Erika Zink graduated from Washington State University with a B.S. in Biology with minors in Chemistry and Psychology in 2004. While an undergraduate, she conducted research in the analysis of the components of noble fir oil and its antibacterial properties at Columbia Basin College. Her work in this area was presented in poster form at the 60th annual regional meeting of the ACS. In her senior year she participated in the DOE Faculty and Student Team internship program at Pacific Northwest National Laboratory, where she worked with a professor and a fellow student to determine if saliva could be used as a medium to test for chemical exposure. This work was presented at the AAAS National Conference in 2005.

Ryan Clark is a sophomore attending Columbia Basin College (CBC) where he is currently pursuing pharmacy with intent to go into pharmacology research. He is involved in undergraduate research on natural resource chemistry, which is being presented at the national American Chemical Society meeting (2005). During his student research appointment at Pacific Northwest National Laboratory as a member of a Faculty and Student Team (FaST), he helped work on developing a method for recovering the metabolites of pesticides and other compounds of interest for creating a process to test for exposure of these chemicals non-invasively.

Karen Grant serves as head of the new joint Bachelor of Science in Chemistry program currently being developed between Columbia Basin College and Washington State University – Tri-Cities. She obtained B.S. degrees in chemistry and in physics from Bates College in 1967 and an M.S. degree in chemistry from the University of Wisconsin in 1969. She has more than ten years experience as a Faculty Research Fellow in the Advanced Organic Analytical Methods Group at the Pacific Northwest National Laboratory and has developed a successful undergraduate research program at Columbia Basin College, a community college in Pasco, WA. In 2001, she was awarded the American Chemistry Council's National Catalyst Award. Her research interests include natural product chemistry and development of analytical methods for analysis of trace organic species in non-invasive biological matrices.

Jim Campbell is a Laboratory Fellow at Pacific Northwest National Laboratory (PNNL) in Richland, Washington. He received his Ph.D. in Analytical Chemistry from Montana State University in 1983. Jim has studied the development of analytical techniques for the detection and quantification of organic components in difficult matrices, both environmental and biological. Most recently his work has involved evaluating saliva as a non-invasive biomonitoring matrix for the determination of exposure to organophosphate insecticides and other hazardous chemicals. In addition, his interests lie in the area of metabolomics.

Eric Hoppe is a Senior Research Scientist in the Chemical and Biological Sciences Group at Pacific Northwest National Laboratory (PNNL) in Richland, Washington. He received his B.S. in chemistry from Colorado State University. His research interests include gas and liquid chromatographic techniques using various inlets, interface systems, and detectors including mass spectrometry and infrared spectroscopy. Other interests include analytical methods development for components of radioactive mixed waste and other highly complex matrices, ordnance compounds, and EPA priority pollutants in a variety of materials. He has developed real-time analytical systems for monitoring of air, gaseous process effluents, engine exhausts, off-gas of in-situ remediation techniques involving thermal and oxidative destruction methods. He regularly tests and develops materials used in advanced engineering programs.

ANALYTICAL METHODOLOGIES FOR DETECTION OF GAMMA-VALEROLACTONE, DELTA-VALEROLACTONE, ACEPHATE, AND AZINPHOS METHYL AND THEIR ASSOCIATED METABOLITES IN COMPLEX BIOLOGICAL MATRICES

