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ORIGINAL PAPER

New spot-on formulation containing chlorpyrifos for controlling horn flies on cattle: laboratory model of insecticide release and field trial

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Abstract A highly viscous formulation containing chlorpyrifos (RET) was evaluated under laboratory, pre-field, and field conditions, and compared against ear tags with organophosphorus insecticides. Laboratory bioassays were performed using *Musca domestica* L. and a thin layer chromatography (TLC) plate of reversed phase silica gel modeling a lipophilic surface. Insects were exposed to the insecticide vapors of both formulations, directly and indirectly to test for lateral diffusion. Knockdown time 50% (KT_{50}) values were determined as toxicological indicators of insecticide release. Minimum KT_{50} values of the direct effect of both formulations on horn flies were reached 4 weeks after being applied. The KT_{50} effect of migrated insecticides showed that RET formulation had a maximal effectiveness between the fourth and tenth last week. The KT_{50} effect of the insecticide migrating from ear tags decreased during the last 2 weeks of the experiment, and the KT_{50} effect of the laterally migrated insecticide was significantly higher for the RET formulation during this period. A pre-field bioassay was performed by exposing pieces of rabbit leather with both formulations and recording the KT_{50}. At the end of the experiment, the KT_{50} effect of laterally migrated insecticide was significantly higher for the RET formulation. Regarding vapor emission, as a general trend the KT_{50} effect of ear tags was greater than for the RET formulation. To evaluate the horn fly infestation in the field bioassays, photographs of the animal were taken. The results shows that RET provided significant control for 11 weeks while the ear tags provided protection until the 12th week.

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Introduction

External cattle parasites include several species of insects, mites, and ticks. Most of these feed on blood, although some feed only on the skin. External parasites infest cattle of all ages, but the associated economic loss is usually measured in terms of decrease in weight gain in growing animals (DeRouen et al. 1995).

The horn fly (*Haematobia irritans* L.) is one of the most important external parasites of beef and milk cattle. Heavy infestations of this blood-sucking insect irritate and stress animals, reducing both beef and milk production (Kunz et al. 1984; Schreiber et al. 1987). A calf infested with more than 200 horn flies gains 15-20 pounds less than uninfested animals over a period of 4-6 months. Likewise, horn flies can reduce the production of milk in dairy cows by up to 20%.

The most important tools for the chemical control of horn flies include insecticide-impregnated ear tags and formulations for pour-on or spot-on treatments. Insecticide-impregnated ear tags have been the most popular alternative used against horn fly infestations due to their excellent residual control (Campbell and Thomas 1992).

A spot-on formulation is a solution of active ingredient (s) that typically contains a co-solvent and a spreading agent. The principal active ingredients in spot-on products for fleas, GI parasites, and heartworm control in dogs and cats include fipronil, imidacloprid, selamectin, pyriproxyfen, ivermectin, and moxidectin. Spot-on formulations are also available for controlling lice on cattle. The physicochemical properties of the active ingredient are important determinants of topical or transdermal behavior. To a great extent, the topical activity against ectoparasites depends on the active ingredient spreading, mixing with the sebum on the skin and hair, and forming depots in pilosebaceous units. The mechanism of percutaneous drug absorption varies between species and is not completely understood. However, low molecular weight and a high lipid/water partition coefficient tend to favor the drug penetrating the skin (Kahn and Scott 2005).

During the last two decades, a greater resistance to pyrethroids, the main form of control against this parasite, has been reported (Orayzún et al. 2008). The widespread nature of horn fly resistance to pyrethroids has increased with the development of ear tags that provide long-term control (until 20 weeks), and their use for several consecutive seasons has resulted in high insecticide pressure (Campbell et al. 2006). In this context, ear tags containing organophosphorus insecticides were developed and despite certain problems of use and cost, they remain an important option for producers to control pyrethroid-resistant populations (Byford et al. 1988; Anziani et al. 1998; Anziani et al. 2000). In addition to the relative high cost of the eartags with respect to other insecticide formulations, there are common problems in their use such as the stress of the animals during the application and the frequent lost of them after placed.

