Evaluation of Derivatization to Improve the Detection of Human Recombinant Erythropoietin using Liquid Chromatography – Mass Spectrometry

By

LAUREN TSEFREKAS

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Forensic Science

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

_____

Benjamin Moeller, Chair

_____

Robert Poppenga

_____

Glendon Parker

Committee in Charge

2025

ABSTRACT

The abuse of erythropoiesis stimulating agents (ESAs), such as recombinant human erythropoietin (rhEPO), is of great concern to the safety, integrity, and fair competition of horseracing. The current methods of definitive rhEPO detection utilize Liquid Chromatography-Mass Spectrometry (LC-MS) to unambiguously identify the presence of human-specific peptides in equine samples. However, confirmation and quantitation become challenging with rhEPO concentrations less than 0.1 ng/mL. This project concentrated on the derivatization of tryptic peptides of rhEPO ^{46}VNFYAWK^{52} (T6) and ^{144}VYSNFLR^{150} (T17) to increase ionization efficiency and lower current limits of detection. A total of eleven derivatization reagents were tested: 5-dimethylamino naphthalene-1-sulfonyl chloride (Dansyl Chloride), nα-(2,4-dinitro-5fluorophenyl)-l-valinamide (DNFP-L-V), (carboxymethyl)trimethylammonium chloride hydrazide (Girard's Reagent T), 2,4,6-triphenylpyrylium tetrafluoroborate (TPP), 2,4,6-trimethylpyrylium tetrafluoroborate (TMP), (n-succinimidyloxycarbonyl-methy)tris(2,4,6-trimethoyxlphenyl)phosphonium bromide (TMPP-Ac-OSu), cyanine3-nhs ester (CY3-NHS), dipyrrolidino(n-succinimidyloxy)carbenium hexafluorophosphate (HSPyU), n,n,n′,n′-tetramethyl-o-(n-succinimidyl)uronium tetrafluoroborate (TSTU), tandem mass tag pro zero label reagent (TMTpro Zero), and 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate (AQC). Following the selection of AQC as the optimal derivatization reagent, the reaction protocol was optimized through a basic buffer selection and sample clean-up process. To assess method performance, equine serum samples without any detectable rhEPO were spiked with an rhEPO standard solution at various concentrations. The samples were purified and rhEPO isolated using immunopurification, followed by tryptic digestion, solid phase extraction cleanup using Hydrophilic-Lipophilic Balance cartridges, derivatization with AQC in a sodium tetraborate

buffer, and analysis using LC-HRMS. Accuracy and precision of the method could not be calculated because integrated ion area counts for all multiply charged species and associated qualifying ions expected from derivatized peptides were not present.

Table of Contents

Abbreviations

i. nα-(2,4-dinitro-5fluoropheyl)-l-valinamide — DNFP-L-V

ii. 5-dimethylamino naphthalene-1-sulfonyl chloride —Dansyl Chloride

iii. (carboxymethyl)trimethylammonium chloride hydrazide — Girard's Reagent T

iv. 2,4,6-triphenylpyrylium tetrafluoroborate — TPP

v. 2,4,6-trimethylpyrylium tetrafluoroborate — TMP

vi. (n-Succinimidyloxycarbonyl-methy)tris(2,4,6-trimethoyxlphenyl)phosphonium bromide — TMPP-Ac-OSu

vii. cyanine3-nhs ester — CY3-NHS

viii. dipyrrolidino(N-succinimidyloxy)carbenium hexafluorophosphate — HSPyU

ix. n,n,n′,n′-tetramethyl-o-(n-succinimidyl)uronium tetrafluoroborate — TSTU

x. tandem mass tag pro zero label reagent — TMTpro Zero

xi. 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate — AQC

1. Introduction

Erythropoietin (EPO) is a 34kDa glycoprotein hormone in humans (UniProt protein ID: P01588), and 21kDA in horses (UniProt protein ID: Q867B1), primarily synthesized by the kidneys [1,2]. Erythropoiesis can be stimulated through hypoxia, the lack of oxygen in the blood, or through mechanisms stimulated by exogenous substances including, but not limited to, recombinant human erythropoietin (rhEPO), cobalt chloride, hypoxia-inducible factor stabilizers, and other erythropoietin stimulating agents (ESAs)[3]. Various subtypes of EPO differ from each other due to changes in glycosylation profiles, amino acid sequence, and through pegylation, and are clinically used for the treatment of anemia in humans. rhEPO has been reported to increase maximum oxygen uptake concentrations, hemoglobin, hematocrit concentrations, and the amount of time one is able to tolerate exercise[4]. As such, due to their ability to increase systemic oxygen-carrying capacity, ESAs are often used as performance enhancers in the world of athletics in a practice called "doping," which has been of concern for at least five decades. This practice of exogenous usage of ESAs has also extended to equine athletes. Organizations such as The Association for Racing Commissioners International, the International Federation of Horse Racing Authorities, and Horseracing Integrity and Safety Authority have established regulatory restrictions regarding improper use chemical substances in racehorses[5–7]. They have classified ESAs—including, but not limited to, erythropoietin and synthetics—as prohibited substances to ensure the fair competition, safety, and integrity of the sport.

This study aimed to use the chemical modification of the rhEPO tryptic peptides ^{46}VNFYAWK^{52} (T6) and ^{144}VYSNFLR^{140} (T17) to potentially increase testing sensitivity. Several studies have indicated that the addition of a derivatizing agent increases both sequence coverage and detection[8–13]; others have reported increased ionization efficiency[14]. This study's

protocol was based off already existing, robust research cited in the Materials and Methods section. As data analysis during initial evaluation of derivatization experiments reported a lack of expected results, alternative derivatization schemes were adapted, and methods were revised as seen in **Table 3**. After the optimal derivatization scheme was selected, it was applied to an immunopurification and analysis approach to determine serum EPO adapted from Bailly-Chouriberry et al. (2012)[15].

2. Background

2.1 Erythropoietin

Erythropoietin production is critical to maintenance of oxygen levels. Endogenous production of erythropoietin varies depending on the oxygen levels in the blood. Erythropoiesis can be triggered by hypoxia or through mechanisms stimulated by exogenous substances such as cobalt, hypoxia-inducible factor stabilizers, and ESAs, including recombinant products, EPO fusion proteins, peptidic EPO mimetics, and EPO gene activators[16]. The stimulation of EPO production triggers the production of erythrocytes which contain hemoglobin (Hb), an iron-carrying protein that transports oxygen to tissues in the body. Thus, an increase in EPO concentrations within the body will result in an increase of oxygen carrying capacity of the blood.

In humans, EPO is naturally produced as a 193-amino acid precursor containing a 27-amino-acid signal peptide, along with a C-terminal arginine, that is removed during post-translation processing[1]. After the removal of the signal peptide, glycans are added to three N-linked glycosylation sites and one O-linked glycosylation site[1]. The activation of red blood cell precursor proliferation is stimulated by the interaction of EPO and the erythropoietin receptor (EPOR), by a process in which EPO cross-links to two EPORs to trigger downstream signal

transduction[1]. Through the process of erythroid precursors' proliferation and differentiation to mature red blood cells, EPO concentration maintenance is required for survival[1]. If EPO is absent, or if concentrations are insufficient, erythroid precursors undergo apoptosis, resulting in a decreased production of new erythrocytes.

2.2 Erythropoietin Stimulating Agents and Their Influence on Equine Performance

The first study of blood doping in humans found that reinfusion of blood increased oxygen uptake by 9%, hemoglobin concentration by 13%, and physical performance capacity by 23% over pre-infusion levels[17]. This was further supported by studies with human athletes[18,19]. Blood doping poses a threat to the integrity and safety of endurance athletics as the increase in hemoglobin allows for greater oxygen delivery to muscles and, thus, both greater oxygen consumption and muscle performance. Administration of synthetically produced rhEPO agents can have similar erythropoietic effects as traditional blood doping, such as increased oxygen carrying capacity and running times to exhaustion[4]. There are several different types of rhEPOs that have been developed including epoetin alfa (Epogen®), darbepoetin alfa (Aranesp®), and methoxy polyethylene glycol-epoetin beta (Mircera®) that differ in their glycosylation pattern, amino acid sequence and molecular weight following pegylation[16]. Newer versions have been designed to have longer half-lives with the same beneficial effects as blood reinfusions. While these agents have been useful in the treatment of a number of serious medical conditions, including the treatment of anemia associated with end-stage renal disease and kidney failure[20], the development of erythropoietin stimulating agents, including rhEPO and its analogs, have been abused in competition sports including horseracing.

While the evidence for performance enhancement in humans is clear, the research has been conflicting whether or not performance enhancing effects actually occur for equine athletes.

In a study of four horses subcutaneously dosed with 30 IU rhEPO per kilogram of bodyweight, hematocrit, hemoglobin, and red blood cell count remained unchanged after 60 hours post application[2]. A number of factors may have influenced the lack of response such as the age of the horses, insufficient dosage, or the possibility that rhEPO is poorly recognized by equine erythroblasts[2]. Conversely, one study reported that, with horses dosed with 50 IU rhEPO per kilogram of bodyweight over the course of three weeks at three times a week, there was a significant increase in red cell volume and aerobic capacity[21]. It also reported a decrease in time needed to recover from strenuous exercise, using the graded incremental exercise test one week prior to rhEPO administration, during administration, and one week after the last administration[21].

Regardless, subcutaneous rhEPO administration to horses appears to result in a number of harmful effects. Generally, an increase in the oxygen carrying capacity of the blood increases blood viscosity and reduces blood flow, which can lead to clotting, stroke, or increased heart attack risks[22]. Another concern is that exogenous administration will produce anti-rhEPO antibodies that may cross react with endogenous EPO, as rhEPO is a foreign protein to the horse, and cause the cessation of erythropoiesis. Additionally, administration of high IU amounts of rhEPO has also been associated with the development of non-regenerative anemia[23–25]. This limits oxygen carrying capacity and leads to tissue hypoxia[23]. Lastly, cases of erythroid hypoplasia have been reported in both male and female Standardbred racehorses after high IU doses of rhEPO[24].

2.3 Current Detection Methods

The mature equine EPO protein (UniProt protein ID: Q867B1) is approximately 84.2% homologous to human EPO (UniProt protein ID: P01588)[26]. The difference can be used for the

detection of human-specific peptides in equine samples confirming exogenous administration. To detect and identify these peptides, the intact rhEPO molecule is tryptically digested after samples are screened as suspects using ELISA analysis. Diagnostic human specific tryptic peptides include ^{15}YLLEAK^{20} (T4), ^{21}EAENITTGCAEHC^{45} (T5), ^{46}VNFYAWK^{52} (T6), ^{54}MEVGQQAVEVWQGLALLSEAVLR^{76} (T8), ^{77}GQALLVNSSQPWEPLQLHVDK^{97} (T9), ^{98}AVSGLR^{103} (T10), ^{104}SLTTLLR^{110} (T11), ^{132}TITADTFR^{139} (T14), and ^{144}VYSNFLR^{140} (T17)[27]. Conventionally, analysis of EPO and ESAs in urine and serum are accomplished with initial screening by anti-human EPO based ELISA assays[27]. An excellent screening tool, ELISA can detect rhEPO down to two to three mlU/ml, or approximately twenty to thirty picograms per milliliter. Following suspect detection using techniques such as ELISA, confirmatory analysis using liquid chromatography with tandem mass spectrometry (LC-MS/MS) or high field asymmetric waveform ion mobility tandem mass spectrometry coupled with liquid chromatography tandem mass spectrometry (LC-FAIMS-MS/MS) can detect peptides of choice. The LC-MS/MS approach is preferred as it provides unambiguous identification. Other methods of confirmation include isoelectric focusing (IEF), sodium dodecyl sulfate polyacrylamide (SDS-PAGE) followed by a western blot, or MAIIA lateral flow isoform testing[15,28].

The tryptic peptides T6 and T17 from human EPO are often used to confirm the presence of rhEPO or analogous ESAs. The development of a LC-MS/MS method focused on the sensitive and reliable confirmation of both was reported in 2007[29]. Though previous studies had reported LC-MS methods for the tryptic digests of rhEPO and darbepoetin-α (DPO), they were not sensitive enough for *in vivo* samples due to the concentrations in plasma being so low[29]. In the study, rhEPO and DPO were extracted from equine plasma by anti-rhEPO antibodies linked to magnetic beads, tryptically digested, and analyzed by LC-MS[29]. As the phosphate buffer and

polyethylene glycol interfered with the tryptic digestion of rhEPO and DPO, the buffer was changed to ammonium bicarbonate and samples were purified utilizing molecular weight cut-off filters[29]. The analytical method was validated by the optimization of LC-MS/MS conditions, resulting in a limit of confirmation of 0.2 nanograms per milliliter, and a limit of detection (LOD) of 0.1 ng/mL for rhEPO and DPO in equine plasma. This was the first LC-MS method that definitively confirmed the presence of rhEPO and DPO in plasma samples.

There have also been recent developments in attempts to lower detectable peptide concentrations. Ultra-performance liquid chromatography and an internal standard of recombinant canine EPO was utilized to develop and validate an improved LC-MS/MS confirmatory procedure for rhEPO, DPO, and continuous erythropoietin receptor activator (CERA) in urine[27]. Adapting Guan *et al.* (2007)'s methods for plasma sample preparation, sample extraction, and enzymatic digestion, the study analyzed the plasma of three Thoroughbred horses administered 20,000 IU of rhEPO, 40,000 IU of rhEPO, and 60 mg of DPO, respectively. After LC-MS analysis of resulting signature peptides in plasma, the study lowered limits of detection of rhEPO (peptides T5 and T6), CERA (peptides T5 and T6), and DPO (peptides T6 and T9) to 0.1, 0.25, and 0.05 ng/mL respectively—all of which are equal to or lower than previously published methods—and demonstrated that the method also worked in equine urine.

Within the last year, in an attempt to improve the workflow of the analysis of rhEPO in equine plasma by optimizing the detection window of epoetin-β, four horses were dosed, one week apart, with 10,000 IU of epoetin-β[30]. Building off the work of Scarth *et al.* (2011), the study screened samples by ELISA, and confirmed rhEPO in suspect plasma samples by capillary flow LC-MS/MS[30]. The limit of quantitation for rhEPO peptides were similar at 0.05 ng/mL for

T6, T11, and T17 and 0.1 ng/mL for T4, but the plasma volume needed was lowered to 2.5 mL[30] from 4.0 mL[27]. This is useful in cases if the amount of available plasma is low, but the study is too recent to already be common in laboratories, and the limit did not change by any appreciable amount.

Generally, the detection of rhEPO below 0.1 ng/mL is challenging without the use of nano-flow and capillary-flow rates coupled with MS/MS based detection[31]. This comes with its own challenge of reliable operation and a disadvantage of long run times per sample, which delays the timely reporting of results. As such, methods amenable with and validated on LC-MS/MS systems might find more favorable results.

2.4 Advancing Detection Methods with Derivatization

The current detection limit of diagnostic rhEPO peptides, indicative of exogenous EPO usage, by LC-MS/MS is in the 0.1 ng/mL range, but rumors of "micro-dosing" in the horse racing industry now require those numbers to be even lower. In addition, plasma EPO assays can only report positive results up to seventy-two hours after administration, even though rhEPO can have a doping effect if dosed one to two weeks before competition[32]. Additionally, once hemoglobin has been raised by recombinant ESAs, micro-doses or more infrequent administration can be used to keep those levels raised[16]. Clearly, robust, reliable, and more sensitive methods to detect and confirm exogenous substance usage in samples are needed.

One potential method to increase the ionization efficiency of peptides is the use of chemical derivatization. In this process, an analyte is chemically altered into a product of a similar chemical structure called a derivative. Most often, a functional group is targeted to be altered[33]. Derivatization is utilized for a number of other reasons including, but not limited to, improving chromatographic separation, facilitating the ease of structural elucidation, and

stabilizing sensitive analytes. Improving ionization efficiency increases the number of charged ions produced in a sample and detected by a mass spectrometer, which thus increases the resulting signal—all of which allows for better detection of analytes in low-concentration or matrix-suppressed samples. Assessment of different derivatization approaches was the focus of this project.

The three groups that may be targeted by tagging reagents are common functional groups such as amino groups at the N-terminus and carboxyl groups at the C-terminus, peptides with less common amino acids (e.g. hydroxyl and sulfhydryl), and post-translational modifications (e.g. glycosylation and phosphorylation) that can be targeted by dedicated reactions[34]. A specific method has not been reported for the derivatization of rhEPO peptides (e.g. T6, T17), although derivatization approaches should be applicable as the chemical make-up at specific locations on the peptide are the same (e.g., amino groups at the N-terminus of a peptide, carboxyl groups at the C-terminus, etc.). A particularly promising reaction to increase ionization efficiency is the addition of a fixed charge at the N-terminus. Since all peptides are characterized by N- and C-termini, this is a "global" approach that will theoretically work with any peptide regardless of parent protein.

N-hydroxysuccinimide esters are the most common reagents to transfer chemical labels to peptides and proteins and fix this positive charge[34]. Multiple studies have reported that they react simply and mildly[13,14,35]. One such reagent is TMPP-Ac-OSu. In 1999, Huang et al. reacted tryptic digests of β-galactosidase with TMPP-Ac-OSu at a pH of 8 – 8.2. The reaction was carried out in a 5-10 molar excess of reagent and twenty-minute incubation time at room temperature, reporting the derivatization of the N-terminus without the derivatization of lysine side chains[35]. The robustness of the reagent and associated protocol has been validated by

multiple studies[14,36,37]. AQC, another N-hydroxysuccinimide ester, has experimentally been dissolved in acetonitrile and reacted with glycoproteins in a basic sodium tetraborate buffer at a four times molar excess.[13]. As previously mentioned, LC-MS/MS is the most definitive form of identification and quantitation for diagnostic peptides. In the study, the electrospray mass spectrometry (ESI-MS)—an electrospray source coupled to LC-MS system—experiments showed a signal intensity increase by a factor of approximately ten, which could be applicable to rhEPO peptide confirmation used in anti-doping laboratories.

Other derivatization reagents that are able to increase the ionization efficiency of peptides include, but are not limited to, quaternary ammonium salts, pyridinium salts, and acyl chlorides[33]. Nα-(2,4-dinitro-5-fluorophenyl)-l-valinamide (DNFP-L-V) is of interest as derivatized cathinone enantiomers were able to be resolved and quantified at sub-nanogram levels in equine serum and urine[8]. Using 2,4,6-trimethylpyrylium tetrafluoroborate (TMP) to derivatize primary amines on protein complexes resulted in a fixed positive charge to the N-termini and lysine residues, as well as improved sequence coverage for peptides[12]. 2,4,6-triphenylpyrilium tetrafluoroborate (TPP) derivatized peptides resulted in sub-femtomolar (10^{-15}) levels of detection, which far surpasses current detection limits[11]. Carboxymethyl trimethylammonium chloride hydrazide (Girard′s Reagent T) found favorable results from derivatization with N-glycans with excellent reliability, reproducibility, as well as improved resolution and sensitivity[38]. All mentioned reagents, in addition to other succinimide esters as specified in **Section 3**, will be applied to this study to attempt to lower the limits of detection for peptides T6 and T17.

3. Materials and Methods

3.1 Chemicals, Reagents, and Equipment

HSPyU, TSTU, TMP, TPP, Girard's Reagent T, DNFP-L-V, trifluoracetic acid, dimethylformamide (DMF), triethylammonium bicarbonate buffer, and triethylamine were purchased from Sigma Aldrich Inc (Atlanta, GA, USA). TMPP-Ac-OSu was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). TMTpro Zero was purchased from Thermo Fisher Scientific (Waltham, MA, USA). AQC was purchased from MedChem Express (Monmouth Junction, NJ, USA). Formic acid, acetonitrile, methanol, and HPLC-grade water were purchased from Honeywell – Burdick & Jackson (Muskegon, MI, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA).

The reference standard for VYSNFLR (GenScript, Piscataway, NJ), Dansyl Chloride (Sigma Aldrich, St. Louis, MO, USA), sodium tetraborate decahydrate (Sigma Aldrich, St. Louis, MO, USA), HPLC-grade ammonium acetate (Thermo Fisher Scientific, Waltham, MA, USA), sodium bicarbonate (Sigma Aldrich, St. Louis, MO, USA), Pepstatine A (Sigma Aldrich, St. Louis, MO, USA), ethylenediaminetetraacetic acid disodium salt dehydrate (Sigma Aldrich, St. Louis, MO, USA), octyl-beta-D-glucopyranoside (Sigma Aldrich, St. Louis, MO, USA), tris(2-carboxyethyl)phosphine (Sigma Aldrich, St. Louis, MO, USA), erythropoietin working solutions (United States Pharmacopeia, North Bethesda, MD, USA), and erythropoietin internal standard (Promise Proteomics, Grenoble, France) were provided by the Equine Analytical Chemistry Laboratory (California Animal Health and Food Sciences, Davis, CA, USA). A 50% hydroxylamine buffer for TMT experiments was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

The EasyPep™ MS Sample Prep Kit was purchased from Thermo Scientific (Waltham, MA, USA). The 12 x 75mm and 16 x 100 Fisher brand borosilicate glass disposable culture tubes used were manufactured by Fisher Scientific (Waltham, MA, USA). The autosampler vials with inserts used were manufactured by Agilent Technologies (Santa Clara, CA, USA). 50mL centrifuge tubes were manufactured by Corning Incorporated (Corning, NY, USA). Pierce™ C18 Tips, 100 µL (Thermo Scientific, Waltham, MA, USA) as well as three and ten thousand molecular weight cut-off filters (Sigma Aldrich Inc, St. Louis, MO, USA) were provided by the Equine Analytical Chemistry Laboratory (California Animal Health and Food Sciences, Davis, CA, USA). Deionized Nanopure water used for buffer solutions and column conditioning and washing was obtained from an in-house NanoPure system (Thermo Scientific, Waltham, MA, USA).

3.2 Preparation of Standards and Stock Solutions

3.2.1 Standards

VYSNFLR (T17) was prepared at a concentration of 1 mg/mL in HPLC-grade water and serially diluted to 100 µg/mL and 10 µg/mL in water. DNFP-L-V and Dansyl Chloride were prepared at a concentration of 1 mg/mL in acetone, respectively. Girard's Reagent T was dissolved at a concentration of 0.1 mg/mL in 10% acetic acid. TPP was dissolved in DMF at a concentration of 1 mg/mL. TMP was prepared at a concentration of 0.05 mg/mL of triethylammonium bicarbonate buffer. TMPP-Ac-OSu was prepared at a concentration of 1 mg/mL in 10% acetonitrile with 0.1% TFA in HPLC-grade water for initial testing, and at a concentration of 1 mg/mL in 100% acetonitrile for later testing. CY3-NHS, HSPyU, and TSTU were prepared at a concentration of 1 mg/mL in acetonitrile, respectively. TMTpro Zero was

prepared at 25 mg/mL in acetonitrile. AQC was prepared at a concentration of 3 mg/mL in acetonitrile.

3.2.2 Stock Solutions

Ammonium acetate was prepared at 50mM by measuring out an appropriate amount of reagent, dissolving with water, adjusting to a pH of 8.5 or 9.0, depending on the reagent to be used with, with ammonium hydroxide, and diluting to a final volume of 50mL. Triethylammonium bicarbonate (100mM) buffer was prepared by aliquoting 3mL into water and diluting to a final volume of 30mL. An ammonium acetate buffer (1M) was prepared by dissolving an appropriate amount into water, adjusting the pH to 8.5, and diluting to a final volume of 25mL. Sodium bicarbonate buffers (100mM, 200mM, and 1M) were prepared by dissolving reagent into water, adjusting with sodium hydroxide to pH 8.8 if necessary, and diluting to a final volume of 50mL. Ammonium bicarbonate solution (200mM) was prepared by dissolving reagent in water, adjusting the pH with ammonium hydroxide, and diluting to a final volume of 25 mL with water. A 10% acetic acid solution was prepared by aliquoting acetic acid at a 10:90 ratio (v:v) to water. A 10% acetonitrile with 0.1% TFA solution was prepared by aliquoting acetonitrile and TFA to a graduated cylinder and diluting to a final volume of 40mL with HPLC-grade water. A sodium tetraborate buffer was prepared at 50mM by measuring out an appropriate amount of reagent and dissolving in 50mL of water. A Pepstatine A solution (1.46 mM) was prepared by dissolving 1 mg of Pepstatine A into 900mL of ethanol and 100mL acetic acid, and vortex mixing for two hours to completely dissolve the reagent into solution. An EPAB solution (50 mM) was prepared by mixing 500mL of nano-pure water, ammonium bicarbonate (49.8mMol), ethylenediaminetetraacetic acid disodium salt dehydrate (12.7mMol), and Pepstatine A solution (1mMol); the solution was adjusted to a pH of 7.9 with 32% sodium

hydroxide. A Octyl-beta-D-glucopyranoside (OGP) solution was prepared at a 20 mg/mL solution (68.4mM) by dissolving octyl-beta-D-glucopyranoside into EPAB 0.05M solution at pH 7.9. A tris(2-carboxyethyl)phosphine (TCEP) solution was prepared at a 2 mg/mL solution (7mM) by dissolving tris(2-carboxyethyl)phosphine into EPAB 50mM solution at pH 7.9. Ammonium bicarbonate (100mM) with 0.1% OGP and 0.1% TCEP was prepared by adding appropriate amounts of ammonium bicarbonate reagent, OGP solution (68.4mM), and TCEP solution (7mM). An ammonium bicarbonate buffer (100mM) was also prepared by mixing an appropriate amount of ammonium bicarbonate into water.