Erika Zink, Ryan Clark, Karen Grant, James Campbell, and Eric Hoppe

ABSTRACT

Non-invasive biomonitoring for chemicals of interest in law enforcement and similar monitoring of pesticides, together with their metabolites, can not only save money but can lead to faster medical attention for individuals exposed to these chemicals. This study describes methods developed for the analysis of gamma-valerolactone (GVL), delta-valerolactone (DVL), acephate, and azinphos methyl in saliva and serum. Liquid chromatography/mass spectrometry (LC/MS) operated in the negative and positive ion mode and gas chromatography/mass spectrometry (GC/MS) were used to analyze GVL and DVL. Although both analytical techniques worked well, lower detection limits were obtained with GC/MS. The lactones and their corresponding sodium salts were spiked into both saliva and serum. The lactones were isolated from saliva or serum using newly developed extraction techniques and then subsequently analyzed using GC/MS. The sodium salts of the lactones are nonvolatile and require derivatization prior to analysis by this method. N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) was ultimately selected as the reagent for derivatization because the acidic conditions required for reactions with diazomethane caused the salts to undergo intramolecular cyclization to the corresponding lactones. *In vitro* studies were conducted using rat liver microsomes to determine other metabolites associated with these compounds. Azinphos methyl and acephate are classified as organophosphate pesticides, and are known to be cholinesterase inhibitors in humans and insects, causing neurotoxicity. For this reason they have both exposure and environmental impact implications. These compounds were spiked into serum and saliva and prepared for analysis by GC/MS. Continuation of this research would include analysis by GC/MS under positive ion mode to determine the parent ions of the unknown metabolites. Further research is planned through an *in vivo* analysis of the lactones and pesticides. These methodologies could be extended for further analysis of other similar compounds.

INTRODUCTION

Non-invasive biomonitoring for chemicals of interest in law enforcement and similar monitoring of pesticides together with their metabolites can not only save money but can lead to faster medical attention for individuals exposed to these chemicals. This study describes methods developed for the analysis of gamma-valerolactone (GVL), delta-valerolactone (DVL), acephate, and azinphos methyl (structures are shown in Figure 1) in saliva and serum with extension to metabolic studies. Currently, studies are being done on these chemicals and their toxicities, but very little is known about their metabolism.

Approximately five billion tons of pesticides were used in the United States in 1997 [1]. Azinphos methyl and acephate are classified as organophosphate pesticides, and are known to be cholinesterase inhibitors in humans and insects, causing neurotoxicity [2, 3]. For this reason they have both exposure and environmental impact implications. Farm workers are often exposed to these pesticides and therefore, it has been recommended that they be tested for the accumulation of these chemicals. In Washington State, bioassays on individuals are required after fifty hours of exposure over a thirty-day span [1]. Currently, these pesticides are

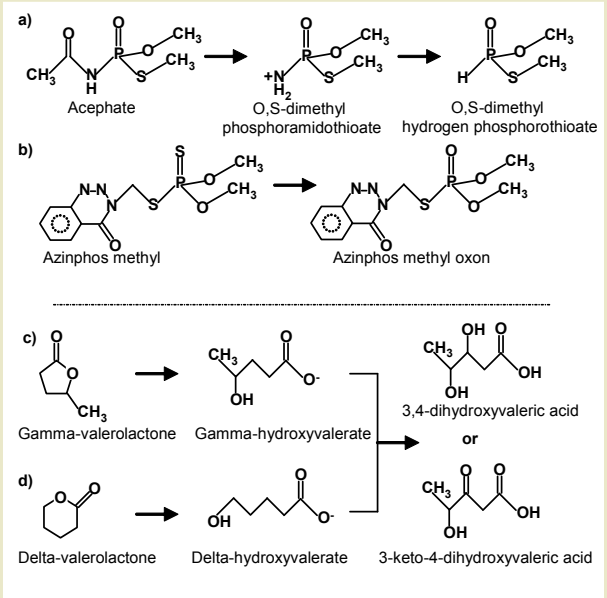

Figure 1. Metabolism possibilities for a) acephate, b) azinphos methyl, c) gamma-valerolactone and d) delta-valerolactone.

GC/MS Conditions		
Parameter	Lactones	Pesticides
Inj. Port Temp	220 C	250 C
Initial Temp	50 C	80 C
Initial Time	2.00 min.	1.00 min.
Solvent Delay	5.00 min.	5.00 min.
Rate	10 deg/min.	20 deg/min.
Final Temp	300 C	300 C

Table 1. General settings for GC/MS for analysis of lactones and pesticides.

monitored by analysis of their metabolites in biological matrices (i.e. blood and urine). These metabolites degrade very quickly and leave the body within a short-time period [4, 5]. The cost to the farmer in terms of testing and time lost per field worker is estimated to be two to three thousand dollars. Detection of the metabolites (see Figure 1a and 1b for possible metabolic routes) could lead to real-time analysis of exposure to these chemicals, resulting in a substantially lower cost.

