REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
30-08-2016	Final Report	1-Sep-2015 - 31-May-2016

4. TITLE AND SUBTITLE
Final Report: Atmospheric Nitration of Proteins in Urban Air and Links to Agent Detection Strategies

5a. CONTRACT NUMBER
W911NF-15-1-0416

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
611102

6. AUTHORS
John A. Huffman

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
University of Denver
2199 S. University Blvd.

Denver, CO 80208 -4711

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS (ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
67813-EV-II.1

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
Urban pollutants O3 and NO2 can nitrate proteins in the atmosphere by forming 3-nitrotyrosine, and it has been shown that allergies can be heightened by nitrated proteins. The optical properties of nitrated proteins have also been shown to change upon nitration, and these reactions could influence how fluorescence is used to detect bioparticles threat agents. Here we developed a new procedure to selectively detect nitrated Amb-a1 protein using a sandwich ELISA protocol. Second, we extracted measurable amounts of protein from pollen spiked onto filters. A lower limit of ~10% of protein mass extracted from Ambrosia artemisiifolia (ragweed) pollen was the highly

15. SUBJECT TERMS
protein nitration, atmospheric oxidation, ELISA, quantification, immunoassay, pollen, fluorescence, spectroscopy, bioaerosol, WIBS, allergies, biowarfare threat agents, ambrosia, ragweed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			John Huffman
UU	UU	UU	UU		19b. TELEPHONE NUMBER 303-871-4404

Report Title

Final Report: Atmospheric Nitration of Proteins in Urban Air and Links to Agent Detection Strategies

ABSTRACT

Urban pollutants O3 and NO2 can nitrate proteins in the atmosphere by forming 3-nitrotyrosine, and it has been shown that allergies can be heightened by nitrated proteins. The optical properties of nitrated proteins have also been shown to change upon nitration, and these reactions could influence how fluorescence is used to detect bioparticles threat agents. Here we developed a new procedure to selectively detect nitrated Amb-a1 protein using a sandwich ELISA protocol. Second, we extracted measurable amounts of protein from pollen spiked onto filters. A lower limit of ~1% of protein mass extracted from Ambrosia artemisiifolia (ragweed) pollen was the highly allergenic Amb-a1 protein. These efforts culminated in a proof-of-concept study to investigate whether polluted air could sufficiently nitrate pollen proteins. We exposed ragweed pollen and Amb-a1 to mildly polluted outdoor air (22 ppb NO2, 49 ppb O3) for 7 days. Using the sandwich ELISA we show that ~1% of protein mass extracted from spiked filters of pollen was Amb-a1 nitrated by ambient air. This suggests that atmospheric reactions may be able to nitrate anemophilous pollen protein sufficiently to influence human allergies. Lastly, we showed dramatic reduction in fluorescence intensity of four proteins nitrated by an aqueous nitration reaction.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received	Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received	Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

B. Swanson, K. Pierce, A. Alhalwani, E. Biesiada, A. Dondero, M. Gosselin, N. Savage, J. A. Huffman, Analysis of optical properties of nitrated proteins potentially relevant to the atmosphere, 35th Annual Meeting of the American Association of Aerosol Research (AAAR), Portland, OR, October 2016.

Number of Presentations: 1.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received	Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Nicole J. Savage	0.00	
Benjamin E. Swanson	0.33	
Marie I. Gosselin	0.67	
FTE Equivalent:	**1.00**	
Total Number:	**3**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
John A. Huffman	0.12	
FTE Equivalent:	**0.12**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Kyle Pierce	0.93	Chemistry
Emmalee Biesiada	0.00	Chemistry
Anna Dondero	0.00	Chemistry
FTE Equivalent:	**0.93**	
Total Number:	**3**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	2.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	2.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	2.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):.....	2.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:.....	1.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attachment

Technology Transfer

1. Foreword

n/a

2. Table of Contents

n/a

3. List of Appendixes, Illustrations and Tables

Table 1	Ambient pollen and protein exposure	Page 5
Reaction 1	Heterogeneous nitration of tyrosine with O_3 and NO_2	Page 2
Reaction 2	Aqueous nitration of tyrosine with TNM	Page 2
Figure 1	ELISA calibration curves for nBSA	Page 3
Figure 2	ELISA calibration curves for Amb and nAmb	Page 4
Figure 3	HPLC calibration curve for BSA	Page 4
Figure 4	Schematic flow diagram for ambient exposure of pollen	Page 5
Figure 5	UV-vis and fluorescence spectra of native and nitrated proteins	Page 7
Figure 6	Changes in UV-vis absorption spectra of nBSA with time	Page 8
Figure 7	Changes in WIBS fluorescence intensity of Amb with nitration	Page 8

