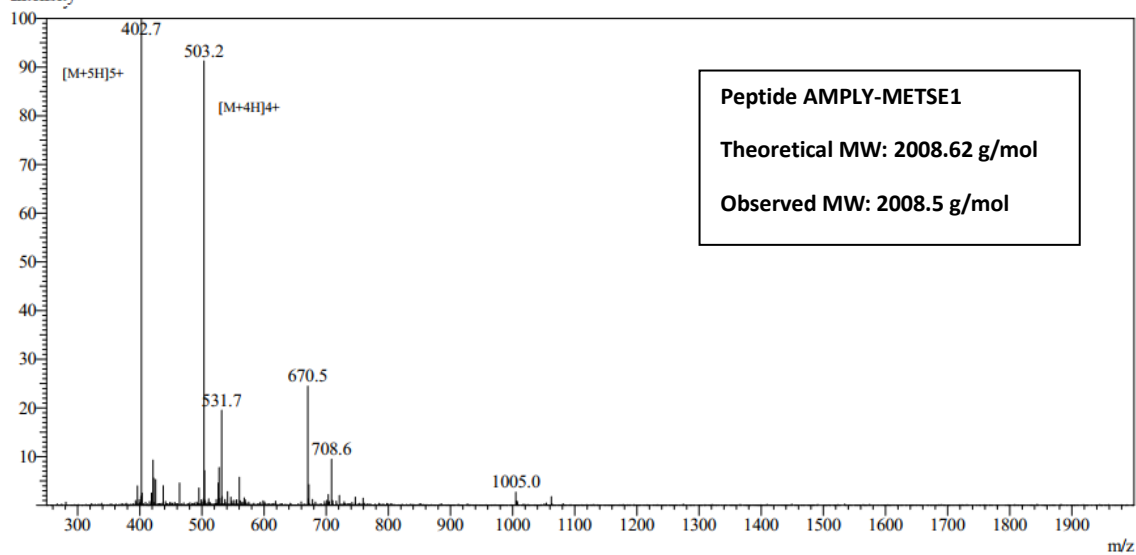
Positive
Intensity



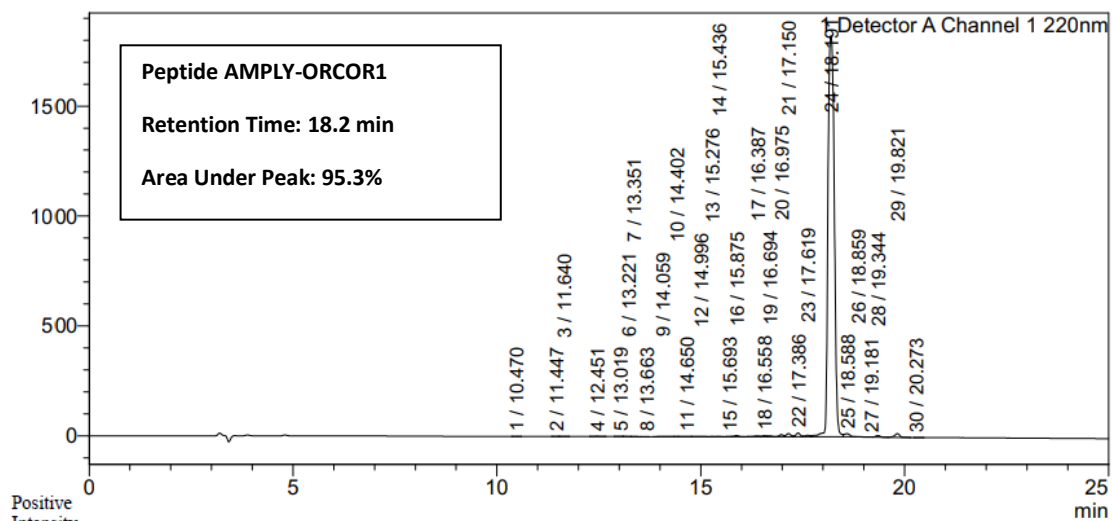
mV



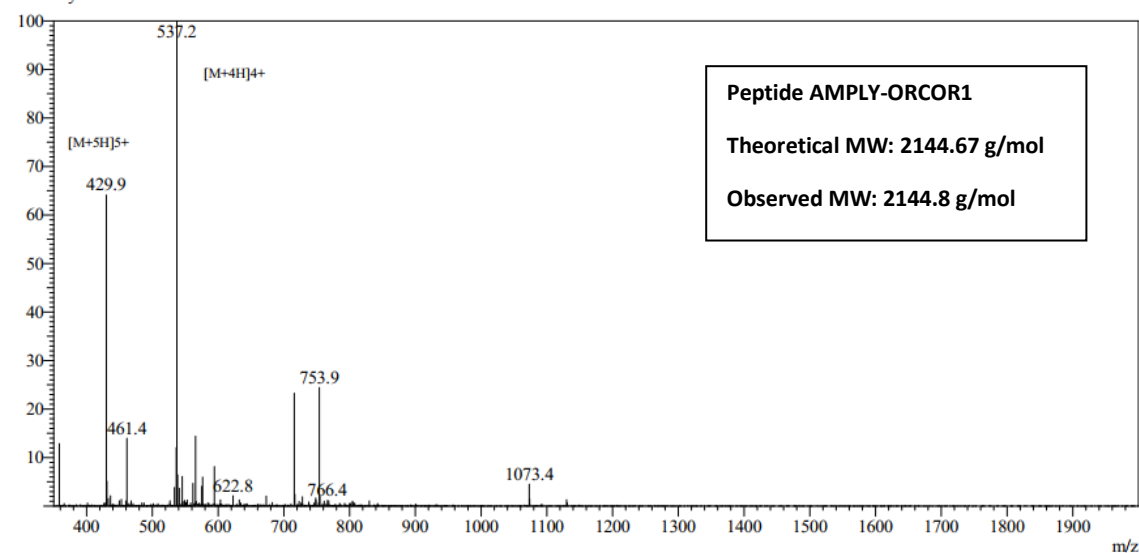
Positive
Intensity



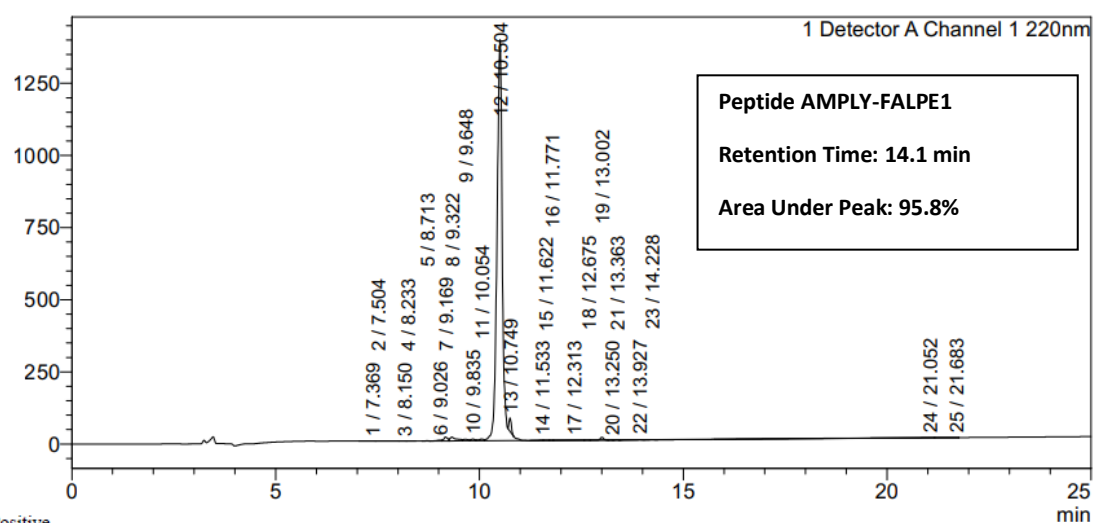
mV



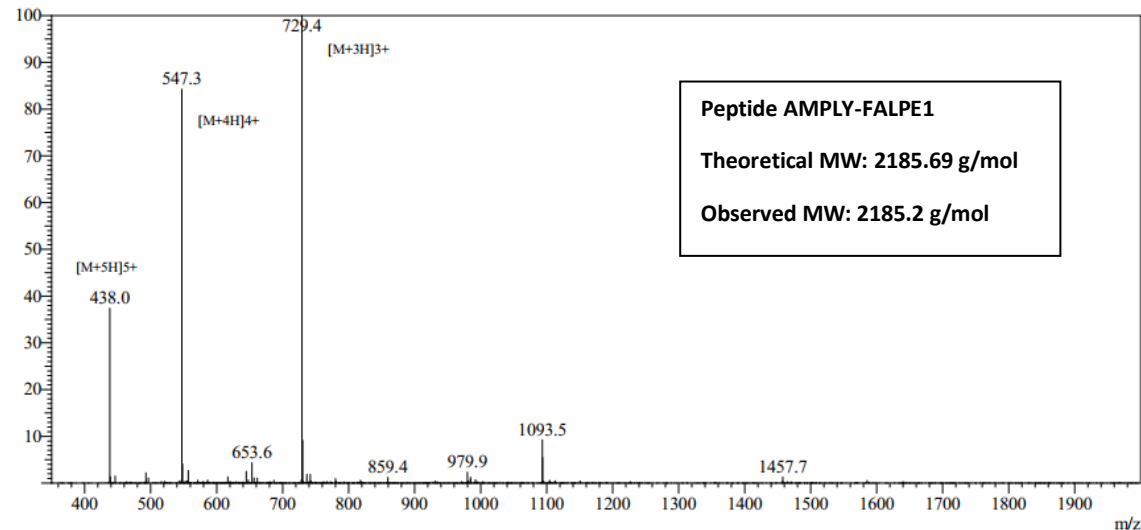
Positive Intensity



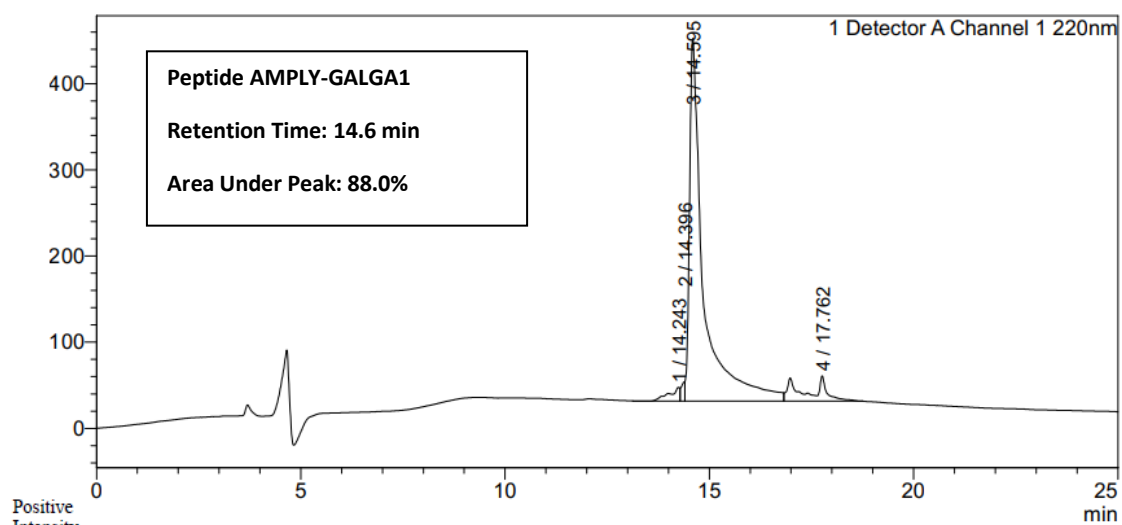
mV



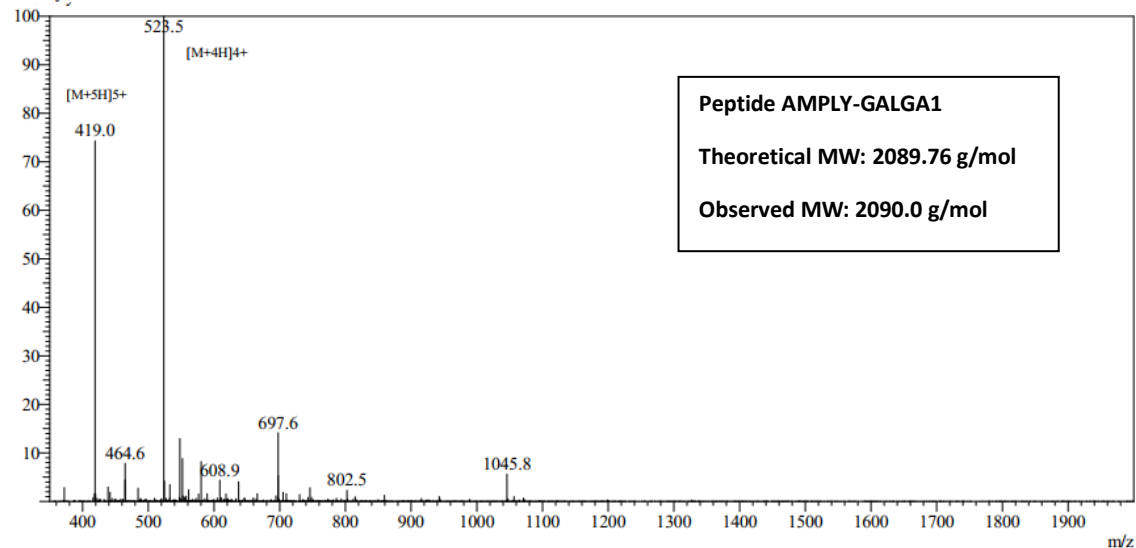
Positive
Intensity



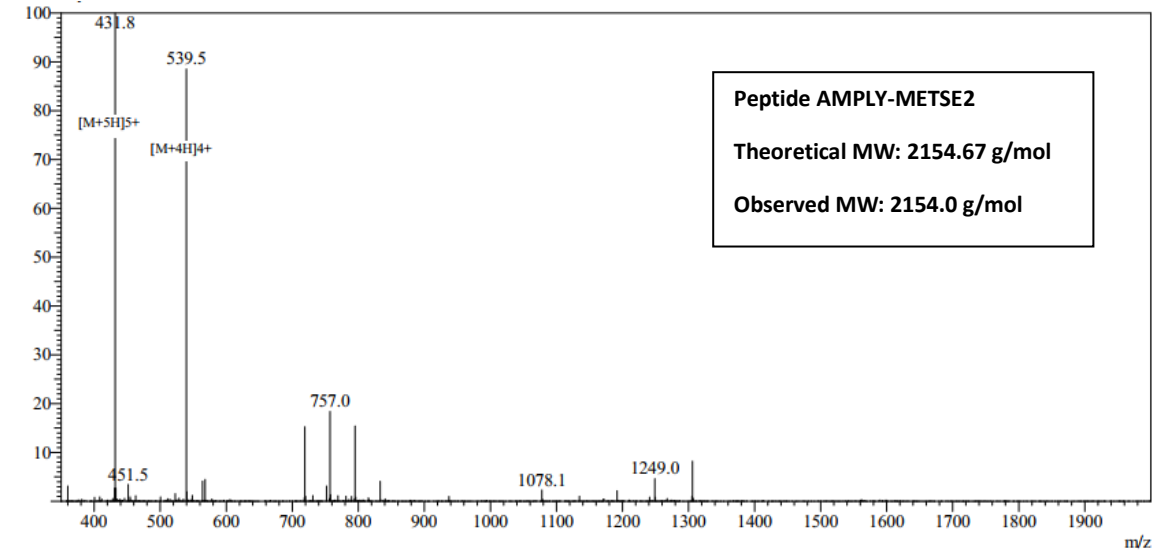
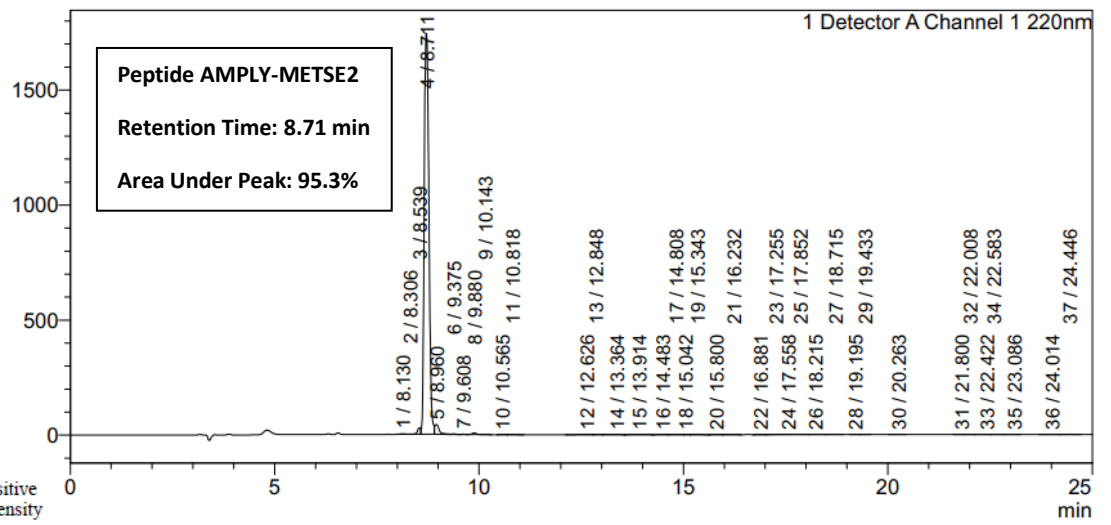
mV

