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Analysis of Iophenoxic Acid Analogues in Small Indian Mongoose (Herpestes Auropunctatus) Sera for Use as an Oral Rabies Vaccination Biological Marker

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Dr. Ronald Myers, Editor

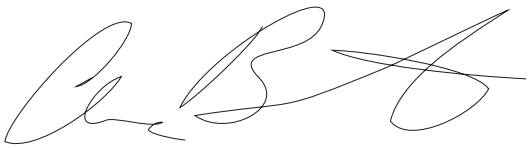
Dear Dr. Myers:

Please find attached our revised manuscript number JoVE59373. The title has been modified to reflect reviewers' comments and more accurately describe the objectives of the study to read: "Analysis of iophenoxic acid analogues in small Indian mongoose (*Herpestes auropunctatus*) sera for use as an oral rabies vaccination biological marker."

We thank the reviewers for their helpful and insightful comments and have addressed them to the best of our ability. This has resulted in considerable changes to the manuscript which we believe now presents a more comprehensive picture of our objectives, methods and results. It is important to point out that this research was a proof of concept to develop a method to evaluate iophenoxic acid residues in mongoose serum and its suitability as a biomarker in practical field distribution of oral rabies vaccine baits. Our laboratory methods were an iterative process where the et-IPA study group provided valuable information on the initial methods, which were modified as needed, resulting in the final method used for the me-IPA study group. It is this final method presented in detail in this manuscript.

Thank you for your consideration.

Sincerely,



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TITLE:

Analysis of Iophenoxic Acid Analogues in Small Indian Mongoose (*Herpestes Auropunctatus*) Sera for Use as an Oral Rabies Vaccination Biological Marker

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KEYWORDS:

biological marker, *Herpestes auropunctatus,* iophenoxic acid, rabies, small Indian mongoose, LC-MS/MS

SUMMARY:

We offered captive mongooses placebo oral rabies vaccine baits with ethyl or methyl iophenoxic acid as a biomarker and verified bait uptake using a novel liquid chromatography with tandem mass spectrometry (LC-MS/MS) method.

ABSTRACT:

The small Indian mongoose (*Herpestes auropunctatus*) is a reservoir of rabies virus (RABV) in Puerto Rico and comprises over 70% of animal rabies cases reported annually. The control of RABV circulation in wildlife reservoirs is typically accomplished by a strategy of oral rabies vaccination (ORV). Currently no wildlife ORV program exists in Puerto Rico. Research into oral rabies vaccines and various bait types for mongooses has been conducted with promising results. Monitoring the success of ORV relies on estimating bait uptake by target species, which typically involves evaluating a change in RABV neutralizing antibodies (RVNA) post vaccination. This strategy may be difficult to interpret in areas with an active wildlife ORV program or in areas

where RABV is enzootic and background levels of RVNA are present in reservoir species. In such situations, a biomarker incorporated with the vaccine or the bait matrix may be useful. We offered 16 captive mongooses placebo ORV baits containing ethyl-iophenoxic acid (et-IPA) in concentrations of 0.4% and 1% inside the bait and 0.14% in the external bait matrix. We also offered 12 captive mongooses ORV baits containing methyl-iophenoxic acid (me-IPA) in concentrations of 0.035%, 0.07% and 0.14% in the external bait matrix. We collected a serum sample prior to bait offering and then weekly for up to eight weeks post offering. We extracted Iophenoxic acids from sera into acetonitrile and quantified using liquid chromatography/mass spectrometry. We analyzed sera for et-IPA or me-IPA by liquid chromatography-mass spectrometry. We found adequate marking ability for at least eight and four weeks for et- and me-IPA, respectively. Both IPA derivatives could be suitable for field evaluation of ORV bait uptake in mongooses. Due to the longevity of the marker in mongoose sera, care must be taken to not confound results by using the same IPA derivative during consecutive evaluations.

INTRODUCTION:

Rabies virus (RABV) is a negative sense single stranded lyssavirus, and circulates among diverse wildlife reservoir species within the orders Carnivora and Chiroptera. Multiple species of mongoose are reservoirs of RABV, and the small Indian mongoose (*Herpestes auropunctatus*) is the only reservoir in Puerto Rico and other Caribbean islands in the Western Hemisphere[1-3]. The control of RABV circulation in wildlife reservoirs is typically accomplished through a strategy of oral rabies vaccination (ORV). In the United States (US), this management activity is coordinated by the USDA/APHIS/Wildlife Services National Rabies Management Program (NRMP)[4]. Currently no wildlife ORV program exists in Puerto Rico. Research into rabies vaccines and various bait types for mongooses has been conducted with promising results suggesting an ORV program for mongooses is possible[5-8].

Monitoring the impact of ORV relies on estimating bait uptake by target species, which typically involves evaluating a change in RV antibody seroprevalence. However, this strategy may be challenging in areas with an active wildlife ORV programs or in areas where RV is enzootic and background levels of RABV neutralizing antibodies (RVNA) are present in reservoir species. In such situations, a biomarker included in the bait or the external bait matrix may be useful.

Various biological markers have been used to monitor bait uptake in numerous species, including raccoons (*Procyon lotor*)[9,10], stoats (*Mustela ermine*)[11,12], European badgers (*Meles meles*)[13], wild boars (*Sus scrofa*)[14], small Indian mongooses[15] and prairie dogs (*Cynomys ludovicianus*)[16,17], among others. In the US, operational ORV baits often include a 1% tetracycline biomarker in the bait matrix to monitor bait uptake[18,19]. However, drawbacks to the use of tetracycline include a growing concern over the distribution of antibiotics into the environment and that detection of tetracycline is typically invasive, requiring tooth extraction or destruction of the animal to obtain bone samples[20]. Rhodamine B has been evaluated as a marker in a variety of tissues and can be detected using ultraviolet (UV) light and fluorescence in hair and whiskers[10,21].

Iophenoxic acid (IPA) is a white, crystalline powder that has been used to evaluate bait consumption in coyotes (*Canis latrans*)[22], arctic fox (*Vulpes lagopus*)[23], red fox (*Vulpes vulpes*)[24],

raccoons[9,25], wild boar[14], red deer (*Cervus elaphus scoticus*)[26], European badgers[12] and ferrets (*M. furo*)[27], among several other mammalian species. Retention times of IPA varies by species from less than two weeks in some marsupials[28,29], to at least 26 weeks in ungulates[26] and over 52 weeks in domestic dogs (*Canis lupus familiaris*)[30]. Retention times may also be dose-dependent[31]. Iophenoxic acid binds strongly to serum albumin and was historically detected by measuring blood iodine levels[32]. This indirect approach was supplanted by high-performance liquid chromatography (HPLC) methods to directly measure iophenoxic acid concentrations with UV detection[33], and eventually with liquid chromatography and mass spectrometry (LCMS)[34,35]. For this study, a highly sensitive and selective liquid chromatography with tandem mass spectrometry (LC-MS/MS) method was developed that utilizes multiple reaction monitoring (MRM) to quantify two analogues of iophenoxic acid. Our objective was to use this LC-MS/MS method to evaluate the marking ability of 2-(3-hydroxy-2,4,6-triiodobenzyl)propanoic acid (methyl-IPA or me-IPA) and 2-(3-hydroxy-2,4,6-triiodobenzyl)butanoic acid (ethyl-IPA or et-IPA) and when delivered in an ORV bait to captive mongooses.

Mongooses were live captured in cage traps baited with commercially available smoked sausages and fish oil. Mongooses were housed in individual 60 cm x 60 cm x 40 cm stainless steel cages and fed a daily ration of ~50 g commercial dry cat food, supplemented twice per week with a commercially available chicken thigh. Water was available *ad libitum*. We delivered two derivatives of IPA, ethyl-IPA and methyl-IPA, to captive mongooses in placebo ORV baits. All baits were composed of a 28 mm x 20 mm x 9 mm foil blister pack with an external coating (hereafter "bait matrix") containing powdered chicken egg and gelatin (**Table of Materials**). Baits contained 0.7 mL of water or IPA derivative and weighed approximately 3 g, of which ~2 g was the external bait matrix.

We offered 16 captive mongooses et-IPA in three concentrations: 0.14% (2.8 mg et-IPA in ~2 g bait matrix; 3 males [m], 3 females [f]), 0.4% (2.8 mg et-IPA in 0.7 mL blister pack volume; 3m, 3f), and 1.0% (7.0 mg ethyl-IPA in 0.7 mL blister pack volume; 2m, 2f). The overall dose of 2.8 mg corresponds to a dose rate of 5 mg/kg[27,36] and is based on an average mongoose weight of 560 g in Puerto Rico. We selected 1% as the highest concentration as research suggests taste aversion to some biomarkers may occur at concentrations >1% in some species[37]. We only offered the 1% dose in the blister pack as flocculation prevented the solute from dissolving in the solvent sufficiently to be evenly incorporated into the bait matrix. One control group (2m, 1f) received baits filled with sterile water and no IPA. We offered baits to mongooses in the morning (~8 a.m.) during or prior to feeding of their daily maintenance ration. Bait remains were removed after approximately 24 hours. We collected blood samples prior to treatment, one day post-treatment and then weekly up to 8 weeks post-treatment. We anesthetized mongooses by inhalation of isoflurane gas and collected up to 1.0 mL of whole blood by venipuncture of the cranial vena cava as described for ferrets[38]. We centrifuged whole blood samples, transferred sera to cryovials and stored them at -80 °C until analysis. Not all animals were sampled during all time periods to minimize the impacts of repeated blood draws on the health of the animals. Control animals were sampled on day 0, then weekly for up to 8 weeks post-treatment.

We delivered me-IPA in three concentrations: 0.035% (0.7 mg), 0.07% (1.4 mg) and 0.14% (2.8

mg), all incorporated into the bait matrix, with 2 males and 2 females per treatment group. Two males and two females received baits filled with sterile water and no IPA. Bait offering times and mongoose anesthesia are described above. We collected blood samples prior to treatment on day 1, and then weekly up to 4 weeks post-treatment.