Despite their cost, cattle ear tags are used intensively whereas high viscosity formulations for localized spot-on treatments are seldom applied for controlling cattle ectoparasites. The main veterinary use for this type of formulation is restricted to small animals (Kahn and Scott 2005).

The object of the present study was to evaluate the insecticide release of a new experimental spot-on polymeric matrix formulation containing chlorpyrifos developed in Argentina, under laboratory and pre-field models. Furthermore, we evaluated the field performance of a spot-on localized application of the formulation on cattle withers in similar amounts to ear tags against horn fly. Evaluations were performed comparing the experimental formulation to ear tags containing organophosphorus insecticides.

Materials and methods

Formulations

The experimental spot-on formulation was prepared by Chemotecnica S.A., Buenos Aires, Argentina. It contained ethylene vinyl acetate copolymer, turpentine, methyl esters of soybean oil and 20% chlorpyrifos (hereinafter, RET).

Ear tags contained 40% diazinon of a total weight of 16.5 g per tag (Over®) and were obtained from Organización Veterinaria Regional SRL, Santa Fe, Argentina.

Insects

Three-day-old *Musca domestica* L. males extracted from a susceptible colony reared at Centro de Investigaciones de Plagas e Insecticidas (CIPEIN) were used in all the bioassays. Emerging flies were sexed by examining the ventral surface of the abdomen and immediately removed by suction using a glass tube with a piece of gauze on one end attached to a plastic tube extension. The insects were kept in a cage made with metal mesh walls and provided ad libitum access to water and food (sugar, milk powder, and dry yeast).

H. irritans were collected from untreated cows in the same paddock where the field trial was carried out, located in Vicente Casares, Buenos Aires, Argentina. The collection method was adapted from Steelman et al. (2003). Individual cows were restrained in a working chute and approximately 100 adult horn flies were collected from each cow by moving an aerial net along their backs from withers to tail. Five cows were sampled in each bioassay. Collected flies from each group of cows were mixed in a cage made with metal mesh walls and immediately transported to the CIPEIN, Villa Martelli, Buenos Aires, Argentina. They were kept unfed at 25°C and not less than 80% HR for at least 12 h before conducting any bioassays. Only flying horn flies were aspirated from the cages for the bioassay tests.

Laboratory bioassay with *M. domestica*

Housefly KT_{50} times for RET formulation and ear tags were evaluated following direct exposure to vapors or laterally diffused insecticide on reversed phase silica gel. For this, a 2 g cylindrical piece of ear tag obtained with a hole puncher, or 2 g of RET formulation delivered with a syringe, were applied on the surface of a 250 μm layer of reversed phase C-18-silica gel 60 on pre-coated 20×20 cm

glass plates (Merck, Darmstadt, Germany). RET formulations were applied on the surface in the middle of the plate, 2 cm from the side. The semi-solid drop of RET did not surpass a diameter of 2 cm during the entire experiment. The pieces of ear tag were clasped onto the silica gel C-18 plates.

To determine the KT_{50} effect of laterally diffused insecticides on house flies, a plastic container (7.5 cm diameter at base, 8 cm diameter at mouth, 6 cm high) was placed over the plates, keeping a distance of 10 cm between the edge of the receptacle and the center of the RET spot or cylindrical piece of ear tag to avoid any superposition between container and sample. Immediately after placing the plastic container over the C18 silica gel plate, ten individuals were introduced through a hole in the side of the plastic container, which was then closed with a cotton wool cap. KT_{50} effect of insecticide vapor was determined in similar plastic containers as those used for lateral diffusion, but for this bioassay the contact of the houseflies with the formulations was avoided by a tulle placed inside the container 0.5 cm above the treated plate. The number of knocked-down flies was registered every 5 min. Flies lying down on the surface that were unable to walk were considered knocked-down. Three independent replicas were performed for each bioassay. Control bioassays were carried out in the same way, but using untreated surfaces.

Treated and control C18 silica gel plates were maintained in an acclimatized chamber at 25°C throughout the entire bioassay and post-treatment period. Determinations of knocked-down insects were carried out on weeks 1, 4, 8, and 10 after each treatment.

