Enhancing Pesticide Exposure Monitoring in Agricultural Workers Using Dried Blood Spots and Adductomics

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Abstract

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The use of chemical agents to eradicate pests and weeds is prevalent across both developing and developed countries, and it plays a crucial role in the agricultural sector by enhancing crop yields and minimizing losses. However, the Environmental Protection Agency (EPA) estimates that over 20,000 agricultural workers in the United States suffer from pesticide poisoning annually, making them one of the most vulnerable occupations to chemical-related injuries worldwide. In light of this alarming statistic, it is imperative to monitor the exposure of workers in the agricultural industry to organophosphates (OP), which are commonly present in pesticides, to ensure their safety and well-being. Despite the importance of monitoring OP exposure, the conventional biomonitoring methods that rely on measurement of cholinesterase inhibition via the Ellman colorimetric assay have several limitations, including the requirement of measuring pre-exposure baseline activity levels, reduced accuracy at lower levels of inhibition, and a

demanding sample collection and shipping process. To address these drawbacks, this study aims to 1) evaluate the use of advanced mass spectrometry technology for biomonitoring OP exposures, which offers higher sensitivity than the standard Ellman colorimetric assay, and 2) assess the use of dried blood spots (DBS) as a biospecimen for monitoring OP exposures, which allows for simpler and less-invasive sample collection and storage.

The results obtained from the current study showed a correlation between the OP adducts in DBS and plasma samples, indicating that DBS can serve as a reliable and convenient biospecimen for biomonitoring OP exposures. Furthermore, the mass spectrometry-based analysis demonstrated higher sensitivity in detecting OP exposure compared to the standard Ellman assay. In conclusion, the use of DBS paired with OP protein adduct analyses by mass spectrometry provides a more efficient and practical method for assessing worker health and safety in the agricultural industry.

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Introduction

1.1 History of Organophosphates

Organophosphorus compounds (OPs) are a diverse group of toxic man-made substances that have various applications, ranging from pesticides and chemical weapons to natural OP toxins and anti-wear agents. Among these, OP insecticides are the most widely used chemicals for personal use and occupational applications.[1] This type of pesticide, when applied responsibly, offers several benefits, such as controlling pests and disease vectors and addressing concerns with food security and safety.

OPs trace back to the mid-19th century, with the synthesis of the first OP insecticide with anti-cholinesterase properties, called tetraethyl pyrophosphate.[2] It was not until the late 1930s that stable OP insecticides started to be marketed. Since then, hundreds of different types of OP insecticides have been commercialized worldwide, becoming one of the most used classes of pesticides.[3] The use of OP insecticides in the US has decreased by 70% since the 1960s driven by factors such as input prices, pesticide regulations, and genetically engineered seeds.[4] However, approximately 80 million pounds of OP insecticides are still sprayed annually in the United States with 75% of their use in the agricultural sector.[5] OP insecticides are a major cause of many occupational poisoning cases.[6] Consequently, farmers and other agricultural workers are at the highest risk of OP toxicity due to the continuous use and exposure to these pesticides. Occupational OP insecticide handlers are at an elevated risk of OP toxicity through dermal contact, inhalation, or accidental ingestion.[7] These exposures could occur during mixing and applying pesticides, or harvesting crops with pesticide residue, which can lead to harmful levels in the body through high exposure events or chronic lower-level exposures. In 2020, the global

agricultural worker population was approximately 860 million, with 44% of those workers experiencing pesticide poisoning each year.[8] Although there are concerns about the negative impact of OP insecticide use on human health and the environment, they continue to be widely used due to the benefits of providing relief from pests and food production efficiency for a growing human population worldwide (**Figure 1**).

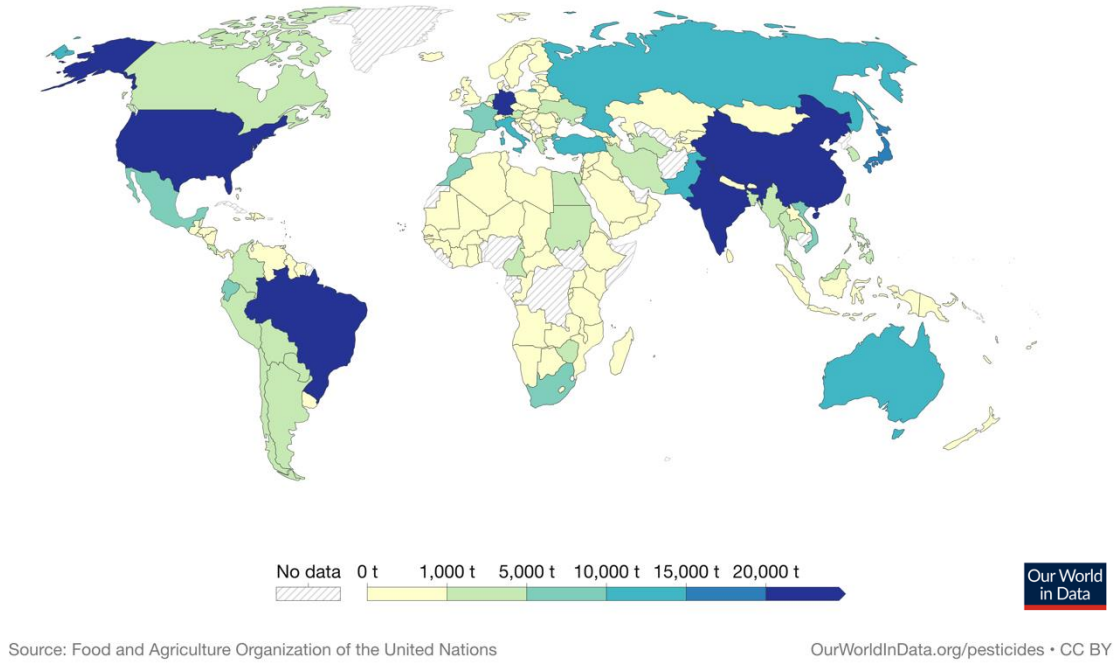

Figure 1. Insecticide use (2020). Annual quantity of insecticides used in agriculture, measured as tons of active ingredient.

Over the years, concerns have been raised about the toxicity of OP insecticides and their impact on human health and the environment. In response to these concerns, several countries worldwide have restricted the use of insecticides. Chlorpyrifos, diazinon, and malathion are among the insecticides that have been banned or have limited use due to their high application rates. For example, the European Union (EU) banned the use of chlorpyrifos in 2020, while in the United States (US), household use was restricted in 2001, with a nationwide ban in 2021.[9] Similarly, in the US, diazinon was banned for household use in 2004, and the EU prohibited its

use in 2006. Malathion is currently used in both the US and the EU under restricted conditions that were introduced in 2016 and 2010, respectively. While regulatory actions have been taken in some countries to restrict or ban insecticides such as chlorpyrifos, their continued use in other regions, particularly in developing countries, highlight the need for global efforts to address its potential adverse effects on human health and the environment (**Figure 1**). These countries often rely heavily on agricultural practices for food production and economic growth, making pesticide use prevalent. However, inadequate regulations, limited resources, and lack of awareness about the potential impacts to the environment and human health pose challenges.

Despite the bans and restrictions on OP insecticides, these chemicals continue to persist in the environment and can continue to pose a risk to human health and the ecosystem. Although OP insecticides can rapidly degrade through hydrolysis in sunlight, air, and soil, trace amounts may still be present in food and drinking water which can lead to unintended exposures.[5] Furthermore, the use of banned pesticides in other countries can result in contaminated food products being imported, further increasing the risk of exposure. The persistence of these chemicals and the potential for exposure highlights the need for ongoing monitoring and research to better understand their impacts and to develop strategies for reducing their use and exposure.

1.2 Exposure to organophosphates

OP insecticides can enter the human body through various routes, including ingestion, inhalation, and dermal absorption. Once inside the body, these pesticides undergo metabolic biotransformation predominately in the liver by cytochrome P450s to an extremely reactive and toxic oxon metabolite,[10] followed by other enzymatic metabolic processes, such as hydrolysis, mediated by esterases.[11] The metabolism of OP insecticides to oxon metabolites involves the

removal of a leaving group, resulting in a highly reactive product that readily binds to serine hydrolase enzymes, such as acetylcholinesterase (AChE), leading to inhibition of the enzyme and cascading toxic effects in the body (**Figure 2**). The inhibition of these enzymes involves the oxon metabolites inhibiting serine hydrolases by forming a covalent bond between the phosphor-alkoxy component of the OP and the hydroxyl group of the active-site serine. This interaction leads to the subsequent inactivation of serine hydrolase enzymes. Following the initial formation of the adduct between the oxon metabolite and the protein, a process known as aging occurs, leading to a structural reorganization. Aging is an irreversible process where one alkyl group dissociates over variable lengths of time, depending on the OP adduct.[12] This aging process transforms the initial protein adduct into a highly stable form, which remains in circulation and attached to the protein for an extended duration until degradation of the protein takes place, based on the protein's half-life in the body.

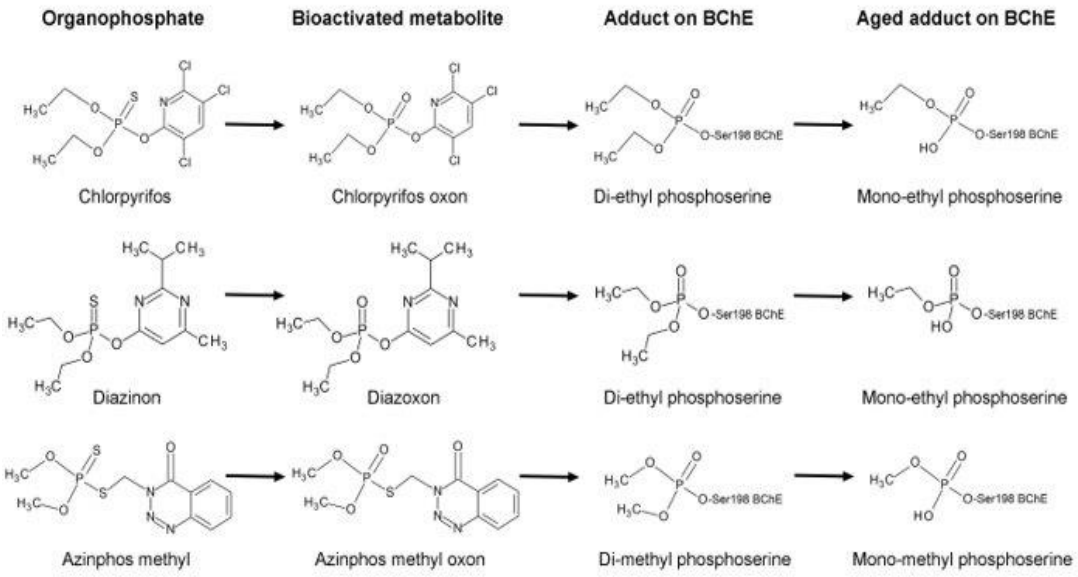

Figure 2. Biotransformation of organophosphate pesticides (Marsillach et al. 2013).

1.3 Health effects of organophosphate exposure

OPs are potent inhibitors of the active site serine on serine hydrolases such as AChE and butyrylcholinesterase (BChE). Inhibition of these enzymes can exert severe effects with subsequent accumulation of the neurotransmitter acetylcholine (ACh). ACh is involved in several important biological processes, including muscle movement, learning and memory, and regulation of the autonomic nervous system, which controls involuntary body functions such as heart rate, digestion, and respiration.[13] AChE is responsible for terminating impulse transmission in the synaptic cleft by rapid hydrolysis of ACh. However, OPs can inhibit AChE by covalently bonding to the enzyme, rendering it incapable of terminating the impulse and leading to an influx of ACh and prolonged excitation of the nervous system (**Figure 3**). Excitation of the nervous system may lead to symptoms such as muscle twitching, blurred vision, and difficulty breathing. In severe cases, there may be seizures, respiratory failure, and/or death.