The lactones, GVL and DVL, have physiological effects similar to those of certain controlled substances of interest to law enforcement agencies. Based on the structure of these compounds and the environment created by ingestion, it is believed that these compounds would first metabolize to the anion (Figure 1c and 1d). Since this structure is similar to that of a fatty acid, a good prediction is that they would undergo beta-oxidation as well, producing the metabolites shown in Figure 1. If analytical techniques could be developed for the detection of GVL, DVL, and their metabolites, the results would provide more effective drug enforcement and litigation, as well as the required first aid due to exposure.

Due to the short-lived nature of these analytes, the development of techniques for their detection will eventually lead to more immediate analysis, particularly if obtained from a non-invasive sampling such as saliva. This would enable agricultural industries to save money on exposure testing for pesticides and allow first aid to be administered within the narrow window of opportunity for such chemicals.

The objective of this study was to determine if any unique metabolites exist and if saliva is a viable matrix for non-invasive biomonitoring. This was done by initially developing techniques for the detection of the analytes in standard solutions. This was followed by the development of techniques for the detection of GVL, DVL, azinphos methyl, and acephate in complex biological matrices, in this case, saliva and serum. Ultimately, an *in vitro* study with rat liver microsomes was conducted where the analytes were spiked into the S-9 fraction, incubated, and then analyzed for possible metabolites. Comparisons of recoveries and different techniques were then drawn between the analytes. Further research is planned through an *in vivo* analysis of the lactones and pesticides.

Methods have been developed for the analysis of GVL, DVL, acephate, and azinphos methyl in blood, saliva, and the S-9 fraction from rat livers. Preliminary results indicate no metabolism occurring in rat blood. However, for DVL and azinphos methyl, there are differences between the control (no metabolism) and the sample undergoing metabolism with the S-9 fraction. The metabolic products are in the initial stages of identification.

MATERIALS AND METHODS

The chemicals used in this study included gamma-valerolactone, GVL (Sigma-Aldrich), delta-valerolactone, DVL (Lancaster Synthesis, Inc.), acephate (Chem Services, Inc), and azinphos methyl (Chem Services, Inc). The serum used was ordered through Sigma-Aldrich and saliva was collected. The livers used for the *in vitro* study were extracted from rats ordered from Charles River Laboratory (Hollister, CA).

The sodium salts of the two lactones were prepared through saponification of 0.001 M solutions of GVL or DVL by adding a solution of 0.001 M of sodium hydroxide in a methanol solvent to the sample. The resulting salts were dried under a nitrogen stream for 30 minutes and then dried overnight with silica beads before purifying by washing the crystals with acetone. Once purified, they were once again blown down to dryness under nitrogen gas and dried overnight as before [6]. The derivatizing agent used, N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) was ordered from Pierce Biotechnology, Inc.

All samples were analyzed by gas chromotagraphy/mass spectrometry (GC/MS) using an HP5890 gas chromatograph with a 30 m x 0.25 mm x 0.25 µm DB5 column (Agilent) and a JEOL SX-102/SX-102 tandem, four sector, high resolution mass spectrometer. The parameters for the analysis of the lactones and the pesticides can be found in Table 1, unless otherwise stated. For those samples that were lyophilized, a Labconco Lyph·Lock 4.5 Freeze Dry System was utilized. The samples that were analyzed by liquid chromatography/mass spectrometry (LC/MS) were done using a ThermoFinnigan TSQ 7000 triple-quadrupole mass spectrometer equipped with an Agilent LC binary pump to perform separations using a variety of different columns. A Harvard Apparatus Model '22' syringe pump was used for direct infusion of the sample into the mass spectrometer. All samples were run under electrospray ionization (ESI) in both the positive ion and negative ion modes.

