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Aptamer-Based Assay For Detection Of Ochratoxin A

Amanda Nicole Bartley
abart043@fiu.edu

FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

APTAMER-BASED ASSAY FOR DETECTION OF OCHRATOXIN A

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by

Amanda Nicole Bartley

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To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This thesis, written by Amanda Nicole Bartley, and entitled Aptamer-Based Assay for Detection of Ochratoxin A, having been approved in respect to style and intellectual content, is referred to you for judgement.

We have read this thesis and recommend that it be approved.

_____
DeEtta Mills

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Yi Xiao

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John P. Berry, Major Professor

Date of Defense: November 8, 2018

The thesis of Amanda Nicole Bartley is approved.

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Dean Michael R. Heithaus
College of Arts, Science and Education

_____
Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2018

DEDICATION

I dedicate this thesis to my family. Without their support, love, and encouragement, the completion of this work would not have been possible.

ACKNOWLEDGMENTS

I wish to thank the members of my committee for their support, patience, and guidance. Dr. DeEtta Mills for use of laboratory instrumentation and biology related questions. Dr. Yi Xiao for guiding methodology and continuously providing support with aptamers and instrumentation. Finally, I would like to thank my major professor, Dr. John P. Berry. From the beginning, he has had confidence in my abilities and pushed me to work harder towards completion.

ABSTRACT OF THE THESIS

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Amanda Nicole Bartley

Florida International University, 2018

Miami, Florida

Professor John P. Berry, Major Professor

Ochratoxin A (OTA) is a potent mycotoxin found in a wide range of agricultural products that has been linked to mitochondrial damage and renal disease. The standard methods for OTA analysis currently rely on the use of high-performance liquid chromatography (HPLC) coupled to fluorescence detection or mass spectrometry. Toward a high-throughput analysis of OTA, a single-stranded DNA aptamer, modified with a fluorophore, coupled to a complementary sequence, modified with a FRET-based quencher that dissociates in the presence of the target toxin, is proposed. In order to integrate target trapping, aptamer immobilization methods were explored to mediate interference issues. Assays were evaluated using wine and blood serum matrices. A solution-based assay in a 96-well plate format provided a limit-of-detection of 2.7 ng/mL which would be suitable for many of the proposed applications. Immobilized aptamer formats, however, were not reliable, and a range of limitations to applications of the assay were identified.

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ABBREVIATIONS AND ACRONYMS

AuNP- Gold nanoparticle

BEN- Balkan Endemic Nephropathy

Cy5- Cyanine 5 (excitation 648nm; emission 668nm)

ELISA- Enzyme Linked Immunosorbent Assay

FAM- Fluorescein (excitation 495nm; emission 520nm)

FD- Fluorescence detection

FRET- Fluorescent Resonance Energy Transfer

HPLC- High-Performance Liquid Chromatography

IAbRQ- Iowa Black RQ dark hole quencher

IARC- International Agency for Research on Cancer

LOD- Limit of detection

LOQ- Limit of quantitation

MS- Mass Spectrometry

OTA- Ochratoxin A

RSD- Relative standard deviation

SELEX- Systematic Evolution of Ligands by Exponential Enrichment

TAMRA- Tetramethyl-6-Carboxyrhodamine (excitation 559nm; emission 583nm)

CHAPTER 1: INTRODUCTION

Ochratoxin A (OTA) is a fungal mycotoxin that is produced as a secondary metabolite of fungi belonging to the genera *Aspergillus* and *Penicillium* (*1*). Ochratoxin A was first discovered by Vanderme et al., in 1965 (*2*). Structurally, OTA is phenylalanine bound to a dihydroisocoumarin moiety through an amide bond (Figure 1).

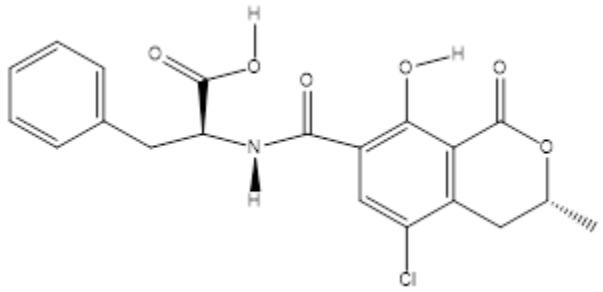

Figure 1: Chemical structure of Ochratoxin A

Ochratoxin A is one of the most common mycotoxins found as an agricultural contaminant of fruits and grains, and products of these (e.g., bread, beer, and wine). Ochratoxin A is absorbed into the gastrointestinal tract and binds to plasma proteins. It is excreted from the body with a half-life of about 35 days (*1*). Toxicologically, OTA is hepatoxic, nephrotoxic, and mutagenic to mammals (*3*), and shown to have inhibitory effects on several enzymes that use phenylalanine as a substrate (*4*), as well as to cause mitochondrial damage. Epidemiologically, OTA has been linked to the Balkan Endemic Nephropathy (BEN) renal disease (*5*). Linked to renal cancer, the International Agency for Research on Cancer (IARC) has classified OTA as Group 2B, or a possible human carcinogen (*1*) (*5*). However, there are few nations that have set regulatory standards to

limit OTA presence in foodstuffs, such as Brazil, Israel, Switzerland, Uruguay, and the European Union (*1*).

Wine is of a growing concern among agricultural products, shown to be contaminated with OTA, because of increased consumption worldwide. Factors which drive the occurrence of OTA in wine remain to be fully understood. The two most common species of fungi found in grapes and wine are *A. niger* and *A. carbonarius*. It was founded that these OTA producing fungi grow well under a wide pH range, 5.0-6.5, and were influenced by water availability (*6*). Higher concentration and contamination levels of OTA in red wines than white wines, from the same growing region, have been reported and suggested to be related to wine making techniques: production of red wine requires a mash ("must") to be produced that includes the skin and pulp of grapes, while white wines discard the skin, potentially increasing exposure to OTA (in skins) during the fermentation process (*7*). Furthermore, climate was found to have possible effects on the production of OTA: southern climates with dry, hot summers had an increased presence (*7*). The European Union (EU) has established regulatory limits on OTA for wine, and the EU regulatory limits prohibit levels above two parts-per-billion (2 ng/mL).

Additionally, the presence of OTA in human serum has been related to the consumption of contaminated foods. Ochratoxin A binds to albumin with an unusually high affinity, resulting in 99.5% of OTA in the circulatory system (*8*). Lino et al. (2008) conducted a study of three areas in Portugal, collecting 104 serum samples, identifying OTA concentrations between 0.14 and 2.49 µg/L based on the consumption of a variety of contaminated goods (*9*). Furthermore, a correlation between certain medical issues,

such as the Balkan Endemic Nephropathy and urinary system tumors, have been related to an increase presence of OTA in foodstuffs and in serum levels. It was founded that several factors affect plasma concentrations of OTA including person to person variation, region variability, and seasonality (*10*).

The biosynthetic pathways of OTA have not been fully characterized and are currently being investigated. Polyketide Synthase (PKS) and Non-Ribosomal Peptide Synthetase (NRPS) are multimodular enzymes involved in the biosynthesis of many secondary metabolites. The dihydroisocoumarin moiety of OTA (Figure 1) is believed to be catalyzed by PKS. The dihydroisocoumarin is linked to L-phenylalanine via amide bond, which is catalyzed by NRPS (*11*). Polyketides and non-ribosomal peptides are, in fact, linked to many mycotoxins.

Analytical Methods for OTA

A variety of analytical methods have been explored for quantitative analysis of OTA in various foodstuffs. The most common methods currently include enzyme-linked immunoassays (ELISA), and methods based on high-performance liquid chromatography coupled to either fluorescence detection (HPLC-FD) or mass spectrometry (HPLC-MS). However, these methods often require extensive extraction and/or sample clean-up steps.

ELISA for OTA

There are multiple ELISA formats that have been used to detect OTA, the most common being a “sandwich style” format. Detection in ELISA results from the

interaction between antibodies and antigens specific to the target, (e.g., OTA) (*12*). In the sandwich format, antigens representing the target (i.e., OTA) are immobilized to the bottom surface of multiwell (e.g., 96-well) plates, and with the addition of an OTA-specific antibody, form a complex. Subsequently, a second antibody that is bound to a "reporter" (typically colorimetric, e.g., horseradish peroxidase) enzyme is added, and free OTA added to the well competes, in turn, with the antigen for the enzyme conjugate. Upon addition of the colorimetric enzyme substrate, a color change results, and OTA can be quantitated as an inverse relationship between concentration and color signal (*13*).

Although ELISA kits for OTA are commercially available, they can be relatively expensive, and accuracy of the technique has frequently been suggested to be rather low. Limits of detection for OTA by way of ELISA methods tend to be around 0.2 ng/mL, fairly close to HPLC-FD methods. However, inter-assay coefficient of variation can range from 4.78-16.67%. Additionally, it has been shown that there are several food matrix effects that can interfere with ELISA, by way of non-specific interactions or cross reactivity (*14*). Using wine as a sample matrix has, indeed, led to mixed results as there are interfering species in wine, particularly including a range of phenolics, which may interfere with antibody-binding (*15*). The addition of polyvinylpyrollidone (PVP) has, therefore, been used for removing polyphenols, and reducing interferences (i.e., "false positives") in immunoassays (*15*).

HPLC-FD and HPLC-MS of OTA

High-Performance Liquid Chromatography (HPLC) enables analytical separation of OTA from other components of sample matrices, and therefore, can increase

selectivity and reliability. When coupled to fluorescence detection, HPLC-FD has shown sensitive detection and quantitation of OTA in foodstuffs as low as 0.01-0.30 µg/kg depending on sample matrices (*16*). In a recent study, a simplified liquid/liquid extraction (LLE) method – eliminating more extensive sample preparation and clean-up steps - was developed and applied to HPLC-FD detection OTA in wine, De Jesus et al. (2018). In this method, De Jesus et al. (2018) used simple extraction with chloroform (followed by concentration *in vacuo*) which was found to be sufficient for recovery of OTA, and subsequent HPLC-FD. The method achieved LOD and LOQ of 0.1 and 0.3 µg/L, respectively, and was applied to 41 wine samples sourced from the United States, showing that more than 85% (of the 41 wines tested) were above the LOD (0.1 µg/L), and 31 of these OTA-positive wines were above the LOQ (0.3 µg/L) (*17*). The sensitivity of this simple LLE is, therefore, well below the EU's regulatory limit of 2 ng/mL in wine, and can be classified as "quick, easy, cheap, effective, rugged, and safe" (QuEChERS).

High-Performance Liquid Chromatography coupled to Mass Spectrometry (HPLC-MS) has been used as a confirmatory method for the detection of OTA and, in fact, multi-mycotoxin analysis in foodstuffs (*18*). Typically, ^{13}C-labeled internal standards are used to compensate for matrix effects when identifying and quantifying mycotoxins by this technique. As an example of using ^{13}C-labeled internal standards, Al-Taher et al. (2013) surveyed 143 red and white wines from 4 major growing regions by means of a simple dilution of wine (and beer) at a 1:5 ratio, and subsequently injected into the UHPLC-MS/MS system for multi-mycotoxin analysis (*19*). It was founded that OTA, among other mycotoxins, were affected by ion enhancement with spiked white wine samples. The methodology applied in this study shows that the LOD and LOQ

values for both red and white wine were 0.1 and 0.5 ng/mL, respectively, and are well below the EU's regulatory limit of 2 ng/mL (*19*).

Aptamer-Based Analysis for OTA

An increasing number of studies have utilized aptamers for detection of OTA and a wide range of analytical applications. Aptamers derive from the Latin word *aptus*, "to fit", and are typically single or double stranded oligonucleotides. Aptamers bind to a specified target molecule and are more chemically stable and cost effective than antibody-based (i.e. ELISA) methods. Aptamers are created through a process known as SELEX, or Systematic Evolution of Ligands by Exponential Enrichment (*20*). An aptamer sequence, specific for OTA, was previously identified by Cruz-Aguado et al. (2008) by way of SELEX. The aptamer sequence is 5′-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3' (*21*). Most analytical methods employing aptamers for OTA detection and quantitation utilize this aptamer sequence with few modifications.

Gold Nanoparticles

Assays for OTA detection and quantitation using aptamers bound to gold nanoparticles (AuNPs) have been previously developed (*22*) (*23*) (*24*). Gold nanoparticles are colorimetric indicators whose size and dispersion changes in the presence of salt, resulting in a measurable color shift from red to blue. In assays, target-specific aptamers are bound to the surface of an AuNPs, and in the presence of OTA, a structural change occurs revealing the AuNP's surface, allowing interactions with salts, and therefore, the corresponding quantifiable color change (*2*). As an example, a study

conducted by Luan et al. (2015) developed an assay using AuNP's and a cationic polymer, polydiallyldimethylammonium chloride (PDDA) to colorimetrically detect OTA in Chinese distilled liquor. In this assay, the OTA aptamer is free to form a duplex with PDDA in the absence of OTA, and therefore, no color change will occur. However, when OTA is present, the aptamer forms a "G-quadruplex" structure, and PDDA aggregates the AuNP's. The reported linear concentration range for this assay is 0.05-50ng/mL with an LOD of 0.009ng/mL (*23*).

Direct Detection of OTA by Aptamers in Solution

Assays that use the direct interaction between OTA and aptamers in solution was recently developed by Chen et al. (2012). The assay specifically utilizes a so-called "structure switching," aptamer formation in which a signaling aptamer specific for OTA, bound to a fluorophore (i.e., fluorescein, or FAM) is paired with a quencher (i.e., TAMRA) modified complementary sequence. Upon addition of OTA, a conformational structure change occurs, therefore, displacing the complementary quencher sequence, and enabling emission of the fluorescent signal (Figure 2) (*25*).

The basic premise of the assay is as follows: two DNA oligomer sequences - the aptamer and complementary "quencher" strand - form a duplex (following heating and cooling). Through a FRET-based mechanism, the quencher strand (via TAMRA modification) attenuates the emission of fluorophore (i.e., FAM) on the aptamer sequence, thus resulting in a very low level of base fluorescence. Aptamers, in turn, have the ability to form a complex with the target (i.e., OTA) allowing a conformation change. Specifically, upon addition of the target OTA, the fluorophore-modified aptamer strand

forms the complex with the target, and releases the quencher strand (Figure 2) enabling a measurable increase in fluorescent emission.

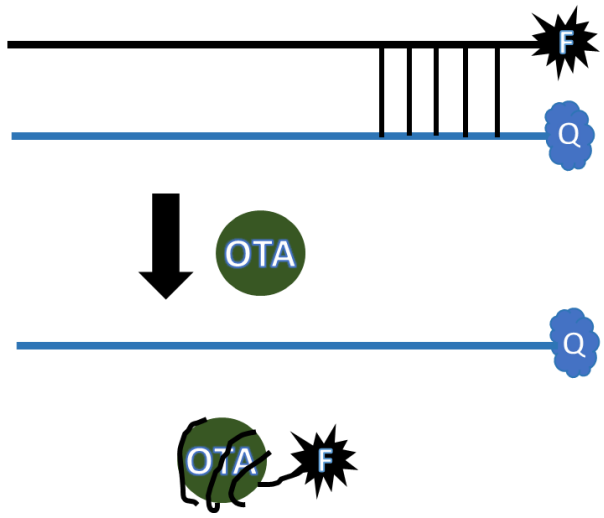

Figure 2: Depiction of conformation change of aptamers with addition of OTA.