4. Statement of Problem Studied

Urban areas worldwide experience ubiquitous traffic pollution that often leads to high concentrations of gas-phase secondary pollutants such as ozone (O_3) as well as nitrogen dioxide (NO_2) and other oxides of nitrogen produced primarily from vehicle exhaust. These pollutants can serve directly as irritants to humans, animals, and vegetation, but also participate in oxidative gas- or heterogeneous-phase chemistry. The role that nitrogen oxide chemistry plays in reactions on biogenic particulate matter in the atmosphere is significantly less studied than the role of ozone [e.g. 1, 2]. There is evidence, however, that NO_2 combined with O_3 in polluted environments may play a critical role in the nitration of proteins on the surface of pollen and other bioparticles [3, 4]. It has been shown that human interaction with nitrated proteins produces a significantly heightened allergenic response compared to native proteins [5]. The optical properties of nitrated proteins have also been shown to be altered compared to native proteins, and it is therefore likely that these reactions could influence the use of fluorescence to detect micron-sized bioparticles, including threat agents [6]. We proposed two main project areas to be investigated during the 9-month exploratory period. These were to: (A) develop a protocol by which we may quantify the concentration of nitrated proteins suspended in the atmosphere, and (B) investigate changes in optical properties of a select set of nitrated proteins and bioparticles. Four specific goals were outlined and outcomes are discussed. Over the short project period we succeeded in achieving initial results in all major areas proposed and have momentum that we anticipate will carry us forward towards further understanding. Note that specific goal numbers listed below are ordered based on the proposal text, but are addressed here in a modified order.

5. Summary of the Most Important Results

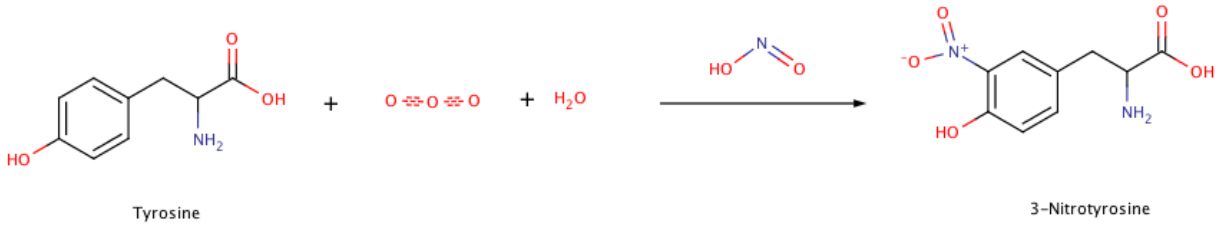
Project Area A: Protocol Development

The first step in the exploratory project was to develop and optimize protocols for nitration of proteins in the lab and for nitrated protein detection. To maximize available financial and equipment resources, the relatively simple and inexpensive detection technique of enzyme-linked immunosorbent assay (ELISA) was chosen for method development. ELISA is a wet chemical binding technique in which either one or two protein antibodies are utilized to selectively capture a specific protein, or alternatively its nitrated analog. For the STIR phase of the project we focused on the protein Amb a 1 from *Ambrosia artemisiifolia* (ragweed) pollen, because the pollen is one of the most ubiquitous and allergenic

anemophilous (airborne) pollen species in both the United States and Europe [7, 8]. Amb a 1 has been identified as the key allergen in the pollen and contains 6 tyrosine residues, similar to the well-studied Bet v1 protein from birch pollen, which contains 7 tyrosine residues [9, 10].

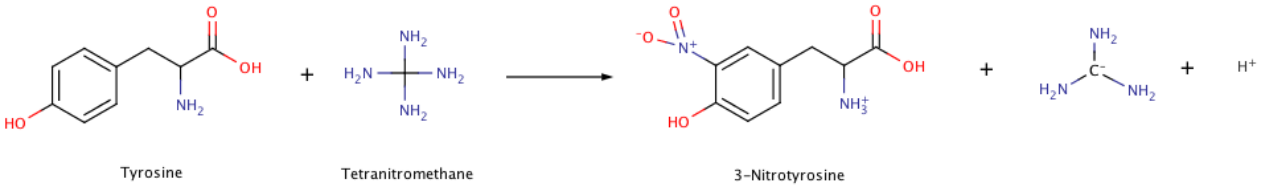
Goal A-0: Nitrate protein in the laboratory

The first step in the process of detecting nitrated protein was to optimize an aqueous-phase nitration reaction. The heterogeneous nitration mechanism utilizing atmospherically relevant concentrations of O_3 and NO_2 has been theorized as the pathway by which proteins are nitrated in the atmosphere. When the surface tyrosine resides are exposed to O_3 and NO_2, the tyrosine undergoes abstraction of a hydrogen atom and the addition of an NO_2 group at the three position, resulting in the 3-nitrotyrosine product (Reaction 1). This reaction does not taken place at embedded tyrosine residues, because these are sterically hindered by the surrounding conformational structure.



Reaction 1: Heterogeneous nitration of tyrosine with O_3 and NO_2

Generation of nitrated proteins by the heterogeneous mechanism described above was not practical our laboratory at this stage, however, because O_3-generation equipment and NO_2 or O_3 detection instrumentation were not available. Aqueous reaction using a mixture of tetranitromethane (TNM) and methanol also selectively produces 3-nitrotyrosine products and has been used widely as a laboratory proxy for the heterogeneous reaction [11]. So to begin our investigation of protein nitration we utilized the aqueous TNM reaction (Reaction 2) for all proteins nitrated in the lab [12].



Reaction 2: Aqueous nitration of tyrosine with TNM

Aqueous nitration of Amb and three other proteins was performed in the lab under varying reaction conditions, as will be discussed later.