The dosing solutions of the analytes for the *in vitro* studies were prepared by making 40,000-ppm solutions. Approximately 0.040 mg of GVL or acephate was added to 1 mL of Milli Q water. In the

BCA Protein AssayStandards 081604					
desc.	mg/mL protein	absorbance protein	Avg. absorbance	mg/mL Calculated	analyzed/ calc (%)
Std 1	0.000	0.235	0.235	-0.091	
Std 2	0.212	0.392	0.392	0.255	120.085
Std 3	0.530	0.547	0.547	0.595	112.322
Std 4	1.060	0.813	0.813	1.180	111.324
Std 5	1.590	0.937	0.937	1.453	91.360
Std 6	2.120	1.049	1.049	1.699	80.133

Table 2. BCA protein assay standards.

case of DVL and azinphos methyl, the solubilities in water were low, so 0.040 g of DVL was added to 2 mL ethanol and 0.040 g of azinphos methyl was added to 2 mL of methanol and each were sonicated to aid solvation.

The buffer solutions used in the preparation of the liver microsomes were prepared prior to the extraction of the rat livers. The phosphate buffered saline (PBS) was prepared by adding 1 tablet (Sigma cat#P-4417) to 200 mL of water (distilled, de-ionized). The homogenization buffer was prepared by measuring 250 mL of stock phosphate buffer in a 500 mL graduated cylinder and adding to this 42.78 g of sucrose. To this was also added 0.190 g of ethylenediam-inetetraacetic acid (EDTA) and enough water to make a total volume of 470 mL. The pH was then adjusted to 7.4-7.5 with 1 N NaOH. The solution was then brought up to 500 mL with water. Lastly, the stock phosphate buffer was prepared by measuring 400 mL of water in a graduated cylinder and adding to this 5.77 g K_2HPO_4 (potassium phosphate, DIBASIC, Fisher/Acros Cat#42419-5000), then 2.08 g KH_2PO_4 (potassium phosphate, MONOBASIC, Fisher/Acros Cat#42420-5000). The pH was then adjusted to 7.4-7.5 using either monobasic or dibasic crystals (slowly), the solution was then brought up to a total of 500 mL with water.

Eight male Spraque Dawley rats were received on 07/27/04 and acclimated to the lab for 7 days. They were euthanized with CO_2 and their body weights were recorded. Blood was extracted from each animal and the livers were first perfused with cold PBS via the vena cava prior to extraction. With some it was necessary to perform an external perfusion by rinsing each lobe of the liver with cold PBS after it had been extracted. The weight of the liver was recorded after removing any extraneous tissues and were found to vary from 11.6 to 15.0 grams. The livers were then rinsed with cold PBS prior to being submersed in 30 mL of ice-cold homogenization buffer. The tissue was then minced into small pieces with scissors while keeping it in the buffer and on ice. The tissues were then homogenized with a Teflon/glass homogenizer (10 strokes) on a drill press. The homogenate was then spun for 20 min at 4°C at 9000 x g (~11,000 rpm). The supernatant was saved and stored at -80°C until used.

The protein content of the S-9 fractions that were isolated from the rats was found to be around 30 mg/ml (Tables 2-4). About 1680 µL of the phosphate buffer solution was added to 330 µL of the S-9 fraction to create a solution with approximately 10 mg of protein per mL. This solution was warmed for approximately two minutes before 10 µL of the dosing solutions described previously were added. Once spiked with the analytes, microsomes were incubated for one hour in a 37°C water bath, and vortex mixed every ten minutes. The reactions were quenched with 2 mL of ethyl acetate, after which they were vortexed and centrifuged. After removing the top organic layer, each sample was washed two more times with 2 mL of ethyl acetate to extract the analytes. The combined organic layers were then blown down to approximately a 1 mL volume under nitrogen and dried with anhydrous sodium sulfate.

S-9 protein assay: 20 x dilution

sample	absorbance	average absorbance	diluted mg/mL protein	final mg/mL protein	Average protein mg/ml
#1	0.867	0.867	1.299	25.975	29.134
#2	0.756	0.756	1.055	21.095	22.627
#3	0.783	0.783	1.114	22.282	24.320
#4	0.894	0.894	1.358	27.162	25.221
#5	0.919	0.919	1.413	28.261	24.672
#6	1.095	1.095	1.800	35.999	29.640
#7	0.9	0.9	1.371	27.426	27.112
#8	1.006	1.006	1.604	32.086	35.157

Table 3. Analysis of samples at 20x dilution.