Acute or chronic exposure to OP insecticides can also pose a significant risk to human health, leading to various long-term adverse effects. Prolonged exposure to low levels of OPs can cause neurophysiological effects including decreased cognitive function, mood disorders, and depression.[14] Additionally, chronic exposure to OPs has been associated with an increased risk of Parkinson's disease, a disorder of the nervous system that affects movement. Moreover, studies have linked chronic OP exposure to respiratory and cardiovascular diseases, and risk of some cancers. The severity of the adverse outcomes of chronic exposure to OPs depends on various factors, including the duration, frequency, and level of exposure. Despite the rapid

metabolization and excretion of OPs, long-term exposure can have significant impacts on human health, particularly in sensitive populations, such as pregnant women, children and elder.

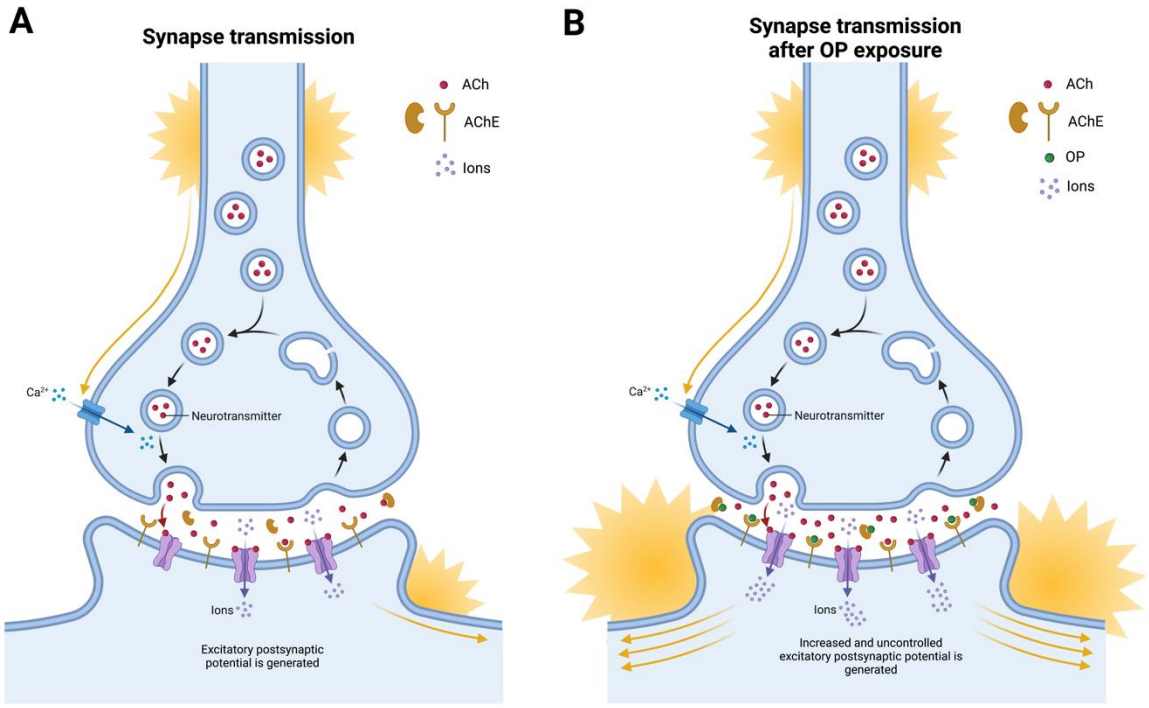

Figure 3. Nervous system effects due to organophosphate exposure. The Figure depicts a cholinergic synapse between two neurons. Upon normal release of the neurotransmitter acetylcholine from the synapse in response to calcium stimulation, (**A**) acetylcholinesterase modulates the signal transmission by binding and degrading acetylcholine; (**B**) organophosphates inhibit acetylcholinesterase, leading to an increase in acetylcholine in the synapse and resulting in overstimulation (created with BioRender). ACh, acetylcholine; AChE, acetylcholinesterase, OP, organophosphates.

1.4 Current OP biomonitoring programs

The historical and current usage of OPs in the United States highlights their significant role in enhancing crop yields and minimizing losses in the agricultural industry. In the past 30 years, the average annual usage of pesticides has been recorded at 1.58 kg per hectare, 0.37 kg per person, and 0.79 kg per thousand international dollars of agricultural production.[15] According to the Food and Agriculture Organization (FAO), OPs have been extensively applied in agriculture due to their broad-spectrum efficacy against various pests. For example, in 2020, the Americas have

imported 1.1 million tonnes of pesticides worth around 6.9 million USD.[15] However, the use of OPs in agriculture has raised concerns about the potential health risks posed to agricultural workers. These workers are particularly vulnerable to OP exposure due to their close proximity to pesticides. The National Institute for Occupational Safety and Health (NIOSH) emphasizes that agricultural workers represent a high-risk group for pesticide poisoning, with estimates indicating that in 2011 over 20,000 agricultural workers in the United States experience pesticide-related injuries annually. [16] Furthermore, there have been cases of migrant agricultural workers that were possible exposed to pesticides during the maximum sensitivity period of their pregnancies that led to adverse birth defects. Given the potential risks associated with OP exposure, it becomes crucial to monitor the exposure level of agricultural workers to assess their overall health and safety.

Biomonitoring involves evaluating human exposure to chemicals and associated health risks by analyzing the presence of toxicants or their metabolites in bodily fluids such as blood, urine, or feces. This approach enables the early detection of exposures, allowing for timely interventions before severe symptoms can manifest, as well as monitoring the recovery of individuals who have been exposed to these chemicals. Assay of AChE activity serves as a diagnosis of potential organophosphate pesticide exposure. The standard method for biomonitoring OP exposures involves using the Ellman colorimetric enzymatic assay technique that was developed in the early 1960s.[17] The Ellman assay is widely used in various studies due to its simplicity, cost-effectiveness, and rapid generation of results. The assay uses acetylcholine or butyrylthiocholine for determining AChE or BChE activity. The photometric method measures enzyme activity by following the increase of a yellow color produced when thiocholine from acetylthiocholine reacts

with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). The reaction produces 5-thio-2-nitrobenzoate that has a yellow color due to the shift of electrons to the sulfur atom.[18] The intensity of the yellow color is proportional to the cholinesterase activity in the sample.

While the Ellman assay is accurate and reliable for determining cholinesterase activity at high inhibition levels, it has limitations when it comes to lower levels of inhibition (20% or less). At such low concentrations, the assay may not provide precise measurements, thus, the sensitivity of the assay decreases. Furthermore, even though 50% inhibition of neural AChE is necessary for the manifestation of anticholinergic symptoms, research has shown that OPs can have adverse health effects and impact neurodevelopment with low levels of cholinesterase inhibition due to delayed or chronic toxicity and sensitivity.

To ensure the accuracy of the Ellman assay, pre-exposure determination of baseline cholinesterase activity is necessary to account for intra- and inter-individual variability. Therefore, a major drawback of the Ellman assay is the need for a baseline (pre-exposure) sample to determine cholinesterase activity after an exposure event. The Ellman assay also requires careful control of the pH of the assay buffers and the substrate concentration is essential to obtain consistent and reliable results. Additionally, the temperature at which samples are stored is an important consideration in the context of biomonitoring OP insecticide exposures. It has been observed that some OP inhibitors can undergo spontaneous reactivation of the inhibitor-enzyme complex at ambient temperatures. This reactivation can lead to an underestimation of the levels of inhibition when samples are not stored under appropriate conditions.

Another limitation for the Ellman assay is its inability to identify the specific OP compound responsible for the inhibition of AChE. This lack of compound identification hinders the ability to pinpoint the exact source the subject was exposed to. Furthermore, there is the possibility of rapid replacement of the inhibited enzyme by newly synthesized proteins, which can occur relatively quickly. This rapid turnover of enzyme activity poses challenges in accurately assessing past exposures, making the Ellman assay less suitable for retrospective analyses. Considering these factors, it is important to exercise caution and consider the limitations of the Ellman assay when utilizing it for exposure biomonitoring. Integrating proper complementary techniques and methodologies can help to enhance the overall accuracy and reliability of assessing OP insecticide exposures using the Ellman assay.

1.5 Biomarkers of OP exposures

Biomarkers are a measurable indicator or characteristic used to assess exposure to various substances, predict health effects, or monitor the progress of a disease or a treatment process. Biomarkers of exposure to OP insecticides have been widely detected in human populations, with the most recognized target being the AChE enzyme. Additionally, long-term neurological consequences in chronically exposed workers are a major concern, given the variability in sensitivity, susceptibility, and vulnerability between different populations. Analysis of OP insecticide levels in blood allows for direct measurement of parent compounds rather than metabolites, providing a more accurate representation of the dose to the target tissue. However, very sensitive analytical methods are required to measure them due to the low concentrations of OPs in blood compared to their metabolites in urine.

Adverse health effects from acute exposures to pesticides are easily diagnosed due to visible signs and symptoms. However, adverse outcomes from chronic exposure such as neurodegenerative effects, are more difficult to diagnose due to the duration of symptom onset.[7] Therefore, monitoring for OP insecticide exposure is important to avoid the acute and chronic health consequences stated earlier, and to quantify the depression of cholinesterase in an individual. For OP insecticides, two biomarkers have been widely studied and validated, butyrylcholinesterase (BChE) from plasma and acetylcholinesterase from erythrocytes. Both blood cholinesterases serve as a reference measurement for the nervous-system acetylcholinesterase activity. BChE is the most used biomarker in the field although this form of cholinesterase is not known to be directly involved in the cholinergic process. However, BChE has been shown to be more reliable and reproducible between laboratories as well as having more correlation when compared to cholinergic AChE activity in the CNS. Human BChE is synthesized by the liver and has a half-life of 11 days in plasma. The 85 kDa tetrameric glycoprotein plays a crucial role in the detoxification process of OPs that are ingested or inhaled. It accomplishes this by binding to OP molecules in a stoichiometric manner, effectively neutralizing their toxic effects.

1.6 History of Dried blood spots

In 1963, Robert Guthrie introduced the dried blood spot sample (DBS) technique for screening children for intellectual disabilities. This process was pioneered by Dr. Ivar Christian Bang, who used blood collected on filter paper to measure glucose and nitrogen levels in the early 1900s.[19,20] DBS samples have proven to be incredibly useful and have made a significant impact on public health. The establishment of population-based newborn screening programs worldwide

utilizing DBS samples is considered one of the most influential achievements in public health.[21] By incorporating DBS into these screening programs, early detection and intervention for a vast range of conditions have become possible, leading to improved health outcomes. The utilization of DBS as a screening tool has not only facilitated the identification of infectious diseases such as congenital rubella, hepatitis B, and syphilis, but it has also paved the way for the identification of health effects such as genetic, metabolic, and endocrine disorders.[22–24] This population-based approach ensures that all infants have equal access to early detection and treatment of diseases, ultimately reducing the burden of disease and improving the overall health of people in the population. Additionally, the systematic archiving of residual newborn DBS samples provides an invaluable resource for ongoing research in epidemiology. Thus, enabling a deeper understanding of the risk factors, prevalence, and long-term outcomes associated with various health factors.