Chen et al. (2012) indicated that the fluorescent signal is linear from the range 1 to 100 ng/mL with a reported limit of detection (LOD) of 0.8 ng/mL. The repeatability of the assay, using the relative standard deviation (RSD), was reported as 2.4% and reproducibility (reported an RSD of eight separate samples with the same concentration) was 4.7%. Selectivity was measured by adding other mycotoxins, including aflatoxin B_1 and zearalenone, and the structurally related ochratoxin B (OTB), into the mixture, and measuring fluorescent intensity. The addition of OTB resulted in a slight increase in fluorescent intensity, while aflatoxin B_1 and zearalenone effects were negligible (*25*).

Immobilization of aptamers

A number of approaches have employed immobilization of aptamers, for a wide range of matrices, as part of assay formats. Immobilization has the potential for target "trapping," and consequently integrated sample clean-up alongside detection.

One such strategy is immobilization of aptamers on 96-well (or other multiwell) plates enable a high-throughput format similar to ELISA. Demirkol et al. (2016) created a fluorescence assay using 96-well microtiter plates with immobilized aptamers to detect *E.* coli. In this case, 96-well plates coated with poly-l-lysine were specifically used to immobilize thiol-modified aptamers by way of sulfo-SMCC (sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate) cross-linking chemistry. Sulfo-SMCC reacts with the NH_2 groups from poly-l-lysine, and forms a maleimide activated carrier, and upon addition of the thiol-modified aptamers, in turn, a carrier-aptamer conjugate is formed, thereby covalently linking the aptamers to the solid support. Addition of *E. coli* and concurrently quantum dots, enabled fluorescent detection (*26*).

Specifically relevant to the current study, Duan et al. (2011) created an aptamer-based assay for detection of OTA whereby two complementary strands, including one (Seq1) immobilized by way of avidin/biotin binding, and one (Seq2) labeled with fluorescent (i.e., FAM) reporter, were effectively used to measure OTA (*27*). In the absence of OTA, a strong background fluorescent signal was measured. In the presence

of OTA, Seq1 was bound to the OTA, therefore releasing some of Seq2, and resulting in a decrease in fluorescent signal, shown in Figure 3.

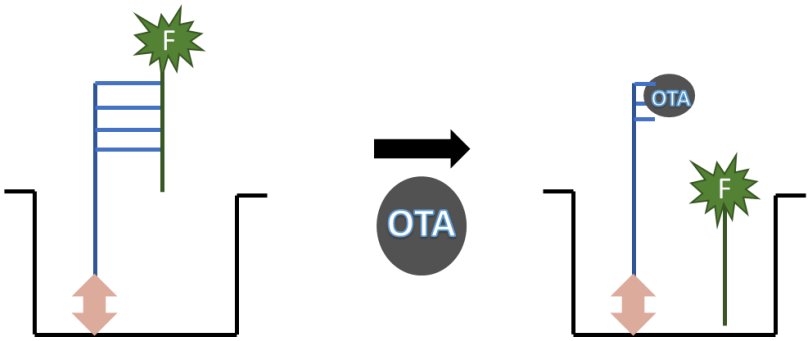

Figure 3: Depiction of Duan, et al. (2011) methodology, whereby a high fluorescent signal is present in the absence of OTA, and a decrease in signal results upon addition of OTA and release of FAM modified strand.

The LOD of the assay was calculated to be 0.01 ng/mL, or 0.01 ppb (well below the EU regulatory guidelines). The authors mention that binding of OTA to aptamers relied heavily on Mg^{2+} and Ca^{2+} ions and less of K^{+} (as reflected in the buffers used) (*27*).

Objectives

The use of fluorescently labeled aptamer, paired with complementary quencher, as described in previous studies (*25*), was adapted in the current study to develop a multiwell, (i.e., 96-well), plate format assay for OTA. The advantage to using a 96-well plate is the ability to perform multiple analyses at the same time with measurements by a plate reader. The present research, specifically, utilizes aptamer sequences and fluorophore/quencher pairs (i.e., FAM/TAMRA), employed in this and other (*27*), studies.

In addition, the use of immobilization techniques (for multiwell microtiter plates) as described in previous studies (*26*), (*27*), were, in turn, explored as a means of

developing a functional assay, whereby sample preparation (i.e., “clean-up”) could be integrated alongside detection. Approaches included the use of sulfo-SMCC cross-linker chemistry to covalently immobilize, and biotin/avidin interactions to non-covalently immobilize, aptamers to assay plates.

Finally, the use of the developed assays, in turn, was assessed with respect to OTA in multiple relevant matrices. These included wine, as a representative (and increasingly relevant) agricultural product known and described above. In addition, blood serum – and specifically commercially available fetal bovine serum – was investigated towards adapting the technique for biomedical applications. This aspect of the study enabled both operational assessment of the assay, and evaluation of the potential for a practical application.

Accordingly, this study had the following specific aims:

Aim 1: Develop a multiwell plate format aptamer-based assay, specifically using fluorescence/quenching, for OTA detection and measurement

Aim 2: Develop a multiwell plate format assay that uses an *immobilized* aptamer, and fluorophore/quencher interactions, for “target trapping” prior to detection and measurement of OTA

Aim 3: Evaluation of the developed assay for detection and measurement of OTA in relevant matrices including wine and blood serum

CHAPTER 2: AN APTAMER-BASED MULTIWELL PLATE ASSAY FOR OTA

Introduction

Aptamers, previously described in the literature, have been shown to be highly specific and increasingly sensitive for target detection. When coupled to a multiwell plate format, the assay allows high-throughput analysis of samples with relatively simple instrumentation (i.e., plate reader). Figure 4 depicts the proposed assay format from the view of a single well. The fluorophore and quencher modified sequences form a duplex structure with relatively low fluorescent signal (Figure 4). Upon addition of the target, OTA, the fluorophore and quencher modified sequences dissociate, and the fluorophore modified sequence forms a "G-quadruplex" structure with OTA, while the quencher sequence remains in solution (Figure 4).

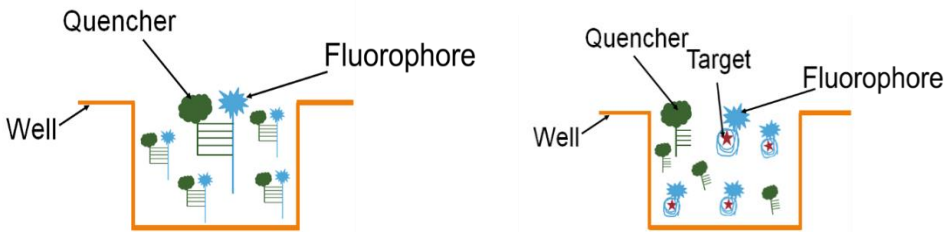

Figure 4: Proposed multiwell in solution aptamer-based assay

The G-quadruplex structure has been described as the proposed folding mechanism, due to high amounts of guanine bases in the aptamer sequence. G-quadruplex structures have been shown to have increased sensitivity and selectivity. Basic G-quadruplex structures rely on hydrogen bonding and presence of certain metal ions such as as potassium (K^+) and sodium (Na^+) (*28*).

The fluorophore modified aptamer (e.g., FAM) and the complementary quencher (e.g., 10 base pair TAMRA modified) sequence that showed the lowest signal to noise ratio in the Chen et al. (2012) study were selected to create a multiwell plate assay for the detection and quantitation of OTA. Furthermore, the optimum molar ratio between fluorophore modified aptamer and quencher sequences (1:3) were considered for adequate quenching of the duplex (*25*).

Materials and Methods

Materials

Fluorophore and quencher modified aptamer sequences were purchased from Integrated DNA Technologies (IDT) in Coralville, Iowa with HPLC purification. The fluorophore modified sequence was 5'- GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-FAM-3', and the quencher modified sequence was 5'-TAMRA-TGT CGG ATG C-3'.

Corning™ Falcon™ 96-well black/clear tissue culture treated plates were purchased from Fisher Scientific. Greiner Bio-One 96-well black/black plates were purchased from VWR International. Trizma® hydrochloride (Tris-HCl), potassium chloride (KCl), and calcium chloride ($CaCl_2$) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride (NaCl) was purchased from Fisher-Scientific (Hampton, NH). Water was purified with a Labconco Water Pro PS system at 18.2 megaohms. Ochratoxin A, 99+% was purchased from ACROS Organics™. Oligonucleotides and OTA stored -20°C.

Instrumentation

Measurements were taken on a Tecan Infinite® M1000 microplate reader with excitation 495nm and emission 520nm. The gain was set to automatically adjust according to the well with the highest signal (100nM aptamer only), and the z-position was automatically set based on scanning volume in wells.

Assay Procedure

Stock solutions of the fluorophore modified sequence were made to 10uM and quencher modified sequence to 20uM with addition of purified water. The binding buffer used for the reaction between duplex and OTA was same as Chen et al. (2012) reported: 10 mM Tris, pH 8.5, 120 mM NaCl, 5 mM KCl, and 20 mM $CaCl_2$ (*25*). Stock solutions of OTA were prepared by initially dissolving in methanol, and subsequent dilution in binding buffer to give final concentrations (when diluted 50-fold into test wells) equivalent to 1, 2, 5, 10, 15, 20 and 30 ng/mL.

A range of aptamer concentrations (2.5, 5, 10 and 20 pmol per well) were evaluated; however, a final concentration of 5 pmol per well of fluorophore modified aptamer was found to be sufficient to generate a linear response in the range of 1-20 ppb OTA. Likewise, a 1:2 molar ratio of aptamer to quencher was found to be similarly efficient to 1:3 molar ratio employed by the Chen et al. (2012) study, in terms of low background fluorescence, and used in the present study. The final working volume of the assay was 50 µL with a 1:2 molar ratio (or 100nM:200nM of fluorophore- to quencher-modified sequences). To prepare the equivalent volume of duplex for each well, 0.5 µL of fluorophore strand (10uM) and 0.5 µL of quencher strand (20uM) were added (in a

500 µL Eppendorf tube) to 48 µL of binding buffer. The duplex was heated to 88 °C for 5 minutes and cooled at room temperature for 30 minutes. The duplex (49 µL) was added to the 96-well plate, and initial measurements were taken in 1-minute intervals for 10 minutes (stability of signal). Next, OTA was added (1 µL) to each well with final concentrations ranging from 1, 2, 5, 10, 15, 20, 30 ng/mL (i.e., 1 µL diluted in 49 µL of assay buffer). Measurements were taken in 1-minute intervals over 10 minutes with the first measurement made within 60 seconds of addition of OTA to the assay.

Calculations and Statistical Analyses

All data were normalized, subtracting the buffer control signal, therefore, reducing the percent change in fluorescent intensity influenced by the buffer composition. The percent change in fluorescent intensity (RFU) was calculated as [(final-initial)/initial]*100. The LOD was calculated as 3*(std. error of y-values/slope) and the LOQ as 10*(std. error of y-values/slope). The reproducibility and repeatability were measured by the %RSD or the (standard deviation/mean) *100. The accuracy was measured by comparing the measured value to the actual value as a percentage of error. Subsequent, ANOVA and t-tests were conducted using Excel, data shown in corresponding Appendices.

Results

A concentration-dependent increase in the percent change in fluorescence was observed over the range of 1 to 10 ng/mL OTA, and a linear ($R^2 = 0.9648$) relationship was observed from 1 to 20 ng/mL (Figure 4). The method was further validated in terms of sensitivity (i.e., LOD and LOQ) and precision including reproducibility and

repeatability (Table 1). Limits of detection and quantitation were calculated as 2.7 ng/mL and 8.9 ng/mL, respectively. The accuracy was measured as a 2.65% error, comparing the expected and the true experimental values. The reproducibility (n = 8, 10 ng/mL) was calculated as a RSD% of 8.8%, whereas repeatability (n = 10, 10 ng/mL) was measured as a RSD% of 5.9% (Table 1).

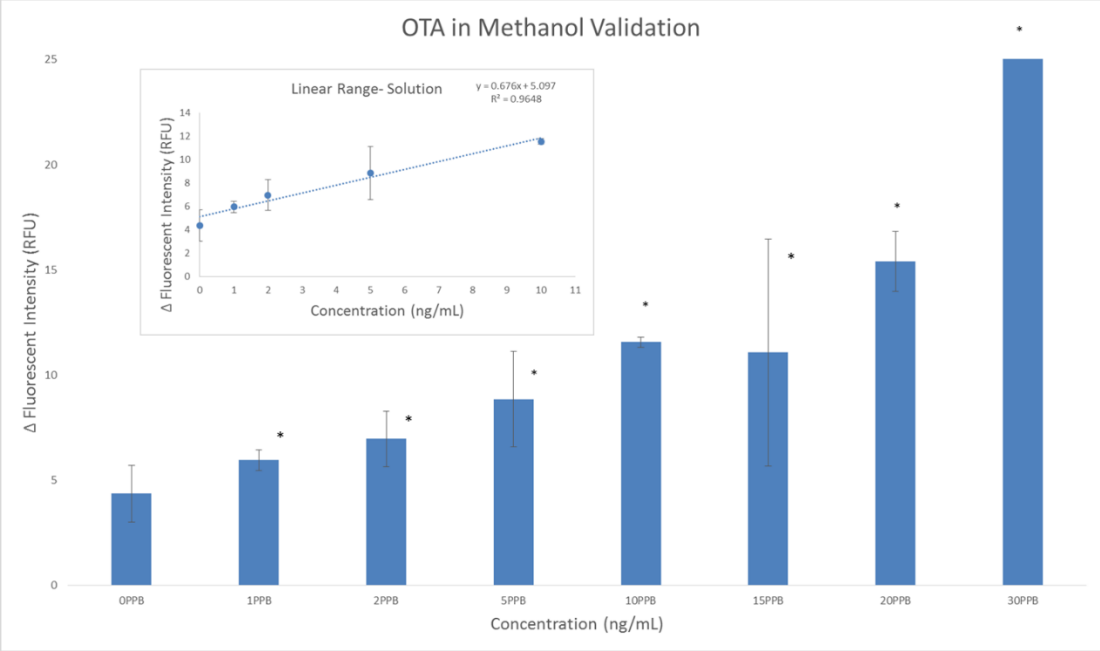

Figure 5: Bar graph showing increasing change in fluorescent intensity with increasing concentration, with calibration including dynamic range (0-10ng/mL)

Table 1: Figures of Merit for solution-based assay

Figures of Merit	
LOD	2.7 ng/mL
LOQ	8.9 ng/mL
Reproducibility	8.8% RSD
Repeatability	5.9% RSD
Accuracy	2.65% Error

Analysis of Variance (ANOVA) was conducted to determine whether there were significant differences between the percent change in fluorescent signals of spiked OTA concentrations. The normality of the variables were tested using Kurtosis and Skewness values, verifying that Kurtosis values between ± 2 and the Skewness values between ± 1. The subsequent ANOVA output for the comparison of group means of 0, 1, 2, 5, 10, 15, 20 ng/mL with 5 replicates is shown in Appendix 2: Table 5. The null hypothesis is that all group means are equal. The F value is greater than the critical value of F meaning that the null hypothesis is rejected ($15.49 > 2.45$). Furthermore, the p-value is $9.39 \times 10^{-08} < 0.05$ indicating a significant difference between group means, or percentage change in fluorescent signal. Additional t- tests were performed to determine which groups were different from each other. Appendix 2: Table 6 shows the t-test results for the comparison of the percent change in fluorescent intensity signal for 0 and 1 ng/mL and the percent change in fluorescent intensity signal for 1 and 5 ng/mL. The p-value for the t-tests comparing 0 and 1 ng/mL and 1 and 5 ng/mL were $0.0366 < 0.05$ and $4.88 \times 10^{-08} < 0.05$, respectively, depicting a significant difference in the percent change of fluorescent signal. Furthermore, Appendix 2: Table 7 shows the t-tests for the comparison of 1 and 15ng/mL (left) and the comparison of 1 and 20ng/mL (right) with p-values $0.0683 > 0.05$ and $7 \times 10^{-07} < 0.05$, respectively. The p-values chosen are the ones for the two-tailed test which determines if there is any significant difference between the signals, including both greater than or less than. The difference between 1 and 15ng/mL was shown as not significant; however, the value was significant for the one-tailed test ($0.0341 > 0.05$).