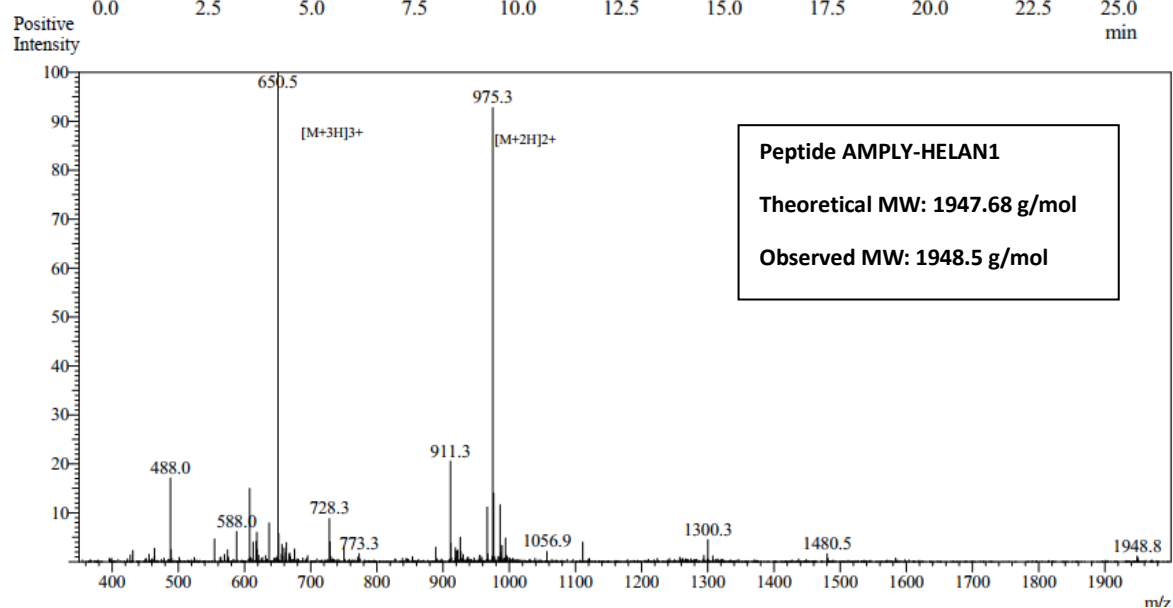
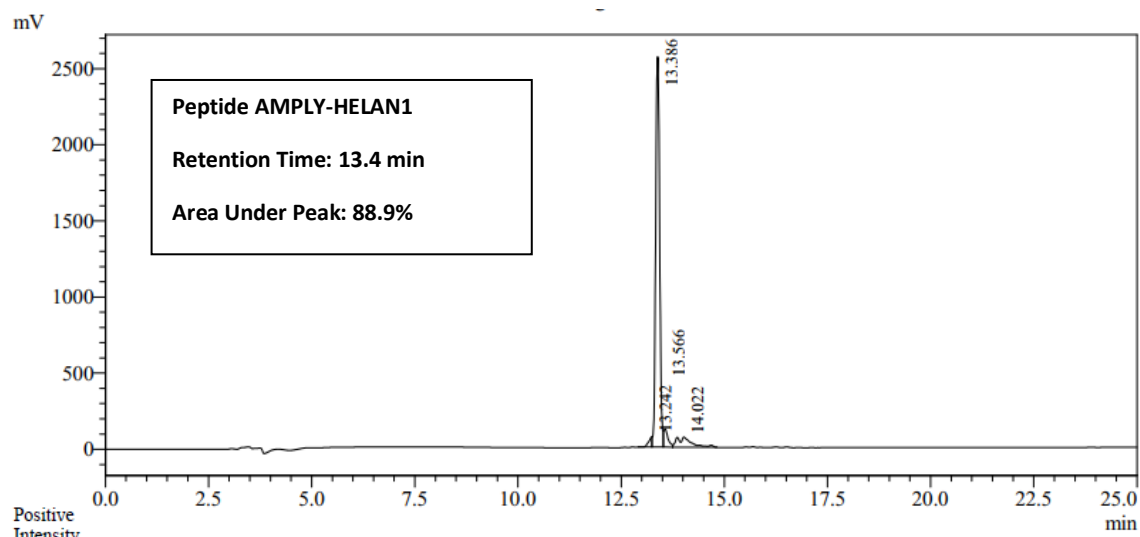


