Research Square

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Stage-Specific detection of Cypermethrin in Lucilia sericata (Diptera: Calliphoridae) reared on experimentally fortified porcine tissues: A QuEChERS- HPLC assessment

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Research Article

Keywords: Cypermethrin, Pork tissues, Lucilia sericata, Temperature, QuEChERS- HPLC

Posted Date: November 24th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11610370/v1

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Additional Declarations: No competing interests reported.

Abstract

The present study aimed to evaluate the distribution and quantification of the pesticide cypermethrin throughout the developmental stages of *Lucilia sericata* larvae, demonstrating the utility of entomological specimens in forensic entomotoxicology in the absence of conventional human tissues. In particular, the effects of tissue type (liver/ muscle) and rearing temperature (20°C, 25°C and 30°C) in larvae reared on cypermethrin spiked pork tissue were investigated using a QuEChERS- HPLC analysis.

The QuEChERS-HPLC method successfully detected and quantified cypermethrin in both tissue and larval samples. All control samples tested negative, which confirmed the specificity of the assay. Muscle tissue retained higher cypermethrin concentrations (4.89–4.94 µg/g) than liver tissue (4.72–4.83 µg/g), with residue levels declining slightly as the temperature increased. While cypermethrin was undetectable in first- and second-instar larvae, it was consistently present from the third instar through the post-feeding stage. Peak concentrations occurred in third-instar larvae, measuring 4.73–4.64 µg/g for those fed on liver and 4.79–4.66 µg/g for those fed on muscle. Finally, a two-way ANOVA confirmed that both tissue type and temperature had significant effects on the residue concentrations (F (2,12) = 30, p = 0.001). The third instar larvae had the highest detectable concentrations of cypermethrin under the experimental conditions and may be a useful stage for toxicological screening.

1. Introduction

In forensic investigation, necrophagous insects can serve as a complementary evidence for tissues or bodily fluids in toxicological studies. Examining insect larvae in cadavers can aid in identifying chemicals or illicit drugs. Chemical substances like drugs and poisons can affect insects' behavior, morphology, physiology, and development. Forensic entomotoxicology studies the effect of toxicants on insect growth, the presence of toxicants in insects that feed on carcasses, and the persistence of toxicants, to provide valuable information that may aid in the determination of the post-mortem interval (PMI), to provide insight into possible cause and/or manner of death [4] etc.

Considering environmental factors that influence developmental timelines is also crucial in forensic investigations to ensure accurate post-mortem interval (PMI_{min}) estimations. Insect physiological functions are highly dependent on temperature [5]. Temperature directly affects insect activity and development. Insects are unable to fly below a specific temperature threshold, preventing them from laying eggs or depositing larvae. Heat accelerates development. Another major factor that affects post-mortem interval (PMI_{min}) estimations is the host species on which the larvae feed which significantly alters larval growth rate [5]. For example, the development of *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) fed lung, liver, and heart, from both cows and swine were compared. Larvae grew significantly faster on porcine than on bovine tissue. Furthermore, development varied when reared on lung and heart compared with the liver of the same animal.

Pyrethroids are now widely utilized in veterinary medicine, human medicine, forestry, wood, and textiles, in addition to crop protection because of their low environmental persistence and pest resistanc [11]. Among these, cypermethrin is one of the most extensively used pyrethroids because of its broad-spectrum activity and cost-effectiveness [23]. But, the widespread and often unregulated use of cypermethrin has led to an increase in accidental, occupational, and intentional poisoning cases [12]. Despite its frequent occurrence in forensic investigations, information regarding the detection and distribution of cypermethrin in necrophagous insects remains limited.