Discussion

The aptamer-based method developed in the present study demonstrated a concentration-dependent fluorescent response for detection and quantitation of OTA with sensitivity which is potentially sufficient for agricultural and biomedical applications. The LOD and LOQ for the solution-based assay of spiked OTA samples were 2.7 ng/mL and 8.9 ng/mL, respectively. The EU's regulatory guidelines prohibits levels in wine, for example, is currently 2 ng/mL, and the developed method would enable detection at this limit. On the other hand, current guidelines for OTA in various commodities for the United States, Canada, and the European Union are depicted in Table 2. The United States does not currently have regulations or maximum limits on OTA in foodstuffs. However, both Canada and the European Union have implemented limits using current and past research. Both Canada and the European Union have limits at 3 ng/mL for grains of direct consumption and cereal products.

*Table 2: Maximum concentrations for commodities as provided by the United States (no guidelines), Canada (*29*), and the European Union (*30*).*

		Max concentration (ng/mL)	
Commodity	United States	Canada[a]	European Union [b]
Grains- direct consumption	-	3	3
Derived cereal products	-	3	3
Cereal food for infants	-	0.5	0.5
Dried vine fruit	-	10	10
Roasted coffee	-	-	5
Grape juice and related products	-	2	2
Wine	-	-	2
[a] Canada Proposed Maximum Limits 2009			
[b] European Commission Maximum Levels 2006			

Additionally, a study testing OTA in serum was conducted by Malir et al. (2013) with 100 pregnant women in their first trimester, ages 19-40 years, in the Czech Republic. The blood serum levels were tested by HPLC-FD and compared with dietary consumption. The range of incidence of OTA in blood serum was between 0.1-0.35 ng/mL, with an average of 0.15 ng/mL. However, these values are similar to those who were not pregnant and eating comparable foodstuffs, indicating the relationship between contaminated foods and serum levels (*31*). Furthermore, urine has been used as a matrix to measure OTA levels in humans. Pascale et al. (2001) measured spiked human urine levels between 0.05-1ng/mL by HPLC-FD (*32*).

The LOD/LOQ for the current assay were, however, lower than previously reported by Chen et al. (2012) for a similar approach (i.e., 0.08 ng/mL). Potential explanations for the higher values (and lower sensitivity) may be related to the difference between the optical capabilities of the two formats (i.e., use of a cuvette versus a 96-well plate format). More specifically, it may be affected by the plates used. There are, in fact, a variety of microtiter plates for 96-well plate assays. However, black plates with black (i.e., opaque) or clear bottoms are most often used for fluorescent assays. Both black/black and black/clear plates were tested in the current study to observe differences in percentage change of fluorescent intensity by way of bottom or top read. The solution-based assays used black plates with clear bottoms and the immobilized assays used black with black plates. Precision of the assay was estimated by taking measurements to calculate reproducibility and repeatability. The reproducibility (%RSD) of the assay was determined to be 8.8% whereas repeatability was 5.9%, and may be, likewise, dependent

upon the optics and precision of the instrumentation, i.e., plate reader (discussed further in Chapter 3).

The concentration of fluorophore modified aptamer was also tested at a range of concentrations including 2.5, 5, 10, and 20 pmol per well. The 5 pmol/well concentration was chosen in the study because it had the most stable and linear increase in relationship among various spiked OTA concentrations, whereas 2.5 pmol/well concentration of fluorophore-modified aptamer did not show a significant difference between aptamer and duplex signals. Additionally, 10 pmol and 20 pmol concentrations were tested, however, 5 pmol was found to be similarly efficient in terms of detection of spiked OTA in relevant ranges of concentrations (Figure 7). The result agrees with the previous studies, Chen et al. (2012), which employed an equivalent concentration of fluorescently labeled aptamer. The Duan et al. (2011) study uses 50nM concentration of aptamer, however, fails to mention the working volume of the assay. Finally, the larger distinction between 0-5 ng/mL in the 5pmol concentration provides the opportunity for greater sensitivity between those concentrations.

Taken together, these results suggest that the proposed assay may, in principle, provide a format suitable for high-throughput detection of OTA. To further assess this potential, relevant matrices must be assessed. However, anticipating a potential contribution of interfering species within these complex biological matrices, I first investigated the possible use of aptamer immobilization within the current assay format as a means of integrated sample preparation via “target trapping” (Chapter 3).

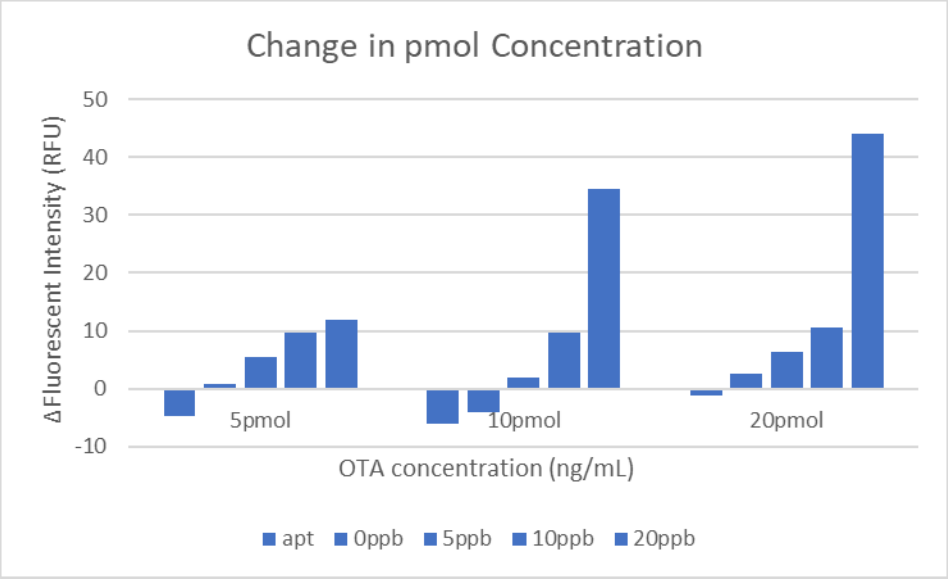

Figure 6: Comparison of fluorophore-modified aptamer pmol concentrations

CHAPTER 3: A MULTIWELL PLATE ASSAY FOR OTA BASED ON IMMOBILIZED APTAMER

Introduction

Modification of the aptamer fluorescence quenching assay (as discussed in Chapter 2) based on the immobilization of the OTA-specific aptamer was investigated. It was proposed that immobilization of aptamers would enable a simplified and integrated sample preparation and clean-up by removal of potential interferences by way of target-trapping and subsequent washing steps. The proposed approach is depicted in Figure 7, where the duplex is immobilized to 96-well test plates by way of cross-linker (i.e., biotin-avidin or thiol chemistry), and upon addition of OTA, the quencher strand is released, and can be removed (by washing), to enable specific measurement of fluorescent signal generated by presence of OTA.

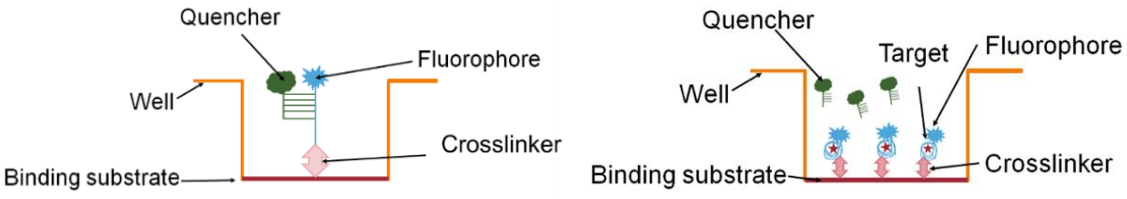

Figure 7: Depiction of immobilization of fluorophore/quencher sequence

Three sets of aptamer configurations and immobilization techniques were explored in which the fluorophore modified sequence was immobilized by either thiol-based (i.e., SMCC) chemistry at the 5' end, or by biotin modifications at either the 5' or 3' ends (see Appendix 1).

Materials and Methods

Materials

Aptamer sequences modified with fluorophore (FAM) and quencher (TAMRA), and modifications (i.e., thiol and biotin) were purchased from Integrated DNA Technologies (IDT) in Coralville, Iowa with HPLC purification. The fluorophore-labeled sequences were as follows: 5'-/Biotin-TEG/GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-FAM-3' (5BIOTIN), 5'-/56-FAM/ TGA TCG GGT GTG GGT GGC GTA AAG GGA GCA TCG GAC A /3BioTEG/-3' (3BIOTIN), and 5'-/ThioMC6-D/ GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA/36-FAM/-3'. The quencher sequences were as follows: 5'-TAMRA-TGT CCG ATG C-3' (5TAMRA) and 5' CCC ACA CCC GAT T /36-TAMSp/-3' (3TAMRA).

Pierce™ Streptavidin Coated High Capacity Plates, 96-well plates, black/black were purchased from ThermoFisher Scientific and stored at -20℃. Corning Poly-D-lysine coated 96- well polystyrene plates, black/clear were purchased from Sigma Aldrich (St. Louis, MO). Chemicals including magnesium chloride ($MgCl_2$), tris(2-carboxyethyl) phosphine (TCEP), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Trizma® hydrochloride (Tris-HCl), potassium chloride (KCl), and calcium chloride ($CaCl_2$) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium Chloride (NaCl) and Sulfo-SMCC No-Weigh Format (8 X 2mg microtubes) were purchased from Fisher-Scientific (Hampton, NH). Water was purified with a Labconco Water Pro PS system (18.2 megaohms). Ochratoxin A, 99+% was purchased from ACROS Organics™. Oligonucleotides and OTA stored -20℃.

Instrumentation

Measurements were taken on a Tecan Infinite® M1000 microplate reader with excitation 495 nm and emission 520 nm. The gain was set to automatically adjust using the well with the highest signal (100 nM aptamer only), and the z-position was automatically set based on scanning volume in wells. Additionally, comparative measurements were taken on a Biotek Synergy HT or Biotek Synergy 2 plate-reader, specifically using filters for 485/20 nm excitation and 528/20 nm emission. Likewise, the gain was set on these instruments to the well with the highest signal (100 nM aptamer only), and subsequently used throughout the procedure.

Immobilization of Aptamers

Aptamers were immobilized on 96-well plates using either biotin/streptavidin interactions, or covalent linkage via sulfo-SMCC reaction with poly-D-lysine coating on plates. Both approaches were subsequently evaluated in assays of OTA.

For biotin-modified aptamers, an excess of quencher (1:2 molar ratio for fluorophore to quencher aptamer) was used; accordingly 0.5 µL of 10 µM 5BIOTIN or 3BIOTIN aptamer, and 0.5 µL of 20 µM of 5TAMRA or 3TAMRA quencher strand were combined in a 500 µL Eppendorf tube, with 49 µL binding buffer. Stock solutions (10 µM biotinylated aptamer, 20 µM TAMRA-modified quencher) were prepared in sterilized water, and stored at -20℃. The binding buffer used for 5BIOTIN/5TAMRA was 10 mM Tris, pH 8.5, 120 mM NaCl, 5 mM KCl, and 20 mM $CaCl_2$, the same as the solution-based assay. The binding buffer for 3BIOTIN/3TAMRA was 10 mM HEPES, 120 mM NaCl, 5 mM KCl, 20 mM $MgCl_2$, 20 mM $CaCl_2$, pH 7.0. Aptamer/quencher

mixtures were heated to 88℃ for 5 minutes, and allowed to cool for 30 minutes at room temperature, to form duplexes. The duplex (50 µL) was subsequently added streptavidin-coated plate, and incubated for 60 min at room temperature.

Secondarily, an immobilization procedure using poly-lysine and sulfo-SMCC was investigated (*26*). TCEP (1mM, IDT recommendation 100x excess) was used to break the disulfide bonds on the thiolated aptamer, and prepare aptamer for subsequent immobilization. A solution of sulfo-SMCC (2 mg/mL) in water was prepared, and 50 µL of this solution was added to each well of a poly-D-lysine coated, 96-well plate, which was placed on a rotary shaker for 2 hours at room temperature. Wells were rinsed with water, and allowed to dry. Following similar procedures as used for biotin-modified aptamers, a 1:2 molar ratio of fluorophore modified sequence (0.5 µL of 10 uM solution), to quencher modified sequence, (0.5 µL of 20uM solution) were added to a 500 µL Eppendorf Tube with 10 mM Tris, pH 8.5, 120 mM NaCl, 5 mM KCl, and 20 mM $CaCl_2$ binding buffer (49 µL). The aptamers were incubated on plates overnight at room temperature before being used for assays.

Assay Procedure

Aptamer assays were generally conducted similar to the technique described in Chapter 2. Measurements (n=10) of immobilized duplexes were taken at 1-minute intervals for 10 minutes to acquire the initial fluorescent readings. The wells, containing immobilized aptamers, were then washed with 0.01M PBS, and refilled with binding buffer. Measurements (n=10) at 1-minute intervals were then taken to determine the change in signal due to possible loss of non-immobilized following washing step. Finally,

1 µL aliquots of OTA, prepared at a range of concentrations (0-20 ppb) were added to the test wells, and a final set of 10 measurements (at 1-minute intervals) were made.

Calculations and Statistical Analyses

All data were normalized by subtracting the buffer control signal, therefore, reducing the percent change in fluorescent intensity influenced by the buffer composition. The percent change in fluorescent intensity (RFU) was calculated as [(final-initial)/initial]*100. Subsequently, ANOVA and t-tests were conducted using Excel (output of these analyses are shown in corresponding Appendices).

Results

To show that the quenching mechanisms work for the immobilization procedures of 5BIOTIN and 3BIOTIN, the relative fluorescent units were compared for the aptamer signal, the buffer signal, and the duplex signal (aptamer and quencher combined). Figure 8 shows the fluorescence of aptamer with and without quencher from 10 measurements. When the aptamer and quencher sequences are added together, a significant reduction in fluorescence was observed, and approached the signal intensity of buffer-only controls.