We tested serum concentration data for normality and estimated means for serum IPA concentrations of different treatment groups. We used a linear mixed model to compare mean serum et-IPA concentrations pooled across individuals. Bait type (matrix/blister pack) was a fixed effect in addition to experimental day, whereas animal ID was a random effect. All procedures were run using common statistical software (**Table of Materials**) and significance was evaluated at $\alpha = 0.05$.

PROTOCOL:

All procedures were approved by the USDA National Wildlife Research Center's institutional Animal Care and Use Committee under approved research protocol QA-2597.

NOTE: The following protocol describes the analysis procedure to detect methyl-iophenoxic acid in mongoose serum. This method is the final version of an iterative process that began with analysis of ethyl-iophenoxic acid in mongoose serum. During the initial evaluation of ethyl-iophenoxic acid minor modifications were made to the methods, resulting in the final protocol presented below. Representative results include those obtained during both iterations.

1. Preparation of solutions and standards

1.1. Purchase me-IPA and et-IPA.

1.2. For mobile phase A, prepare 1 L of 0.1% (v/v) formic acid in water by combining 1 mL of formic acid with 1 L of ultrapure water (≥ 18 MΩ). For mobile phase B, prepare 1 L of 0.1% (v/v) formic acid in acetonitrile (ACN) by combining 1 mL of formic acid with 1 L of ACN.

1.3. For diluent, prepare 200 mL of 0.5% (v/v) trifluoroacetic acid (TFA) in ACN by combining 1 mL of TFA with 200 mL of ACN.

1.4. Prepare concentrated IPA stock solutions of me-IPA and et-IPA in ACN at concentrations of approximately 1000 µg/mL.

1.4.1. Weigh approximately 10 mg of me-IPA on a microbalance and record the mass to ± 0.0001 mg. Quantitatively transfer the me-IPA to a 10 mL Class A volumetric flask using 45 mL ACN. Sonicate 1 min to dissolve all solids, and then bring to volume with ACN.

1.4.2. Transfer ~8 mL of each stock to amber 8 mL glass vials with poly-tetrafluoroethylene (PTFE)-lined caps. Store at room temperature (RT). Transfer the remaining stock to hazardous waste.

1.5. For the 25x-7 me-IPA stock (**Table 1**), prepare a stock of me-IPA in ACN at approximately 200 µg/mL. Example: Transfer 1 mL of the me-IPA concentrated stock from step 1.4.2 to a 5 mL Class A volumetric flask using a 1000 µL glass syringe. Dilute to volume with ACN. Transfer the stock to an amber 8 mL glass vial with PTFE-lined cap. Store at RT.

1.6. Prepare the six additional 25x me-IPA Stocks described in **Table 1**. For each stock, combine the volumes indicated using a repeat pipettor in an amber 8 mL amber glass vial with PTFE-lined cap. Store each stock at RT.

1.7. For the 25x surrogate stock, prepare a surrogate stock of me-IPA in ACN at approximately 10 µg/mL from the concentrated stock prepared in step 1.4.2. Transfer 0.100 mL of the concentrated me-IPA stock to a 10 mL Class A volumetric flask using a 100 µL glass syringe, and then dilute to volume with ACN.

1.7.1. Transfer ~8 mL to an amber 8-mL glass vial with PTFE-lined cap. Store at RT. Transfer the remaining stock to hazardous waste.

1.8. Prepare 4x stocks containing both analytes in 2 mL screw-top glass autosampler vials as described in **Table 2**.

1.8.1. For example, to prepare stock 4x-7, to a 2 mL vial, add 0.20 mL of the 25x-7 me-IPA stock from step 1.5 using a repeat pipettor with 0.5 mL capacity tip. Add 0.20 mL of the 25x surrogate et-IPA stock from step 1.7 using a repeat pipettor with 0.5 mL capacity tip.

1.8.2. Add 0.85 mL of ACN using a repeat pipettor with 1 mL capacity tip. Cap the vial securely and invert 5x to mix.

1.9. Prepare the standard curve in 2 mL screw-top autosampler vials as described in **Table 3**.

1.9.1. For example, to prepare standard 7 (Std 7), to a 2-mL vial, add 0.20 mL of the 4x-7 Stock from step 1.8.2 using a repeat pipettor with 0.5 mL capacity tip. Add 0.60 mL of ultrapure DI water using a repeat pipettor with 1 mL capacity tip. Cap the vial securely and invert 5x to mix.

2. Sample preparation

CAUTION: Personnel performing this procedure must have received the full series of rabies pre-exposure prophylaxis and have a documented rabies antibody titer above 0.5 IU from a Federal Occupational Health designated medical facility. Personnel must wear lab coats and eye protection at all times while performing the extraction. CAUTION: Perform steps 2.3−2.6 in a class II biosafety cabinet.

2.1. For each sample, prepare a 1.5 mL microcentrifuge tube containing 200−300 mg of NaCl. Arrange the tubes in an 80-position plastic rack. Set aside for use in step 2.6.

NOTE: A micro scoop (or other small measuring device) is recommended for large numbers of samples.

2.2. For each sample, label two 1.5 mL microcentrifuge tubes: one as “A” and the other as “B”. Arrange the tubes in an 80-position plastic rack.

2.3. Place the following materials and equipment needed for serum extraction in a class II biosafety cabinet: microcentrifuge tubes (in racks) prepared in steps 2.1 and 2.2, a vortex mixer, repeat pipettor with 0.5 mL and 5 mL capacity tips, 100−1000 µL air displacement pipette with 1000 µL tips, containers with approximately 100 mL each of diluent and ultrapure DI water, and a biohazard waste container.

2.4. Remove serum samples from frozen storage and warm to RT in the biosafety cabinet. Vortex mix each serum sample prior to sampling.

2.5. Using a repeat pipettor with 0.5 mL capacity tip, dispense 0.050 mL of mongoose serum into tube “A” and add 0.050 mL of 25x surrogate stock. Then add 0.950 mL of diluent to tube “A” using a repeat pipettor with 5 mL capacity tip. Cap securely and vortex mix for 10−15 s.

2.6. Dispense the pre-weighed NaCl from step 2.1 into tube “A” and vortex mix 3x for 8−12 s. Wipe down the outside surfaces of the vial rack containing tube “A” using 70% (v/v) isopropanol.

NOTE: The rack of samples may now be removed from the class II biosafety cabinet.

2.7. Centrifuge tube “A” at 12,000 x *g* for 1 min to separate the aqueous and ACN phases. Pipette 0.80 mL of the upper ACN phase to tube “B” using a 100−1000 µL air displacement pipette. Transfer the remaining solution in tube “A” to hazardous waste and discard the empty tube in a biohazardous waste container.

2.8. Remove ACN and TFA from tube “B” with a gentle flow of N_2 gas in a 45 °C water bath.

2.9. Add 0.250 mL of ACN to tube “B” using a repeat pipettor, vortex mix for 4−5 s, and then centrifuge briefly (2−4 s) at 12,000 x *g* to collect the liquid in the bottom of the tube.

2.10. Add 0.750 mL of ultrapure DI water to tube “B” using a repeat pipettor with 5 mL capacity tip, vortex mix for 4−5 s, and then centrifuge for 1 min at 12,000 x *g* to clarify the sample.

2.11. Transfer 0.75 mL of the supernatant to an autosampler vial using a 1000 µL air displacement pipette. Discard pipette tips in biohazard waste container.

2.12. Cap autosampler vials securely and analyze by LC-MS/MS (section 4). Transfer the remaining solution in tube “B” to hazardous waste and discard the empty tube to a biohazardous waste container. Dispose of all biohazardous waste by autoclaving or incineration.

3. Quality control samples

CAUTION: Follow the cautionary statements described in section 2.

NOTE: The following procedure describes the minimum number of quality control (QC) samples required for an analysis. Replicates at each level are recommended if sufficient control mongoose serum is available.

3.1. Prepare four 1.5 mL microcentrifuge tubes containing 200−300 mg of NaCl. Arrange the tubes in an 80-position plastic rack.

3.2. For each QC sample, label two 1.5 mL microcentrifuge tubes: one as “A” and the other as “B”. Arrange the tubes in an 80-position plastic rack.

3.3. Repeat step 2.3.

3.4. Remove control mongoose serum from frozen storage and warm to RT in the biosafety cabinet. Vortex mix the control serum prior to sampling.

3.5. Dispense 0.050 mL of control mongoose serum into the four 1.5-mL “A” tubes using a repeat pipettor with 0.5 mL capacity tip.

3.6. Fortify each of the four QC samples as specified in **Table 4** using a repeat pipettor with 0.5 mL capacity tip. Cap each QC sample securely and vortex mix for 10−15 s.

3.7. Perform the extraction procedure as described in steps 2.6−2.12.

4. LC-MS/MS analysis

4.1. Configure the LC-MS/MS with all parameters described in **Table 5**. Power on the LC-MS/MS and allow the column to reach 70 °C before setting the flow rate to 0.800 mL/min.

4.2. Set up a sequence in the data acquisition software (**Table of Materials**) to inject the standard curve before and after each batch consisting of quality control samples and unknown samples.

4.3. Inject all standards and samples and acquire MRM ion chromatograms using parameters listed in **Table 5**.

4.4. After sequence completion, turn off the LC-MS/MS and dispose of all autosampler vials as hazardous waste.

5. Quantification

5.1. Use the data analysis software to generate a calibration curve of relative responses versus

relative concentrations for me-IPA using et-IPA as the internal standard. Calculate the relative responses from the quantifier MRM transition for me-IPA (556.6 → 428.7) divided by the MRM transition for et-IPA (570.7 → 442.7). Construct a 7-level calibration curve using a second order quadratic function that is weighted 1/x and ignores the origin.

5.2. Calculate the serum concentration (C_{serum}) of me-IPA using the following equation:

$$C_{serum} = \frac{(c_{instrument})(1.25)(V_{final})}{V_{serum}}$$

where $c_{instrument}$ is the concentration determined by the instrument from the calibration curve in units of µg/mL, 1.25 is the dilution factor ($\frac{1\ mL}{0.8\ mL} = 1.25$), V_{final} is the final sample volume (1.0 mL), and V_{serum} is the serum volume in mL (0.050 mL nominal).

