PENETRATION AND METABOLISM OF SPINOSYN A IN LEPIDOPTEROUS LARVAE

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Abstract

Spinosyn A, the principal component of Tracer® (spinosad) possesses many highly desirable characteristics for an insect control agent including pyrethroid levels of activity and a very favorable mammalian and environmental profile. However, the initial action of spinosyn A is somewhat slower than some pyrethroid insecticides such as cypermethrin. Understanding spinosyn A penetration and metabolism may provide approaches to further improving spinosad and the spinosyns as insect control agents. Studies comparing the injection versus topical toxicity of spinosyn A and cypermethrin in *Heliothis virescens* (tobacco budworm) larvae show that spinosyn A is as active as cypermethrin by injection, but about 5-fold less active than cypermethrin when applied topically. This apparent difference in the rate of penetration is confirmed by *in vivo* studies in last stadium *Trichoplusia ni* (cabbage looper) larvae examining the penetration of spinosyn A versus permethrin; at 4 hr posttreatment >30% of applied permethrin was internal while <10% of the applied spinosyn A was internal. Likewise, for topically applied spinosyns A, B and D, only 1.5 - 4% of the applied dose was present in the hemolymph 3 hr posttreatment. As with *T. ni*, radiotracer studies with *H. virescens* larvae showed that spinosyn A penetrates at a slower rate (2% in 3 hr) than does cypermethrin (42% in 3 hr). Studies of spinosyn A metabolism in *H. virescens* midguts and induced rat liver homogenates suggest that spinosyn A is highly stable to oxidative metabolism. In vivo metabolism of the acaricide, fenazaquin, and spinosyn A showed fenazaquin to be readily metabolized while there was no detectable metabolism of spinosyn A. Likewise, the co-application of piperonyl butoxide with spinosyn A did not alter activity in adult house flies while a six-fold increase in toxicity was noted with piperonyl butoxide + permethrin. Thus, available information suggests that while spinosyn A is initially slower to penetrate into lepidopterous larvae, once it is internalized it is relatively stable to metabolism thereby contributing to it's high level of activity.

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Introduction

Insect resistance management (IRM) is central to cotton insect control, especially for the tobacco budworm (Sparks 1980, Sparks et al. 1993). Fundamental to IRM is the availability of safe and effective insect control agents. The spinosyns are a novel class of microbially derived macrolides that possess insecticidal activity (Kirst et al. 1991, Sparks et al. 1995, 1996, Thompson et al. 1995). Tracer® (common name, spinosad, Fig. 1) is a naturally occurring mixture of spinosyn A (about 85%) and spinosyn D (about 15%). Tracer® represents a whole new genre of naturally derived materials that not only possess pyrethroid levels of activity against a variety of cotton insect pests, including the tobacco budworm (Table 1) , but also exhibits exceptionally favorable environmental and mammalian toxicity profiles (Sparks et al. 1995, Thompson et al. 1995, Table 1).

Topical bioassays of spinosyn A (the principal component of Tracer®) produced a LD_{50} value comparable to a variety of pyrethroids and superior to a wide range of organophosphorus, carbamate and other insect control agents (Table 1, Sparks et al. 1995). However, efficacy against the target organism is not the only measure by which a molecule is judged; environmental and mammalian safety also help define the utility of a molecule. Although possessing some limitations (Hollingworth 1976), including dependence on pest species and application method, the vertebrate selectivity ratio (VSR; acute rat oral LD_{50} mg/kg / insect LD_{50} µg/g, Hollingworth 1976), a type of therapeutic index, provides one measure of the relative selectivity of an insecticide for the target insect species (in a particular cropping system) versus non-target mammals. Certainly, many of the older cotton insecticides such as endrin and methyl parathion are comparatively toxic to mammals (VSR = < 1.0), especially when contrasted against some members of the pyrethroids which can have VSR's in excess of 100 (Table I). However, it is important to note that while there are general trends, there can also be a great deal of variation within a class as is observed among the organophosphorus and pyrethroid insecticides. The VSR of spinosyn A (2627-3906), as derived in this particular instance, is among the most favorable known for any cotton insect control agent to date (Table I). Thus, the already excellent mammalian safety profile of spinosad is further enhanced by its high level of efficacy towards the pest target, thereby conferring an outstanding margin of safety for this market.