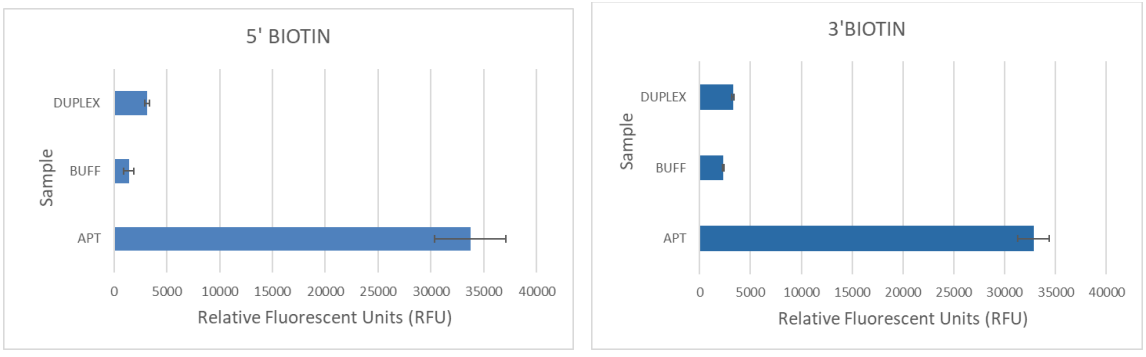

Figure 8: Quenching mechanism significantly reduces RFU for 5BIOTIN assay (left) and 3BIOTIN assay (right).

Figure 9 shows the calculated percent change in fluorescent intensity upon addition of spiked OTA, in methanol, to 5BIOTIN/5TAMRA duplex. A single factor ANOVA was used to assess the significant differences in the percent change of fluorescent intensity across various concentrations (0, 1, 5, 10, 20 ng/mL). The ANOVA provided a p-value of 0.3211 > 0.05, showing that there was no significant difference among percent change in fluorescent intensity for various concentrations (Appendix 3: Table 8).

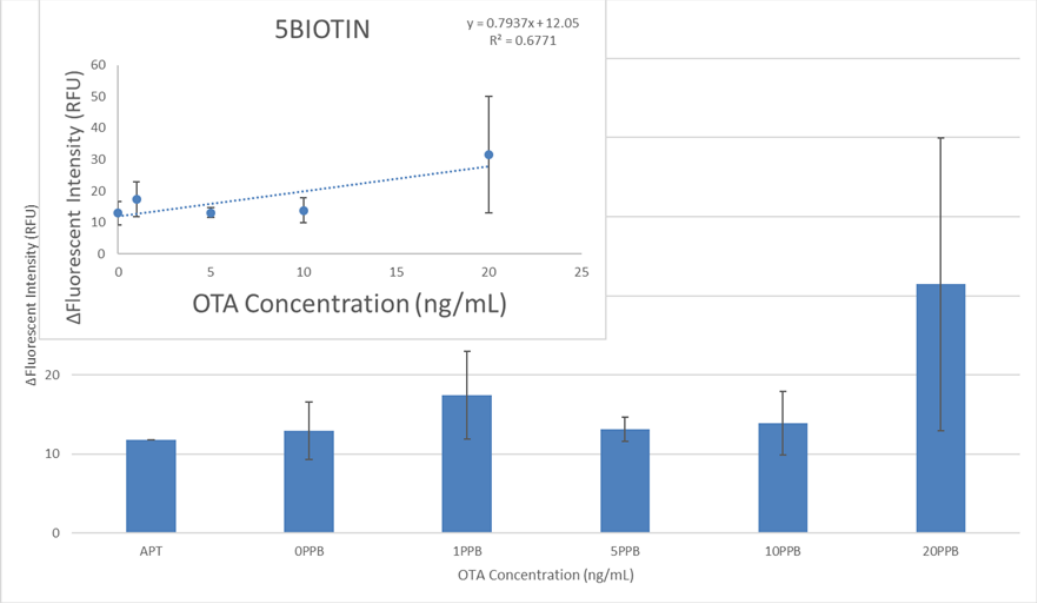

Figure 9: Change in relative fluorescent intensity upon addition of OTA to immobilized aptamers

To further investigate, the 5BIOTIN assay was evaluated on a similar (Biotek 2) instrument. Figure 10 shows that the change in fluorescent intensity was found to increase, but all measurements curiously represented negative changes in fluorescence. Potential reasons for this negative change in fluorescence could include the conformational change of the aptamers upon addition of the OTA was unsuccessful or there were instrumental issues.

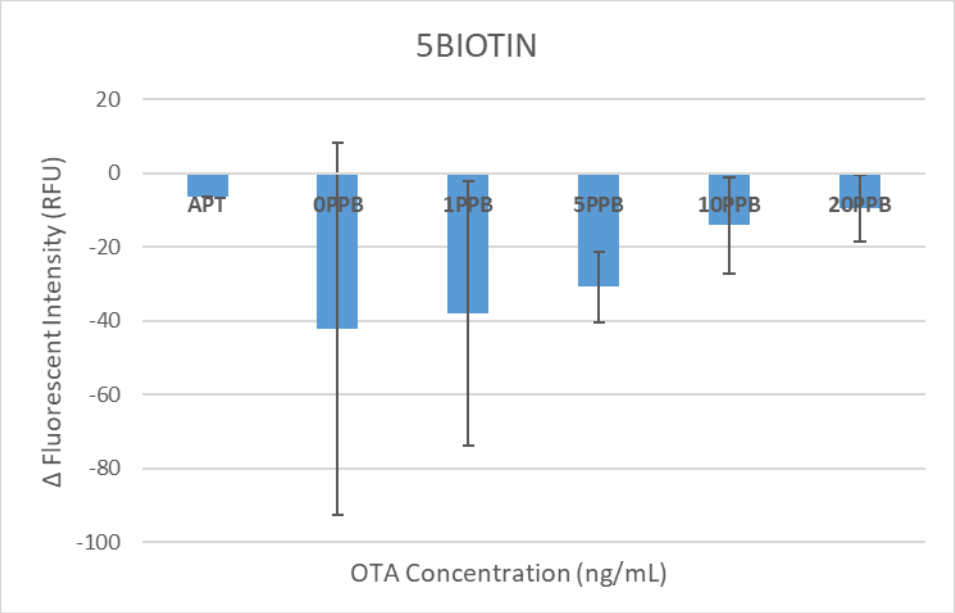

Figure 10: 5BIOTIN assay with increasing change of fluorescent intensity.

Immobilization and assay procedures were next conducted for 3BIOTIN/3TAMRA in duplicate. Figure 11 shows the relative change in fluorescent intensity for 0, 1, 5, 10 and 20 ng/mL OTA. An apparent effect on concentration on fluorescence response was observed over this range. An ANOVA single factor was conducted to test if there were any significant differences between concentrations, however, the p-value 0.196 > 0.05 indicated no significant difference among the percentage change in fluorescent intensity (Appendix 3: Table 9).

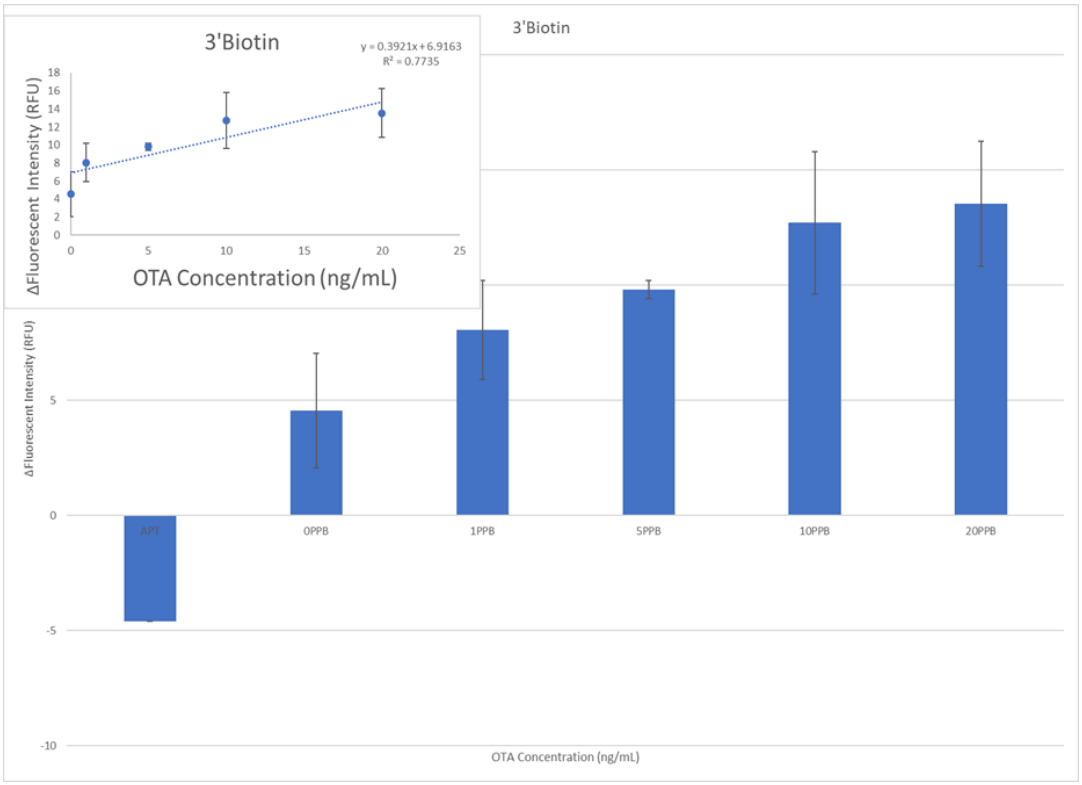

Figure 11: Change in fluorescent intensity of 3BIOTIN

Two buffers, namely TRIS and HEPES, were evaluated in the 5BIOTIN assay (Figure 12). Results confirmed a lack of concentration-dependent increase in fluorescence, as expected for the proposed assay, and moreover, showed no significant difference between the two buffers. A two-factor ANOVA was performed to analyze the statistical significance of Tris vs. HEPES buffer on each of the assays respectively (Appendix 3: Table 10). The p-value for concentration was 0.6506 > 0.05, concluding that there was no significant difference in the percent change of fluorescent intensity in concentrations for the two buffers. Furthermore, the p-value for the percent change in fluorescent intensity comparing buffers among concentrations was 0.4123 > 0.05, concluding the buffers were not statistically different. Lastly, the p-value for the

interaction between concentration and buffer was 0.1112 > 0.05, indicating there is no significant interaction between concentration and buffer type (Tris vs. HEPES).

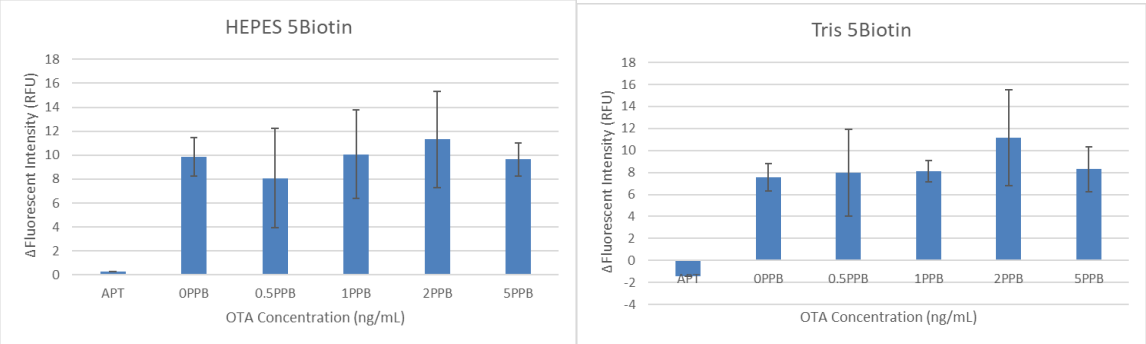

Figure 12: Comparison of HEPES and Tris buffers used in 5Biotin assay

Finally, immobilization of thiol-modified aptamer via SMCC-linking chemistry was investigated. However, the duplex did not appear to effectively immobilize to the bottom of the well, and washed off during the 1st and 2nd washing steps (PBS1, PBS2) as shown in Figure 13. The readings after each washing step show a significant decrease in signal from binding buffer (BB) to PBS1 and PBS 2, where almost a complete loss of relative fluorescent signal was observed.

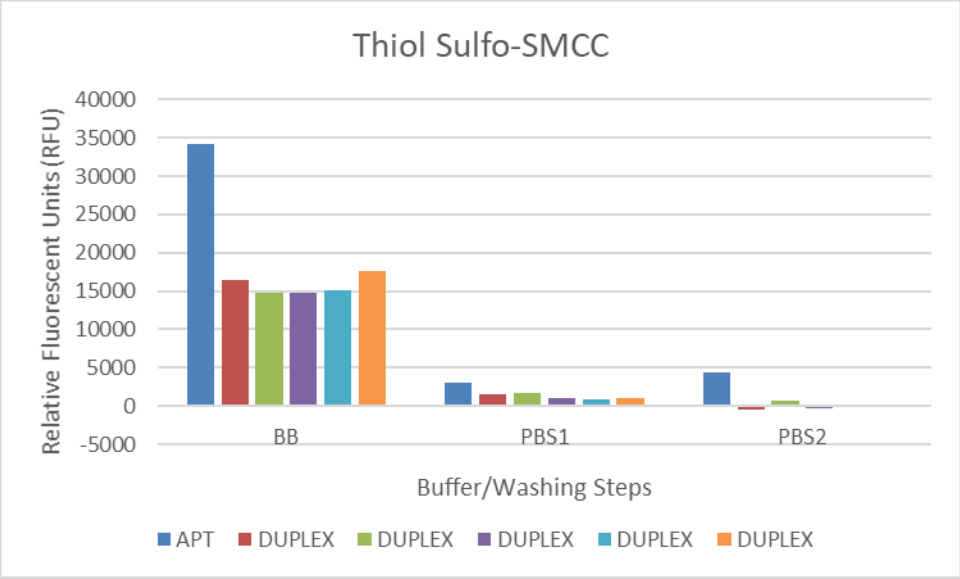

Figure 13: Average relative fluorescent units (RFU) indicating loss of fluorescent signal over two washing steps

Discussion

Fluorescence response of the immobilized 5BIOTIN/5TAMRA duplex did not indicate a linear relationship with OTA concentration upon initial assessments (Figure 9). The average percent change in fluorescent intensity for 0, 5, and 10 ng/mL spiked samples resulted in values of 12.92, 13.11, and 13.88%. This indicates a lack of concentration-dependent relationship with OTA. Additionally, the standard deviation for the percent change in fluorescent intensity of 20 ng/mL provides a range of signal from 12-50%, indicating the lack of sensitive detection, overlapping with the concentrations 0, 5, and 10 ng/mL (Figure 9). When testing the 5BIOTIN assay on a separate plate reader (Figure 10), all signals were negative with decreasing negativity as concentrations increased. Reasons for this could be instrumentational issues or that the quencher is working incredibly well, so the conformational change upon addition of OTA and subsequent fluorescent signal, was being quenched.