3.3 Initial Evaluation of Derivatization

A total of eleven reagents were tested for their ability to derivatize rhEPO peptide T17 and tested in replicates. Alternate pathways using ammonium acetate or ammonium bicarbonate were selected for reagents originally using sodium bicarbonate. Working off Loganathan et al. (2021)'s derivatization procedure, DNFP-L-V in acetone was added to a 10 μg/mL solution of rhEPO peptide T17 and sodium bicarbonate buffer, and incubated at room temperature for three hours[8]. Similar to Anari et al. (2002)'s method, Dansyl Chloride was prepared in acetone, added to 100 μL of a 10 μg/mL solution of rhEPO T17 peptide solution and 100 mL of sodium bicarbonate buffer (100 mM, pH 10.5), vortex mixed for one minute, and then incubated at 60°C for three minutes[9]. Based off Naven and Harvey (1996)'s method, Girard's Reagent T was dissolved in a 10% acetic acid solution and added to a 100 μg/mL solution of rhEPO peptide T17 so that the reagent would be in seventeen times excess, and the entire solution was heated at 75°C for three hours[10]. An alternate scheme of Girard's Reagent T was also tested based off Gil et al. (2008)'s experimental design by replacing the N-glycan sample with peptide and heating at the same temperature but for four hours[38]. TPP was dissolved into DMF and added to a 10

μg/mL solution of rhEPO T17 peptide solution and triethylamine based off Siwińska et al. (2020)'s experimental design[11]. The reaction mixture was incubated for one hour at 60°C, evaporated under nitrogen, and reconstituted with 5% acetonitrile and 0.2% formic acid. TMP was dissolved into 100mM triethylammonium bicarbonate (TEAB) buffer, pH 8.5. The solution was added to a 100 μg/mL solution of rhEPO T17 peptide and 100mM TEAB buffer so that the reagent would be in ten times molar excess of the peptide. The protocol was based off experimental methods detailed by Polasky et al. in 2018[12]. Initially, TMPP-Ac-OSu was dissolved in 10% acetonitrile and 0.1% trifluoroacetic acid, combined with peptide working solution two and sodium bicarbonate and reacted as described by Shen et al. (2015)[37]; latter experiments dissolved the reagent in acetonitrile at 1 mg/mL and added to a 1 mg/mL solution of rhEPO peptide T17 and 50mMol ammonium acetate, and incubated at 55°C for twenty minutes. CY3-NHS, HSPyU, and TSTU were dissolved in acetonitrile at 1 mg/mL, added to a 1 mg/mL solution of rhEPO T17 peptide and 50 mM ammonium acetate, and incubated at 55°C for twenty minutes in individual schemes, similar to protocol as described by Ullmer *et al* (2006)[13]. TMTpro Zero was dissolved in acetonitrile at 25 mg/mL according to Thermo Fisher TMTpro Zero package instructions[39], added to a solution of rhEPO peptide T17 at 12.5 μg/mL in TEAB, reacted at room temperature for one hour before the addition of a 5% hydroxylamine buffer, cleaned using an EasyPep™ MS Sample Prep kit, evaporated under nitrogen, and reconstituted with 0.1% formic acid in water. AQC was dissolved in acetonitrile, also based off Ullmer et al.'s protocol, added to a basic sodium tetraborate or ammonium acetate buffer and a 100 μg/mL rhEPO T17 peptide solution, and heated in the same manner[13].

3.3.1 Direct Infusion Parameters

Evaluation of the initial derivatization experiments employed a Thermo Scientific Quantum Ultra Triple Stage Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled with an Agilent 1100 Series HPLC (Agilent Technologies, Inc., Santa Clara, CA, USA) with an electrospray ionization source. Mobile phase A was prepared by mixing acetonitrile with 0.1% formic acid, and mobile phase B was prepared by mixing HPLC-grade water with 0.1% formic acid. Reaction solutions were introduced into a T-injection line at a flow rate of 5 μL/min, and combined with 50% mobile phase A and 50% mobile phase B at a rate of 400 μL/min to be infused onto the system. The spray voltage, capillary offset, and tube lens offset were set at 3971, 35, and 131V, respectively. The spray current was set at 3A. The sheath gas pressure, aux gas pressure, and ion sweep gas pressure were set at 40, 10, and 0 arbitrary units. The flow rate for direct injection was set to 5 μL/min, and the scan parameters were set to full scan MS from 200 – 1500 m/z. Ions with discernable peaks that matched, or were close to matching, expected ions were fragmented at a collision energy of 20eV to ascertain if quantifying ions associated with rhEPO peptide T17 were present. The LC-MS system was controlled by EZ Tune (Thermo Fisher Scientific, Waltham, MA, USA) and the system was operated using positive mode ESI. Data was processed and analyzed using Qual Browser (Thermo Fisher Scientific, Waltham, MA, USA).

3.4 Optimization of AQC

The derivatization and detection of rhEPO should both be a process amenable to LC-MS/MS system over time, as well as a simple and effective series of steps that can be added to existing rhEPO confirmation protocols. To achieve this, care was taken to optimize both solvents and add a series of steps that were easily able to be integrated into the confirmatory protocol

used by the Equine Analytical Chemistry Laboratory (California Animal Health & Food Safety Laboratory, Davis, CA, USA).

Peptide derivatization was evaluated under a variety of conditions. First, AQC was added to a mock-version of the digestion solution that resulted from the Equine Analytical Chemistry's (EACL) EPO confirmation method. Following results, the both the use of Pierce™ C18 Tips (100 µL), 3kDa molecular weight cut-off filters, and Hydrophilic Lipophilic Balance columns for sample clean-up was evaluated. Samples were also tested with different amounts of trypsin, including no trypsin, to determine if the enzyme interfered with peptide derivatization. Derivatization efficacy was also evaluated with four different buffers: 50mM sodium tetraborate, 50mM ammonium acetate, 100mM ammonium bicarbonate, and 100mM ammonium bicarbonate buffer with 0.1% OGP and 0.1% TCEP.

3.4.1 LC-MS/MS Parameters

Method development for AQC optimization employed a Thermo Scientific Quantum Ultra Triple Stage Quadrupole Mass Spectrometer coupled with an Agilent 1100 Series HPLC with an electrospray ionization source. The analytical column used was an Avantor ® ACE® C18 column (Radnor, PA, USA) with a particle size of 3.0 µM, a length of 100 mm, and a 2.1 mm inner diameter. The column was held at room temperature. Analytes' precursor and product ions, collision energy, and tube offset were recorded in the compound optimization window of the EZ Tune program by direct infusion and in positive mode ESI. A reverse-phase gradient eluted the analytes at a flow rate of 0.4mL/min, with a total injection volume of 5 µL into the HPLC system. Mobile phase A was prepared by mixing acetonitrile with 0.1% formic acid, and mobile phase B was prepared by mixing HPLC-grade water with 0.1% formic acid. Initial conditions were 5% mobile phase A gradually ramped to 95% at 6.0 minutes, held until 8.0

minutes, and ramped back down to 5% for a total run time of 15 minutes. The mass spectra acquisition data window started at 2 minutes and continued for 6 minutes with a total of two scan events. After chromatographic separation, the analytes were introduced to the electrospray ionization source using a heated electrospray ionization source. The capillary temperature was set at 320°C. The spray voltage, capillary offset, and tube lens offset were set at 3971, 35, and 131V, respectively. The spray current was set at 3A. The sheath gas pressure, aux gas pressure, and ion sweep gas pressure were set at 40, 10, and 0 arbitrary units. The system was controlled using XCalibur (Thermo Fisher Scientific, Waltham, MA, USA). Data was processed and analyzed using Qual Browser (Thermo Fisher Scientific, Waltham, MA, USA).

3.5 Sample Clean-Up by Hydrophilic-Lipophilic Balance (HLB) Columns

Oasis HLB 3cc (60mg) extraction cartridges were purchased from Waters Corp (Milford, MA, USA). Set on a 48-well positive pressure manifold (Cera Inc., Baldwin Park, CA, USA), the columns were conditioned with 3 mL of methanol and then 3 mL of Nanopure water. Samples were applied to the column and allowed to drip through at a rate of one drop per second. After sample application, the columns were washed with 3 mL Nanopure water and dried at maximum nitrogen flow for two minutes. Retained peptides were eluted with 3 mL methanol into 12 x 75mm borosilicate tubes. After elution, samples were dried with nitrogen gas in a TurboVap LV Evaporator (Caliper Life Sciences, Hopkinton, MA, USA) for approximately thirty-five minutes at a pressure of 5 – 10 PSI and a temperature of 45°C. Dried samples were reconstituted in 50 mM sodium tetraborate buffer (pH 8.9) and AQC solution, and heated at 55°C for twenty minutes in order to derivatize the peptides in solution.

3.6 Immunopurification Performance and Validation

Method validation followed the EACL's in-house procedure for the identification and detection of rhEPO and darbepoetin from horse samples by LC-MS based on methodology from Bailly-Chouriberry et al. (2012)[15]. A MAIIA EPO Purification Kit was purchased from MAIIA Diagnostics (MAIIA AB, Uppsala, Sweden), used for the rapid purification and concentration of recombinant EPO from spiked serum samples. Briefly, a serum sample that was demonstrated by analysis to have no detectable rhEPO was thawed at room temperature, and 2 mL aliquots were spiked with an Epogen standard solution at a linearly increasing rate as shown in **Table 1.** Two solvent blanks with and without aliquots of AQC, a negative control, and a positive control were also included. All samples except negative controls were spiked with EPO-SIL Internal Standard to correct for analyte loss. Samples were loaded onto MAIIA columns, and the antibodies bound to the solid substrate captured, purified, and concentrated EPO. The EPO was then eluted from antibodies. Then, samples were filtered with 10kDa molecular weight cut-off filters, reduced, and denatured at 95°C. Following reduction, samples were digested with 10 µL of trypsin at 37°C. To clean samples of trypsin and aid with derivatization, samples were diluted with 2 mL of Nanopure water and cleaned with HLB columns as outlined in **Section 3.5.** The dried samples were reconstituted with sodium tetraborate buffer and 20 µL of a 3 mg/mL AQC solution prepared immediately before use.

The final method validation experiment included a series of quality control (QC) levels as outlined in **Table 1.** Two different solvent blanks were utilized—one with the addition of AQC and the other without—to ensure that the AQC working solution had not been contaminated.

Table 1: Epoetin Spike Information

Tube	Vol of EPO Working Solution (µL)	Equivalent EPO in Serum (ng/mL)	Vol of Internal Standard (µL)	Equivalent Internal Standard in Serum (ng/mL)
50ng	1[1]	50	20[2]	10
100ng	2[1]	100	20[2]	10
200ng	4[1]	200	20[2]	10
500ng	10[1]	500	20[2]	10
1000ng	20[1]	1000	20[2]	10
QC_{1-6}	4[1]	200	20[2]	10
Solvent Blank Derivatized (SBD)	0	0	20[2]	10
Solvent Blank Underivatized (SBU)	0	0	20[2]	10
Negative Control (NC)	0	0	0	0
Positive Control (PC)	20[2]	1	20[2]	10
1 = 100 ng/µL 2 = 1000 pg/µL				

3.6.1 LC-HRMS-MS/MS Parameters

Method performance samples were run on a Q-Exactive HF LC-MS/MS system. Continuing the in-house protocol, an Avantor ® ACE® Excel Column with C18 column (Radnor, PA, USA) with a particle size of 3.0 µM, a length of 100mm, and a 2.1mm inner diameter was used. The column was held at 40°C. A reverse-phase gradient eluted the analytes at a flow rate of 0.25 mL/min, with a total injection volume of 40 µL into the HPLC system. Mobile phase A was prepared by mixing acetonitrile with 0.2% formic acid, and mobile phase B was prepared by mixing HPLC-grade water with 0.2% formic acid. Initial conditions were 5% mobile phase A with a linear gradient to 65% at 12.0 minutes, another ramp from 65% to 90% from 12.0 minutes to 12.50 minutes, and then back down to 5% for a total run time of 20 minutes. XCalibur™ software (Thermo, San Jose, CA, USA) controlled the system and data

processing on a Microsoft Windows platform. Analytes were expected to be detected at mass-to-charge (m/z) ratios shown in **Table 2.**

Table 2: Expected Underivatized, Expected AQC-Derivatized , and Qualifying Ions for rhEPO Peptides and Internal Standards

	Expected Mass (m/z) Underivatized by AQC	Expected Mass (m/z) Derivatized by AQC	Qualifying Ions (m/z)
VNFYAWK (T6)	646.2400	549.24	714.360, 214.118, 828.401
VNFYAWK Internal Standard	468.2469	553.2469	722.374
VYSNFLR (T17)	449.7340	534.743	636.3460, 235.144, 799.409
VYSNFLR Internal Standard	545.7468	539.7468	646.354

4. Results

4.1 Evaluation of Derivatization

Theoretical reactions were drawn out using ChemSketch (Advanced Chemistry Development, Inc., Toronto, ON, Canada), based on literature reviews regarding derivatization approaches in electrospray and MALDI mass spectrometry[33]. Reaction solutions were introduced into the LC-MS/MS system by direct infusion and examined to see if resulting ion chromatograms provided mass-to-charge ions that matched drawn-out reactions. Throughout infusion, mass-to-charge values at 449 m/z and 898 m/z were monitored as they indicated the presence of underivatized rhEPO peptide T17 in +2 and +1 charge states. An overview of derivatization reagents, initial reaction conditions, and experiment success is provided in **Table 3.**

Table 3: Derivatization Reagents, Initial Reaction Conditions, and Experiment Success

Derivatization Reagent	Initial Reaction Conditions	Experimental Success
DNFP-L-V	1. DNFP-L-V in acetone with sodium bicarbonate buffer. Incubation: 3 hr, RT. 2. DNFP-L-V in acetone with ammonium acetate buffer. Incubation: 3 hr, RT. 3. DNFP-L-V in acetone with ammonium bicarbonate buffer. Incubation: 3hr, RT.	No
Dansyl Chloride	Dansyl Chloride in acetone with sodium bicarbonate buffer (100mM, pH 10.5). Incubation: 3 min, 60°C.	No
Girard's Reagent T	1. Girard's Reagent T in 10% acetic acid at 17x excess. Incubation: 3 hr, 75°C. 2. Girard's Reagent T in 10% acetic acid at 5mMol. Incubation: 3hr, 75°C.	No
TPP	1. TPP in DMF and the addition of N,N,N-triethylamine. Incubation: 1 hr, 60°C, followed by evaporation under nitrogen and reconstitution. 2. TPP in DMF and the addition of N,N,N-triethylamine. Incubation: 1 hr, 60°C, followed by addition of 10% formic acid, evaporation under nitrogen, and reconstitution.	No
TMP	TMP in 100mM TEAB. Incubation: 24 hr, RT.	No
TMPP-Ac-Osu	1. TMPP-Ac-OSu in 10% ACN with 0.1% TFA followed by addition of 200 mM sodium bicarbonate buffer. Incubation: RT for 1 hr followed by addition of 0.1% TFA. 2. TMPP-Ac-OSu in ACN with ammonium acetate buffer. Incubation: 20min, 55°C.	No
HSPyU	HSPyU in ACN with ammonium acetate buffer. Incubation: 20 min, 55°C.	No
TSTU	TSTU in ACN with ammonium acetate buffer. Incubation: 20 min, 55°C.	No
CY3-NHS	CY3-NHS in ACN with ammonium acetate buffer. Incubation: 20 min, 55°C.	No
TMTpro Zero	TMTpro Zero in ACN followed by addition of a 5% hydroxylamine buffer. Incubation: 60 min, RT.	No
AQC	1. AQC in ACN with ammonium acetate buffer. Incubation: 20 min, 55°C. 2. AQC in ACN with sodium tetraborate buffer. Incubation: 20 min, 55°C.	Yes

After evaluating duplicate results against each other, the difference in ion abundances appeared to be negligible, if not entirely non-existent. For example, the difference mass-to-charge ratios appeared at the same width of 68 m/z with the first and second experiments with DNFP-L-V, indicating intense byproduct formation or potential hydrolysis. These patterns and particular m/z ions appeared at the same numbers for each run, though ion abundances were subject to change. For this reagent, theoretical ions did not match ions observed.

Figure 1: Theoretical Reactions of DNFP-L-V with rhEPO Peptide T17.

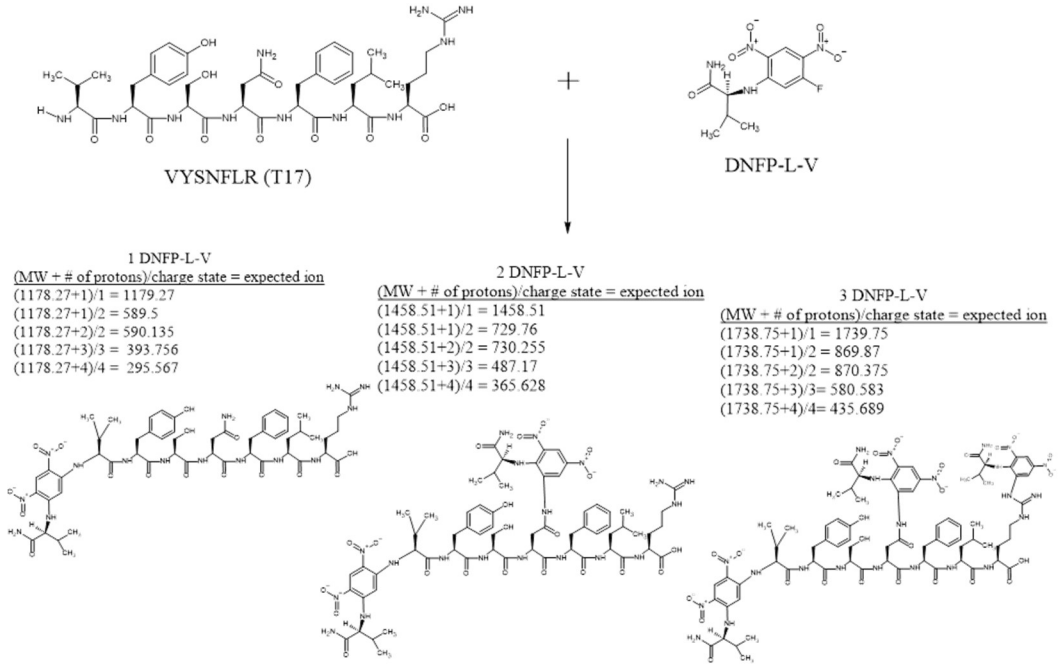
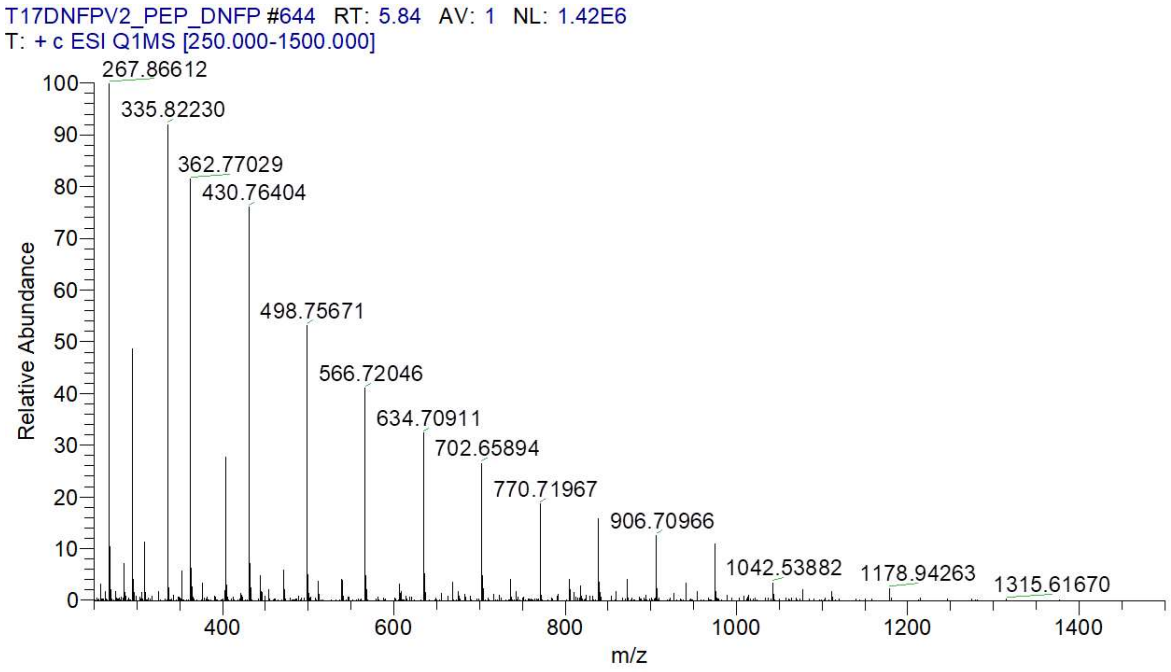


Figure 2: Mass Spectra of DNFP-L-V Reaction Solution Infusion



The spectra of Dansyl Chloride did not have any ions observed in the full scan MS spectrum that corresponded to rhEPO peptide T17 during either the first or second infusions. Both spectra were fairly clean and had a few discernible ions, but they did not match any of the m/z values theorized by ChemSketch.

Figure 3: Theoretical Reactions of Dansyl Chloride with rhEPO Peptide T17

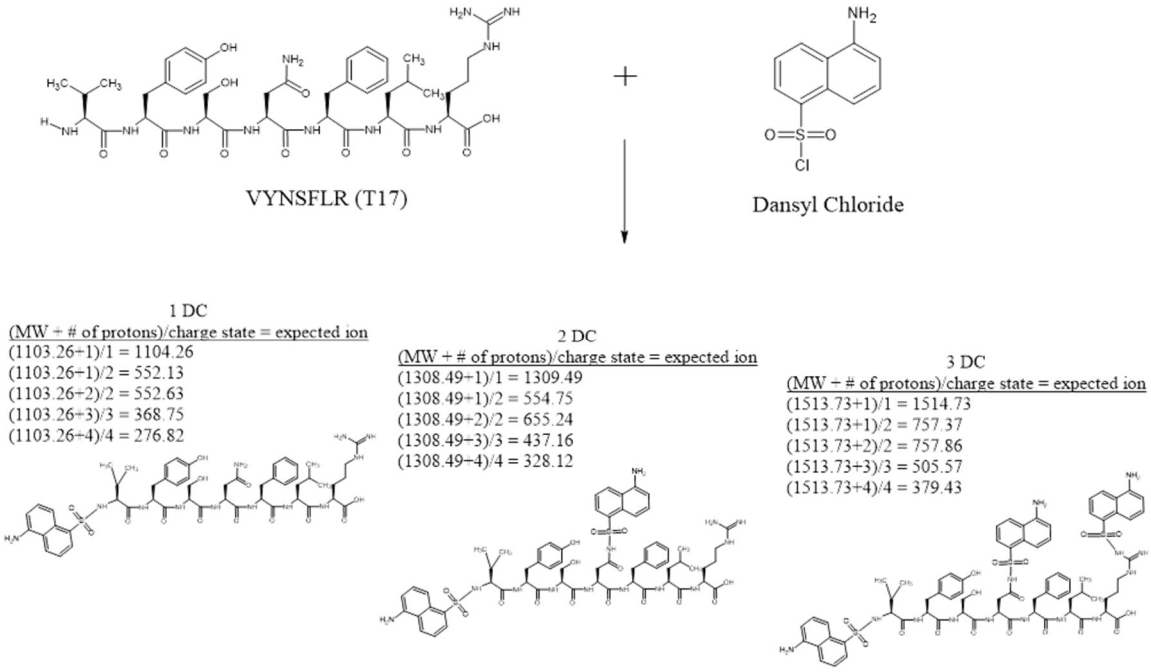
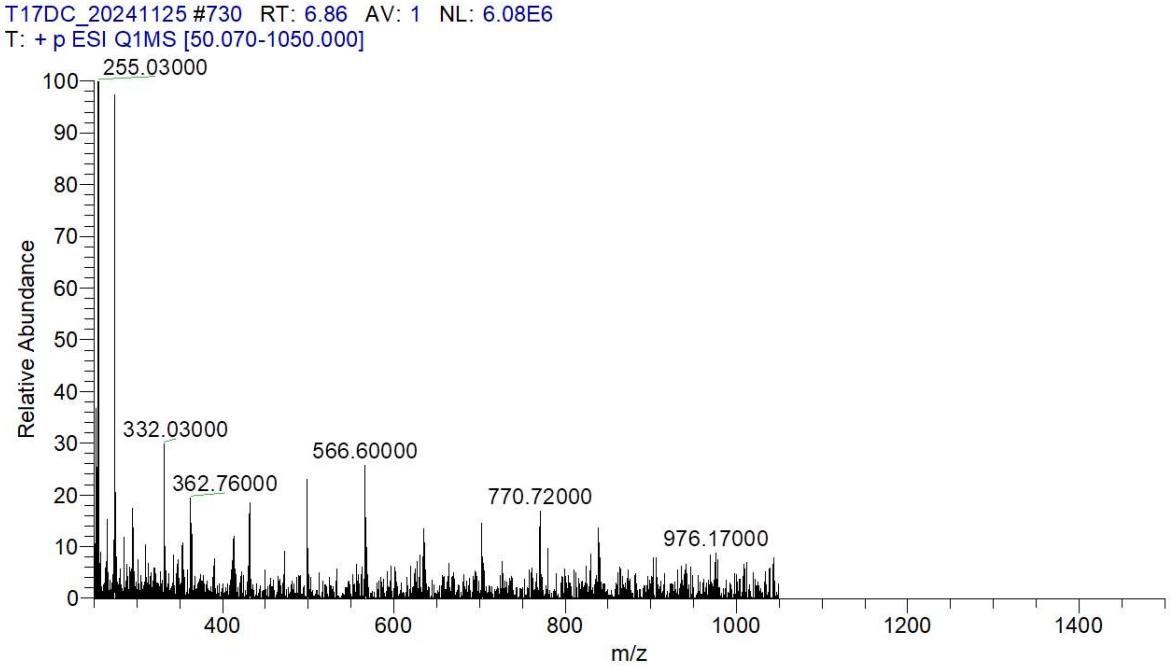


Figure 4: Mass Spectra of Dansyl Chloride Reaction Solution Infusion



Girard's Reagent T has been reported to modify peptides at the C-terminus and attach a fixed charge[33] or otherwise derivatize aldehydes and ketones. Theorized masses were calculated based on either the N- or C-terminus derivatization as exemplified by Zaikin and Borisov (2022) and Ko and Brodbelt (2012). Major mass-to-charge ratios in the infusion spectra did not match theorized mass values—whether derivatized at the N- or C-terminus—and the presence of a sizeable peak of 449 m/z indicated that peptide T17 was not being derivatized.