Positive
Intensity

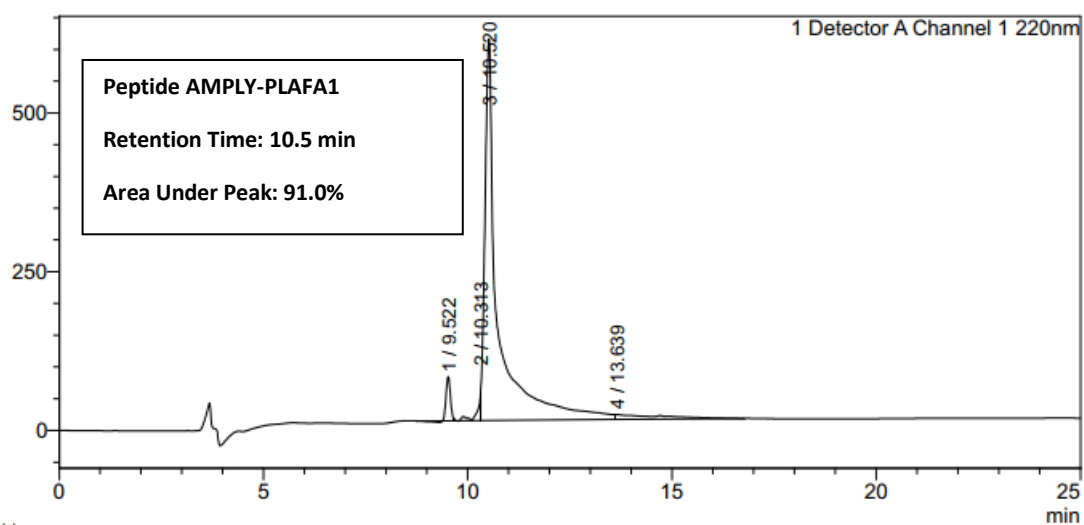


mV

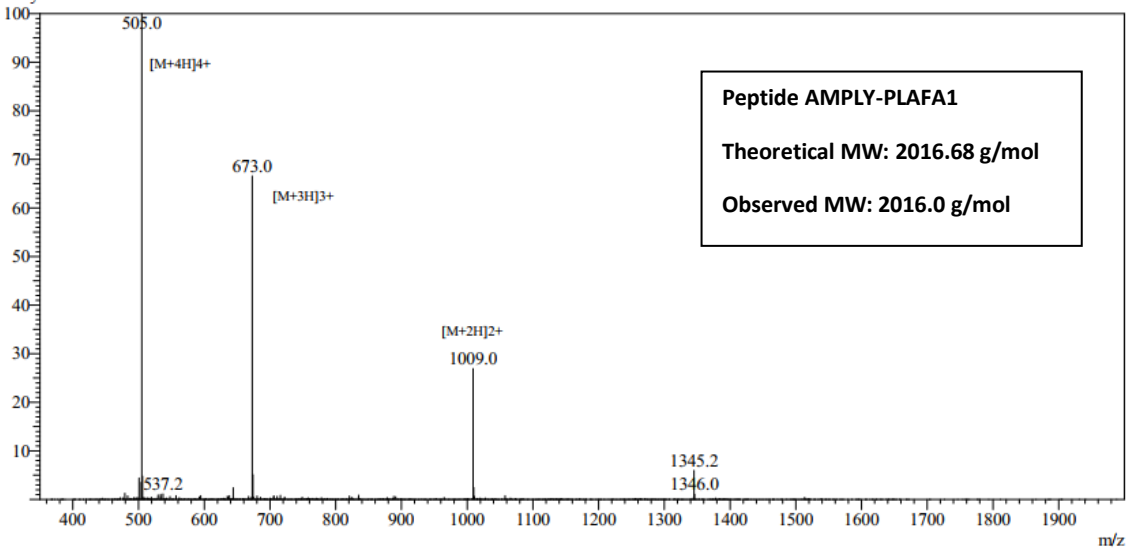


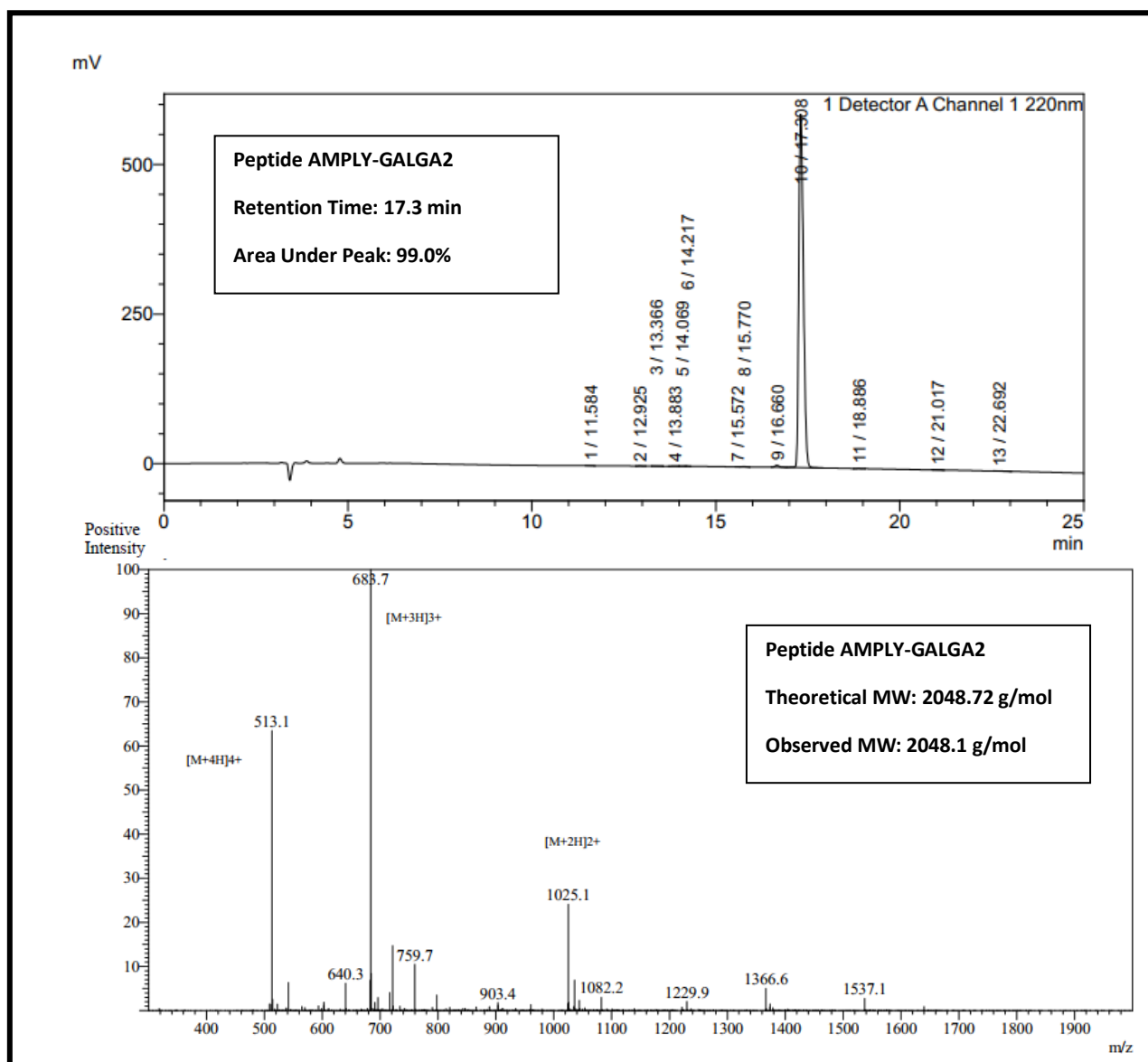


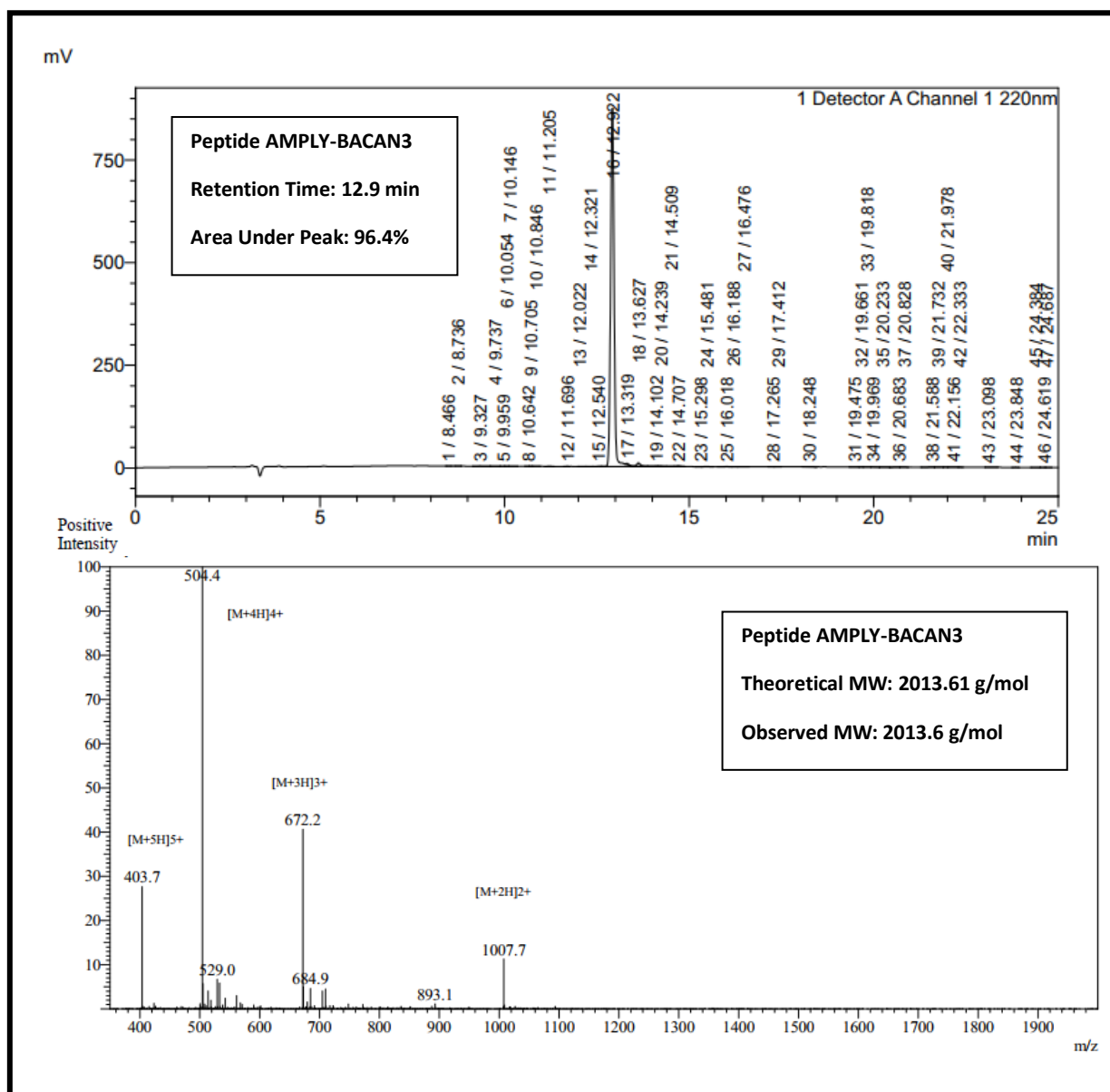
mV

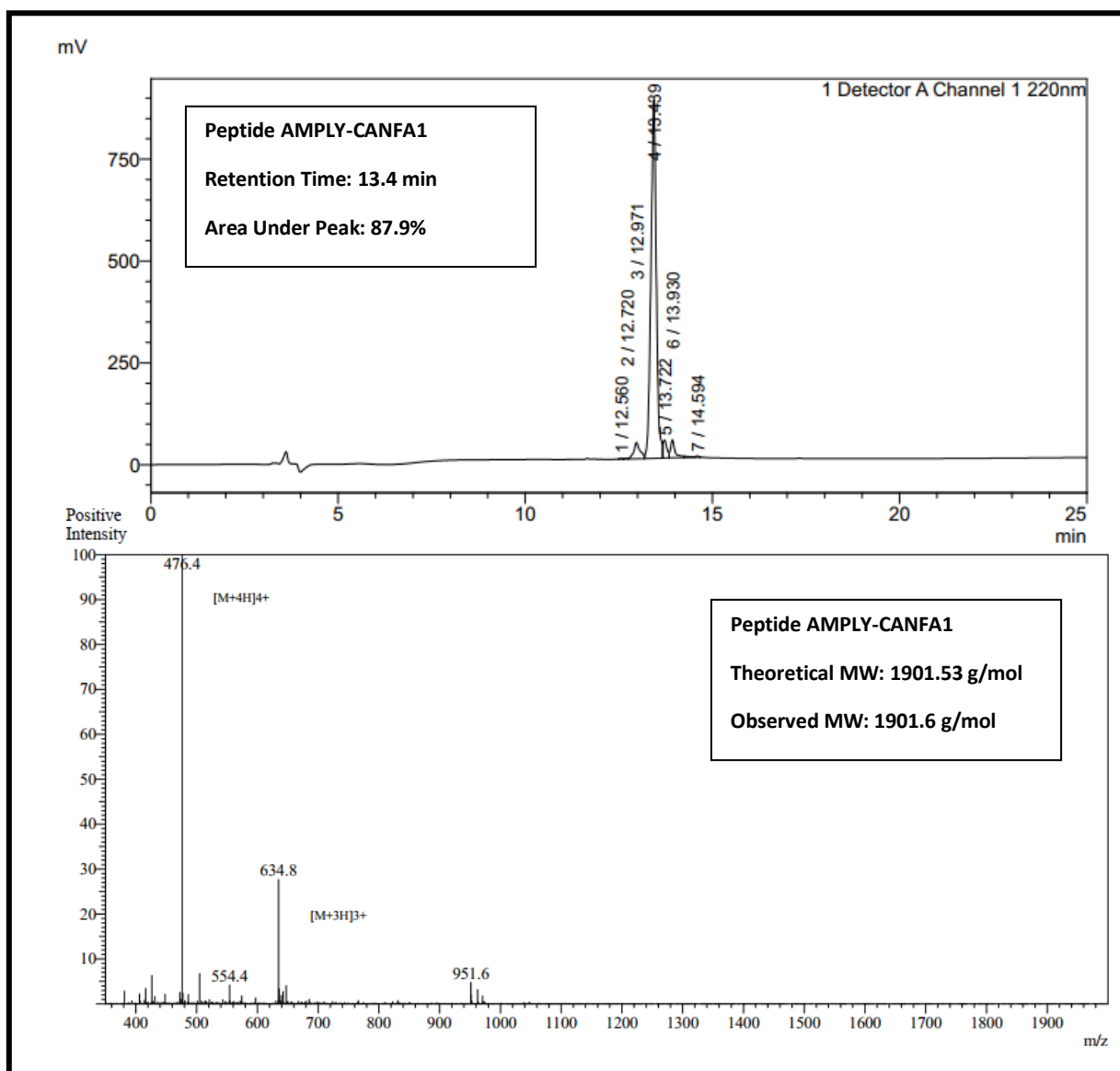


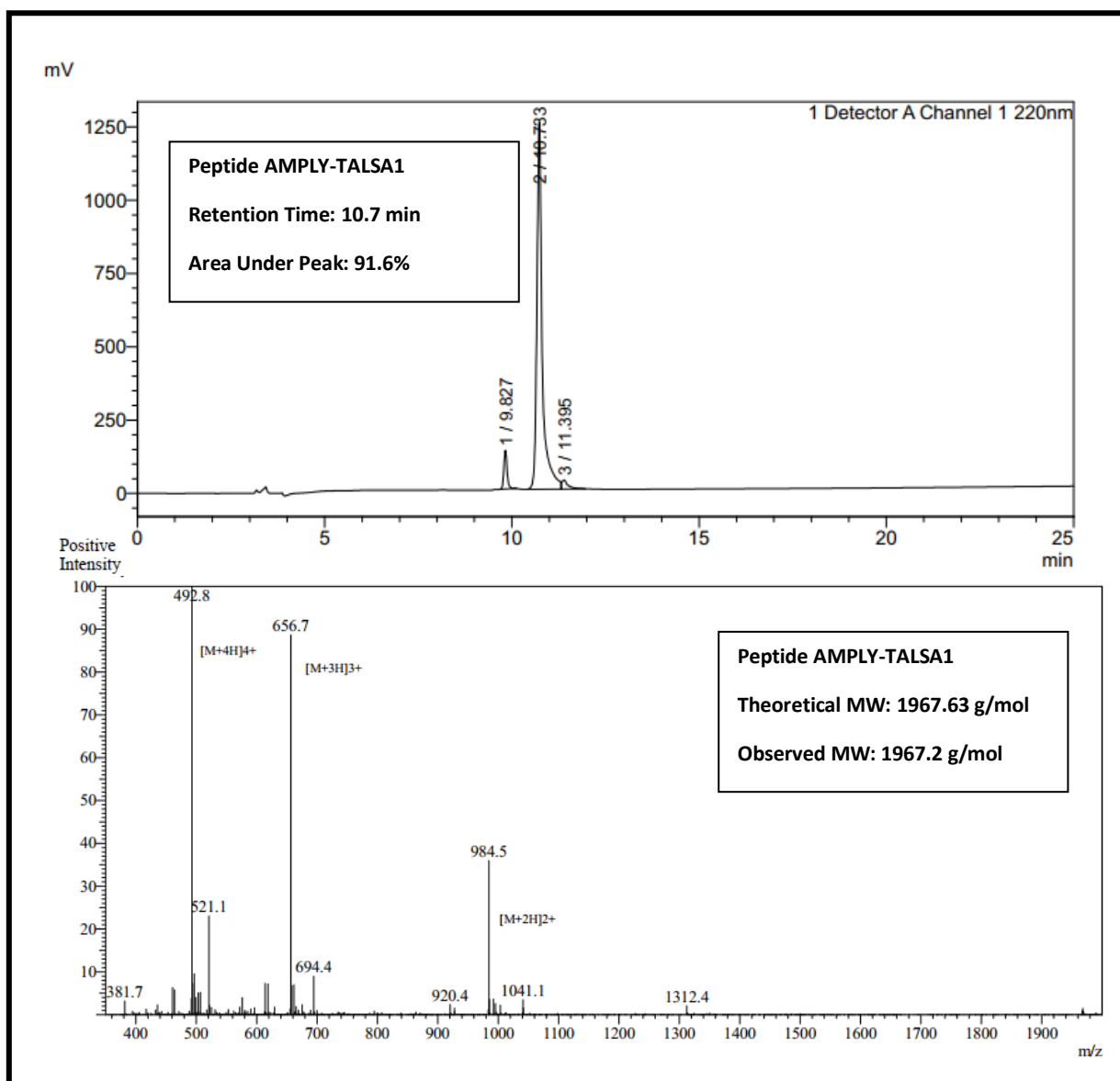
Positive
Intensity

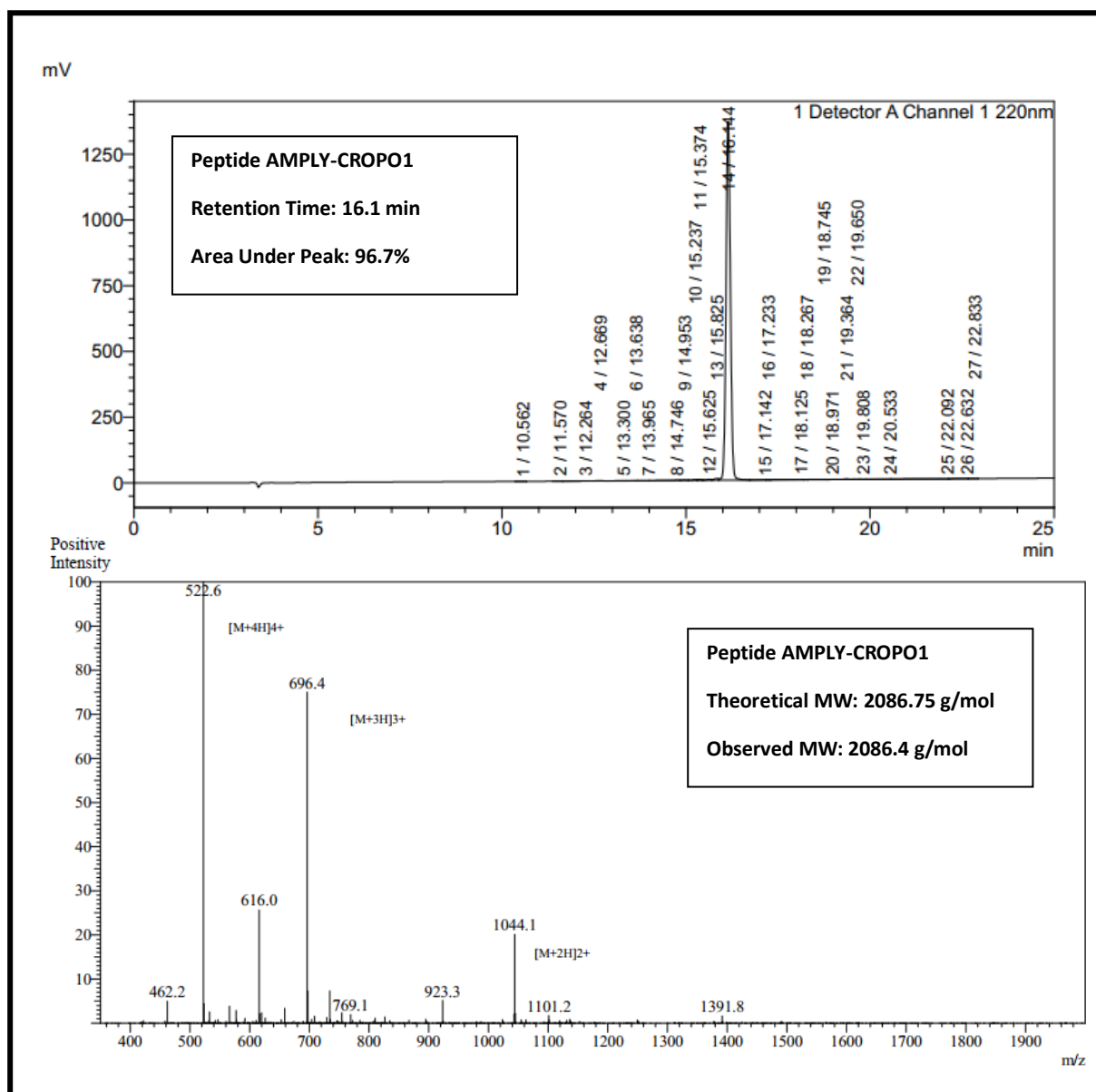