Upon further review of the Duan et al. (2011) study, I decided to incorporate the approach of heating the 96-well plate prior to assays. Heating aptamers enables unraveling and opening of conformational structure to assist in the binding of OTA and displacement of the quencher strand. Due to instrumentation downtime, this method was not attempted on the Tecan M100 Infinite, but instead evaluated on a Biotek Synergy HT plate reader. Upon heating this assay for 40 minutes at 45℃ and using the relevant percentage change in fluorescent intensity calculation, no significant change in fluorescent signal with increasing OTA concentration was detected. The percentage change in fluorescent signal for the duplex with 0 ng/mL OTA increased, more so than those duplexes spiked with 1, 5, 10, and 20 ng/mL OTA. The reason for this is unknown, but it is possible that the heating step dissociated some portion of the quencher from this duplex, therefore, increasing the fluorescent signal upon measurement.

Based on the lack of success of the 5BIOTIN assay, an alternative aptamer configuration was evaluated, specifically the addition of the biotin modification to the 3' was investigated (3BIOTIN). However, similar results were achieved in which a lack of concentration-dependency was noted.

Optically, the immobilization of aptamers to the bottom of a multiwell plate may restrict the emitted fluorescent signal, in terms of the solution dynamics within the test well. The excitation wavelength in this case needs to travel through the solution to the bottom of the well, potentially posing interference or scattering effect before reaching the aptamer duplexes. The distribution of duplexes on the bottom of the well is not known, or characterized here, and may limit emission and, thus, measured signal.

Changing the aptamer configuration to a biotin modification on the 3' end, and the fluorophore modification to the 5' end, required a new quencher sequence that would be complementary. As the quencher for the solution-based assay and the 5BIOTIN assay appeared to be functional, a similar complementary 13-base pair sequence was selected for the 3BIOTIN assay. Although a 10-base pair sequence would have been more similar to the 5BIOTIN assay, for purposes of adding the quencher (TAMRA) to the 3' end certain base pair additions were required (i.e. TAMRA requires T following). Results indicated a lack of concentration-dependent fluorescent change, potentially resulting from the quencher sequence. The quencher sequence was chosen based on functionality of the 5BIOTIN assay, however, shorter or longer sequences may be more suitable.

Finally, use of SMCC-linking chemistry was investigated. The thiol modified assay lost over 98% in signal from washing which suggests that the immobilization procedures were not successful. Immobilization issues could have resulted from the operational variables including reconstitution of aptamer in purified water, and then subsequent dilution with concentrated TCEP (to break disulfide bonds), instead of initial reconstitution in TCEP.

Taken together, immobilization techniques investigated here were not shown to be effective either in terms of sufficiently stable immobilization, or utility for detection of OTA-induced fluorescence changes of the fluorophore/quencher aptamer pairs. Subsequent studies, therefore, focused on solution-based approaches to the aptamer assays.

CHAPTER 4: APPLICATION OF APTAMER-BASED ASSAY FOR OTA TO RELEVANT MATRICES

Introduction

The overarching aim of the study was development of aptamer-based methods to quantitate OTA for application to relevant biological matrices. Included among the matrices investigated were wine, as a relevant agricultural product, and blood serum in relation to biomedical applications (i.e., detection of OTA exposure). The growing market for wine consumption, as discussed in Chapter 1, has brought around an emerging need for mycotoxin detection in wine samples. As a result of OTA in various agricultural products, this and other mycotoxins have been found in human blood serum indicating exposure, and furthermore, enabling investigation of the contribution of this exposure - and assessment of exposure levels - relative to possible health effects (e.g., carcinogenicity).

Contamination of foodstuffs is highly dependent on the type of crop, and the stages of processing. The two most common fungi species responsible for OTA production are *Aspergillus* and *Penicillium* (*11*). During the wine-making process, the key factors that influence OTA contamination is water availability, temperature, and pH (*6*) (*33*). OTA has been detected in a variety of wines around the world, although most recently, a study of U.S. wines showed both widespread occurrence, and high levels, of OTA (*17*).

With respect to human exposure, and possible health effects, OTA has been reported in blood serum, and linked to occurrence of negative health outcomes (e.g.,

nephropathy, cancer). Khlifa et al. (2008), for example, conducted a study where 105 serum samples were taken from healthy patients, and 131 serum samples were taken from nephropathic patients (reported to be related to OTA consumption), in Tunisia. Of the 105 healthy subjects, 28% tested positive for OTA with a mean of 0.49 ± 0.67 ng/mL. Of the 131 nephropathic patients, 61% tested positive for OTA with a mean of 0.98 ± 1.15 ng/mL. The study concluded that chronic interstitial nephropathy appeared to be associated with higher levels of OTA in the serum (*34*).

A variety of high-throughput approaches have been explored for effective extraction of OTA from complex matrices such as wine and fetal bovine serum. Both of these matrices present challenges. It has been previously shown, for example, that phenolics present in wines may interfere with analyses, and specifically aptamer (and immunoaffinity, e.g., ELISA) based techniques (*15*) as developed in the present study. On the other hand, it has been shown that OTA binds readily to serum albumin proteins, and represents a challenge to extraction techniques for this matrix. Alongside assessment of the aptamer-based assay (developed in the current study) for quantitative analysis of OTA in wine and blood serum, extraction methods for each matrix were evaluated by conventional methods (i.e., HPLC-FD; De Jesus et al., 2018). Taken together, the goal was to develop and assess the aptamer-based assay technique for application to these relevant biological matrices.

Materials and Methods

Materials

Fluorophore and quencher sequences were purchased from Integrated DNA Technologies (IDT) in Coralville, Iowa with HPLC purification. The fluorophore sequences are as follows: 5'-/Biotin-TEG/GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-FAM-3' (5BIOTIN), 5'-/56-FAM/ TGA TCG GGT GTG GGT GGC GTA AAG GGA GCA TCG GAC A /3BioTEG/-3' (3BIOTIN). The quencher sequences are as follows: 5'-TAMRA-TGT CCG ATG C-3' (5TAMRA) and 5' CCC ACA CCC GAT T /36-TAMSp/-3' (3TAMRA).

Pierce™ Streptavidin Coated High Capacity 96-well plates (black/black) were purchased from Thermo-Fisher Scientific, and stored at -20℃. Chemicals including magnesium chloride ($MgCl_2$), tris(2-carboxyethyl) phosphine (TCEP), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Trizma® hydrochloride (Tris-HCl), potassium chloride (KCl), and calcium chloride ($CaCl_2$) were purchased from Sigma-Aldrich (St. Louis, MO). Water was purified with a Labconco Water Pro PS system (18.2 megaohms). Ochratoxin A, 99+% was purchased from ACROS Organics™. Oligonucleotides and OTA stored -20℃. Eppendorf tubes (1.5 mL capacity) were purchased from Aquarious. Centrifuge tubes (15 mL capacity) and autosampler vials (1.8 mL capacity) were purchased from VWR International. Autosampler 12 x 32 mm screw top caps, and 200 µL glass inserts were purchased from Microsolv Technology Corporation. Solvents including acetonitrile, acetic acid, water (HPLC grade), chloroform, ethyl acetate, and hydrochloric acid were purchased from Fisher Chemical.

Internal standard for HPLC, 7-methoxycoumarin, was purchased from SigmaAldrich (St. Louis, MO).

Red wine (Yellowtail® Merlot 2015) was purchased from a local (Publix) supermarket. As a proxy for blood serum, fetal bovine serum (from USDA approved countries) was purchased from Sigma-Aldrich, and stored, until use, at -20℃.

Assay Instrumentation

Measurements were taken on a Tecan Infinite® M1000 microplate reader with excitation 495 nm and emission 520 nm. The gain was set to automatically adjust using the well with the highest signal (100 nM aptamer only), and the z-position was automatically set based on scanning volume in wells. Additionally, some measurements were taken on a Biotek Synergy 2 plate reader, whereby the filter was set to 485/20 nm excitation and 528/20 nm emission. The gain was, likewise, measured as the well with the highest signal (100nM aptamer only), and subsequently used throughout the procedure.

Aptamer Assay Procedure

Aptamer assays were generally performed as previously described (in Chapter 2) with modifications, as discussed below. Briefly, an equivalent volume of duplex was prepared for each well, and consisting of 0.5 µL of fluorophore strand (10uM) and 0.5 µL of quencher strand (20uM) added) to 48 µL of binding buffer (in a 500 µL Eppendorf tube). The duplex was heated to 88 °C for 5 minutes, and cooled at room temperature for 30 minutes. The duplex (49 µL) was added to the 96-well plate, and initial measurements were taken in 1-minute intervals for 10 minutes (stability of signal). Measurements were

taken in 1-minute intervals over 10 minutes (n = 10). Next, OTA-containing sample (i.e., spiked wine or serum, or extracts) was added in 1 µL to each well to give appropriate concentration ranges (i.e., 1 µL diluted in 49 µL of assay buffer). Measurements were taken in 1-minute intervals over 10 minutes, with the first measurement made within 60 seconds of addition of OTA to the assay.

Wine

A variety of methods were investigated for extraction of OTA, and introduction into assays. Initially, spiked wine was added directly to the assay, however, to mitigate potential interferences other methods were explored. One method included simple dilution: 450 µL of red wine was diluted to 45 mL in Tris binding buffer, creating a 1% wine solution, to dilute possibly interfering species (e.g., phenolics). Secondly, wine was extracted using a previously developed, De Jesus et al. (2018), liquid/liquid extraction (LLE) method specifically using chloroform, and reconstitution of extracted OTA in either binding buffer or organic solvent (i.e., methanol).

Recognizing that phenolics present in wines may interfere with aptamer-based detection, a method to remove phenolics from wine was investigated. Specifically, the addition of polyvinylpyrollidone (PVP) to wine samples as previously reported (*15*). Briefly, 400 µL of a 20 ug/mL solution of PVP-40 in water was added to a 2 mL aliquot of wine, shaken at room temperature for 5 minutes, centrifuged for 10 minutes at 6000 rpm, pH was adjusted to 7.5 by addition of 1M sodium hydroxide (NaOH), and filtered with 0.22 micron filter (*15*).

Fetal Bovine Serum

Fetal bovine serum (FBS) was employed to investigate the ability to extract, recover and measure OTA using the aptamer assay. Aliquots of FBS were portioned and stored in 15-mL polypropylene centrifuge tubes for subsequent studies. Sub-aliquots of FBS were spiked with relevant concentrations of 0, 50, 250, 500 and 1000 ng/mL OTA (0, 1, 5, 10, 20 final concentration in assay with 1:50 dilution) in methanol.

Two extraction methods for serum were investigated for application to the aptamer assays. The first method utilized a simple LLE with chloroform whereby a 500 µL aliquot of OTA-spiked serum was extracted with 1mL of chloroform, and subsequent operating procedures similar to that used for LLE of wine (i.e. vortex, remove supernatant, dry, reconstitute). After verifying by HPLC-FD that this method was insufficient to extract OTA from serum a multi-step extraction, previously reported (*35*), was explored. In this method, 1mL of serum spiked with OTA concentration was combined with 5 mL of a 1:1 mixture of 0.2 M magnesium chloride ($MgCl_2$) and 0.1 M hydrochloric acid (HCl), and mixed for 1 minute (*35*). After mixing, 3 mL of chloroform was added to the mixture, shaken, and centrifuged at 768xg RCF for 5 minutes. The bottom chloroform layer was removed and dried down under a stream of air. The water layer was removed and the extraction was conducted again by adding 3 mL of chloroform. The chloroform layer was removed and added to the other, dried down under a stream of air, and reconstituted with 1 mL of methanol.

HPLC-FD of OTA

To evaluate the effectiveness of extraction techniques for wine and serum, a previously described HPLC-FD method, De Jesus et al. (2018), was employed to quantify OTA. For analyses, samples were reconstituted in either binding buffer or methanol, and effectiveness of both were subsequently compared by HPLC-FD. Calibration curves, covering a relevant range of OTA concentrations, were generated. Specifically, OTA was reconstituted and diluted in 200 µL of HPLC mobile phase (i.e., water, acetonitrile and 2% glacial acetic acid, 49.5:49.5:1), and transferred to HPLC amber vials containing 200 µL inserts.

For HPLC-FD measurement of OTA, a Shimadzu Prominence UFLC system with an autosampler and fluorescence detector, and equipped with a Sonoma C18(2) column (5µm, 100 Å pore size, 250mm x 4.6mm, ES Industries, West Berlin, NJ, USA), was used. The injection volume was 20 µL, and separation was achieved by an isocratic elution (water, acetonitrile and 2% glacial acetic acid, 49.5:49.5:1) at 1.0 mL/min. Fluorescent detection was conducted using 333nm excitation and 460nm emission.

HPLC-UV/Vis/FD of Phenolics

To assess phenolics in wine and potential application of PVP for removal of phenolics(see above), in HPLC-UV/Vis/FD technique was modified from Rodriguez-Delgado et al. (2001) and employed. Briefly, 1 mL of wine was transferred to a 15 mL polypropylene centrifuge tube to which 1 mL of diethyl ether was added, followed by vortexing (10 s), and equilibration (10 min). The organic supernatant layer was removed, and dried under a stream of air. The phenolic extract was reconstituted in methanol: water

(1:1), and analyzed by HPLC-UV/VIS/FD using a Waters Nova-Pak® C16 column (4µm, 3.9 x 300mm, Ireland). Analytical separation was achieved using a gradient elution of solvent A (methanol:acetic acid:water, 10:2:88) and solvent B (methanol:acetic acid:water, 90:2:8) as follows: 0 to 15 min, 100% to 85% solvent A; 15 to 25 min, 85% to 50% solvent A; 25 to 34 min, 50% to 30% solvent A. Phenolics were measured using a photodiode array detector (200-450nm), and fluorescence detection (excitation 278nm, emission 360nm), as described (*36*).

Calculations and Statistical Analysis

All data were normalized, subtracting the buffer control signal, therefore, reducing the percent change in fluorescent intensity influenced by the buffer composition. The percent change in fluorescent intensity (RFU) was calculated as [(final-initial)/initial]*100. The LOD was calculated as 3*(std. error of y-values/slope) and the LOQ as 10*(std. error of y-values/slope). The reproducibility and repeatability were measured by the %RSD or the (standard deviation/mean) *100. Recoveries were calculated based on the linear regression of ΔRFU versus OTA concentration from calibration curves (RFU= m [OTA] + b, where m and b are slope and y-intercept of the regression), and percent recovery was calculated based on the measured versus the actual concentration of OTA. Statistical tests, including ANOVA and t-tests, were conducted using Excel (output is given in Appendices).

Results

OTA in Wine

Ability of the aptamer-based assay to measure OTA in wine was assessed in toxin-spiked wine samples. The wine used in these studies was confirmed by HPLC-FD not to have OTA aside from that spiked into aliquots. The wine was subsequently spiked with OTA at a concentration range of 0, 50, 250, 500, 1000, and 1500 ng/mL (giving final concentrations, after 1:50 dilution, of 0, 1, 5, 10, 20, and 30ppb). Initially, aliquots (1 µL) of spiked wine samples were diluted into test wells. No clear concentration dependent increase in fluorescence was observed, although, both 20 and 30 ng/mL have positive changes (Figure 14).

