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**TECHNICAL NOTE**

**PATHOLOGY/BIOLOGY**

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# Standardization of Histological Procedures for the Detection of Toxic Substances by Immunohistochemistry in Dipteran Larvae of Forensic Importance*

**ABSTRACT:** Immunohistochemistry (IHC) technique is an alternative toxicological analysis to detect drugs in insects of forensic importance, but it requires thorough histological procedures. In this study, we tested different fixatives—phosphate-buffered paraformaldehyde 4% (PP), Carnoy's fluid (CF), Kahle's solution (KS), ethanol in different concentrations, and ethanol associated to PP and CF, time of fixation and histological processes for dipteran larvae's tissue, aiming to develop a sample preparation protocol for IHC application. A suitable fixation was achieved using PP for 12 and 24 h, CF for 3 h, 70% ethanol for 19 days, and 70% ethanol/CF for 2 h/3 h. Postfixation using negative pressure, two immersions in xylene for 30 min each, and one in xylene plus paraffin for 45 min increased tissue preservation. An immunohistochemical test for cocaine detection was performed using monoclonal benzoylecgonine antibody from mouse, peroxidase-conjugated anti-mouse IgG and visualized by 3,3′-diaminobenzidine method showed these histological procedures didn't compromise antigenicity.

**KEYWORDS:** forensic science, forensic entomology, entomotoxicology, histological technique, immunohistochemical analyses, fixatives

Necrophagous insects feed on organic remains of dead animals to obtain the protein source needed to ovarian development and to stimulate oviposition/larviposition. These larvae develop on carcasses affecting the decay process (1). Therefore, these entomofauna can be useful in medico-criminal investigations, litigations on human and animal pests, and stored food contamination, subjects approached by forensic entomology (2,3).

Sarcosaprophagous entomofauna can be also an alternative source to provide information about toxicological analyses when human tissues, blood, or urine are not available due to the advanced stage of decomposition of a corpse (4). Entomotoxicology aim at detecting toxic substances in larvae tissue and investigating the effect and interference caused by these drugs in the development of insects with the purpose to estimate a postmortem interval more accurately (5,6).

Detection of chemical substances in necrophagous insects is possible because immature specimens feed on contaminated human tissues introducing into their metabolism drugs and toxins that can be accumulated allowing their identification (6). The techniques performed to insect specimens are basically the same used to human tissues and biologic fluids: gas chromatography/mass spectrometry, liquid chromatography/mass spectrometry, thin-layer chromatography, radioimmunoassay, and immunohistochemistry (IHC) (7–28).

Immunohistochemical technique is a promising alternative approach to drug detection in insect, because it is more specific than the traditional assays, and a great advantage of this methodology is the information concerning the pattern of topographic distribution of drugs in larval tissues, generating pharmacodynamics and pharmacokinetics data (29). Bourel et al. (21), for example, studying morphine metabolism in *Calliphora vomitoria* L. (Diptera: Calliphoridae) larvae demonstrated, by IHC, the accumulation of this drug in the area between endo- and exocuticle. Furthermore, it is known that such substances as morphine can be rapidly and efficiently excreted by larvae, but a low concentration, usually lower than that observed in the food source, can be distributed and sequestered into different parts of the larval body, such as hemolymph, fat bodies, and/or cuticle, remaining incorporated within the larval tissues through the different stages of development (30).

A fact that must be observed in preparation of entomological samples for histological studies is that substantial differences related to morphological and integumental characteristics among different fly species may require a new kind of standardization. The literature on this issue is rare, and there is no specific data for histology of necrophagous dipterans (31–34). Besides, choosing the correct fixative and time of fixation that

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provides maximal preservation of tissue morphology with minimal loss of antigenicity is a constant concern in immunological analysis (35).

Entomotoxicology still needs protocol standardization and validation (36), so information generated by this analysis can be valid in the courtroom. Aiming at standardization of fixation protocols for preservation of dipteran larval tissue structure for further IHC assays, we evaluated different fixatives for immature specimens of *Chrysomya albiceps* (Wiedemann), *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae), and *Sarcophaga* (*Liopygia*) *ruficornis* (Fabricius) (Diptera: Sarcophagidae), flies of forensic importance in Brazil.