REPRESENTATIVE RESULTS:

Representative ion chromatograms from a me-IPA analysis are presented in **Figure 1**. The control mongoose serum (**Figure 1A**) illustrates the retention time of et-IPA (surrogate analyte) and the absence of me-IPA at the indicated retention time. The quality control sample (**Figure 1B**) illustrates the baseline separation of me-IPA from et-IPA as well as the quantifier and qualifier transitions for me-IPA. **Figure 1C** shows a representative sample from the study with an observed serum concentration of 33.5 µg/mL me-IPA.

A representative calibration curve from a me-IPA analysis is presented in **Figure 2**. The 7-level, 14-point standard curve ranges from 0.00202 to 8.27 µg/mL me-IPA with a correlation coefficient (r^2) of 0.9998. Correlation coefficients ranged from 0.9998 to 0.99997 for the five me-IPA analyses. The surrogate analyte et-IPA concentration was 0.502 µg/mL in all standards.

Table 6 presents the accuracy and precision results for control mongoose serum fortified with 0, 1.3, 31, and 82 µg/mL me-IPA (n = 10 at each level). The results were collected from five separate analyses. Percent recoveries ranged from 96.9% to 109%. The percent relative standard deviation (% RSD) at the three fortification levels was 3.4%, 1.7%, and 2.3%, respectively.

The signal-to-noise ratio (S/N) observed in quality control samples (n = 10 negative controls; n = 10 at 1.3 µg/mL me-IPA) was used to determine the detection limit (DL; 3 x S/N) and quantitation limit (QL; 10 x S/N). The DL and QL for me-IPA in mongoose serum were 0.012 µg/mL and 0.042 µg/mL, respectively.

The peak area responses of the qualifier MRM transition divided by the quantifier MRM transition ($\frac{556.6 \rightarrow 126.9}{556.6 \rightarrow 428.7}$) was calculated for all standards and samples. This ratio for each sample was then divided by the average ratio observed in the calibration standards to determine the qualifier percent match. The qualifier ratio for the sample shown in **Figure 1C** was 0.439, with a 96.3% match.

The recovery of et-IPA surrogate analyte was calculated for all QC samples and unknown samples by dividing the et-IPA peak area response by the average et-IPA peak area response observed in the calibration standards. Average surrogate analyte recoveries were 91.0% (negative controls), 91.4% (1.3 µg/mL), 92.8% (31 µg/mL), and 95.4% (82 µg/mL).

No interference peaks for either the quantifier or qualifier transitions of me-IPA were observed in control mongoose serum (**Figure 1A**).

The extraction procedure and instrumental conditions used to determine et-IPA in mongoose serum (**Figure 3** and **Table 7**) was identical to the me-IPA method, but with the following changes. Propyl-iophenoxic acid (pr-IPA) was used as the surrogate analyte and an older, less sensitive LC-MS/MS was used. The source drying gas temperature was 350 °C with a flow of 12 L/min and a nebulizer pressure of 35 psi. The capillary voltage was -2500 V. The source had no sheath gas or means to adjust nozzle voltage. The quantifier MRM transition for et-IPA was 570.7 → 442.8 (584.7 → 456.8 for pr-IPA). The fragmentor was 80 V and the collision energy was 10 V for both analytes. The qualifier MRM for et-IPA was 570.7 → 126.8 with a collision energy of 40 V.

The testing of all mongoose serum samples for et-IPA was performed over eight analyses. The 7-level calibration curve ranged from 0.00207 to 8.48 µg/mL with correlation coefficients (r^2) ranging from 0.9990 to 0.9999. The surrogate analyte pr-IPA concentration was 0.512 µg/mL. **Table 7** presents the accuracy and precision results for control mongoose serum fortified with 0, 1.3, 13, 32, 85, and 170 µg/mL me-IPA. The results were collected from eight separate analyses. Percent recoveries ranged from 89.5% to 115%. The % RSD at the five fortification levels was 4.3%, 1.5%, 2.3%, 5.6%, and 1.1%, respectively. The S/N observed in quality control samples (n = 21 negative controls; n = 21 at 1.3 µg/mL me-IPA) was used to determine the DL and QL. The DL and QL for me-IPA in mongoose serum were 0.12 µg/mL and 0.42 µg/mL, respectively. The average surrogate analyte recovery from quality control samples was 86.8% (n = 75). No interference peaks for either the quantifier or qualifier transitions of et-IPA were observed in control mongoose serum.

All mongooses offered et-IPA baits consumed ≥25% of the bait within the 24 hour time constraint and had quantifiable levels of et-IPA in their sera (**Table 8**). From the mixed model analysis, overall mean serum IPA concentrations were marginally higher from baits with 2.8 mg of biomarker in the bait matrix (17.5 µg/mL, 95% CI 11.7−23.3 µg/mL) in comparison to the blister pack (9.8 µg/mL, 95% CI 4.0−15.6 µg/mL) (F = 3.6, P = 0.07). Concentrations for both bait types decayed with experimental day (β = -0.15 ± 0.04, F = 14.4, P = 0.0005). Individual level variability in serum IPA concentrations was observed (animal ID covariance parameter estimate = 46.6 ± 20.7). All mongooses consumed 100% of control baits with only the empty foil blister pack remaining. Mean concentration of et-IPA residue in serum was variable by time period and did not appear to decrease consistently over time (**Figure 3**).

All mongooses offered me-IPA baits consumed ≥ 25% of the bait within the 24 hour time limit and had quantifiable levels of me-IPA in their sera (**Table 9**). Mean serologic concentration of et- and me-IPA in mongoose sera appeared dependent upon the concentration of IPA in the bait.

Higher concentrations of IPA in the bait resulted in higher serum residues even in cases where the overall dose (2.8 mg in the case of et-IPA) remained the same. Interestingly, me-IPA appeared to produce a more even degradation pattern over time with an initial spike at day 1, followed by a steady decline until day 14 where concentrations appeared to plateau (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative ion chromatograms. (**A**) Representative MRM ion chromatogram of control mongoose serum fortified with surrogate analyte ethyl-iophenoxic acid (et-IPA). The arrow indicates the retention time for methyl-iophenoxic acid (me-IPA). (**B**) Representative MRM ion chromatogram of control mongoose serum fortified with 31 µg/mL me-IPA. The relative intensities of the quantifier (Quant) and qualifier (Qual) transitions for me-IPA are shown. (**C**) Representative MRM ion chromatogram of a mongoose serum sample with an observed me-IPA concentration of 33.5 µg/mL.

Figure 2: Representative calibration curve. An original calibration curve generated by the data analysis software for methyl-iophenoxic acid (me-IPA).

Figure 3: Mean serum ethyl IPA (et-IPA) concentration by bait type and concentration over time.

Figure 4: Mean serum methyl IPA (me-IPA) concentration by bait concentration over time. All me-IPA concentrations were incorporated into the external bait matrix.

Table 1: Preparation of 25x me-IPA stocks in ACN (in 8-mL amber glass vials).

Table 2: Preparation of 4x IPA stocks in ACN (in 2-mL autosampler vials).

Table 3: Preparation of me-IPA standards in 75/25 water/ACN (in 2-mL autosampler vials).

Table 4: Quality control sample fortification (prepare in 1.5-mL microcentrifuge tubes).

Table 5: LC-MS/MS parameters. Quantifier product ions are bolded.

Table 6: QC results for me-IPA in mongoose serum (µg/mL).

Table 7: QC results for et-IPA in mongoose serum (µg/mL).

Table 8: Mean (stand deviation [SD]) ethyl-IPA serum concentration by bait type. ND = not detected, NA = Not applicable.

Table 9: Mean (SD) methyl-IPA serum concentration by dose. ND = Not detected, NA = Not applicable. All methyl IPA concentrations were incorporated into the external bait matrix.

DISCUSSION:

The LC-MS/MS method developed for the studies utilized the selectivity of multiple reaction monitoring to accurately quantify me-IPA and et-IPA in mongoose serum. The selectivity of MS/MS detection also allowed for a simple clean-up protocol relying solely on acetonitrile to precipitate proteins from serum prior to analysis.

Iophenoxic acids are soluble in ACN but are practically insoluble in water. To exclude water from the ACN extraction, sodium chloride was added to force a clear water:ACN phase separation by increasing the ionic strength of the aqueous (serum) phase. The volatile acid trifluoracetic acid (TFA) was also added to ensure that iophenoxic acids were protonated during the extraction and would be more readily solubilized in the ACN phase. TFA is removed during the dry-down step prior to LC-MS/MS analysis.

Blood draws from mongoose were approximately 1 mL, yielding 0.5 mL or less of sera. To perform replicate analyses of each sample, a micro-scale sample preparation procedure was required that used microcentrifuge tubes rather than typical analytical laboratory glassware such as Class A volumetric pipettes and flasks. To achieve accurate and precise results it is necessary that analysts be proficient in the use of glass microliter syringes and positive-displacement repeat pipettors with microliter-size tips.

A limitation of this method is that it requires costly LC-MS/MS instrumentation and analysts trained in its use and maintenance. However, because me-IPA and et-IPA are baseline resolved using the HPLC conditions described, the method could potentially be adapted to a single-quadrupole LCMS or HPLC with UV detector provided no interferences were observed.

The difference in mean serum concentrations between mongooses that consumed baits with et-IPA in the blister pack and bait matrix at the 2.8 mg dose suggests spillage when the marker is contained in the blister pack, rather than incorporated into the bait matrix. This may have implications for the purpose of estimating vaccine as opposed to bait uptake. For example, incorporating the marker into the external bait matrix may inflate the estimation of vaccine coverage if the animal eats the matrix but vaccine spills out. However, regulatory restrictions may preclude the ability of mixing a biological marker directly with a vaccine within the blister pack. In the cases in which <100% consumption was recorded, three cases were with baits containing et-IPA in the blister pack, two of which were the higher 1% concentration. This suggests potential taste aversion when et-IPA is at higher concentrations. When baits containing 1% (20.0 mg) et-IPA incorporated into the bait matrix were offered to mongooses, all animals rejected the bait.