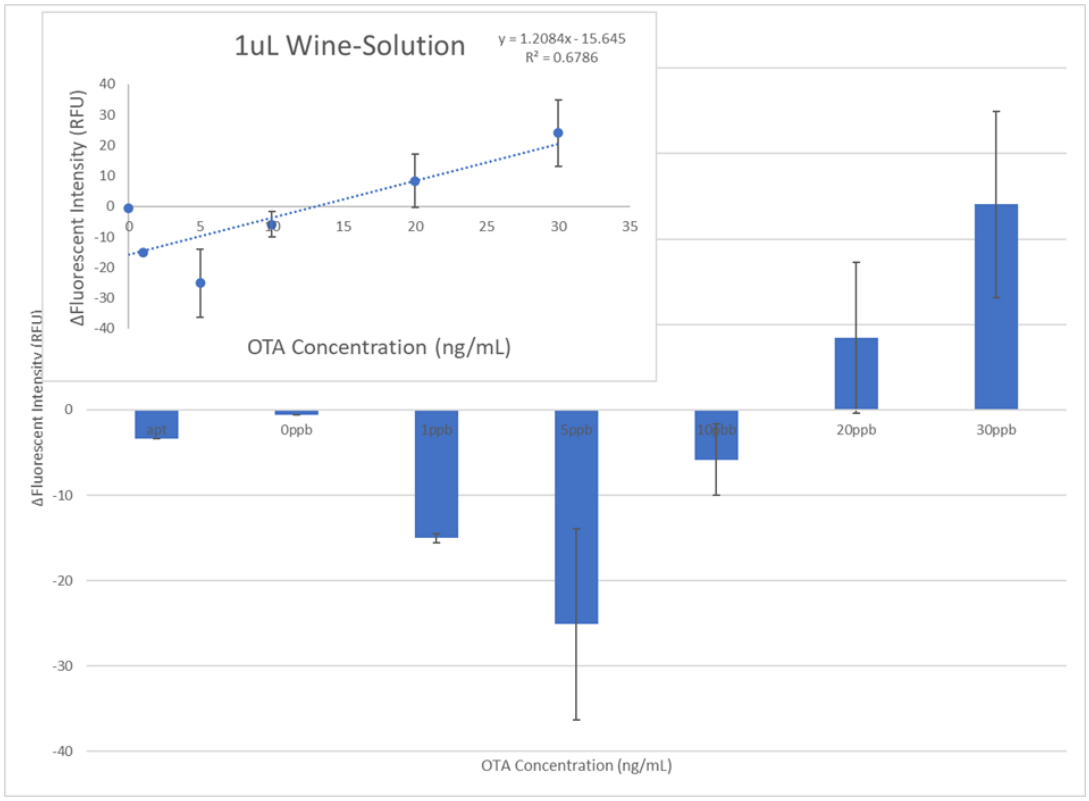

Figure 14: Wine samples (neat) spiked with OTA added to wells in 1 µL volumes

As an alternative approach, wine was subsequently diluted to 1% with binding buffer, and then spiked with varying OTA levels, which were subsequently diluted once more, upon addition to 96-well plate. Back calculating, 5 ng/mL would, therefore, correspond to a 500 ng/mL concentration of OTA in the wine. Upon addition of 10 µL of 1% wine with no OTA, there was still detectable background signal. However, there appears to be a concentration-dependent increase in the measured fluorescent response (Figure 15).

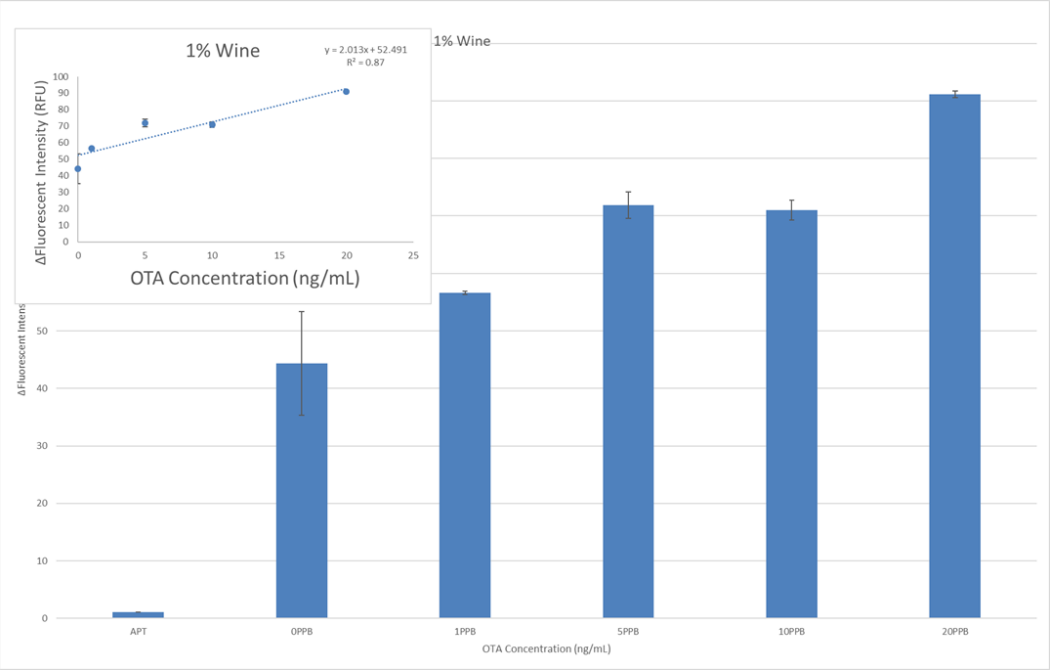

Figure 15: Wine diluted 100 fold with binding buffer and spiked with OTA

Next, wine samples were spiked and extracted following previously developed (De Jesus et al., 2018) method of LLE. Initialy, extract was reconstituted and concentrated with 100 µL of binding buffer. However, no concentration-dependent increase in fluorescence was discernible, and very high error (i.e., standard deviation) in the response was observed (Figure 16).

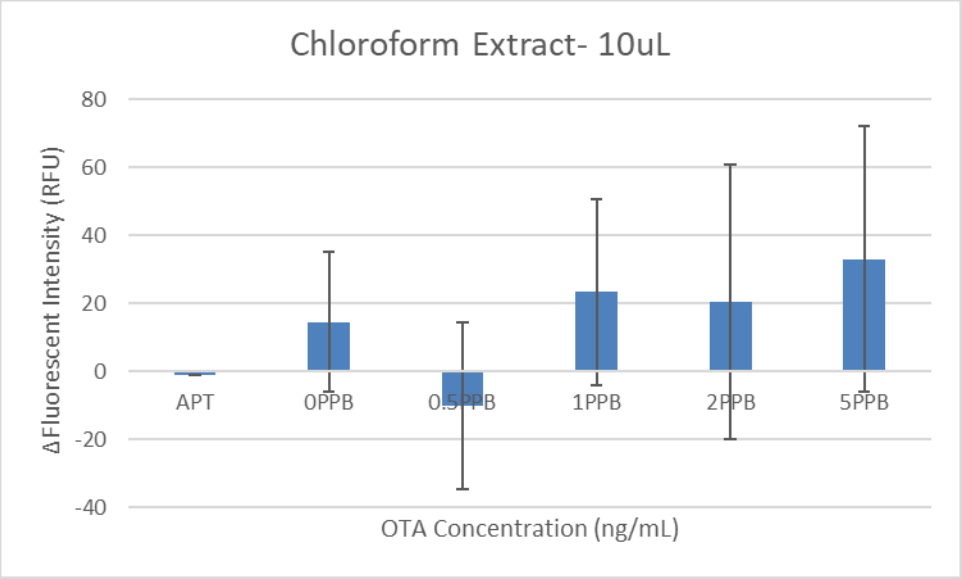

Figure 16: Extracted OTA using liquid/liquid extraction added in 10 µL volumes

To investigate the potential interference of phenolics present in wine and extracts, PVP was added to wine to remove potentially interfering phenolics as previously described as a "fining" method for wine (*15*), (*37*). Addition of PVP to wine showed no improvement of the concentration-dependency of the fluorescence response for either directly diluted wine or wine extracts (Figure 17).

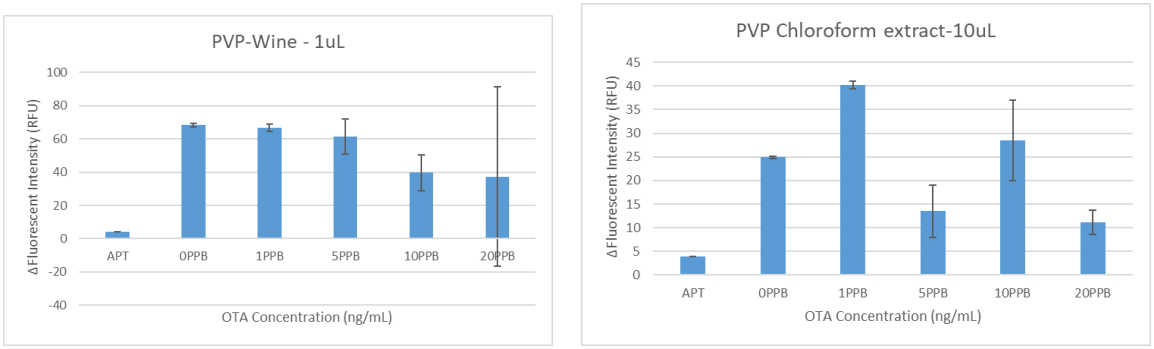

Figure 17: In solution assay using PVP/wine/chloroform extract method

To further assess the ability of PVP to reduce phenolics, HPLC-UV/Vis/FD was utilized to analyze PVP treated and untreated wine. As shown in Figure 18, the addition of PVP to wine had no discernible effects on the levels or composition of the major phenolic components.

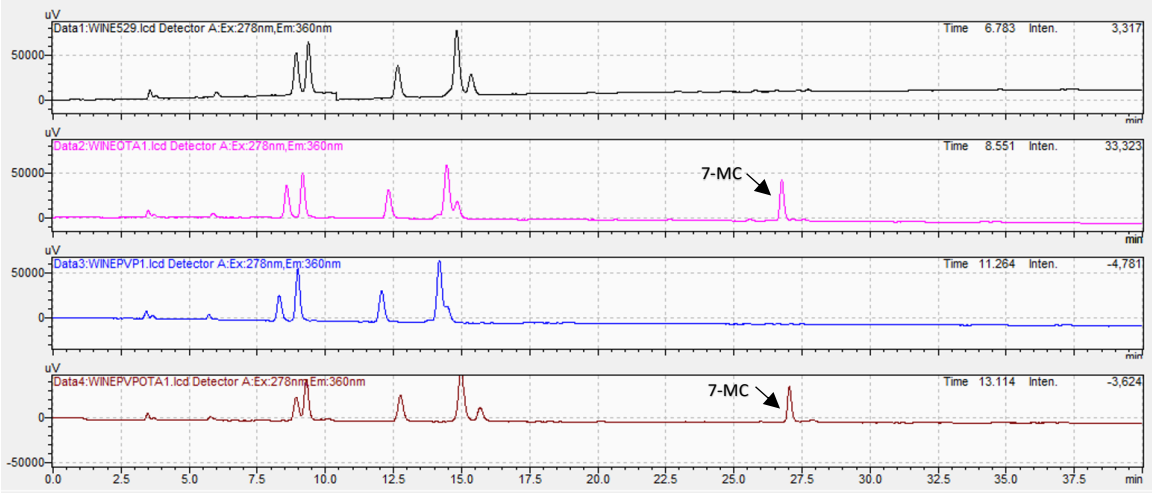

Figure 18: Individual chromatograms for phenolic separation with PVP extracted wine

Finally, to evaluate the effect of the solvent used to reconstitute OTA in wine extracts, the use of binding buffer (as initially employed) was compared, by HPLC-FD, to methanol as a recovery solvent. As shown in Figure 19, methanol was clearly a more effective solvent for recovery of OTA following extraction (corresponding chromatograms are given in Appendix 4 (Figure 25 and 26). Recoveries for methanol were approximately 78-90%, whereas when reconstituted in binding buffer, only 0-4% of OTA was recovered (Table 3).

Figure 19: Comparison of wine reconstitution with OTA calibration curve

Table 3: Recoveries of wine in methanol and binding buffer

	Concentration (ng/mL)			
Recoveries %	50	250	500	1000
Wine Meth	78.7374	82.1552	89.6555	87.3264
Wine BB	0	2.68792	2.6199	3.76197

Based on the obvious improvement of OTA recovery using methanol, the aptamer assay was repeated with extracts of wine reconstituted in methanol. A clear concentration-dependent response was observed, and a highly linear relationship (R^2= 0.97) between fluorescent change and OTA concentration was found in the relevant range of 0-20ppb (Figure 20). The calculated LOD and LOQ were found to be 4.4 ng/mL and 14.7 ng/mL, respectively, and higher than observed for OTA alone (See Chapter 2).

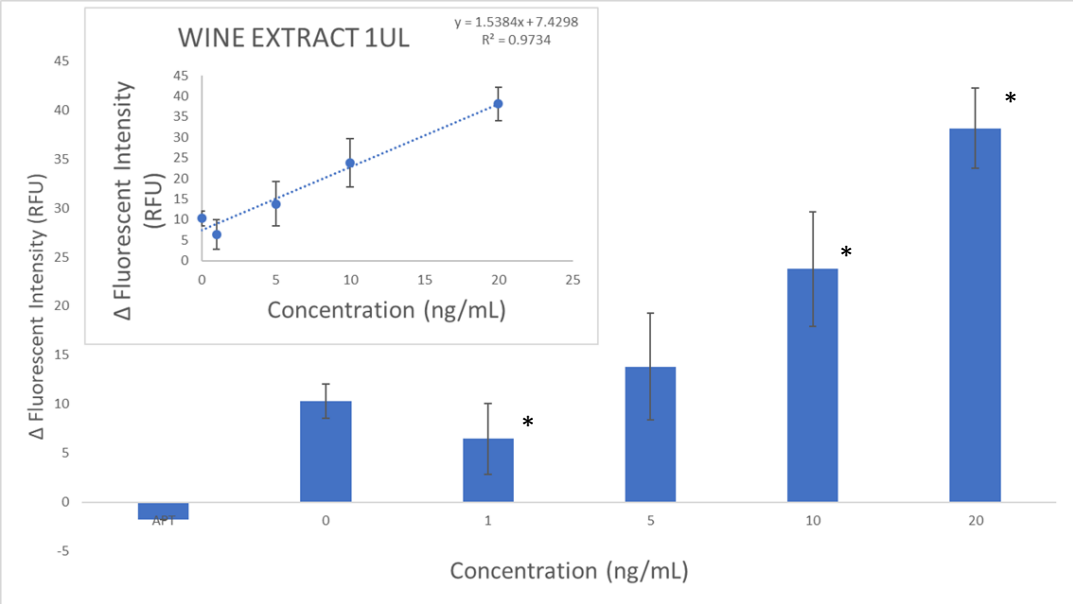

Figure 20: Bar graph showing increasing change in fluorescent intensity with increasing concentration, with calibration including dynamic range (0-20ng/mL)

OTA in Serum

As a proxy for blood serum, commercially available FBS was spiked with various concentrations of OTA (from 0, 50, 250, 500, 1000 ng/mL, and corresponding to final concentrations of 0, 1, 5, 10, 20 ng/mL in the assays). As for serum, spiked FBS (1µL) was initially diluted directly into assay wells. As shown in Figure 21, no concentration-dependent increase in fluorescent change was observed.