Athough the effects of pesticides including cypermethrin on insect development and morphological parameters have been previously reported [6, 9, 13, 14], the present study addresses a different analytical questions: the detection and quantification of cypermethrin distribution across the developmental stages of *L. sericata*, as well as in porcine tissues (muscle and liver). Conventional approaches, including liquid-liquid extraction (LLE) [9] or solid-phase extraction (SPE) [8] which are labour-intensive, solvent-heavy, have been used for the isolation of toxicants from biological and insect matrices. Pesticide residue analysis in biological and food matrices has also been done using the QuEChERS method (quick, easy, cheap, effective, rugged, and safe) [3]. Its application to insect specimens might provide a useful method for sample preparation, but the effectiveness of an extraction process must be evaluated in the matrices being studied rather than presumed from its application in other sample types. Although previous studies have examined the effects of pesticides on necrophagous insects and the detection of toxicants across different developmental stages, analytical data regarding cypermethrin residues in *Lucilia sericata* reared on various porcine tissues remains limited. Specifically, research is needed to determine whether parent cypermethrin can be detected in different immature stages, and whether residue concentrations vary between liver-fed and muscle-fed cohorts under different rearing temperatures.

In order to examine cypermethrin residues in experimentally treated porcine liver and muscle as well as in specific *L. sericata* developmental stages, the current study used a QuEChERS-based extraction technique in conjunction with HPLC-PDA. The specific goals

were to: (i) evaluate the procedure's analytical performance in the matrices under investigation; (ii) ascertain the concentrations of cypermethrin in treated porcine tissues; and (iii) investigate the detection and measured concentrations of cypermethrin across insect developmental stages at 20, 25, and 30°C. The study was planned as a supplementary analytical inquiry carried out concurrently with the developmental study that had already been published.

2. Methodology

2.1. Establishment of *L.sericata* colony

For the study, wild-type adult male and female flies were captured using sweep nets. The preferable sites for the collections were animal faeces, vegetable and fruit remains, and garbage waste. The captured flies were identified using a taxonomic identification key developed by Williams and Villet [16]. The adult *L. sericata (Meigen, 1826)* flies were placed in a 30cm x 30cm x 30cm insect cage and transported to the Forensic Science Laboratory, School of Basic and Allied Health Sciences, Sharda University, India.

The flies were provided with water, and milk +sugar solution (1:1) continuously, and a fresh chicken breast and liver (50gm each) for oviposition medium. The eggs of these flies, along with the breast and liver sections, were collected and carefully placed on a glass plate. The glass plate was placed on damp wood shavings within a larger wooden box (29x22x10 cm), covered with a fine net, and tightly sealed using an elastic band. The eggs hatched, and the development of the larvae was tracked until they reached the pupation stage. After being manually separated from the wood shavings, the pupae were moved to a new rearing chamber. This procedure was repeated as the continuous replenishment of the colony. Adult flies from various wild populations were periodically added to the colony to reduce the possibility of inbreeding. In order to produce enough larvae for experiments and to acclimate the flies to laboratory conditions, they were cultivated for three generations.

2.2. Preparation of Cypermethrin- treated tissues

A 100 ml stock solution of analytical grade cypermethrin (99% purity) was prepared by dissolving (10 mg) of cypermethrin in acetonitrile, creating a stock solution with a concentration of 0.1 mg/mL (100µg/mL). Fresh porcine tissues (liver and muscle (loin) were purchased from a local butcher and stored at -20^{o}C until use. Before the experiment, 100 g of each of the aliquots of pork tissues—liver and muscle—were defrosted at room temperature and minced using a mechanical grinder (Foshan XGreek, stainless steel bowl). Following the guidelines provided by Magni [9], minced tissues were homogenized with cypermethrin solution at a ratio of 10ml per 100g tissue using a laboratory homogenizer (Remi Lab World, RQ-127_AD, 50 Hz), yielding a nominal loading concentration of 0.01mg/g in tissue. Control tissues were treated with acetonitrile without cypermethrin.

The cypermethrin concentration was selected to ensure measurable analytical recovery and to produce observable developmental effects under controlled laboratory conditions. This concentration serves as an experimental model for assessing insect toxicokinetics and developmental modulation. Control tissues received equivalent volumes of acetonitrile to account for solvent effects. Following homogenization, the tissues were equilibrated under a fume hood for 30 minutes to allow the solvent to evaporate before larval introduction. This procedure minimized solvent-associated toxicity and maintained tissue consistency across all experimental groups.

