ACCUMULATION OF ORGANOPHOSPHATE PESTICIDES IN PORCINE TISSUE OF PIGS RAISED IN EASTERN NORTH CAROLINA: ANALYSIS BY MALDI-TOF-MS

by

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Abstract

The incidence rate of diabetes and obesity is increasing each year, as well as organophosphate pesticides (OPs) in the United States. OPs are toxic and can cause numerous acute effects, but the long-term chronic exposure to OPs has not been investigated. Therefore, the goal of this project is to determine if common OPs utilized in eastern North Carolina (ENC), as well as their common metabolites, accumulate in the kidneys of pigs, and therefore, accumulate in humans. This study is important because it is the first step in analyzing the correlation between Type II Diabetes and OP exposure in eastern NC. Porcine kidneys were analyzed using MALDI-TOF-MS in CHCA positive ion mode, CHCA negative ion mode, and DHB negative ion mode to determine OP and metabolite identities. In all three modes, key differential m/z peaks were determined to fall within the targeted m/z range of approximately 130-380 Da. More specifically, key m/z peak values corresponded to the molecular weights of targeted OPs coumaphos, diazinon, and malathion (raw m/z values of 350.5, 363, and 330.6 Da, respectively). Therefore, this preliminary data suggests that OPs could be present in the porcine tissues and potentially accumulate in humans to manifest as Type II Diabetes.

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Introduction

Organophosphate Pesticides (OPs)

Organophosphate Pesticides (OPs) comprise a specific class of pesticides that are biodegradable and readily available to the common population to use as insecticides. Over 1.1 billion pounds of OPs are currently used in the United States per year; roughly 90% of these OPs are utilized for agricultural purposes.[1] Some of the most commonly used OPs in the United States are chlorpyrifos and diazinon (**Figure 1**). Chlorpyrifos is known to be toxic to humans, and human exposure has been linked to acute neurological disorders and autoimmune responses.[2] Diazinon, an insecticide used both as an indoor and outdoor commercial pest control product, is one of the few OPs that has significant lipid solubility, which can potentially lead to storage in fatty tissues and therefore delay toxicity.[3]

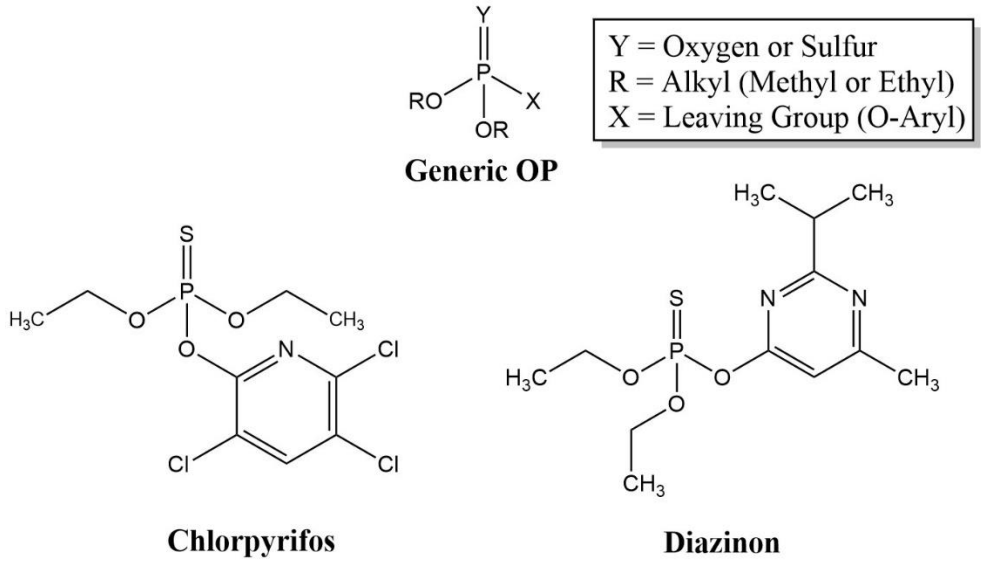

Figure 1. Chemical structures for chlorpyrifos, diazinon, and a generic OP.

These toxic compounds can leach into the environment and enter the food chain through agriculture, water sources, and livestock. Since the mode of pesticide application is not localized, there are concerns about the environmental and human health risks related to chronic pesticide

exposure, in particular Type II diabetes. According to the U.S. Agricultural Health Study from 1993 to 2003, which was a 10-year cross-sectional study of pesticide exposure in the United States, there was a significantly higher incidence rate of diabetes in those who were exposed to these toxic OPs than those who were not exposed.[4] Eastern North Carolinians are highly vulnerable to pesticide exposure through agriculture and livestock farming, the primary method of income in this region. It is likely that the estimated 10 million eastern NC "factory-farmed" hogs are exposed to these toxic OPs and store them in their adipose and organ tissues.