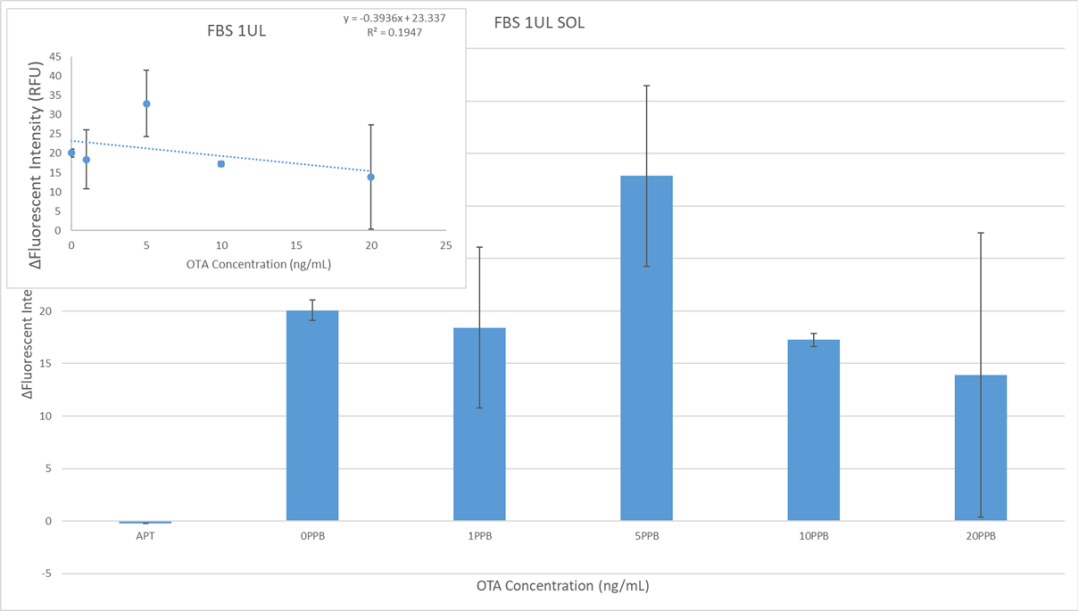

Figure 21: Change in fluorescent intensity upon addition of spiked BFS in solution-based assays for 2 replicates

Extraction of OTA-spiked serum was investigated. Initially, a simplified LLE (based on that used for wine) method with chloroform extraction and reconstitution in binding buffer, was evaluated. As shown in Figure 22, there was clearly no concentration-dependent increase in fluorescent response in the assay. Measurement of

OTA in serum extracts (redissolved in binding buffer) suggested very little recovery by this technique.

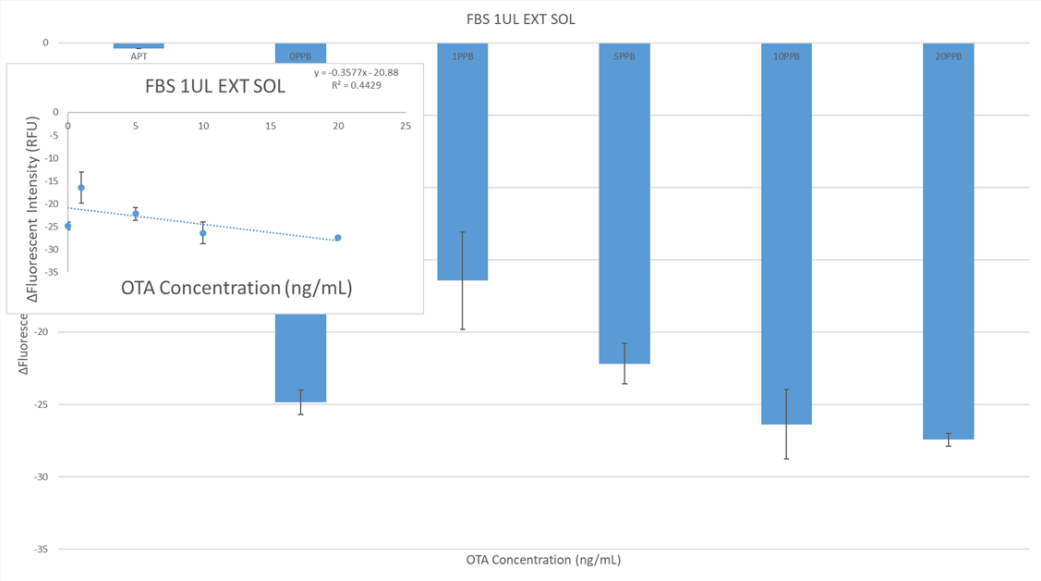

Figure 22: Change in fluorescent intensity upon addition of extracted BFS spiked samples.

It has, in fact, been found in multiple studies, Grajewski et al. (2012); Khlifa et al. (2010); Malir et al. (2013), that OTA binds readily to proteins (i.e., albumin) in serum. Accordingly, an alternative extraction method was investigated, and specifically, a previously reported method, Grajewski et al. (2012), which utilizes addition of $MgCl_2$ and acidification (with HCl) prior to chloroform extraction. Samples were reconstituted in either binding buffer or methanol and analyzed for OTA by HPLC-FD to compare recoveries (Figure 23). Observed recovery of OTA by either binding buffer or methanol was, however, quite low; although, the use of methanol was more effective (12-27%) compared to binding buffer (1-3%) (Figure 23 and Table 4).

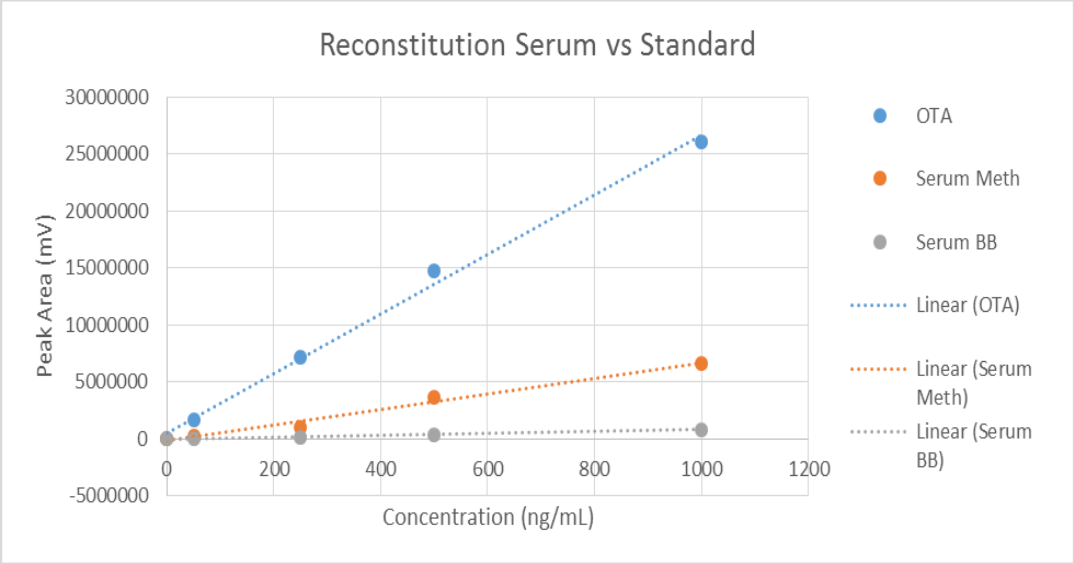

Figure 23: Comparison of serum reconstitution with OTA calibration curve

Table 4: Recoveries of serum in methanol and binding buffer

	Concentration (ng/mL)			
Recoveries %	50	250	500	1000
Serum Meth	12.4381	14.2197	26.8138	24.9787
Serum BB	2.40254	1.26028	2.30345	3.11586

The aptamer assay was repeated with serum that was extracted (with addition of $MgCl_2$ and acidification), and specifically reconstituted in methanol. A linear concentration-dependent increase in fluorescent response was observed (R^2=0.93) over the relevant range of 0-20 ppb, however, calculated LOD (8.0ng/mL) and LOQ (26.8 ng/mL) were higher than observed for wine (Figure 24). Corresponding chromatograms of samples in binding buffer and methanol are given in Appendix 4 (Figure 27 and 28).

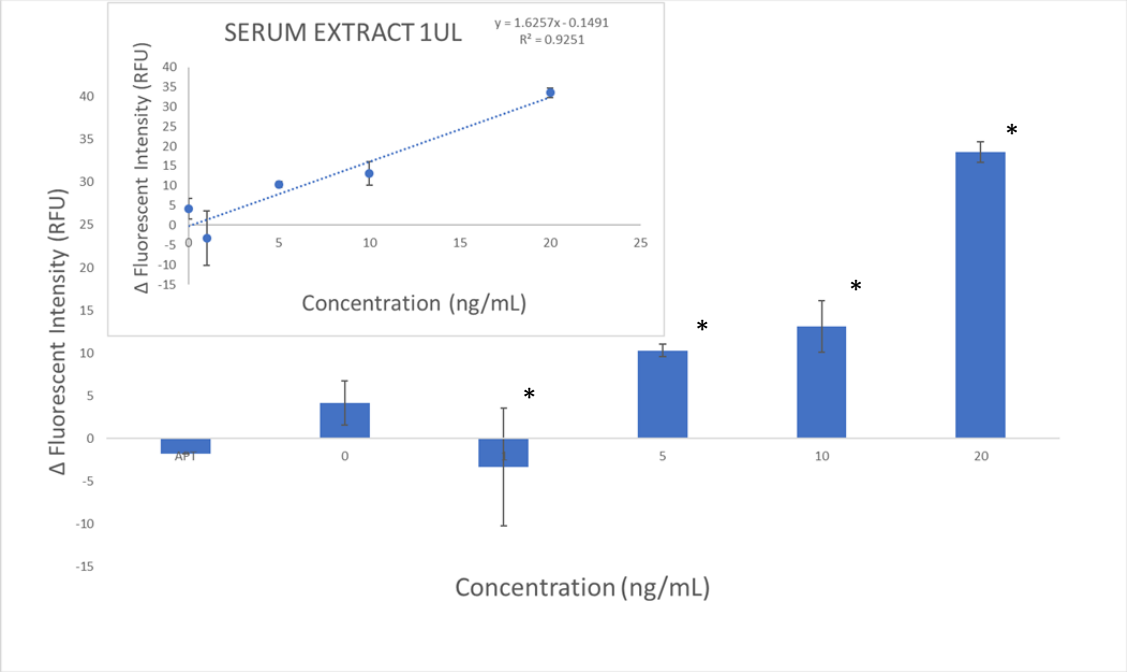

Figure 24: Bar graph showing increasing change in fluorescent intensity with increasing concentration, with calibration including dynamic range (0-20ng/mL)

Discussion

Toward adaptation of the aptamer-based assay, previously developed in this research, for high-throughput analysis of OTA in relevant biological matrices, the assay was evaluated with respect to wine, as a relevant agricultural product, and blood serum (i.e., FBS), as a biomedically relevant matrix. These matrices were found to present multiple challenges to the proposed assay approach. Although some of these challenges were overcome by relatively simple extraction and other sample preparation techniques, a number of limitations to these methods were found.

OTA in Wine

Several strategies for assay-based analysis of OTA in wine were explored including direct introduction into assays, dilution (in assay buffer) prior to assays,

removal of interfering phenolics (by PVP prior to assays), and finally simplified extraction and recovery of OTA from wine. Direct introduction of wine did not provide a concentration-dependent response in the assays (Figure 14). It has been previously reported, Vidal et al. (2012); Mavrikou et al. (2018), that phenolics (e.g., flavonoids, phenolic acids) from grapes which contribute both "color" and other characteristics to the wine that can interfere with aptamer-based detection, and in fact, similar affinity-based methods (e.g., ELISA). Interference, in the current context, could include both cross-reactivity of aptamers with phenolics, and possible overlap in the fluorescence excitation/emission wavelengths between phenolics and fluorophore (i.e., FAM) used in the assay. With respect to the latter point, anthocyanins, which are primary phenolics associated with wine color, absorb in the visible range (465-560nm), as well as UV range (265-275nm) (*38*), and would likely interfere with excitation/emission wavelengths used in the assay (i.e., 495 and 520nm, respectively). The use of PVP which has been shown to bind and remove phenolics was investigated as a means to reduce these interfering compounds; however, subsequent HPLC-FD analysis suggested little capacity to remove phenolics.

Dilution of wine (in binding buffer) prior to assays did enable relatively precise concentration-dependent response in the assay (Figure 15). However, this approach requires a 100-fold dilution of wine and would likely limit the effectiveness of the assay with respect to environmental and biomedically relevant OTA concentrations which are typically in low (0-10) ppb ranges. In the case of wines, for example, the currently established EU regulatory limit is 2ppb.

Finally, extraction of OTA from wine was investigated and specifically a simplified LLE method previously developed for application to HPLC-FD (*17*). In addition, the suitability of recovery solvent (use to reconstitute extracts) was assessed. It was found that a simple LLE with chloroform was, when coupled to OTA reconstitution in methanol, relatively effective in the recovery of OTA from wine (Figure 19), and furthermore, enabled concentration-dependent fluorescent response in the aptamer assay (and thus, potentially quantitative application) for OTA in wine (Figure 20). However, sensitivity was approximately 2-fold lower, based on calculated LOD/LOQ, compared to results observed for OTA alone (in development of the assay; See Chapter 2).

OTA in Serum

As a biomedically and forensically relevant sample application, blood serum (FBS) was evaluated as a sample matrix. OTA has been found in human serum in association with exposure to OTA via contaminated food or other agricultural products and in relation to asserted human health effects (e.g. nephropathy, cancer) (*1*), (*5*), (*9*), (*10*), (*31*).

Similar to wine, introduction of OTA-spiked FBS by both direct dilution into assays, and following solvent extraction, were evaluated. Direct introduction (dilution) into assays was clearly unable to illicit a concentration-dependent (and, therefore, quantitative) response in assays (Figure 21). Although a relatively simple solvent (i.e. LLE) based method for extraction was developed, and found to improve recovery and assay performance including a concentration-dependent response (Figure 24), the calculated LOD/LOQ suggest a highly reduced sensitivity of the assay (with a 4-fold

decrease, compared to OTA alone; See Chapter 2). This is likely due to the low recovery of OTA from serum (Figure 23 and Table 4). Indeed, previous studies have shown that OTA readily binds to albumin in serum and likely limits the recovery in the extraction and assay developed here. Future studies should focus on refinement of extraction techniques to overcome this limitation, and otherwise, improve sensitivity of the assay.

CHAPTER 5: CONCLUSION

Ochratoxin A is a widespread and potent mycotoxin that contaminates a wide range of crop plants, and thus, foods and other agricultural products (*29*) (*39*) (*40*) (See Chapter 1). The current research focused on development of novel and sensitive aptamer-based method for detection and quantitation of OTA in relevant sample matrices. Specifically, the approach developed herein utilized a fluorescence quenching of complementary oligonucleotide aptamers, and more specifically, adaptation of this approach to a high-throughput (e.g., 96-well plate) format. A solution-based aptamer assay showed promising results (Chapter 2) with a calculated LOD of approximately 2.7 ng/mL and LOQ of 8.9 ng/mL which would potentially enable detection and quantitation in parts-per-billion concentration ranges relevant to applications for assessment of OTA in agricultural products, medically and/or forensically relevant samples.