DBS refers to the technique of applying whole blood onto filter paper and allowing it to dry. Blood samples can be obtained through various methods, commonly by pricking a finger or the heel for newborns using a sterile-safe lancet. The blood is then deposited onto paper, either directly or using a capillary tube or pipette. The filter paper card is then dried and can be stored for an extended period with minimal degradation, depending on the analyte of interest. DBS offers several advantages over venous puncture-based whole-blood samples, including minimal invasiveness, and the ability to be collected by personnel with basic training. Standard DBS cards feature pre-printed circles of approximately 12 mm in diameter, which can hold between 50-70 µL of whole blood. This small blood volume requirement is particularly beneficial when working with pediatric patients and newborns. Additionally, the long-term stability and compact

storage requirements make DBS a viable option for sample preservation beyond what is feasible for whole-blood samples collected through venipuncture methods.

1.7 Occupational use of dried blood spots

The convenience and cost-effectiveness of collecting and analyzing DBS make them a valuable tool for assessing occupational exposures consistently. One notable example is the study of wildland firefighters in the United States, where DBS samples were repeatedly collected through the workday to analyze biomarkers of exposure to woodsmoke.[25,26] By collecting samples at regular intervals during the work shift, researchers can gain insights into the temporal patterns of exposure and identify specific areas or tasks that contribute to the high levels of exposure. This knowledge allows for the implementation of interventions and other targeted controls to minimize concentration or duration of exposure in those specific areas or tasks.

Despite the Occupational Safety and Health Administration (OSHA) requiring biological monitoring for specific chemicals in certain work settings, routine sampling at the national level is not currently mandated and other countries may not have this requirement. For instance, hazardous occupations may not have mandatory routine biomonitoring depending on the country, region, or specific industry. Moreover, traditional venous blood sampling for biomonitoring can be expensive due to the sample storage requirements and transportation procedures. Generally, these samples should be kept in containers that are leak-proof, durable, and capable of withstanding impact. For short term storage, the samples need to be stored at 4-8°C to slow enzymatic activity and prevent degradation of certain analytes or biomarkers in the samples. For long-term storage, these samples should be kept frozen preferably at -80°C.[27] Furthermore,

shipping liquid blood samples is more expensive due to factors such as larger volume and weight, specialized packing requirements, and the need for temperature regulation. These factors contribute to costly shipping costs associated with liquid blood samples compared to the more cost-effective and convenient transportation of DBS samples. Consequently, resource-limited, or remote regions that are implementing venipuncture-based monitoring may then have a challenging time abiding to the storage and transport requirements.

The less-invasive nature of DBS sampling, combined with the ease of storing and transporting samples, presents opportunities for widespread application and consistent exposure measurement. By increasing occupational biomonitoring sampling, future screenings can capture peak exposures that may overwhelm defense mechanisms and induce adverse health effects. While DBS has been primarily used for monitoring, diagnosis, and epidemiological studies of viruses, further research should explore and validate its use in comparison to whole blood measures, allowing for broader application in human biomonitoring studies.[26] Therefore, future investigations should continue to expand our understanding of DBS and explore its applicability to different chemical stressors and industries, broadening its potential for occupational exposure assessment.

1.8 Hypothesis

Our long-term goal is to improve the identification of environmental exposures to pesticides using protein adductomics. Adductomics is the measurement of all exposure-driven post translational modifications of proteins. The overall objective of this project is to improve upon the current cumbersome biospecimen collection process for biomonitoring and enhance the

identification of OP insecticide exposures among agricultural workers. Our first hypothesis is that the use of a more sensitive technology, such as mass spectrometry, to identify OP adducts in plasma, will allow us to determine exposure to OPs without the need for a baseline sample. Furthermore, our second hypothesis will focus on identifying OP adducts in DBS using mass spectrometry, proving that the use of DBS is a valid alternative compared to current venous blood drawing methods.

1.9 Specific Aims

Aim 1: Identify OP-adducted BChE in plasma using an immunopurification bead protocol followed by targeted high-resolution mass spectrometry (LC-MS/MS).

Our OP insecticide of interest was Chlorpyrifos as it was the most used OP insecticide in WA agricultural fields at the time of sample collection. Oxon's are organic compounds derived from a chemical in which a phosphorus-sulfur bond in the parent compound is replaced by a phosphorus-oxygen bond. Chlorpyriphos does not directly cause toxicity but rather the liver metabolizes the pesticide and creates an oxon derivative of chlorpyrifos that is highly reactive and toxic. Thus, *in vitro* pure BChE inhibited (and non-inhibited) with chlorpyrifos oxon were used as standards for MS. The Marsillach lab had previously developed a fast and accurate protocol for the purification of biomarker proteins that successfully identified chlorpyrifos exposures in 128 agricultural workers from WA.[29] The developed method included a rapid immunopurification protocol for plasma BChE coupled with high resolution mass spectrometry (LC-MS/MS). Since this method has shown promising results, it will be utilized for the identification of OP-BChE in the plasma samples. The immunopurification protocol used magnetic beads coupled to commercially available mouse monoclonal anti-human plasma BChE antibodies to immuno-purify BChE from plasma. The samples were prepared by using our two-

step protocol by targeting OP protein adducts on the active-site serine (serine 198) of BChE. Bead-bound BChE was then digested with chymotrypsin and OP adducts were targeted based on their active site serine peptide using high resolution mass spectrometry.

Aim 2: Identify OP-adducted BChE in DBS using an immunopurification bead protocol followed by targeted high-resolution mass spectrometry (LC-MS/MS).

Archived DBS samples, collected in conjunction with the plasma samples in Aim 1, served as a valuable resource to validate the efficacy of using DBS for the identification of OP-adducted BChE and assess their usefulness as a biospecimen in the cholinesterase monitoring program. To extract proteins from the DBS cards, a protocol was implemented involving the extraction of 12 3-mm punches from the DBS cards. These punches were then incubated overnight to facilitate the extraction of proteins from the DBS punches. The subsequent extract followed the Marsillach lab's immunopurification protocol and high-resolution mass spectrometry stated previously, targeting the same OP adducts on the BChE active site serine peptides.

Materials and Methods

2.1 Agricultural worker samples

In order to assess pesticide exposure and its impact on cholinesterase activity, the Simpson lab (DEOHS, UW) conducted a comprehensive field sampling campaign involving 13 orchard workers in Washington who were involved in the application of chlorpyrifos. This study presented a unique approach compared to previous pesticide exposure studies as it involved the collection of a time series of samples from each individual worker.

The sampling process consisted of collecting samples at different time points throughout the pesticide handling process. Specifically, samples were obtained from workers prior to handling chlorpyrifos, at the beginning of the spraying activity, at the conclusion of the spraying season, and one month after the completion of spraying (**Figure 4**). This sequential sampling approach allowed for the assessment of temporal changes in cholinesterase activity and pesticide exposure within each worker.

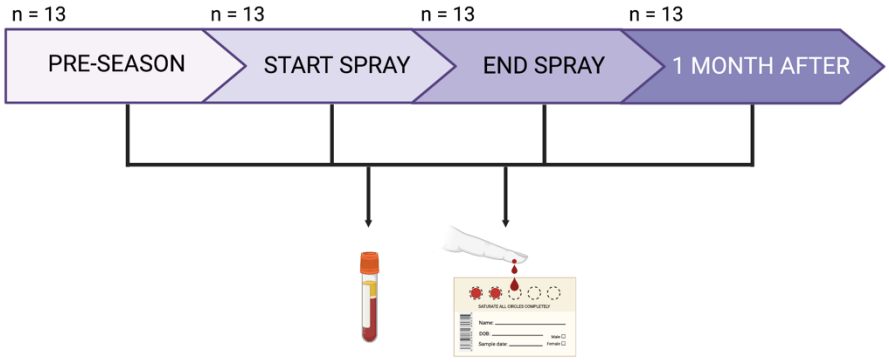

Figure 4. Timeline of agricultural worker biospecimen collection.

Within the scope of our study, we have undertaken a focused investigation into the start and end of the spray season samples collected from the agricultural workers. These time intervals have been chosen to provide a direct comparison between the biospecimen collected at the start of the spray season and the ones obtained immediately after the spray season. The rationale behind this choice is rooted in the theoretical expectation that the post-season samples would likely exhibit a higher concentration of adducts. This is attributed to the fact that during the active spraying period, workers are continuously exposed to pesticides, potentially resulting in a higher accumulation of adducts in their bodies. Through a comparative analysis of these distinct time points, our objective was to evaluate the extent of CPO exposures in plasma and DBS samples. This comparative assessment allowed us to determine the validity of utilizing DBS as an alternative approach for assessing OP exposures, with an initial focus on chlorpyrifos.

2.2 Biospecimen collection

To enable a direct comparison between the less invasive DBS finger prick method and the traditional venipuncture method, both DBS and venipuncture samples were collected from each of the 13 participants in the field. Blood obtained via venipuncture was drawn by a certified phlebotomist using plasma collection tubes with lithium heparin (BD Vacutainer®). DBS samples were obtained using a sterile lancet, applying a few drops of blood onto Whatman® protein saver cards (MilliporeSigma), allowing for a simple and less invasive collection method.

To gather additional information and potential confounding factors related to cholinesterase activity, subjects completed a survey at the time of each sample collection. The survey included questions regarding pesticide application practices, job tasks performed by the workers, and other relevant factors that may influence the cholinesterase activity levels of the agricultural workers. All subjects included were informed about the research project and gave their written consent before being included in the study, under protocols approved by the Institutional Review Board (IRB) at the University of Washington.

This comprehensive field sampling campaign aimed to provide a thorough assessment of pesticide exposure and its impact on cholinesterase activity in orchard workers. By collecting a time series of samples, employing both DBS and venipuncture samples, and considering relevant factors through participant surveys, the Simpson lab aimed to obtain a comprehensive understanding of the relationship between pesticide exposure and cholinesterase activity in the agricultural sector.

2.3 Ellman assay

Plasma BChE activity was assessed following the standard methodology described by the Ellman assay with some modifications.[17] To initiate the reaction, 100 µL of diluted BChE or plasma was combined with 100 µL of a substrate solution consisting of 10.7 mM butyrylthiocholine and 10.3 mM DTNB in 0.1 M sodium phosphate buffer at pH 8.0. The enzymatic activity was monitored at 405 nm every 30 seconds for a duration of 3 minutes at a controlled temperature of 25°C using a SpectraMax® i3x Multi-Mode Microplate Reader (Molecular Devices, San Jose CA). All reagents utilized in the assay were sourced from Sigma-Aldrich ensuring consistent quality and reliability. By employing this standardized and well-established procedure, we can accurately measure plasma BChE activity, providing valuable insights into the functional status of this enzyme and its potential modulation of OP exposure. Furthermore, the Ellman assay was used as a verification measure for the presence of BChE and the efficacy of the magnetic beads.

2.4 Plasma BChE immunoprecipitation

We have successfully developed a streamlined protocol for the immunopurification of plasma BChE to improve purification efficiency (**Figure 5**).[29,30] The purification process is essential for isolating BChE from plasma samples and obtaining highly purified protein for further analysis. In this optimized method, we reduced the volume of plasma required to 100 µL and incubated the plasma overnight to ensure binding. This reduction in volume is particularly advantageous when working with limited sample quantities, allowing for more efficient utilization. All steps were performed using Protein LoBind Eppendorf Tubes to minimize protein loss.