2.3. Experimental design and larval sampling

For each of the experimental units (two tissue substrates (pork liver and muscle), two groups- cypermethrin- treatment and control- reared at three temperatures; 20, 25 and 30^{o}C), with three independent replicate batches of 200 newly hatched larvae were individually transferred onto the treated pork tissues (muscle (loin) and liver), yielding 36 batches and 7,200 larvae in total.

Larvae were maintained in a BOD incubator under a 12:12 L: D photoperiod and 55% relative humidity. Larval growth was monitored every 8 hours by sampling 5 larvae per replicate for length and width using a digital Vernier caliper and larval body mass by a digital balance with an accuracy of 0.0001g, by immersing them in hot water (2–3 sec), followed by preservation in 80% of ethanol [7]. In parallel, at each of the developmental stage, including the first, second, third instar, post-feeding, and pupal stages, 10 larvae per replicate were collected, rinsed in distilled water, and stored at − 20°C for toxicological analysis [2], consuming a fixed 150 larvae per batch and totaling 1,800 larvae across the study. The developmental stage of larvae was identified through morphological examination of the posterior spiracles.

2.4. Sample Preparation

Sample extraction followed the QuEChERS method [3]. Briefly, 2.0 g of tissue was macerated with a mortar and pestle, followed by the addition of 3.6 mL water, 5.0 mL acetonitrile, and 2.14 mL ethyl acetate to the mixture. The sample was vortexed for 1 minute at 2200 rpm,

followed by addition of 2.86 mg $MgSO_4$ and 0.71 mg sodium acetate. It was vortexed again for 1 minute and then centrifuged for 11 minutes at 4000 rpm.

The supernatant was stored at − 20°C overnight, and then centrifuged again for 5 minutes at 4000 rpm. A 5 mL aliquot was transferred to a micro-centrifuge tube containing 150 mg $MgSO_4$, 30 mg PSA, and 30 mg C18. The mixture was stirred for 1 minute at 2200 rpm, and then centrifuged for 12 minutes at 9000 rpm. A 20 µL portion of the clarified extract was used for HPLC analysis. The same extraction procedure was applied for the sampled immature stages (first, second, and third instar, post-feeding, and pupal stages) of *Lucilia sericata* (Meigen, 1826).

2.5. High-Performance Liquid Chromatography (HPLC)

Quantification was performed using a Breeze QS HPLC system quipped with the photodiode array detector (with a 10 µAU noise specification). An Atlantis dC18 column (4.6 × 150 mm, 5 µm) was utilized for this separation. The mobile phase consisted of acetonitrile (designated as phase A) and HPLC grade water (designated as phase B) at a 70:30 v/v ratio. The flow rate was set at 1 mL/min, with a column temperature of 30°C, and detection was performed at a wavelength of 204 nm. The injection volume was 20 µL for both standards and samples.

Using **Cmatrix=(Cextract×Vfinal)/ Msample**, where Cextract is the HPLC-derived concentration (µg/mL), Vfinal is the final extract volume (mL), and Msample is the extracted sample mass (g), cypermethrin concentrations in the original biological matrices were calculated from the concentration obtained from the calibration curve.

For pork tissue and larval samples, 2 g of sample was extracted and the final extract volume was 5 mL; therefore, the conversion factor was 2.5.

2.6. Method Validation

The QuEChERS extraction protocol and HPLC analytical conditions used in this study were adapted from previously validated methods reported for pesticide residue analysis. To confirm their applicability to pork tissue and *Lucilia sericata* larval matrices, a method verification was conducted to assess linearity, LOD, LOQ, selectivity, and recovery [24].