S-9 protein assay: 50 x dilution

sample	absorbance	average absorbance	diluted mg/mL protein	final mg/mL protein
#1	0.57	0.57	0.646	32.29
#2	0.496	0.496	0.483	24.16
#3	0.516	0.516	0.527	26.36
#4	0.488	0.488	0.466	23.28
#5	0.468	0.468	0.422	21.08
#6	0.488	0.488	0.466	23.28
#7	0.52	0.52	0.536	26.80
#8	0.624	0.624	0.765	38.23

Table 4. Analysis of samples at 50 x dilution.

A control was created with the analyte present by first adding 2 mL of ethyl acetate to the microsome-buffer solution to prevent any metabolism from occurring and then allowing them to heat for 2 minutes before adding the analyte. These were incubated for one hour before the compounds of interest were extracted using the same technique as described for the samples above. Adding 2 mL of ethyl acetate to the microsome-buffer solution and extracting three times (just as the previous samples) created a method blank. The microsome samples that were left after being extracted were then lyophilized and derivatized with MTBSTFA to test for any other possible metabolites. The extracted samples were analyzed by GC/MS for acephate, azinphos methyl, and GVL. The derivatized sample extracts containing azinphos methyl and GVL were also analyzed by GC/MS.

To verify that the enzymes in the microsomes were functioning properly, they were tested with chlorpyrifos (CLP). This was done by creating a sample, a duplicate, an analyte blank and a solvent blank using a similar method to that used for the analytes of interest. One of the samples was derivatized with MTBSTFA to determine if the metabolite, trichloropyridinol (TCP), was present and compared to a standard of TCP derivatized with MTBSTFA. The results showed that metabolism had occurred and that the microsomes were functioning as expected [10].

The blood that was extracted previously was used for incubation with the metabolites, using a method similar to that used for the microsome incubations. In this case, the dosing solutions were prepared by adding approximately 10 mg of the GVL, DVL, and acephate to 10 mL of Milli Q water, and 10 mg of azinphos methyl to 10 mL of methanol. A sample, duplicate and a method blank were created using 40 µL of the water solutions and 20 µL of the methanol solution. Also, for these samples, ethyl acetate was used as the quenching solvent, except for GVL, which was quenched with dichloromethane. Two method blanks were also created using ethyl acetate and dichloromethane. All the samples were extracted three times and analyzed by GC/MS.

RESULTS AND DISCUSSION

GVL and DVL.

Saponification of the lactones to produce the sodium salts produced 89.7 mg of Na-GHV, giving about a 63% yield. A similar yield was observed with the formation of Na-DHV from DVL. Analysis by GC/MS of the analyte standards produced reasonable responses, as shown in Figure 2. The responses of the lactones were found to be low compared to that of the derivatized sodium salts. This could have been due to the elevated injection port temperature, which was consistent with studies done on the acids of the analogs of the lactones. The lactones are reported to be thermally labile [8]; the effects of which could be tested using lower temperatures and by on-column analysis. The base ion for GVL is believed to result from the loss of the methyl group attached to the ring; whereas the base ion for DVL is the lactone (Figure 3c and 3d). The derivatized sodium salts produced mass spectra that had the same base ion with an m/z of 189, shown in Figure 3e and 3f.

When GVL was analyzed by LC/MS, it was found that the pH of the analyte solution could have a tremendous effect on the results. When deionized water was used, which had a pH of around 8.6, the lactone appeared to open up and polymerize with a mass difference of 140 between each increment. It was also found that at this pH, the NIM for GVL was the same as the NIM for Na-GHV and the PIM for GVL was the same as the PIM for Na-GHV. However, when Milli-Q water was used, which had a pH of around 7.9, the NIM for GVL was found to show no recognizable results, but the PIM showed a small amount of the sodium adduct (m/z 122.7) and no polymerization. When the electrospray capillary temperature was increased from 200°C to 250°C the main response was at m/z 122.7.

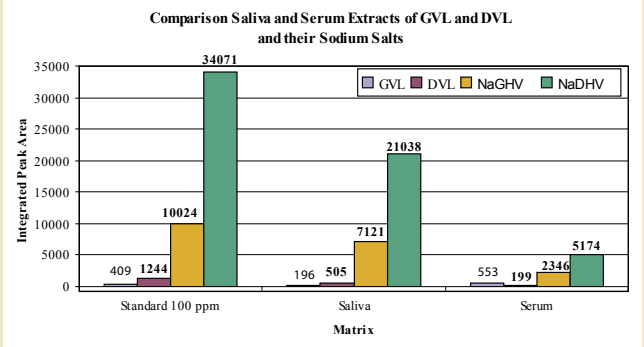

Figure 2. Results of the lactones and their sodium salts in saliva and serum.