Goal A-1: Develop ELISA method for protein quantification

Stated Goal #1 of the project was to determine the most efficient ELISA reaction procedures, including whether to utilize a direct or sandwich ELISA process for detection, in order to produce a reliable calibration curve to quantify both non-nitrated (Amb) and nitrated (nAmb) forms of natural Amb a 1. The direct ELISA process involves capturing a protein onto the bottom of a reaction vessel and selectively attaching a biotinylated, chromophoric antibody to the protein. Direct ELISA can be used to quantify total nitrated tyrosine on all proteins in the sample by using an antibody specific to 3-nitrotyrosine or to quantify a specific protein, such as Amb, by using a protein-specific antibody. The magnitude of the color change produced is read by a UV-vis spectrometer plate-reader (Infinite M1000 Pro, Tecan) and scales

with the concentration of protein detected. The sandwich process is similar, but involves selectively capturing a protein onto a first "capture" antibody and then attaching a second "detection" antibody (biotinylated and chromophoric). The sandwich ELISA process allows for added selectivity by requiring two stages of antibody coupling. By utilizing a capture antibody selective to a given protein and a detection antibody selective to 3-nitrotyrosine we hypothesized that we could selectively quantify the amount of nitrated Amb a 1 in a sample.

The scope of the proposed project was sufficiently broad and the timeline short enough that we were not able to support a single student full-time without interrupting the students' degree progress. As a benefit, this allowed us to begin the investigations of all goals as a team effort. The undergraduate students in our laboratory who were in charge of the ELISA portion of the project were relatively new to research involving ELISA techniques. So, the first few months of the 9-month project period were utilized getting acquainted with best practices applied to bovine serum albumin (BSA) and nitrated BSA (nBSA) as inexpensive protein targets. For example BSA can be purchase in 100 g quantities for ca. $300 (Sigma Aldrich), while Amb a 1 is purchased in 250 µg quantities for $565 (Indoor Biotechnologies).

After optimizing the TNM-based nitration procedure on a variety of proteins, we transitioned to working on ELISA protocol development. We optimized a number of ELISA procedures in the following order: (1) direct BSA, (2) direct nBSA using nitrotyrosine antibody (α-nTyr), (3) sandwich nBSA using two α-nTyr antibodies, and finally (4) sandwich nBSA using αBSA capture antibody and α-nTyr detection antibody (e.g. Figs. 1a-c). Figure 1a shows an example of a direct ELISA calibration curve for nBSA detection. Each calibration curve attached here shows the UV-vis absorbance response on the y-axis and the antigen (protein) concentration on the x-axis. The curves typically exhibit approximately sigmoidal relationships over a wide range of concentrations (two orders of magnitude or more), but the UV-vis signal typically saturates the detector before this is achieved (e.g. Fig. 1a). Of equal importance to achieving proportional detector response to varying protein concentration is the need to achieve low blank signal, which shows that the detector antibody was not bound to unexpected material to produce a false positive signal.

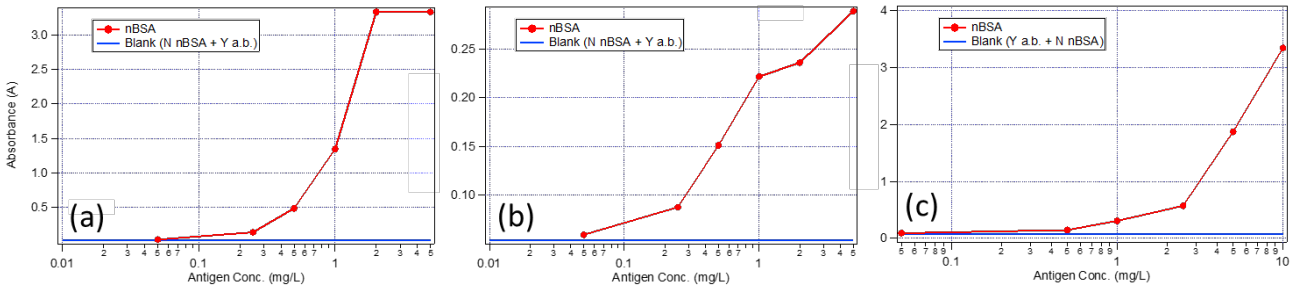


Figure 1: ELISA calibration curves for nBSA. (a) Direct, (b) Sandwich (two α-nTyr), (c) sandwich (αBSA capture, α-nTyr detection).

Once were confident that we could reliably quantify nBSA concentrations sandwich ELISA, we transitioned to Amb a 1 protein. We followed the same general pathway as with BSA, but starting with direct Amb detection and utilizing antibodies from competing vendors to optimize results. Similar to the BSA sequence, we optimized the following ELISA procedures: (1) direct Amb, (2) direct nAmb using α-Amb, (3) direct nAmb using α-nTyr, (4) sandwich Amb using two αAmb, and finally (5) sandwich nAmb using α-Amb capture and α-nTyr detection. Figures 2a-d show associated calibration curves for Amb detection. The last of these (Fig. 2d) shows one example of the sandwich ELISA for selective detection of nitrated Amb a 1, which was the most important achievement within proposed Goal #1. With a successful method developed to quantify nAmb a 1 in solution using this procedure we were able to investigate the concentration of protein in more complex samples. Each ELISA experiment requires a number of

experimental steps, and each can add uncertainty to the overall results. The overall results suggest the protocols are effective at protein quantification, but additional experimentation will be required to improve reproducibility and reduce uncertainty. For example, further investigation will be necessary to improve our understanding of dilution ratios and incubation times required at multiple steps in the process in order to optimize quantification in concentration ranges most important for nitrated products in atmospheric pollen samples.