Figure 5: Theoretical Reaction of Girard Reagent T with rhEPO Peptide T17

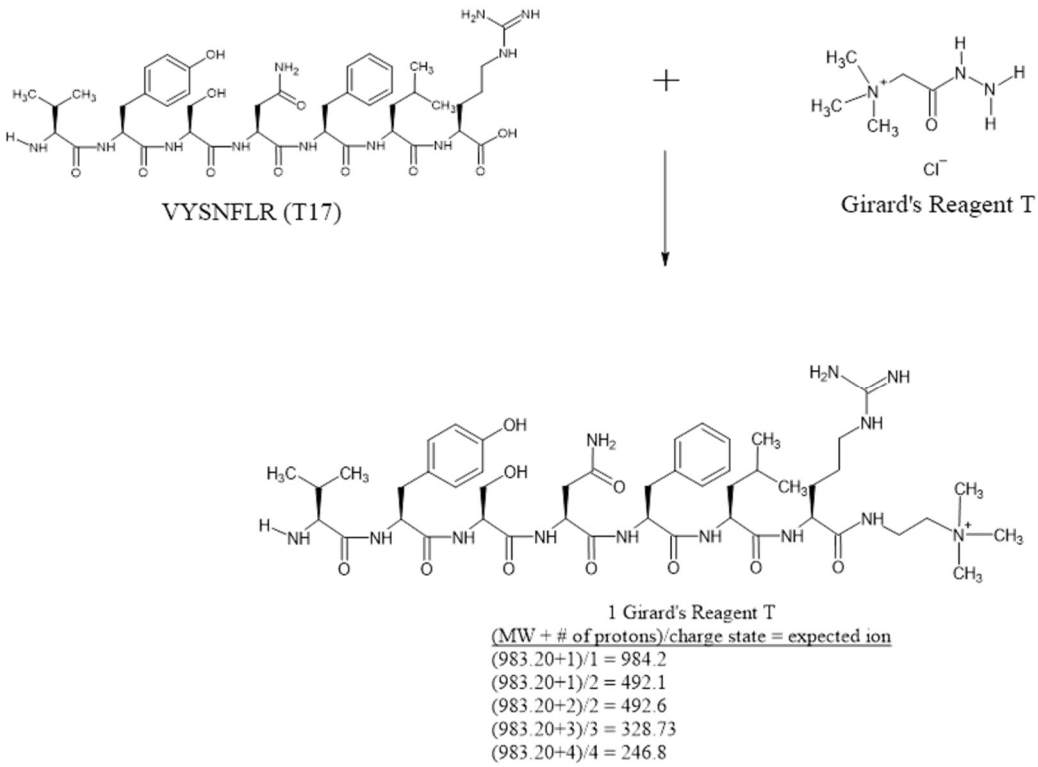
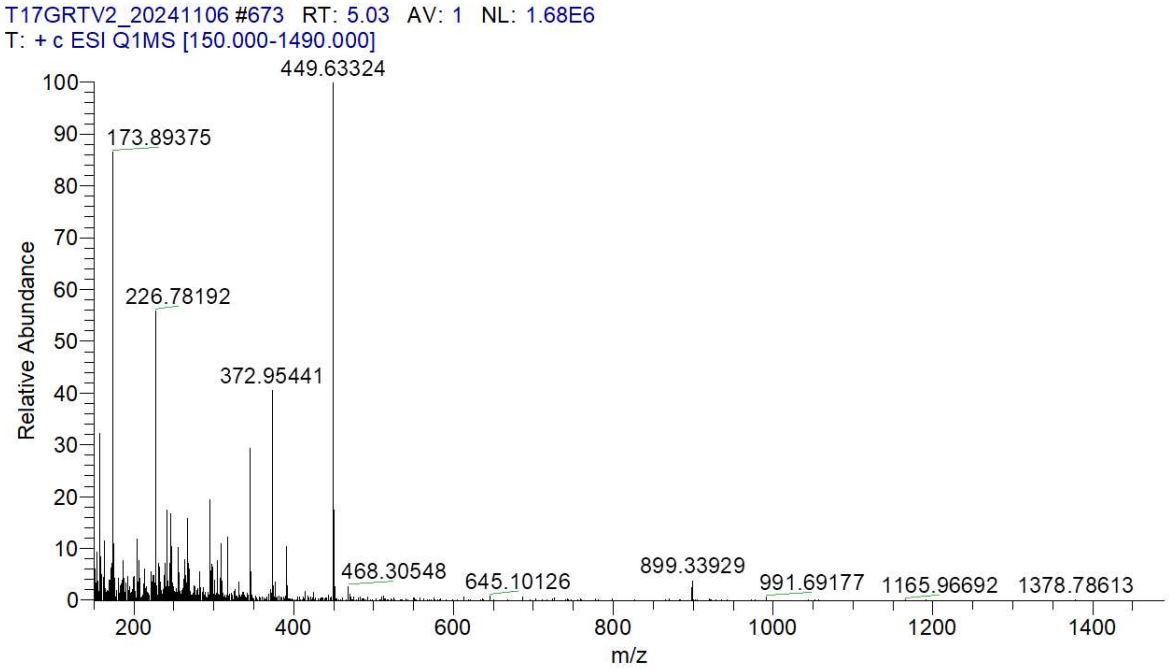


Figure 6: Mass Spectra of Girard's Reagent T Reaction Solution Infusion



For the pyridinium salts, the spectra provided discernible major ions. TPP was tested in two replicates and then once with the addition of 10% formic acid to stop the reaction. Unsurprisingly, the major ions were compared and found to be similar, even if ion abundances varied slightly, and none of the ions matched expected values calculated. TMP was tested in duplicate in the same reaction conditions, and with freshly prepared reagent in solution. The presence of low abundance ions in the mass spectrum indicated that peptide was present in solution based off +2 and +1 charge state m/z values (449 m/z and 898 m/z); otherwise, the theorized masses of the peptide-pyridinium complex were not present, or not present at any appreciable abundance.

Figure 7: Theoretical Reactions of TPP with rhEPO Peptide T17

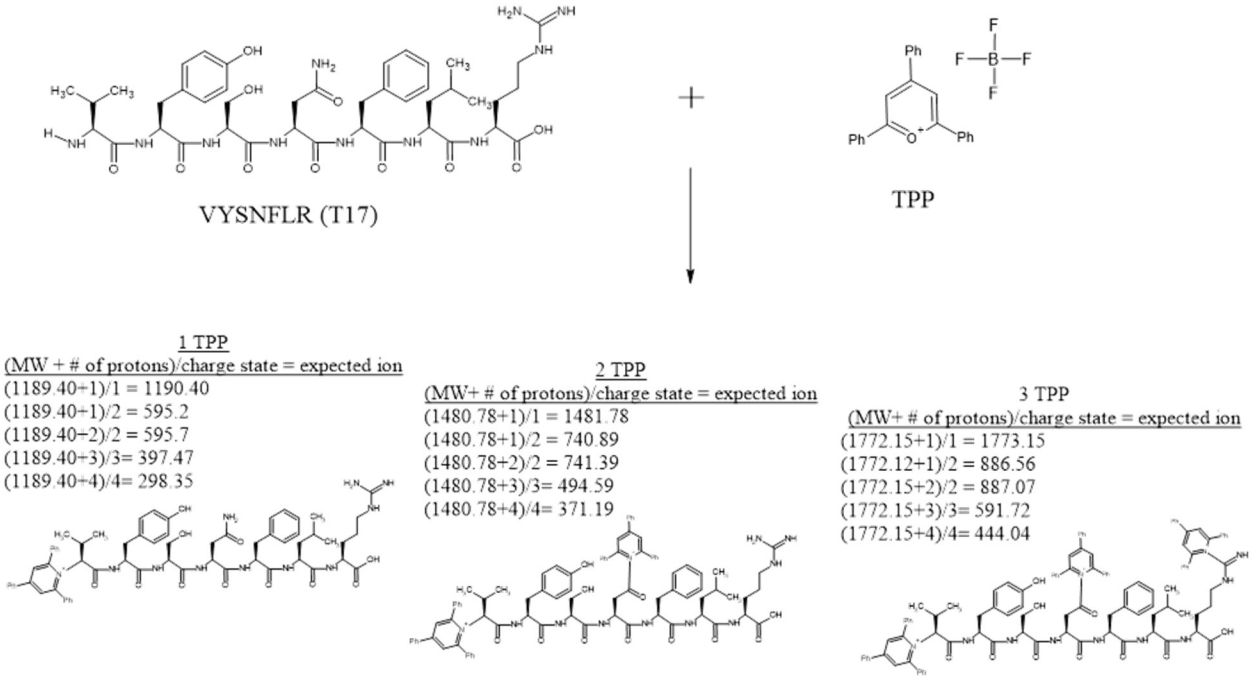


Figure 8: Mass Spectra of TPP Reaction Solution Infusion (Reaction Protocol 1)

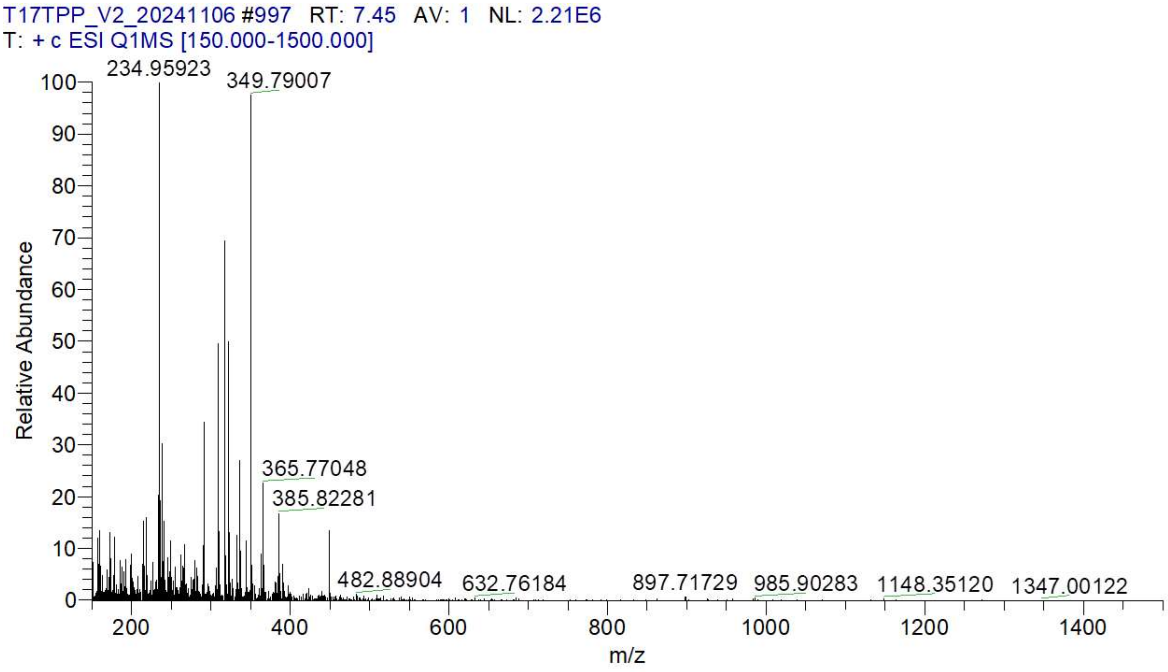


Figure 9: Theoretical Reactions of TMP with rhEPO Peptide T17

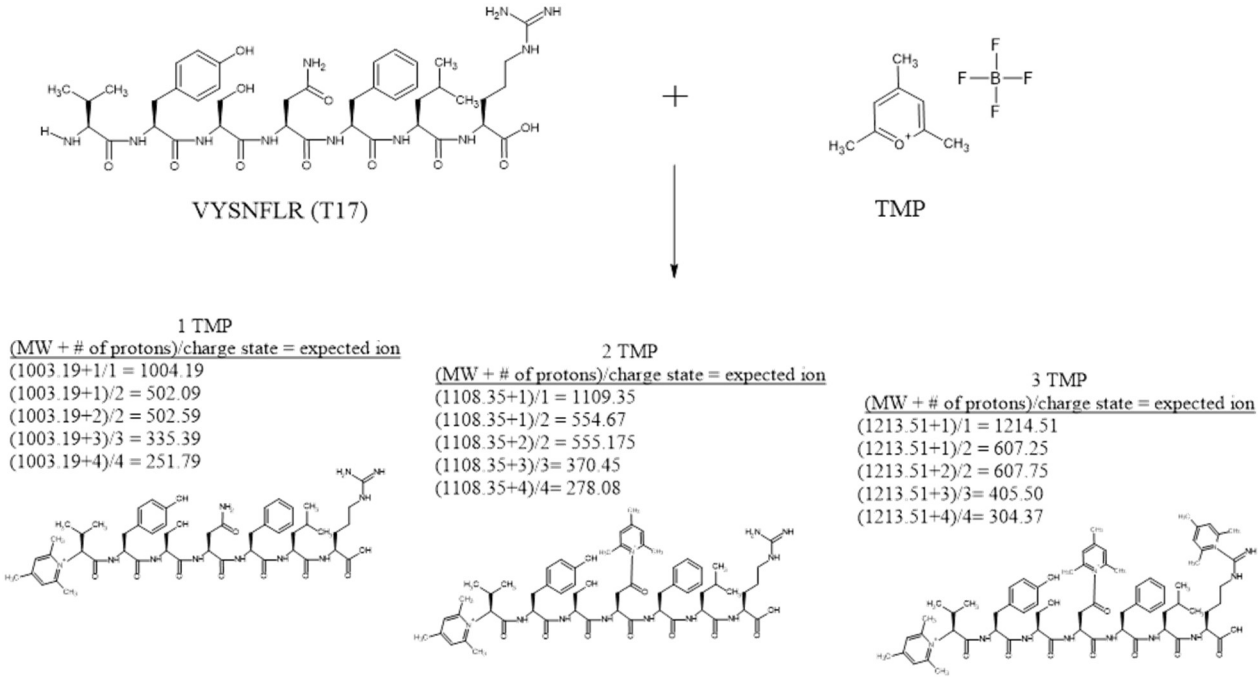
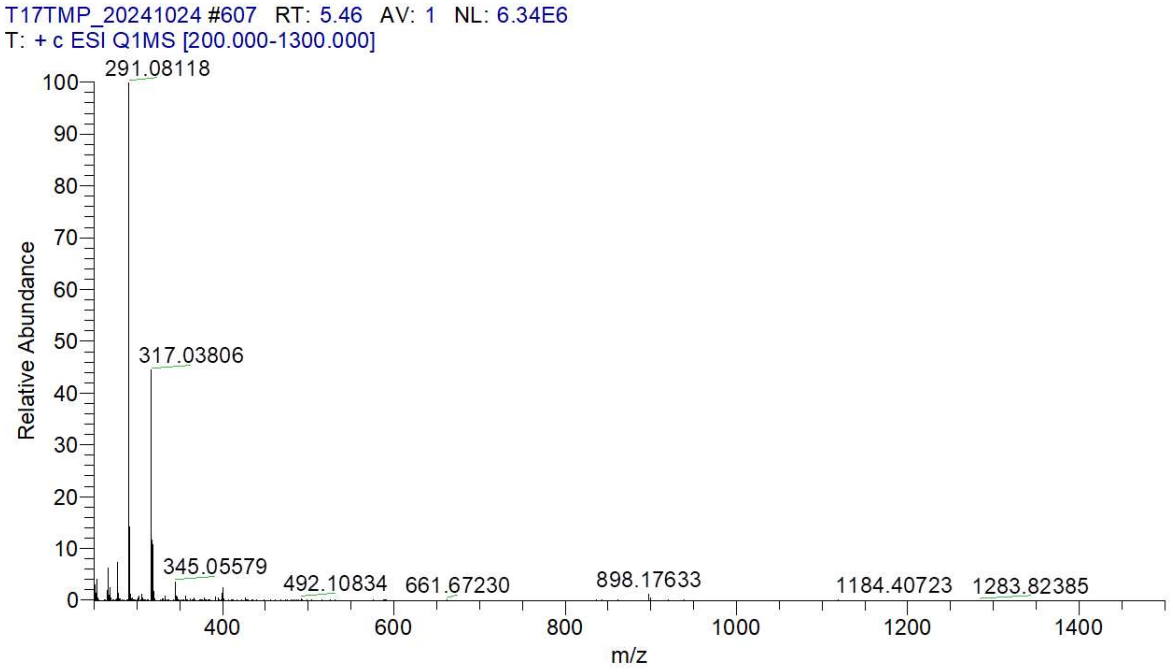


Figure 10: Mass Spectra of TMP Reaction Solution Infusion



The initial spectra of TMPP-Ac-Osu was also polymeric in nature—that is, each peak was separated by a mass-to-charge ratio of approximately 68 m/z. The reaction proceeded in polypropylene tubes for the first reaction, and then borosilicate culture tubes for the second. The spectra exhibited the same qualities in both experiments. Dissolving the reagent in acetonitrile and testing it with the same reaction conditions as AQC, TSTU, and HSPyU produced different results; a major peak appeared at 589 m/z and a minor peak at 735 m/z. As 735 m/z was close to the theorized ion drawn in ChemSketch, it was fragmented using collision energies from 0 – 40eV, with a fragmentation from collision energy of 25eV (**Figure 13**).

Figure 11: Theoretical Reactions of TMPP-Ac-OSu with rhEPO Peptide T17

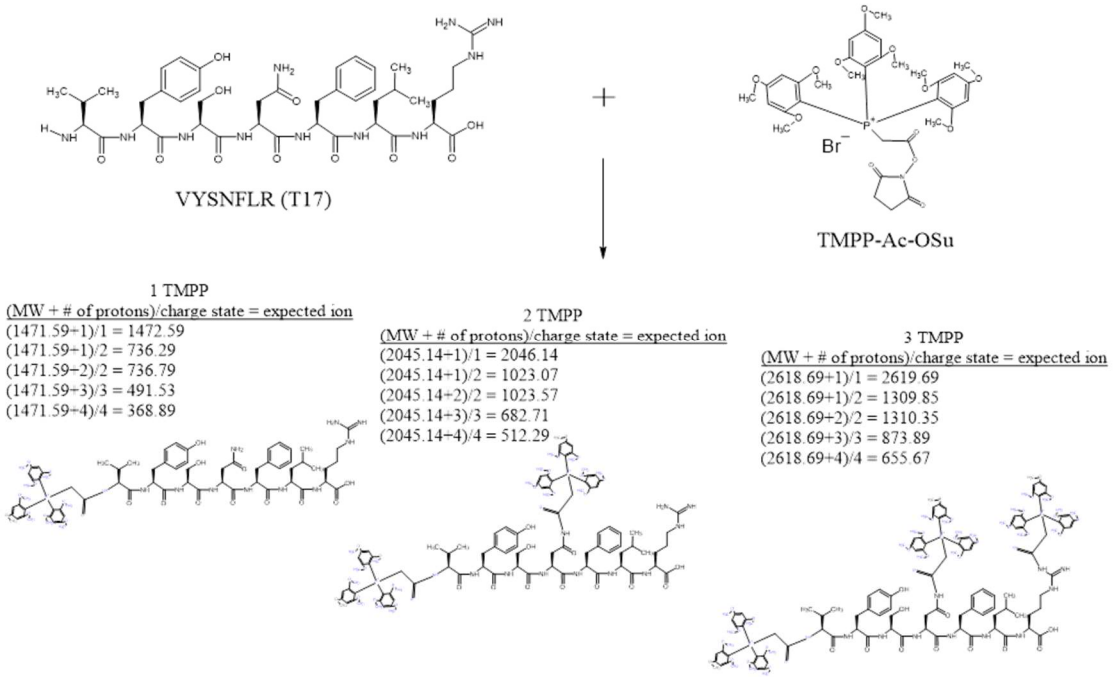


Figure 12: Mass Spectra of TMPP-Ac-OSu Reaction Solution (ACN/Ammonium Acetate Buffer) Infusion

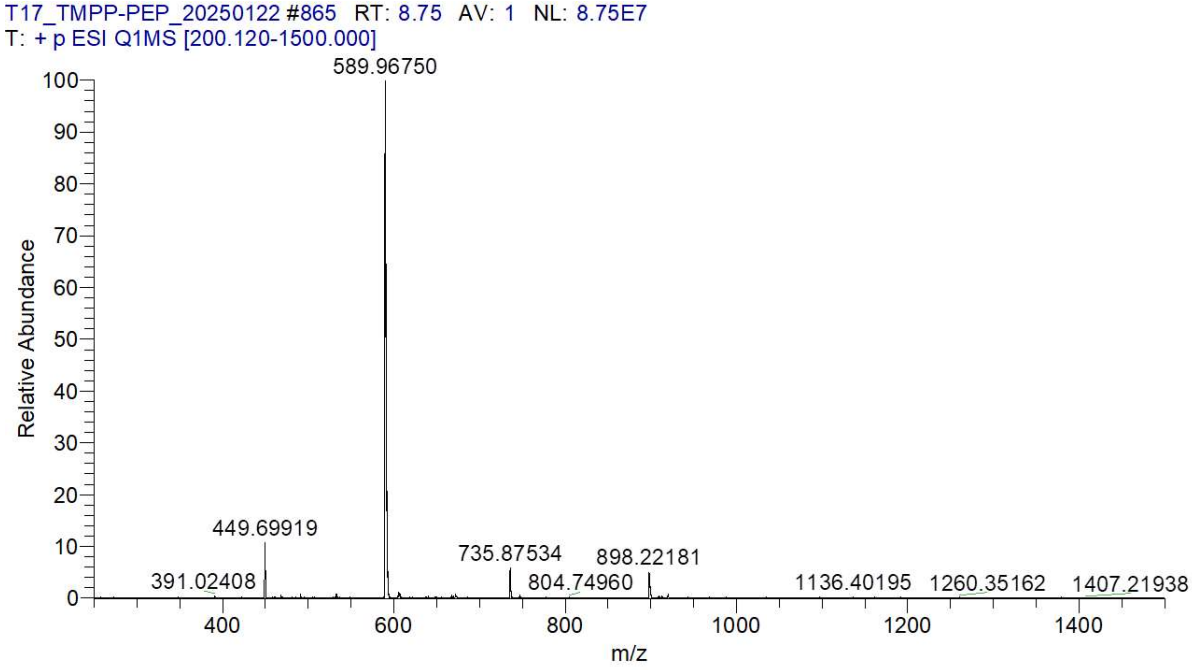
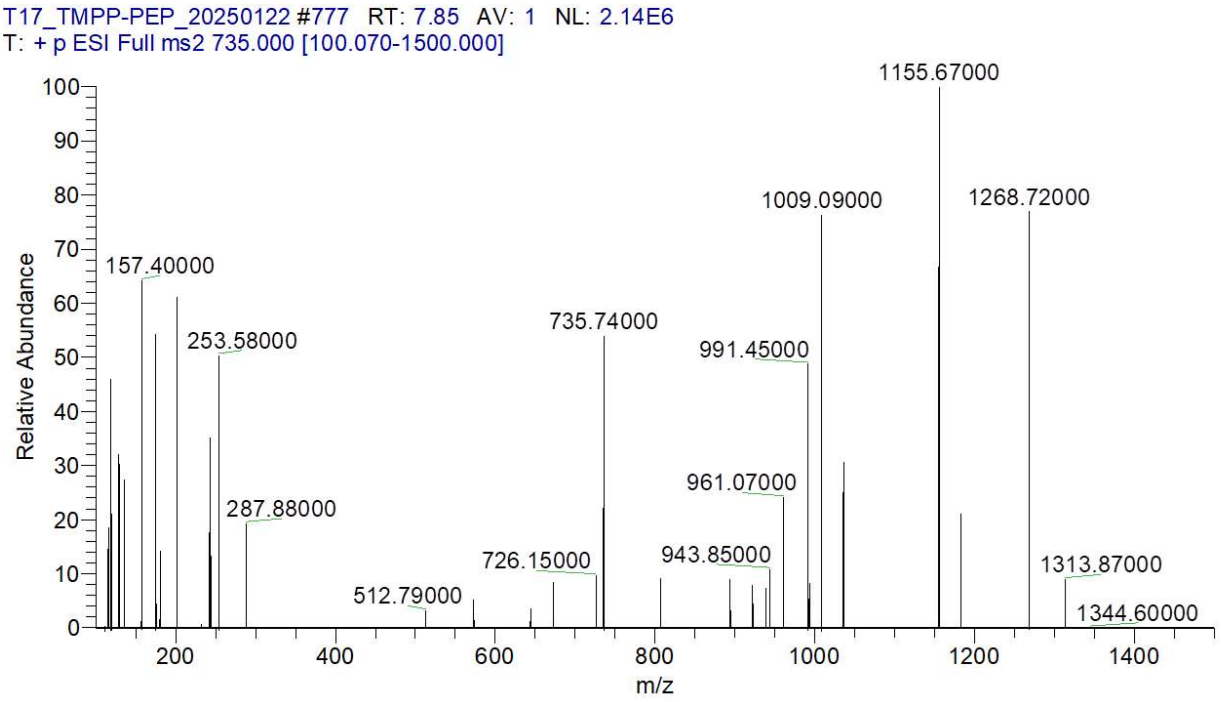


Figure 13: Full MS2 Scan Mass Spectra of TMPP Reaction Solution with an Expected Ion of 735 m/z at 25eV



CY3-NHS, HSPyU, and TSTU ion abundances also did not match mass to charge ratios calculated on ChemSketch. Fragmenting the large ions closest to the expected mass of the derivatized peptide in each individual spectra indicated that derivatization was not successful, as quantifying ions such as 799 m/z, 636 m/z, or 235 m/z would be present. Fragmentation was not performed for spectra that reported high ion abundances for 449 m/z and 898 m/z (e.g., for TSTU) as that indicated the presence of singly and doubly charged rhEPO peptides. To check the validity of the protocol, glycine was used as a positive control and derivatized by TMPP-Ac-Osu, TSTU, HSPyU, and CY3-NHS. Expected masses matched, though ion abundances were low, indicating that the protocol was successful, but not particularly effective for those four succinimide esters when reacted with the T17 peptide.