## Materials and Methods

Adults of *C. albiceps*, *C. megacephala,* and *S.* (*L.*) *ruficornis* were collected in field (Campinas, São Paulo, Brazil—22º54′21″ S: 47º03′39″W), identified using taxonomic keys (37,38), housed in screened plastic cages in a controlled-temperature room at 12-12 h photoperiod; 25 ± 1°C; 70 ± 10% relative humidity and fed with raw bovine liver, sugar, and water.

Raw ground beef was offered for oviposition/larviposition, and the maggots obtained were removed from this substrate using a thin brush and transferred to nylon-covered plastic vials containing artificial diet (39). These vials were maintained in a growth chamber (model 387, Fanem™, São Paulo, São Paulo, Brazil) with the same conditions as described for adults, until the larvae reached third instar, when they were removed from the diet, washed in water and immersed in the fixative forming two groups: (i) entirely fixed larvae or (ii) larvae cut transversely in the sixth body segment before the fixation process to verify whether there was difference in penetration of the fixatives in the larval tissues. After being fixed, entire larvae were also cut transversely in the sixth body segment before embedding in paraffin. Considering all the tested conditions in this study, it was used a total of 230 third instar larvae of *C. albiceps, C. megacephala*, or *S.* (*L.*) *ruficornis*.

Phosphate-buffered paraformaldehyde 4% (PP), Carnoy's fluid (CF), Kahle's solution (KS), and ethanol in different concentrations (70%, 80%, 90%, and absolute) were the tested fixatives.

PP was prepared by diluting 8 g of paraformaldehyde (Merck™, Darmstadt, Hessen, Germany) in 20 mL of distilled water at 80°C on a heating magnetic stirrer until the solution got crystal clear when six drops of sodium hydroxide 1 N was added, followed by 50 mL of buffer phosphate 0.2 M, and the volume was completed with distilled water up to 100 mL. To ensure the quality of this fixative, it is important to keep the temperature at 80°C during all the process. CF was made by mixing 30 mL of chloroform, 10 mL of glacial acetic acid, and 60 mL of ethanol (31). For KS preparation, 30 mL of 95% ethanol, 12 mL of formaldehyde, 4 mL of glacial acetic acid, and 60 mL of distilled water were mixed (40).

The experimental intervals of fixation, it means the time samples were kept in the fixative, were 3, 6, 9, 12, 20, 22, 24, 36, and 48 h for PP; 30 min, 1, 2, 3, 4, and 5 h for CF; 18, 20, and 22 h for KS; 48 h and 19 days for 70%, 80%, 90%, and absolute concentrations of ethanol. Tests using two of these fixatives for the same sample were also carried out to simulate a real situation when a crime scene investigator collect the samples in vials containing 70% ethanol, and these larvae are transferred to vials containing one of the chosen fixatives when they get to the lab: 70% ethanol for 2 h then PP for 12 h; 70% ethanol for 2 h then PP for 24 h, and 70% ethanol for 2 h then CF for 3 h.

Postfixation samples were washed in running water for 15 min, dehydrated in an increasing gradual alcoholic concentration series: 70%, 80%, 95%, and three repetitions of 100% ethanol for 30 min each immersion, diaphanized (=cleared) in 100% ethanol/xylene 1:1 for 30 min, and in two repetitions of pure xylene for 15 min each. A test was performed by increasing the time of the two pure xylene repetitions for 30 min each in different vials aiming to reduce the contamination from the previous reagents on the procedure elapse. Then, the samples went through a sequence of xylene/paraffin 1:1 for 30 min and two immersions in paraffin for 60 min each. In this phase, another

TABLE 1—*Synthesis of fixation and postfixation procedures tested, including number of samples used in each step.*