Bioassay with *H. irritans* on rabbit hide samples

Knockdown activity on horn flies produced by direct exposure or laterally diffused insecticide was evaluated on rabbit leather samples with RET formulation or ear tags.

A 2 g cylindrical piece of ear tag obtained with a hollow punch or 2 g of RET formulation delivered with a syringe were applied on the surface of 20×20 cm pieces of desiccated but not tanned rabbit hide (Curtiembre Emprocar S.A., Gualeguay, Entre Rios, Argentina). Knockdown activity on horn flies produced by direct exposure or laterally diffused insecticide was evaluated on leather rabbit samples with RET formulation or ear tags. The rabbit skins were only washed and dried while the stretch, but with the hair.

Application of the formulations, determination of KT_{50}, and effect of laterally diffused insecticides on horn flies and controls were performed as described above.

Treated and control rabbit hide samples were exposed outdoors during the entire bioassay, and KT_{50} activity was registered on weeks 1, 2, 3, 4, 5, and 10 after the treatments. Maximum temperature, relative humidity, and rainfall values were recorded weekly.

Field trial

A trial was conducted on three lots of animals, each located in different plots within the Serendip S.A. dairy farm located in Vicente Casares, Buenos Aires Province, Argentina (35º 1′ S, 58º 34′ W). The test began on December 16, 2008. Lots 1 and 2, of approximately 2 and 4 ha, respectively, were separated by a minimum distance of 200 m. Untreated control animals were allocated to lot 1 and animals treated with RET to lot 2. Lot 3, of about 12 ha, was approximately 500 m away from lot 1 and 700 m from lot 2. Ear tag-treated animals were allocated to this plot. Weekly maximum temperature, relative humidity, and rainfall values were recorded throughout the trial.

Thirty-three Holland pregnant heifers of similar weight (around 500 kg) were selected for the field trial and individually marked on their left ear with numbered tags. Animals were sorted to be randomly divided into three lots. The number of cows was 10, 12, and 11 for RET, ear tag, and control groups, respectively. No insecticide treatments had been applied to these cows for at least 1 year before starting the field trial. All heifers were individually marked on their left ear with numbered tags and had access to mineral supplementation and water during the entire study.

RET spot-on formulation was administered on the withers using a syringe at a dose of 16 g per cow. Insecticide tags were attached on the inside middle area of the right ear (one tag per cow).

Horn fly infestation on each heifer was estimated on every cow in each group 1 day after beginning the treatment (week 0), and thereafter on a weekly basis for 11 weeks. The heifers were held for no more than 5 min in a cattle chute until they stood quietly and flies settled on them. Horn fly infestation was estimated by taking a photograph of an 80×40 cm area on the withers from the neck backwards, perpendicular to the cow, and then manually counting the number of flies in the picture. To evaluate the accuracy of this method, we compared some results with the real number of flies counted in a series of pictures covering the entire animal and found that the single photograph method provided estimates of about 81-88% of the actual number of flies.

Statistical analysis

KT_{50} values were calculated with their respective intervals of confidence (IC95%) using an "*ad hoc*" software, Probit 3.0 based on the probit method (Litchfield and Wilcoxon 1949). According to the criterion widely used in previous studies, KT_{50} values were considered significantly different

Table 1 KT_{50} determined in house flies (*M. domestica*) exposed to insecticide vapors or laterally diffused insecticide from ear tags and RET formulation on reversed phase TLC plates

Weeks after treatment	KT_{50} (minutes)			
	Vapor emission		Lateral diffusion	
	RET	Ear tag	RET	Ear tag
1	195.4 (185.7-204.1)	116.9 (95.1-130,1)	167.2 (157.2-175.7)	99.5 (91.7-105.3)
4	112.7 (102.0-119.9)	80.4 (70.3-85.5)	123.5 (112.5-130.8)	68.5 (58.1-75.0)
8	161.2 (150.4-167.6)	237.8 (224.7-247.6)	151.4 (140.9-156.4)	167.9 (158.0-173.3)
10	122.8 (111.7-129.2)	230.9 (212.8-244.4)	132.9 (121.7-139.2)	174.5 (162.5-182.8)

whenever the IC95 did not overlap ($P<0.05$; Toth and Sparks 1988).