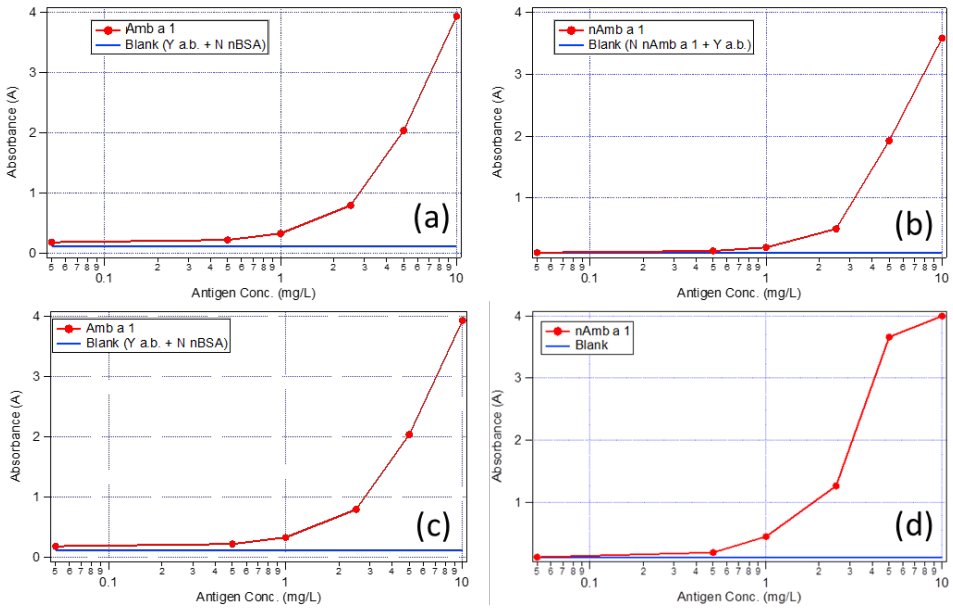


Figure 2: ELISA calibration curves for Amb a 1 and nitrated Amb a 1. (a) Direct Amb a 1, (b) Direct nAmb a 1 (α-nTyr), (c) sandwich (two α-Amb a 1), (d) sandwich (α -Amb a 1 capture, α -nTyr detection). Red line shows signal response. Blue line shows blank measurement.

Goal A-2: Develop HPLC method for measuring nitration degree

An associated sub-goal (Goal #2) was to develop an HPLC method (high-performance liquid chromatography) for measuring nitration degree. We began this process and achieved a calibration of nBSA nitration degree using HPLC (Fig. 3). As we transitioned to measure the degree of Amb a 1 nitration produced in lab, progress was limited on two fronts. The first was that the HPLC instrument developed a hardware problem and was down for more than a month. During that time we transitioned to other sub-goals of the project, but decided not to return to this task, because of the time and protein material cost that would have been required to produce large enough volumes of nitrated product (ca. 100 µg) for each of 3-6 points on the calibration curve. If funded to continue work in this area we will explore the merit of investigating the degree to which we are able to nitrate the Amb a 1 protein using both TNM and gas-phase reactions.

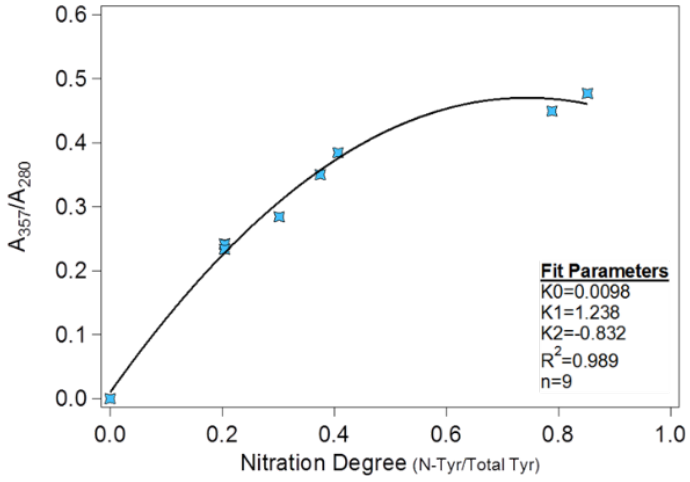


Figure 3: HPLC calibration curve showing ratio of absorptivity at 357 nm (nTyr) to absorptivity at 280 nm (Tyr) as a function of BSA nitration degree.