Figure 14: Theoretical Reactions of CY3-NHS with rhEPO Peptide T17

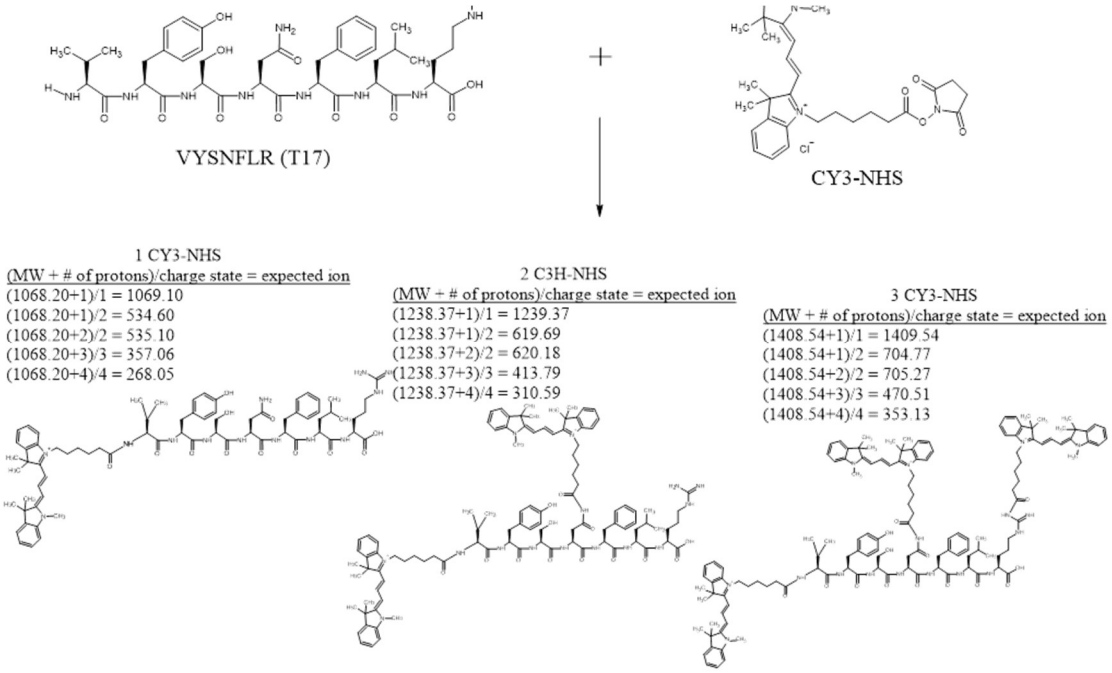


Figure 15: Mass Spectra of CY3-NHS Reaction Solution Infusion

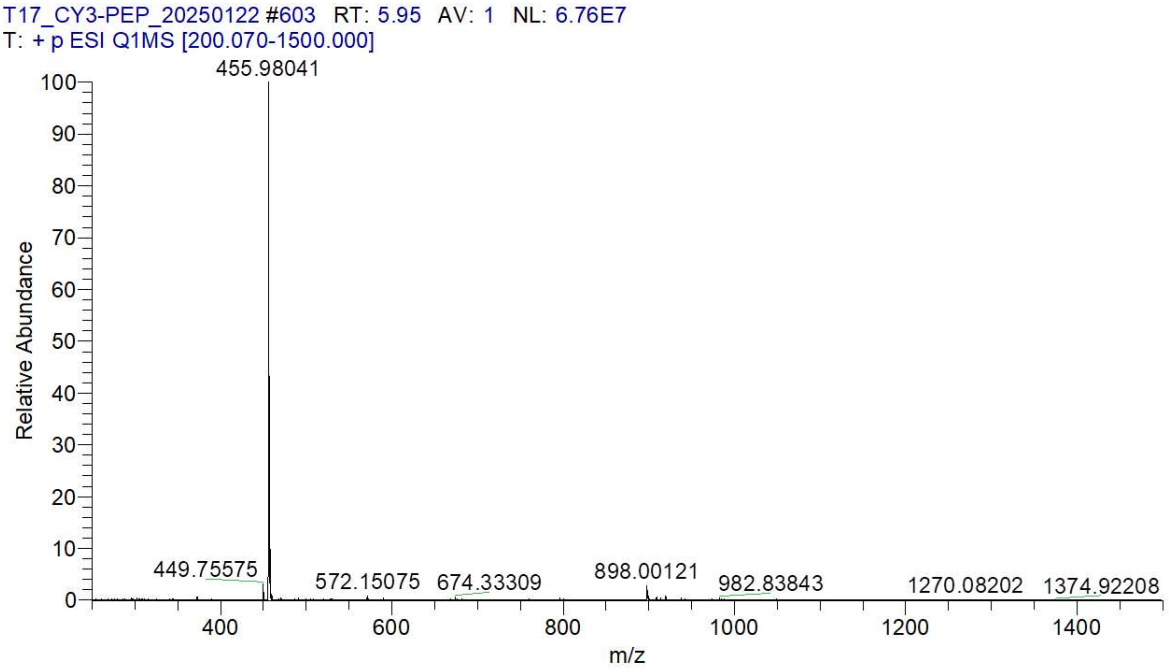


Figure 16: Theoretical Reactions of HSPyU with rhEPO Peptide T17

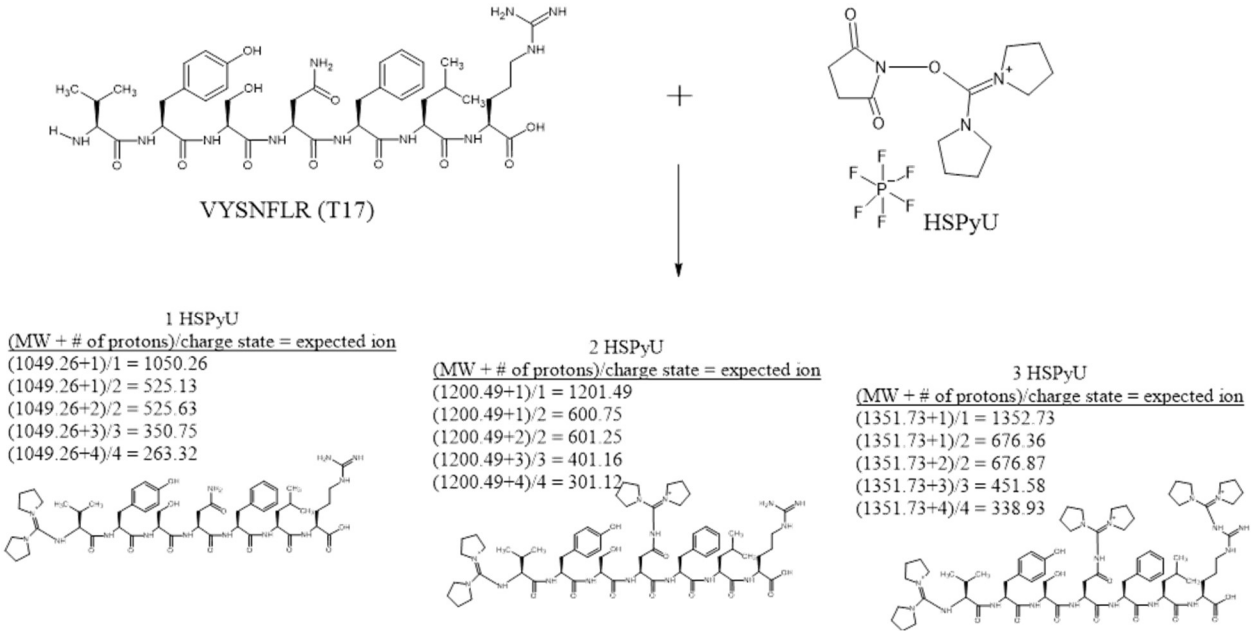


Figure 17: Mass Spectra of HSPyU Reaction Solution Infusion

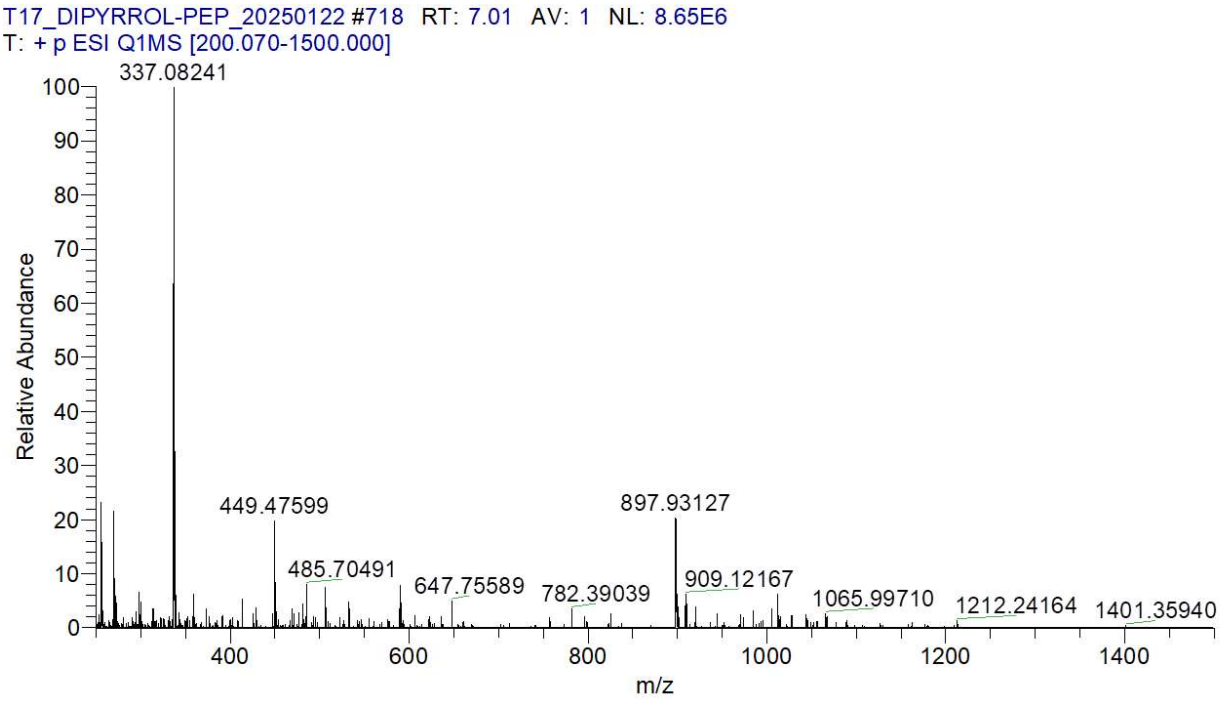


Figure 18: Theoretical Reactions of TSTU with rhEPO Peptide T17

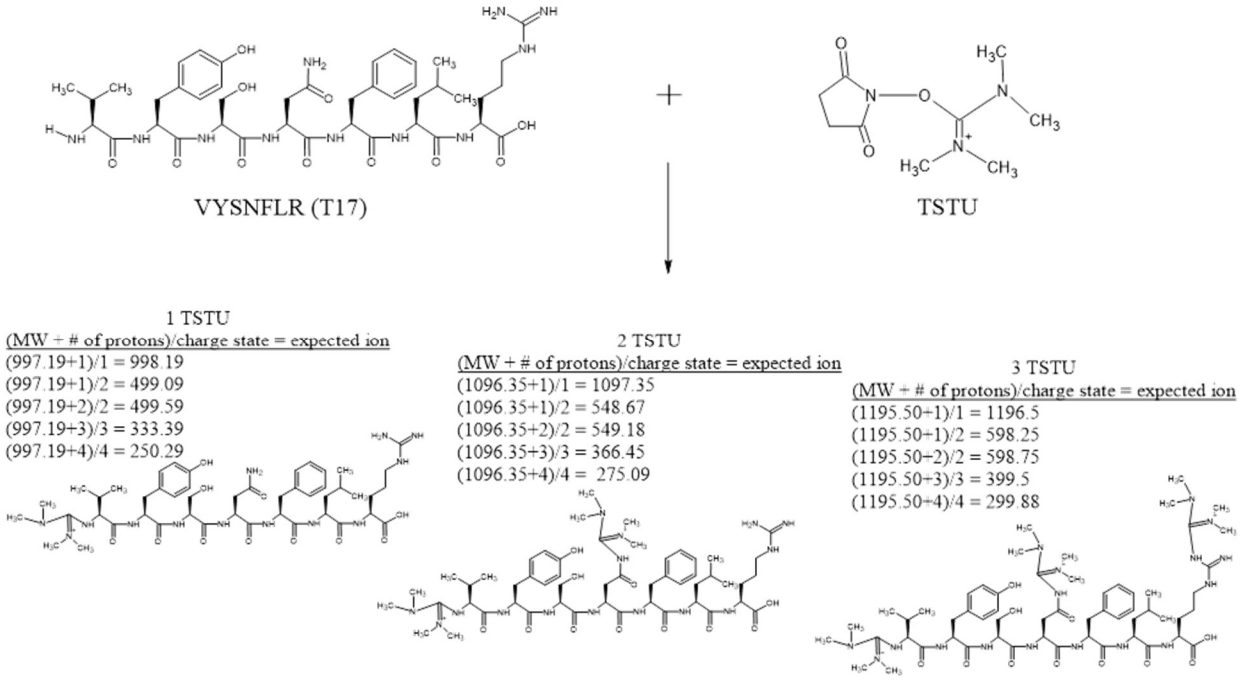
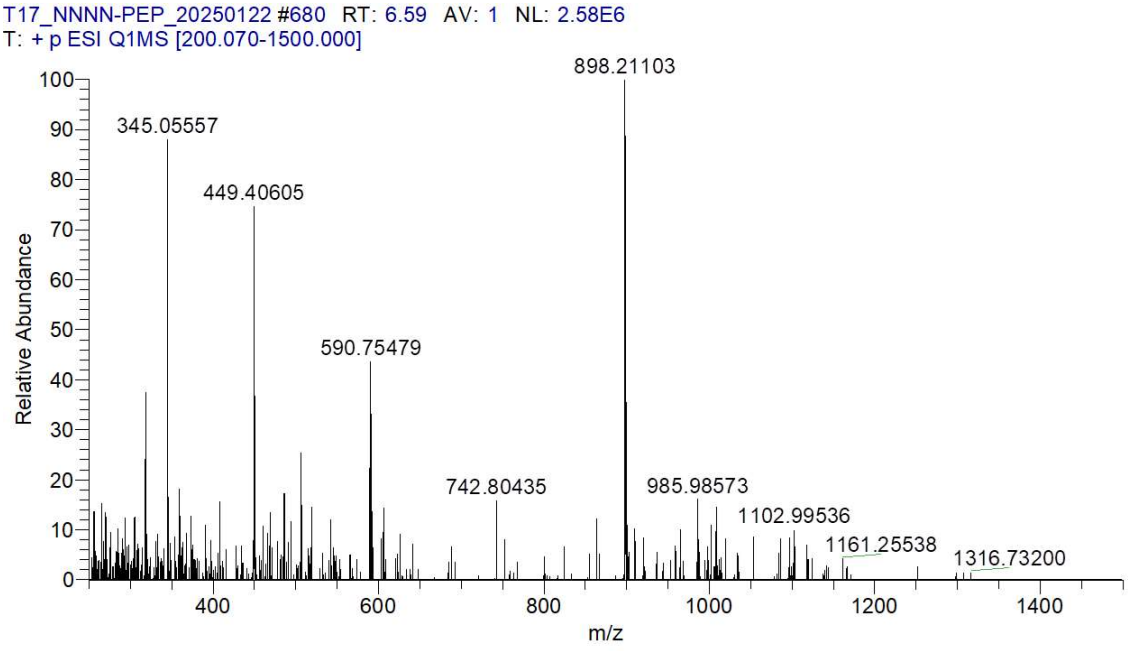


Figure 19: Mass Spectra of TSTU Reaction Solution Infusion



According to Thermo Fisher Scientific User Guide, the peptide mass modification of TMTpro multiplex reagents is 304.2071 Da. However, infusing both the original reaction solution and a one-to-ten dilution in 0.1% formic acid in water garnered the same results (**data not shown**). The total ion chromatograms revealed a lack of outstanding mass to charge ratios that could definitively report the singly- or double-charged derivatized peptide.

Out of eleven possible derivatizing reagents, only AQC provided expected results. During initial infusions, the expected m/z value of the peptide-AQC complex matched the reported value on the total ion chromatogram, and the fragmentation of the suspected AQC-peptide complex also revealed quantifying ions associated with rhEPO peptide T17 (799 m/z and 636 m/z) shown in **Figures 20, 21, and 22.**

Figure 20: Theoretical Derivatization of rhEPO Peptide T17 with AQC

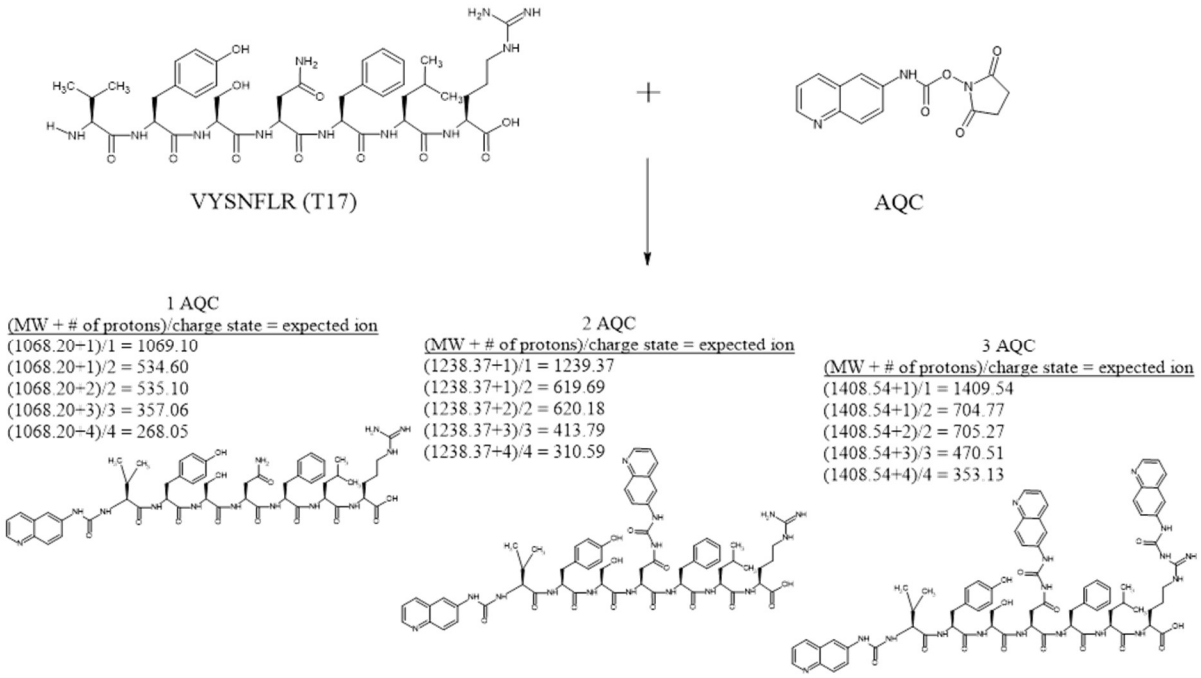


Figure 21: Full Scan Mass Spectra of AQC Reaction Solution Infusion

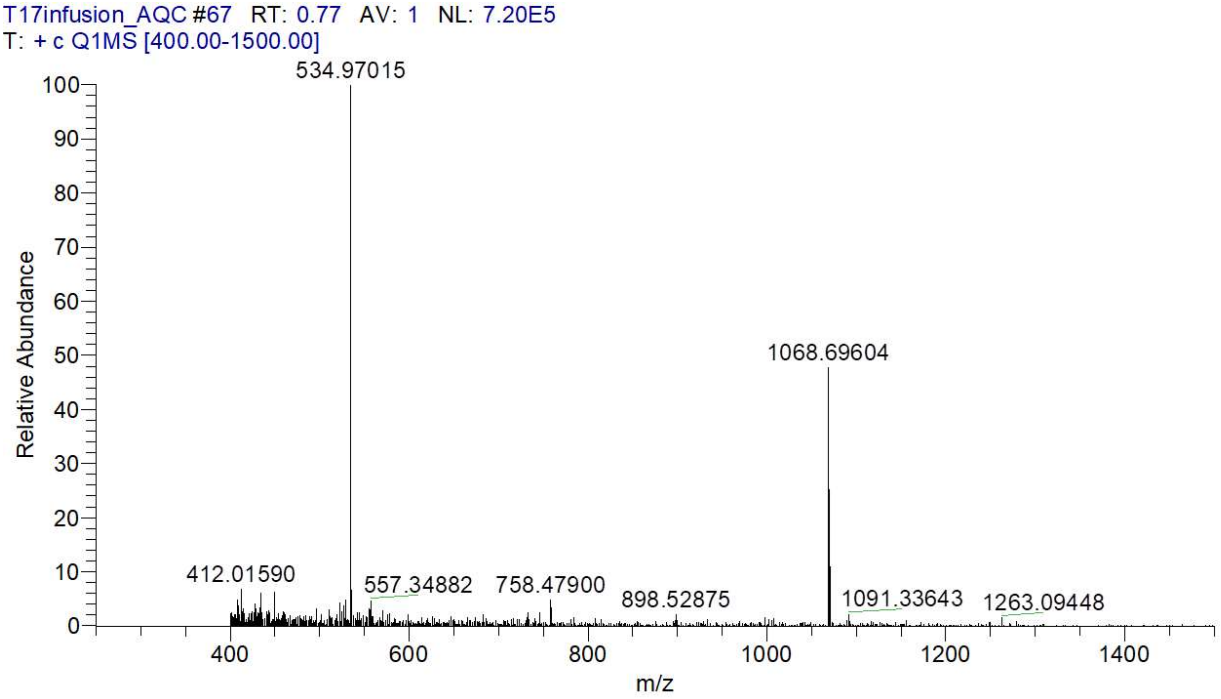
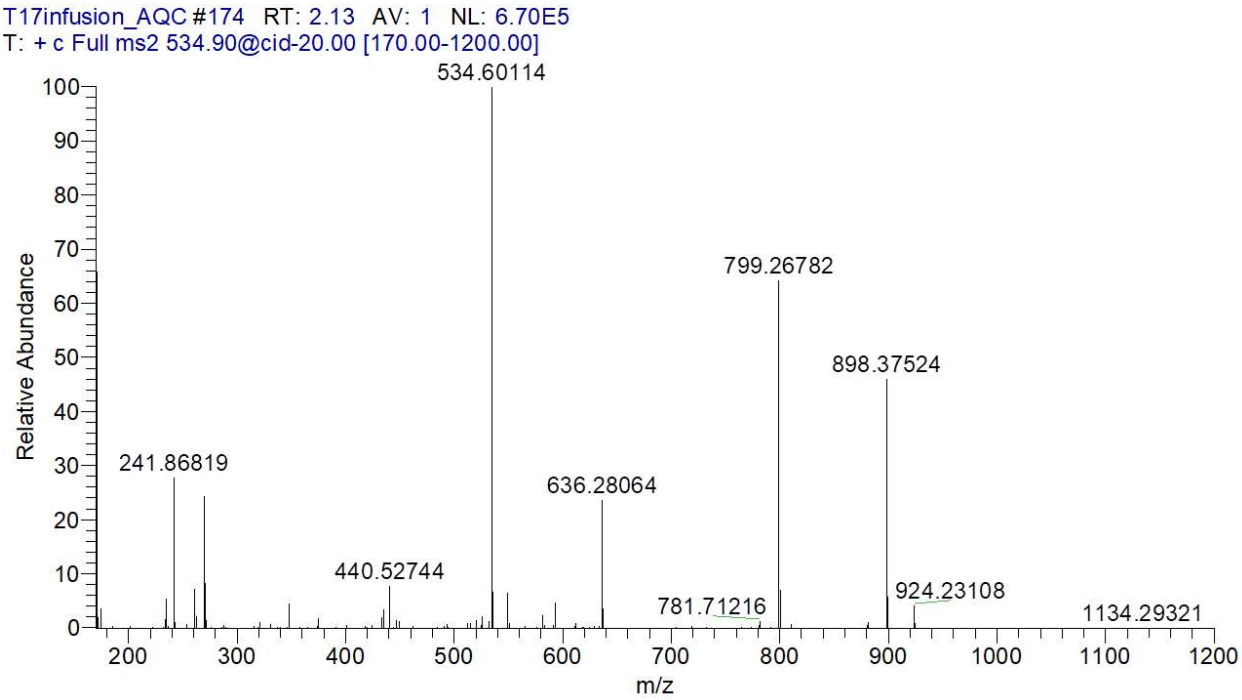


Figure 22: MS2 Scan Mass Spectra of AQC Reaction Solution Infusion at CID 20eV



4.2 Evaluation of AQC Optimization

Further method development employed the same Thermo Scientific Quantum Ultra Triple Stage Quadrupole Mass Spectrometer coupled with an Agilent 1100 Series HPLC with an electrospray ionization source. Analyte precursor and product ions, collision energy, and tube offset were recorded in the compound optimization window of the EZ Tune program (Thermo Fisher Scientific, Waltham, MA, USA) by direct infusion and in positive mode ESI. Analyte optimization settings are included in **Table 4** comparing the turn-over of underivatized peptide to derivatized peptide**.**

Table 4: ESI Mode and Precursor Ion, Product Ions, and Optimized Collision Energy

Analyte	ESI Mode and Precursor Ion (m/z)	Product Ion (m/z)	Optimized Collision Energy (eV)
VYSNFLR (T17)	Positive 449.338	235.140 646.300 799.400	20 20 20
VYSNFLR (T17)-AQC Derivative	Positive 534.747	120.016 136.027 170.996 242.092 270.091	44 42 35 20 22

After selecting AQC as the best-performing derivatization reagent, the next step was to optimize the reaction protocol. The mock-digest solution was tested first by aliquoting 20µL AQC into the tryptic digest solution with 6 µL of a 10 µg/mL rhEPO T17 peptide solution (equivalent to a molar concentration of 1000 ng/mL of erythropoietin), as that would be the simplest and easiest way to integrate derivatization into the confirmation method. Based on the results, it was determined that not all the peptide in solution was being derivatized. The molar ratio of AQC to T17 peptide was in 3191-times excess, and so different molar ratios were tested in order to determine if the amount of AQC would affect the peptide-AQC complex output. For

this analysis, 6 μL of a 10 μg/mL solution of peptide T17 was reacted with AQC at a concentration of 3 mg/mL, 1 mg/mL, 100 μg/mL, and 10 μg/mL in four respective experiments in a mock-digestion solution. As expected, as AQC concentration decreased, so did the amount of derivatized peptide (**Figures 23-26)**. The working solution of AQC was kept at 3 mg/mL throughout the rest of the optimization process.