| Step | Chemical Compound | Time of Exposure | Conditions | Number of Samples |
|---|---|---|---|---|
| Fixation | Phosphate-buffered paraformaldehyde 4% | 3, 6, 9, 12, 20, 22, 24, 36, 48 h | Environmental | 5 larvae for each time of exposure |
| | | | Negative pressure | 5 larvae for each time of exposure |
| | Carnoy's fluid | 30 min, 1, 2, 3, 4, 5 h | Environmental | 5 larvae for each time of exposure |
| | | | Negative pressure | 5 larvae for each time of exposure |
| | Kahle's solution | 18, 20, 22 h | Environmental | 5 larvae for each time of exposure |
| | | | Negative pressure | 5 larvae for each time of exposure |
| | Ethanol (70%, 80%, 90%, and absolute) | 48 h, 19 days | Environmental | 5 larvae for each concentration |
| | | | Negative pressure | 5 larvae for each concentration |
| | 70% ethanol/paraformaldehyde | 2 h/12 h, 2 h/24 h | Environmental | 5 larvae for each time of exposure |
| | | | Negative pressure | 5 larvae for each time of exposure |
| | 70% ethanol/Carnoy's fluid | 2 h/3 h | Environmental | 5 larvae |
| | | | Negative pressure | 5 larvae |
| Postfixation | Water | 15 min | Environmental | 230 larvae |
| | Gradual alcoholic concentration series: 70%, 80%, 95%, and 3x absolute | 30 min each immersion | Environmental | 115 larvae |
| | | | Negative pressure | 115 larvae |
| | 100% ethanol/xylene 1:1 | 30 min | Environmental | 115 larvae |
| | | | Negative pressure | 115 larvae |
| | Two repetitions of pure xylene | 15 min each | Environmental | 58 larvae |
| | | | Negative pressure | 57 larvae |
| | | 30 min each | Environmental | 58 larvae |
| | | | Negative pressure | 57 larvae |
| | Xylene/paraffin 1:1 | 30 min | 60°C | 115 larvae |
| | | 45 min | 60°C | 115 larvae |
| | Two repetitions of paraffin | 60 min each | 60°C | 230 larvae |

test was accomplished increasing the time of the tissues in xylene/paraffin 1:1 to 45 min. The embedding process occurred in a stove at approximately 60°C. Paraffin blocks were made with an anterior and a posterior half of each sample, and they were kept in refrigerator overnight. Fixation and postfixation process were performed under normal environmental conditions and also tested under negative pressure (vacuum), using a small desiccator and a vacuum pump (DOA-P104E-AA from Thomas Scientific™, Swedesboro, NJ) (Table 1).

Embedded samples were sectioned transversely at 5 μm, between the fourth and eighth body segment, and each slide contained three sections of the tissue. They were kept in a stove overnight at approximately 30°C. For staining, the slides went through a sequence of two pure xylene repetitions for 5 min each, in a decreasing gradual alcoholic concentration sequence with two 100% ethanol repetitions, one of 95%, 80%, and 70% for 3 min each, distilled water for 15 min, Ehrlich's hematoxylin for approximately 1 min, another repetition of distilled water for 15 min, eosin for approximately 1 min, a pass through 95% and 100% ethanol, two 100% ethanol/pure xylene repetitions for 3 min each, then proceeded to the assembly with cover slip and Canadian balm or a low-viscosity mounting medium.

An immunohistochemical test was performed for the detection of cocaine. PP 4% for 24 h was the fixative of choice, and the fixation process was carried out under environmental conditions. Postfixation samples were washed in water for 15 min, dehydrated in an increasing gradual alcoholic concentration series: 70%, 80%, 95%, and three repetitions of 100% ethanol for 30 min each immersion, diaphanized in 100% ethanol/xylene 1:1 for 30 min and in two repetitions of pure xylene for 30 min each. These steps were performed under negative pressure. Then, the samples went through a sequence of xylene/paraffin 1:1 for 45 min and two immersions in paraffin for 60 min each. The embedding process occurred in a stove at approximately 60°C. Paraffin blocks were made with an anterior and a posterior half of each sample, and they were kept in refrigerator overnight. Prior to the section of the larval tissue, that were cut transversely at 5 μm between the fourth and eighth body segment, slides were prepared with poly-L-lysine. The sections passed through the same process of deparaffinization and dehydration as the ones that were submitted to Ehrlich's hematoxylin staining. Then, the endogenous peroxidase was blocked using 0.3% hydrogen peroxide, washed with phosphate buffered saline (PBS), and incubated with 1% bovine serum albumin (BSA) to avoid nonspecific bindings (41). They were washed in PBS, then 1% PBS-BSA, and incubated with monoclonal benzoylecgonine antibody from mouse (EastCoast Bio, Inc., North Berwick, ME) (dilution 1:100). The secondary antibody used was a peroxidase-conjugated anti-mouse IgG (Sigma Inc., St. Louis, MO) (dilution 1:100) that is already standardized for mammal tissues (41). Antigen-antibody reaction was visualized using a peroxidase substrate solution containing 3,3′-diaminobenzidine and hydrogen peroxide. The slides were counterstained with Harris' hematoxylin.