Project Area B: Initial Test Measurements

Goal B-4: Measure nitrated proteins on atmospheric filters

Having sufficiently optimized protocols to nitrate Amb a 1 and quantify both native and nitrated Amb a 1 protein, we turned our attention towards applying the ELISA process towards detection of proteins nitrated under atmospheric conditions. The first step here was to learn how to extract the protein from the *Ambrosia artemisiifolia* pollen grains (Bonapol, Czech Republic). Following a hybrid of literature procedures we allowed pollen grains to soak in an aqueous phosphate buffered saline (PBS) solution until the grains swelled and ruptured [4]. The mixture was filtered through 25 mm diameter polypropylene syringe filters with 0.45 µm pores (VWR) and then centrifuged using membrane filter with cut-point at 30 kDa (Merck Millipore). Pollen extracts with molecular mass >30 kDa were analyzed using the bicinchoninic acid assay (BCA, Sigma) using BSA protein standard to measure the total protein content and the Amb a 1 concentration was quantified through the direct ELISA protocol optimized previously. Using these tests we calculated that approximately 1% of the extracted pollen mass was attributable to the sum of measured proteins and that ca. 0.7% of the detected protein mass was Amb a 1. We also subjected the filter extracts of Ambrosia pollen to the TNM nitration reaction and measured the resultant concentration of nitrated Amb a 1. Using this procedure we estimated that we nitrated 28% of the tyrosine residues on the extracted Amb a 1 protein. Each of these experiments were performed only twice, however, based on the short project period, and so more optimization will be required to increase the protein yield of the extraction and to improve uncertainty through experimental replication.

Following the experiments on pollen extraction, we performed one round of proof-of-concept tests using Ambrosia pollen and Amb a 1 protein exposed to polluted ambient air. Nineteen samples of pollen and two samples of protein were spiked onto 47 mm (Whatman) and 2.1 mm (Pall) glass microfiber filters and loaded into Pall filter housings matching filter diameter (Fig. 4). HEPA-filtered ambient air from a semi-urban, high traffic road junction near Parker, Colorado was pumped across ten filters in parallel at a total flow rate of 7.8 lpm (0.5 – 01.0 lpm per filter). The approximate concentration of NO_2 was 22 ppb and of O_3 was 49 ppb. The sampling period included several ozone alert days. Direct measurements of O_3, NO_2, and relative humidity (RH) were not possible, because we did not have access to detectors for these species. Instead, we chose a sampling site that was 12 miles from an EPA sampling site and report gas concentration from the publically available EPA site as averages for the sampling period.

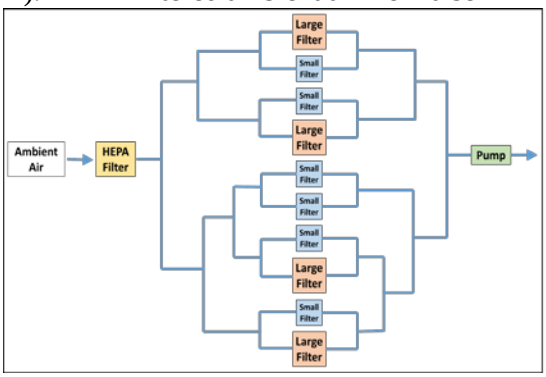


Figure 4: Schematic flow diagram for the exposure of pollen-spiked filters

After sampling was completed, filters were retrieved and subjected to extraction procedures determined earlier in the project. Extracts were analyzed for total protein (BCA assay) and nAmb a 1 (sandwich ELISA). Results are presented in Table 1 and summarized here. After analyzing three filters, an average of 85 µg protein was recovered, or approximately 9.1 mg protein per gram of pollen. Nitration by ambient air resulted in ca. 0.11 µg of nAmb a 1 per filter, or 10 mg nAmb a 1 per gram of protein detected. Exposure to pure Amb a 1 protein deposited onto the filter resulted in a higher rate of nitration, 66 mg nAmb a 1 per gram of protein detected.

	Ambrosia Pollen	Amb a 1 Protein
Total Protein Mass	80 - 91 mg	17.4 µg
Protein Mass / Pollen Mass (g/g)	0.0078 - 0.010	-
nAmb Mass (µg)	0.093 - 0.13	0.15
nAmb Mass / Protein Mass (g/g)	0.0077 - 0.011	0.066

Table 1: Summarized results filters spiked with pollen or pure protein and exposed to ambient air. Total protein mass determined by BCA assay; nAmb mass determined by sandwich ELISA.

Goal B-3: Measure protein optical properties

Four proteins were nitrated by the aqueous TNM reaction discussed: BSA, OVA (Ovalbumin from chicken egg), Amb a 1, and Asp f 1 (protein from mold allergen *aspergillus fumigatus*). The first two proteins were chosen, because they are well studied and relatively inexpensive. The second two were chosen, because they are key aeroallergens.

The UV-vis absorbance spectra and fluorescence emission spectra of each protein was measured in both native and nitrated forms, as shown in Figure 5. In all four cases, the nitrated proteins exhibited a marked drop in the absorptivity at the 350 nm peak characteristic of native tyrosine, indicating that little non-nitrated tyrosine remains on the proteins. Fluorescence emission spectra were taken of all four protein samples by scanning excitation wavelength and reporting measurements as an excitation emission matrix (EEM). Again, each of the four proteins exhibited a dramatic decrease in fluorescence emission of the peak at λ_{Ex} 280 nm, λ_{Em} 350 nm (72.7% reduction for OVA; 95.2 – 98.8% reduction for three others). Reaction conditions were approximately the same, but differed slightly, because the concentration of the purchased protein solutions varied from 0.2 mg/mL to 1.0 mg/mL. One experiment was conducted in which the timing of the timing of the BSA nitration reaction was monitored, showing that more than half of the growth in the 350 nm absorption peak occurs during the first 30 minutes, but that the peak was still growing at the last measurement point of 90 min (Fig. 6).