Literature Review & Project Goals

Preliminary studies have shown a connection between OP usage and elevated rates of obesity, as well as the disruption of glycolysis and increased cellular oxidative stress in animal and human models[5-8]. However, none of them have researched the longitudinal effects of this OP exposure in eastern NC. Therefore, the goal of this research is to determine if OPs used in eastern NC and their common metabolites accumulate in the kidney tissues of pigs, and consequently, humans. The Spuches lab proposes that if there is a bioaccumulation of OPs in porcine tissue, then there may be an accumulation in human tissues as well. These OPs include chlorpyrifos, coumaphos, diazinon, dichlorvos, malathion, phorate, terbufos, and trichlorfon, as well as common metabolites such as diethyl phosphate, diethylthiophosphate, dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate, and diethyldithiophosphate. To detect these molecules, Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) was employed to analyze porcine kidney samples in order to obtain preliminary data. This research is crucial because there is little known regarding OP accumulation in the tissues of hogs raised in ENC. The methods developed in this study will then be extended to human tissue.

Materials and Methods

Porcine Tissue Sample Collection

Porcine kidney samples were acquired from Acre Station Slaughter House (Pinetown, NC). A total of 10 kidneys from 10 adult pigs raised in eastern NC (Beulaville, NC) were collected. Kidney sample identity numbers were labeled as follows: KD4, KD7, KD9, KD11, KD13, KD14, KD15, KD18, KD20, and KD22. Numbering was determined by the order in which kidney samples were processed. The kidney samples were stored at -80 ℃ until homogenization, which ensured an accurate representation of the kidney samples during MALDI-TOF-MS analysis. Kidneys were homogenized in a stainless-steel laboratory grade blender (Conair™ Waring™ Stainless Steel Blender, Thermo Fisher Scientific) and lyophilized to remove excess water. Samples were stored at -80 ℃ until analysis by MALDI-TOF-MS.

Porcine Tissue Preparation & OP Extraction

The porcine tissue OP extraction method utilized involved a simple extraction with acetonitrile. A sample of each porcine kidney (6 mg) was dissolved in acetonitrile (0.200 mL) for OP extraction at a concentration of 30 mg/mL. Each sample was vortexed for five minutes and stored at -40 ℃ for 3 days to break down cellular matrix and promote release of parent compounds and metabolites. After cellular breakdown, samples were centrifuged (approximately 7000 rpm) to collect excess cellular matrix. The aqueous layer was then used for OP analysis by MALDI-TOF-MS. All reagents used in this study were of HPLC grade (99% purity) from Sigma-Aldrich.

MALDI-TOF-MS Analysis

MALDI-TOF-MS is an analytical technique that has been used in pesticide residue analysis in both plant and animal matrices. To optimize detection of OPs, two sample matrices, α-cyano-4-

hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB), were utilized at a concentration of 10 mg/mL in a mixture of 70:30 acetonitrile to water and 0.3% TFA. Since target OP and metabolite mass values fell within the optimum detection regions for both CHCA and DHB, both matrices were employed to optimize signal quality. Triazine Pesticides Standard Mix (TPS) certified reference material was used as the control pesticide at a known concentration of 10 mg/mL (Supelco^{TM}, Lot # XA25879V). This standard was utilized to confirm and validate the MALDI-TOF analysis method in the two matrices.

The dried droplet technique was utilized to analyze extracted OP samples, where equal volumes (1 µL) of both matrix and sample are spotted onto an MTP 384 ground steel well plate, which is the sample introduction system for MALDI-TOF-MS analysis. The two matrices, CHCA and DHB, were spotted onto the plate and allowed to dry for approximately five minutes. Pig samples and the control standard, TPS, were then spotted on to the corresponding matrix location and allowed to dry for approximately five minutes. All samples were recrystallized prior to MALDI measurement. **Figure 2** shows an example of the MALDI-TOF-MS spotting plate.

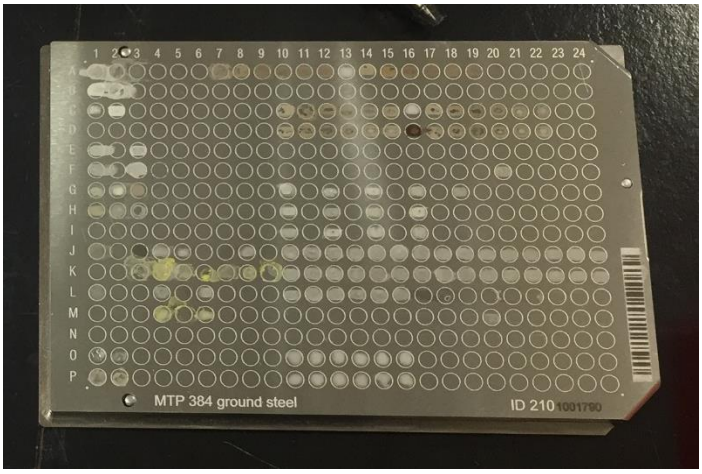

Figure 2. MTP 384 ground steel well plate utilized for MALDI-TOF-MS analysis.

MALDI-TOF-MS was performed in a mass range from 100-2000 Da on an ABI Voyager DE Pro MALDI-TOF Mass Spectrometer equipped with a modified pulsed all solid-state laser (355 nm laser excitation, Bruker, Germany). Calibration of the instrument was carried out with the TPS OP reference standard. Key instrument parameters for OP detection include: ion source 1, 19 kV; ion source 2, 16.65 kV; lens, 8.2 kV; reflector 1, 21 kV; reflector 2, 9.7 kV. Custom processing scripts for AutoXecute^{TM} was utilized for automated mass acquisition. Each sample analysis pulled 5 random points on each spot on the plate and an averaged raw MS spectrum was generated. An example of a raw MS spectrum is showcased in **Figure 3** below.