Calibration and Linearity was evaluated by analysing cypermethrin standard solutions prepared by dissolving 1 mg of cypermethrin in 10 ml of acetonitrile. The solution was diluted to 0.1, 0.5, 2, 20, and 100 µg/ml, and 20µl were collected and injected into the HPLC. Calibration curves were created by plotting the peak area ratios of cypermethrin.

The **limit of detection (LOD) and limit of quantification (LOQ)** were determined based on signal-to-noise ratios of 3:1 and 10:1, respectively. Method repeatability was assessed through six replicate injections of sample preparations and one standard preparation, and results were expressed as relative standard deviation (% RSD).

Method selectivity was assessed by comparing chromatograms of blank pork tissues and *L. sericata* larvae extracts with those spiked with known concentrations of cypermethrin. No significant interfering peaks were observed at the retention time of cypermethrin in blank samples as shown in Fig. 2. Peak purity was confirmed using the Photodiode array (PDA) detector by comparing the UV-vis absorption spectrum of the analyte in the samples with that of the analytical standards.

Extraction recovery was determined by comparing the peak area ratios of samples fortified with cypermethrin before the QuEChERS extraction with those of blank matrix extracts fortified after extraction. Recovery experiments were conducted at (10 µg/mL) fortification level using three independent replicates. Matrix-specific recovery was evaluated separately for pork muscle, pork liver and larval matrices.

2.7. Statistical Analysis

Statistical analyses were performed using Graph Pad Prism 10.2.3 (Graph Pad Software, USA). Cypermethrin concentrations in treated pork liver and muscle tissues at different temperatures were expressed as mean ± standard deviation (SD) unless otherwise stated. Differences in cypermethrin concentrations among temperature groups and between tissue types were analyzed using two-way analysis of variance (ANOVA), with temperature and tissue types as fixed factors, followed by Tukey's post hoc test for multiple comparisons. The statistical significance threshold (α) was set at 0.05 for all tests. All analyses were performed in three independent biological replicate batches to ensure reproducibility and minimize experimental error. Mean values and standard deviations were calculated for all quantitative HPLC results to evaluate the reliability and variability of the data.

3. Results

3.1. Validation of the method

The method performance was assessed by calibration curves established under optimized HPLC-PDA conditions by plotting its peak-area ratio against concentration. The regression equation was y = 237,956.4x- 402030.8 with a strong linearity (R^2= 0.9958, Fig. 1).

The method exhibited a limit of detection LOD of 0.03 µg/mL, and a limit of quantification LOQ of 0.1 µg/mL, indicating high senstivity. The extraction efficiency of the QuEChERS method was further confirmed through matrix-specific recovery assessment. The recovery values ranged from 75 to 80% across different matrices, with pork liver showing 76.5 ± 1.8%, pork muscle 78.2 ± 2.1%, and larval samples 79.1 ± 2.3% (n = 3). The summary of the validation parameters is reported in the Table 1.

Table 1
Shows the validation parameters of the QuEChERS-HPLC method for cypermethrin detection.

Parameters	Value
Linearity Range	0.1–100 µg/mL
Correlation coefficient (R^2)	0.9958
Limit of Detection (LOD)	0.03 µg/mL
Limit of Quantification (LOQ)	0.1 µg/mL
Precision (RSD %)	< 3%
Extraction recovery	75–80%
	Pork liver- 76.5 ± 1.8% (n = 3)
	Pork Muscle – 78.2 ± 2.1% (n = 3)
	Larval Matrices-79.1 ± 2.3% (n = 3)
Selectivity	No interfering peaks observed

3.2. Cypermethrin detection in Pork tissuesµ

The results of the analysis showed that cypermethrin was detected and quantified in liver and muscle pork tissues samples.

The measured cypermethrin concentrations in experimentally treated pork tissues at various temperatures are shown in Table 2. Concentrations are expressed as µg/g of tissue and were calculated from HPLC-derived extract concentrations after QuEChERS extraction, accounting for the final extract volume and sample mass. These measured concentrations may differ from the nominal fortification concentration because of extraction recovery and analytical variability.