Figure 23: Peptide T17 Reacted with a 3 mg/mL Solution of AQC

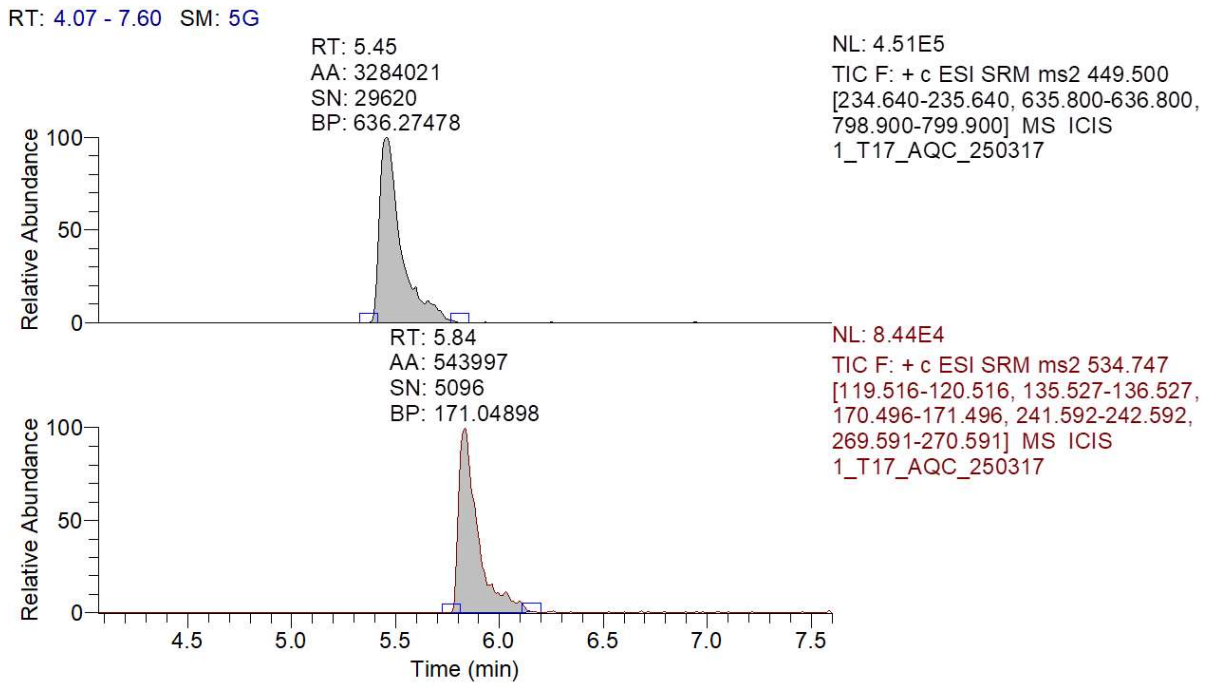


Figure 24: Peptide T17 Reacted with a 1 mg/mL Solution of AQC

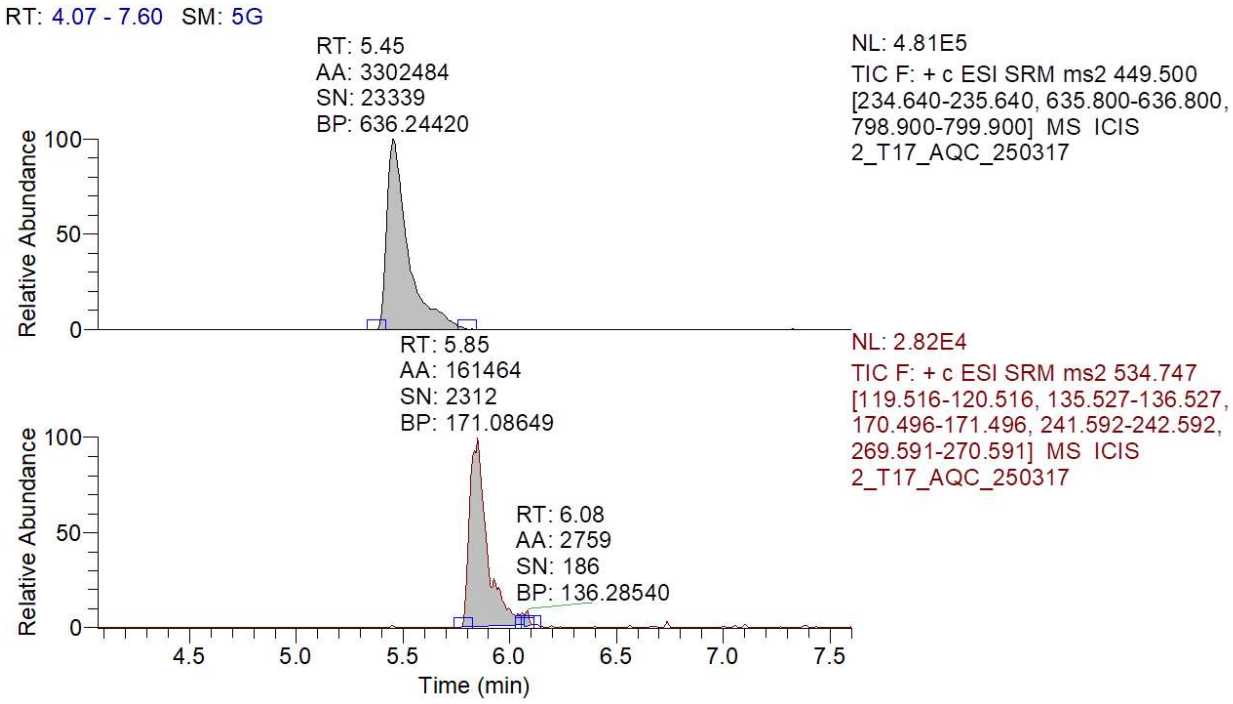


Figure 25: Peptide T17 Reacted with a 100 µg/mL Solution of AQC

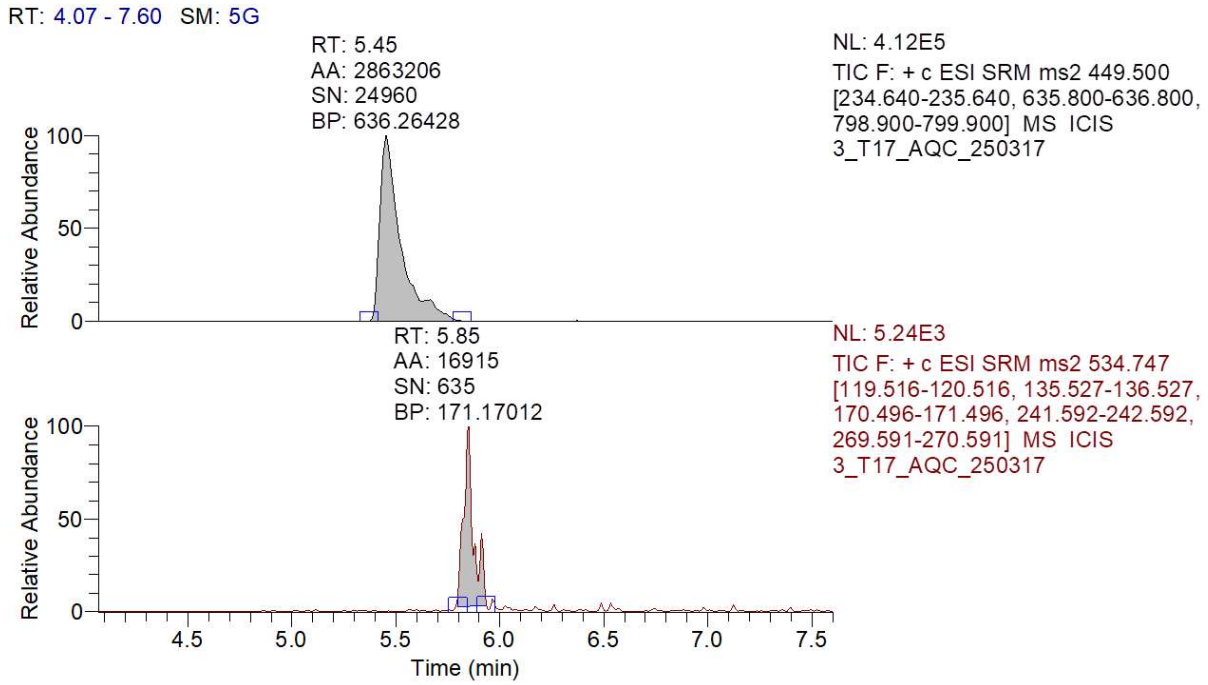
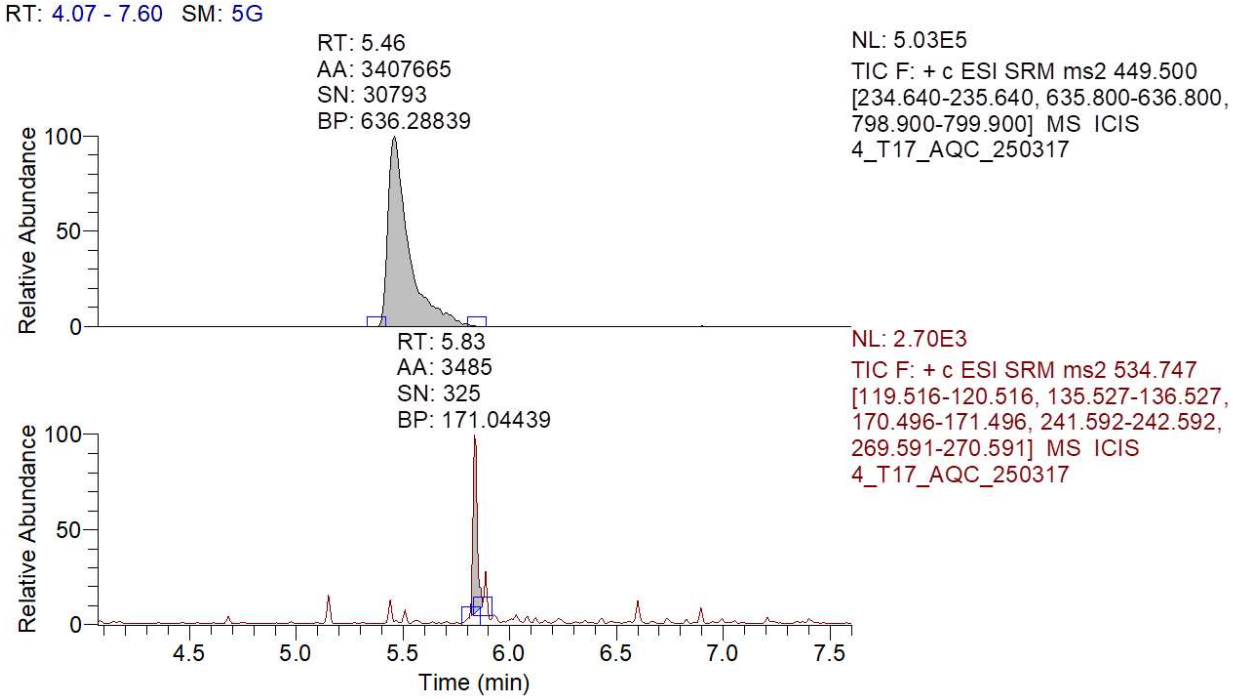
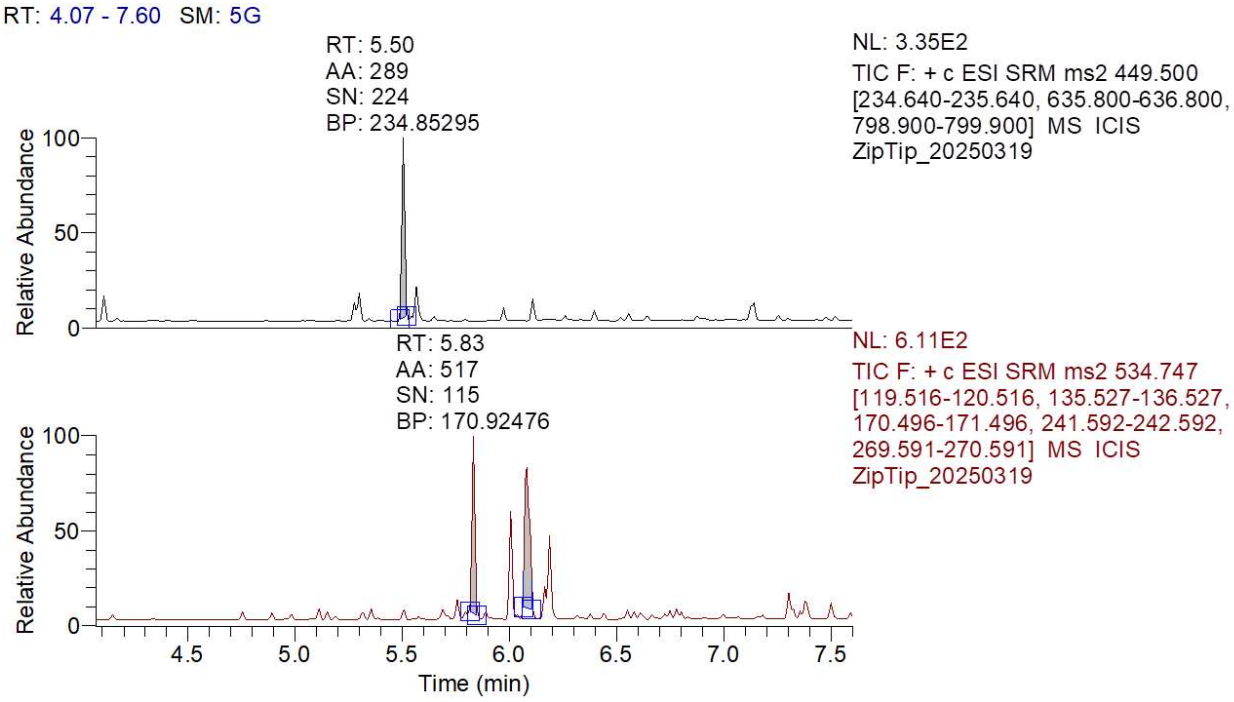


Figure 26: Peptide T17 Reacted with a 10 µg/mL Solution of AQC



Sample clean-up utilized Pierce™ C18 Tips (100 mL), and chromatographic results showed that both peptide and derivatized peptide loss in the sample were significant when compared to a pre-clean-up reaction solution. These results were seen both with the use of C18 Tips before and after derivatization. Thus, the next method was to modify the amount of trypsin in solution.

Figure 27: Sample Clean-Up Using Pierce™ C18 Tips (100 mL) ("ZipTip") After Derivatization



Trypsin concentration and the use of HLB columns were tested in the same run. rhEPO peptide T17 was reacted with AQC in a sodium tetraborate and 1 µL of trypsin (**Figure 28)**. A mock-digest solution with 10 µL of trypsin was cleaned using an HLB column, and rhEPO peptides were eluted from the column with methanol, dried under nitrogen, and reconstituted with sodium tetraborate and a 3 mg/mL AQC solution as outlined in **Section 3.5**. Results for

HLB are shown in **Figure 29.** Examining the comparison of integrated ion areas for the derivatized peptide saw a significant increase in normalization levels and integrated ion areas for the experiment using HLB. Additionally, ammonium bicarbonate with 0.1% OGP and 0.1% TCEP appeared to derivatize peptides better in experimental methods where lesser amounts of trypsin was used as compared to sodium tetraborate buffer, seeing slightly higher integrated ion areas for derivatized peptides as opposed to the sodium tetraborate buffer. The amount of derivatized peptide in a solution of rhEPO peptide T17, ammonium bicarbonate with 0.1% OGP and 0.1% TCEP, AQC, and 1 μL of trypsin were unable to be quantified (**Figures 30 and 31**).

Figure 28: Derivatized Reaction Solution with Sodium Tetraborate ("Borate") and 1μL of Trypsin

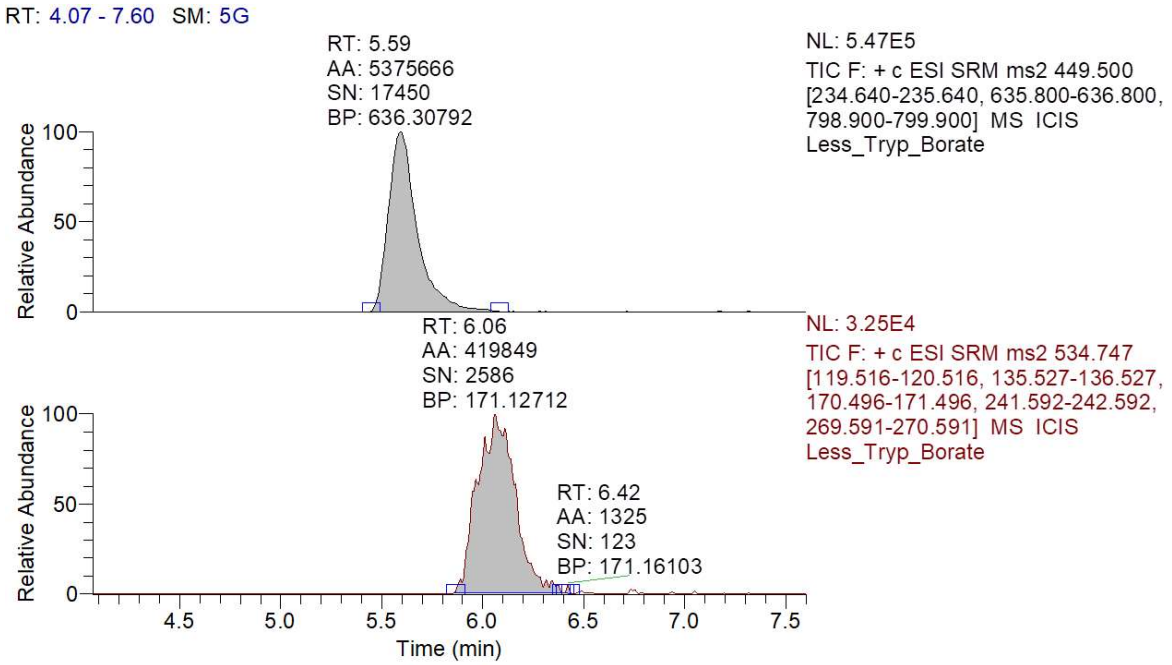


Figure 29: Derivatized Reaction Solution with Sodium Tetraborate After HLB

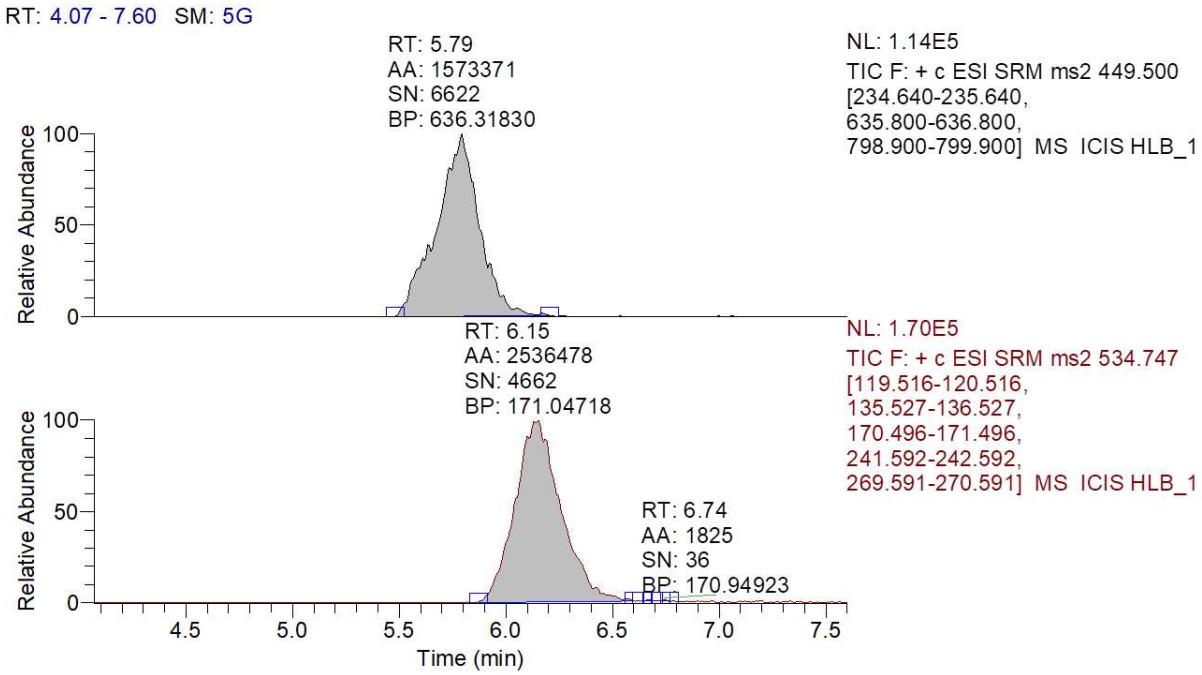


Figure 30: Derivatized Reaction Solution with Ammonium Bicarbonate with 0.1% OGP and 0.1% TCEP ("ABi") and 1mL of Trypsin

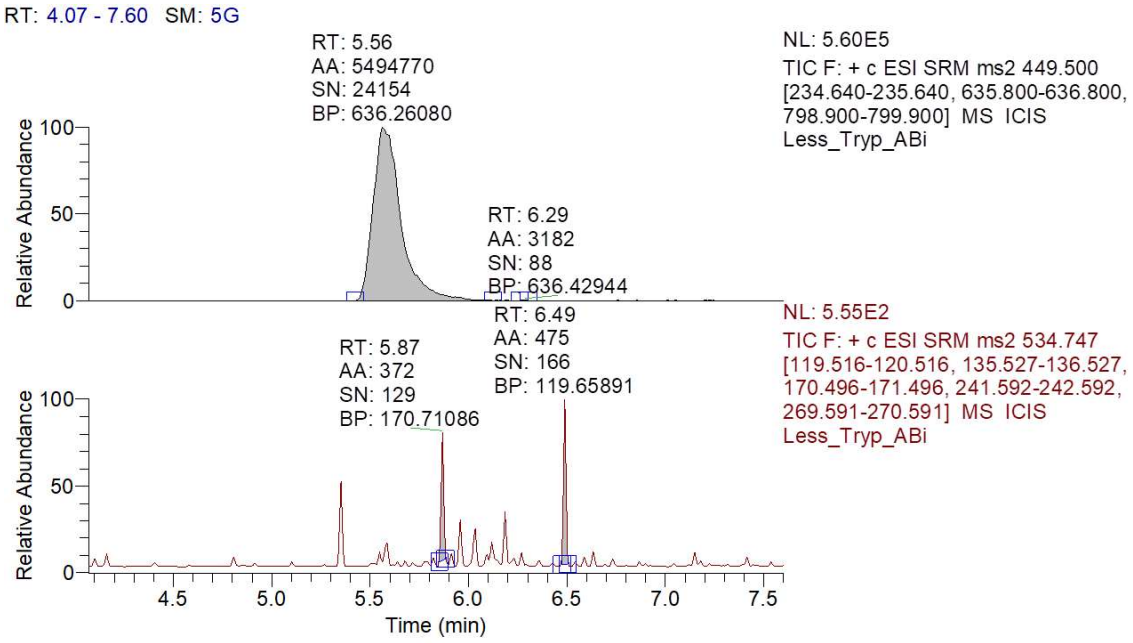
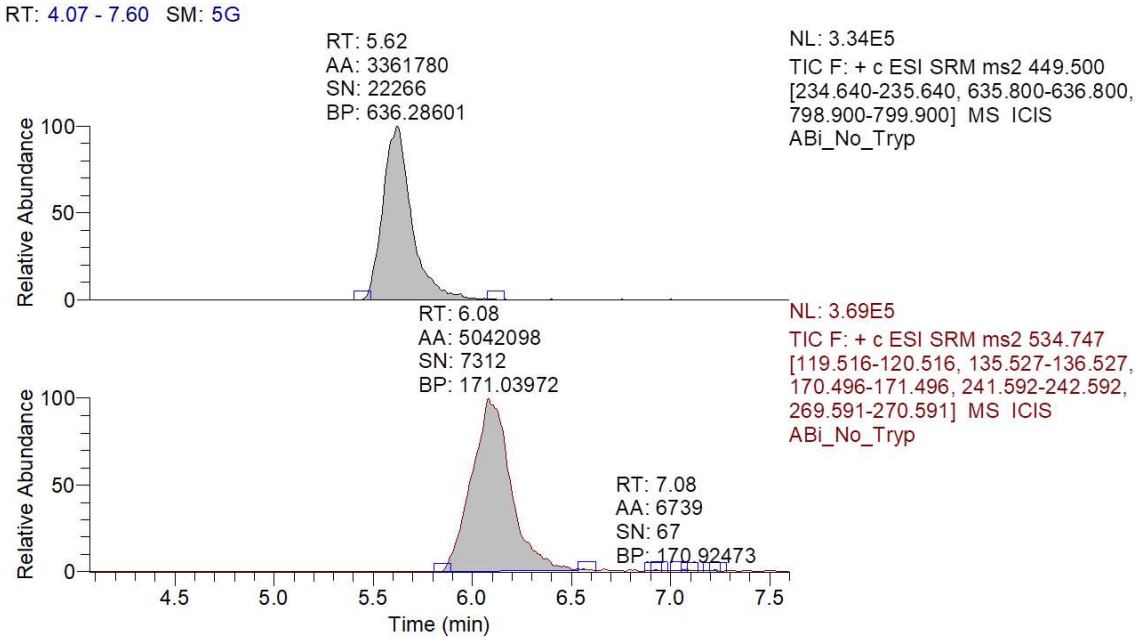


Figure 31: Derivatized Reaction Solution with Ammonium Bicarbonate with 0.1% OGP and 0.1% TCEP ("ABi") After HLB



However, moving forward with the first version of the method validation experiment—as described in **Section 3.6**—determined that ammonium bicarbonate dissolved in water, without OGP or TCEP, did not facilitate the derivatization of peptides as efficiently as expected. Thus, ammonium bicarbonate pH 8.9 (100mMol) ("ABi"), ammonium bicarbonate (100mMol) with 0.1% OGP and 0.1% TCEP ("ABi_OT"), and sodium tetraborate (50mMol) ("Borate") buffer were compared against each other, using the TSQ Quantum Ultra, to optimize the reaction further. During this experiment, the incubation time of the reaction solution at 55°C was also tested. Results are organized by buffer type and time (**Figures 32 – 37).** For this experiment, it was concluded that sodium tetraborate buffer was the best choice to move forward with regarding method performance and validation. The difference in incubation time resulted in negligible area counts comparatively.