All horn fly infestation data determined in the field trial were subjected to one-way analysis of variance (ANOVA; Brown et al. 1992). Differences between treatment means were assessed by the Unequal N Tukey HSD test ($P<0.05$) for multiple comparisons. Control was considered adequate when horn fly counts remained below 50% control.

Results

A thin layer C18 silica gel was used as a laboratory lipophilic surface to model the lateral diffusion of insecticides released by RET formulation and ear tags on cattle skin. Using *M. domestica* as a biosensor, we recorded the KT_{50} effect after exposure over the spot, but not in direct contact with the formulation, to evaluate vapor emission, and beside the spot to assess insecticide migration on the TLC surface. Houseflies were not used as a surrogate of horn fly to evaluate insecticide toxicity but as a standardized insect to compare insecticide vapor emission and migration by using KT_{50} as an indicator of toxic concentration in a laboratory model.

Table 1 shows the toxic profile of the effect of vapor emissions from RET formulation and ear tags as a function of post-application time. As can be seen, the insecticide vapors of both formulations reach their maximum toxic effect 4 weeks after application. Despite the variability of the KT_{50} effect at different post-treatment times, the results of weeks 4 and 10 suggest that the decay of the insecticide vapor effect of ear tags is more pronounced than for RET formulation.

Table 1 also shows the KT_{50} effect on houseflies of the laterally migrated insecticide. As can be seen, the KT_{50} variation profile was different for both formulations. The KT_{50} of laterally migrated RET formulation did not show a clear tendency of variation with post-treatment time. The KT_{50} effect shows show fluctuating results with maximal effectiveness on weeks 4 and 10 (last week) of the experiment. On the contrary, the KT_{50} effect for ear tags decreased during the last 2 weeks of the experiment (weeks 8 and 10). Moreover, in these last 2 weeks, the faster knock down effect of RET than of the ear tags indicated a higher residual efficacy of laterally migrated insecticide for the RET formulation than for ear tags (Table 1).

Table 2 KT_{50} determined in horn flies (*H. irritans*) exposed to insecticide vapors or laterally diffused insecticide from ear tags and RET formulations on rabbit leather

Weeks after treatment	KT_{50} (minutes)			
	Vapors		Lateral diffusion	
	RET	Ear tag	RET	Ear tag
1	80.4 (74.2-84.3)	60.9 (55.3-64.6)	208.4 (198.3-213.8)	85.2 (74.4-94.0)
2	24.3 (17.7-28.1)	43.3 (39.9-46.9)	144.8 (131.8-154.9)	NCR
3	21.3 (17.8-24.8)	13.4 (10.4-16.4)	118.8 (107.3-140.7)	55.9 (49.0-61.6)
4	28.9 (22.7-34.1)	20.3 (17.7-24.1)	119.3 (99.9-153.8)	85.2 (60.1-99.9)
5	43.9 (39.9-47.4)	20.8 (15.5-23.4)	45.0 (22.5-62.8)	45.7 (30.6-57.6)
10	55.2 (48.8-60.8)	23.4 (20.3-26.6)	94.3 (88.4-99.2)	139.1 (114.2-206.1)