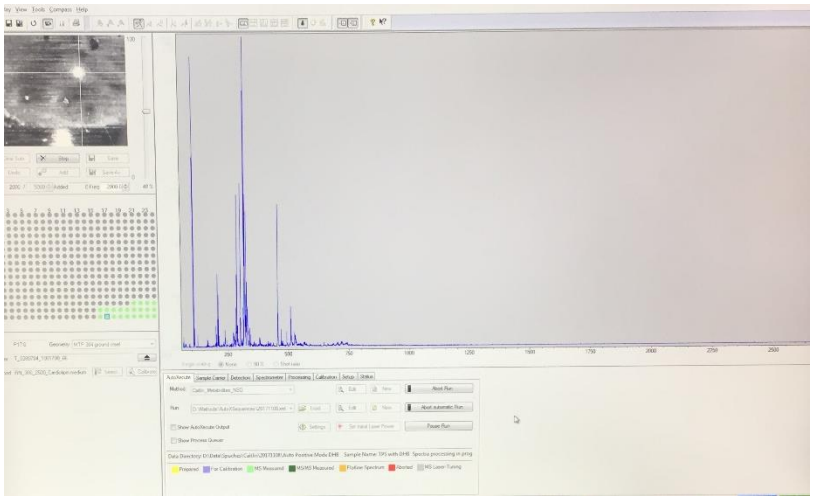

Figure 3. Raw, averaged MS spectrum for KD 14 in Negative Ion Mode.

Accompanying flexControl software was used for controlling the laser and spotting plate optimization, and flexAnalysis were utilized for data processing. Notable parameters for MALDI analysis was measurement in both positive and negative mode for CHCA matrix samples and negative mode for DHB matrix samples. Both modes were utilized in CHCA to optimize signal quality of OPs and metabolites. Negative mode analysis in DHB was chosen to increase ideal signal conditions.

Results

The MALDI-TOF-MS spectra obtained in this study were imported into flexAnalysis, which is provided by Bruker, for data processing and generation of characterization profiles. Baseline subtraction, normalization of spectral data, peak definition, and comparison of multiple spectra were automatically calculated and performed by the flexAnalysis software. Principle Component Analysis (PCA) was used to reduce the dimensionality of the kidney data set and report mass to charge ratio (m/z) variance without losing key data. As a result, principle components (PCs) were utilized to reduce m/z intensity redundancies from MALDI-TOF-MS spectral data.

Multivariate analysis, such as MALDI-MS-outputs, often requires a substantial number of correlated variables. Principle Component Analysis is a dimension reduction tool that can be utilized to reduce a large set of variables to a small set of information, while still retaining most of the information in the larger set. This mathematical procedure transforms a number of correlated variables into a smaller number of uncorrelated variables called principle components. The first principle component accounts for as much of the variability in the data as possible, as each succeeding component accounts for the remaining variability as possible. In the following sections, PCA and PC scores will be reported for the three modes analyzed.

CHCA Positive Ion Mode

All kidney samples were analyzed in CHCA Positive Ion Mode and are reported in this section. The CHCA references the matrix utilized to introduce the sample into the gaseous phase, while the positive ion refers to the type of ion created after laser ionization; in this case, positive ions were formed. The classification of the kidney samples in CHCA positive ion mode was established using the first two PCs in the PCA of the kidney set, shown in **Figure 5.** The

contribution of PC1 and PC2 in the generation of the characterization plot of variance were 57.5% and 12.2%, respectively. To determine the differential m/z peaks, PC analysis was used to create a dimensional visual view of the m/z peak plot for CHCA positive ion mode. **Figure 5A** illustrates the spatial scattering of the differential m/z peaks and therefore the key m/z values. Data points farther away from the center of the graph are the differential peaks, showing elevated levels of the given m/z value compared to the other kidney samples. **Figure 5B** assembles this information into groupings based on which kidney samples showcase elevated m/z peaks relative to other kidney samples. **Figure 4** displays two of the larger differential peaks in each kidney sample compared to the other samples. **Figure 4A** displays samples KD4, KD7, and KD9, which have an elevated m/z value of 379.0 compared to the other remaining kidney samples. **Figure 4B** showcases samples KD11, KD13, KD14, KD15, KD18, KD20, and KD22, all which have an elevated m/z value of 379.1 Da as compared to the other remaining kidney samples. These two values are of interest because the target OP and metabolite m/z values range from 130-380 Da.