Table 2
shows the cypermethrin concentrations (µg/g) in experimentally treated pork tissues at different temperatures *[Mean ± SD, n = 3]*

Tissue	20 ºC	25 ºC	30 ºC
Pork Liver	4.83 ± 0.01	4.72 ± 0.01	4.80 ± 0.03
Pork Muscle	4.93 ± 0.01	4.91 ± 0.02	4.89 ± 0.01

Two-way ANOVA was performed to evaluate the effect of temperature and tissue type on cypermethrin concentration, including their interaction. The tissue × temperature interaction was [significant] (F (2,12) = 30, p = 0.001). Tukey's post-hoc test was subsequently used for pairwise comparisons.

As according to the Table 2, across all the temperature conditions, muscle tissue consistently exhibited higher cypermethrin concentrations than liver tissue. Concentrations remained relatively stable across temperatures, with slight reductions at 30^{O}**C** in liver samples.

Table 3
cypermethrin concentration (µg/g) in *L. sericata* immature stages reared on experimentally treated pork tissues at different temperatures *[Mean ± SD, n = 3]*

Temperature	Pork Liver tissue		Pork Muscle tissue	
	3rd instar	Post-feeding (pf)	3rd instar	Post-feeding (pf)
20°C	4.73 ± 0.01	4.53 ± 0.03	4.71 ± 0.01	4.56 ± 0.02
25°C	4.67 ± 0.09	4.58 ± 0.02	4.79 ± 0.04	4.62 ± 0.01
30°C	4.64 ± 0.01	4.48 ± 0.01	4.66 ± 0.05	4.53 ± 0.01

Residue analysis of larvae and pupae revealed that cypermethrin was undetected in first, second instar, pupae and in all control groups. In contrast, it was consistently detected and quantified in the third, and post-feeding larvae across all temperatures. The highest larval concentrations were generally observed in the third instar stage irrespective of the temperature regime (Table 3).

Cypermethrin concentrations were highest in third-instar larvae in both liver-fed and muscle-fed groups. In liver-fed larvae, concentrations decreased from 4.735 µg/g at 20°C to 4.644 µg/g at 30°C, while muscle-fed larvae showed 4.719, 4.794, and 4.665 µg/g at 20, 25, and 30°C, respectively. Post-feeding larvae exhibited lower but detectable concentrations, ranging from 4.489–4.587 µg/g in the liver-fed group and 4.563–4.625 µg/g in the muscle-fed group.

4. Discussion

1. **Cypermethrin concentration between porcine liver and muscle**

Detectable amount of Cypermethrin were found in both treated pork liver and muscle. In all tested temperature conditions, the measured concentrations of cypermethrin were numerically higher in muscle than in liver. The absolute differences were, nevertheless, negligible. These findings show that, under the experimental conditions, the two examined matrices had different measured residue concentrations.

Owing to its lipophilic nature, and partitioning into tissue components may influence its distribution (13). However, tissue lipid content, tissue-specific binding, and pesticide partition coefficients [18] were not directly measured in this study. Therefore, lipid partitioning or variations in tissue metabolism cannot be definitively linked to the higher measured concentrations in muscle. These explanations are still theories that need further research. The findings of the study done on heavy metal bioaccumulation in rabbit organs and *Chrysomya albiceps* (Wiedemann, 1819) larvae [1] differs from the present study, which reported the highest concentration of mercury and cadmium in the liver tissue followed by muscle. However, these changes were not pronounced, indicating relative stability of cypermethrin in the treated tissues prior to larval exposure. Cypermethrin concentrations decreased with rising temperatures across all tested temperature ranges, most likely as a result of faster chemical degradation in warmer conditions [17], however, these mechanism were not directly measured in the present study.

2. Stage-specific detection in

During the first, second instar or pupal samples, the cypermethrin was not detected. Reduced uptake during early larval development may be linked to variations in body size, cuticular characteristics, feeding intensity differences in the amount of substrate consumed, sample composition or analytical recovery [19]. The cypermethrin concentration was highest during the third instar in both treated pork tissues, correlating with active feeding, but declined during the post-feeding and was not even detected during the pupal stages.