NCR non-consistent results between five replicates

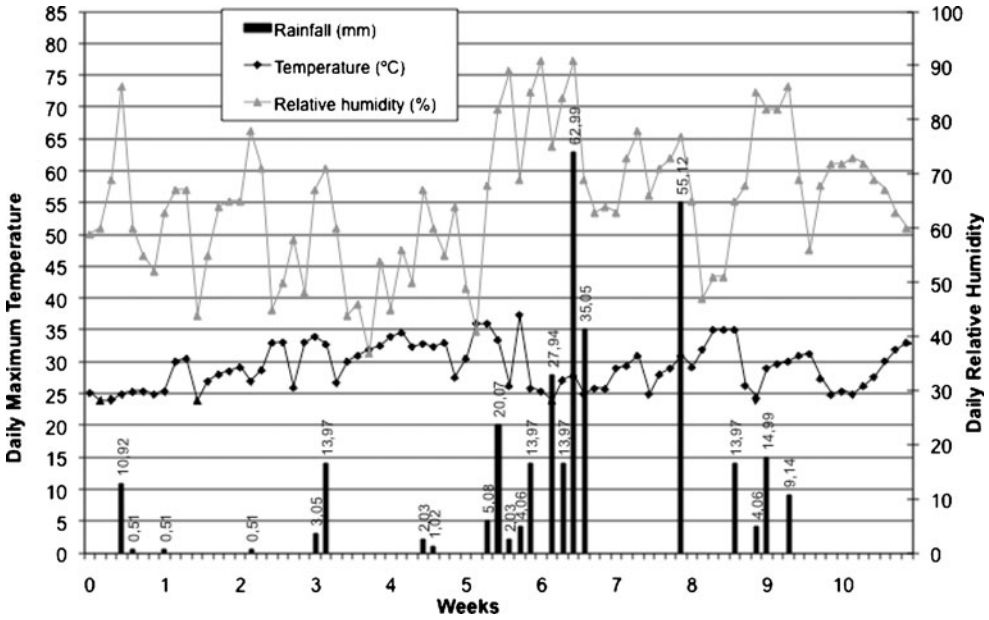

Fig. 1 Weather conditions during the insecticide activity experiment for RET and ear tag formulations applied on rabbit leather

A 20×20 cm of piece of rabbit hide was used to evaluate the process of insecticide release on a biological material following a localized application of insecticide formulations. Although rabbit leather is not a surrogate for cattle skin could be considered as a simplified model with some similarity with cattle skin in its general structure and chemical composition. In fact, both materials have an epidermis and dermis, the two settled on a layer of subcutaneous fat and with a porous matrix constituted by collagen, an amphoteric fibrous protein, along with some noncollagenous matter such as hair, epidermis, and flesh layers, soluble proteins, fats, etc (Panduranga Rao et al. 1995; Table 2).

KT_{50} values for horn flies were obtained as a function of post-treatment time. Flies were exposed to the treated leather samples and knockdown times were registered for insects placed directly on the surface and insects placed on the side to assess lateral insecticide migration. Rabbit leather samples were left outdoors, exposed to weather conditions throughout the experiment. Weekly maximum temperature, relative humidity, and rainfall values registered during the assay are shown in Fig. 1.

After the formulations were applied, the insecticide spread out on the rabbit leather producing finite values of KT_{50} with maximal knockdown effect in week 5. At the end of the experiment (10 weeks), KT_{50} of laterally migrated insecticide was significantly smaller for the RET formulation compared to the ear tag.

Regarding vapor emission, as a general trend, ear tags showed a significantly greater KT_{50} effect than the RET