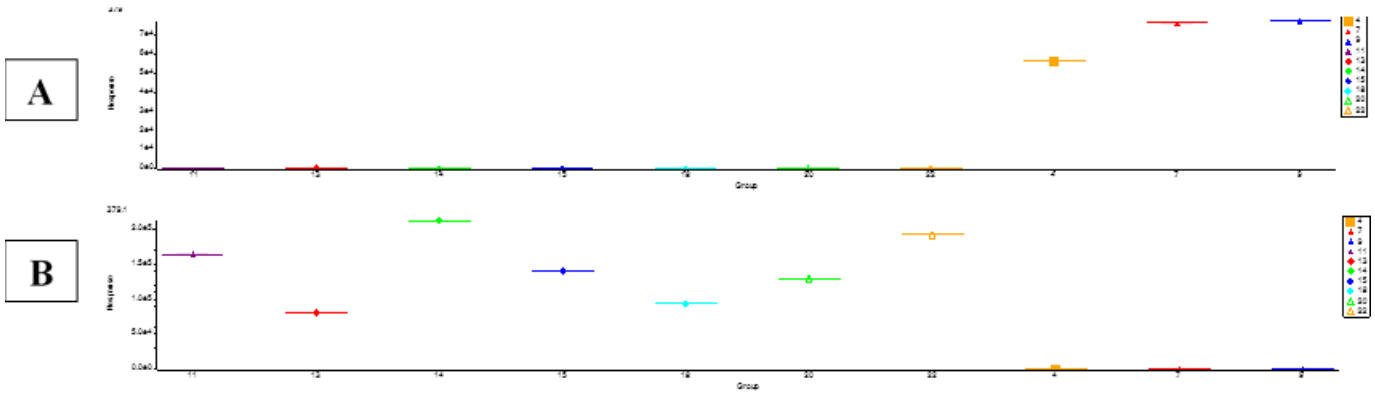

Figure 4. Individual kidney panels for two differential m/z peak values. These values are relative to the other kidneys and therefore only provides qualitative data. **A**. Differential peak m/z intensities for 379.0 Da. KD4, KD7, and KD9 have an elevated m/z value of 379.0 compared to the other kidney samples. **B**. Differential peak m/z intensities for 379.1 Da. KD11, KD13, KD14, KD15, KD18, KD20, and KD22 have an elevated m/z value of 379.1 compared to the other kidney samples.