Figure 32: Ammonium Bicarbonate (100mMol) ("ABi") Derivatization Solution with a Reaction Time of 10 Minutes

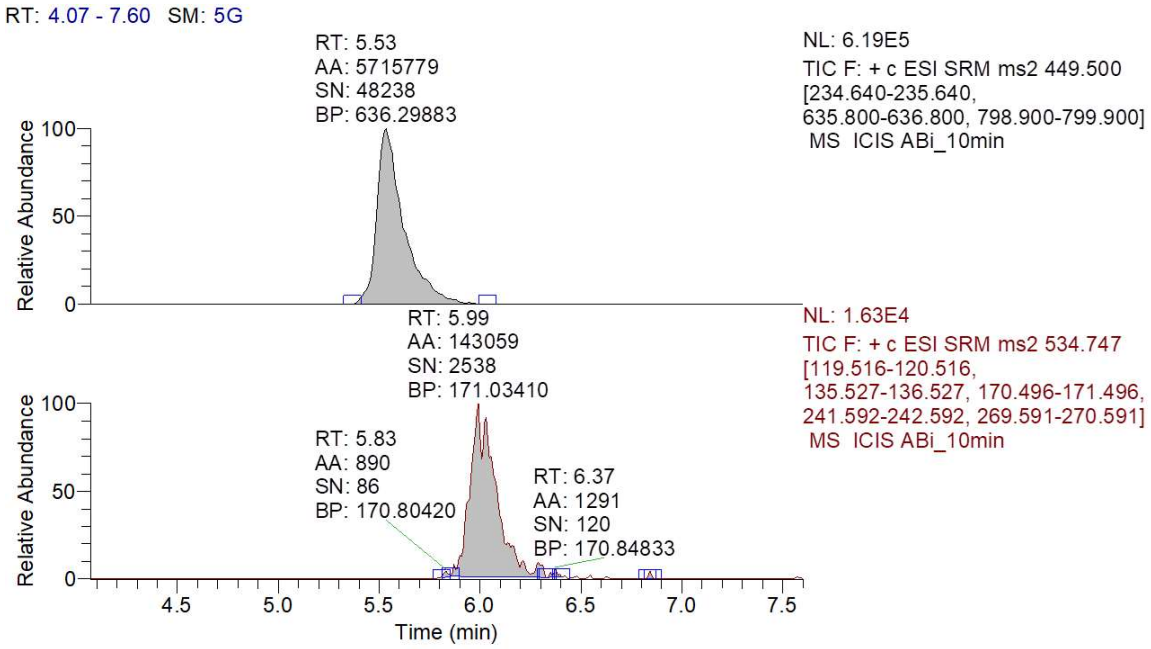


Figure 33: Ammonium Bicarbonate (100mMol) ("ABi") Derivatization Solution with a Reaction Time of 20 Minutes

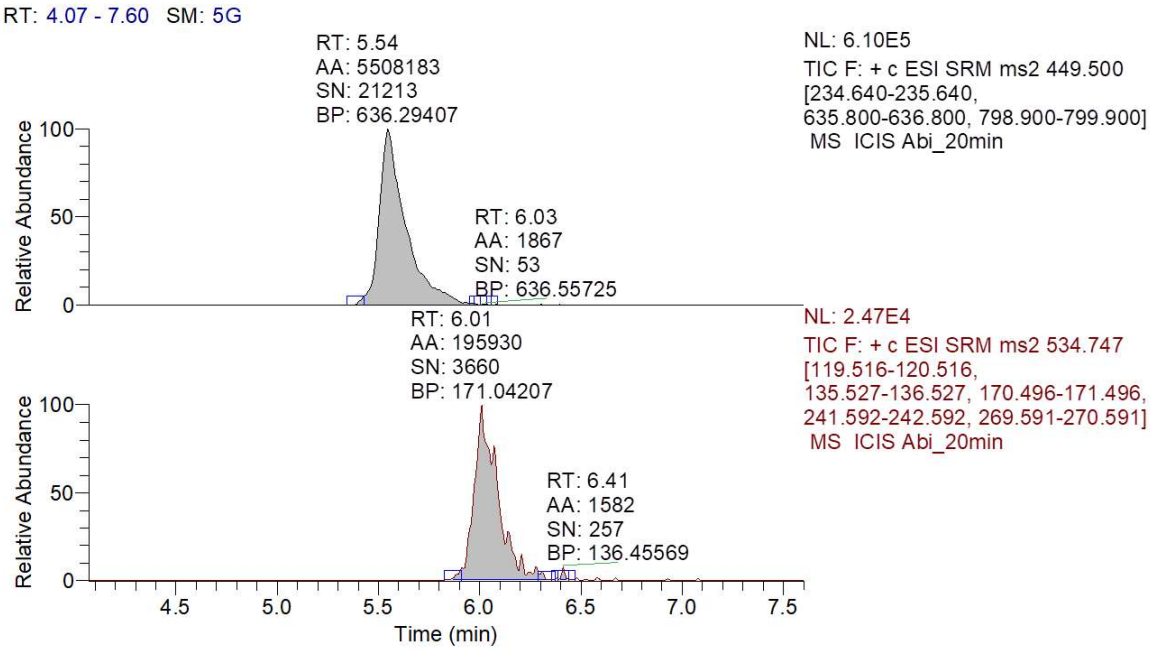


Figure 34: Ammonium Bicarbonate (100mMol) with 0.1%OGP, 0.1% TCEP ("ABi_OT") Derivatization Solution with a Reaction Time of 10 Minutes

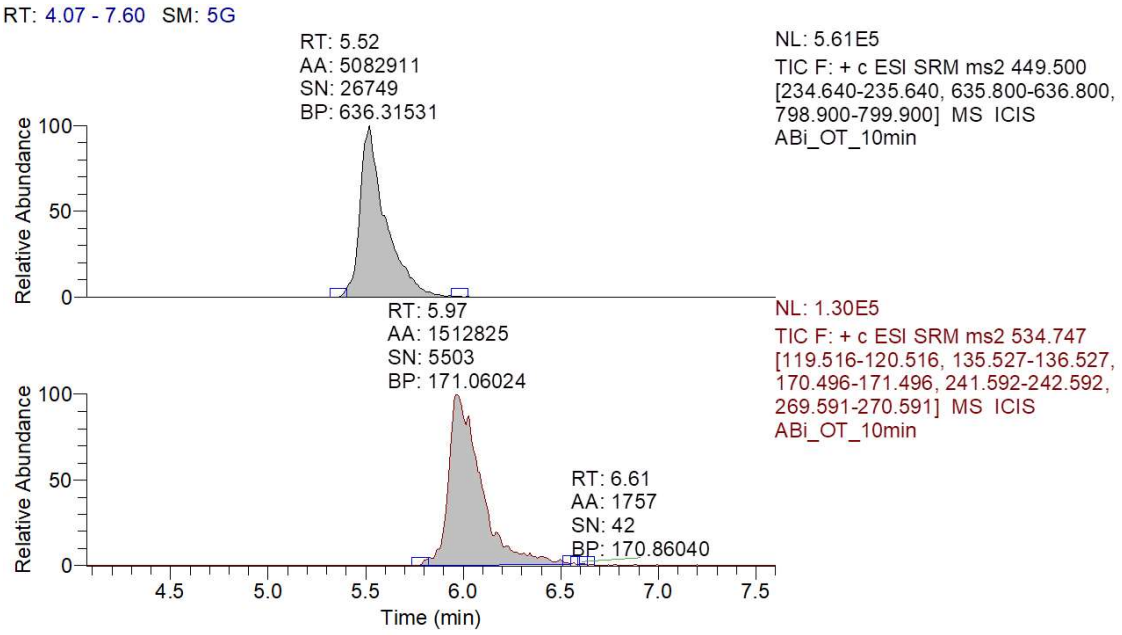


Figure 35: Ammonium Bicarbonate (100mMol) with 0.1%OGP, 0.1% TCEP ("ABi_OT") Derivatization Solution with a Reaction Time of 20 Minutes

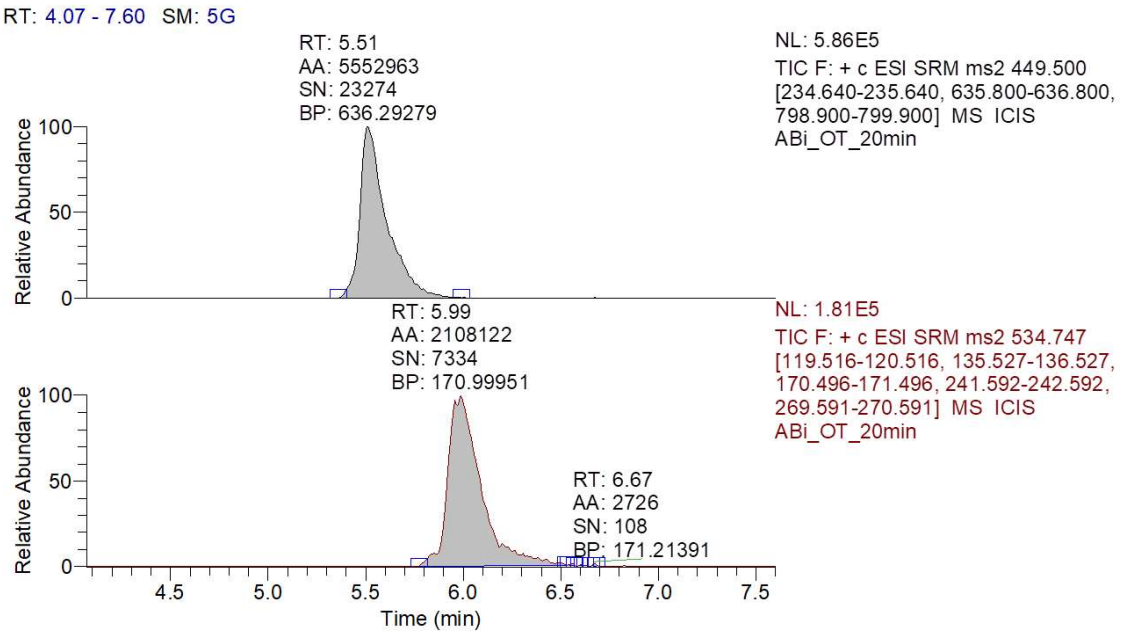


Figure 36: Sodium Tetraborate ("Borax") Derivatization Solution with a Reaction Time of 10 Minutes

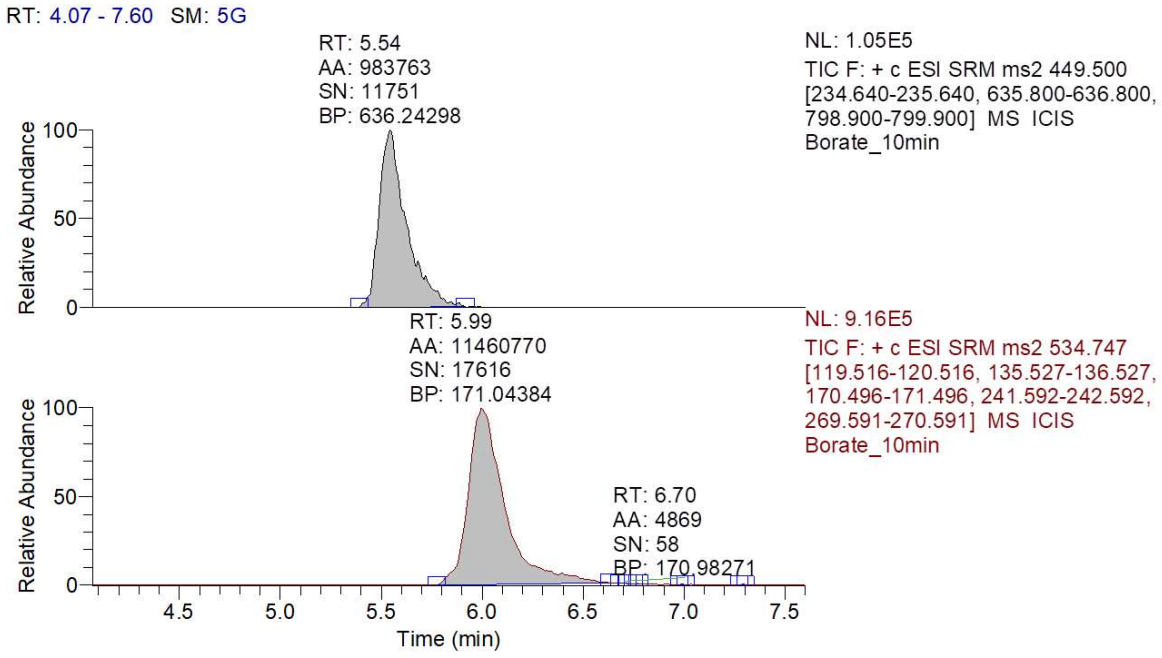
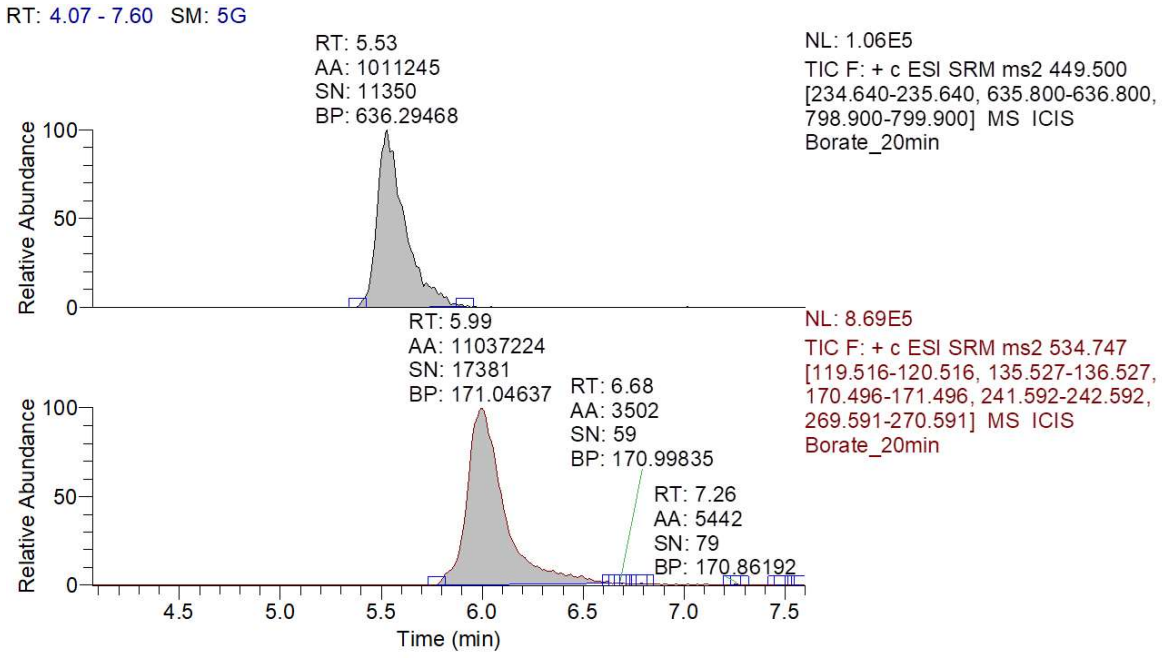


Figure 37: Sodium Tetraborate ("Borax") Derivatization Solution with a Reaction Time of 20 Minutes



4.3 Immunopurification Performance and Evaluation

After reaction parameters were optimized as much as possible, the method was performed twice according to the in-house protocol with the addition of a series of quality control (QC) spikes at 20 ng/mL. Although the peptide concentration ranged from 0.5 – 100 ng/mL, an amount usually detectable by HRMS systems, the integrated ion areas were low, and derivatization yield was both insufficient and inconsistent. The integrated ion areas also did not increase linearly for the calibration curve of the first or second performance experiment. One experiment utilized 3kDa molecular weight cut-off filters to remove trypsin out of solution, but resulting integrated area count results were as poor as the HLB-column area counts performed during the first set of immunopurification performance experiments.

To ensure that enough data would be obtained to calculate accuracy and precision of the method, all spike levels were increased to ensure consistent integrated ion areas. However, while sample clean-up, buffer optimization, and derivatization after immunopurification without HLB were successful separately **(Figures 29, 32-37,** and **38** respectively), the integration of all optimization steps into the EACL's confirmatory procedure for rhEPO and DPO was not successful enough to obtain validation results. The final method performance experiment yielded inconsistent and inconclusive results as shown below in **Figures 39 – 46.** As spiked internal standard was not detected, extracted ion chromatograms underivatized and derivatized rhEPO peptides T6 and T17 are shown. Underivatized peptides and associated ions, as described in **Table 2,** are shown on the left of extracted ion chromatograms, and derivatized peptides and associated ions on the right. One quality control example is provided as the representative QC to show that the method was unsuccessful. A standard for derivatized T17 peptide is also provided.

Figure 38: Successful Performance of Derivatization Following In-House Immunopurification Procedure