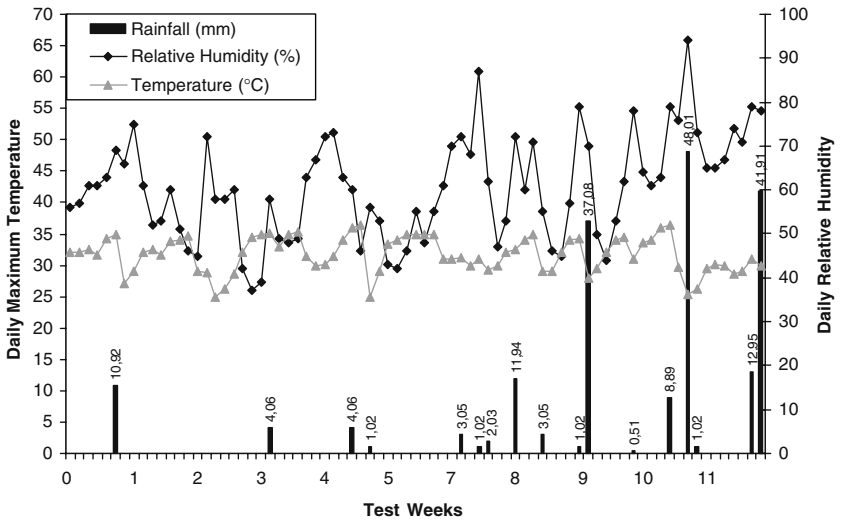

Fig. 2 Weather conditions during the field trial

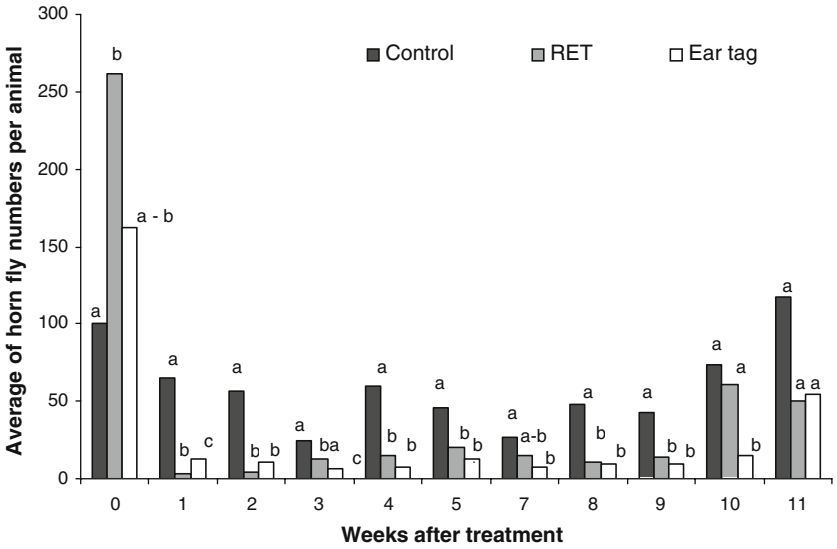

Fig. 3 Arithmetic mean of the percentage reduction of *H. irritans* in cows treated with ear tags, RET formulation or untreated controls. Bars with different letters within the same group are significantly different ($p<0.05$)

formulation, particularly in the last two recorded weeks of the experiment (weeks 5 and 10).

Horn fly control by ear tags and the novel RET formulation applied on cows with a syringe was evaluated under field conditions. Figure 2 shows the weather conditions during the field trial. Figure 3 shows the arithmetic mean of the number of *H. irritans* in cows treated with diazinon-impregnated ear tags, RET formulation with chlorpyrifos and controls, and their corresponding statistical differences. The results of the ANOVA show that both the ear tags and RET formulation were effective from the first week of treatment. The lack of a significant control caused for RET formulation during week 3 could be due to the low level of infestation observed in the control group, probably caused by the dry weather conditions during the first weeks of the field trial (Fig. 2). The effect of the RET formulation was significant ($p<0.05$) until week 9. On the other hand, ear tags remained effective until week 10 compared to the control group ($p<0.05$). Although both treatments provided house fly control until the ninth week of the experiment, significant differences between both treatments were observed in weeks 1, 3, and 7 ($p<0.05$). RET formulation showed more effective control than ear tags during the first week, while ear tags proved to better in weeks 3 and 7.

Discussion

The way insecticides are delivered from ear tags or spot-on formulations to produce fly control in cattle are almost unknown. It is currently accepted that the impregnated insecticide in ear tags is slowly released on the animal's coat. The natural movement of the animal and its ear could bring about this migration.

In 1983, Miller et al. characterized the pattern of release for fenvalerate, permethrin, and deltamethrin ear tags on cattle (Miller et al. 1983). They assumed that the release of insecticides is solely determined by the rate of diffusion of active principles to the surface of the tag, and that this surface is maintained at zero concentration by the subsequent transference to the animal surface. The study indicated that the release of pyrethroids from ear tags follow Fick's laws of diffusion. Working with dye-impregnated pads attached to ear tags, Beadles et al. (1977) demonstrated that self-grooming and movements for fending off horn flies produce a visible transfer of dye to limited areas of the body surface of cows such as the neck, shoulder, and flanks.