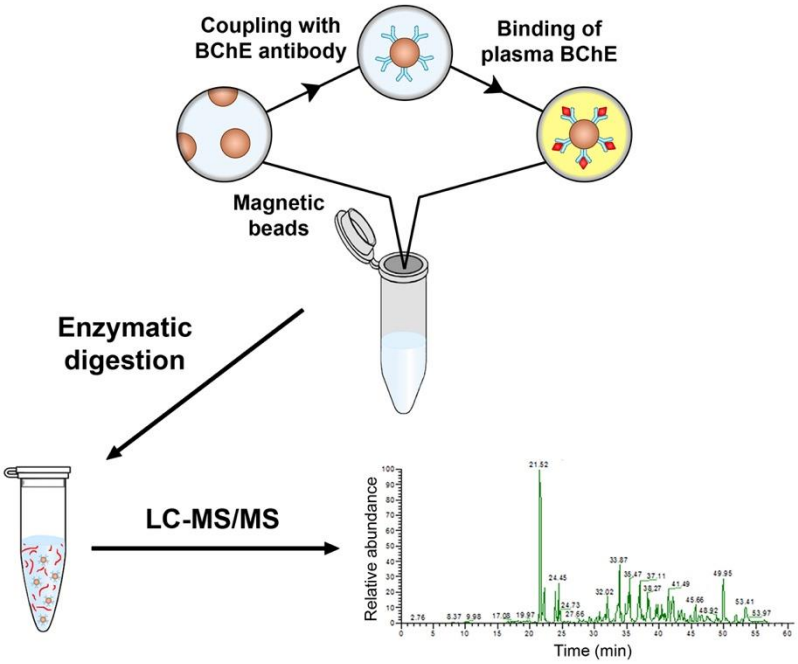

Figure 5. Immunoprecipitation process to isolate and enrich BChE from plasma.

To achieve BChE immunopurification, we coupled 10 µg of mouse monoclonal anti-human BChE antibody (Thermo Fisher Scientific™) to 1 mg of epoxy-coated magnetic beads according to the manufacturer's instructions (Dynabeads Antibody Coupling Kit, Thermo Fisher Scientific™). These antibodies specifically bind to BChE, facilitating its isolation from plasma. These BChE antibody-coupled beads were stored at 4°C until further use. The plasma samples obtained from agricultural workers in Washington state were diluted 1:1 v/v in 100 mM NaCl, 10 mM NaPO4 pH 7.4 buffer and incubated with 0.4 mg of antibody-coupled beads rotating at 4°C overnight. After the overnight incubation, BChE activity in the diluted plasma before and after beads was measured using the Ellman assay to ensure the incubation led to a significant reduction of enzymatic activity in the plasma samples. A significant reduction of enzymatic activity in samples was identified as approximately 10% or less BChE activity remaining in the

diluted plasma samples with beads when compared to the same plasma that did not undergo incubation with beads.

After incubation, BChE-bound beads were thoroughly washed four times with phosphate-buffered saline to remove any non-specifically bound proteins. After the thorough washing of the BChE-bound beads, the beads were then resuspended in 15 µL of the surfactant 0.1% w/v RapiGest SF (Waters) in 50 mM ammonium bicarbonate pH 7.8 buffer. To denature the protein, the resuspended BChE-bound beads were heated in a dry bath for 10 min at 90°C. This denaturing step facilitates the unfolding of the protein structure, making it more accessible for the subsequent enzymatic digestion and analysis. After this step, we saved 5 µL of the supernatant for gel electrophoresis analysis.

Following denaturation, the protein was reduced by incubating the beads with 5 mM dithiothreitol (DTT; ThermoFisher Scientific) at 50°C for 30 min. This reduction step with DTT breaks disulfide bonds within the protein, by providing electrons to react with the sulfur atoms in cysteine residues. By breaking these disulfide bonds, it ensures optimal peptide fragmentation.

To prevent the reformation of disulfide bonds, the reduced protein was then alkylated by incubating the beads with 15 mM iodoacetamide (IAA; ThermoFisher Scientific) in the dark at room temperature for 30 min. Iodoacetamide reacts with the thiol groups of cysteine residues, effectively preventing the reformation of the disulfide bonds and locking the resided cysteine residues into their modified state. It is important that this step is performed in the dark to reduce the risk of unwanted oxidation and reactivity. Light, particularly in the presence of oxygen, can

promote the oxidation of cysteine residues, leading to the undesired formation of disulfide bonds and potentially compromising the integrity of the protein sample.

The protein bound to the beads was then subjected to on-bead enzymatic digestion using a 1:50 ratio of chymotrypsin (Promega). The digestion was carried out by shaking (600 rpm) the samples at 37°C for 2 h using a MultiTherm™ Shaker (Benchmark Scientific). Chymotrypsin is a proteolytic enzyme that cleaves peptide bonds specifically at aromatic amino acid residues, such as tyrosine, phenylalanine, and tryptophan, generating smaller peptide fragments for subsequent analysis.

Following the digestion, the samples were separated from the beads using a DynaMag™-2 Magnet (ThermoFisher Scientific), allowing the supernatant containing the digest to be collected. To hydrolyze the RapiGest SF surfactant present in the buffer, 37% hydrochloric acid was added to a final concentration of 250 mM. The mixture was then incubated for another 45 min at 37°C. This hydrolysis step results in the breakdown of the surfactant and the formation of a cloudy precipitate. The sample was then centrifuged for 10 min at 13,000 g and the supernatant was collected to remove the hydrolysis surfactant. To preserve the integrity of the peptides for mass spectrometry analysis, the supernatant was frozen at -20°C until further processing.

2.5 DBS BChE immunoprecipitation

To purify BChE from DBS extract samples, the same immunoprecipitation approach used for plasma samples was employed. DBS samples were prepared by extracting 12 punches of 3 mm diameter from the DBS cards. Each punch was transferred to a Protein LoBind Eppendorf Tube. DBS punches were incubated vigorously on a shaker with 0.1% Triton-X 100 in pure water for 1

h at room temperature. Afterwards, the samples were transferred to a shaker at 4°C for overnight incubation to maximize the amount of protein that is extracted from the DBS punches.

After the incubation with 0.1% Triton-X 100 in water, the same immunoprecipitation and digestion procedure was followed as described for the plasma samples (**Figure 6**). Specifically, the extracted proteins were incubated with 0.4 mg of the BChE antibody-coupled beads, rotating overnight at 4°C to facilitate the binding of BChE to the antibody-coupled beads. The subsequent steps include washing the beads, resuspending the bound BChE in RapiGest SF, and the digestion process using chymotrypsin. By using the BChE immunoprecipitation method, we were able to specifically extract and enrich BChE from DBS samples, enabling further analysis of OP-adducted BChE via mass spectrometry. Samples were kept at -20°C until analysis via mass spectrometry.

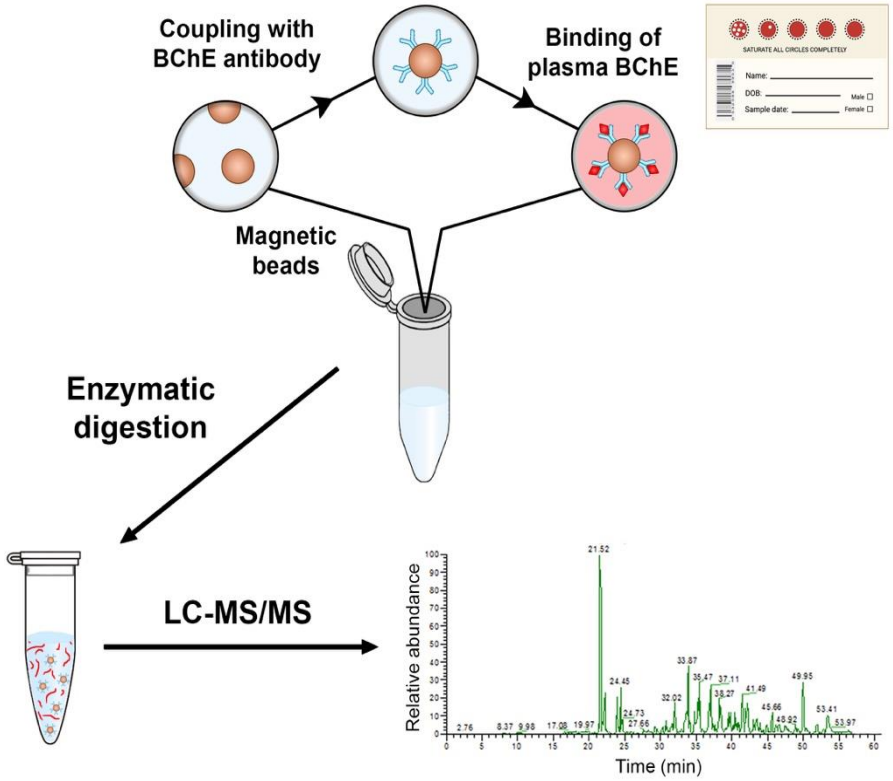

Figure 6. Immunoprecipitation process to isolate and enrich BChE from DBS.

2.6 Positive control for MS method development

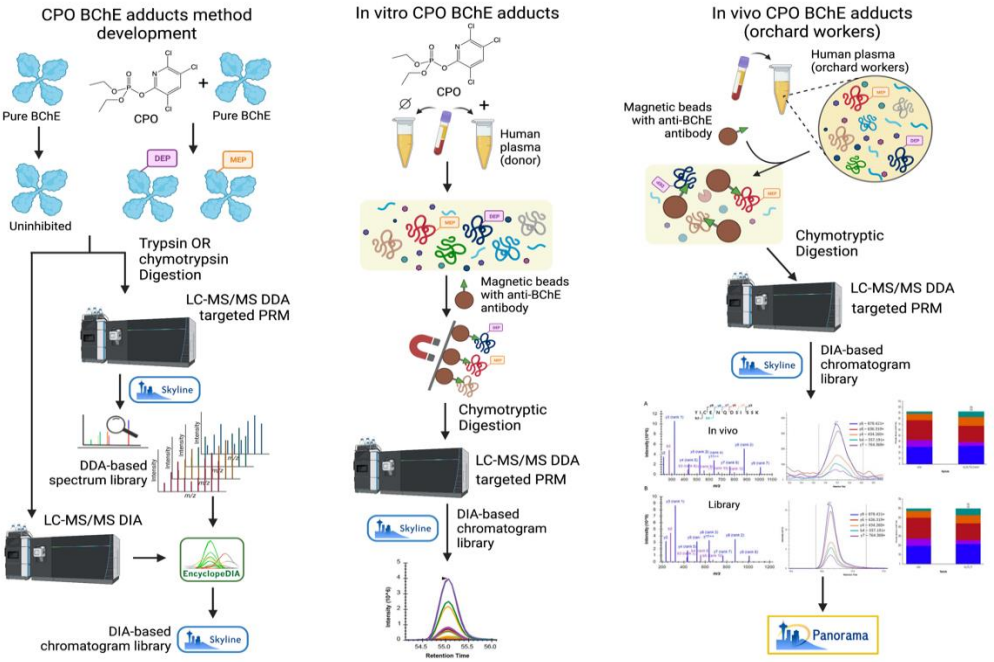

Figure 7. Method development plan for the quantification of OP-adducted BChE in plasma.

To prepare our positive controls for mass spectrometry method development, we used purified human BChE from frozen Cohn fraction IV-4 from pooled human plasma, kindly gifted by Dr. Oksana Lockridge.[31] Purified BChE was inhibited using CPO (CAS 5598-15-2; 98% purity, Chem Service) to serve as positive controls for OP exposure. A 30 mM mixture of stock CPO in methanol was made and stored at 4°C prior to use. The mixture was then brought into an aqueous solution (pure water) prior to incubation with pure BChE. Pure BChE was also incubated with water and used as negative control for CPO exposure.