For immunohistochemical test, two kinds of negative controls were made: one of the three sections of each slide was not incubated in the primary antibody but in saline and, then, in the secondary antibody, and the other control was to perform the immunohistochemical process in tissue from larvae that were reared in a diet that did not contain the drug.

Slides from all tests were observed in a common optic microscope (Motic™, Wetzlar, Hessen, Germany), and the photographs were produced in a digital image system with the support of an image capture software Leica IM50™, using a common optic microscope (Axioplan2, Carl Zeiss™, Oberkochen, Baden-Württemberg, Germany) with a camera (Leica DFC280, Leica Microsystems™, Wetzlar, Hessen, Germany) attached.

## Results

A qualitative pattern to evaluate the results of the histological slides was adopted considering the larval tissue integrity being classified as satisfactory those presenting most of structures preserved, regular those partially preserved and unsatisfactory those which structures presented too fragmented, partially or totally indistinguishable (Table 2).

The best results (Fig. 1*A,B*), for example, with histological sections well defined and all larval tissues with distinctness, were obtained from the samples treated with PP during 12 and 24 h, 70% ethanol during 19 days, CF during 3 h and 70% ethanol/CF. Results considered regular presented the cuticle and some of the muscle structure distinguishable (Fig. 1*C,D*), while the slides considered unsatisfactory, it was possible to visualize only minced fragments (Fig. 1*E,F*). As it wasn't possible to obtain slices from tissues treated with PP during 36 and 48 h, and KS during 18, 20 and 22 h, due to the hardness of the samples which made the tissues to shatter, they were also considered unsatisfactory.

The process of cutting the larvae in halves before immersing in the fixative showed better conservation of the tissues when compared with the ones entirely fixed. Postfixation in PP and

TABLE 2—*Summary of the obtained results related to the different fixatives and fixation intervals.*

| | | | Results | |
|---|---|---|---|---|
| Fixatives | Time | Satisfactory* | Regular† | Unsatisfactory‡ |
| Phosphate-buffered | 3 h | | X | |
| Paraformaldehyde | 6 h | | X | |
| 4% | 9 h | | X | |
| | 12 h | X | | |
| | 18 h | | X | |
| | 20 h | | X | |
| | 22 h | | X | |
| | 24 h | X | | |
| | 36 h | | | X |
| | 48 h | | | X |
| Carnoy's fluid | 30 min | | X | |
| | 1 h | | X | |
| | 2 h | | | X |
| | 3 h | X | | |
| | 4 h | | | X |
| | 5 h | | | X |
| Kahle's solution | 18 h | | | X |
| | 20 h | | | X |
| | 22 h | | | X |
| 70% ethanol | 48 h | | | X |
| | 19 days | | | |
| 80% ethanol | 48 h | | | X |
| | 19 days | | X | |
| 90% ethanol | 48 h | | | X |
| | 19 days | | X | |
| absolute ethanol | 48 h | | | X |
| | 19 days | X | | X |
| 70% ethanol | 2 h + 3 h | X | | |
| + Carnoy's fluid | | | | |
| 70% ethanol | 2 h + 12 h | | | X |
| + Phosphate-buffered | 2 h + 24 h | | | X |
| Paraformaldehyde 4% | | | | |

*Sections presented tissues and structures preserved.
†Sections presented fragmented tissues and structures.
‡It was not possible to obtain the sections or tissues, and structures were extremely fragmented.