Spatial and temporal variations of pyrethroid residues on different areas of the cattle following ear tag treatment were attributed to insecticide release, spreading rate and degradation on hair coat (Taylor et al. 1986; Yeung et al. 1989; Mwangala et al. 1993). According to these studies, the head and neck were the areas with the highest levels of pyrethroid residues and 10-12 weeks were the longest post-treatment times with residue detection. According to Wright et al. (2006), ear tag insecticide is transferred onto the back and flanks by a natural grooming behavior as a result of its fat solubility that allows it to migrate through the lanolin in the hair.

Spot-on formulations contain a high concentration of active principle and its release can either penetrate the skin and act systemically or spread over the skin surface and act by contact. Topical activity against ectoparasites depends to some extent on the active ingredient spreading, mixing with the sebum coating the skin and hair, and forming deposits in the pilosebaceous units (Kahn and Scott 2005).

Although numerous studies have dealt with the efficacy of insecticidal ear tags, none have considered the use of

laboratory models to evaluate the lateral migration of active principles.

We used lipophylic surfaces to observe passive diffusion and vapors emissions of organophosphorus insecticides released from the polymeric matrix of ear tag or RET formulation. The objective of bioassay tests on lipophylic surfaces was to perform a comparison between insecticide released by both formulations in equivalent amounts recommended by the fabricants. Because the amount recommended of RET per animal for horn fly control is equivalent to the ear tag (RET: 16 g/cow; ear tag, one ear tag of 16 g per cow) the comparison was performed between the same weights of both formulations.

A simple lipophylic surface of reversed thin layer chromatography plates of C18-silica gel was the first model to observe organophosphorus insecticides released from the polymeric matrix of ear tag or RET formulation.

Our results of the KT_{50} effect of organophosphorus insecticides against houseflies in laboratory conditions showed that RET formulation and ear tags deliver active principles as vapor emission and by lateral diffusion on the reversed phase C-18-silica gel.

As a general trend, we found that housefly KT_{50} values of insecticide release from RET and ear tag formulations on C18-silicagel suggest that the RET formulation emits a more constant rate of insecticide in vapor emission and surface migration. Insecticide release from ear tags had an initial higher rate of vapor emission and surface diffusion with respect to RET formulation, as suggested by lower KT_{50} values registered in weeks 1 and 4, but in both cases effectiveness decayed in the last 2 weeks of the experiment (weeks 8 and 10).

The KT_{50} effect on horn flies exposed to laterally diffused insecticide from RET formulation or ear tags applied on rabbit leather under outdoor conditions suggest that a rapid and efficient migration was sustained throughout the duration of the experiment (10 weeks). The KT_{50} produced by the active principles released from both formulations showed a different pattern of activity during the post-treatment period. In the case of ear tags, the effect of released diazinon did not show any tendency during the first 5 weeks, with variability attributable to the complex process of lateral diffusion and fluctuating outdoor conditions. The last determination of KT_{50} in week 10 showed a decreased efficacy for migrated insecticides that can be attributed to a decrease in the ear tag content of active principles. The KT_{50} effect of chlorpyrifos released from RET formulation as function of post-treatment time suggests a growing efficiency during the first 5 weeks which then decreases in the tenth week, probably due to a reduction in insecticide concentration.

As a general trend, the insecticide activity of RET formulation against the housefly and horn fly in the laboratory and scaled-up model systems shows a good performance for chlorpyrifos in its delivery profile, with slightly better residual results than observed for diazinon release. Therefore, a field trial was carried out to compare the actual real efficacy in horn fly control.

Different durations of efficacy of organophosphorus ear tags have been reported in the field control of horn flies, with values ranging between 6 and 7 weeks (Lysyk and Colwell 1996), 8-11 weeks (Campbell et al. 2006), 15-20 weeks (Crosby et al. 1991), and 19-20 weeks (Cocke et al. 1990; Spradbery and Tozer 1996). Statistical analysis of our field trial results showed a decay in the control performance of ear tags during week 11, while the RET formulation showed a residual activity until week 10.

Taking into account the residual activity of the RET formulation controlling *H. irritans* in field conditions and its simple and safe application method on cows, this novel chlorpyrifos formulation could be considered a useful alternative for controlling ectoparasites in cattle.

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