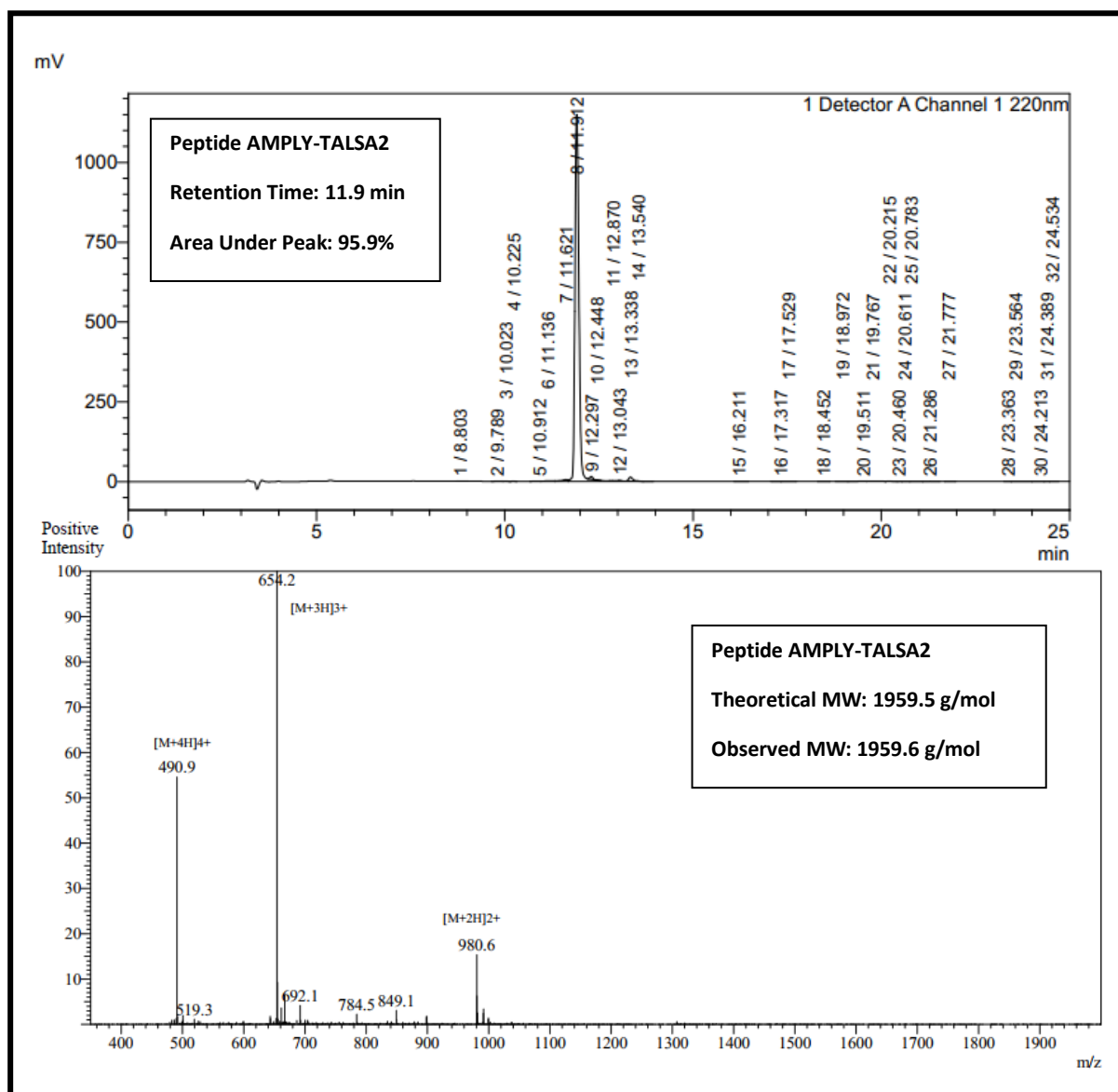


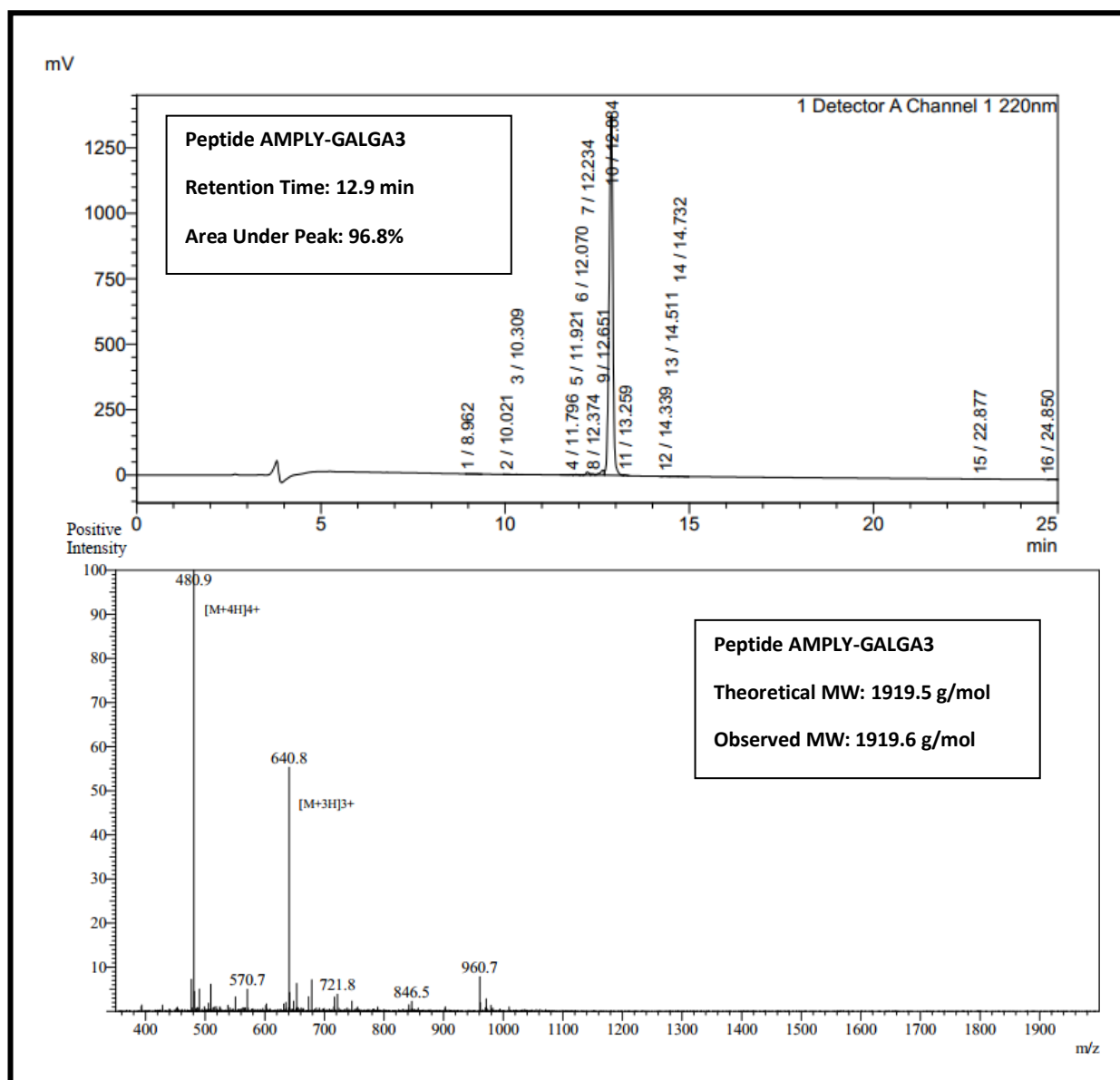


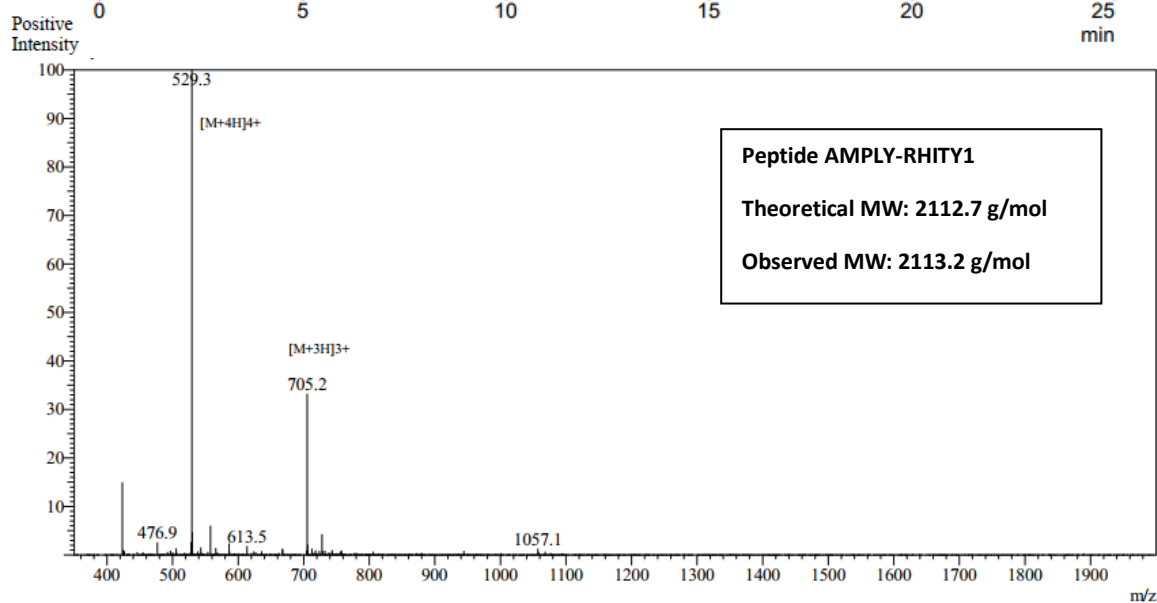
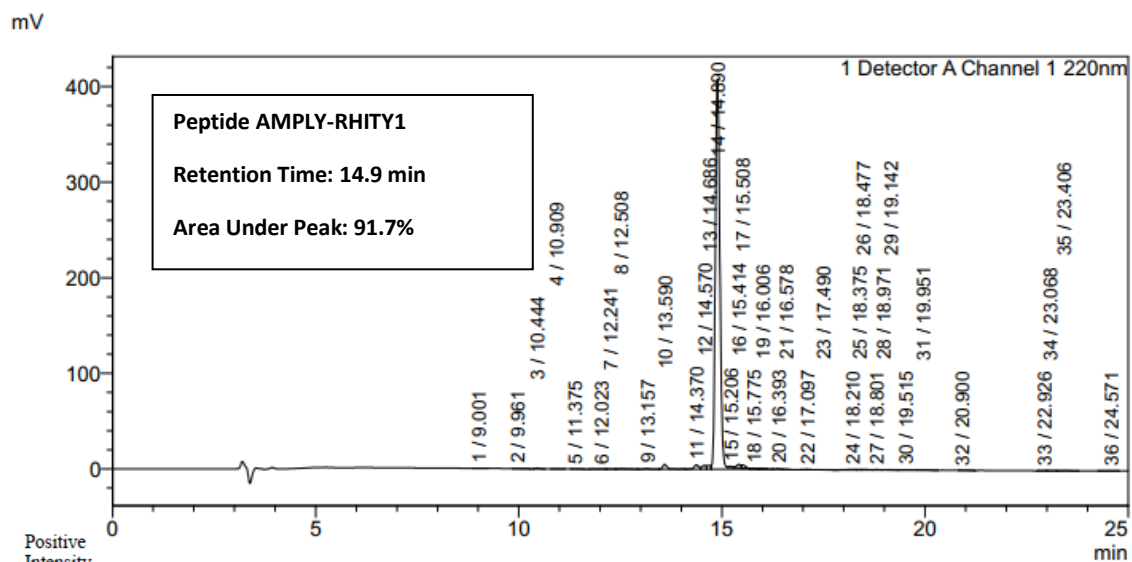


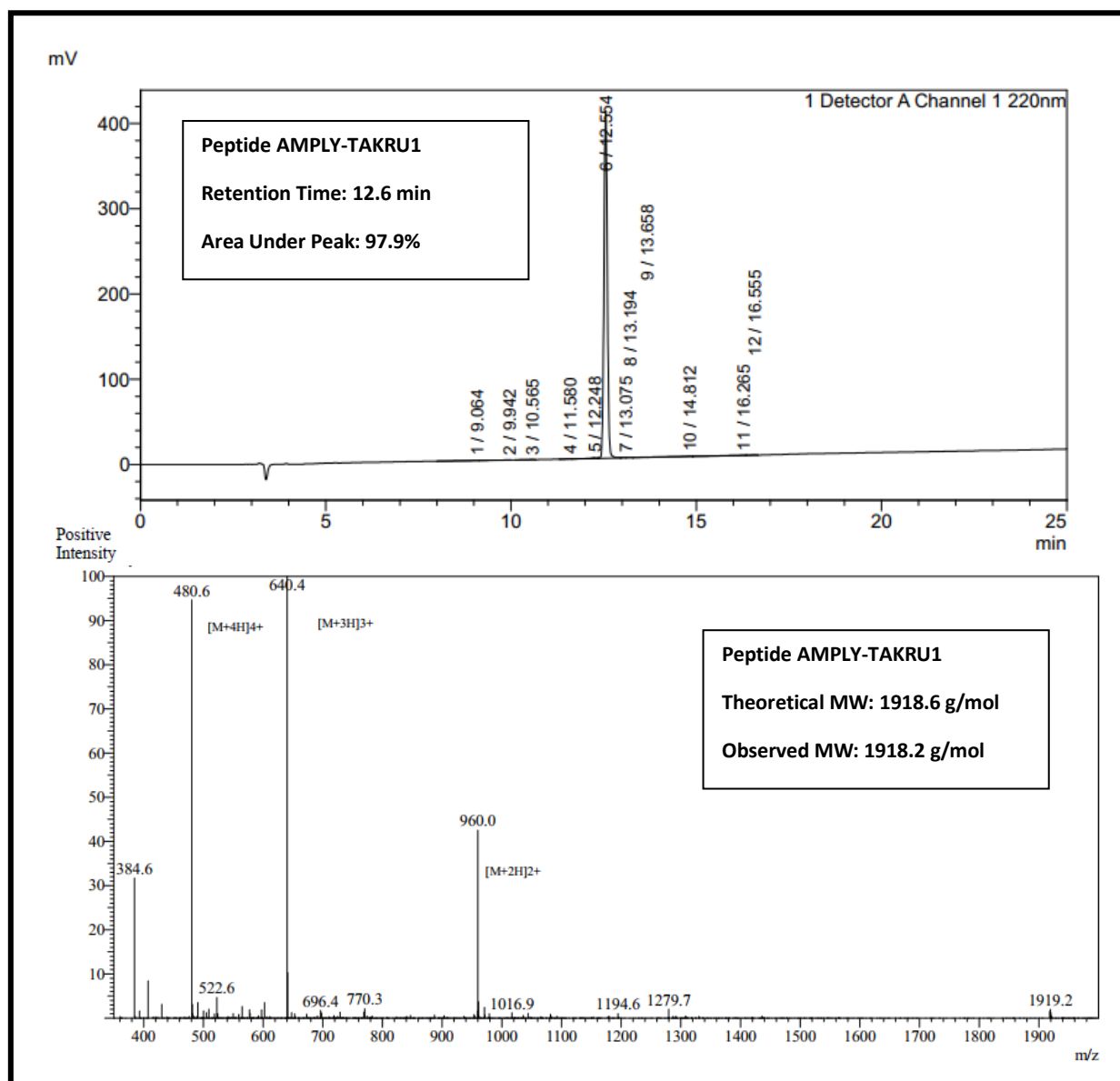


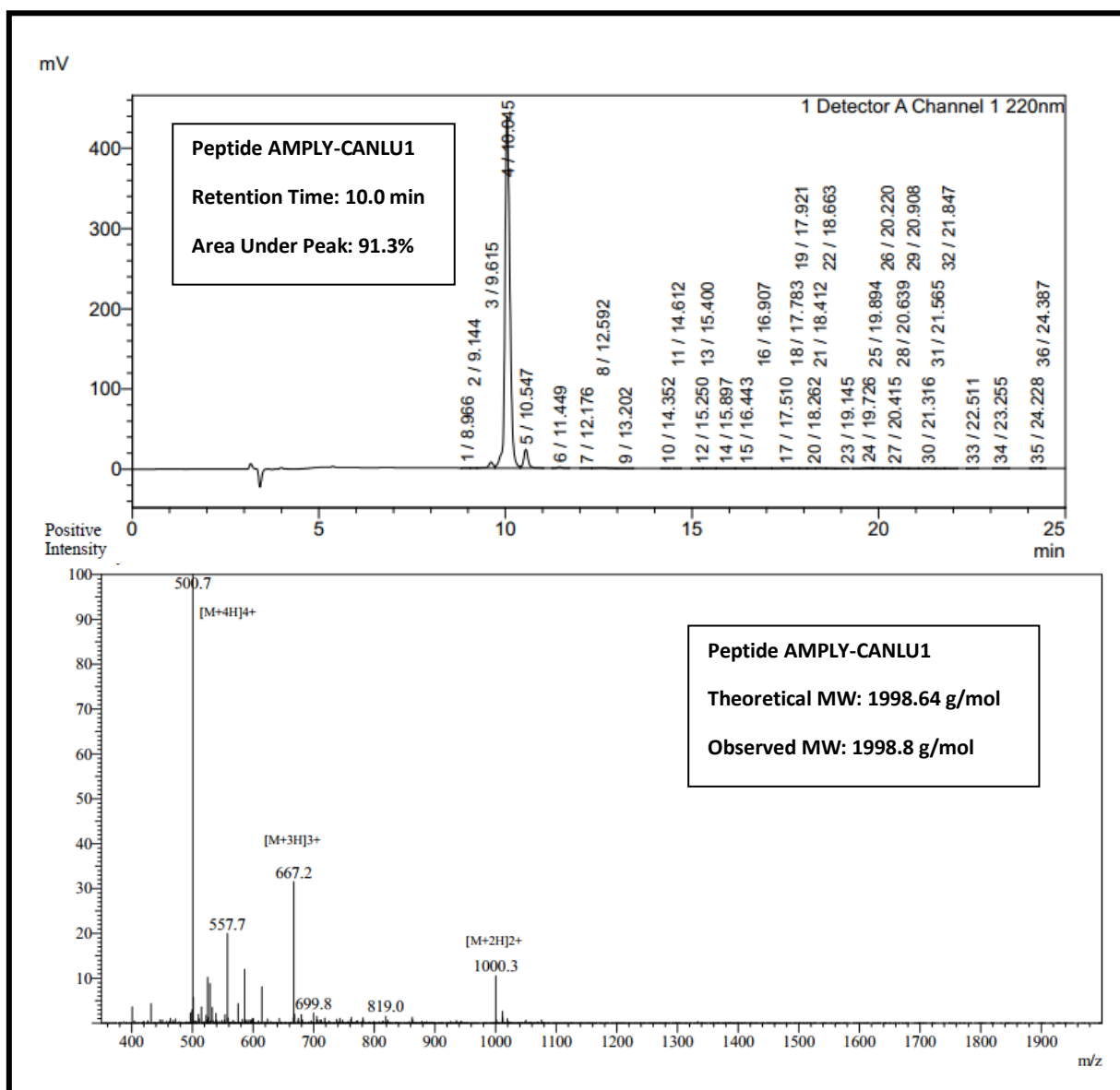


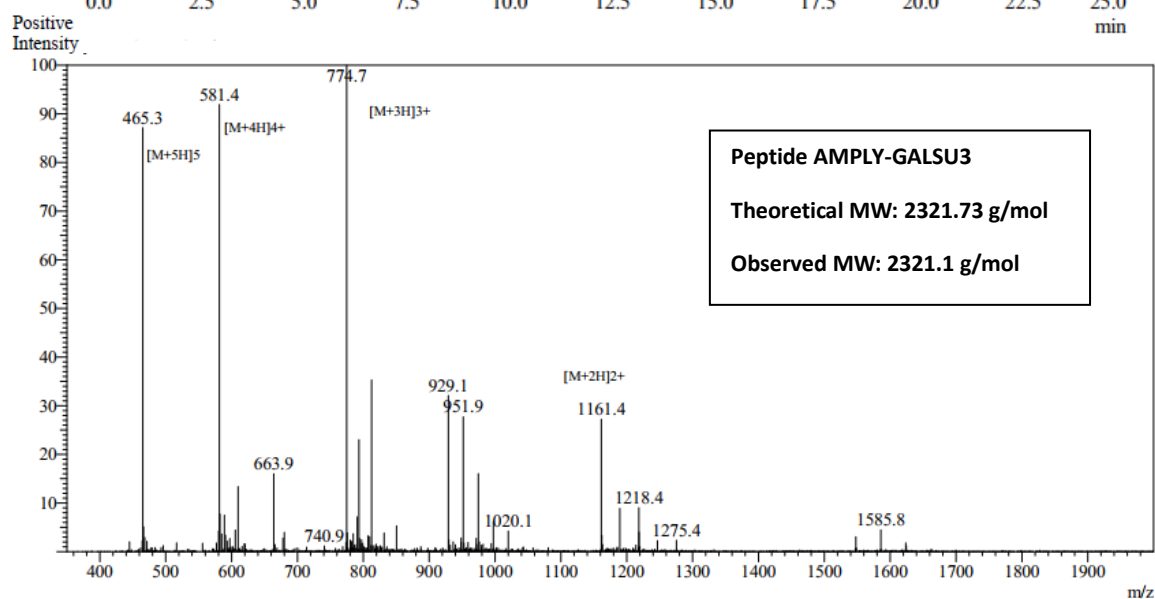
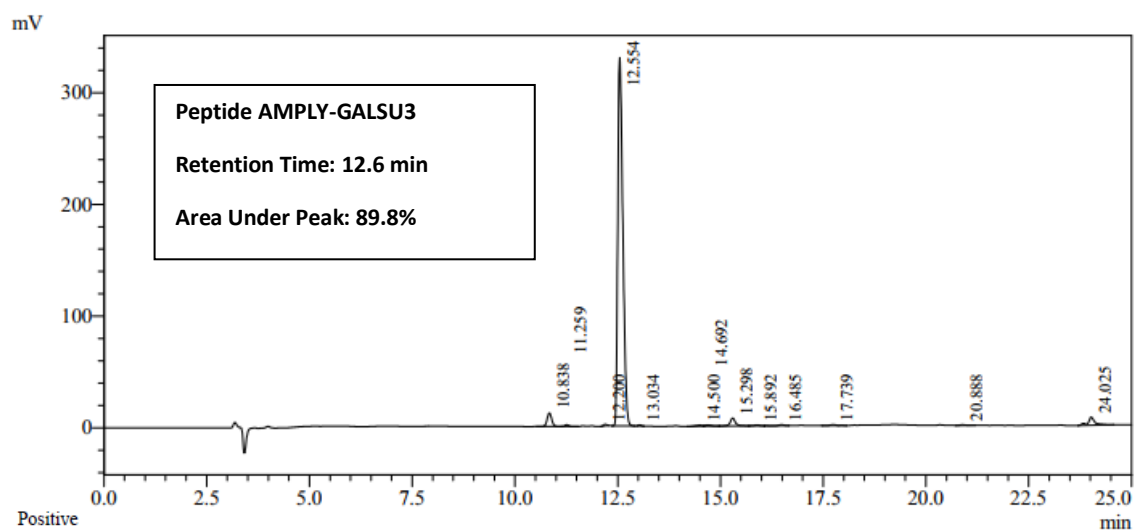