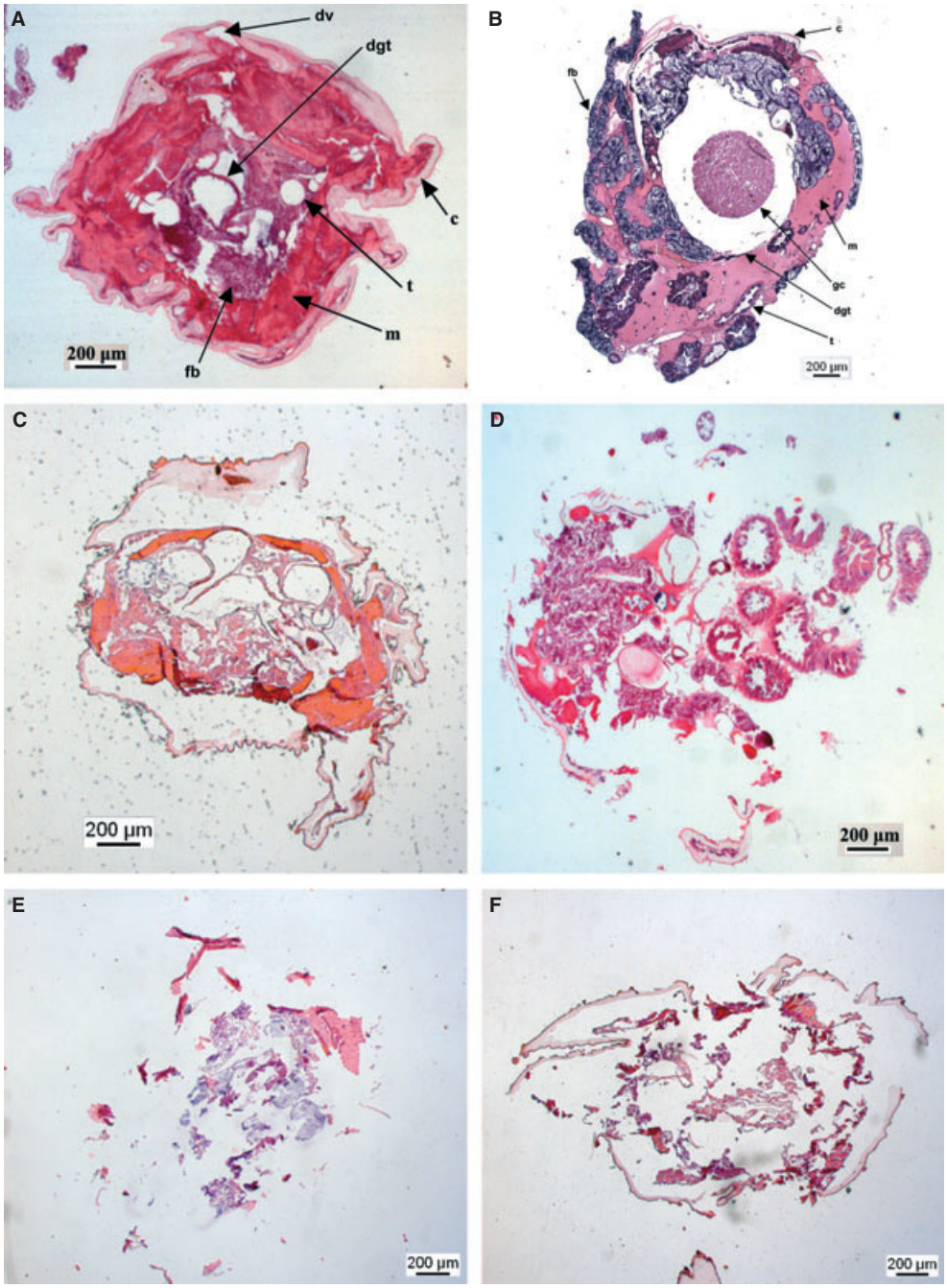


FIG. 1—*Satisfactory (A,B), regular (C,D), and unsatisfactory (E,F) results of histological analyses of dipteran larvae. (A)* Chrysomya megacephala *larvae fixed in phosphate-buffered paraformaldehyde 4% during 24 h; (B)* C. megacephala *larvae fixed in 70% ethanol for 2 h and Carnoy's fluid fixation mixture by 3 h; (C)* Chrysomya albiceps *larvae fixed in 80% ethanol for 19 days; (D)* C. megacephala *larvae fixed in phosphate-buffered paraformaldehyde 4% during 22 h; (E)* C. albiceps *larvae fixed in Carnoy's fluid fixation mixture during 5 h; (F)* C. albiceps *larvae fixed in 80% ethanol for 48 h (C). dv, dorsal vessel; dgt, digestive tube; c, larval cuticle; t, tracheole; m, muscular fibers; fb, fat bodies; gc, gut content.*

dehydrating process performed under negative pressure (vacuum) had better results than under environmental condition. On the other hand, fixation under negative pressure did not improve the process. Cuticle area was more preserved when the samples were immersed in pure xylene during 30 min each repetition and in xylene/paraffin 1:1 for 45 min, than when immersed in pure xylene for 15 min and in xylene/paraffin for 30 min.

The complete methodology of fixation and postfixation of the more suitable procedures with the best results of tissue integrity preservation and, consequently, more appropriate to IHC technique, is recommended in Table 3.