In general, some pyrethroids such as cypermethrin may possess somewhat better contact activity than spinosyn A (drench, contact and topical bioassays), but spinosyn A is generally more active than these pyrethroids in assays that have an oral component (diet-egg, leaf-dip, leaf spray; Sparks et al. 1995). To better understand the activity of the spinosyns, studies were carried out to investigate the rate at which externally applied spinosyn A can in

penetrate into and be metabolized by selected lepidopterous larvae.

Materials and Methods

Chemicals

All biochemicals were from Sigma. Technical samples of spinosyn A, spinosyn B, spinosyn D and spinosyn J were provided by DowElanco as were the H^3-(81.9 Ci/mmol) and C^{14}-(51.6 mCi/mmol) spinosyn A and H^3 -fenazaquin (60 Ci/mmol). Permethrin and cypermethrin were from Chem Service, and the piperonyl butoxide was from Aldrich Chemical. C^{14}- labeled (2-5 mCi/mmol) spinosyn B and D were provided by Lilly Research Laboratories. C^{14}-permethrin (26.7 mCi/mmol) was a gift from ICI Americas. H^3-Inulin (1.73 Ci/mmol) was from Amersham.

Insects

Larvae of the cabbage looper, *Trichoplusia ni*, were reared on a pinto bean diet (diet #1, Roe et al. 1982), at 27±1 C, 40±10% relative humidity with a photoperiod of 14:10 (L:D). Larvae of the tobacco budworm, *Heliothis virescens*, were reared on a pinto bean diet at 27±1 C, 40±10% relative humidity with a photoperiod of 14:10 (L:D).

Insecticide Bioassays

Third (20-30 mg) or last (fifth, 200-250 mg) instar larvae of the tobacco budworm were treated topically on the thoracic dorsum as described previously (Leonard et al. 1988) with each compound in 1 µl of acetone; Injections into the mid-dorsum of the abdomen (last instar larvae) were made with a sharpened 10 µl Hamilton syringe. Controls in all bioassays received only acetone (1 µl). Larvae were held at 27° +1° for 72 h, and examined for mortality 24, 48 and 72 h post-treatment. The criterion for mortality was the inability of the larvae to move or change position within 30 s after prodding. Fifteen to twenty larvae were treated with each concentration of the insecticide. Five to six concentrations were used to estimate the LD_{50}. The LD_{50} values were estimated by probit analysis (Finney, 1971). The non-overlap of the 95% fiducial limits was used as the criterion of statistical significance.

In Vivo Penetration and Metabolism

Third or last stadium *H. virescnes* or last *stadium T. ni*, larvae (4-5 per time point) were topically treated with radiolabeled material (typically 1µl/larva) in acetone and then held individually in 20 ml glass scintillation vials. At selected posttreatment time intervals each larva removed from the holding vial, rinsed with acetone (2 x 2 ml), and then either homogenized (in 500 µl methanol) or had hemolymph removed (via a clipped anal proleg). The radioactivity present in the holding vial and in the combined rinses (after the solvent was evaporated off) were quantified by liquid scintillation counting (lsc). For the penetration studies, 100 µl of the methanolic fraction was quantified by lsc. For metabolism studies the methanoic homogenate was centrifuged (2000 x g) for 10 min. The supernatant was then removed, the pellet was resuspended (with vortexing) in 500 ul of methanol and then re-centrifuged. The two supernatants were combined and two 100 ul aliquots were analyzed by lsc. The pellets were resuspended in lsc fluid and counted. The remaining volume of each supernatant was reduced under a stream of nitrogen to an approximate volume of 100 ul and analyzed by thin layer chromatorgaphy (tlc) or high pressure liquid chromatography (hplc). For tlc analyses, 25 ul from each sample was spotted on a prelayered Whatman L5KDF tlc plate (5 x 20 cm, 4 channels), and then developed in either toluene-methanol (90:10) or hexane-acetone (60:40). Chromatorgaphic standards spinosyn A, spinosyn B (Sparks et al. 1995), spinosyn J (Sparks et al. 1995) and (for the fenazaquin studies) fenazaquin. The radioactivity present on the tlc plates was analyzed using a tlc scanner. Hplc analyses were carried out on a Waters hplc system using a Sepelco 15 cm, C18 column eluted with methanol - water at 1 ml/min.