A total of 20 µg of pure BChE enzyme was incubated with a final concentration of 25 µM CPO for 3 h at room temperature. Another 20-µg aliquot of pure BChE was incubated with water to

use as a non-inhibited control. Following the incubation period, an Ellman BChE activity assay was performed to confirm that the activity of the enzyme was completely inhibited, compared with the non-inhibited sample. This step ensured that the protein activity was null, indicating a successful BChE inhibition by CPO.

To enable downstream processing and mass spectrometry analysis, the pure BChE samples underwent a buffer exchange procedure. The Zeba™ spin desalting columns (7K molecular weight cutoff, ThermoFisher Scientific) were used to remove any excess CPO from the sample. These columns utilize a chromatography principle, allowing big proteins such as BChE to pass through and retaining small molecules. The sample buffer was replaced with a 50 mM ammonium bicarbonate buffer at pH 7.8. Following the Zeba™ Spin Desalting Columns step, the eluate containing the recovered inhibited BChE was stored at 4°C overnight to allow adequate time for the aging process. The pure BChE sample incubated with water went through the same steps as the CPO-inhibited sample (spin desalting column and overnight aging). Since the pure BChE samples did not need to go through any enrichment step, CPO-inhibited and non-inhibited pure BChE samples were denatured, reduced, alkylated, and digested with chymotrypsin as described above. Once acidified with HCl to hydrolyze RapiGest, samples were kept at -20°C until mass spectrometry analyses.

2.7 Plasma positive and negative controls

In order to test the developed MS method for CPO adduct identification on BChE, we carried out in vitro inhibitions of a plasma sample from a donor using CPO (**Figure 7**). Plasma incubated with water was used as a negative control for OP exposures.

A total of 250 µL of plasma were incubated with a final concentration of 25 µM CPO or water for 3 h at room temperature, as described with pure BChE above. Then, we used the Ellman assay to confirm full inhibition of BChE activity by CPO, compared with the non-inhibited plasma sample. We did not use Zeba^{TM} spin desalting columns for removing excess CPO in plasma samples because the immunoprecipitation protocol will take care of that. CPO-inhibited and uninhibited plasma samples were aged at 4°C overnight.

BChE from non-inhibited and inhibited plasma samples was immunoprecipitated using BChE-coupled magnetic beads, followed by denaturing, reduction, alkylation, chymotryptic digestion, and surfactant hydrolysis as described above. Samples were kept at -20°C until mass spectrometry analyses were carried out.

2.8 DBS positive and negative controls

As similarly described earlier with plasma samples, we used whole blood from a donor to prepare DBS positive and negative controls to test the developed MS method (**Figure 8**).
A total of 200 µL of whole blood were incubated with CPO (25 µM final concentration) or water for 3 h at room temperature, as described above. Due to the presence of hemoglobin, we were not able to use the Ellman assay to confirm full inhibition of BChE activity by CPO, as the red color interfered with the formation of yellow, and thus, with the spectrophotometric readings.

However, we followed the exact same CPO inhibition protocol used for plasma and pure BChE that reliably fully inhibits BChE. As with plasma, we did not use Zeba^{TM} spin desalting columns

for removing excess CPO in DBS extracts because the immunoprecipitation protocol will take care of that. Inhibited and uninhibited blood samples were aged at 4°C overnight. Then, we

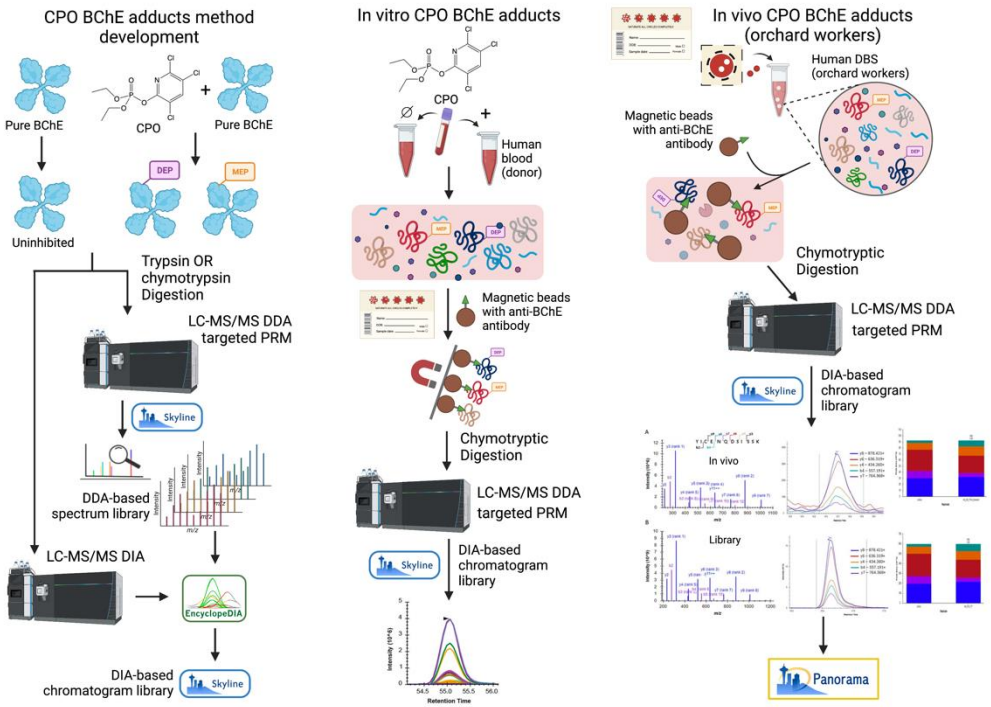

Figure 8. Method development plan for the quantification of OP-adducted BChE in DBS.

created DBS by transferring the aged CPO-inhibited and uninhibited whole blood samples to Whatman® protein saver cards (MilliporeSigma). We used the exact same filter paper cards used to collect the agricultural worker samples. A total of ~30 µL of blood were pipetted on top of each pre-printed circle, ensuring the circles were fully saturated with blood. We let the cards air dry for 4h at room temperature and we stored them individually at -80°C in zipper bags with one desiccant sachet (to protect from moisture) until use.

For BChE immunoprecipitation, frozen filter paper cards were acclimated to room temperature for a few minutes, and 3-mm punches (12 total) were obtained and transferred to a Lo-bind Eppendorf tube with 0.1% Triton X-100 in water, as described above. Once the DBS extracts

were obtained, BChE was immunoprecipitated using anti-BChE magnetic beads, followed by denaturing, reduction, alkylation, chymotryptic digestion, and surfactant hydrolysis, as detailed above. The final samples were stored at -20°C until mass spectrometry analyses were carried out.

2.9 Electrophoresis gels

Electrophoresis gels were employed as a crucial tool in our study to assess the effectiveness of the BChE immunoprecipitation method and verify the presence of our target protein in our samples. The gels allowed us to visualize and separate the proteins based on their molecular weight. By comparing the protein bands obtained from the immunoprecipitation samples with those from the known molecular weight ladder, we were able to confirm the successful enrichment of BChE and validate its presence in our samples. This gel-based analysis served as an essential quality control step, ensuring the reliability and accuracy of our subsequent mass spectrometry analysis of OP-adducted BChE. Samples were prepared and loaded onto denaturing polyacrylamide gels (4-12% NuPAGE® Bis-Tris gels, Thermo Fisher Scientific) following manufacturer instructions. Gels were stained using the Pierce® Silver Stain kit (Thermo Fisher Scientific).

2.10 MS analyses of BChE OP adducts

Mass spectrometry experiments were conducted in Dr. Michael MacCoss' laboratory (Genome Sciences, UW). Data-independent acquisition, data-dependent acquisition (DDA), and parallel-reaction monitoring (PRM) are integral components of our experimental approach. We conducted mass spectrometry experiments using a Thermo Vanquish Neo UHPLC System coupled with a Thermo Orbitrap Eclipse mass spectrometer (ThermoFisher Scientific). Peptides

were separated using a PepSep column (150 µm ID x 25 cm) packed with 1.9 µm ReproSil C18 beads (Brucker, 1892479), while a PepMap Neo Trap column (300 µm ID x 5 mm) packed with 5 µm C18 beads (ThermoFisher Scientific; 174500) was utilized in trap-and-elute mode. Solvent A, consisting of 0.1% formic acid in water, and solvent B, consisting of 0.1% formic acid in 80% acetonitrile, were used for the mobile phase gradient elution. For each injection, a sample was loaded and eluted using a 30-minute gradient from 4% to 40% B at 1 µL/min (**Table 1**).

Table 1: Mobile phase gradient settings for the mass spectrometer.

No.	Time	Duration (min)	Flow (µL/min)	%B	Volume (µL)	No. of Column Volumes
1	0.000	Run				
2	0.000	0.000	1.300	4.0	0.00	0.00
3	0.700	0.700	1.300	6.0	0.91	0.51
4	1.000	0.300	0.800	6.5	0.32	0.18
5	56.700	55.700	0.800	40.0	44.56	25.09
6	57.200	0.500	1.300	55.0	0.53	0.30
7	58.700	1.500	1.300	99.0	1.95	1.10
8	58.700	Column Wash				
9	63.000	4.300	1.300	99.0	5.59	3.15
10	63.000	Stop Run				
11	63.000	Column Equilibration				

DDA data was acquired using the data-dependent acquisition method on the same LC-MS system. This approach was employed to generate a DDA-based spectrum library using a pure BChE sample (non-inhibited and CPO-inhibited). The precursor spectra ranging from 300 to 1,600 m/z were acquired at a resolution of 60,000, with the normalized automatic gain control (AGC) target set at 100% and a maximum injection time of 50 ms. The RF lens was carefully adjusted to a value of 40%, enhancing the ion transmission and overall sensitivity of the mass spectrometer. Moreover, we employed a monoisotopic precursor selection (MIPS) filter with the Peptide mode, enabling the selection and isolation of precursor ions with high efficiency and specificity. To capture detailed fragment spectra, we operated the mass spectrometer at a resolution of 30,000 at m/z 400 to 2,000 ensuring accurate and precise measurements. The

automatic maximum injection time was utilized to dynamically adjust the injection duration, allowing for optimal signal intensity while avoiding saturation effects. Additionally, we maintained the normalized AGC target at 100%, ensuring consistent ion population control during the acquisition process.

For DDA, we employed an isolation width of 1.4 m/z to ensure accurate precursor isolation. The normalized collision energy was set at 27 to promote effective fragmentation of the peptides. To optimize the data quality, charge exclusion was enabled to include only precursor charges ranging from +2 to +7, thus filtering out irrelevant charge states. To avoid reanalysis of similar precursor ions, dynamic exclusion was implemented with a time window of 30 seconds, effectively suppressing redundant data acquisition. To process the DDA data, the RAW files were converted to the mzML format, which facilitated subsequent analysis. The converted files were then searched against a human BChE sequence using MSGF Plus.[28] To account for potential modifications, dynamic methionine oxidation and static cysteine carbamidomethylating were considered during the search, ensuring comprehensive detection of post-translational modifications. To ensure high-confidence identifications, the search results were filtered to a 1% peptide-level false discovery rate (FDR) using Percolator (version 3.1). This stringent filtering step improved the reliability of the identified peptides. Based on the identified peptides, a DDA-based spectrum library was constructed using Skyline/Bibliospec software. This library served as a comprehensive reference for subsequent analysis. To facilitate data integration and comparison with DIA data, the DDA-based spectrum library was then converted into a DIA-based chromatogram library, enabling an accurate extraction and quantification of targeted peptides in the following DIA analysis.