Post-feeding larvae showed lower chemical concentrations than third-instar larvae, likely due to reduced exposure after active feeding stopped. However, since cypermethrin metabolites, excretion rates, and detoxification enzyme activities were not measured, the concentration decline cannot be definitively linked to metabolic clearance, transformation, or excretion. Although previous entomotoxicological research highlights the stage-dependent detection of drugs and metabolites in necrophagous larvae [6, 8, 9, 20, 14, 22, 24], a direct comparison with the current study is difficult. This is due to distinct differences in the targeted compounds, insect species, sample matrices, and analytical platforms.

3. Temperature and measured residue concentrations

Concentrations in the porcine tissues and insect samples varied across the three rearing temperatures. Although concentrations were generally lower at 30°C than at 20°C, this pattern was not uniform across all tissues and developmental stages. Insect feeding behavior, developmental rate, and physiological processes can all be impacted by temperature. The stability of chemical compounds in the feeding substrate may also be impacted [21]. Nevertheless, insect metabolic activity, cypermethrin metabolite formation, and pesticide degradation in the substrate were not directly measured in this experiment. Therefore, rather than being indicative of increased metabolic clearance or temperature-dependent degradation, the observed differences should be characterized as temperature-associated variation in measured concentrations. The persistence of cypermethrin in fully developed larvae confirms that this pesticide is retained across life stages; however, residue concentrations are clearly influenced by developmental stage and environmental temperature, both of which should be considered when interpreting entomotoxicological evidence.

These findings need to be interpreted in the light of the limitations of the study. However, the critical validation factor of extraction recovery was evaluated, but the internal standard was not used and the metabolite was not identified, which may affect the quantitative accuracy. Future research including metabolite profiling would be of great value to insect toxicokinetics, biotransformation pathways and stage specific susceptibility. This kind of work is important to improve entomotoxicological interpretation, especially in complex forensic cases where pesticide exposure could interfere with developmental time lines.

Conclusion

This study used a QuEChERS-based extraction method in conjunction with HPLC-PDA to examine cypermethrin residues in experimentally treated porcine liver and muscle as well as in Lucilia sericata developmental samples. Third-instar and post-feeding larval samples, as well as porcine tissue matrices, contained cypermethrin. At the method's stated analytical detection limit, it was not found in pupal, first-instar, or second-instar samples. Third-instar larvae typically had the highest measured concentrations in the insect samples. This has important forensic implications: because cypermethrin is not detectable in early instars and pupae, a negative toxicological finding at these life stages should not be interpreted as evidence of lack of exposure, and ambient temperature should be considered when interpreting residue levels in field-collected larvae. Future work should include metabolite identification to elucidate clearance pathways and validation of this simplified QuEChERS-HPLC method for other pesticide classes, necrophagous species and temperature ranges to further support its use in forensic entomotoxicology casework.

Declarations

Author Contribution

Usha Sisodia: Conceptualization, methodology, investigation, experimental work, data collection, formal analysis, data curation, and writing – original draft.Sally Lukose: Supervision, conceptualization, validation, interpretation of results, critical review, and editing of the manuscript.Both authors contributed to the revision of the manuscript, read and approved the final version, and agreed to be accountable for all aspects of the work.