Mean IPA concentrations for et- and me-IPA at day 14 were approximately 17 and 19 µg/mL, respectively. Similar research performed at Instituto de Investigación en Recursos Cinegéticos, Ciudad Real, Spain, using butyl and pentyl-IPA found day 14 concentrations of approximately 45 and 10 µg/mL in mongoose sera when delivered at the same concentration and bait formulation as in our study. These differences suggest that different derivatives of IPA may metabolize at different rates in mongooses, which can be useful when marker retention (either short or long-term retention) is a concern.

Differences in the physiology of the gastrointestinal system may affect absorption and excretion of IPA in different species. The plasma elimination half-life of IPA in domestic cats (*Felis catus*) when delivered at 1.5 mg/kg was 107 days whereas the rate in brushtail possums at the same dose rate was approximately one day[28]. When IPA was given to domestic goats (*Capra aegagrus hircus*) at a dose rate of 1.5mg/kg the terminal elimination half-life of IPA was 81 days, although investigators continued to find elevated iodine concentrations up to 160 days following administration[39]. The gastrointestinal system of members of *Vivveridae* (the family to which mongooses had previously been assigned[40]) is described as similar to that of the domestic cat[41], which may explain the retention time of IPA in mongooses. Research also suggests IPA may be metabolized differently in marsupials, allowing for more rapid excretion than in eutherian species[29].

Both derivatives of IPA evaluated in this study provided long-term (4−8 weeks) marking ability in mongooses. The use of IPA as a biological marker in mongooses should take into consideration the study objectives and the desired duration of marking. In cases where animals are to be marked from the same study sites during consecutive time periods, different derivatives of IPA could be used to ensure results from one marking event are not confounded by marking during previous events. As part of operational ORV for wildlife in the US, sampling the target species and testing for the presence of RVNA and biomarker is typically conducted 4−6 weeks following ORV distribution[42]. From a practical standpoint, it appears either et- or me-IPA can be readily detected during this time period. Future research to evaluate the residue decay function in mongooses offered various concentrations of both congeners of IPA evaluated in this study would be useful.

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DISCLOSURES:

Authors AV and SO are fulltime employees of an oral rabies vaccine bait manufacturer.

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Figure 1

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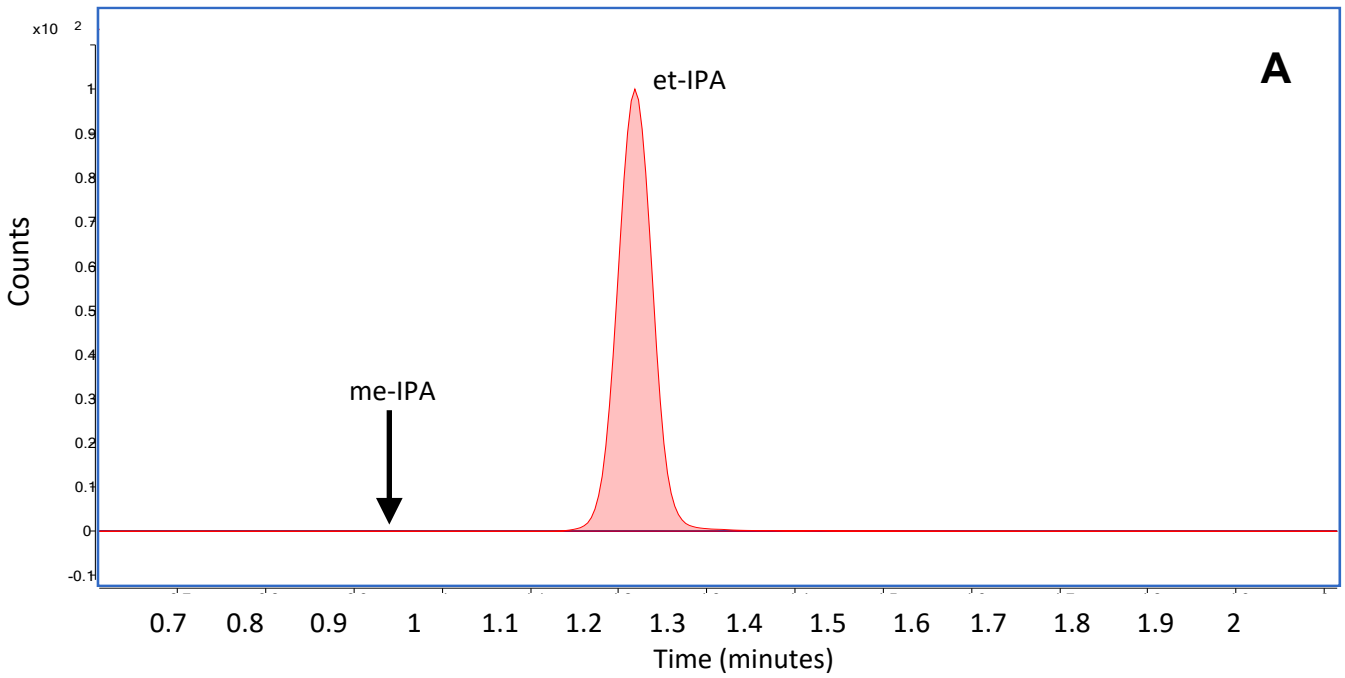