Immunostain in larvae used on preliminary test with cocaine demonstrated accumulation of the metabolite benzoylecognine in different tissues (Fig. 2).

## Discussion

To IHC, it is important to have larval tissues and structures preserved because, as confirmed in our preliminary test

TABLE 3—*Fixation and postfixation protocol recommended to larval samples aiming immunohistochemical analysis.*

| Step | Procedure | Observations |
|---|---|---|
| 1. Fixation | Immersion of larval sample in fixative liquid (choose one of the following fixatives):<br>i) Phosphate-buffered paraformaldehyde 4% for 12 h;<br>ii) Phosphate-buffered paraformaldehyde 4% for 24 h;<br>iii) Carnoy's fluid for 3 h;<br>iv) 70% ethanol for 19 days;<br>v) 70% ethanol for 2 h + Carnoy's fluid for 3 h. | Larvae must be divided into halves before immersion in the fixative liquid.<br>Do it under environmental conditions. |
| 2. Postfixation | a. wash samples in running water for 15 min;<br>b. dehydrate samples in a gradual alcoholic concentration series (30 min each immersion): 70%, 80%, 95%, and three repetitions in 100%;<br>c. diaphanize for 45 min in 100% ethanol plus xylene (1:1) and for 30 min each in two repetitions of pure xylene;<br>d. begin embedding process with sample immersion in xylene plus paraffin (1:1) for 45 min and two sample immersions in paraffin (60 min each);<br>e. make paraffin blocks with two halves of each sample. | Do it under negative pressure.<br>Repetitions of pure xylene must be in different vials.<br>Diaphanization must be performed under negative pressure.<br>The embedding process must be carried out in a stove at approximately 60ºC.<br>Keep paraffin blocks in refrigerator overnight before making slices. |