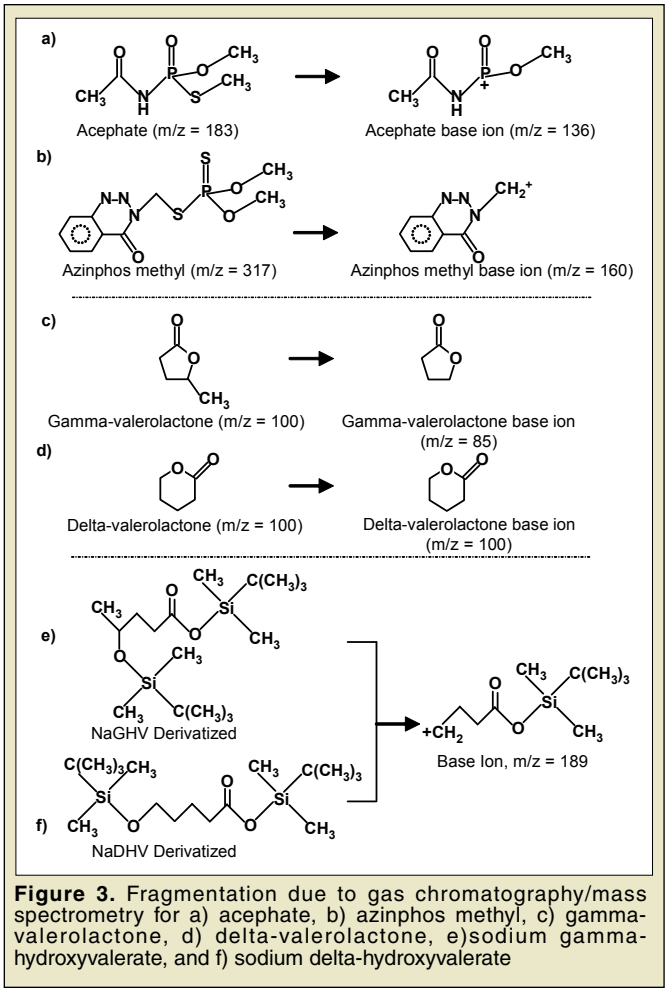

Figure 3. Fragmentation due to gas chromatography/mass spectrometry for a) acephate, b) azinphos methyl, c) gamma-valerolactone, d) delta-valerolactone, e)sodium gamma-hydroxyvalerate, and f) sodium delta-hydroxyvalerate

Analysis of the lactones spiked into human saliva collected from volunteers produced reasonable results by GC/MS (Figure 5). At an injection port temperature of 220°C, the response was lower for GVL than when the injection port temperature was lowered to 150°C. For DVL, an injection port temperature of 220°C was used, which showed good results. A saliva blank, extracted with dichloromethane was analyzed by GC/MS, showed a fairly clean sample with no large peaks. The GVL and Na-GHV saliva samples that were analyzed by LC/MS gave a weak signal under NIM with a peak at m/z 116.8, while nothing was seen under PIM conditions. The sodium salts of the lactones, when analyzed by GC/MS after lyophilization and derivatization with MTBSTFA for the saliva samples, showed an adequate response (Figure 2).

Serum was initially found to have a pH between 8 and 9, which initially produced a weak response when the lactones were extracted with dichloromethane. When the pH was lowered to between 7 and 8, a better recovery was achieved. The pH was further adjusted to between 6 and 7 and the use of ethyl acetate as an alternative solvent each produced improved results, as shown in

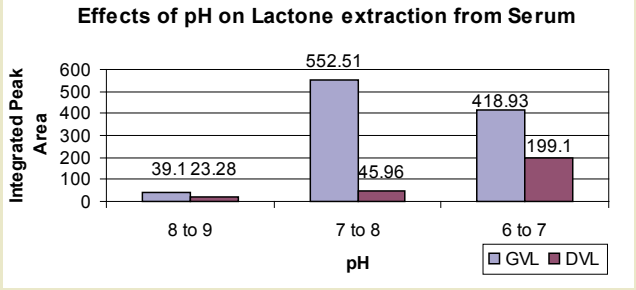

Figure 4. Effects of pH on the lactones in serum.