We employed the chromatogram library workflow, previously described in detail for DIA methods.[32,33] The DIA approach allowed us to comprehensively detect all BChE peptides and investigate the reproducibility of chymotrypsin peptide digestion. By studying the digestion reproducibility, we aimed to identify the peptides without adducts that exhibited the best digestion reproducibility and demonstrated strong signal intensity. To ensure accurate retention time alignment and quantification, Pierce™ Peptide Retention Time Calibration (PRTC; ThermoFisher Scientific) mixture peptides were introduced into the samples as internal reference peptides for the purpose of indexed retention time (iRT) calibration. In this process, Thermo Orbitrap Eclipse MS was employed, and staggered 12 m/z narrow precursor isolation windows were acquired in the range of 400 to 1,000 m/z, following a previously described methodology.[33] These windows were subsequently deconvoluted to yield active 6 m/z narrow precursor isolation windows.[34] To generate a comprehensive DIA-based chromatogram library, we used the EncyclopeDIA software suite (version 1.12.31).[32] The library was constructed based on the DDA-based spectrum library obtained from pure BChE samples. For each peptide identified with a 1% FDR determined by Percolator,[35] retention time models were created.

Our experimental setup involved the collection of precursor spectra in the range of 400 to 1,600 m/z, employing a resolution of 30,000. To ensure optimal performance, we set the standard AGC target and allowed for automatic maximum injection time. The RF lens was set at 40% to fine-tune the ion transmission efficiency. For fragment spectra, we selected a scan range of 500 to 2,000 m/z, acquiring them at a resolution of 15,000. The AGC target was set to the standard value, and we utilized the automatic injection time for efficient data acquisition. The isolation

width was set to 2 m/z with unscheduled time mode and the normalized collision energy was set at 30%. In our analysis, we carefully optimized the collision energy to achieve efficient fragmentation of the precursor ions. We set the normalized collision energy at 30% for the fragmentation of adducted peptides, ensuring effective dissociation and generation of informative fragment ions. The detection of these fragmented ions was performed using the high-resolution Orbitrap mass analyzer. To promote targeted analysis, we generated an inclusion list containing the m/z values of protonated precursor peptide ions for both adducted and unmodified forms. This inclusion list was generated using the Skyline-daily software, allowing us to selectively monitor these specific peptide ions during our experiments. Skyline is an open-source software for building quantitative methods and analyzing the resulting mass spectrometer data (MacCoss lab, Dept. Genome Sciences, UW).

For the fragmentation of adducted peptides, we employed the collision-induced dissociation (CID) technique with a normalized collision energy set at 17%, which promotes the fragmentation of the peptide backbone and the release of characteristic fragment ions. The Orbitrap mass analyzer was used for the detection of these fragmented ions, enabling accurate mass measurement and identification. In contrast, for unmodified peptides, we employed the higher-energy collision dissociation (HCD) technique with a normalized collision energy set at 30%, which generates more extensive fragmentation and provides additional structural information. Again, the Orbitrap mass analyzer was used for the detection of the fragmented ions.

To ensure accurate identification and quantification, we generated a spectral library using pure BChE incubated with CPO, serving as a reference for the OP-adducted BChE peptides.

Additionally, Thermo PRTC peptides were employed to generate an iRT reference, enhancing the accuracy of the peptide retention time alignment across multiple samples. For the detection of agricultural workers samples, we designed a final targeted method that combined an inclusion list generated from three selected peptides exhibiting good digestion reproducibility with the inclusion of adducted peptides (along with their unmodified forms) (**Table 2**). This approach allowed us to focus on the relevant peptide targets and ensure comprehensive coverage of both adducted and unmodified forms (**Figure 7**). This approach was then applied to the DBS samples for the method development as well (**Figure 8**).

Table 2. Inclusion list.

	Chymotryptic peptide	Modification	m/z
BChE active site peptides	GESAGAASVSL	None	474.7353
	GES*AGAASVSL	Di-ethyl phosphate	542.7497
	GES*AGAASVSL	Mono-ethyl phosphate	528.7341
	FGESAGAASVSL	None	548.2695
	FGES*AGAASVSL	Di-ethyl phosphate	626.2839
	FGES*AGAASVSL	Mono-ethyl phosphate	602.2683
BChE control peptides	VGVNKDEGTAF	None	568.7828
	KIFFPGVSEF	None	585.8133
	GGGFQTGTSSLHVY	None	705.836

2.11 Data analyses

For the agricultural worker plasma samples and the agricultural worker DBS extracts, the acquired data from the mass spectrometer was uploaded to Skyline for pre-processing to ensure accurate and reliable data analysis. Raw data was processed via Skyline. While Skyline

automatically selects the peak areas corresponding to the inclusion list imported, we verified each selected peak and, when needed, we manually adjusted the peak areas corresponding to the target peptides of interest. These areas-under-the-peaks were then exported into Excel for further analysis. Peak areas were acquired for the two BChE active-site peptides pertaining to our aged and unaged modifications, as well as for the non-modified active-site peptide. These peak areas were obtained from the mass spectrometer data using the selected ion chromatograms for each peptide. Additionally, we included three library BChE peptides as controls, which were peptides derived from BChE but expected to remain unmodified by CPO or other OP exposures. These control peptides were used to normalize the amount of BChE protein in each sample loaded to the mass spectrometer. Normalization allowed us to account for any variations in sample preparation and instrument response, ensuring comparability between samples. In Excel, we calculated the percentage of adducted BChE in each sample using the following equation:

$$\%\ adducted\ BChE = \left(\frac{\frac{A\ GES^{*}(+108)}{A\ control}}{\frac{A\ GES^{*}(+108)}{A\ control} + \frac{A\ GES(unmod)}{A\ control}}\right) + \left(\frac{\frac{A\ FGES^{*}(+108)}{A\ control}}{\frac{A\ FGES^{*}(+108)}{A\ control} + \frac{A\ FGES(unmod)}{A\ control}}\right) \times 100$$

Where:

A is the total area-under-the-peak obtained from Skyline.

$GES^{*}(+108)$ corresponds to the active-site chymotryptic BChE peptide GES*AGAASVSL with an added mass of 108 Da on the active-site Serine amino acid (Ser 198).

$control$ corresponds to one of the 3 chymotryptic BChE peptides not modified by OP exposures, used to normalize the amount of BChE injected to the LC-MS/MS.

$GES(unmod)$ corresponds to the unmodified active site chymotryptic BChE peptide GESAGAASVSL.

$FGES^{*}(+108)$ corresponds to the active-site chymotryptic BChE peptide FGES*AGAASVSL with an added mass of 108 Da on the active-site Serine amino acid (Ser 198).

$FGES(unmod)$ corresponds to the unmodified active site chymotryptic BChE peptide FGESAGAASVSL.

Results

3.1 BChE immunoprecipitation from plasma and DBS extracts

BChE immunoprecipitation from both plasma and DBS was performed to enrich the target protein for further analysis. We aimed to evaluate our immunoprecipitation method and if there was an impact from the on-bead digestion following immunoprecipitation, as compared to immunoprecipitation alone. The immunoprecipitated samples were used for electrophoresis and the resulting gels provided visual insights into the efficacy of the immunoprecipitation method.

The gel image showcased distinct protein bands at ~80 and ~160 kDa, corresponding to the BChE monomer and dimer, respectively, in both immunoprecipitations alone and the immunoprecipitations followed by on-bead digestion when using plasma or DBS extracts (**Figure 9**). It should be noted that the immunoprecipitation followed by on-bead digestion showed additional bands at around 25 and 50 kDa, which correspond to the antibody that was coupled to the beads. While the plasma and DBS extract immunoprecipitations also displayed non-BChE bands (~60 kDa, likely albumin; ~15 kDa, likely hemoglobin), our

immunoprecipitation method provided samples highly enriched in BChE from plasma and DBS extracts, compared to the non-immunoprecipitated samples (**Figure 9**).

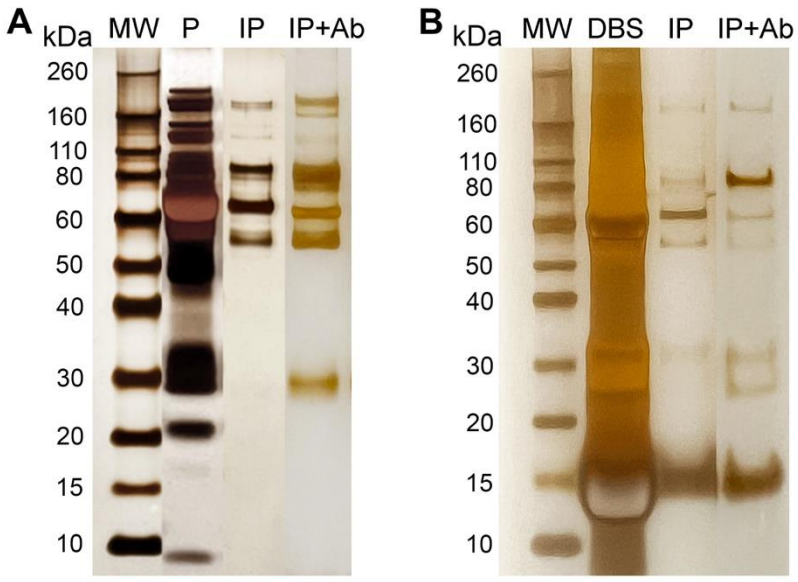

Figure 9. Electrophoresis gels before and after immunoprecipitation process from (**A**) plasma and (**B**) DBS extracts. MW, molecular weight; P, Plasma; IP, immunoprecipitation; IP+Ab, immunoprecipitation following on-bead digestion.

3.2 Pure plasma BChE MS

The spectra obtained from our pure BChE samples provided valuable insights into the active-site peptides we monitored (GESAGAASVSL and FGESAGAASVSL). These peptides served as key indicators of BChE modifications. By looking at the spectra, we focused on the multiple b-ions and y-ion series as it enables more reliable identification and quantification of the peptides when their peaks are overlapped (**Figure 10**). Furthermore, each peak exhibited a specific retention time during chromatographic elution. This specific elution pattern provided a basis for comparison with the retention times of the agricultural worker samples that were analyzed later. We anticipated that the peaks in the samples would elute around the same retention times as

observed in our pure plasma BChE samples, ensuring consistency, and facilitating accurate identification.