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Figures

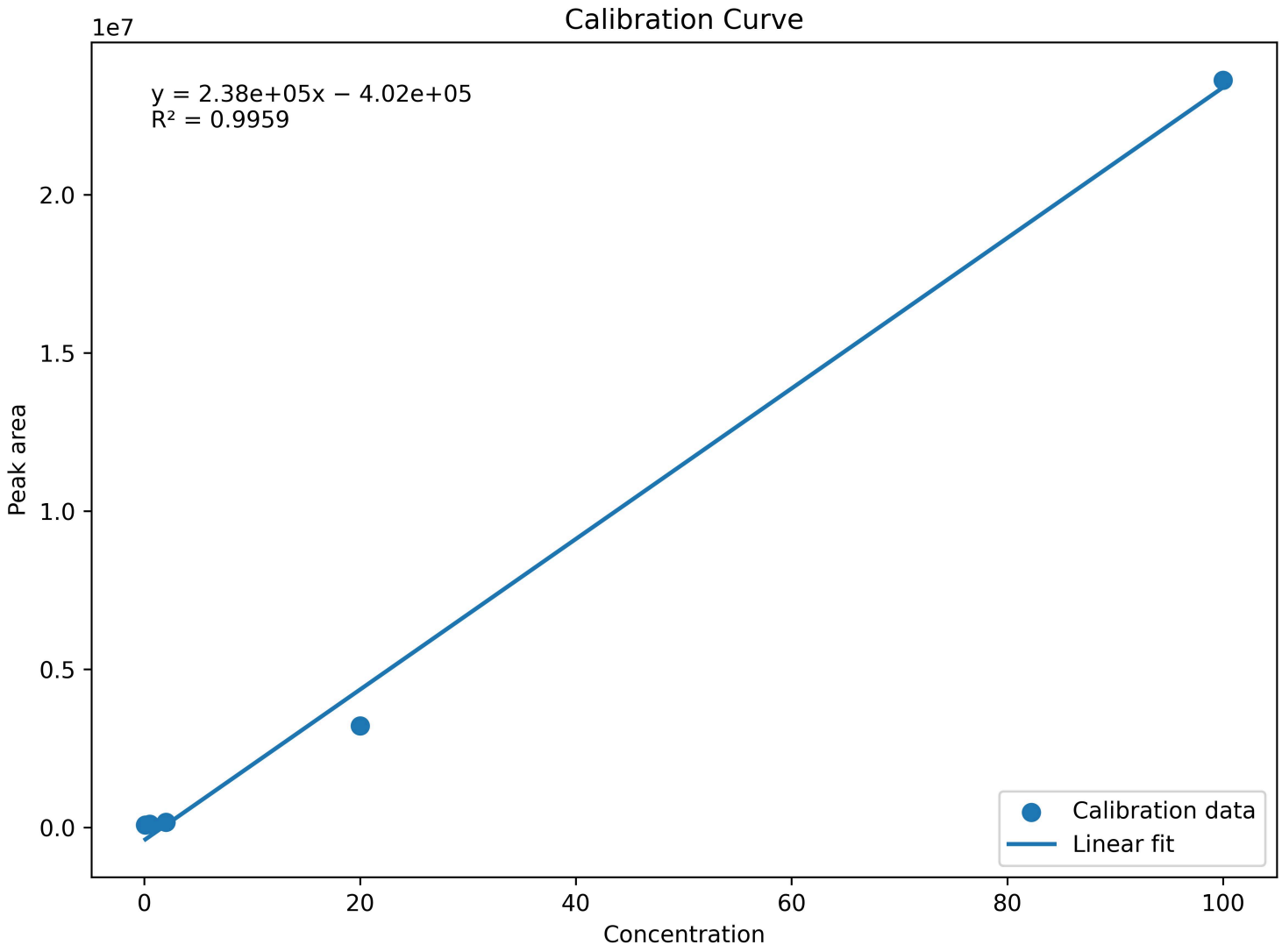
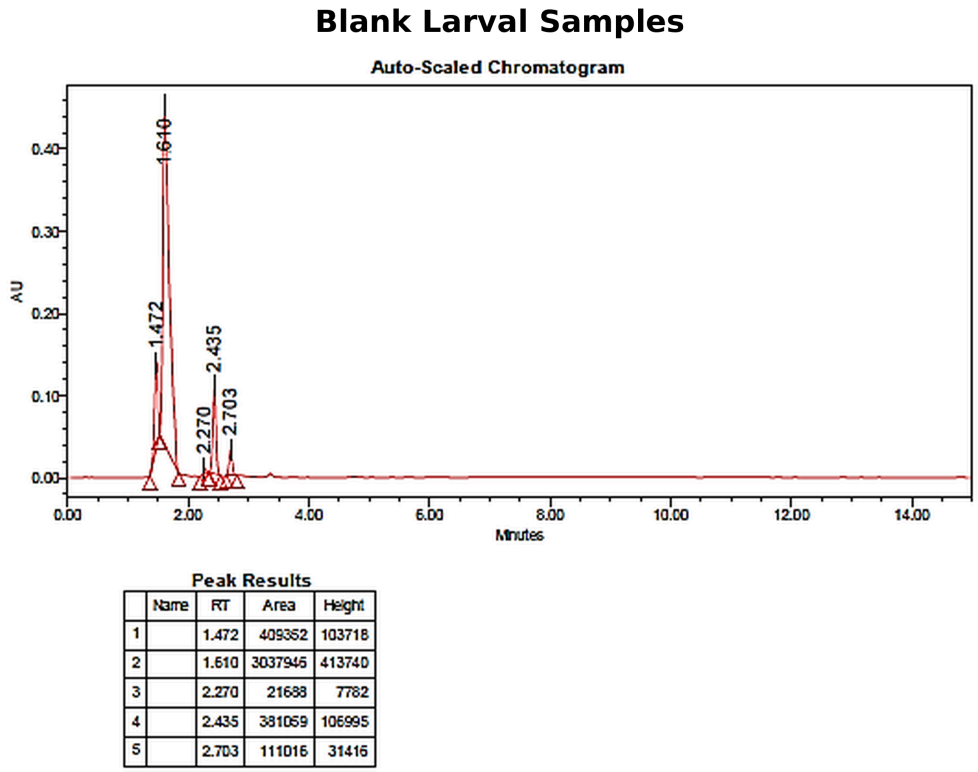