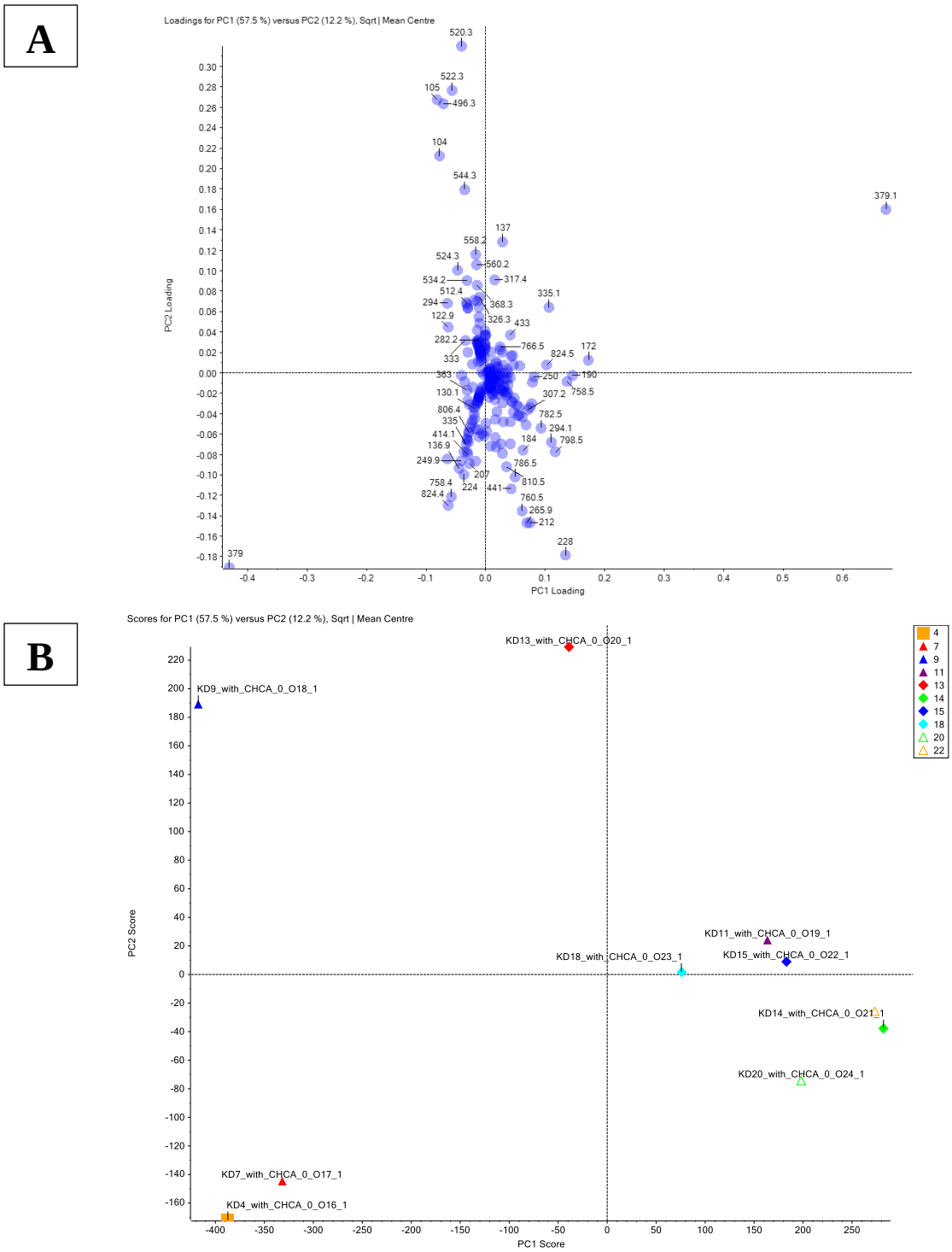

Figure 5. Porcine kidney sample analysis in CHCA Positive Ion Mode. **A**. Plot of PC2 Score vs. PC1 Score. This figure showcases the dimensional image from PCA, which illustrates the mass intensities for all kidney samples on a single plot. Darker blue circles correspond to mass values that are found in all samples and therefore are less likely to be OP/metabolite m/z values. Lighter blue circles correspond to higher intensities (concentrations) of m/z values in certain kidney samples compared to the other kidney groupings. **B**. Plot of characterization of groupings of kidney samples according to m/z values based on PC1 and PC2 scores. Three groupings appear: in the first grouping, KD11, KD14, KD15, KD18, KD20; the second grouping, KD7 and KD4; and the third grouping, KD9 and KD4 (with least correlation). Groupings showcase the m/z ions that are found in higher concentrations within the grouping relative to the other groups/kidney samples.

CHCA Negative Ion Mode

All kidney samples were analyzed in CHCA Negative Ion Mode and are reported in this section. The CHCA references the matrix utilized to introduce the sample into the gaseous phase, while the negative ion refers to the type of ion created after laser ionization; in this case, negative ions were formed. Porcine kidney samples in CHCA in negative ion mode were analyzed in the same manor as CHCA positive ion mode, using the first two PCs in the PCA. **Figure 6** graphically represents the contributions of PC1 and PC2, which were 77.1% and 6.2% respectively. To spatially represent the m/z values in all kidney samples, PC analysis was utilized to create a dimensional visual view of the m/z peak plot for CHCA negative ion mode. Key m/z values were determined in the same fashion as above, with the most deviation from the center as the differential peaks. **Figure 6A** showcases the spatial scattering of the m/z peaks as well as the key differential peaks for the kidney samples. **Figure 6B** transfers this information into kidney groupings to showcase which kidney samples correspond to elevated key differential m/z peaks. A couple examples of key individual differences between kidney samples in CHCA negative mode is displayed in **Figure 7** at m/z values of 143.8 and 282.8 Da respectively. Again, these two values fall within the m/z intensity interest range of 130-380 Da.