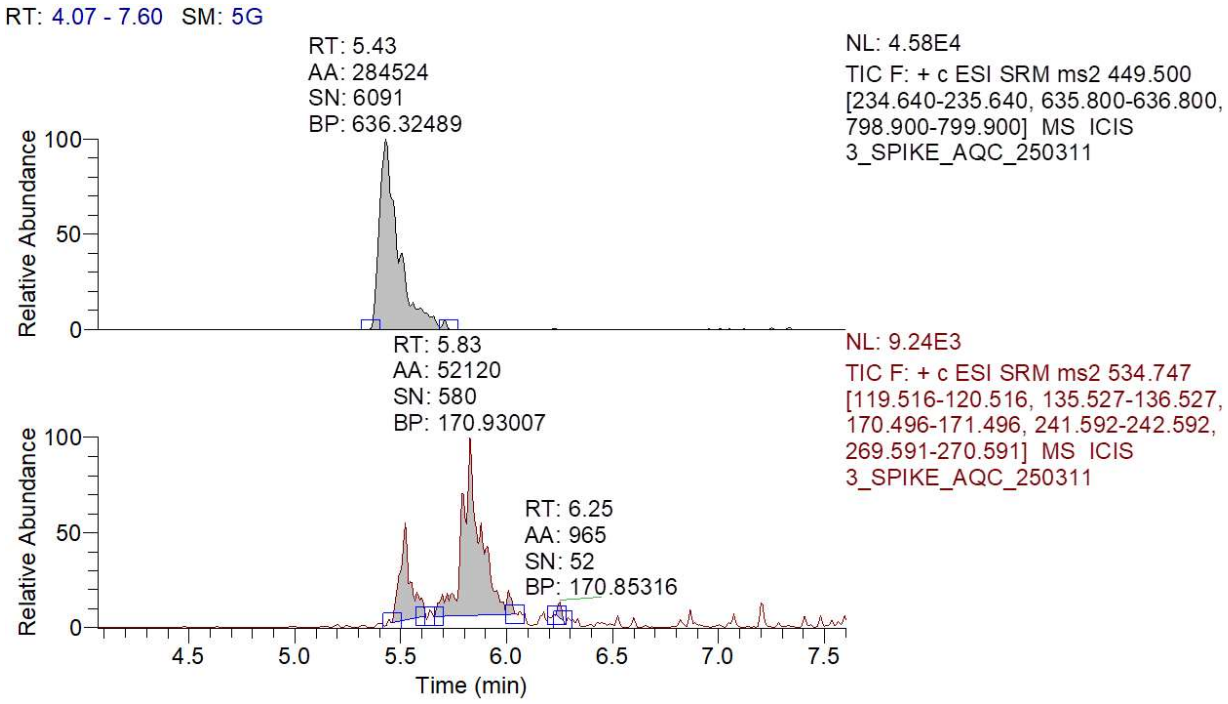


Figure 39: Negative Control Extracted Ion Chromatogram for rhEPO Peptide T6

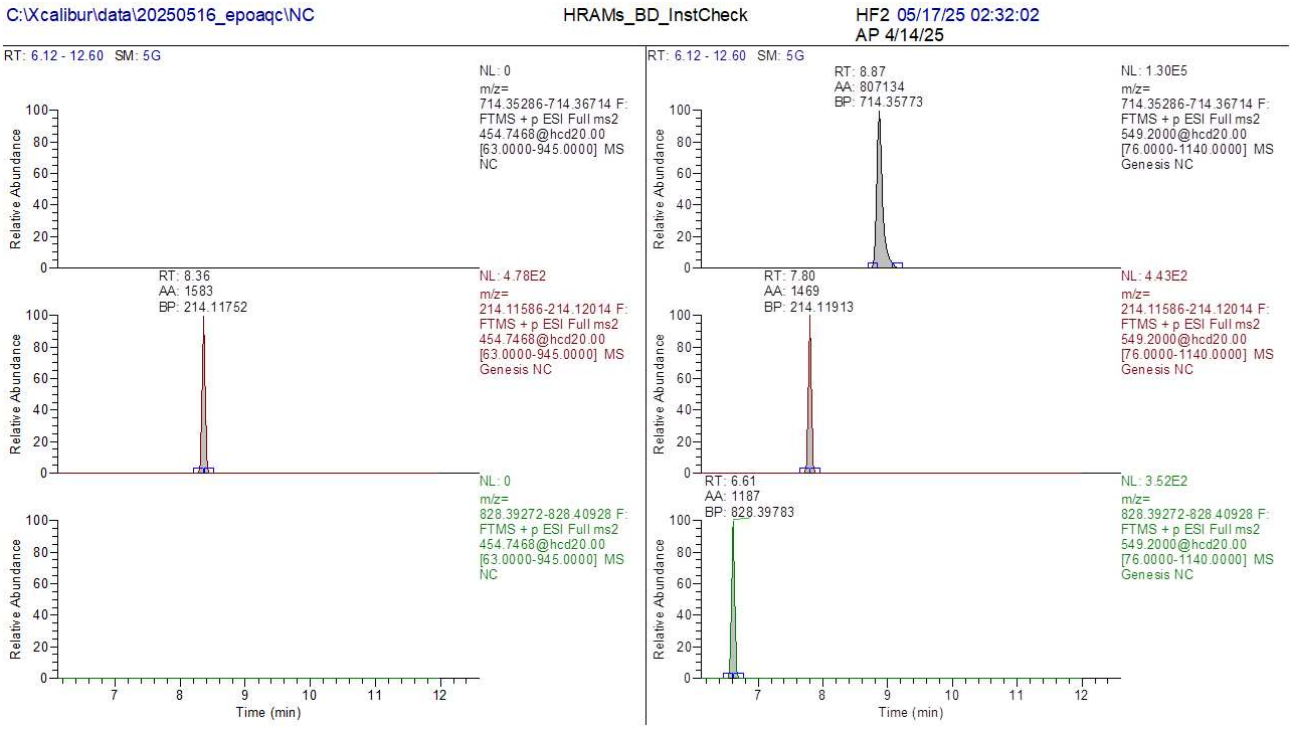


Figure 40: Positive Control Extracted Ion Chromatogram for rhEPO Peptide T6

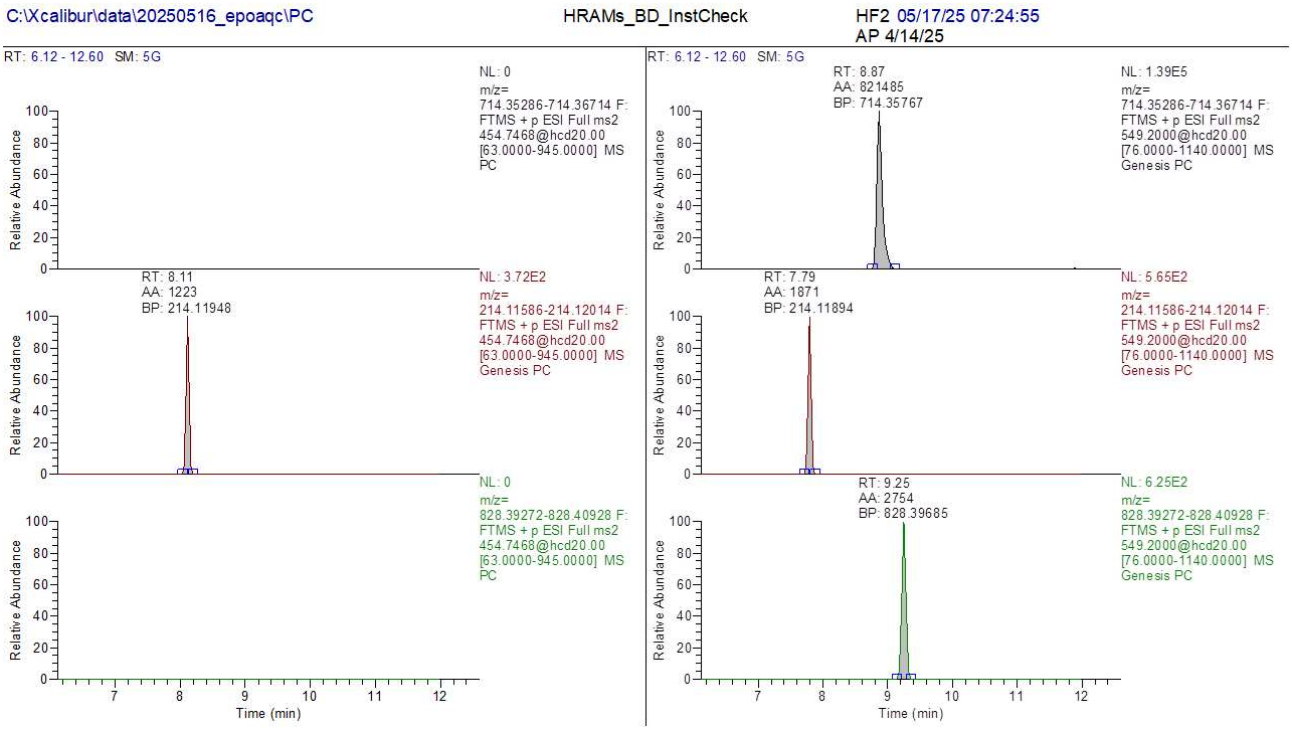


Figure 41: 1000ng Spike Extracted Ion Chromatogram for rhEPO Peptide T6

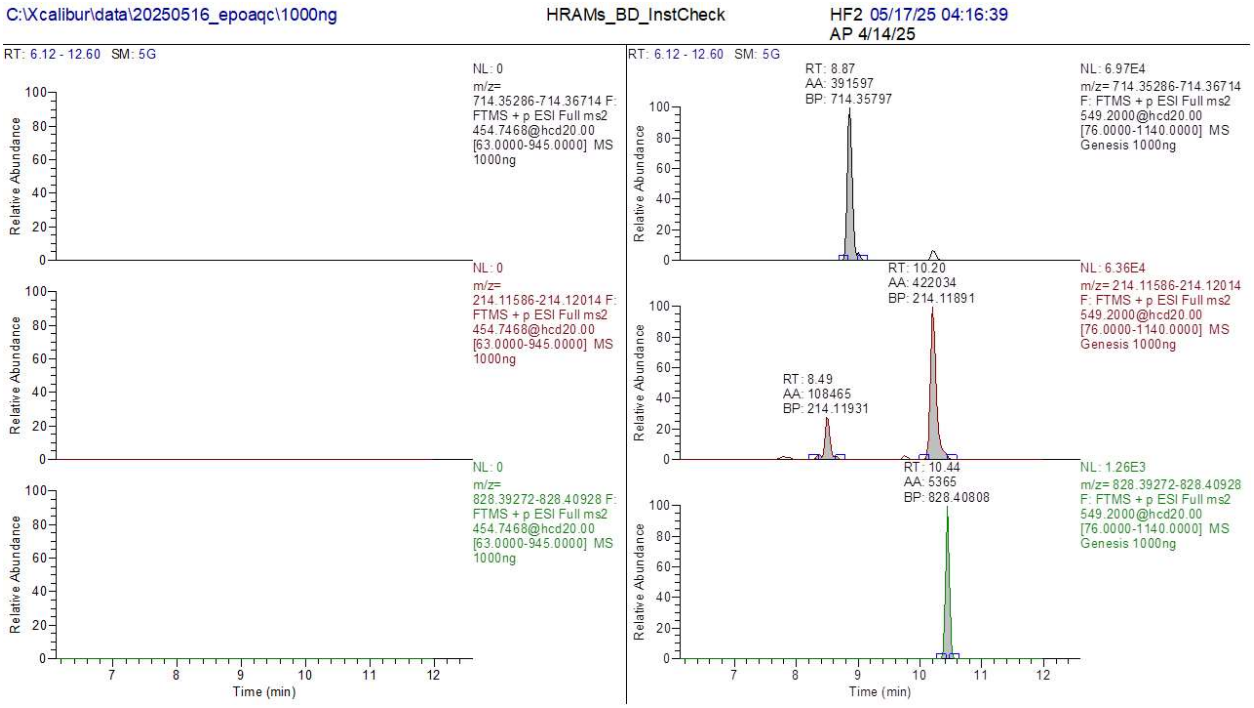


Figure 42: Representative Quality Control Extracted Ion Chromatogram for rhEPO Peptide T6

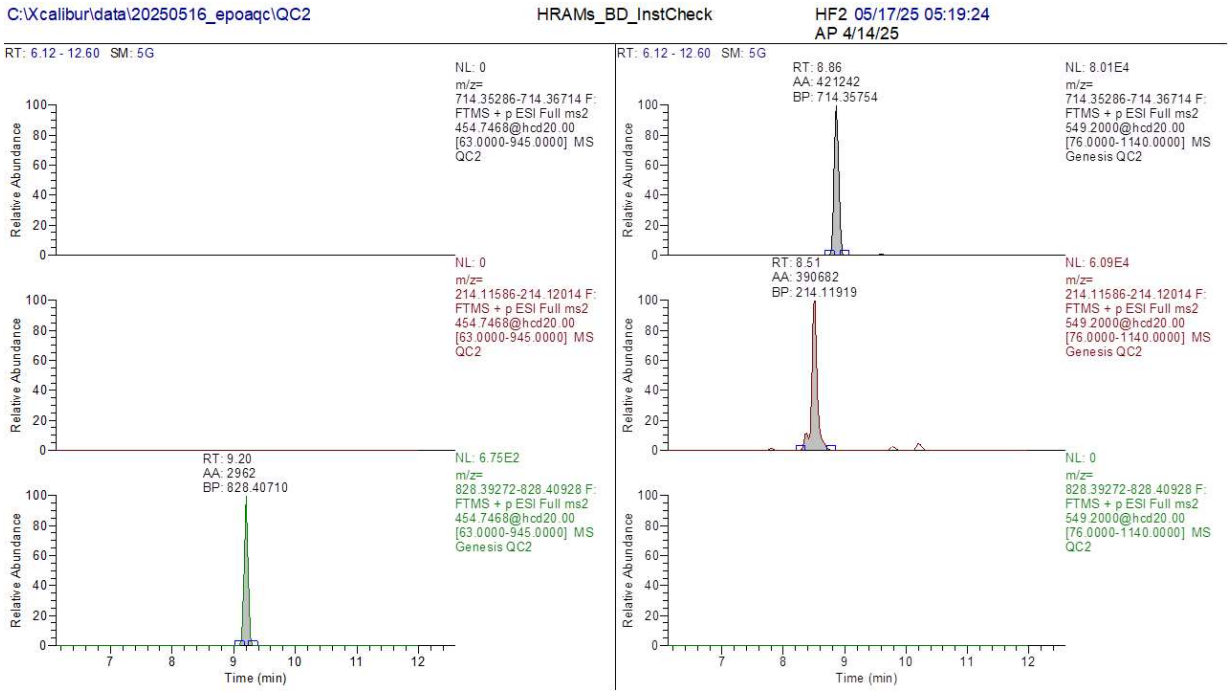


Figure 43: Negative Control Extracted Ion Chromatogram for rhEPO Peptide T17

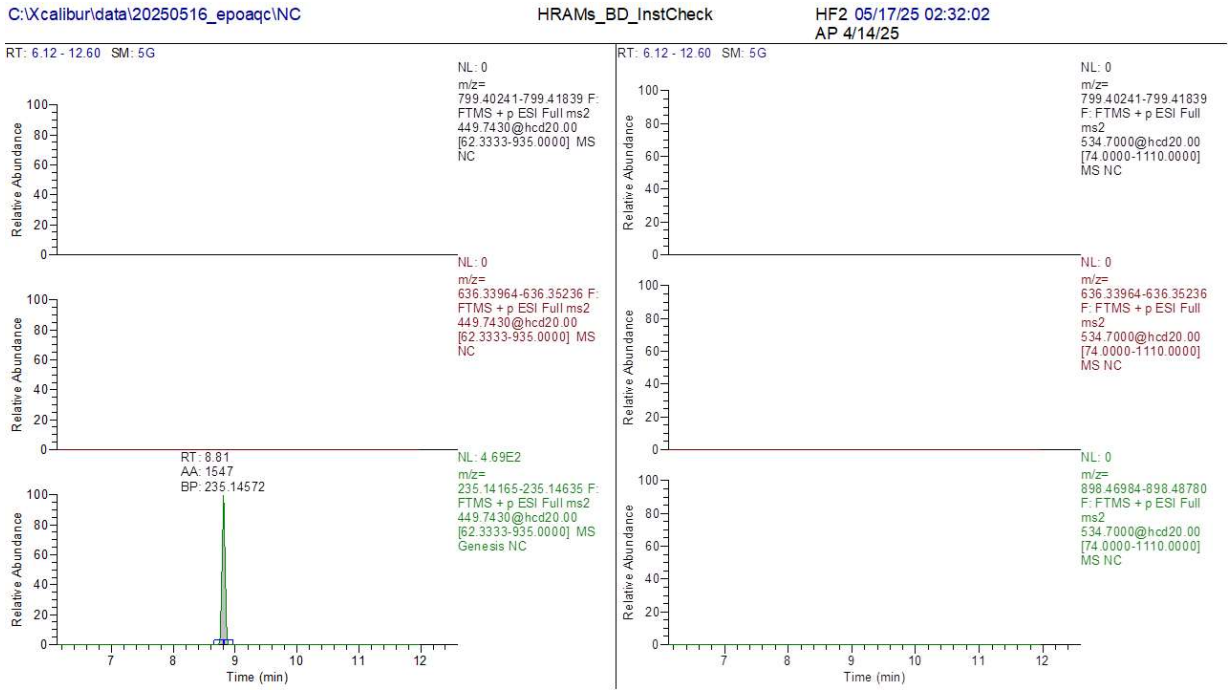


Figure 44: Positive Control Extracted Ion Chromatogram for rhEPO Peptide T17

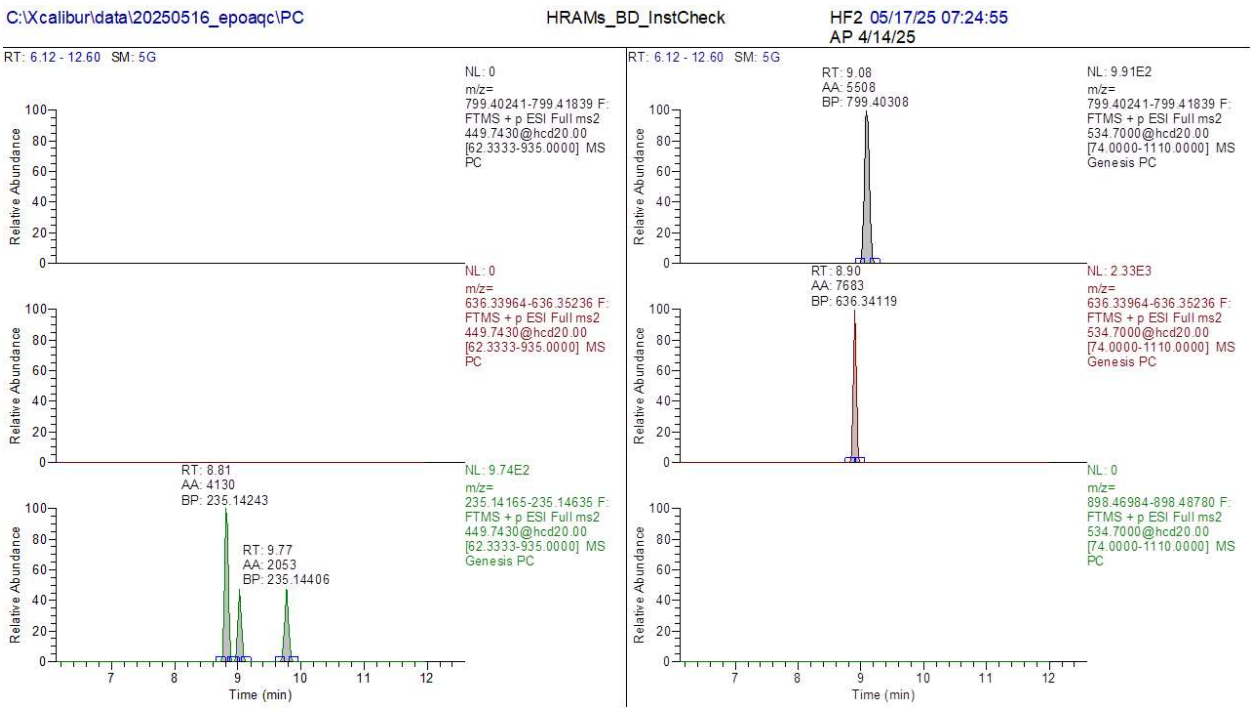


Figure 44: 1000ng Spike Extracted Ion Chromatogram for rhEPO Peptide T17

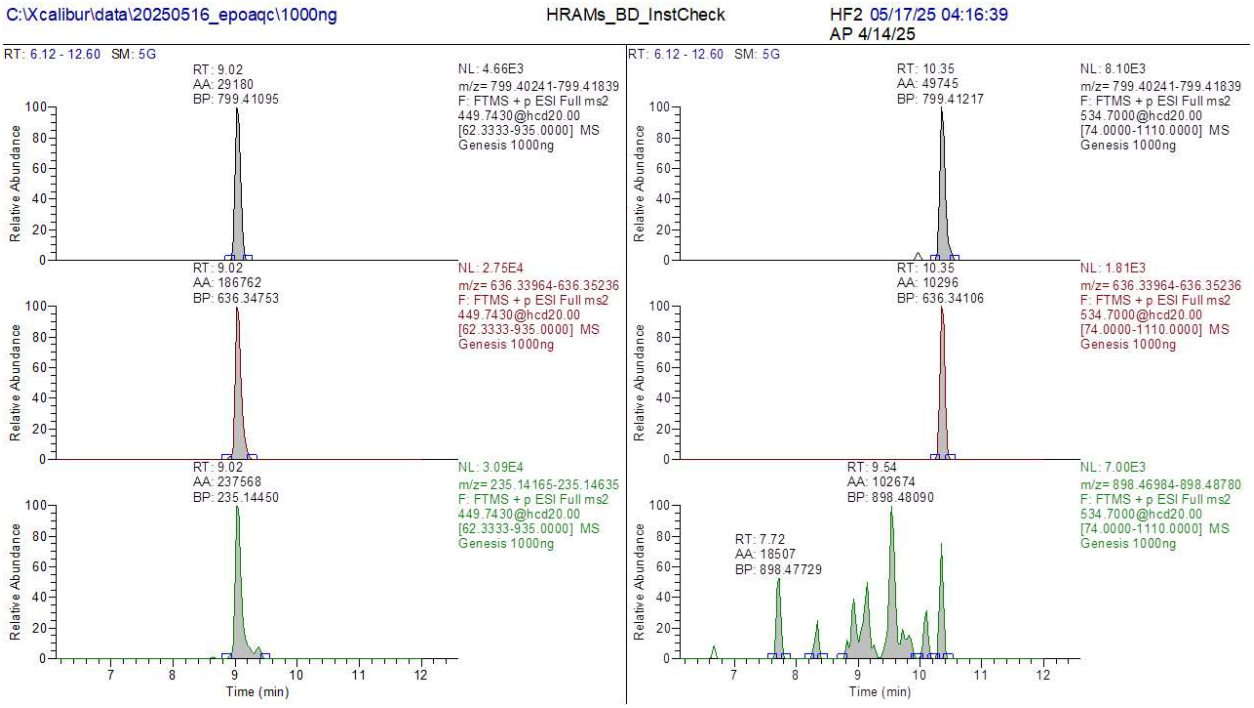


Figure 45: Representative Quality Control Extracted Ion Chromatogram for rhEPO Peptide T17

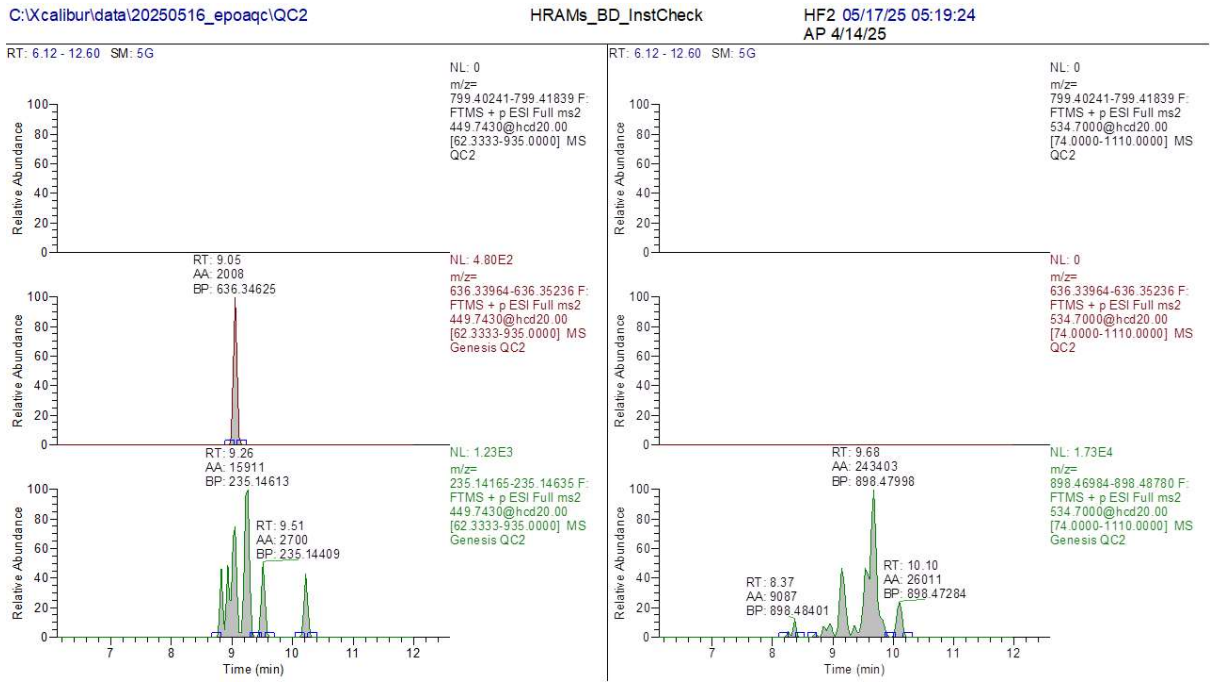
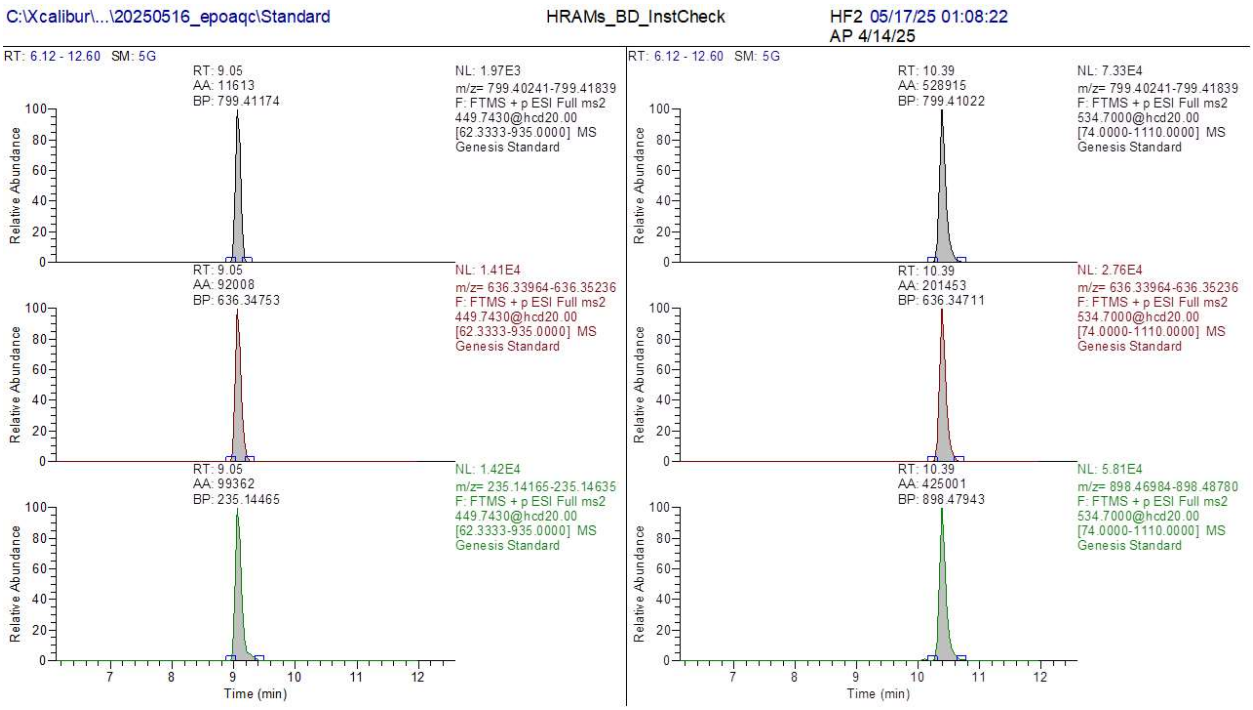


Figure 46: Extracted Ion Chromatogram for Derivatized rhEPO Peptide T17 Standard



An expected multiply charged species associated with derivatized rhEPO peptide T6 was set to be detected at 549 m/z, but a resulting extracted ion chromatogram lacked the qualifying ions associated with the peptide: 714 m/z, 214 m/z, and 828 m/z. Additionally, the ubiquitous presence of "T6" indicated that the expected ion associated with a doubly-charged, derivatized species was not correct. This is shown best in **Figure 39 – 42.** For rhEPO peptide T17, ion integration areas also reported inconsistencies in presence, poor peak integration, and poor peak separation at bases.

5. Discussion

5.1 Derivatizing Reagents

Detection of rhEPO in a horseracing sample would result in considerable sanctions for the responsible persons, including large fines, extensive suspensions, and/or lifetime bans from racing. As the presence of the tryptic peptides VNFYAWK and VYSNFLR are used to confirm

the presence of rhEPO, the data generated from these analyses must meet strict identification criteria as defined by the Association of Official Racing Chemistry guidelines for identification using mass spectrometry: at least three qualifying ions and with a ratio of 20% relative and 5% absolute to a known standard, as well as a matching retention time within ±2% or ±12 seconds to the reference sample[40]. As such, a method for the derivatization of such peptides should be sensitive, reproducible, and include such qualifications. The resulting MS2 spectra should provide sufficient product ions to meet the qualitative identification criteria set forth by the AORC.

Experiments were tested in either duplicate—if sodium bicarbonate buffer was not replaced by ammonium bicarbonate or ammonium acetate—or in triplicate. For each experiment, standards were made fresh, in their respective solvents, in case the reagent did not fully make it into solution during initial preparation. All derivatization approaches used the T17 tryptic peptide from rhEPO as the peptide to be derivatized. Reagents were picked based on the existing EACL protocol for their ability to derivatize either peptides, glycans, or if they were able to resolve and quantitate analytes at low concentrations.

DNFP-L-V produced results that were structurally similar to those of polymers (**Figure 1 and 2**). This indicates that the peptide broke apart at equidistant or near equidistant points, or the byproducts from the derivatizing reagent dominated the spectra. However, the exact cause was unknown and appeared even when the sodium bicarbonate buffer was substituted, separately and with a remade DNFP-L-V standard, for ammonium bicarbonate or ammonium acetate—all of which were generally mild conditions.

Much of the existing protocol for Dansyl Chloride derivatizes ethinylestradiol[9], or is generally carried out at elevated temperatures and basic pHs[41]. Unlike ethinylestradiol, rhEPO

peptide T17 is more susceptible to hydrolysis in basic conditions, which is then accelerated at higher temperatures. As the signal intensity of the peptide (at a mass of 449 m/z and 898 m/z) was low during infusion (**Figure 4),** it may be safe to hypothesize that the rhEPO peptide was hydrolyzed in some manner, or that byproduct formation otherwise dominated the spectra.

Girard's Reagent T has been reported to derivatize oligosaccharides to increase detection sensitivity[10] and the C-terminus of peptides to improve electron transfer dissociation efficiencies[42]. However, even with a seventeen-fold excess of reagent to peptide, expected ions of a multiply-charged species were not observed with experiments involving Girard's Reagent T (**Figures 5 and 6**). It is possible that the addition of Girard's Reagent T introduced a stable charge—as it has a positively charged nitrogen attached to three methyl groups—and that the entire complex had a charge state outside of calculated expectancies, which would lead to a mass-to-charge ratio outside the scan range utilized in the mass acquisition. It is also possible that the reagent simply did not react with the peptide in any appreciable way, despite a feasible derivatization of the C-terminus as demonstrated by Ko and Brodbelt (2012)[42], and that it was overshadowed by byproduct ions with higher ion abundances on the resulting spectrum.

As reported in **Figures 7 – 10**, both spectra for TPP and TMP did not have observable ions consistent with expect products. TPP and TMP are reported in the literature to attach a stable positive charge to peptides[12,43], primary amines[44], and poly(carboxylic acid)s[45]. While TMP did increase sequence coverage in one study[12], it tethered its charge to lysine (K) side chains—which only one of the two diagnostic peptides has, so it was unsurprising in hindsight that it did not derivatize rhEPO peptide T17. While TPP should theoretically react with peptides, it introduces the addition of three phenyl groups attached to a pyridine molecule. This may have presented too much steric hindrance, especially with an already crowded N-terminus of both

peptides, and, thus, not been able to derivatize the molecule. Indeed, literature reports that pyridinium salts exhibit lower reactivity towards α-amino groups—e.g. the N-terminus of T6 and T17—and favor ε-lysine groups and dicarboxylic acids[45,46]. In future endeavors, the use of the T6 peptide may produce favorable results as the peptide's structure includes a lysine able to be derivatized, but it is important to consider the usefulness of the reagent for both diagnostic peptides.