Techniques for immobilization of aptamers in multiwell plate formats were investigated (Chapter 3), specifically as a means to integrate sample "clean-up" (via "target trapping" and/or removal interfering species by washing steps). Approaches included the use of biotin/streptavidin binding to test plates, and SMCC-based chemistry for covalent binding to plates. None of the approaches provided reproducible, or even concentration-dependent, responses in assay upon addition of OTA. Among other explanations (e.g., apparent lack of effective immobilization by SMCC), it was concluded that limitations of fluorescence detection in assays may relate to the optical properties and specifically efficacy of plate readers for fluorometric measurements, of surface

immobilized nature of the aptamers. Future studies will need to explore this methodological aspect.

Application of the aptamer-based assay was evaluated for relevant matrices including wine and serum (Chapter 4). It was found that extraction from either matrix was required. Extraction techniques that were applied did enable quantitative utility of the assay. However, sensitivity in both cases was lower than expected (from assay development studies; Chapter 2). Calculated limits of detection, and quantitation, were in the ppb range (i.e., LOD= 4.4 ng/mL and 8.0 ng/mL, respectively, for wine and serum), however, were lower (2- and 4- fold respectively, for wine and serum) than OTA alone. This is likely due to the relatively low recovery of OTA (> 90% and > 25% for wine and serum, respectively) afforded by solvent extraction and subsequent reconstitution steps.

Recommendations for future work would include optimization of extraction and sample preparation methods for wine and serum (and other future applications). In addition, the limited potentially "optimistic" result for test formats based on immobilized aptamers would also suggest future value, continue investigation of this approach which would, likewise, potentially improve efficacy of the assay to agricultural, biomedical, forensic, and other (e.g., environmental) applications.

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APPENDICES

Appendix 1

Name/ Purpose	Fluorophore	Quencher
SOLUTION	5’- GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA /36-FAM/-3’	5’-/55-TAMK/ TGT CCG ATG C-3’
5BIOTIN/5TAMRA	5’-/5BiotinTEG/ GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA /36-FAM/-3’	5’-/55-TAMK/ TGT CCG ATG C-3’
3BIOTIN/ 3TAMRA	5’/56-FAM/TGA TCG GGT GTG GGT GGC GTA AAG GGA GCA TCG GAC A/3BioTEG/-3’	5’ CCC ACA CCC GAT T/36-TAMSp/-3’
Cy5/IAbRQ	5’- GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA /3Cy5Sp/-3’	5’-/IAbRQ/ TGT CCG ATG C-3’
Cy5/IAbRQ BIOTINYLATED	5’-/5BiotinTEG/ GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA /3Cy5Sp/-3’	5’-/IAbRQ/ TGT CCG ATG C-3’
THIOL	5’-/5ThioMC6-D/ GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA /36-FAM/-3’	5’-/55-TAMK/ TGT CCG ATG C-3’
MICROCYSTIN	5’- GGC GCC AAA CAG GAC CAC CAT GAC AAT TAC CCA TAC CAC CTC ATT ATG CCC CAT CTC-/36-FAM/-3’	5’-/55TAMK/ GCG GAG ATG G- 3’

Appendix 2

Table 5: ANOVA to determine significant differences between the percent change in fluorescent intensity for spiked OTA concentrations of 0, 1, 2, 5, 10, 15, and 20 ng/mL.

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
0ppb	5	30.2742	6.05484	1.786239		
1ppb	5	38.27047	7.654094	0.24914		
2ppb	5	43.34975	8.66995	1.744987		
5ppb	5	66.73838	13.34768	0.175141		
10ppb	5	74.19832	14.83966	2.458706		
15ppb	5	63.8488	12.76976	29.25187		
20ppb	5	85.54322	17.10864	2.030175		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	500.4297	6	83.40495	15.48787	9.39E-08	2.445259
Within Groups	150.785	28	5.385179			
Total	651.2147	34				

Table 6: t-test difference between 0 and 1 ng/mL (left) and t-test difference between 1-5 ng/mL (right)

t-Test: Two-Sample Assuming Equal Variances		
	0ppb	*1ppb*
Mean	6.05484	7.654094
Variance	1.786239	0.24914
Observati	5	5
Pooled Va	1.017689	
Hypothesi	0	
df	8	
t Stat	-2.50657	
P(T<=t) on	0.018283	
t Critical o	1.859548	
P(T<=t) tw	0.036566	
t Critical t	2.306004	

t-Test: Two-Sample Assuming Equal Variances		
	1ppb	*5ppb*
Mean	7.654094	13.34768
Variance	0.24914	0.175141
Observati	5	5
Pooled Va	0.21214	
Hypothesi	0	
df	8	
t Stat	-19.5454	
P(T<=t) on	2.44E-08	
t Critical o	1.859548	
P(T<=t) tw	4.88E-08	
t Critical t	2.306004	

Table 7: t-test difference between 1 and 15 ng/mL (left) and t-test difference between 1 and 20ng/mL (right)

t-Test: Two-Sample Assuming Equal Variances					
	15ppb	*1ppb*			
Mean	12.76976	7.654094			
Variance	29.25187	0.24914			
Observati	5	5			
Pooled Va	14.7505				
Hypothesi	0				
df	8				
t Stat	2.10605				
P(T<=t) on	0.034146				
t Critical o	1.859548				
P(T<=t) tw	0.068292				
t Critical tv	2.306004				

t-Test: Two-Sample Assuming Equal Variances					
	20ppb	*1ppb*			
Mean	17.10864	7.654094			
Variance	2.030175	0.24914			
Observati	5	5			
Pooled Va	1.139657				
Hypothesi	0				
df	8				
t Stat	14.00308				
P(T<=t) on	3.28E-07				
t Critical o	1.859548				
P(T<=t) tw	6.56E-07				
t Critical tv	2.306004				

Appendix 3

Table 8: ANOVA to determine significant differences between the percent change in fluorescent intensity for various spiked OTA concentrations of 0, 1,5, 10, and 20 ng/mL for the 5BIOTIN assay.

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
0ppb	2	25.8528	12.9264	13.20752		
1ppb	2	34.85917	17.42958	31.01861		
5ppb	2	26.22408	13.11204	2.276456		
10ppb	2	27.76077	13.88038	15.95848		
20ppb	2	62.94928	31.47464	341.9591		
ANOVA						
ce of Varia	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between	496.4382	4	124.1096	1.534414	0.321066	5.192168
Within Gr	404.4202	5	80.88403			
Total	900.8584	9				

Table 9: ANOVA to determine significant differences between the percent change in fluorescent intensity for various spiked OTA concentrations of 0, 1,5, 10, and 20 ng/mL for the 3BIOTIN assay.

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
0PPB	2	16.66571	8.332857	6.168985		
1PPB	2	16.10863	8.054316	4.607152		
5PPB	2	19.63154	9.815768	0.160996		
10PPB	2	25.44309	12.72155	9.580523		
20PPB	2	27.08687	13.54343	7.4149		
ANOVA						
ce of Varia	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between	50.6872	4	12.6718	2.268285	0.19651	5.192168
Within Gr	27.93256	5	5.586511			
Total	78.61976	9				

Table 10: Two factor ANOVA output comparing Tris and HEPES buffer for 5BIOTIN assay

Anova: Two-Factor With Replication						
SUMMARY	0PPB	0.5PPB	1PPB	2PPB	5PPB	Total
Tris						
Count	2	2	2	2	2	10
Sum	15.14871	15.96287	16.24895	22.35158	16.57895	86.29105
Average	7.574355	7.981435	8.124473	11.17579	8.289474	8.629105
Variance	1.55071	15.71641	0.963011	19.0507	4.124713	6.46452
HEPES						
Count	2	2	2	2	2	10
Sum	19.74325	16.14522	20.13756	22.63747	19.27326	97.93676
Average	9.871623	8.072612	10.06878	11.31874	9.636628	9.793676
Variance	2.562701	17.18127	13.5098	16.09151	1.892931	6.891866
Total						
Count	4	4	4	4	4	
Sum	34.89196	32.10809	36.3865	44.98905	35.8522	
Average	8.722989	8.027024	9.096625	11.24726	8.963051	
Variance	3.130283	10.96867	6.084378	11.72088	2.610823	
ANOVA						
ce of Varia	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Buffer	6.781122	1	6.781122	0.731957	0.412279	4.964603
Concentra	23.44351	4	5.860879	0.632625	0.650574	3.47805
Interactio	4.12021	4	1.030052	0.111184	0.975748	3.47805
Within	92.64375	10	9.264375			
Total	126.9886	19				

Appendix 4

Table 11: t-test difference between 20 and 30 ng/mL spiked wine in solution-based assay

t-Test: Two-Sample Assuming Equal Variances				
	30PPB	*20PPB*		
Mean	24.04006	8.409634		
Variance	118.9755	78.43149		
Observati	2	2		
Pooled Va	98.70349			
Hypothesi	0			
df	2			
t Stat	1.573274			
P(T<=t) on	0.128149			
t Critical o	2.919986			
P(T<=t) tw	0.256299			
t Critical t	4.302653			

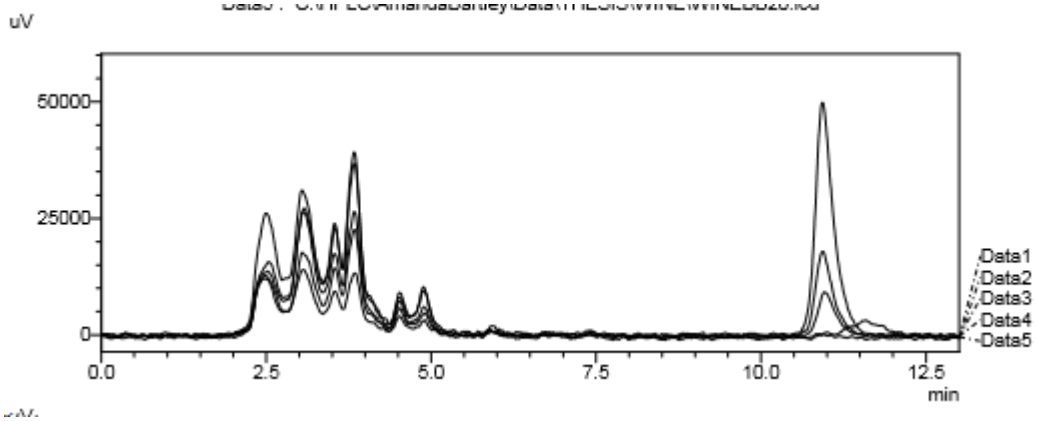

Figure 25: Chromatogram of wine reconstituted in binding buffer

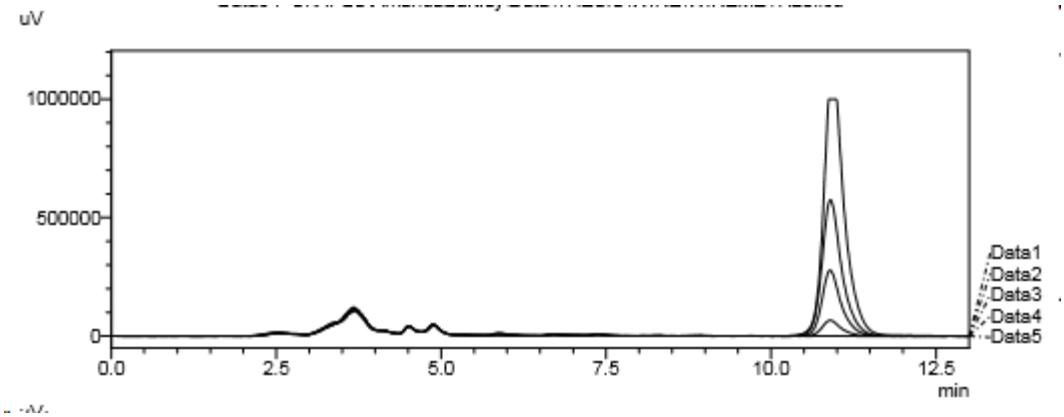

Figure 26: Chromatogram of wine reconstituted in methanol

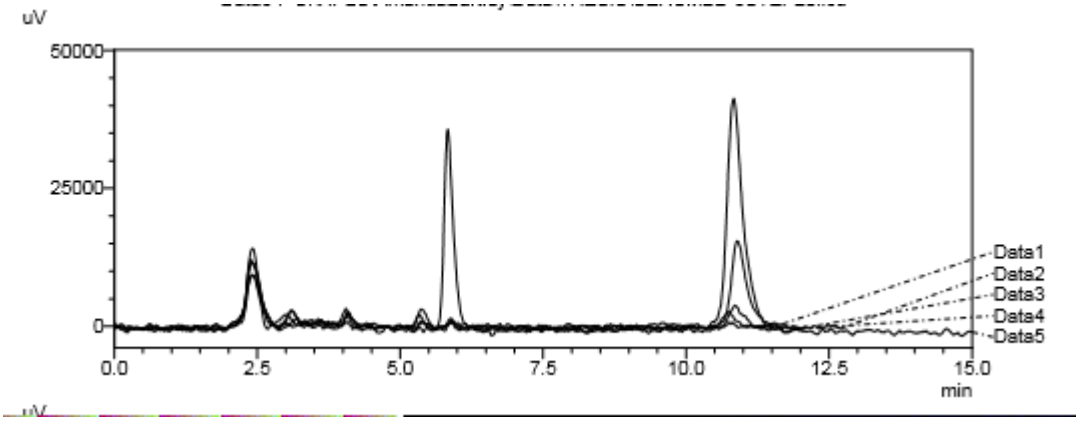

Figure 27: Chromatogram of serum reconstituted in binding buffer

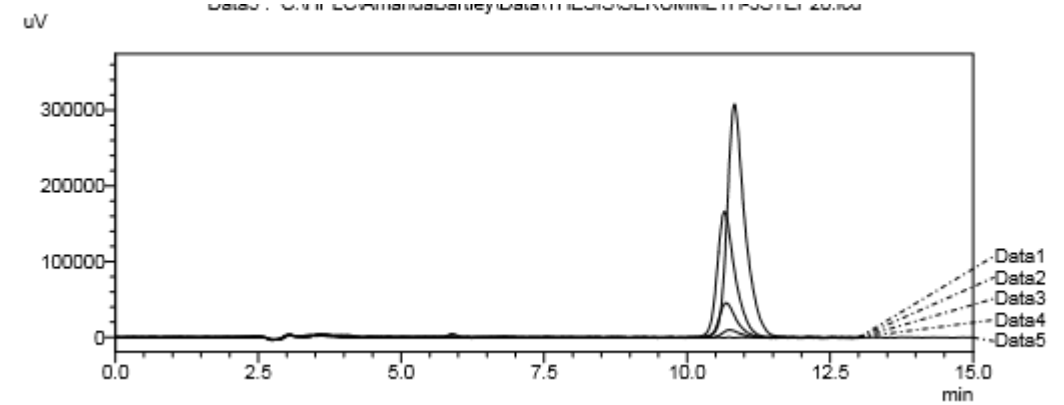

Figure 28: Chromatogram of serum reconstituted in methanol