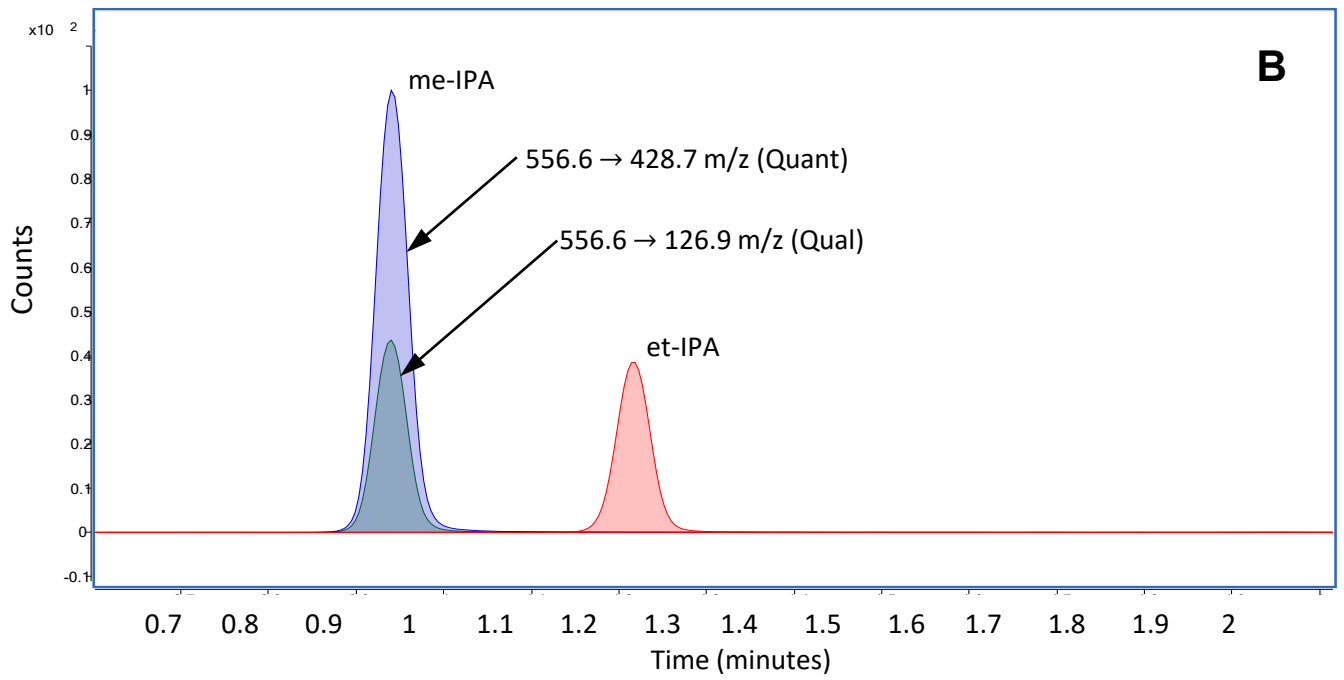

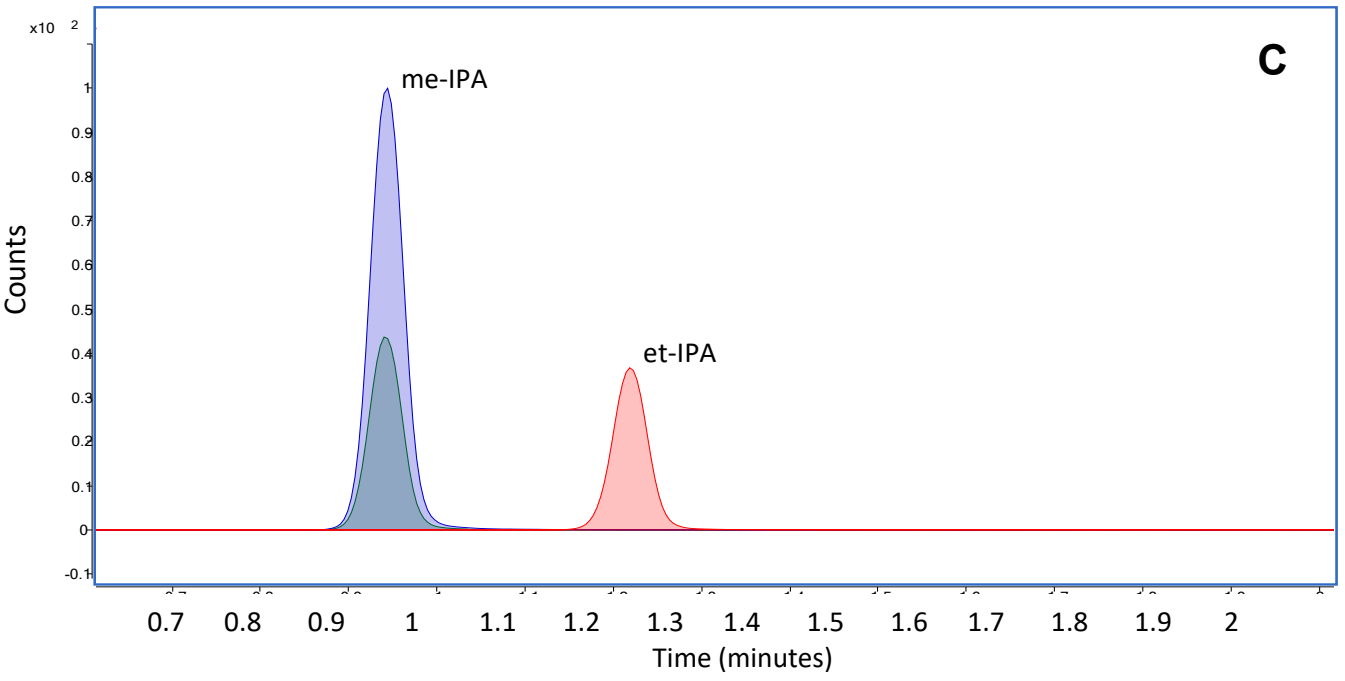

Figure 2

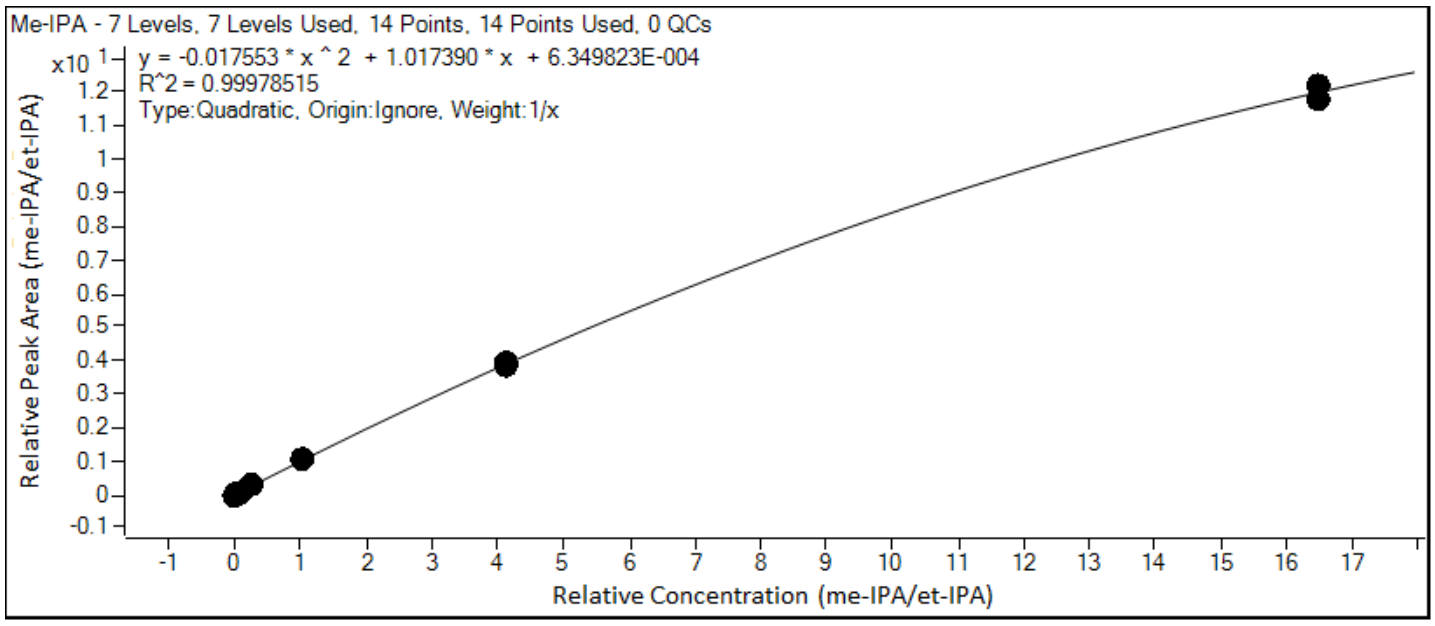

Figure 3

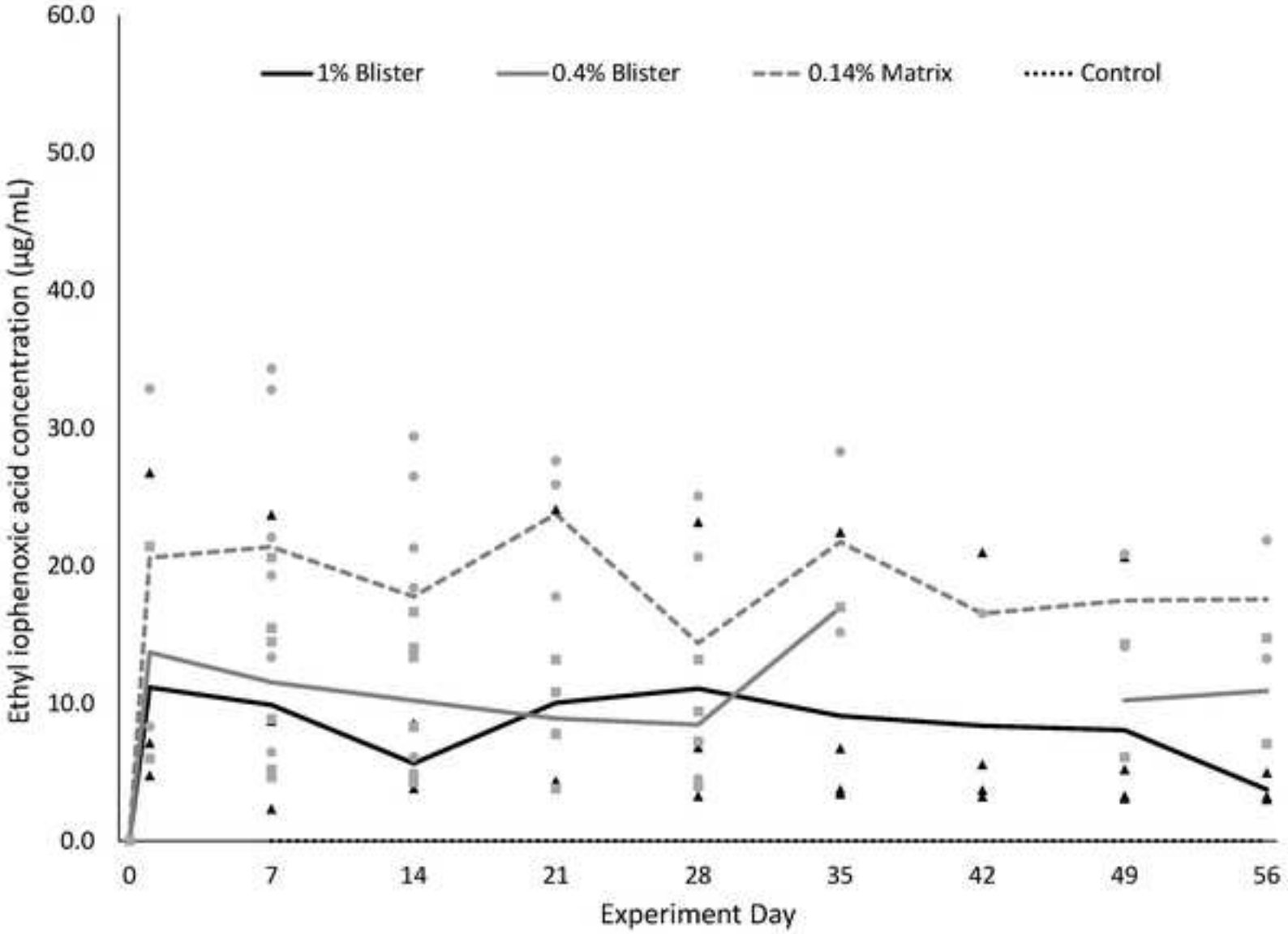

Figure 4

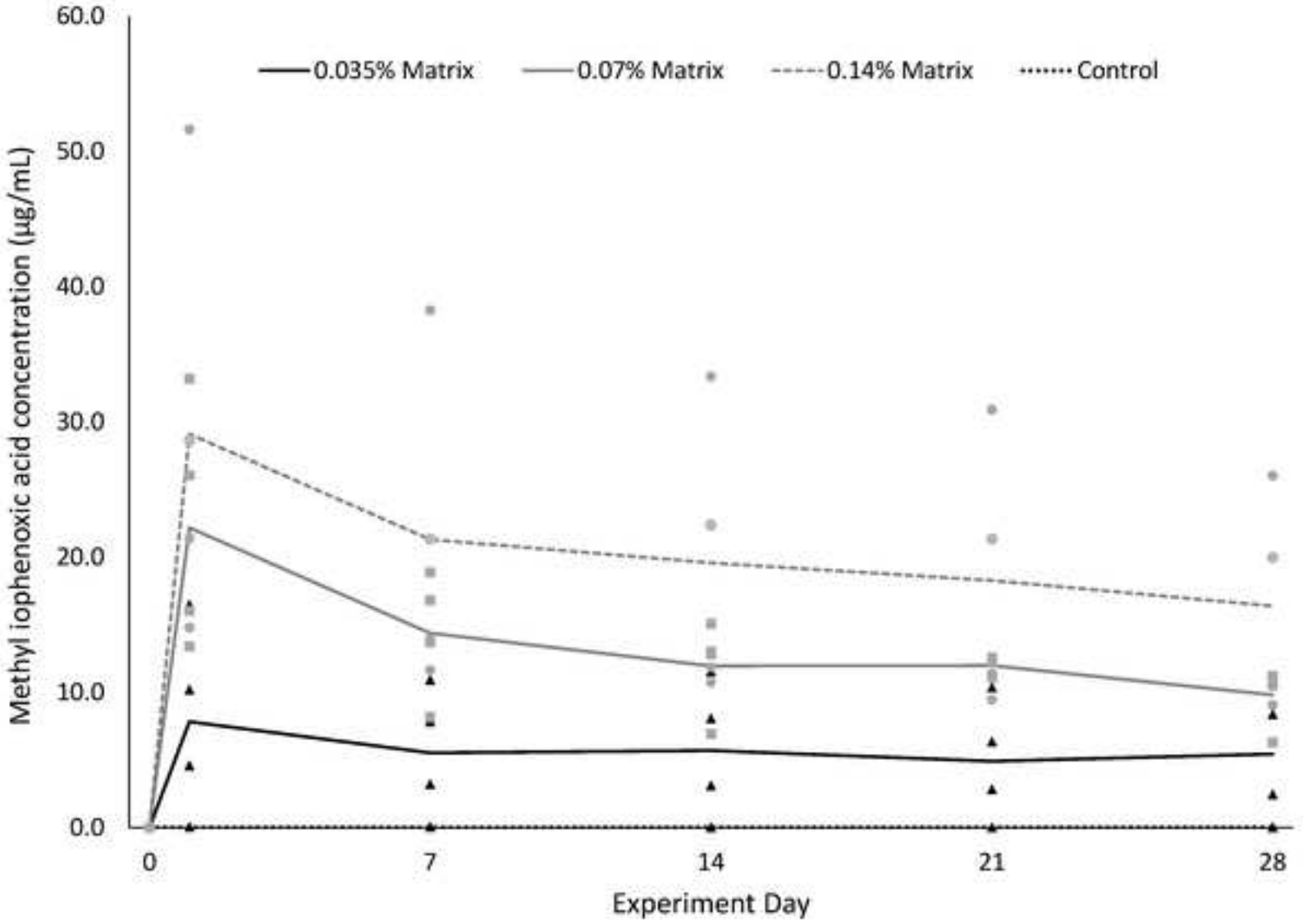

Table 1. Preparation of 25x me-IPA stocks in ACN (in 8-mL amber glass vials).

ID		me-IPA Concentration (µg/mL)
25x-7	Refer to step 1.6	200
25x-6	Combine 1.000 mL 25x-7 Stock with 3.000 mL ACN	50
25x-5	Combine 1.000 mL 25x-6 Stock with 3.000 mL ACN	13
25x-4	Combine 1.000 mL 25x-5 Stock with 3.000 mL ACN	3.1
25x-3	Combine 1.000 mL 25x-4 Stock with 3.000 mL ACN	0.78
25x-2	Combine 1.000 mL 25x-3 Stock with 3.000 mL ACN	0.2
25x-1	Combine 1.000 mL 25x-2 Stock with 3.000 mL ACN	0.049

Table 2. Preparation of 4x IPA stocks in ACN (in 2-mL autosampler vials).

		Concentration (µg/mL)	
ID		me-IPA	et-IPA
4x-7	0.200 mL 25x-7 + 0.200 mL 25x Surrogate + 0.850 mL ACN	32	1.6
4x-6	0.200 mL 25x-6 + 0.200 mL 25x Surrogate + 0.850 mL ACN	8	1.6
4x-5	0.200 mL 25x-5 + 0.200 mL 25x Surrogate + 0.850 mL ACN	2	1.6
4x-4	0.200 mL 25x-4 + 0.200 mL 25x Surrogate + 0.850 mL ACN	0.5	1.6
4x-3	0.200 mL 25x-3 + 0.200 mL 25x Surrogate + 0.850 mL ACN	0.12	1.6
4x-2	0.200 mL 25x-2 + 0.200 mL 25x Surrogate + 0.850 mL ACN	0.031	1.6
4x-1	0.200 mL 25x-1 + 0.200 mL 25x Surrogate + 0.850 mL ACN	0.008	1.6
4x-0	0.200 mL 25x Surrogate + 1.050 mL ACN	0	1.6

Table 3. Preparation of me-IPA standards in 75/25 water/ACN (in 2-mL autosampler vials).

		Concentration (µg/mL)	
ID		me-IPA	et-IPA
Std 7	0.200 mL 4x-7 + 0.600 mL DI water	8	0.4
Std 6	0.200 mL 4x-6 + 0.600 mL DI water	2	0.4
Std 5	0.200 mL 4x-5 + 0.600 mL DI water	0.5	0.4
Std 4	0.200 mL 4x-4 + 0.600 mL DI water	0.13	0.4
Std 3	0.200 mL 4x-3 + 0.600 mL DI water	0.03	0.4
Std 2	0.200 mL 4x-2 + 0.600 mL DI water	0.0078	0.4
Std 1	0.200 mL 4x-1 + 0.600 mL DI water	0.002	0.4
Std 0	0.200 mL 4x-0 + 0.600 mL DI water	0	0.4
Blank	0.200 mL ACN + 0.600 mL DI water	0	0

Table 4. Quality control sample fortification (prepare in 1.5-mL microcentrifuge tubes).

		Concer (µg/mL)
ID		me-IPA
Negative Control	0.050 mL 25x Surrogate + 0.950 mL Diluent	0
Low Fortification	0.050 mL 25x Surrogate + 0.020 mL 25x-4 + 0.930 mL Diluent	1.2
Mid Fortification	0.050 mL 25x Surrogate + 0.030 mL 25x-6 + 0.920 mL Diluent	30
High Fortification	0.050 mL 25x Surrogate + 0.020 mL 25x-7 + 0.930 mL Diluent	80

ntration in serum
et-IPA
10
10
10
10

Table 5. LC-MS/MS parameters.

Liquid Chromatography:	