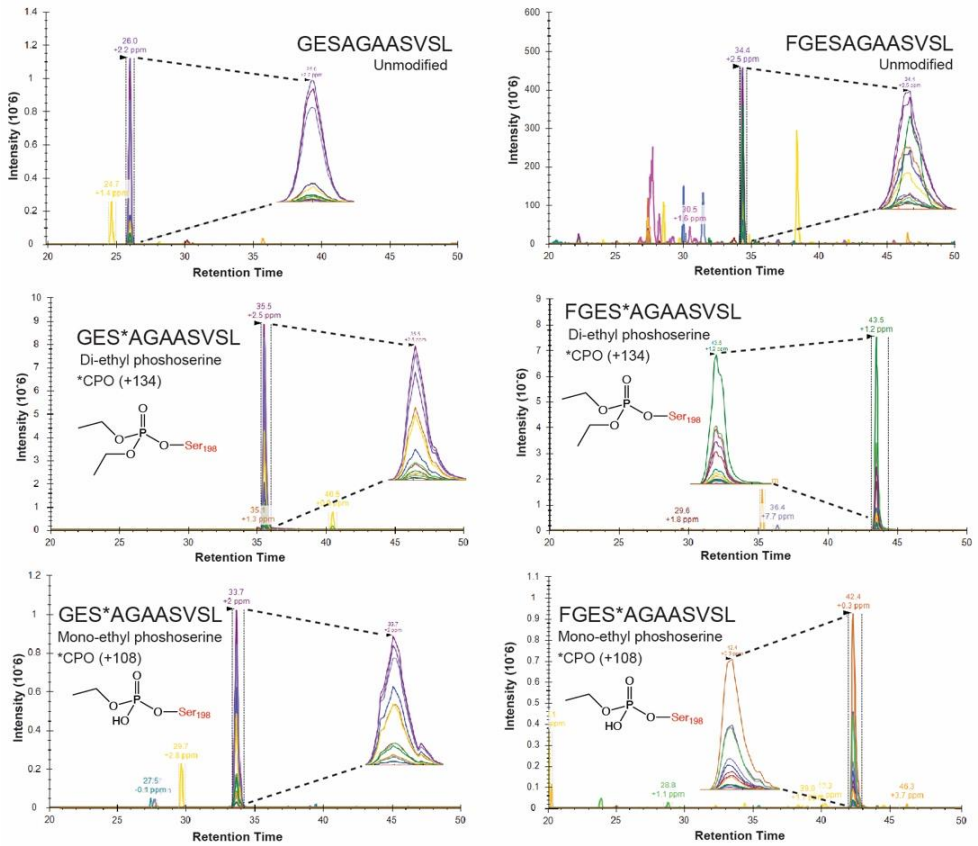

Figure 10. Skyline chromatograms of the eluted active-site BChE chymotryptic peptides GESAGAASVSL and FGESAGAASVSL without modifications and with the di-ethyl phosphoserine (+134 Da) or mono-ethyl phosphoserine (+108 Da) on Serine198 originated from pure BChE uninhibited or inhibited with CPO in vitro. The vertical lines on either side of the major peaks indicate the integration boundaries for the peak. The lines in the zoomed peaks represent the major precursor isotopes as well as various fragment ions (b-ions and y-ions) associated with the precursor in the spectral library.

To identify potential modifications, such as the presence of larger than expected amino acids, we utilized fragment spectra analysis. By analyzing the fragment spectra, we could determine if any amino acids within the peptides were larger than their expected size, indicating a potential modification or adduct formation.

3.3 CPO exposure identification (plasma and DBS)

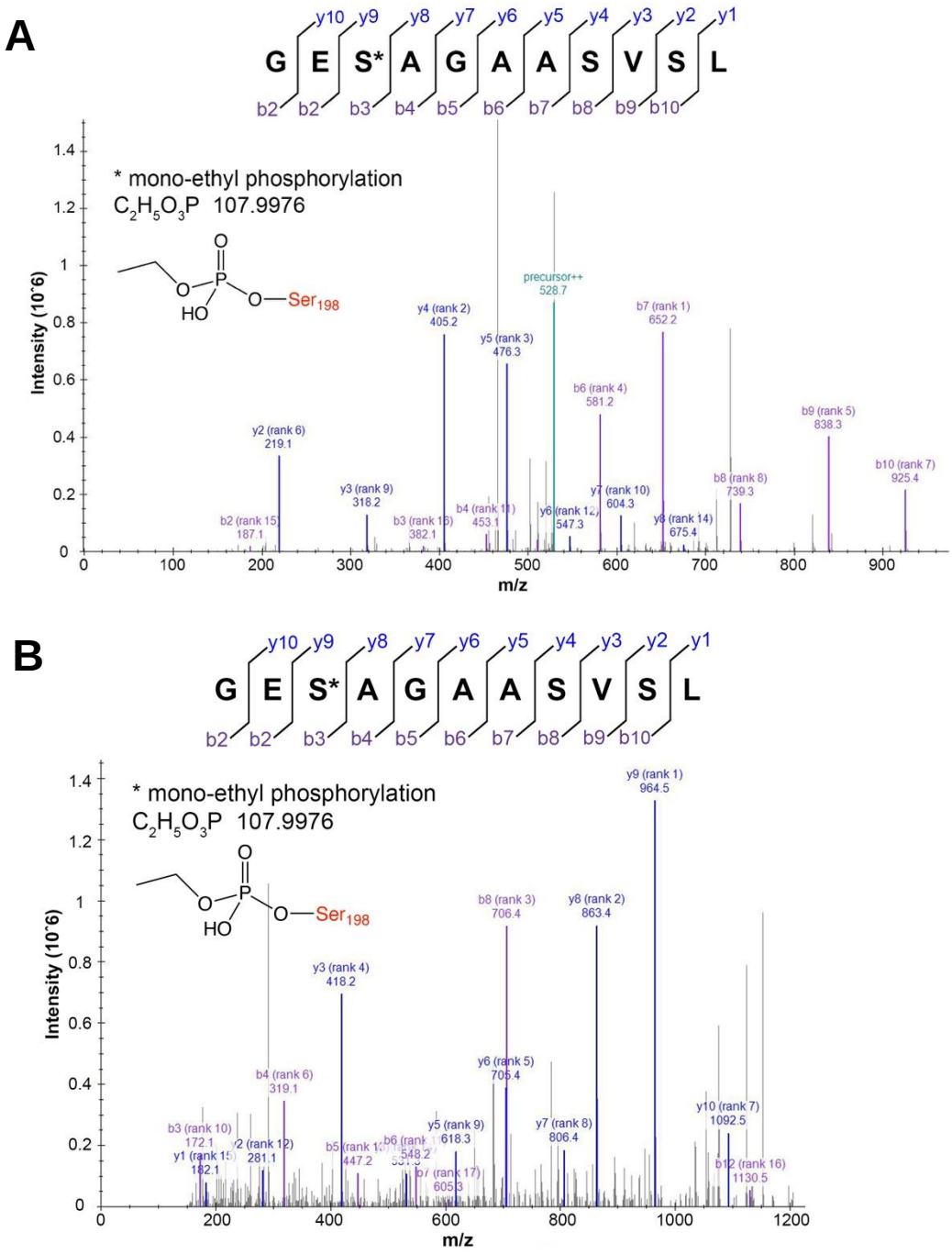

Figure 11. MS/MS spectrum of BChE active site peptide GESAGAASVSL showing mono-ethyl phosphorylation of Serine198 in (**A**) a post-spray season plasma sample of an agricultural worker; and (**B**) a post-spray season DBS sample of the same agricultural worker.

We successfully identified CPO exposure by analyzing plasma and DBS samples using mass spectrometry (**Figure 11**). The MS/MS spectrum of the BChE active site peptide GESAGAASVSL, revealing the presence of mono-ethyl phosphorylation of Serine-198. This specific modification serves as a biomarker of CPO exposure. Notably, we detected the CPO adduct in both the post-spray season plasma sample and the post-spray season DBS sample of the same agricultural worker. The ability to detect CPO adducts in both plasma and DBS samples highlights the potential utility of DBS as a biospecimen to monitor OP exposures.

3.4 Ellman assay vs adducts

In our first Specific Aim, we sought to investigate the relationship between the percentage of inhibited plasma BChE (Ellman assay) and the percentage of adducted plasma BChE (adducts). The results revealed a modest inverse correlation between these two parameters (**Figure 12**). To overcome the limitations of the Ellman assay, we analyzed the measurement of OP adducts using mass spectrometry, which has demonstrated higher sensitivity in detecting OP insecticide

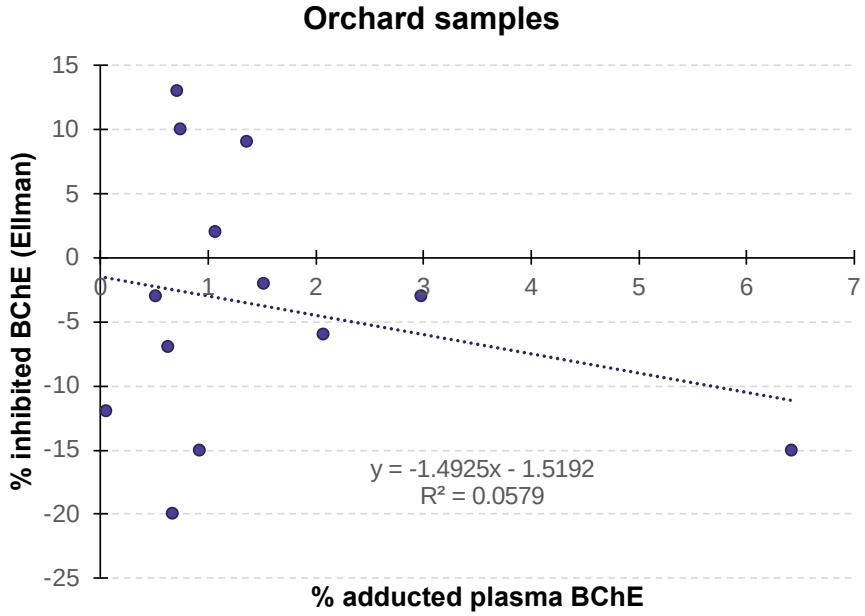

Figure 12. Relationship between % adducted plasma BChE and BChE depression in serial samples collected from Washington State pesticide handlers.

exposures. Our data not only confirmed this higher ended sensitivity but also provided insights into the discrepancies observed between the mass spectrometry results and the Ellman assay. Specifically, we identified CPO adducts in samples that showed a gain in BChE activity during the spray season compared to before the spray season according to the Ellman assay. The increased sensitivity of mass spectrometry can be attributed to its ability to directly detect and quantify the adducts from OP insecticide exposure compared to relying on the measurement of enzymatic activity. By focusing on protein modifications, we were able to capture subtle changes indicative of OP-insecticide adduct formation, even at low levels of exposure (**Figure 12**). The data shows the need for improved methods in accurately assessing pesticide exposures, particularly in situations where the traditional BChE activity assay may yield less reliable results.

3.5 Plasma adducts vs DBS adducts

In comparing the adduct levels in plasma and DBS samples in Specific Aim 2, we observed promising findings that highlight the potential of DBS as an alternative for biomonitoring OP insecticide exposures in agricultural workers. The correlation between the adduct levels measured in DBS and plasma samples indicates comparable ratios of modified BChE to total BChE in both plasma and DBS extracts (**Figure 13**), suggesting that DBS could serve as a reliable biospecimen for biomonitoring OP exposures. The scatter plot graphically represents this relationship, where each point represents a specific sample. Points that lie closer to the trend line demonstrate a higher degree of correlation, suggesting that changes in adduct levels in plasma are mirrored by the corresponding changes in DBS extracts. This observation is noteworthy as it highlights the consistency between the adduct measurements using the two types of

biospecimens, independently of the control peptide used to normalize the samples (**Figure 13A, B and C**).