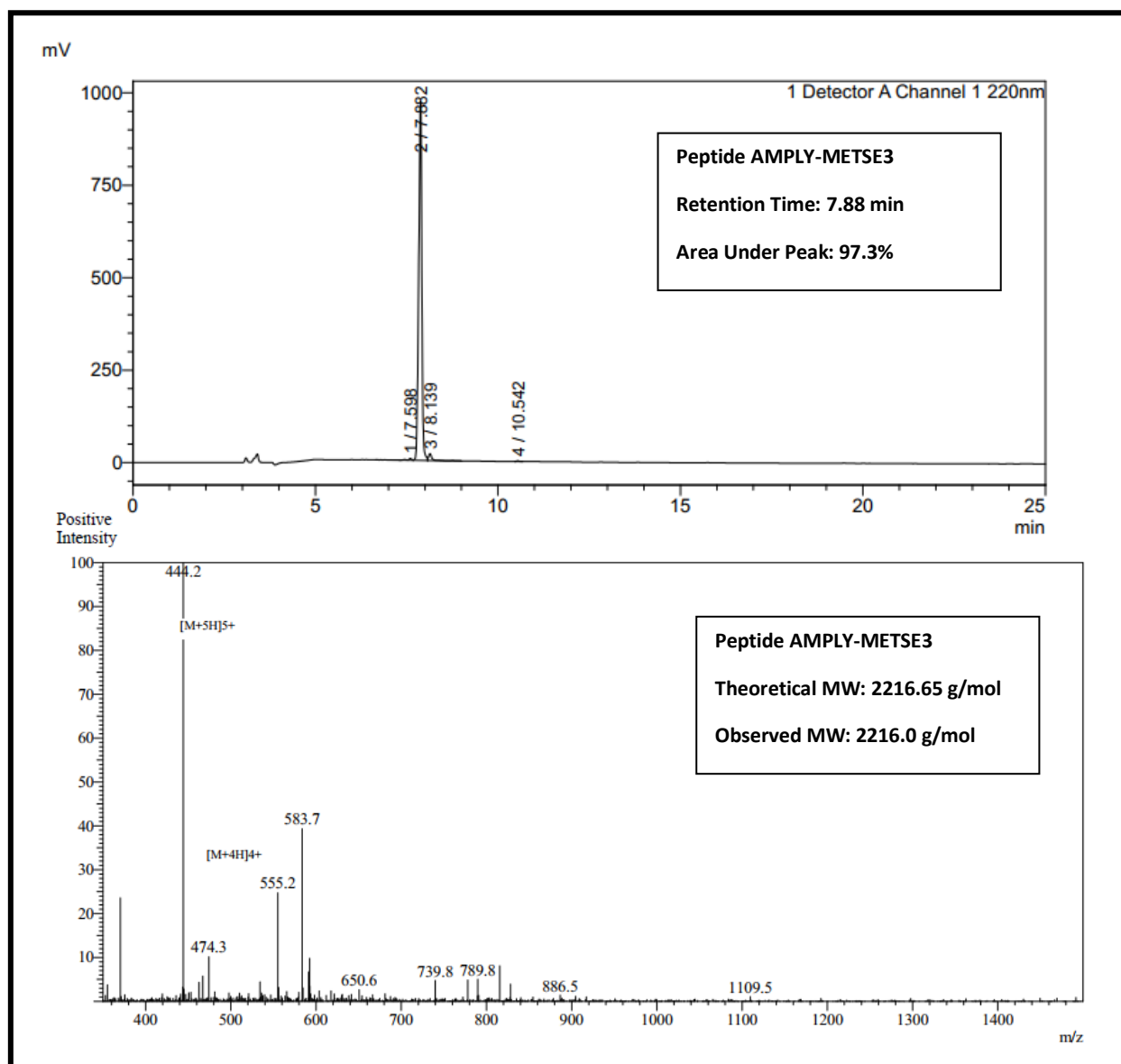


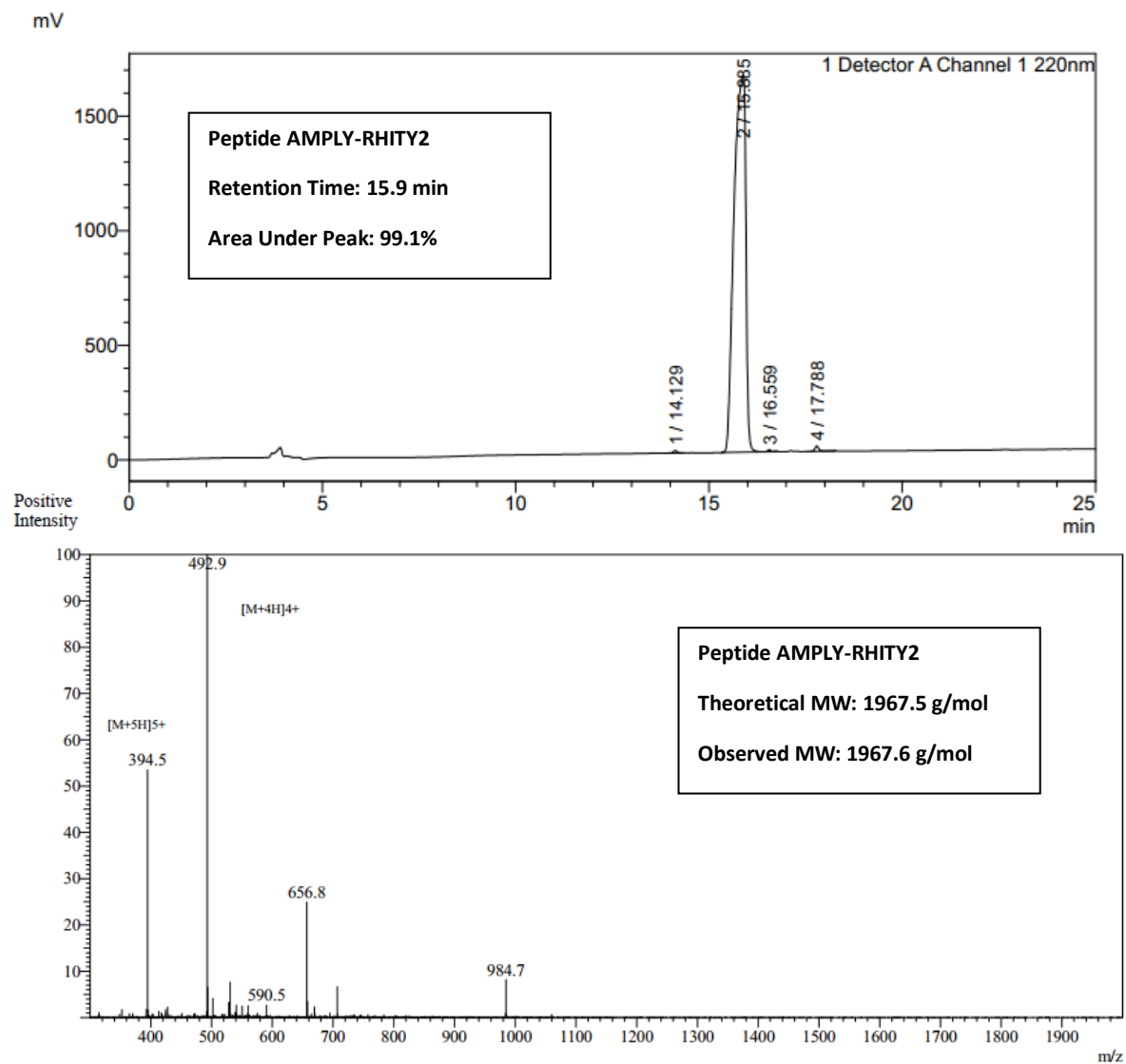


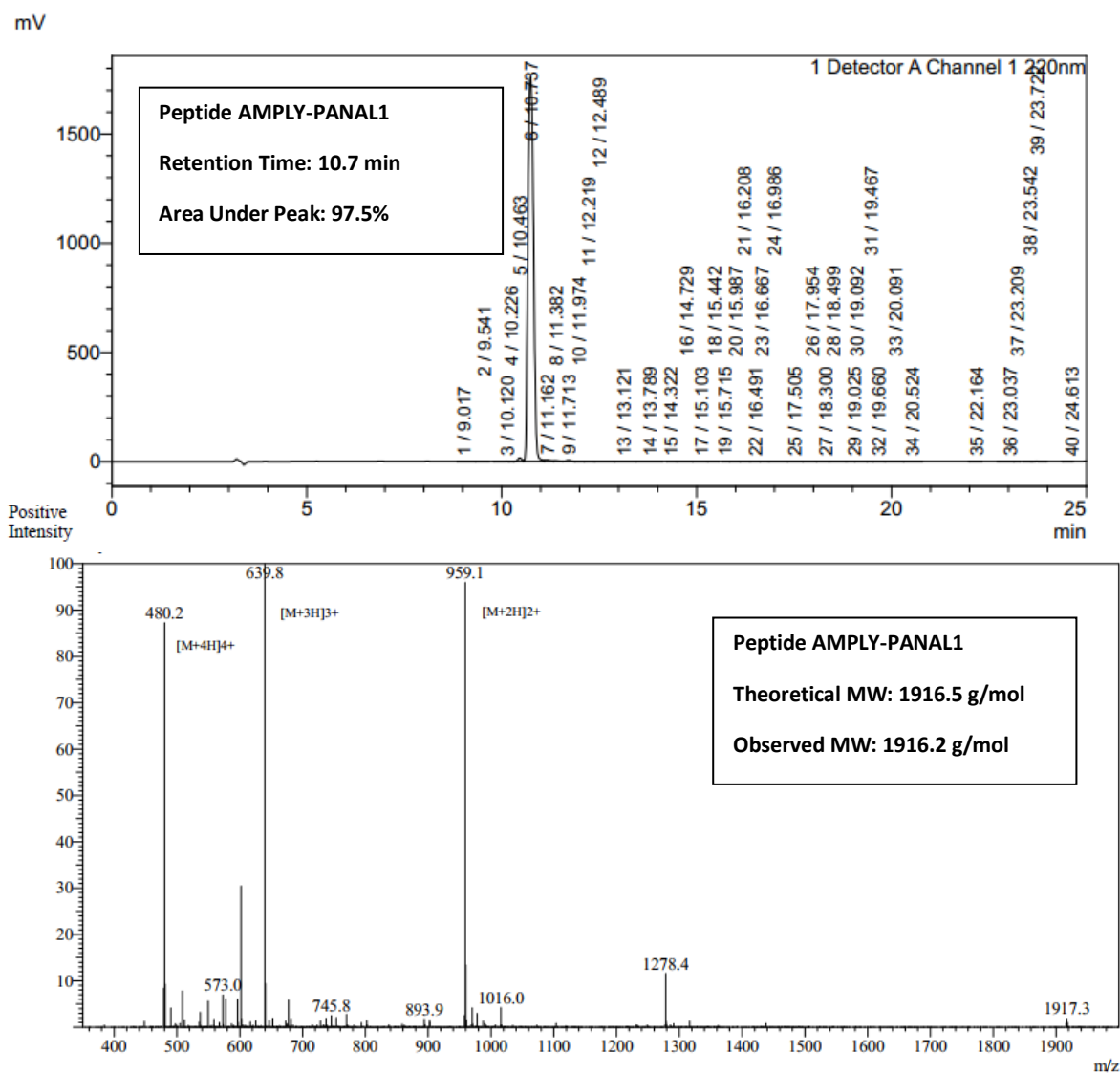


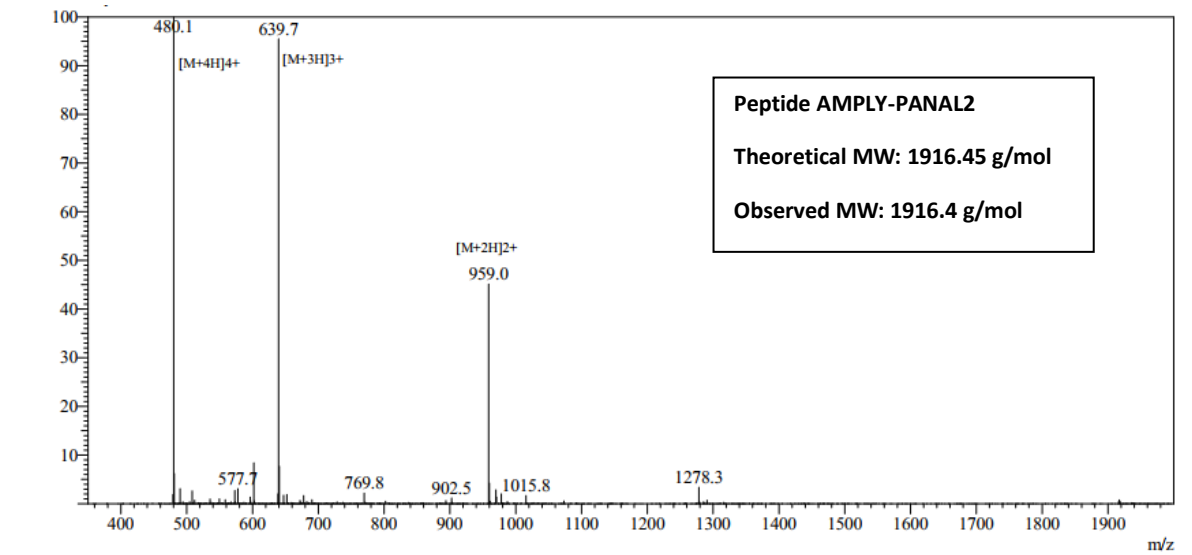
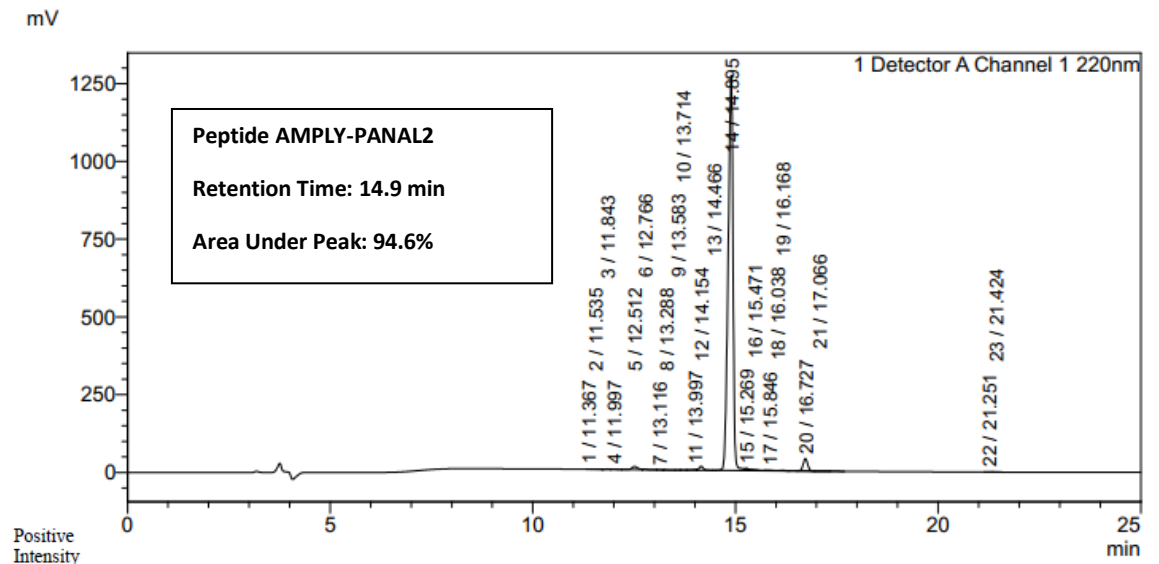