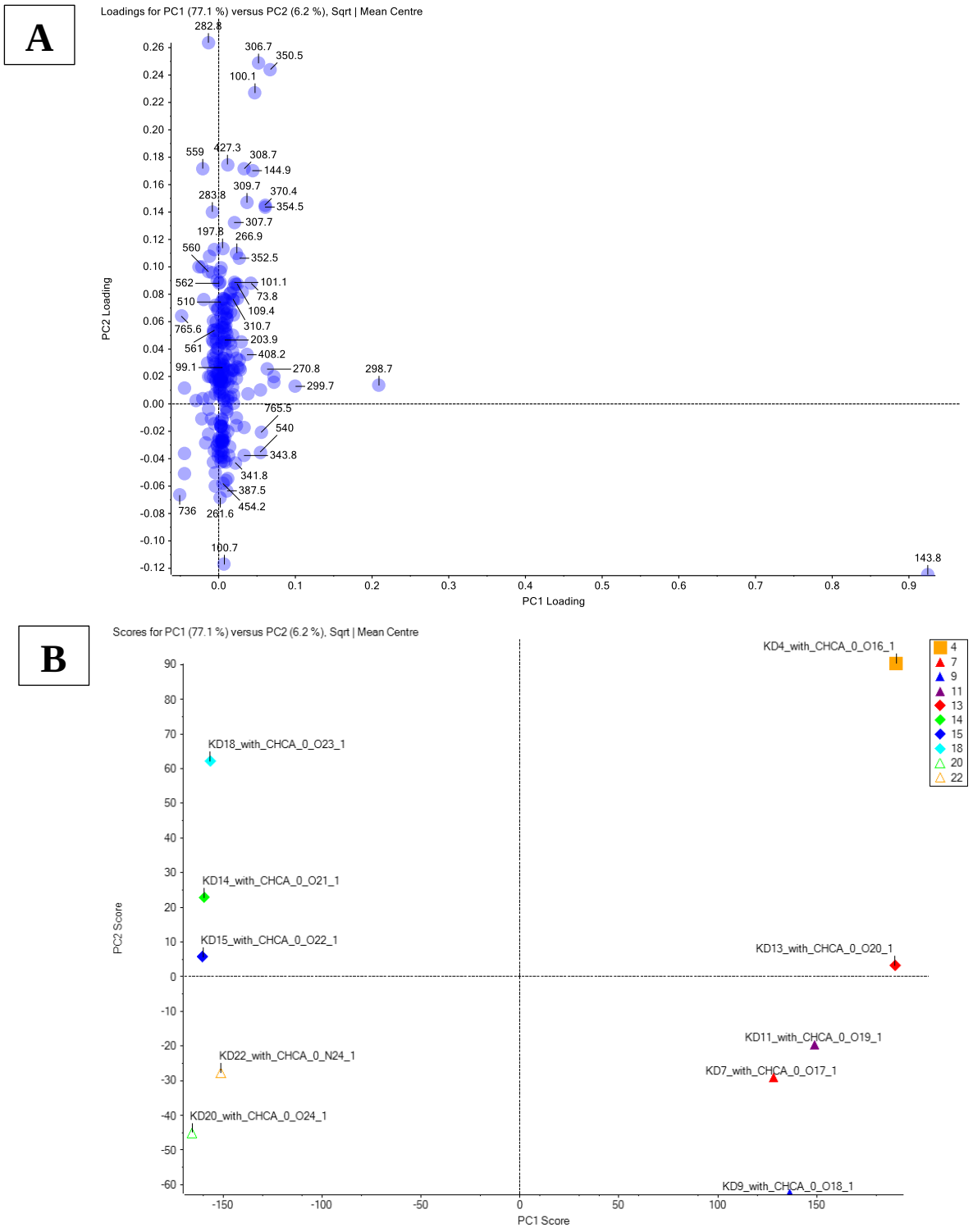

Figure 6. Porcine kidney sample analysis in CHCA negative ion mode. **A**. Plot of PC2 Score vs. PC1 Score for CHCA negative ion mode. Darker blue circles correspond to m/z values that are found in all kidney samples. Greater deviations from the center (lighter blue circles) correspond to increased spectral intensity for m/z values in certain kidney samples relative to the other kidney samples. **B**. Plot of characterization of groupings of kidney samples according to m/z values based on PC scores in CHCA negative ion mode. Three groupings appear: in the first grouping, KD11, KD7, KD13, and KD9; the second grouping, KD18, KD14, KD15, KD20, and KD22; and the third grouping, KD4. These groupings showcase the m/z ions that are found in higher concentrations within the groups relative to the other groupings.

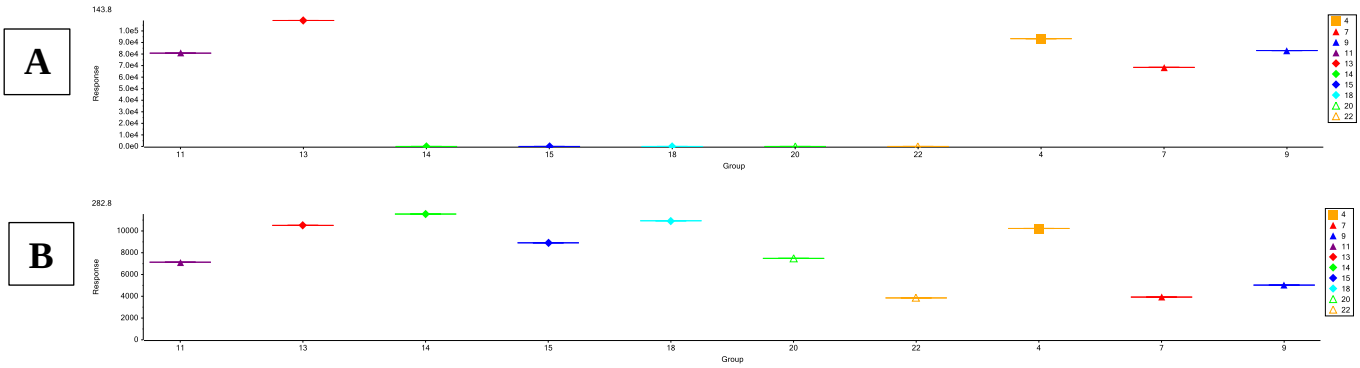

Figure 7. Individual kidney m/z intensity panels for two differential m/z peak values in CHCA negative ion mode relative to the kidney samples, therefore providing quantitative data (i.e., identity). **A**. Differential peak m/z intensities for 379.0 Da. KD4, KD7, KD9, KD11, and KD13 have an elevated m/z value of 143.8 Da compared to the other kidney samples. **B**. Differential peak m/z intensities for 282.8 Da. All samples show an elevated level of this m/z intensity (concentration), though it is found most concentrated in KD4 and KD14.

DHB Negative Ion Mode

All kidney samples were analyzed in DHB Negative Ion Mode and are reported in this section. The DHB references the matrix utilized to introduce the sample into the gaseous phase, while the negative ion refers to the type of ion created after laser ionization; in this case, negative ions were formed. DHB was only analyzed in negative ion mode because this matrix system prefers to ionize smaller compounds, such as alkyl-phosphate groups. As such, these phosphate groups only prefer to be negatively charged, hence the analysis in negative ion mode.

Porcine kidney samples analyzed in DHB negative ion mode were prepared in the same manner as CHCA positive ion mode using the first two PCs in the PCA. **Figure 8** spatially and graphically represents the contributions of PC1, 53.0%, and PC2, 20.8%, to the PCA score. PCA was utilized to create a spatial representation of the m/z values in the kidney samples analyzed in DHB negative ion mode relative to the entire data set. Greater deviations correspond to key m/z values, with the most deviations from the center as the differential peaks. **Figure 8A** illustrates the key differential peaks for kidney samples analyzed in DHB negative mode. **Figure 8B** compiles this information into groupings to showcase which kidney samples correspond to elevated key

differential m/z peaks. To demonstrate some of the key individual differences in DHB negative ion mode, **Figure 9** displays two key differential m/z peaks at 286.7 Da and 343.8 Da, respectively. Again, these two values fall within the m/z intensity interest range of 130-380 Da.