As a more applied investigation into the changes in fluorescence properties, nitrated BSA was aerosolized and analyzed using a wideband integrated bioaerosol sensor (WIBS) designed to detect fluorescing bioaerosols in real-time (Fig. 7). The WIBS instrument detects fluorescence of each individual particle independently in three fluorescence channels: λ_{ex} 280 nm, λ_{em} 320 – 400 nm (FL1); λ_{ex} 280 nm, λ_{em} 410 – 650 nm (FL2); and λ_{ex} 370 nm, λ_{em} 410– 650 nm (FL3). Individual particles are considered fluorescent here if they exceed fluorescent thresholds for any channel, as defined as the average baseline plus 3 standard deviations (σ) of the baseline measurement (vertical blue bars in Fig. 7) [13]. This experiment showed consistent results with the benchtop spectrofluorometer. Specifically, WIBS channel FL1, used to detect protein content and often used as a broad discriminator between bioaerosol and non-bioaerosols, showed a dramatic decrease in fluorescence upon nitration. Approximately 85% of native BSA exhibited fluorescence intensity above the baseline value, while only 3% of nitrated BSA fluoresced above the baseline, and the average intensity of the fluorescence dropped significantly as well.

From these experiments it is clear that protein sufficiently nitrated will exhibit a drastic decrease in fluorescence intensity and could be detected at a much lower efficiency by single-particle bioaerosol instruments such as the WIBS. It is important to extrapolate these results to whole bioparticles, however, such as pollen or bacterial cells. We made a first attempt to nitrate several species of pollen grains using the aqueous TNM reaction, but this effort was hampered by the fact that the pollen swelled and ruptured during the reaction. Results were inconclusive, due to the fact that the rupturing process releases particles that change the fluorescent properties. To investigate these effects in more detail it will be necessary to nitrate bioparticles via the heterogeneous $O_3 + NO_2$ reaction discussed above.