The hemolymph volume was determined by injecting a known quantity of H^3-inulin into the hemocoel of the larva, taking samples (25ul) of the hemolymph at selected intervals and then calculating the hemolymph volume based on the radioactivity present in the sample as determined by lsc. Based on this method the hemolymph volume of last stadium, day 3 (L5D3) *T. ni* larvae was approxiamtely 110 µl.

Results

Bioassays

Spinosyn A was more active to last stadium *H. virescnes* larvae by injection than when applied topically (Table 2). In contrast, cypermethrin equally toxic whether applied by injection or topical application (Table 2). Spinosyn A was as toxic to last stadium *H. virescnes* larvae by injection as cypermethrin, but was about 5-fold less active than cypermethrin by topical application (Table 2), an observation consistent with previous data (Table 1, Sparks et al. 1995).

In addition to spinosyn A, other spinosyns were also examined for injection and topical toxicity towards last stadium larvae of the tobacco budworm. Among the spinosyns spinosyn A was the most active by either injection or topical treatment at 24 hr posttreatment, followed closely by spinosyn D. At 24 hr posttreatment, spinosyn B was much less active than spinosyn A, while spinosyn J was virtually relatively inactive at the highest dose tested (Table 2). By 72 hrs. posttreatment, with the exception of spinosyn J, all of the spinosyns possessed very similar LD50 values for either application method. However, the topical activity of the spinosyns was consistantly less (ca 5x) than by injection (Table 2). These observations suggested that the intrensic activity of

spinosyns A, B or D are equal to that of cypermethrin if the compound could get inside of the larva. Thus, the injection data suggests that spinosyn A may be penetrating into *H. virescnes* larvae at a slower rate than other insect control agents such as cypermethrin.

Third instar *H. virescnes* larvae and adult house flies were bioassayed with spinosyn A with and without piperonly butoxide (10 ug/insect). In both cases, the piperonly butoxide had no effect on the LD_{50} values for spinosyn A (Table 3). In contrast, piperonyl butoxide was co-applied with permethrin to adult house flies, the toxicity of permethrin was increased by more than 6-fold (Table 3).

In Vivo Penetration and Metabolism

C^{14}-Labeled permethrin and H^3-spinosyn A applied topically to last stadium larvae of *T. n*i were found to penetrate at very different rates (Fig. 2). At four hrs posttreatment, more than 30% of the radiolabeled material was in the internal faction in the permethrin treated larvae while less than 10% of the applied radioactivity was in the internal fraction for the spinosyn A treated larvae (Fig. 2).

A comparison of C^{14}-labeled spinosyns A, B and D applied topically to last stadium *T. ni* larvae all had relatively low rates (1.5 - 4 %) of penetration when hemolymph levels were evaluated three hrs posttreatment (Fig. 3). These results are thus consistent with the topical versus injection data.

Evaluation of spinosyn A penetration into third instar larvae of *H. virescens* also suggests a low rate of penetration (ca. 2%) three hrs following topical application (Fig. 4). This rate of penetration is much slower than that observed for a variety of insect control agents (Fig. 4) as determined by both in house studies and reported in the literature. Interestingly, another fermentation derived macrolide, abamectin, is also known to penetrate into *H. virescens* larvae rather slowly (Bull 1986; Fig. 4).