Generally, the N-hydroxysuccinimide esters presented the highest potential for successful derivatization during the reagent selection process. N-Hydroxysuccinimide esters are able to react in aqueous media at ambient or near ambient temperatures and can selectively derivatize the N-terminus. They are the most useful in bio-organic analysis, and, thus, derivatization for ionization enhancement for peptides[33]. Similar to the polymeric spectra of DNFP-L-V, in initial conditions of 10% acetonitrile and 0.1% TFA, the spectra of TMPP-Ac-Osu also reported the same results. The strongly acidic conditions (pH 1 – 2) were likely to have hydrolyzed the peptide before reaction solution was adjusted to a basic pH. As discussed in previous studies[36], the hydrolyzed versions of TMPP-Ac-OSu, as well as its byproducts, dominated the spectra, even if slight amounts of peptide were derivatized. This may have also happened with others, excluding AQC. It was initially hypothesized that, for TMPP-Ac-Osu, that the presence of a polymeric spectra was the product of polymer leech from polypropylene tubes due to the acidic reaction solution, but repeating the experiment in borosilicate culture tubes reported the same spectra. In latter experiments, TMPP was reacted in the same manner as AQC; a major product ion at 589 m/z was theorized to be the hydrolyzed byproduct of the reagent.

Interestingly, the rest of the succinimide esters (HSPyU, TSTU, and CY3-NHS) did not provide expected results using similar reaction conditions as AQC. There is a possibility that the

peptide was derivatized by the succinimide esters, but the result was singly charged and thus the mass-to-charge number was outside the scan window utilized in the mass acquisition. Lastly, since AQC successfully derivatized the peptide at a working solution concentration of 3 mg/mL, it is possible that the other succinimide esters were not in sufficient molar excess to yield expected results. Future work should evaluate whether higher concentrations of succinimide esters in solution can successfully add a positive charge to T17 and T6.

The use of derivatization reactions is commonly applied in the field of quantitative proteomics with the popularization of tandem mass tags utilizing isotopomer labels to permit evaluation of relative abundances of peptides from multiple samples within the same LC-MS acquisition[47]. Many of the TMT tags utilize an amine reactive N-succinimide ester moiety that allows for the covalent binding of the tag to the targeted peptides at available amines. TMT label reagents are useful for the simultaneous analysis of analytes in a single run on MS systems. They generate a reporter ion with a unique mass as they isobarically label samples, and signals can be compared against each other to assess the amount of analyte quantified. Depending on the multiplex reagent set, up to thirty-two samples can be analyzed at the same time. In the context of racing laboratories, one sample takes approximately twenty minutes to run for analysis. Depending on the number of samples that need to be run, this can delay reporting times to appropriate enforcing agencies. One study has also reported that the use of TMT-labeled samples achieved comprehensive quantitative coverage for lower abundance proteins[48]. Clearly, this could improve detection in samples associated with "micro-dosing," where low-level analytes in equine serum would be otherwise undetectable by current mass spectrometry methods. However, the spectra for the derivatized peptide did not yield any outstanding ions that may have reported the success of the reaction (**data not shown).** It is possible that the clean-up, evaporation, and

reconstitution steps resulted in enough loss so that the derivatized product was unable to be quantified during infusion.

5.2 AQC Optimization

The relative solubility of AQC in acetonitrile appeared to be 3 mg/mL, as vortexing constantly for ten minutes yielded a solution with barely-visible particulates, and an hour of using the sonication setting in a Branson Bransonic Ultrasonic Cleaner (Emerson Electric, St. Louis, MO, USA) produced the same results. At the very least, the experiments using different concentrations of AQC proved that AQC prepared at 3 mg/mL resulted in the highest production of derivatized product peptide (**Figure 23),** so the very slight visibility of particulates in solution appeared not to be of concern. During the method performance process, 1.5 mg of AQC was aliquoted into a 4 mL amber vial (Thermo Fisher Scientific, St. Louis, MO, USA) and dissolved with 500 µL of acetonitrile before derivatization. This is because any instance of water in solution will be rapidly scavenged by AQC, which then produces 6-aminoquinoline (AMQ), which will thus react with another AQC molecule to form a slowly-precipitating di-aminoquinolyl urea in acetonitrile[49]. AMQ does not interfere with chromatographic analysis, but injecting precipitate in solution can contaminate the HPLC column potentially increasing back-pressure and shift retention times.

As AQC reacts with primary and secondary amines as reported by multiple studies[13,50] to form a derivative with a quinoline ring attached to free amino groups (e.g. the ε-group of lysine and the α-amino group of the N-terminus amino acid) by a linking group, it was hypothesized that trypsin inhibited the conversion of rhEPO peptide T17 to its derivatized counterpart. Indeed, the conversion of peptide to derivatized peptide appeared lower in experiments with mock-digest conditions cleaned by HLB and in conditions with lowered amounts of trypsin (1 mL) as seen in

Figures 28 – 31. Thus, the use of HLB columns was utilized to remove the excess protein from the trypsin digestion while attempting to isolate and concentrate the targeted peptides. However, further optimization was needed, as the derivatization yield was approximately half of the peptide in solution as shown in **Figures 29 and 31.**

Optimizing the right buffer was particularly challenging as ease of use, correct pH, and the use of a volatile buffer, which is more amenable to ESI-MS following LC systems than non-volatile buffers, needed to be balanced. Cohen (2005) describes the pH range of the coupling of the quinoline tag of AQC to amino acids and other amines as 8.0 – 10.5[49]. Ammonium acetate was an initial choice as it was based off the EACL procedure for the derivatization of fluoxetine in horse urine samples with AQC to allow for additional product ions for qualitative identification (personal communication – B. Moeller). Ammonium bicarbonate with 0.1% OGP, 0.1% TCEP was chosen as it was a part of the resulting digestion solution from the EACL's EPO confirmation method. Ammonium acetate was chosen as it is volatile and amenable to LC-MS/MS systems, and sodium tetraborate had been used by multiple AQC-derivatization studies[13,49,50]. Initial theories suspected that the amines in the reaction buffers would hinder the derivatization process, but compounding factors invalidated this particular hypothesis. It was also hypothesized that OGP and TCEP interfered with the derivatization of peptide in earlier experiments, but salts and detergents have been reported as inconsequential to the labeling reaction[51]. Sodium tetraborate proves to be an optimal reaction buffer as it has reported results with lower background signal from buffer components and good reaction yields[49]. Since this study's experiments optimizing buffer conditions resulted in sodium tetraborate performing the best (**Figures 32-37)**, the final method performance tests utilized it as both the reconstitution and reaction solution.

It has been noted that the amino acids Asp, Glu, Val, and Ala were found to be slow-reacting amino acids with AQC[49]. This may be due to their relatively unreactive side chains as compared to lysine or other amine containing amino acids, and may have contributed to the poor derivatization yield in some manner as the potential derivatization site was on the N-terminus of peptides T6 and T17's valine. The reaction time of twenty minutes was consistent throughout the experiment as literature-based protocols never exceeded this time, but future endeavors may consider optimizing the reaction time. Generally, the turn-over of peptide to derivatized peptide fluctuated throughout the optimization and method confirmation process, although results were never better than approximately 20% of the underivatized peptide left in solution. Low or fluctuating derivatization yield may have been due to a number of factors, including incorrect buffer pH, temperature, or incubation time. The exact cause is unknown, as the protocol used attempted to replicate established literature and followed the addition of sodium tetraborate buffer to AQC dissolved in acetonitrile[13]. Theoretically, as long as the pH of the reaction solution stayed between 8.0 – 10.5, the labeling reaction proceeds, and all buffers were adjusted to this pH range if necessary. The temperature was kept consistent at 55°C for twenty minutes throughout the method development process.

5.3 Immunopurification Performance and Validation

It is unknown why the results of the immunopurification performance experiments varied so significantly. One possible explanation is that the samples were exposed to the MAIIA kit exposure aid and serum buffer for too long, which could have affected the peptides in some manner. This was rectified in the final validation experiment in which the first set of samples was purified according to the EACL's protocol, and exposure aid was only added to the second

set of samples just before they were purified. Otherwise, sample spikes were performed carefully and in the same manner with each method performance.

The lowest point on the calibration curve, 0.5 ng/mL, was too low to be spiked for the first method performance experiment, and so it was increased to 5 ng/mL. For such a low amount, it is likely that analytes were lost during the sample clean-up, drying, or reconstitution process, and were as such undetectable. It is additionally likely that the internal standard was spiked too low; the lowest point on the calibration curve for the final validation experiment was 50 ng/mL, and the internal standard was 10 ng/mL. Although the EACL's confirmatory method requires a final concentration of internal standard at 1 ng/mL, the process also does not require a sample clean-up step, and, as such, tryptic peptides are not lost to the same degree.

6. Conclusion

Out of a total of eleven different reagents were evaluated, 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatized recombinant human EPO peptides T6 and T17 following tryptic digestion. The method was developed on a TSQ Quantum Ultra LC-MS/MS and then evaluated on a Q-Exactive HF LC-MS/MS system. To assess the method performance, equine serum samples without any detectable rhEPO were spiked with an rhEPO standard solution at various concentrations. The samples were purified and rhEPO isolated using immunopurification, followed by tryptic digestion, solid phase extraction cleanup using Hydrophilic-Lipophilic Balance cartridges, and derivatization in a sodium tetraborate buffer with AQC and analysis using LC-HRMS. Accuracy and precision of the method were not able to be calculated as integrated ion area counts were not present for multiply charged species and associated qualifying ions expected from derivatized peptides, especially rhEPO peptide T6.

Although derivatization did not yield complete turn-over and validation was unsuccessful, the presence of additional qualifying ions may be useful in qualitative analyses.

7. References

(1) Elliott, S.; Pham, E.; Macdougall, I. C. Erythropoietins: A Common Mechanism of Action. *Exp. Hematol.* **2008**, *36* (12), 1573–1584. https://doi.org/10.1016/j.exphem.2008.08.003.

(2) Jaussaud, P.; Audran, M.; Gareau, R.; Souillard, A.; Chavanet, I. Kinetics and Haematological Effects of Erythropoietin in Horses. *Vet. Res.* **1994**, *25* (6), 568–573.

(3) Haase, V. H. Regulation of Erythropoiesis by Hypoxia-Inducible Factors. *Blood Rev.* **2013**, *27* (1), 41–53. https://doi.org/10.1016/j.blre.2012.12.003.

(4) Ekblom, B.; Berglund, B. Effect of Erythropoietin Administration on Mammal Aerobic Power. *Scand. J. Med. Sci. Sports* **1991**, *1* (2), 88–93. https://doi.org/10.1111/j.1600-0838.1991.tb00276.x.

(5) Uniform Classification Guidelines for Foreign Substances and Recommended Penalties and Model Rule, 2023. https://www.arci.com/docs/Uniform-Classification-Guidelines-Version-17.0.pdf.

(6) International Agreement on Breeding, Racing and Wagering, 2024. https://www.ifhaonline.org/default.asp?section=IABRW&area=2.

(7) Prohibited List (Rule Series 4000), 2023. https://bphisaweb.wpengine.com/wp-content/uploads/2024/06/4000-Series-1.26.2023.pdf.

(8) Loganathan, D.; Yi, R.; Patel, B.; Zhang, J.; Kong, N. A Sensitive HPLC-MS/MS Method for the Detection, Resolution and Quantitation of Cathinone Enantiomers in Horse Blood Plasma and Urine. *Anal. Bioanal. Chem.* **2021**, *413* (8), 2147–2161. https://doi.org/10.1007/s00216-021-03182-1.

(9) Anari, M. R.; Bakhtiar, R.; Zhu, B.; Huskey, S.; Franklin, R. B.; Evans, D. C. Derivatization of Ethinylestradiol with Dansyl Chloride To Enhance Electrospray Ionization: Application in Trace Analysis of Ethinylestradiol in Rhesus Monkey Plasma. *Anal. Chem.* **2002**, *74* (16), 4136–4144. https://doi.org/10.1021/ac025712h.

(10) Naven, T. J. P.; Harvey, D. J. Cationic Derivatization of Oligosaccharides with Girard's T Reagent for Improved Performance in Matrix-Assisted Laser Desorption/Ionization and Electrospray Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **1996**, *10* (7), 829–834. https://doi.org/10.1002/(SICI)1097-0231(199605)10:7<829::AID-RCM572>3.0.CO;2-Y.

(11) Siwińska, N.; Pasławska, U.; Bąchor, R.; Szczepankiewicz, B.; Żak, A.; Grocholska, P.; Szewczuk, Z. Evaluation of Podocin in Urine in Horses Using Qualitative and Quantitative Methods. *PLoS One.* **2020**, *15* (10). https://doi.org/10.1371/journal.pone.0240586.

(12) Polasky, D. A.; Lermyte, F.; Nshanian, M.; Sobott, F.; Andrews, P. C.; Loo, J. A.; Ruotolo, B. T. Fixed-Charge Trimethyl Pyrilium Modification for Enabling Enhanced Top-Down Mass Spectrometry Sequencing of Intact Protein Complexes. *Anal. Chem.* **2018**, *90* (4), 2756–2764. https://doi.org/10.1021/acs.analchem.7b04806.

(13) Ullmer, R.; Plematl, A.; Rizzi, A. Derivatization by 6-aminoquinolyl- *N* -hydroxysuccinimidyl Carbamate for Enhancing the Ionization Yield of Small Peptides and Glycopeptides in Matrix-assisted Laser Desorption/Ionization and Electrospray Ionization Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20* (9), 1469–1479. https://doi.org/10.1002/rcm.2464.

(14) Gallien, S.; Perrodou, E.; Carapito, C.; Deshayes, C.; Reyrat, J.-M.; Van Dorsselaer, A.; Poch, O.; Schaeffer, C.; Lecompte, O. Ortho-Proteogenomics: Multiple Proteomes Investigation through Orthology and a New MS-Based Protocol. *Genome Res.* **2009**, *19* (1), 128–135. https://doi.org/10.1101/gr.081901.108.

(15) Bailly-Chouriberry, L.; Cormant, F.; Garcia, P.; Lönnberg, M.; Szwandt, S.; Bondesson, U.; Popot, M.-A.; Bonnaire, Y. A New Analytical Method Based on Anti-EPO Monolith Column and LC-FAIMS-MS/MS for the Detection of rHuEPOs in Horse Plasma and Urine Samples. *Analyst* **2012**, *137* (10), 2445. https://doi.org/10.1039/c2an15662h.

(16) Jelkmann, W.; Lundby, C. Blood Doping and Its Detection. *Blood.* **2011**, *118* (9), 2395–2404. https://doi.org/10.1182/blood-2011-02-303271.

(17) Ekblom, B.; Goldbarg, A. N.; Gullbring, B. Response to Exercise after Blood Loss and Reinfusion. *J. Appl. Physiol.* **1972**, *33* (2), 175–180. https://doi.org/10.1152/jappl.1972.33.2.175.

(18) Buick, F. J.; Gledhill, N.; Froese, A. B.; Spriet, L.; Meyers, E. C. Effect of Induced Erythrocythemia on Aerobic Work Capacity. *J. Appl. Physiol.* **1980**, *48* (4), 636–642. https://doi.org/10.1152/jappl.1980.48.4.636.

(19) Brien, J.; Simon, L. The Effects of Red Blood Cell Infusion on 10-Km Race Time. *JAMA* **1987**, *257* (70), 2761–2765.

(20) Eschbach, J. W.; Egrie, J. C.; Downing, M. R.; Browne, J. K.; Adamson, J. W. Correction of the Anemia of End-Stage Renal Disease with Recombinant Human Erythropoietin. *N. Engl. J. Med.* **1987**, *316* (2), 73–78. https://doi.org/10.1056/NEJM198701083160203.

(21) McKeever, K. H.; Agans, J. M.; Geiser, S.; Lorimer, P. J.; Maylin, G. A. Low Dose Exogenous Erythropoietin Elicits an Ergogenic Effect in Standardbred Horses. *Equine Vet. J.* **2006**, *38* (S36), 233–238. https://doi.org/10.1111/j.2042-3306.2006.tb05545.x.

(22) Shaskey, D. J.; Green, G. A. Sports Haematology: *Sports Med.* **2000**, *29* (1), 27–38. https://doi.org/10.2165/00007256-200029010-00003.

(23) Woods, P. R.; Campbell, G.; Cowell, R. L. Nonregenerative Anaemia Associated with Administration of Recombinant Human Erythropoietin to a Thoroughbred Racehorse. *Equine Vet. J.* **1997**, *29* (4), 326–328. https://doi.org/10.1111/j.2042-3306.1997.tb03132.x.

(24) Piercy, R. J.; Swardson, C. J.; Hinchcliff, K. W. Erythroid Hypoplasia and Anemia Following Administration of Recombinant Human Erythropoietin to Two Horses. *J. Am. Vet. Med. Assoc.* **1998**, *212* (2), 244–247. https://doi.org/10.2460/javma.1998.212.02.244.

(25) Schwarzwald, C.; Hinchcliff, K. W. Recombinant Human Erythropoietin as a Cause of Non-Regenerative Anemia in a Stable of Racing Thoroughbreds. In *Sports Medicine*; Denver, CO, USA, 2004; Vol. 50, pp 270–271.

(26) Sato, F.; Yamashita, S.; Kugo, T.; Hasegawa, T.; Mitsui, I.; Kijima-Suda, I. Nucleotide Sequence of Equine Erythropoietin and Characterization of Region-Specific Antibodies. *Am. J. Vet. Res.* **2004**, *65* (1), 15–19. https://doi.org/10.2460/ajvr.2004.65.15.

(27) Scarth, J. P.; Seibert, C.; Brown, P. R.; Teale, P.; Beamon, G. J.; Pearce, C. M.; Sams, R. A. UPLC-MS/MS Method for the Identification of Recombinant Human Erythropoietin Analogues in Horse Plasma and Urine. *Chromatographia* **2011**, *74* (7–8), 593–608. https://doi.org/10.1007/s10337-011-2119-y.

(28) Lönnberg, M.; Carlsson, J. Membrane Assisted Isoform Immunoassay A Rapid Method for the Separation and Determination of Protein Isoforms in an Integrated Immunoassay. *J. Immunol. Methods.* **2000**. https://doi.org/10.1016/s0022-1759(00)00287-8.

(29) Guan, F.; Uboh, C. E.; Soma, L. R.; Birks, E.; Chen, J.; Mitchell, J.; You, Y.; Rudy, J.; Xu, F.; Li, X.; Mbuy, G. LC−MS/MS Method for Confirmation of Recombinant Human Erythropoietin and Darbepoetin α in Equine Plasma. *Anal. Chem.* **2007**, *79* (12), 4627–4635. https://doi.org/10.1021/ac070135o.

(30) Richards, S.; Palmer, D.; Cawley, A.; Wainscott, M.; Keledjian, J. Enhanced Analysis of Equine Plasma for the Presence of Recombinant Human Erythropoietin — Implementation of an Improved Workflow. *Drug Test. Analysis* **2024**, *17* (6), 786–795. https://doi.org/10.1002/dta.3785.

(31) Delcourt, V.; Garcia, P.; Chabot, B.; Caroff, M.; Bache, L.; Popot, M.-A.; Bailly-Chouriberry, L. Fast Confirmatory Analysis of rHuEPOs in Plasma for Horseracing Doping Control by Coupling Evosep One & Q-Exactive HF. *EVOSEP* **2019**.

(32) Souillard, A.; Audran, M.; Bressolle, F.; Jaussaud, P.; Gareau, R. Pharmacokinetics and Haematological Parameters of Recombinant Human Erythropoietin after Subcutaneous Administrations in Horses. *Biopharm. Drug Dispos.* **1996**, *17* (9), 805–815. https://doi.org/10.1002/(SICI)1099-081X(199612)17:9<805::AID-BDD995>3.0.CO;2-H.

(33) Zaikin, V. G.; Borisov, R. S. Options of the Main Derivatization Approaches for Analytical ESI and MALDI Mass Spectrometry. *Crit. Rev. Anal. Chem.* **2022**, *52* (6), 1287–1342. https://doi.org/10.1080/10408347.2021.1873100.

(34) Stefanowicz, P.; Kluczyk, A.; Szewczuk, Z. Derivatization of Peptides for Improved Detection by Mass Spectrometry. In *Amino Acids, Peptides And Proteins*; Ryadnov, M., Hudecz, F., Eds.; Royal Society of Chemistry: Cambridge, 2015; Vol. 40, pp 36–74. https://doi.org/10.1039/9781782622680-00036.

(35) Huang, Z.-H.; Shen, T.; Wu, J.; Gage, D. A.; Watson, J. T. Protein Sequencing by Matrix-Assisted Laser Desorption Ionization–Postsource Decay–Mass Spectrometry Analysis of

theN-Tris(2,4,6-Trimethoxyphenyl)Phosphine-Acetylated Tryptic Digests. *Anal. Biochem.* **1999**, *268* (2), 305–317. https://doi.org/10.1006/abio.1998.3085.

(36) An, M.; Zou, X.; Wang, Q.; Zhao, X.; Wu, J.; Xu, L.-M.; Shen, H.-Y.; Xiao, X.; He, D.; Ji, J. High-Confidence de Novo Peptide Sequencing Using Positive Charge Derivatization and Tandem MS Spectra Merging. *Anal. Chem.* **2013**, *85* (9), 4530–4537. https://doi.org/10.1021/ac4001699.

(37) Shen, H.; An, M.; Zou, X.; Zhao, X.; Wang, Q.; Xing, G.; Ji, J. Evaluation of the Accuracy of Protein Quantification Using Isotope TMPP-Labeled Peptides. *Proteomics.* **2015**, *15* (17), 2903–2909. https://doi.org/10.1002/pmic.201400495.

(38) Gil, G.-C.; Kim, Y.-G.; Kim, B.-G. A Relative and Absolute Quantification of Neutral N-Linked Oligosaccharides Using Modification with Carboxymethyl Trimethylammonium Hydrazide and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Anal. Biochem.* **2008**, *379* (1), 45–59. https://doi.org/10.1016/j.ab.2008.04.039.

(39) TMTpro Mass Tag Labeling Reagents and Kits User Guide (Pub.No. MAN0018773 E), 2024. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0018773_TMTproMassTagLabelingReagentsandKits_UG.pdf.

(40) AORC Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry, 2024. https://www.aorc-online.org/documents/aorc-ms-criteria-ilacg7/.

(41) Takeuchi, T. HPLC of Amino Acids as Dansyl and Dabsyl Derivatives. In *Journal of Chromatography Library*; 2005; Vol. 70, pp 229–241. https://doi.org/10.1016/S0301-4770(05)80010-7.

(42) Ko, B. J.; Brodbelt, J. S. Enhanced Electron Transfer Dissociation of Peptides Modified at C-Terminus with Fixed Charges. *J. Am. Soc. Mass Spectrom.* **2012**, *23* (11), 1991–2000. https://doi.org/10.1007/s13361-012-0458-z.

(43) Polasky, D. A.; Dixit, S. M.; Keating, M. F.; Gadkari, V. V.; Andrews, P. C.; Ruotolo, B. T. Pervasive Charge Solvation Permeates Native-like Protein Ions and Dramatically Influences Top-down Sequencing Data. *J. Am. Chem. Soc.* **2020**, *142* (14), 6750–6760. https://doi.org/10.1021/jacs.0c01076.

(44) Huang, S.; Palanisamy, S.; Yu, X.; Wang, Y.; Liu, D.; Gong, W.; Zhang, X. α-Active Pyrylium Salt 2,4,5-Triphenylpyrylium for Improved Mass Spectrometry-Based Detection of Peptides. *Anal. Chem.* **2021**, *93* (32), 11072–11080. https://doi.org/10.1021/acs.analchem.0c04809.

(45) Kawasue, S.; Sakaguchi, Y.; Koga, R.; Yoshida, H.; Nohta, H. A Pyridinium Derivatization Reagent for Highly Sensitive Detection of Poly(Carboxylic Acid)s Using Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2022**, *33* (8), 1492–1498. https://doi.org/10.1021/jasms.2c00086.

(46) Waliczek, M.; Kijewska, M.; Rudowska, M.; Setner, B.; Stefanowicz, P.; Szewczuk, Z. Peptides Labeled with Pyridinium Salts for Sensitive Detection and Sequencing by Electrospray Tandem Mass Spectrometry. *Sci. Rep.* **2016**, *6* (1), 37720. https://doi.org/10.1038/srep37720.

(47) Thompson, A.; Schäfer, J.; Kuhn, K.; Kienle, S.; Schwarz, J.; Schmidt, G.; Neumann, T.; Hamon, C. Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by MS/MS. *Anal. Chem.* **2003**, *75* (8), 1895–1904. https://doi.org/10.1021/ac0262560.

(48) O'Connell, J. D.; Paulo, J. A.; O'Brien, J. J.; Gygi, S. P. Proteome-Wide Evaluation of Two Common Protein Quantification Methods. *J. Proteome Res.* **2018**, *17* (5), 1934–1942. https://doi.org/10.1021/acs.jproteome.8b00016.

(49) Cohen, S. A. Quantitation of Amino Acids as 6-Aminoquinolyl-N-Hydroxysuccinimidyl Carbamate Derivatives. *J. Chromatogr. Libr.* **2005**, *70*, 242–267. https://doi.org/10.1016/S0301-4770(05)80011-9.

(50) Gray, N.; Zia, R.; King, A.; Patel, V. C.; Wendon, J.; McPhail, M. J. W.; Coen, M.; Plumb, R. S.; Wilson, I. D.; Nicholson, J. K. High-Speed Quantitative UPLC-MS Analysis of Multiple Amines in Human Plasma and Serum via Precolumn Derivatization with 6-Aminoquinolyl- *N* -Hydroxysuccinimidyl Carbamate: Application to Acetaminophen-Induced Liver Failure. *Anal. Chem.* **2017**, *89* (4), 2478–2487. https://doi.org/10.1021/acs.analchem.6b04623.

(51) Stöckmann, H.; Duke, R. M.; Millán Martín, S.; Rudd, P. M. Ultrahigh Throughput, Ultrafiltration-Based *N* -Glycomics Platform for Ultraperformance Liquid Chromatography (ULTRA^3). *Anal. Chem.* **2015**, *87* (16), 8316–8322. https://doi.org/10.1021/acs.analchem.5b01463.