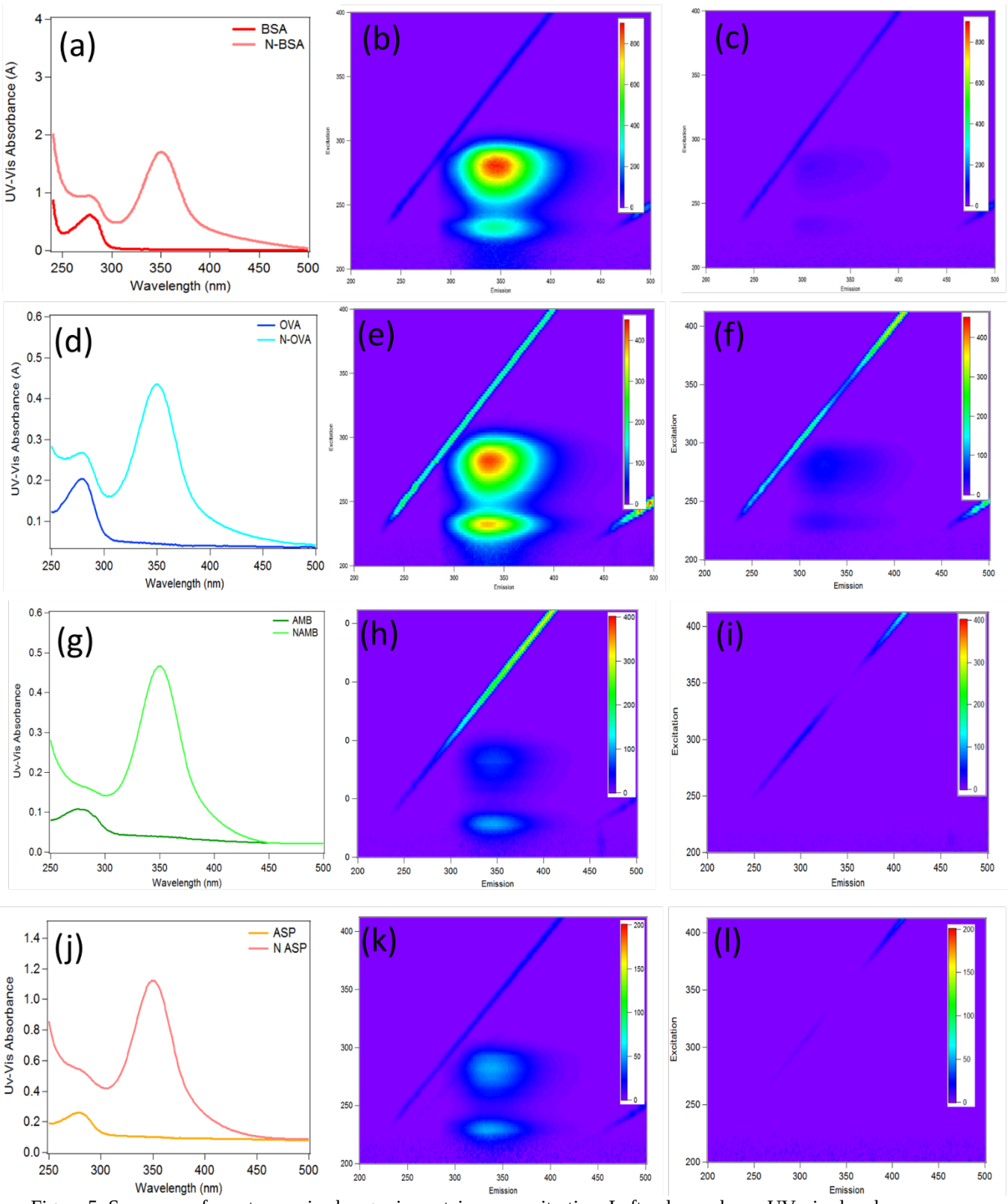


Figure 5: Summary of spectroscopic changes in proteins upon nitration. Left column shows UV-vis absorbance spectra of both native and nitrated proteins. Following columns show fluorescence EEMs of native (middle column) and nitrated (right column) protein.

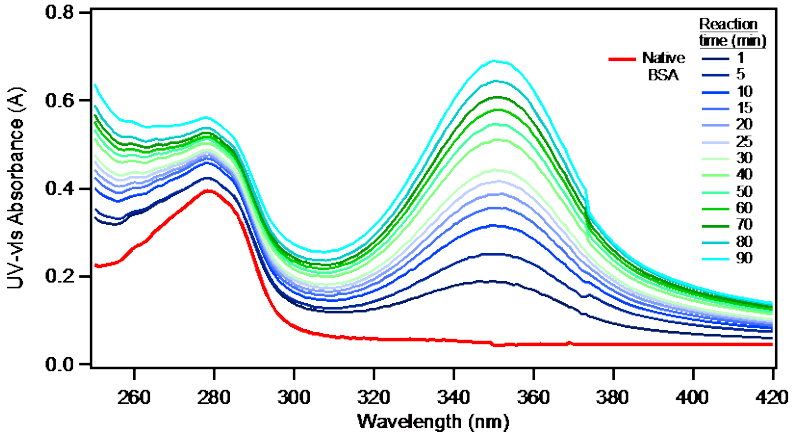


Figure 6: Changes in UV-vis absorbance spectrum of BSA changing as a result of time of aqueous TNM reaction.

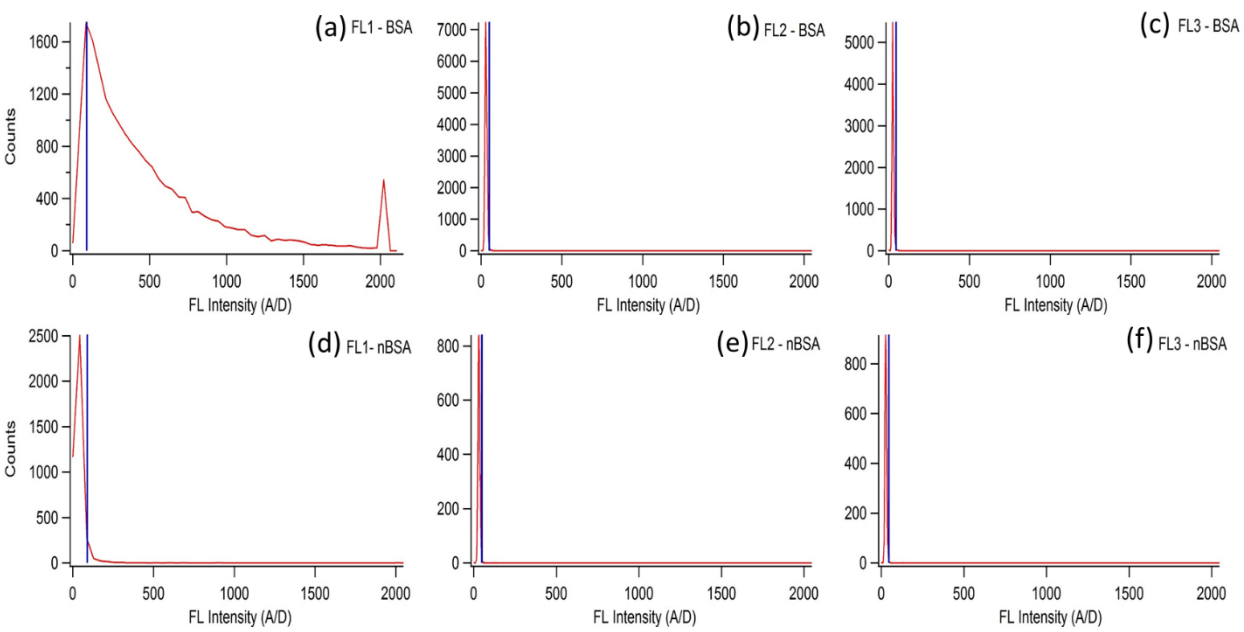


Figure 7: Changes in fluorescence observed by single particles of BSA protein measured by WIBS bioaerosol instrument. Top row shows fluorescence intensity of BSA in each of 3 WIBS channels. Bottom row shows nBSA.

Summary:

The short-term innovative research grant awarded by the ARO provided the opportunity for us to begin investigating the properties of nitrated protein that may have relevance to atmospheric chemistry, human health, and fluorescence-based detection of bioparticle threat agents. The 9-month period completed by a team of graduate and undergraduate students was sufficient to lay the groundwork for more systematic follow-up studies to further investigate how urban pollution impacts airborne proteins that affect basic atmospheric chemistry, human health, and the fluorescence-based detection of bioparticle threat agents.