H^3-fenazaquin, topically applied to third instar *H. virescens* larvae was readily taken up, and by about nine hrs posttreatment nearly 15% of the radioactivity was in the internal fraction (Fig. 5). Starting at 3 hrs posttreatment and plateauing at 6 to 9 hrs posttreatment, a significant proportion of the internal radioactivity were metabolites other than fenazaquin (Fig. 5). At nine hrs after treatment, only about 6-7% of the internal radioactivity was parent (fenazaquin). Thus, at least half of the fenazaquin that had penetrated had been metabolized. suggesting that a significant amount of metabolism was occurring. In contrast, the topical application of C^{14}-spinosyn A to last stadium *H. virescens* larvae again demonstrated a slow rate of penetration with only slightly more than 3% of the applied radioactivity being present in the internal faction at nine hrs posttreatment (Fig. 6). Interestingly, HPLC analysis of the internal radioactivity at all time points (1, 3, 6, 9 and 24 hrs posttreatment) found all of the radioactivity was associated with one peak that co-eluted with the parent, spinosyn A, as demonstrated by the HPLC chromatogram for the nine-hr time point (Fig. 7). All of the radioactivity in the internal fraction was associated with the parent (spinosyn A) and not either of two potential metabolites spinosyn B or spinosyn J.

Discussion

The biological activity of any insecticide is a function of a) inherent reactivity with the target site and b) concentration at the target site as a function of time. Concentration is influenced by the amount applied, metabolism, penetration and excretion. Although the injection activity of spinosyn A is equal to that of cypermethrin (Table 2), as is the oral activity (Sparks et al. 1995), data on topical versus injection activity suggests that the topical activity of spinosyn A may be limited by a slower rate of penetration than is observed for other cotton insect control agents such as cypermethrin (Table 2). These observations are supported by specific penetration assays showing that the rate of penetration into the hemolymph / internal fraction is far less for spinosyn A than is observed for pyrethroids (permethrin, cypermethrin) and other cotton insect control agents. These observation appears to be true for other spinosyns as well (i.e. spinosyn B and D). Hot-probe bioassays for initial speed of action indicate that topically cypermethrin is somewhat faster acting than topically applied spinosyn A (Sparks et al. 1995). Thus, the relatively slow rate of penetration for spinosyn A into larvae of *H. virescens* may be a significant factor limiting the *initial* speed of action. However, the biological activity of spinosyn A is consistently in the range of pyrethroids such as permethrin and cypermethrin (Sparks et al. 1995, 1996, Tables 1 & 2). Thus, other factors must, in part, compensate for the limited amount of spinosyn A that is initially present in *H. virescens* larvae.

Studies in mammals suggest that spinosyn A is readily metabolized, primarily to spinosyns B and J (DowElanco 1994). An examination of spinosyn A's structure presents several potential sites for metabolism (Fig. 8). Toxicity data indicates that none of a variety of potential metabolites (including spinosyns B and J) is any more active than spinosyn A. Thus, bioactivation of spinosyn A to a more active metabolite is unlikely, and virtually any potential metabolite that can be envisioned for spinosyn A will lead to a less active molecule (Fig. 8).

Although both permethrin and cypermethrin have been shown to be readily metabolized in *H. virescens* larvae (Sparks 1996), as is fenazaquin (Fig. 5), spinosyn A appears to be relatively stable (Figs. 6 & 7). These observations are further supported by lack of spinosyn A metabolism observed in *in vitro* metabolism studies with midgut homogenates from *H. virescens* (Sheets, Yarski and Chang, unpublished data). Injected spinosyn A is also poorly metabolized *in vivo* by *H. virescens* larvae. Finally, the piperonyl butoxide mediated inhibition of

monooxygenases, a major enzyme system involved in insecticide metabolism (Dauterman and Hodgson 1990), had no effect on the activity of spinosyn A to house flies , while the activity of permethrin was improved by about six-fold (Table 3), again suggesting a lack of susceptibility to metabolism for spinosyn A. Thus, the lack of spinosyn A metabolism may well be a significant factor in the excellent biological activity obeserved for spinosyn A against *H. virescens* larvae, in part compensating for the slow rate of penetration.