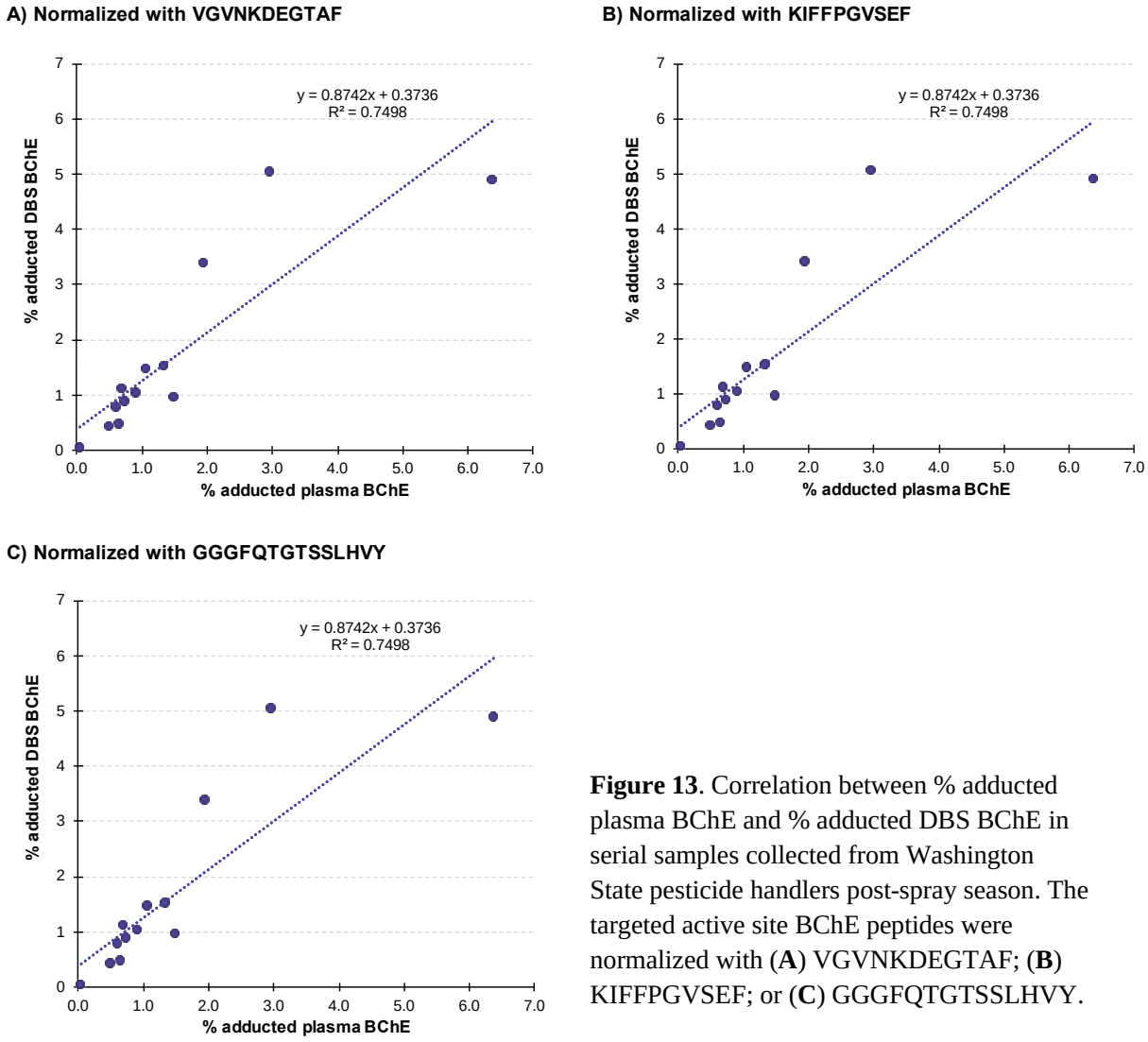

Figure 13. Correlation between % adducted plasma BChE and % adducted DBS BChE in serial samples collected from Washington State pesticide handlers post-spray season. The targeted active site BChE peptides were normalized with (**A**) VGVNKDEGTAF; (**B**) KIFFPGVSEF; or (**C**) GGGFQTGTSSLHVY.

Discussion

In specific aim 1, our focus was on identifying OP-adducted BChE in plasma samples using our established immunoprecipitation bead protocol by targeted high-resolution mass spectrometry (LC-MS/MS). This approach aimed to overcome the limitations of the traditional Ellman assay,

which may not accurately detect low levels of BChE inhibition. The Ellman assay relies on an inhibition threshold that has been associated with a high rate of false-positive measurements, leading to potential inaccuracies in assessing low level exposures (<20% inhibition).

Our study sought to validate the utility of the LC-MS/MS approach by comparing the results obtained with those from the Ellman assay. Our findings revealed notable discrepancies between the two methods, particularly in detecting low levels of BChE inhibition. These findings are consistent with previous studies that have reported false-positive measurements and limitations of the Ellman assay, especially when relying on a baseline sample that exhibits high variability.[36]

In our study, we observed the presence of di-ethyl phosphoserine in pure BChE, and *in-vitro* plasma samples inhibited with CPO, but its absence in the agricultural worker samples. This discrepancy suggests that the natural aging process of BChE, which involves the conversion of the initial phosphorylated serine to di-ethyl phosphoserine, is more efficient *in vivo* compared to *in vitro*. The absence of di-ethyl phosphoserine in the agricultural worker samples indicates there is a more robust and effective aging process that occurs in the human body due to factors such as enzymatic reactions, cellular processes, and physiological conditions. The interplay of these factors can enhance the aging process, resulting in the absence of di-ethyl phosphoserine in the agricultural samples compared to the pure BChE and *in-vitro* plasma samples.

Furthermore, our analysis uncovered an intriguing observation, a subset of individuals in the study exhibited an increase in BChE activity after the spray season, contrary to the expected depression in activity due to OP insecticide exposure. This finding challenges the reliance on

baseline activity samples as a reliable measurement for assessing BChE inhibition, emphasizing the inherent variability in an individual's BChE activity and the need for more dynamic approaches in exposure assessment.

It is worth noting that our study had a relatively small sample size of 13 workers, which can impact the generalizability and the power of the findings. The limited number of participants may not fully represent the diversity of exposure scenarios and variations between individuals. Despite this constraint, our results provide valuable preliminary evidence of the association between the inhibited BChE, and OP adduct formation, emphasizing the need for further research with larger and more diverse cohorts.

In comparing our results to the findings of the study conducted by Dubrovskii and colleagues,[37] several important observations can be made. Our research also encountered challenges associated with the traditional Ellman's assay in quantitatively assessing the degree of enzyme inhibition. As highlighted in the study, the determination of initial enzymatic activity prior to OP exposure is crucial for accurate quantification, but it is often difficult to estimate. Variability in BChE activity among individuals based on factors such as sex, age, and health status can be significant, reaching up to 22%. Furthermore, even within the same individual, there can be considerable variance, ranging from 6-8%.[37] These observations emphasize the limitations of the Ellman assay in reliable assessing low-level exposures. The study also compared the Ellman assay to a mass spectrometry-based analysis, similar to our approach. They found agreement between the two methods, indicating that the mass spectrometry-based method for BChE adduct formation was in concordance with the inhibitory measurements obtained using the Ellman assay. It is noteworthy

that the agreement observed in their study was based on inhibition levels around 70% using the Ellman assay, which has shown to be reliable for detecting higher levels of inhibition. However, for low-level exposures where inhibition is below 20%, the mass spectrometry-based method offers enhanced sensitivity and accuracy.

In our second specific aim, we aimed to investigate the potential of DBS in identifying OP-adducted BChE using the immunopurification bead protocol coupled with targeted high resolution mass spectrometer. The identification and quantification of OP-adducted BChE were successfully achieved using our magnetic bead protocol and LC-MS/MS method. The obtained spectra and peak areas provided valuable insights into the extent of OP exposure and the levels of BChE inhibition. Our findings revealed a strong correlation between plasma and DBS data, indicating similar ratios of modified BChE to total BChE. This correlation provides preliminary evidence supporting the reliability and validity of DBS as a biomonitoring tool for OP exposure. We compared our results to a relevant study that investigated the performance of DBS for the detection of organophosphate nerve agents.[38] This study showcased the excellent performance of DBS in monitoring five nerve agent metabolites with accuracy and precision, reinforcing the potential of DBS as a reliable biospecimen for biomonitoring OP exposure. The strong correlation between DBS and plasma data in our study reinforces the consistency of our results with those of Shaner et al. 2018.[38]

In recent years, there has been a notable shift in the use of OP insecticides, with many countries implementing phase-out strategies due to their adverse effects on human health and the environment. In addition to the phased-out strategies for OP insecticides, it is important to address the broader issue of pesticide safety and regulation. Historically, the conventional

agricultural industry and regulatory agencies have often asserted the safety of pesticides until compelling evidence emerges, leading to their eventual ban or restriction due to their toxicity.[39] An illustrative example is chlorpyrifos, which initially received widespread usage and endorsement but later faced bans and restrictions due to mounting evidence of its harmful effects.[40] This pattern highlights the significance of continuous evaluation and scrutiny of pesticide safety to ensure the protection of human health and the environment. It is also essential to note the shifting dynamics in agricultural practices, with a noticeable increase in herbicide usage such as glyphosate, which has become the most herbicide used pesticide in the United States. While herbicides serve important purposes in weed control, it is critical to closely examine their mechanism of action and their potential health impacts to take a proactive approach to implement effective monitoring programs to address any emerging concerns.

Conclusion

In conclusion, our study highlights the superiority of mass spectrometry in detecting OP adducts in plasma compared to the conventional Ellman assay. The use of mass spectrometry provides a more sensitive and accurate measurement of OP exposure, allowing for the identification and quantification of specific adducts with high precision. This enhanced sensitivity is particularly valuable in assessing low-level exposures that may go undetected by the Ellman assay, thereby providing a more comprehensive understanding of OP-related health risks.

Furthermore, our research demonstrates the utility of DBS as a viable biospecimen collection process for biomonitoring OP exposure using our immunoprecipitation followed by mass spectrometry method. The strong correlation between plasma and DBS data validates the

reliability of DBS as a biomonitoring tool. The ease of DBS collection, storage, and transportation, coupled with the cost-effectiveness of this approach, make it a practical alternative for biomonitoring in both resource-constrained settings and occupational health surveillance programs. The advantages offered by DBS, such as its ease of use and cost-effectiveness, have significant implications for both low- and middle-income countries (LMICs) and employers. In LMICs, where limited resources and infrastructure may impede traditional biomonitoring approaches, the accessibility and practicality of DBS make it an attractive option for assessing OP insecticide exposure. Additionally, the cost-effectiveness of DBS may incentivize employers to monitor worker exposure more frequently, leading to improved occupational health and safety practices.

Overall, our findings underline the importance of employing sensitive analytical methods, such as mass spectrometry, for accurate assessment of OP insecticide exposure. Additionally, the utilization of DBS as an alternative biospecimen collection method offers a promising alternative that combines convenience, reliability, and cost efficiency.

Future Directions

Future research should aim to build upon the less invasive techniques for biomonitoring. Therefore, there are several key directions for future research in the field of biomonitoring OP insecticide exposures using DBS. First and foremost, it is crucial to validate the use of mass spectrometry as an alternative for biomonitoring and DBS as a reliable biospecimen collection method by expanding the study to include a cohort of subjects. This will help establish the

robustness and generalizability of our findings, and further solidify the utility of DBS in accurately assessing OP insecticide exposures.

Moreover, an exciting avenue for future investigation lies in exploring the potential of albumin as a biomarker of exposure using DBS as a collection method. Given its status as the most abundant protein in plasma, albumin presents an attractive target for biomonitoring purposes. The high abundance of albumin negates the need for an enrichment step, simplifying the analysis process. Furthermore, albumin's longer half-life compared to BChE (20 days vs. 11 days) provides a wider window of time to detect exposures, making it a promising candidate for assessing chronic or intermittent exposures to OP insecticides.

Notably, albumin's ability to bind to various chemicals and toxicants opens up the possibility of using it as a biomarker to assess other/multiple exposures beyond OPs. This multifunctional aspect of albumin could allow for a more comprehensive evaluation of individuals' overall chemical exposures and potential health risks. Leveraging the non-invasive nature of DBS, future studies could capitalize on its ease of use for monitoring albumin levels as a broader indicator of chemical exposures in occupational or environmental settings,

Furthermore, exploring the use of newborn DBS for monitoring *in utero* exposures holds significant potential for understanding the impact of maternal exposures on fetal development. By using the achieved DBS samples from child monitoring programs, it becomes possible to retrospectively investigate *in utero* exposures and their potential long-term health implications. This line of research can provide valuable insights into early-life exposures and inform strategies for reducing risks and improving maternal and child health outcomes.

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