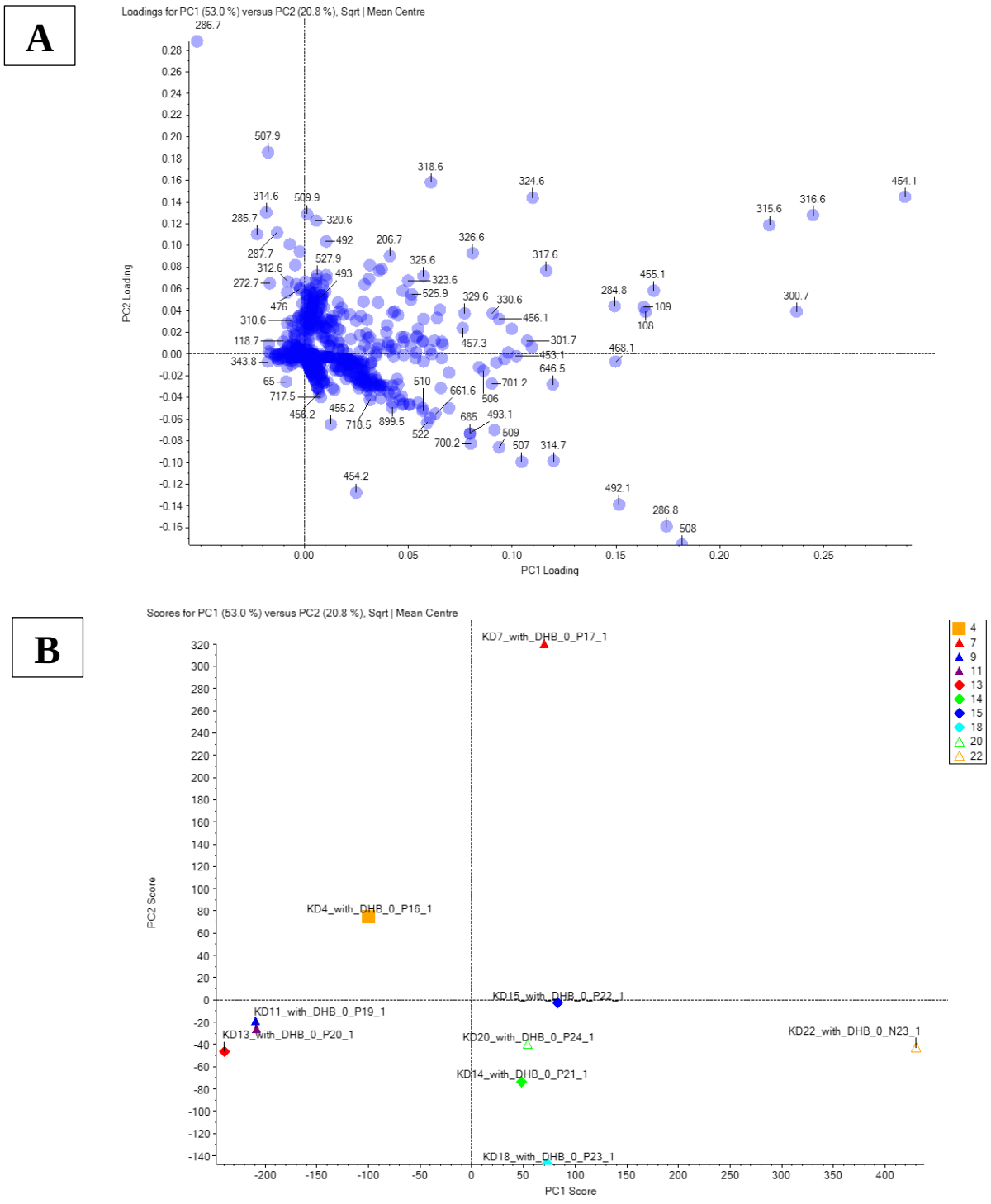

Figure 6. Visual analysis of porcine samples in DHB negative ion mode. **A.** Plot of PC2 Score vs. PC1 Score for DHB negative ion mode. Darker blue circles showcase m/z values that are found in most or all samples with little deviation. Lighter blue circles showcase a greater deviation from other kidney samples (i.e., from the center), thus corresponding to an increased spectral intensity at that m/z value relative to other kidneys. **B**. Plot of the grouping characterizations according to PC scores in DHB negative ion mode. Two (loose) groupings appear: in the first grouping, KD14, KD15, KD18, KD20 and KD22; and the second grouping, KD4, KD7, KD9, KD11, and KD13. These groupings showcase the elevated m/z value concentrations relative to other groupings.