Column:	C18, 2.1 x 50 mm, 2.5 µm partic
Column temperature:	70 °C
Injection volume:	5 µL
Flow rate:	0.800 mL/min
Mobile Phase:	Solvent A:
	Solvent B:

Gradient program:		
Time (min):	0	0.25
% B:	40	40

Needle Wash: ACN, 3 s

MS/MS Source: ESI (negative mode)	
Gas temperature:	300 °C
Gas flow:	5 L/min
Nebulizer:	45 psi
Capillary:	-4000 V
Nozzle voltage:	-500 V
Sheath gas temperature:	250 °C
Sheath gas flow:	7 L/min

MRM Transitions:

Analyte	Precursor Ion (m/z)	Product Ion (m/z) *
Me-IPA	556.6	**428.7**
		126.9
		126.8
Et-IPA	570.7	**442.7**

Time Segments:

Segment	Start (min)	End (min)	
1	0	0.6	
2	0.6	2	
3	2	4.75	

* Quantifier transitions are **bolded**.

cle size

	0.1% formic acid in water
	0.1% formic acid in ACN

2.25	2.26	3.4
55	100	100

	Fragmentor (V)	Collision Energy (V)	Dwell Ti
	74	12	6
		65	6
		61	6
	87	16	6

Type	Diverter	Delta EMV	
MS2 Scan	To Waste	0	
MRM	To MS	-100	
MS2 scan	To Waste	0	

3.41	4.75
40	40

me (ms)	Time Segment
0	2
0	
0	
0	

Polarity	Data Stored
Negative	No
Negative	Yes
Positive	No

Table 6. QC results for me-IPA in mongoose serum (µg/mL).

ID	Day	Target me-IPA	Observed me-IPA	Percent Recovery		
QC-1	1	0	ND	N/A		
QC-2	1	0	ND	N/A		
QC-11	2	0	ND	N/A		
QC-12	2	0	ND	N/A		
QC-21	3	0	ND	N/A		
QC-22	3	0	ND	N/A		
QC-31	4	0	ND	N/A		
QC-32	4	0	ND	N/A		
QC-3	5	0	ND	N/A		
QC-4	5	0	ND	N/A		
QC-13	1	1.25	1.26	101%	Ave $_{(10)}$ =	102%
QC-14	1	1.25	1.23	98.40%	SD =	3.50%
QC-23	2	1.25	1.26	101%	% RSD =	3.40%
QC-24	2	1.25	1.28	102%		
QC-33	3	1.29	1.3	101%		
QC-34	3	1.29	1.25	96.90%		
QC-5	4	1.29	1.37	106%		
QC-6	4	1.29	1.41	109%		
QC-15	5	1.29	1.3	101%		
QC-16	5	1.29	1.34	104%		
QC-25	1	30.1	30.2	100%	Ave $_{(10)}$ =	103%
QC-26	1	30.1	31.2	104%	SD =	1.80%
QC-35	2	30.1	31.1	103%	% RSD =	1.70%
QC-36	2	30.1	31.1	103%		
QC-7	3	31	31.6	102%		
QC-8	3	31	31.4	101%		
QC-17	4	31	32.5	105%		
QC-18	4	31	32.8	106%		
QC-27	5	31	31.7	102%		
QC-28	5	31	32.1	104%		
QC-37	1	80.2	77.8	97.00%	Ave $_{(10)}$ =	101%
QC-38	1	80.2	78.9	98.40%	SD =	2.30%
QC-9	2	80.2	81.8	102%	% RSD =	2.30%
QC-10	2	80.2	79.8	99.50%		
QC-19	3	82.7	83.5	101%		
QC-20	3	82.7	84	102%		
QC-29	4	82.7	84.7	102%		
QC-30	4	82.7	87.2	105%		

QC-39	5	82.7	83	100%		
QC-40	5	82.7	84.1	102%		

Table 7. QC results for et-IPA in mongoose serum (µg/mL).

Target et-IPA (µg/mL)	N	Mean (%)	SD (%)	% RSD
0	21			
1.3	12	103	4.5	4.3
13	9	91.7	1.4	1.5
32	12	106	2.4	2.3
85	12	105	5.8	5.6
170	9	106	1.1	1.1

Table 8. Mean (stand deviation [SD]) ethyl-IPA serum concentration by bait type.