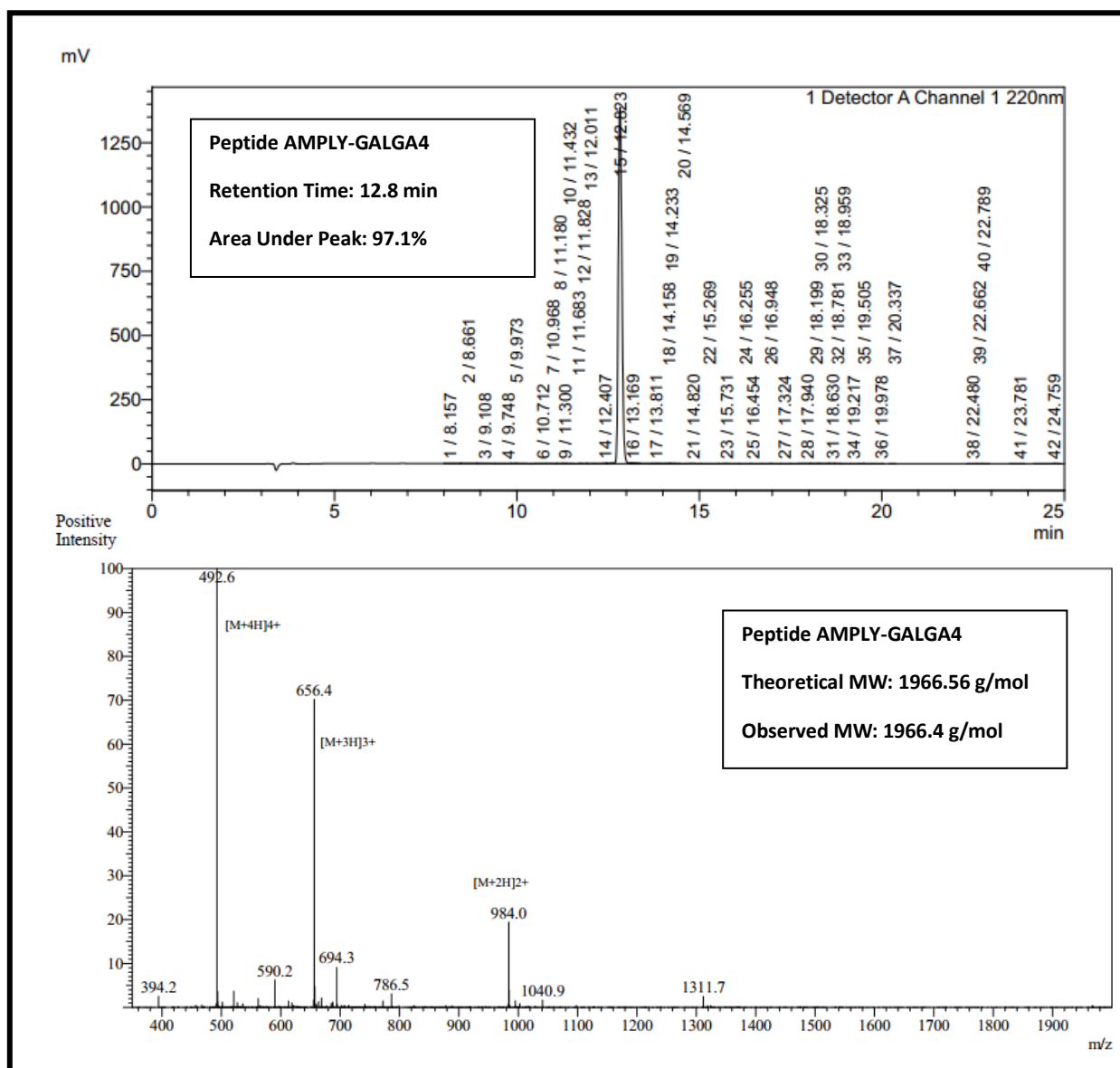


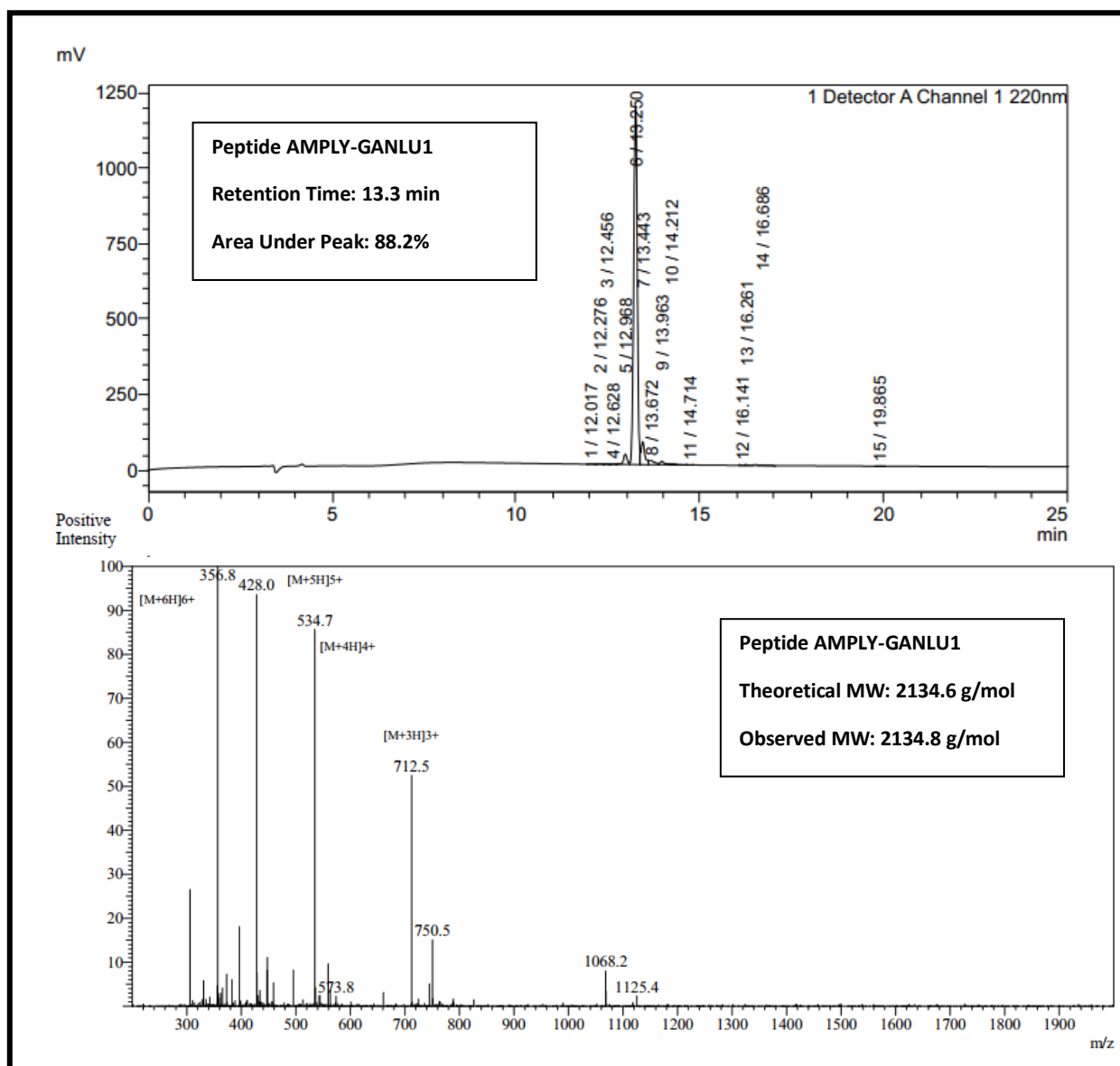


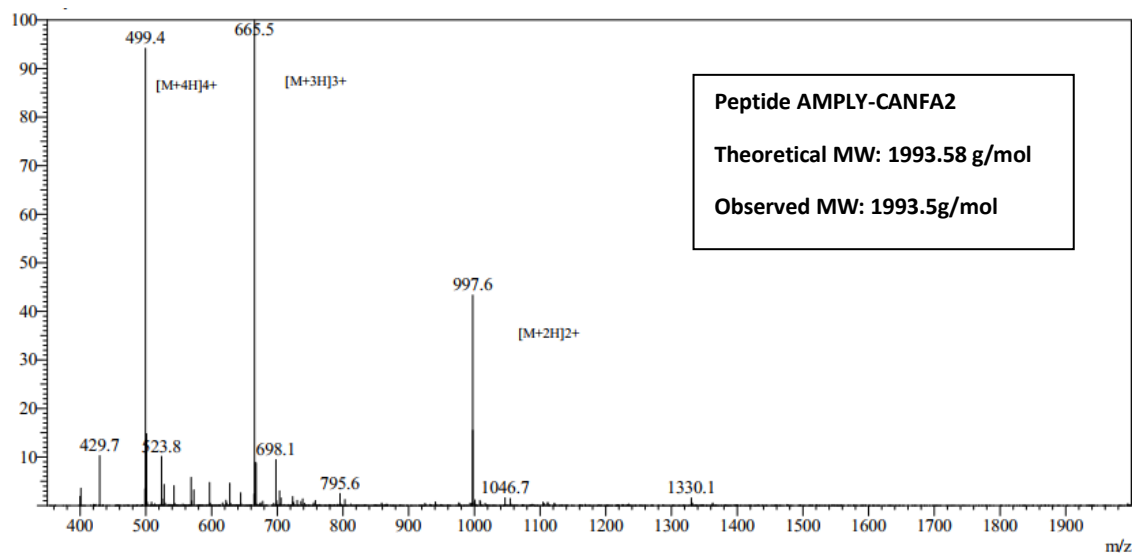
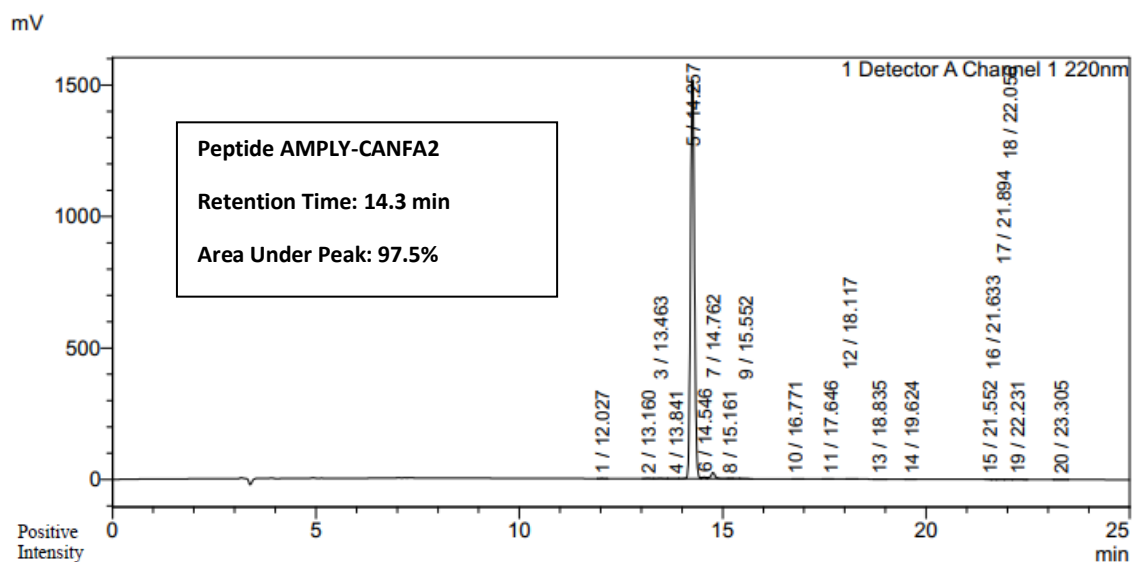


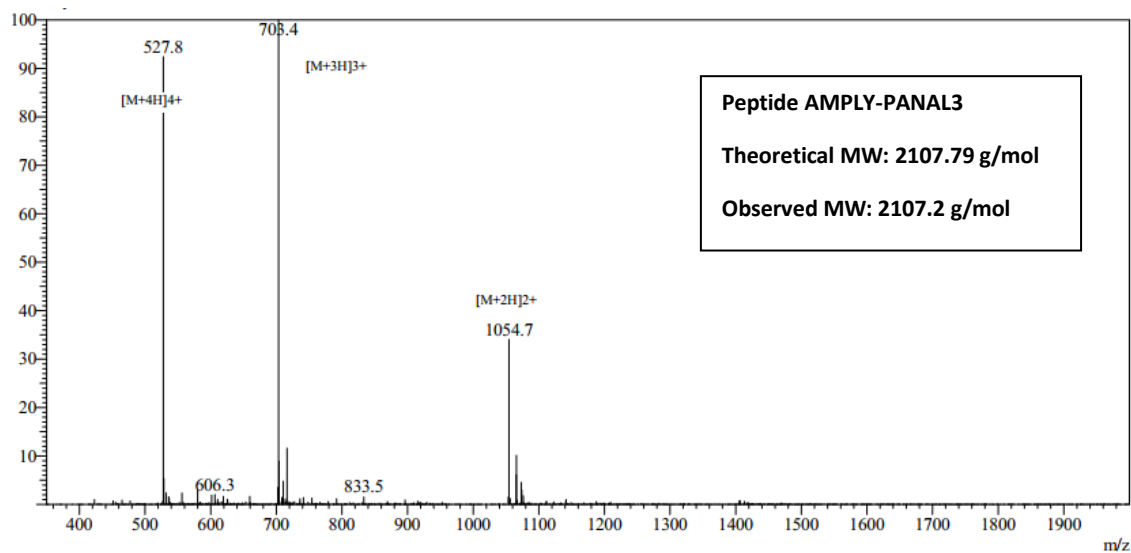
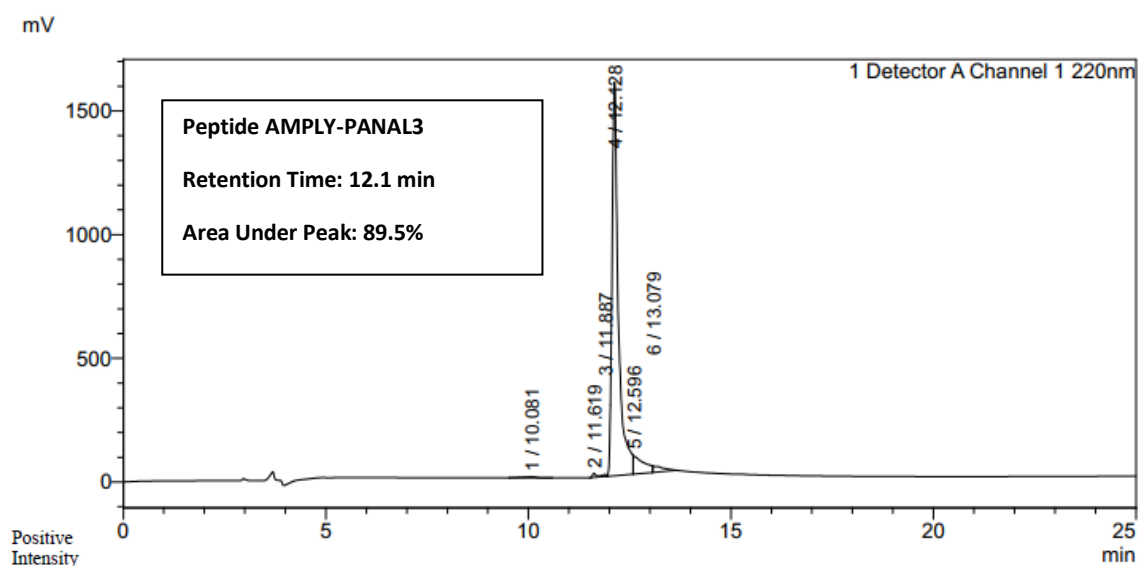