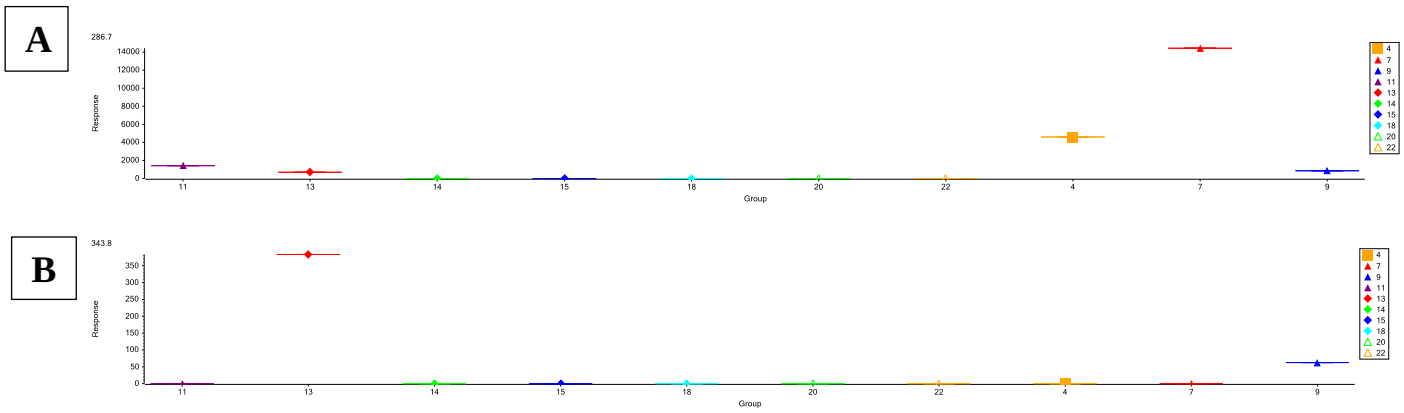

Figure 7. Kidney sample relative m/z intensities for two differential m/z peak values in DHB negative ion mode. **A.** Differential peak m/z intensities for 286.7 Da. KD4, KD7, and KD9 have an elevated m/z value of 286.7 Da compared to the other kidney samples. **B**. Differential peak m/z intensities for 343.8 Da. KD 13 shows a relatively elevated signal intensity compared to the other kidney samples at m/z value 343.8 Da and KD9 showing a slight elevation of signal intensity compared to the other samples.

Discussion

MALDI-TOF-MS was utilized to analyze 10 pig kidney samples in 3 different matrices/modes (CHCA positive ion mode, CHCA negative ion mode, and DHB negative ion mode) to determine optimum analysis of OP and metabolomic identity. The data collected showcases a multitude of m/z value deviations within the kidney samples relative to the data set. These deviations relate to elevated concentrations of the key differential m/z peak values, which corresponds to the m/z value range of the targeted OPs and metabolites. All samples in this study show elevated concentrations for key m/z values within the targeted m/z range, which warrants further research on this data set.

In all three modes, key differential m/z peaks were determined to fall within the targeted m/z range of approximately 130-380 Da. CHCA positive ion mode, for example, had a key differential peak value that corresponded to 363 Da. This value closely resembles the m/z value for the targeted OP coumaphos, 362.77 Da. CHCA negative ion mode also had a key differential peak at 350.5 Da, which closely resembles the m/z value for the targeted OP chlorpyrifos, 350.59

Da. DHB negative mode showcased a key differential m/z value of 330.6 Da, which is very similar to the identity of the OP malathion, 330.36 Da. All three of these OPs are commonly used in eastern NC at high concentrations as herbicides and pesticides. These preliminary results therefore point towards the presence of relatively elevated OPs and metabolite concentrations within the kidney samples.

Further research on this study includes statistical analysis on the data to further determine key differential m/z values. This would include performing t-tests on the collected data in order to solidify confidence in key elevated m/z values, as well as pair down the possible identities for the m/z values. Further research also includes analysis by Liquid Chromatography Mass Spectrometry (LC-MS) to determine definitive concentrations of OPs and metabolites in the sample, as well as confirm identity of the molecules. Though MALDI-TOF-MS is a powerful tool to determine identities of molecules, this instrument only provides qualitative data relative to the sample set. Therefore, analysis by LC-MS would be the next step in analyzing these samples for OPs and corresponding metabolite concentrations, as well as confirming identities.

This data provides insight to the OP usage in eastern NC, as well as provides preliminary data for its correlation with Type II Diabetes. There is little known regarding OP accumulation in the tissues of hogs raised in eastern NC; therefore, this study provides a first step in the analysis of OP accumulation in porcine tissue. The methods developed in this study are also important in the analysis of human tissues regarding OP accumulation, as well as its relationship with Type II Diabetes. Since the preliminary data suggests that OPs could be in porcine tissue, then it is possible that there is a bioaccumulation in humans as well. This bioaccumulation would lead to a higher risk for and incidence rate of Type II Diabetes, which is already a common disease in eastern NC.

Therefore, this research is the first step in analyzing the correlation between OP exposure and Type II Diabetes in eastern NC.

Conclusions

The purpose of this study was to determine if seven common OPs used in North Carolina, as well as their metabolites, accumulate in the kidneys of pigs. These OPs include chlorpyrifos, coumaphos, diazinon, dichlorvos, malathion, phorate, terbufos, and trichlorfon, as well as common metabolites such as diethyl phosphate, diethylthiophosphate, dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate, diethyldithiophosphate. MALDI-TOF-MS was utilized in this study because it is a powerful tool in identifying key differential m/z peak values for identification of key compounds within a sample. The preliminary data collected showcases many elevated concentrations of key differential m/z peak values, which corresponds to the m/z value range of the key OP and metabolites to be identified in this study. A few key differential values determined were 363, 350.5, and 330.6 Da, which corresponds to coumaphos, chlorpyrifos, and malathion, respectively. Further research includes the statistical analysis of the data set, as well as analysis by LC-MS to confirm OP identity and concentrations within the kidney samples. This research showcases the exposure of OPs in porcine tissues, as well as the first step in analyzing the correlation between Type II Diabetes and OP exposure in eastern NC.

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