Time Period	Bait type and ethyl IPA bait concentration (µg/mL)							
	0.4% - Blister		1.0% - Blister		0.14% - Matrix		Control	
	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N
Day 0	ND	5	ND	4	ND	6	ND	3
Day 1	13.7 (10.9)	2	11.2 (10.4)	4	20.6 (17.3)	2	NA	NA
Day 7	11.5 (6.3)	6	9.9 (9.6)	4	21.4 (10.9)	6	ND	3
Day 14	10.2 (5.2)	6	5.7 (2.5)	3	17.8 (10.2)	6	ND	3
Day 21	8.9 (4.1)	4	10.0 (9.5)	4	23.8 (5.3)	3	ND	2
Day 28	8.5 (3.9)	5	11.1 (10.6)	3	17.7 (9.3)	4	ND	3
Day 35	17.0 (NA)	1	9.1 (9.0)	4	21.7 (9.3)	2	ND	1
Day 42	NA	0	8.4 (8.5)	4	16.5 (NA)	1	ND	2
Day 49	10.2 (5.8)	2	8.1 (8.4)	4	17.5 (4.7)	2	ND	2
Day 56	10.9 (5.4)	2	3.8 (1.1)	3	17.6 (6.1)	2	ND	2

Table 9. Mean (SD) methyl-IPA serum concentration by dose.

Time Period	Dose and methyl IPA concentration (µg/mL)							
	0.04%		0.07%		0.14%		Control	
	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N
Day 0	ND (NA)	4	ND (NA)	4	ND (NA)	4	ND (NA)	4
Day 1	7.8 (7.1)	4	22.2 (9.2)	4	29.1 (16.0)	4	ND (NA)	4
Day 7	4.4 (4.8)	4	14.4 (4.6)	4	21.3 (12.0)	4	ND (NA)	4
Day 14	5.7 (5.9)	4	12.0 (3.5)	4	19.6 (10.6)	4	ND (NA)	4
Day 21	4.9 (4.5)	4	12.0 (0.8)	3	18.3 (9.9)	4	ND (NA)	4
Day 28	5.4 (5.0)	4	9.8 (2.4)	4	16.4 (8.1)	4	ND (NA)	4

TABLE OF MATERIALS	
Name	Company
Acetonitrile, Optima grade	Fisher
Analytical balance	Mettler Toledo
C18 column, 2.1 x 50 mm, 2.5-µm particle size	Waters Corp.
ESI Source	Agilent
Ethyl-iophenoxic acid, 97 %	Sigma Aldrich
Formic acid, LC/MS grade	Fisher
LCMS software	Agilent
Methyl-iophenoxic acid, 97 % (w/w)	PR EuroChem Ltd.
Microanalytical balance	Mettler Toledo
Microcentrifuge	Eppendorf
MS/MS	Agilent
N-Evap	Organomation
Oral Rabies Vaccine Baits	IDT Biologika, Dessau Rossleau, Germany
Propyl-iophenoxic acid, 99 % (w/w)	PR EuroChem Ltd.
Repeat pipettor	Eppendorf
Screw-top autosampler vial caps, PTFE-lined	Agilent
Sodium chloride, Certified ACS grade	Fisher
Statistical Software Package	SAS Institute, Cary, North Carolina, USA
Trifluoroacetic acid, 99 %	Alfa Aesar
UPLC	Agilent
Vortex Mixer	Glas-Col
0.2-mL pipettor tips	Eppendorf
0.5-mL pipettor tips	Eppendorf
1.5-mL microcentrifuge tubes	Fisher
1250-µL capacity pipette tips	GeneMate
1-mL pipettor tips	Eppendorf
2-mL amber screw-top autosampler vials	Agilent
5-mL pipettor tips	Eppendorf
80-position microcentrifuge tube rack	Fisher
8-mL amber vials with PTFE-lined caps	Wheaton
70 % (v/v) isopropanol	Fisher
100-1000 µL air displacement pipette	Eppendorf

Catalog Number	Comments
A996	
XS204	
186003085	
G1958-65138	
N/A	Lot MKBP5399V
A117	
MassHunter Data Acquisition and Quantitative Analysis	
N/A	Lot PR0709514717
XP6U	
5415C	
G6470A	
115	
N/A	
N/A	Lot PR100612108RR
M4	
5190-7024	
S271	
N/A	
L06374	
1290 Series	
099A PV6	
30089.413	
30089.421	
14-666-325	
P-1233-1250	
30089.43	
5182-0716	
30089.456	
05-541-2	
224754	
A459	
ES-100	

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Title of Article: Iophenoxic Acid as a biological marker for oral rabies vaccination in the small Indian mongoos

Author(s): AR Berentsen, RT Sugihara, CG Payne, I Leinbach, SF Volker, A Vos, S. Ortmann, AT Gilbert

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CORRESPONDING AUTHOR:

Name:	Are R Berentsen
Department:	
Institution:	USDA APHIS WS National Wildlife Research Center
Article Title:	Iophenoxic Acid as a biological marker for oral rabies vaccination in the small Indian mongoose
Signature:	 Date: 7 November 2018

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Changes to be made by the author(s) regarding the manuscript:

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4. Please revise the protocol (lines 116-129, 199-206, etc.) to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion. Changes made to the text where appropriate
5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
6. 1.5: Please list an approximate volume of these stock solutions to prepare. Changes made to the text where appropriate
7. 2.4: What happens to the aqueous phase? Is it saved for a later step? Changes made to the text where appropriate
8. 2.6: Where is ACN added? To the tube in step 2.5? Changes made to the text where appropriate
9. 2.8: Which supernatant? From step 2.7? Please also specify the volume of supernatant transferred. Changes made to the text where appropriate
10. 4.1: Please reference Table 3 for composition of Standard 6. Please specify how to adjust time segments. Changes made to the text where appropriate
11. 5.1: What are the relative responses? How are they obtained? Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.). Changes made to the text where appropriate
12. Please include single-line spaces between all paragraphs, headings, steps, etc. Changed as requested
13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Highlighted as requested
14. Figure 1: Please label the x- and y-axes. Labelled

15. Figures 3 and 4: Please fix the unit on the y-axis. It should be µg/mL. In the figure legend please describe different curves, in particular the different trendlines. Figures have been modified for clarity and consistency.
16. Table 5: Please remove commercial language (Agilent 1290 Series, Agilent AJS, Xbridge, Agilent 6470). What does % B refer to? Changes made to the text where appropriate
17. Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file. Changed as requested
18. Lines 209-210: Please describe what Figure 1 shows. Changed as requested
19. Lines 219-233: Where are these data? Tables 6 and 7.
20. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: Changes made to the text where appropriate
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
21. References section: Please do not make the reference numbers superscripts. Changed as requested.
22. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment. Changed as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The main aim of this study was to evaluate the efficacy of using ethyl-iophenoxic acid and methyl-iophenoxic acid as a biomarker of vaccine bait uptake in the Indian mongoose (Herpestes auropunctatus), a major reservoir of rabies virus in Puerto Rico. Vaccine bait with the biomarkers and placebo baits were offered to captive raccoons and serum collected prior to ingestion of bait and up to 8 weeks post-ingestion. Detection of bait intake was quantified using LC-MS. The efficacy of wildlife vaccination programs critically depends on bait being ingested by target species. As pointed out by the authors, quantifying bait uptake using biomarkers is a critical component of evaluating vaccination programs, especially in areas with ongoing vaccination and/or enzootic disease (wherein serum antibody levels are not a good indicator of vaccine uptake). Numerous studies using other biomarkers have revealed that numerous factors (e.g., bait competition) can affect bait uptake, and thus compromise field vaccination programs. On the whole I found the manuscript to be an important contribution because it broadens the range of biomarkers that are routinely used in vaccine uptake studies. These markers come with their own advantages and disadvantages. For example, tetracycline is a good marker, but requires the invasive removal of teeth to evaluate uptake.

Comments:

1. Title needs to be reconsidered (including the odd capitalization). Authors may consider something more specific like: Iophenoxic acid as a biological marker for oral rabies vaccine bait uptake in the small Indian mongoose: Changed as requested
2. Line 69-80: Retention times: Added as requested
3. Line 81-86: It is unclear why two derivatives of et-IPA were used. Was there a clear a priori expectation of differences in these two derivatives? The objective of the biomarker evaluation was to evaluate whether IPA was detectible in mongoose serum. Our initial time frame was to see if IPA was detectible approximately 4-6 weeks post bait consumption. Operationally, when oral rabies vaccination is performed, seroconversion is evaluated 4-6 weeks post-vaccination. We were interested in determining whether IPA would be detectible for at least 6 weeks post baiting to coincide with post-ORV trapping. Et-IPA was evaluated first. Once the retention times appeared to extend beyond approximately 2 months (this was not expected, based on shorter retention times found in other small mammals), we decided to evaluate met-IPA as well to see if the retention time was different.