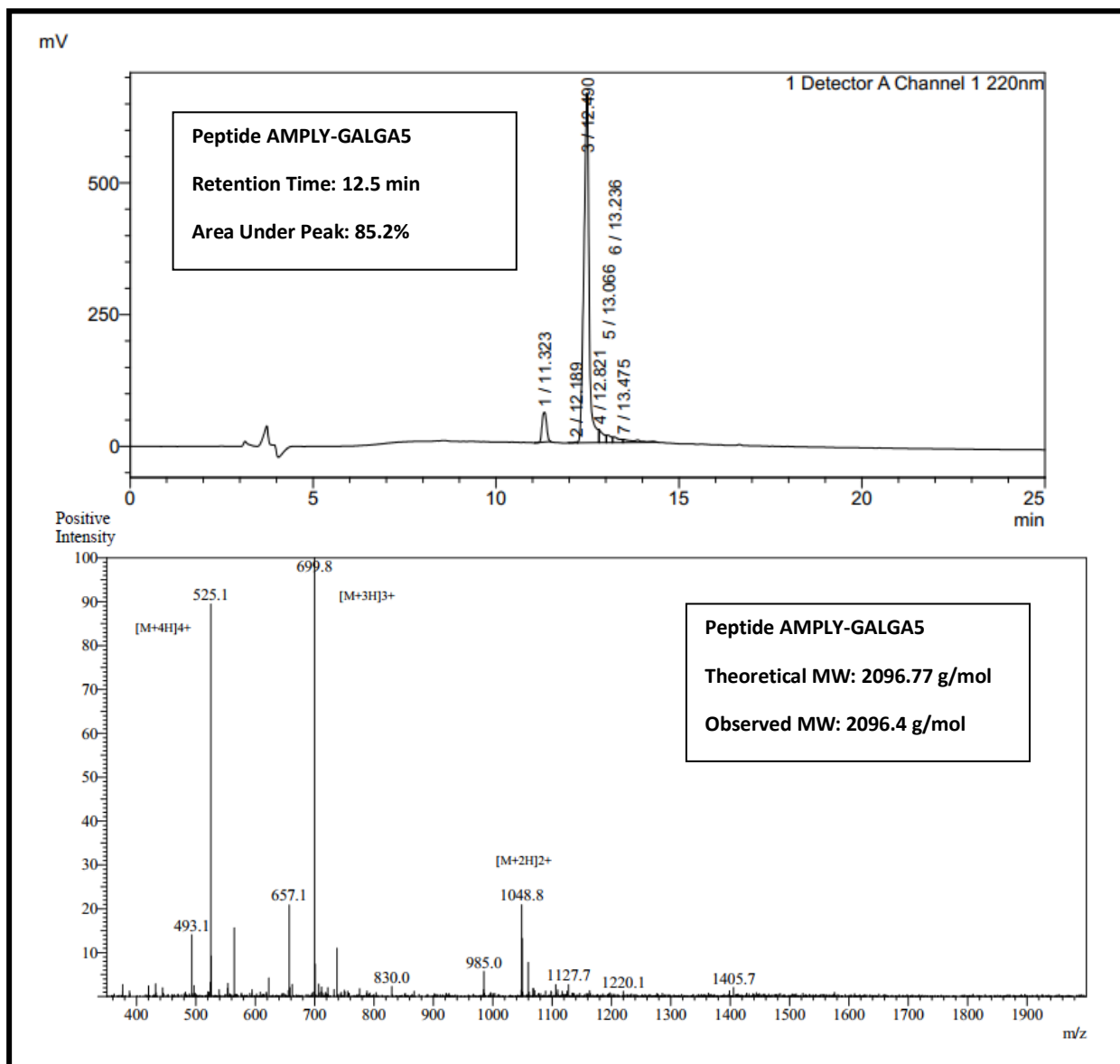


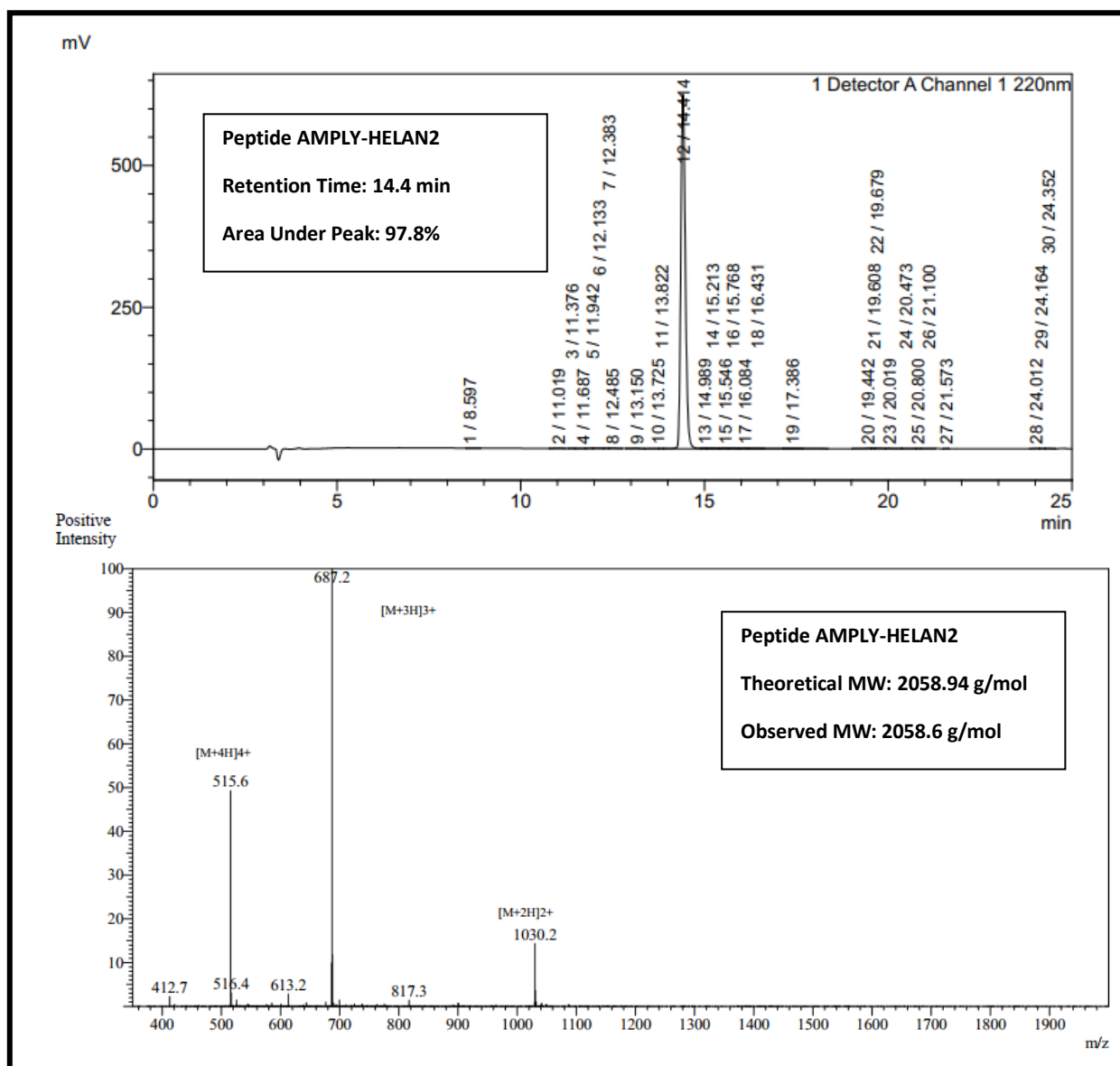


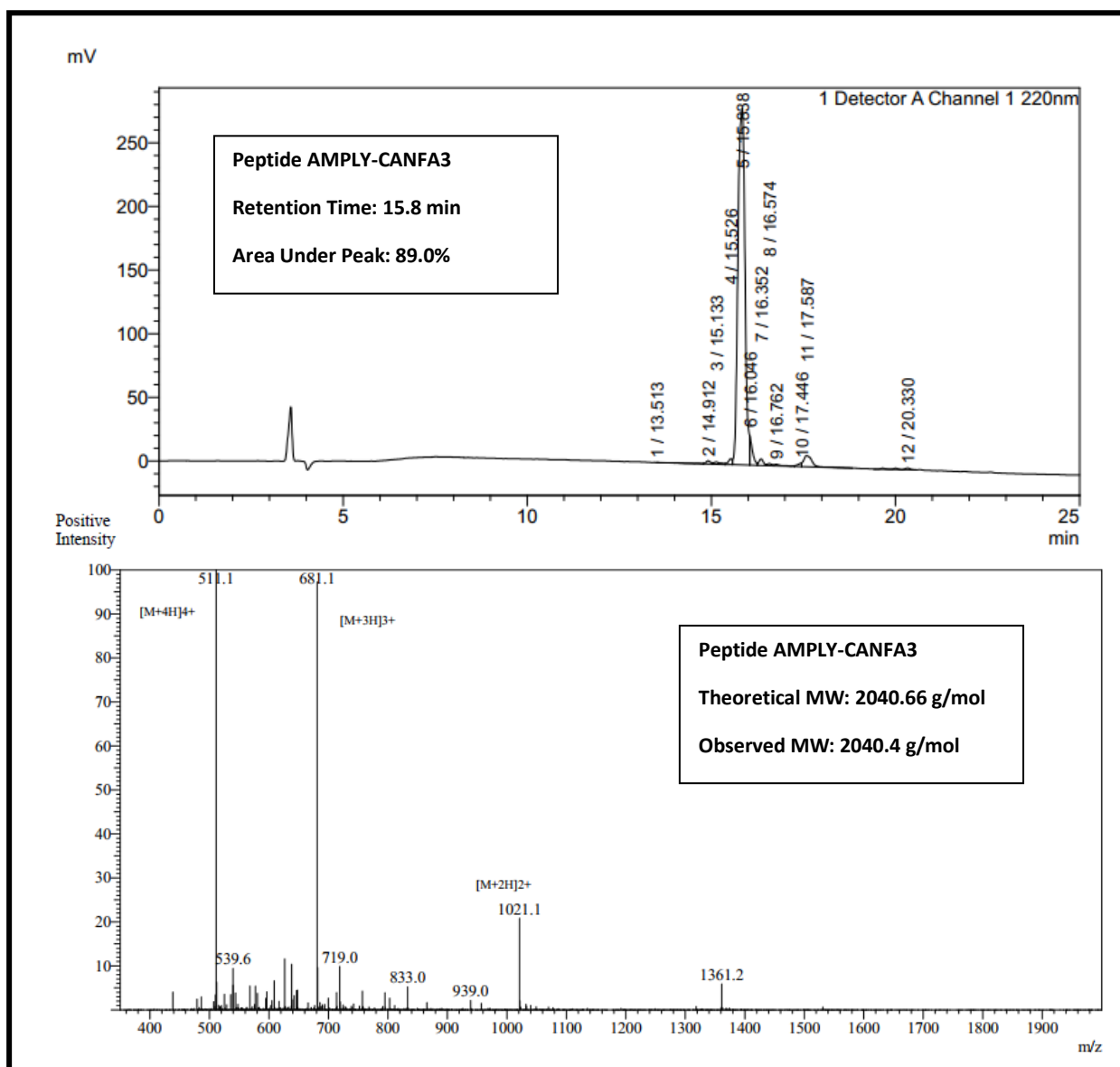


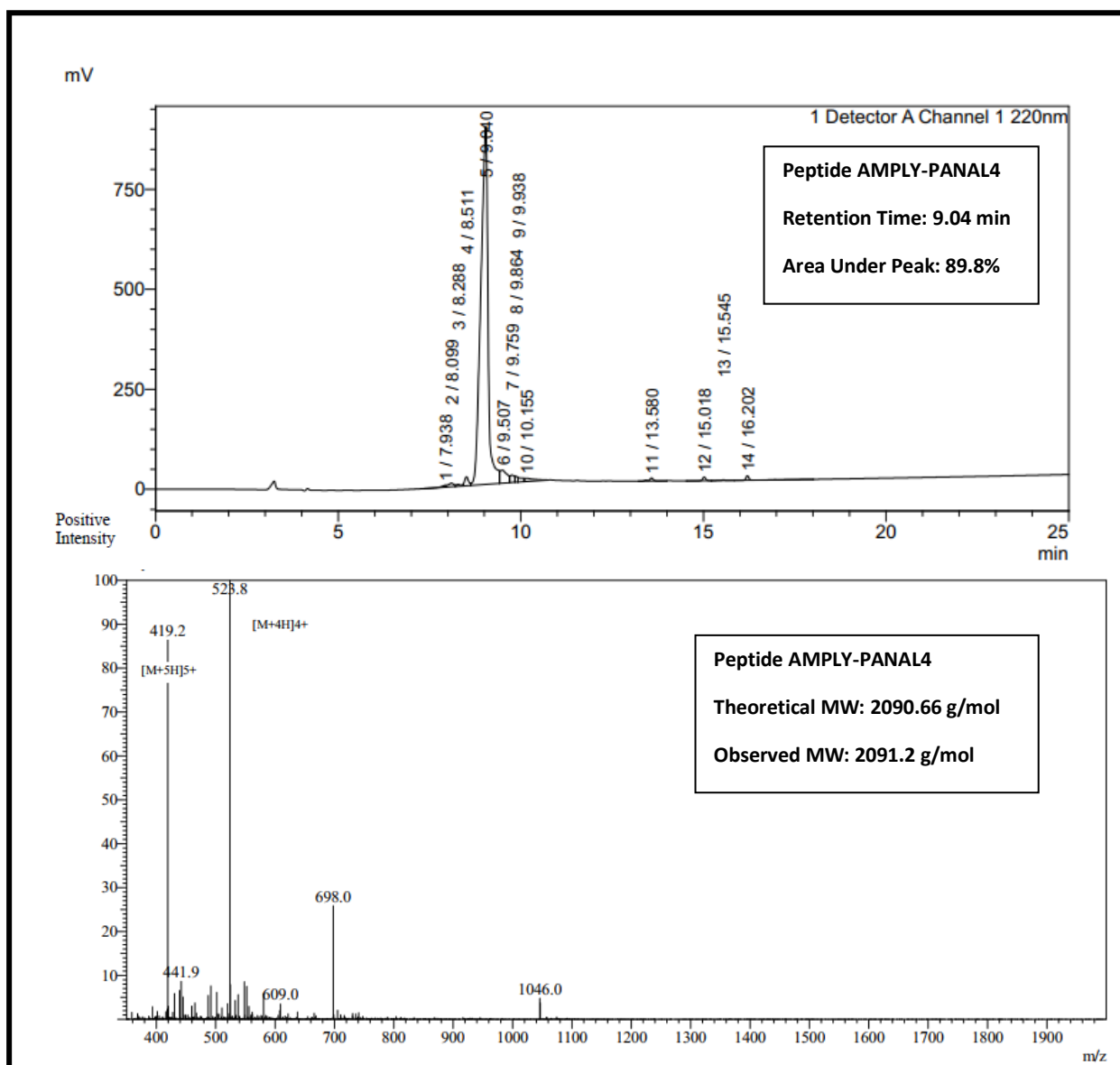


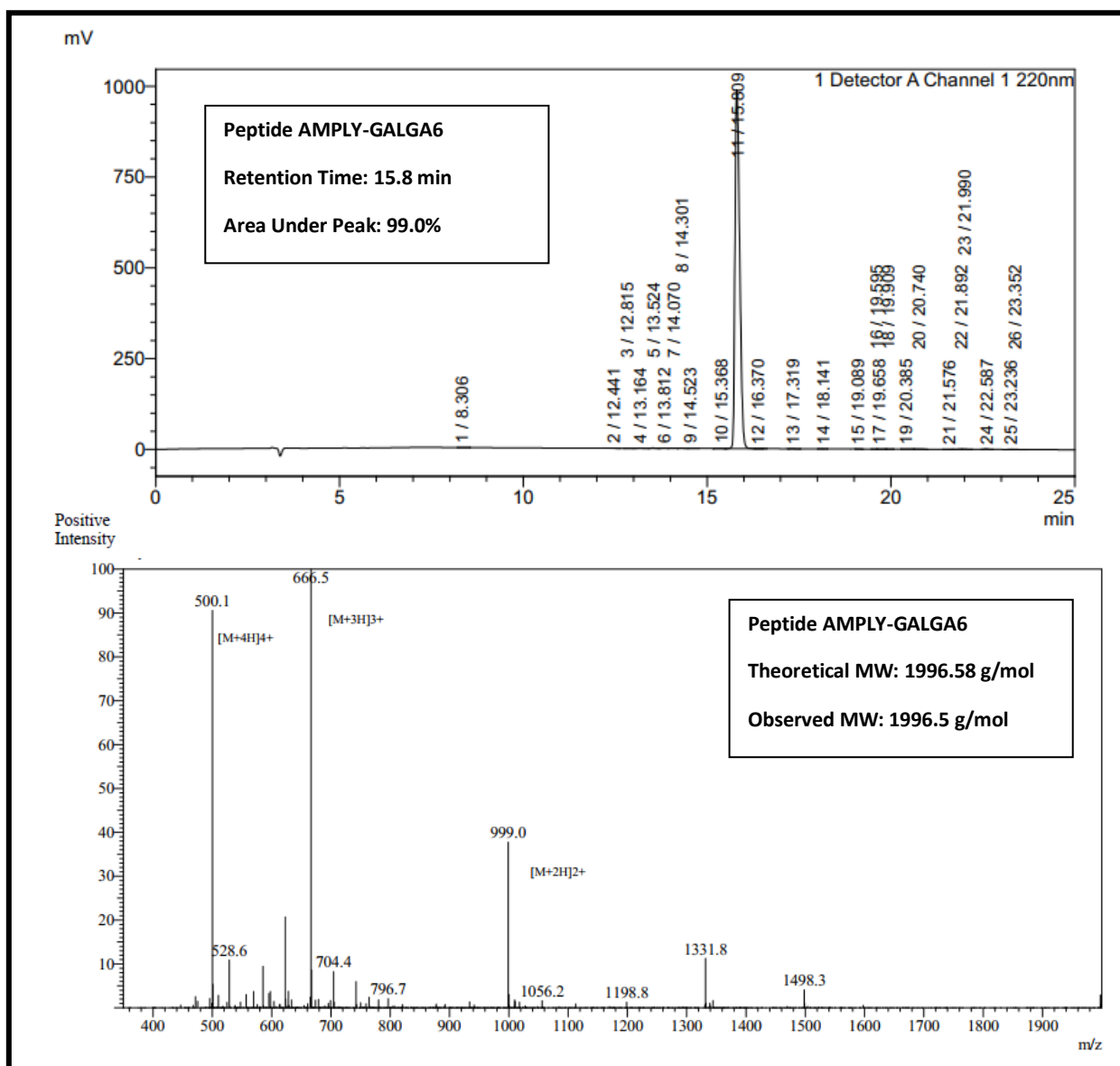