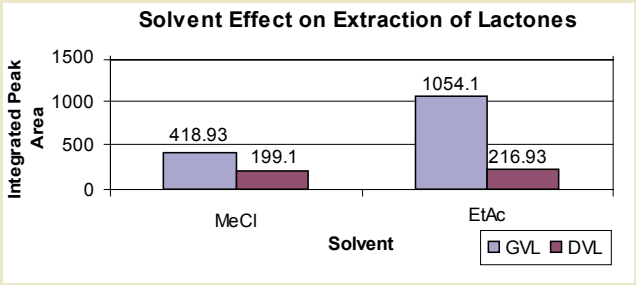

Figure 5. Results of the lactones in saliva and serum.

Figures 4 and 5. It was found with both lactones that ethyl acetate proved to be the better extraction solvent. Lyophilization followed by derivatization with MTBSTFA of the serum samples proved to produce good responses for the sodium salts of the lactones when analyzed by GC/MS (Figure 2).

Azinphos methyl and acephate.

Analysis of the standards showed reasonable responses, which are shown in Figure 6. The base ion of azinphos methyl (m/z = 160) is thought to be due to the loss of the -SPS(OCH_3)$_2$ group from the parent compound, and the base ion for acephate (m/z = 136) is believed to be due to the loss of the –SCH_3 group from the phosphate (Figures 3a and 3b). The results for acephate spiked into saliva and serum produced reasonable results. The response for azinphos methyl in saliva was not nearly as prominent; however the serum extract showed good results (Figure 6).

The overall comparison of metabolized and unmetabolized results for each analyte is illustrated in Figure 7. Results of the *in vitro* tests and comparison the chromatograms from the metabolized and non-metabolized samples showed there were potential nonpolar metabolites for azinphos methyl. A comparison of the chromatograms from metabolized and non-metabolized extracted samples of acephate and GVL showed no unique peaks. Therefore, if a metabolite is being created, it is not being derivatized under these conditions. However, a possible metabolite near 14 minutes was observed for the GVL sample that was derivatized

CONCLUSIONS

The initial results of this study show that unique metabolites may exist for these compounds and that saliva could be a possible matrix for the analysis of exposure to these chemicals. Gas chromatography/mass spectrometry was found to be an effective tool for analyzing the samples although derivatization was required in some cases.

It was found during development that certain solvents worked better than others at extracting the analytes. The use of ethyl acetate was more effective because it is more polar than dichloromethane. It was also determined that the use of diazomethane as a derivatizing agent was inferior for the lactones. This was due to the acidic conditions required for the derivatization reaction which also causes an intramolecular cyclization of the lactones. Also the use of N-methyl-bis(trifluoroacetamide) MTBFA was not as effective as MTBSTFA because it appeared to be affected by the presence of residual water, which caused it to no longer derivatize the sodium salts of the lactones.

The formation of a lactone (cyclic ester) from the corresponding hydroxyl acid is an equilibrium process. Significant amounts of product are usually only obtained when a five- or six-membered ring would result; since these have angles closest to the optimum open chain value and, therefore, less ring strain. In fact, gamma-lactones and delta-lactones undergo intramolecular esterification from their hydroxyl acid counterparts so readily that an acid catalyst is often not needed. However, data presented from a study of hydrolytic equilibria of lactones shows that alkyl substitution on the ring increases the amount of lactone present at equilibrium (demonstrated in Figure 8) [9]. This may explain why there is a reversal in the relative amounts of the gamma- and delta-lactones recovered from serum (shown in Figure 4 for lactone extraction) compared to the