The initial study of a number of diverse goals confirm several positive results. First, we were able to develop procedures to selectively detect both native and nitrated Amb a 1 protein from *Ambrosia artemisiifolia* (ragweed) pollen using ELISA. This portion of the project required many elementary steps and experimental trials, but was eventually achieved. HPLC was utilized to establish the procedure for determining the degree of nitration for BSA protein, and could be used in the future with other proteins, for example, to further understand the nitration process of the heterogeneous reaction pathway. Second,

we were able to extract measurable amounts of protein from pollen spiked on filters. A lower limit of approx. 1% of protein mass extracted was Amb a 1. Third, exposure of Ambrosia pollen and Amb protein to ambient air for 7 days was sufficient to nitrate sufficient amount of Amb a 1 protein to detect using the new sandwich ELISA. We show that approx. 1% of protein mass extracted from spiked filters of pollen was Amb a 1 nitrated by ambient air. This suggests that atmospheric reactions may indeed be able to nitrate anemophilous pollen protein sufficiently to influence human allergies. Lastly, we showed dramatic reduction in fluorescence intensity of proteins nitrated by an aqueous TNM reaction that is frequently used as a proxy for the heterogeneous reaction that is broadly accepted to produce 3-nitrotyrosine products on atmospheric particulate matter.

Follow-up studies will be required to reduce experimental uncertainty in several areas introduced here, including steps in the ELISA protocols for Amb a 1 detection and to most efficiently extract proteins from observed pollen. Further work will also be required to investigate the nitration of pure proteins and whole bioparticles after nitration using the heterogeneous O_3 and NO_2 chemical reactions that mimic real atmospheric conditions. This procedure was not possible at this stage, because O_3-generation equipment and NO_2, O_3 detection instrumentation was not available. Following proof-of-concept experiments here and using the proposed heterogeneous procedures it will be possible to test whether urban pollutant concentrations are sufficient to nitrate whole bioparticles enough to reduce their ability to be detected by real-time single-particle instruments such as the WIBS. Lastly, longer-term follow-up studies will allow collection of ambient samples of particulate matter to quantify the concentration of nitrated proteins, specifically the allergen Amb a 1, in urban air.

6. Bibliography

1. Pan, Y.-L., et al., *Effects of ozone and relative humidity on fluorescence spectra of octapeptide bioaerosol particles.* Journal of Quantitative Spectroscopy & Radiative Transfer, 2014. **133**: p. 538-550.
2. Santarpia, J.L., et al., *Changes in fluorescence spectra of bioaerosols exposed to ozone in a laboratory reaction chamber to simulate atmospheric aging.* Optics Express, 2012. **20**(28): p. 29867-29881.
3. Shiraiwa, M., et al., *Multiphase Chemical Kinetics of the Nitration of Aerosolized Protein by Ozone and Nitrogen Dioxide.* Environmental Science & Technology, 2012. **46**(12): p. 6672-6680.
4. Franze, T., et al., *Protein nitration by polluted air.* Environmental Science & Technology, 2005. **39**(6): p. 1673-1678.
5. Gruijthuijsen, Y.K., et al., *Nitration Enhances the Allergenic Potential of Proteins.* International Archives of Allergy and Immunology, 2006. **141**(3): p. 265-275.
6. Meldal, M. and K. Breddam, *Anthranilamide and nitrotyrosine as a donor-acceptor pair in internally quenched fluorescent substrates for endopeptidases: Multicolumn peptide synthesis of enzyme substrates for subtilisin carlsberg and pepsin.* Analytical Biochemistry, 1991. **195**(1): p. 141-147.
7. Csepe, Z., et al., *Predicting daily ragweed pollen concentrations using Computational Intelligence techniques over two heavily polluted areas in Europe.* Science of the Total Environment, 2014. **476**: p. 542-552.
8. Frenz, D.A., et al., *Seasonal Characteristics of Ragweed Pollen Dispersal in the United States.* Annals of Allergy Asthma & Immunology, 1995. **75**(5): p. 417-422.
9. *The University of Texas Medical Branch, SDAP - Allergen Amb a1*. 2013; Available from: http://fermi.utmb.edu/cgi-bin/SDAP/sdap_12?dB_Type=0&allid=1&seqid=15.
10. Karle, A.C., et al., *Nitration of the Pollen Allergen Bet v 1.0101 Enhances the Presentation of Bet v 1-Derived Peptides by HLA-DR on Human Dendritic Cells.* PLoS ONE, 2012. **7**(2).
11. Sokolovsky, M., J.F. Riordan, and B.L. Vallee, *Tetranitromethane. A Reagent for the Nitration of Tyrosyl Residues in Proteins**. Biochemistry, 1966. **5**(11): p. 3582-3589.
12. Franze, T., et al., *Enzyme immunoassays for the investigation of protein nitration by air pollutants.* Analyst, 2003. **128**(7): p. 824-831.
13. Gabey, A., et al., *Measurements and comparison of primary biological aerosol above and below a tropical forest canopy using a dual channel fluorescence spectrometer.* Atmospheric Chemistry and Physics, 2010. **10**(10): p. 4453-4466.