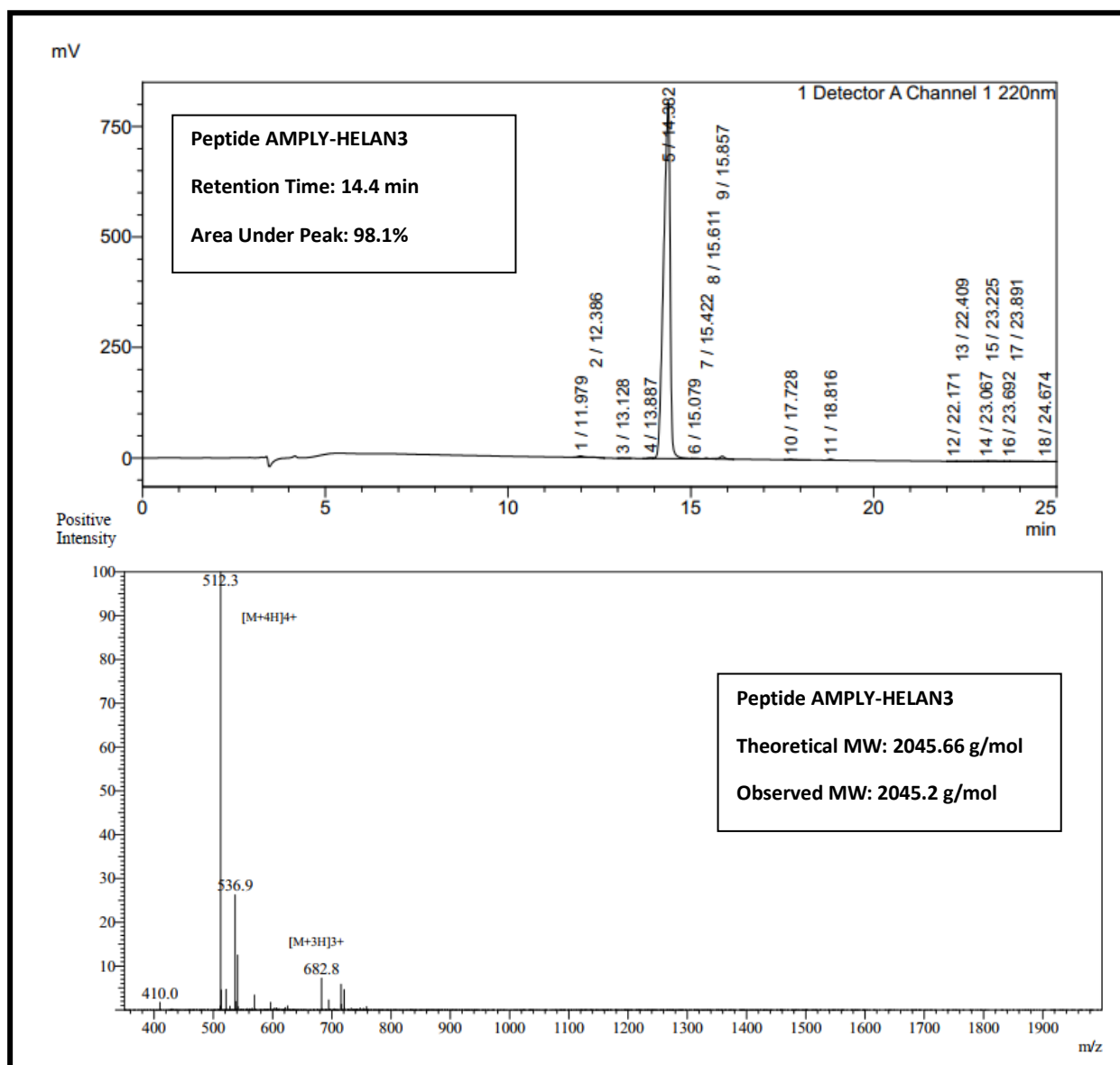


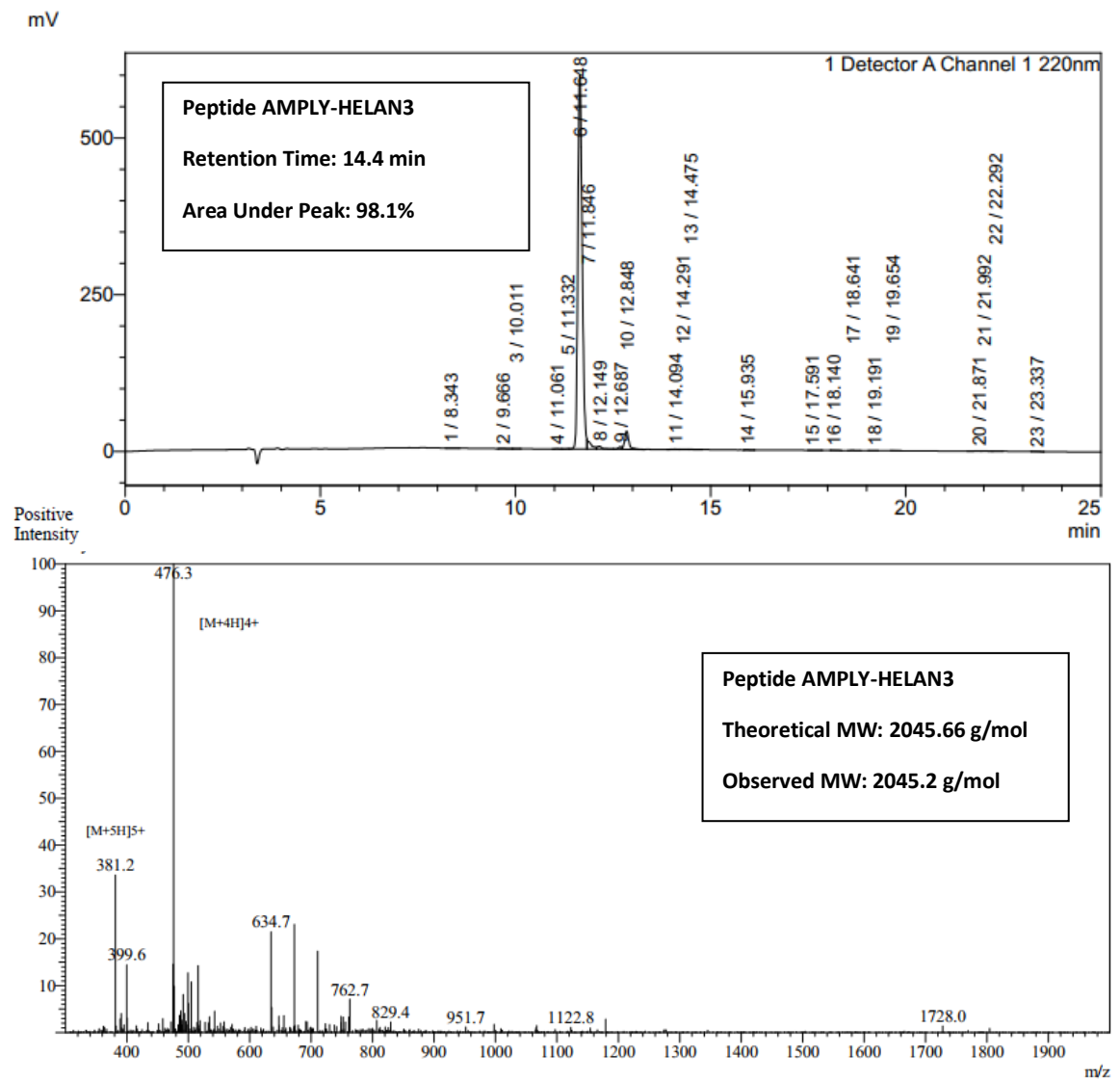


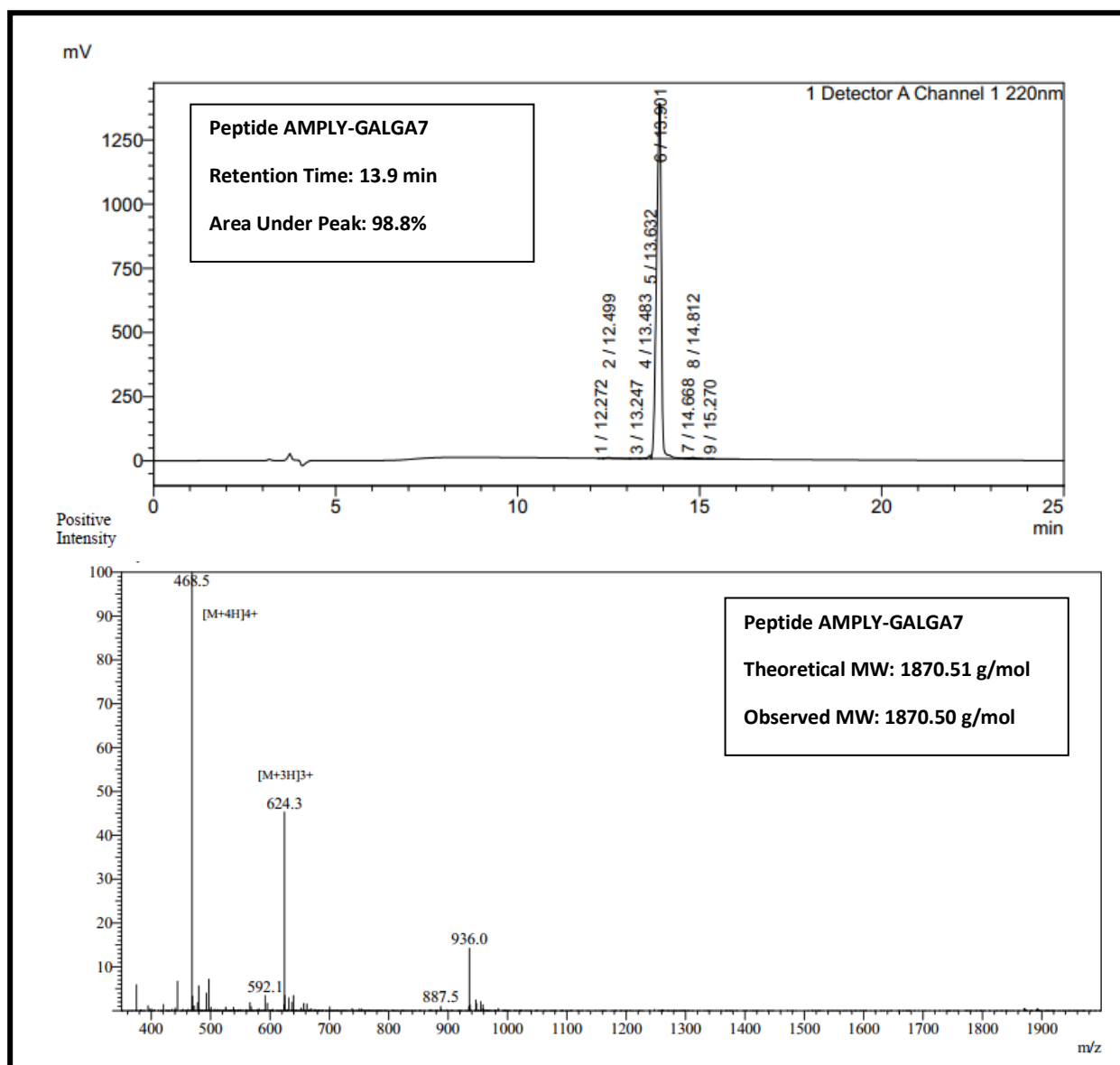




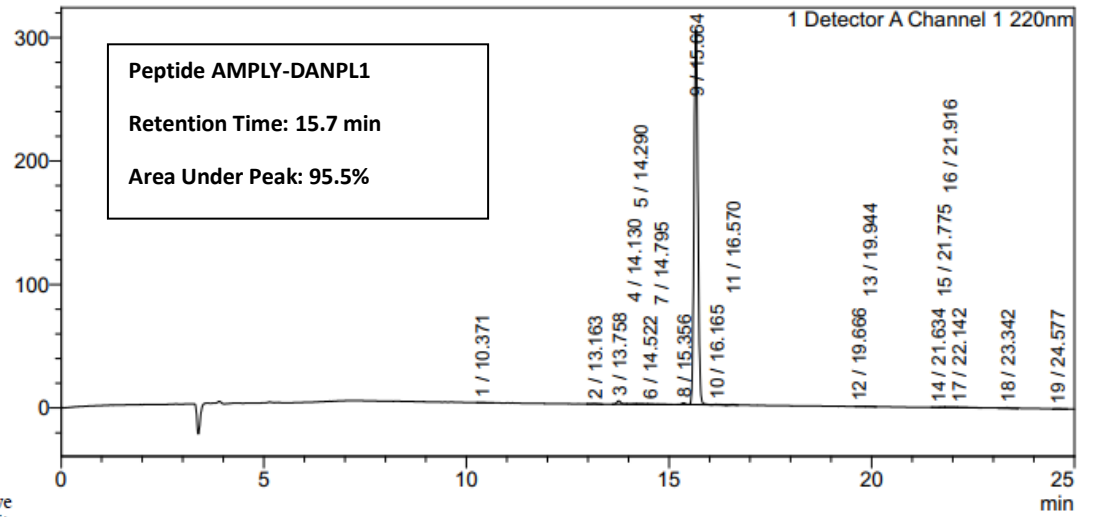








mV



Positive
Intensity