4. Line 86-90:
4a. The logic behind the choice of dosage levels is unclear (except for the 1% dose). For example, was there a particular reason that 5mg/kg was considered optimal. The 5 mg/kg dose corresponds to previous work which successfully used IPA to mark other small mammals, as outlined in references 17 and 36.
4b. Line 86-90: It may be important to clarify what is meant by the "external bait matrix". It is also unclear if these were different baits (i.e., matrix or blister; as indicated in the analyses; Line 109) or different locations of biomarker incorporation in the same bait. Clarification has been added to state: "All baits were comprised of a 28x20x9 mm foil blister back with an external coating (hereafter "bait matrix") comprised of….
4c. This paragraph is also confusing because it says that the biomarkers were incorporated at three concentrations (0.14%, 0.4% and 1%). However, the last one is not actually comparable to the first two because here both the dose and concentration increase. The concentrations of 0.14 and 0.4% correspond to a consistent dose rate of 5mg IPA per kg of mongoose. We used an average mongoose weight of approximately 560g. The concentrations are different because of the differences between the amount or volume of external bait matrix (~2 g) vs the volume of the blister pack (0.7 mL). That is, 2.8mg of IPA is a lower concentration when mixed with 2g of bait matrix than in 0.7 mL of blister pack liquid. The higher concentration of 1% was determined as a taste-aversion threshold in some species as described in reference 35. We were only able to evaluate the 1% concentration when incorporated into the external bait matrix because of a difficulty keeping the IPA in solution when attempting to inject the solution into the 0.7 mL blister pack. Because of the difficulty in keeping the IPA in solution at higher concentrations when added to the liquid blister pack, we decided to evaluate me-IPA solely when incorporated into the external bait matrix, as would be performed in operational use of ORV baits. With the retention times found by delivering et-IPA at 2.8mg (5mg/kg) in the bait matrix we determined it would be valuable to use the 2.8 mg as the highest dose with me-IPA and decrease the dose by 50% (1.4mg) and 75% (0.07mg).
5. Line 96: How were the animals captured prior to isoflurane anesthesia? Information on capture and housing has been added.
6. Line 98: This needs to be made clearer. What was the maximum frequency of draws. The maximum frequency of draws was weekly. At some weekly time periods we were unable to obtain a blood sample. Rather than continue to try (thus risking animal health) we determined it

was best to abandon sample collection during that time period and wait until the next scheduled sampling period a week later.
7. Line 102: Why was me-IPA study design different from et-IPA study design (concentrations, doses and duration of sampling). Such differences in design are especially critical given that this is a techniques paper, and such differences are likely to make it difficult to compare the two biomarkers. It is possible that the authors had different expectations for these markers which necessitated the different designs (also see point 2). The analysis was an iterative process. We first evaluated et-IPA in various concentrations and found longer than expected retention times, based on studies with other small mammals. Based on manufacturing problems (a difficulty in keeping IPA in solution at higher concentrations in the blister pack) we decided to only evaluate me-IPA when incorporated into the external bait matrix which has a higher volume (thus lower concentration but same overall amount of IPA) than inside the blister pack. Once we determined that 2.8mg et-IPA had a retention time exceeding our expectations we evaluated me-IPA at 2.8mg and two lower amounts (0.14 and 0.07mg) all incorporated into the external bait matrix. In operational use of ORV baits biomarkers are typically incorporated into external bait matrices as adding marker to the actual vaccine could change the chemical properties of the vaccine or in some cases have viricidal effects, compromising vaccine efficacy.
8. Line 109: It is surprising that time points were included as a random effect. Given the introduction, I would assume that there was a clear expectation of a reduction over time. Treating time as a fixed effect (and ID as random) would allow for the detection of an interaction of biomarker concentration and time on serum concentrations. I also feel that it would be useful to carry out some analyses to evaluate sensitivity and specificity (e.g., using Receiver Operating Curves etc.). We agree with the reviewer and revised the analysis so that only animal ID was a random effect, with fixed effects of Bait Type (categorical) and Time (continuous). We tested for the interaction term between Bait Type and Time, but it was only marginally significant and similarly only marginally reduced the AICC (difference less than 1) compared to the same model without the interaction term, so we exclude the interaction term in the final analysis presentation.
9. Line 139: Ensure full form of abbreviations come after the first mention (e.g., ACN) Changed as requested.
10. Line 172: Is there a logic to these concentrations in the QC samples? QC fortification concentrations bracket the observed levels in serum from low concentrations to levels beyond the highest observed levels.
11. Line 178: A little more detail on the calibration standards and generation of the calibration curve is required. Additional clarification and details have been added to the text.
12. Line 193: An equal sign is missing in the equation Added
13. Line 198: It is unclear where these numbers and/or criteria for confirmation come from. Again it would be helpful to carry out sensitivity/specificity analyses. References to confirmation have been removed as this topic is more accurately described in the "qualifier percent match" and "surrogate analyte extraction recovery" sections of Representative Results. 14. Line 249: Was the amount of bait consumed incorporated in the analyses (even qualitatively?) Bait consumption was not included in the analysis. There did appear to be a correlation between bait estimated bait consumption and IPA resides (less consumption leading to lower residues) but this was not formally evaluated and the number of animals consuming < 100% of the bait were too small within treatment groups for meaningful analysis. Differing abilities among mongooses to metabolize IPA may also result in differing residues which may be a confounding factor in

attempting to make correlations.
15. Line 253-255: This information is probably more useful in the Introduction/Methods: Moved as recommended
16. Line 261: Poorly worded sentence; Please rephrase: Rephrased to read: "Mean serologic concentration of et- and me-IPA in mongoose sera appeared dependent upon the concentration of IPA in the bait. Higher concentrations of IPA in the bait resulted in higher serum residues even in cases where the overall dose (2.8mg in the case of et-IPA) remained the same."
17. Line 264: It is practically impossible to make out what these figures are depicting. May showing the observed points would help. If possible the authors may consider using color figures. The polynomial trend lines shown are not convincing (for example the predicted peak of the trend line in many cases is not even close to the observed peak values. Figures have been changed to include the observed points. Trend lines have been removed.
18. Quality of the figures is very poor. Different font sizes and fonts, as well as different locations of the axes labels pose issues. The use of a constant level of precision within a particular axis would be helpful. Use of a logged axis in Fig 2 (or inset to show the lower concentrations should be considered. Figures have been modified for clarity and consistency
19. Table 7 & 8: It is not clear how useful an SD is with 2-4 samples. Please also report the ranges of values. Alternatively, adding the actual observed data points to Fig 3 & 4 would be helpful. Observed data points have been added to figures 3 and 4 and the trend lines removed.
20. General: I think there needs to be a more comprehensive discussion of the pros and cons of the proposed methodology and a more comprehensive comparison of these to existing methods. For example, it is unclear to me is the LC-MS techniques are general enough that the serum once access to an LC-MS and personnel trained to use these methods would be a major limiting factor in using this method. Details regarding specialized training/equipment required have been added. With respect to comparing the methods described in this paper to other methods currently employed is challenging as this method is novel with respect to its use in evaluating IPA residues in mongoose serum.

Reviewer #2:

Manuscript Summary:
This is a very well written paper describing usage of biomarker incorporated within the bait matrix to monitor the success of rabies of oral rabies
vaccination (ORV). The findings indicate a potential of introduction of baits containing methyl-iophenoxic acid (me-IPA) in concentrations of 0.035%, 0.07% and 0.14% in the external bait matrix. IPA evaluated in this study provided long-term (4-8 weeks) marking ability in mongooses. I see no need to modify, add to, or correct the manuscript

Reviewer #3:

Manuscript Summary:

Overall impression: I found this manuscript very interesting. Although the idea is not new, the method for analyzing is up-to-date. The authors gave detailed protocol for test performing, experiment design, results presentations and conclusions.

Comment:
1. It is a bit confusing, what is actually the objective of your study? According to the Abstract (line 40), the objective of your study is to evaluate marking ability of et- and me-IPA. In the further text, the objective of study is "Our objective was to use LCMSas a technique to evaluate the marking ability of ethyl- and methyl- IPA when delivered in an ORV" (lines 80-82). Please, specify what are your goals. Then, the objective of study should be clearly defined in Abstract too. Our overall goal was to evaluate as a proof of concept the marking ability of two derivatives of IPA in mongooses, varying in concentration and delivery method. In order to reach our objective we used LCMS for the analysis. The analysis was an iterative process where methods used for et-IPA analysis were modified as needed and those modifications applied to the me-IPA analysis method.

Major Concerns:
1. From the research point of view, the main problem is that there were two groups with the different number of animals included in the experiment. The first group had 16, and the second 12 animals. Even the number of animals in control groups was not the same. This differences had the impact on the statistical data and objectivity of obtained results. The study was designed as a proof of concept, with limited statistical comparisons performed. In our opinion, the results are straightforward to demonstrate proof of concept. For example, even though we employed fewer negative controls compared to treatment animals, controls unequivocally lacked any evidence of the biomarker at any time of study in stark contrast to treated animals. We contend that we limit our conclusions to those readily supported by the data.

A linear mixed model approach to the analysis of different mean serum et-IPA levels accounts for uneven sampling design, so we are unclear about the comment from the reviewer. We do not statistically compare the two different forms of IPA with one another.

2. There is no clear explanation why the authors measured et-IPA for 8 weeks, and me-IPA for 4 weeks in mongooses sera. What is the reason for different length of follow-up in the study? Especially because, as I can realize from the Figures 3 and 4, that the concentration of et-IPA and me-IPA declined, but didn't disappear completely in both groups. I found the lack of this research that the measurement of IPA were not finished to the end, till the undetectable level. In operational use, oral rabies vaccine baits are deposited on the landscape and sero-surveys are conducted pre- and post-vaccination to estimate vaccination rates. Typically it can take up to 30 days for a full immune response to occur. Therefore, post-vaccination sero-surveys are conducted 4-6 weeks post-vaccination. In some areas, such as Puerto Rico, background rabies virus neutralizing antibodies are found in the target species (mongooses), which cannot be differentiated from virus exposure and vaccination. Given no vaccination program for mongooses currently exists the observed antibody titers are likely result of rabies virus exposure. The use of a biological marker may help differentiate between natural virus exposure and antibodies as a result of the vaccine. While it would be useful to learn the endpoint, from a

practical standpoint it was only necessary for us to evaluate marker longevity for 4-6 weeks. Evaluating the endpoint of IPA residues was beyond the scope and objectives of this study. However, the reviewer may be interested to know that recently completed field trials suggest that ethyl-IPA residues can remain detectible in mongooses for at least six months.

Minor Concerns:

1. Authors mentioned three times unpublished data (Lines 92, 319 and 324). It should be avoid this kind of references in peer-reviewed journals. Removed

2. Table 1: It is written Step 1.6.1, but in the part Protocol there is no 1.6.1 but only 1.6. Changed to refer to step 1.6 in the protocol.

3 Table 2: It should be written: Step 1.9 in the empty row. Step 1.9 is referenced in the Protocol. There are no steps referenced in Table 2. We are uncertain about what the reviewer is referring to.