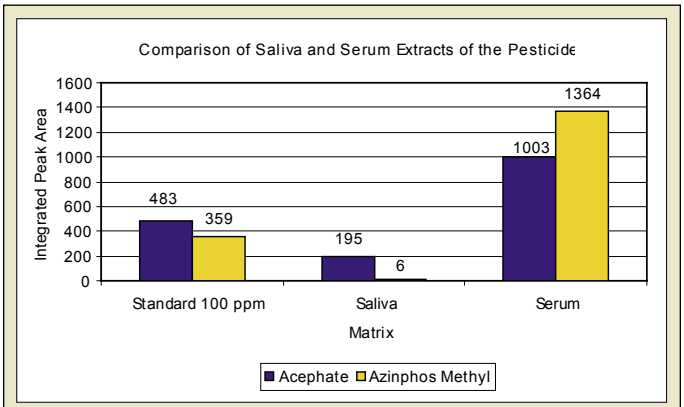

Figure 6. Results of the pesticides in saliva and serum.

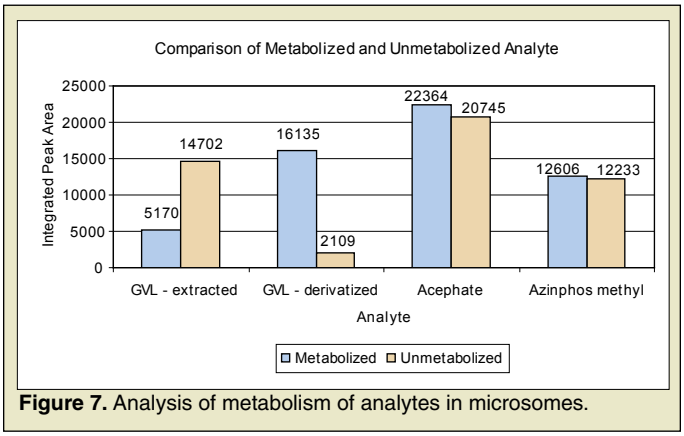

Figure 7. Analysis of metabolism of analytes in microsomes.

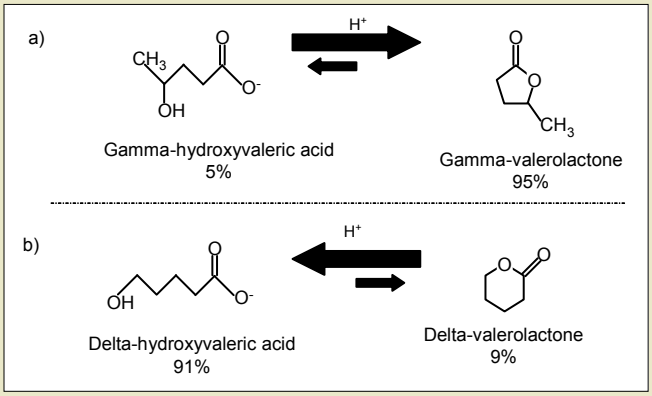

Figure 8. Demonstration of the unequal distribution of gamma- and delta-valeric acid with the corresponding lactones in solution due to the methyl side chain.

gamma- and delta-hydroxyl acids recovered from saliva and serum (shown in Figure 2).

Continuation of this research would also include analysis with GC/MS under positive ion mode. This would help to determine the parent ion (molecular weight) of the unknown metabolites, which would aid in their identification. On-column injection techniques for GC/MS analysis of the lactones would also verify whether thermal lability is affecting the recovery results.

Future work in this research line will also include moving from *in vitro* studies to *in vivo* studies. This research will create techniques that could be used for advance warning tests for other hazardous chemicals, and detection for the use of illegal drugs. This could also be used forensically to determine if particular suspects were either making the warfare agents or the drugs in question. Economically this will create more cost-effective techniques for farmers who must monitor workers for exposure to pesticides and decrease the medical costs for those individuals exposed to such chemicals.

Safety note

MTBSTFA has significant safety risks. These risks include violent reactivity with water which could produce hydrofluoric acid. MTBSTFA is highly flammable and the resulting toxic fumes consist of carbon monoxide, carbon dioxide, silicon oxides, nitrogen oxides and hydrogen fluoride gas. Ingestion, inhalation and contact with the skin must be avoided by using proper measures and precautions found in the material safety data sheet.

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