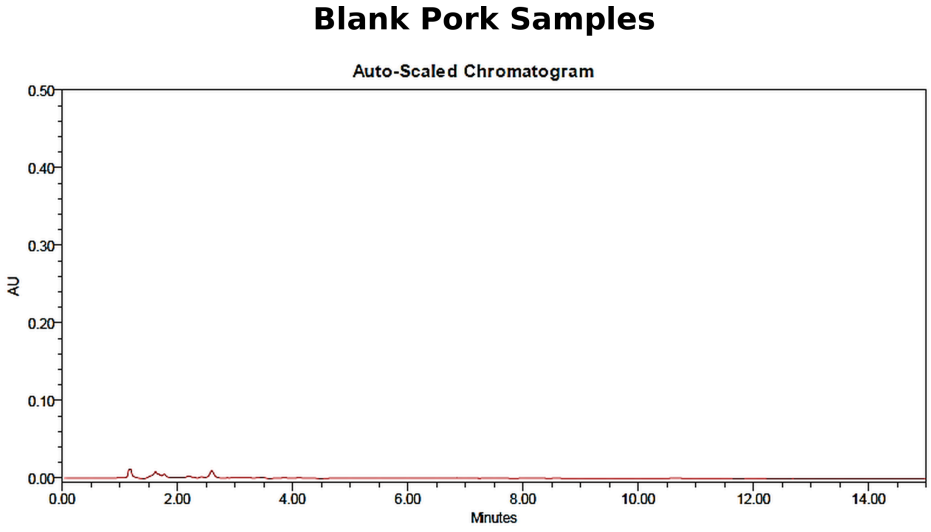
Figure 1

Legend not included with this version



Peak Results

	Name	RT	Area	Height
1		1.472	409352	103718
2		1.610	3037945	413740
3		2.270	21688	7782
4		2.435	381059	106995
5		2.703	111016	31416



Peak Results

	Name	RT	Area	Height
1				

Figure 2

Legend not included with this version

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- fiqures.docx