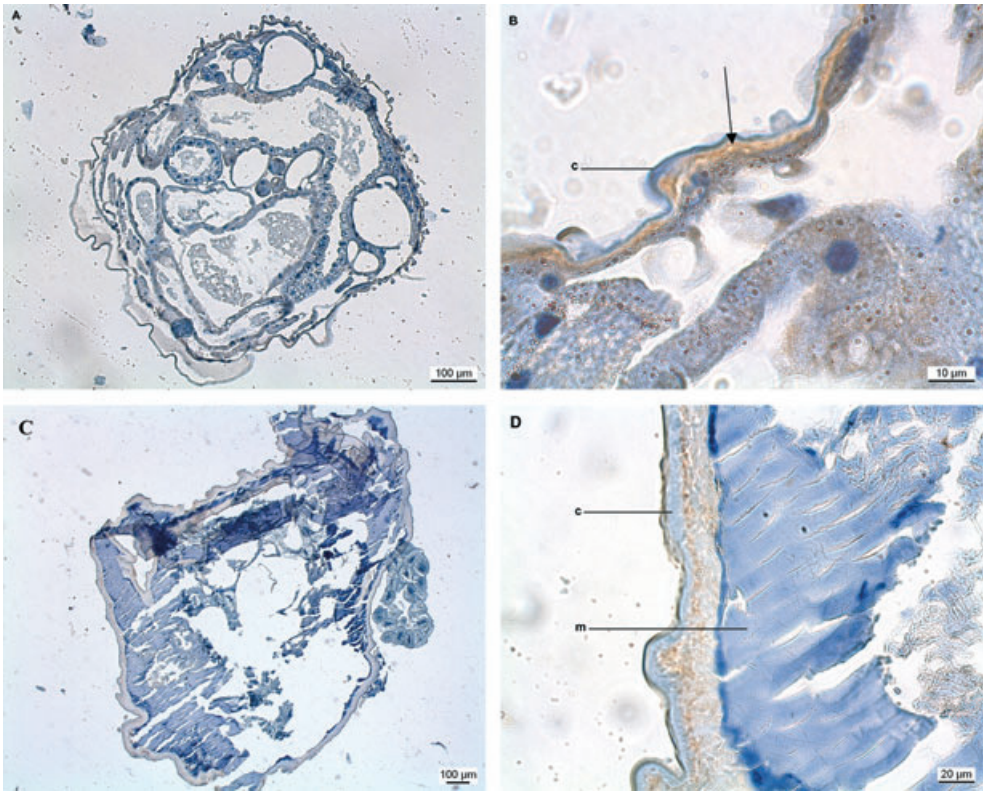


FIG. 2—*Immunohistochemical staining in dipteran larvae tissue for detection of cocaine using monoclonal benzoylecgonine antibody from mouse (dilution 1:100) and a peroxidase-conjugated anti-mouse IgG (dilution 1:100), visualized using a peroxidase substrate solution containing 3,3'-diaminobenzidine and hydrogen peroxide, and counterstained with Harris' hematoxylin. A,B: section of the larva reared in diet containing cocaine (positive samples); C,D: section of the larva reared in diet without cocaine (negative control). arrow = positive staining; c, larval cuticle; m, muscular fibers.*

with cocaine and also by Bourel et al. (21), toxic substances can be accumulated in different larval tissues, such as the cuticle and the muscular fibers, and to find out the accumulation area of a drug all the tissues must be available to analysis.

Despite of the fact that PP, CF, and KS contain paraformaldehyde in their chemical components, these fixatives presented different results. This probably occurred due to the form (solid or liquid) and concentration of paraformaldehyde and also to the preparation procedure of each fixative, which can include a buffer for example.

We suppose that the samples fixed during 36 and 48 h in PP were unfeasible due to the long time of exposition of the samples to the fixative, compared with the period of 12 and 24 h, whose sections proved these to be the periods sufficient and more suitable for a good tissue fixation. To evaluate and try to optimize the use of this fixative, other intervals of time were tested, between 3 and 9 h, but none of them presented the larval cuticle entirely preserved maybe due to the limited time to occur chemical interactions needed to the desirable results of fixation.

CF and KS mixtures are well-known fixatives used for fixation and preservation of insects (31–40), for this reason, they

were tested aiming tissue integrity needed to IHC. In KS's test, it was impossible to make sections of the tissues due to the strong action of this fixative, maybe because of the solvent amount, in the tested periods, so the procedures did not follow-up. Although CF has showed satisfactory results in 3 h, larvae tested in other times were not properly preserved possibly for the solvent issue as mentioned for KS.

The ethanol fixation experiments were accomplished thinking about the work routine of Medico-Legal Institutes (MLI), and crime scene investigators that are responsible for collect samples for laboratory analysis. Despite results with ethanol fixation were satisfactory only in longer periods of exposition, this fixative is a feasible alternative for being commonly available in laboratories, presents no risk of losing samples if they stay for a long time in it, and it is less toxic to environment than the other tested fixatives. Furthermore, the association between 70% ethanol and CF improved the needed time that samples may stay in the fixative representing an extrapolation to practical situations, in which the time in each of the fixatives would correspond to the time elapse between the transportation from the crime scene to the laboratory where they would be prepared for IHC analyses.

To cut larvae in halves improved the fixation process because it allowed a better penetration of the fixatives in the internal structures, keeping tissue integrity during the confection of histological slices. According to the results, it seems possible to collect samples from the crime scene or MLI in vials containing 70% ethanol and divide larvae in halves when the material arrives in the laboratory for analysis.

During postfixation process, to increase time that the samples stay in xylene and xylene-paraffin improved the quality of tissue fixation probably because of larvae tissue constitution that requires a longer time in these substances for diaphanization and solvent action to facilitate paraffin penetration. Besides, the negative pressure created by the vacuum pump potentiated the xylene action aiding to remove water and ethanol used in the former steps, allowing the paraffin to completely penetrate in the tissue during embedding step. These procedures favored histological slices promoting better preservation and visualization of larvae ultrastructure.

Samples of larvae reared in diet containing cocaine were fixed with PP for 24 h, and immunohistochemical test was performed. The results were extrapolated to the other fixatives that showed satisfactory fixation process considering the similarity of tissue integrity obtained, the drug availability and the antibody cost. Immunohistochemical technique enabled us to confirm antigenicity preservation what corroborates that the use of PP, CF, ethanol, or ethanol associated to CF as fixatives of choice do not reduce sample antigenicity significantly. The use of a suitable fixative capable to keep tissue integrity without compromise sample antigenicity is one of the crucial factors for the application of immunohistochemical methodology (42).

Once studies that focus on entomotoxicological analysis by IHC are rare and do not mention methodological specificities of samples fixation and postfixation process, our study aimed to contribute in this area by developing a suitable standardization to insect tissues, generating a valid and practical protocol.

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