The lack of apparent metabolism for spinosyn A may not only contribute to the pyrethroid levels of activity for spinosyn A in *H. virescens* larvae, but may also contribute to relative lack of cross-resistance for spinosyn A in a variety of resistant insect species. Fenvalerate, abamectin and teflubenzuron resistant strains of diamondback moth displayed significant levels of cross-resistance to cypermethrin but not to spinosyn A (Sparks et al. 1995). Interestingly, several of these strains possessed enhanced levels of monooxygenases and glutathione tranferases (Dr. Chih-Ning Sun, National Chung Hsing University, personal communication). Likewise, a variety of *H. virescens* field strains that were resistant to several types of insecticides (including profenofos and cypermethrin) were very well controlled by spinosyn A (Sparks et al. 1995). Thus, available information suggests that spinosyn A is not readily metabolized by a variety of enzyme systems that typically play a role in insecticide resistance in larvae of *H. virescens*, thereby making spinosad an excellent tool in resistance and insect pest management programs. However, as with any new tool, it is important that proper resistance management practices be followed to help prevent the development of resistance to spinosad or any other cotton insect control agent.

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Table 1. Acute Insect (*Heliothis virescens*, topical, 48-72 hr, µg/g) and Mammalian (rat oral, acute, mg/kg; rabbit dermal, mg/kg) Toxicity of Insect Control Agents Tested on or Used for the Control of Cotton Insect Pests

Compound	H.v. LD_{50}	Rat Oral LD_{50}	VSR[b]	Refs
DDT				
DDT	52-152	87	0.9-2.8	3,4,17
Toxaphene-DDT	26.7-85	40*	0.5-1.5*	3,17
Cyclodienes				
Endrin	46.7	3	0.06	3,17
Endosulfan	73.3	18	0.3	3,17
Organophophates				
Me Parathion	11.6-65.0	9	0.1-0.8	7,17
EPN	16.7-33.0	14	0.4-0.8	3,12,17
Azinphosmethyl	29.3-33.3	5	0.2	3,12,17
Chlorpyrifos	79.5	135	1.7	17,18
Monocrotophos	29.7	8	0.3	12,17
Acephate	74.3	866	11.7	13,17
Profenofos	11.0	400	36	7,17
Sulprofos	24.0	107	4.5	12,17
Carbamates				
Carbaryl	136-232	307	1.3-2.2	1,4,17
Methomyl	4.33, 26.7	17	0.6-3.9	7,12,17
Pyrethroids				
Permethrin	1.33-2.79	>4000	>1434-3008	7,12,17
Fenvalerate	0.870-1.89	451	239-1139	7,17
Cypermethrin	0.241-1.61	247	153-1025	7,17
λ-Cyhalothrin	0.929	56	60	7,10,17
Esfenvalerate	0.429	75	174	7,10
Bifenthrin	1.32	55	42	7,10,11
Tralomethrin	0.251	1070-1250	4263-4980	2,6
Avermectins				
Abamectin	1.16	10.6-11.3	9.1-9.7	1,5
Emamectin	0.10	70	700	6,15
Pyrroles				
Chlorfenapyr	4.5	223-459	50-101	8,16
Spinosyns				
Spinosyn A	1.28-1.44	3783-5000	2627-3906	14

a) Approximate year discovered/identified, * indicates year patent filed or compound disclosed.

b) VSR - vertebrate selectivity ratio for Rat oral / Hv toxicity. Not necessarily representative for other insect pests.

Refs: 1- Bull 1986, 2 - Dowd and Sparks 1988, 3 - Graves et al. 1964, 4 - Graves et al. 1967, 5 - Lankas and Gordon 1989, 6 - Larson et al. 1985, 7 - Leonard et al. 1988, 8 - Lovell et al. 1990, 9 - Merck 1995, 10 - Meister 1996, 11 - Neuman 1990, 12 - Polazzo 1978, 13 - Rose and Sparks 1984, 14 - Sparks et al. 1995, 15 - Sparks 1996, 16 - T. C. Sparks, T. V. Worden and M. B. Hertlein 1992-1994 unpublished data, 17 - Ware 1983, 18 - Whitten and Bull 1974.

Table 2. Effect of Application Method on Spinosyn and Cypermethrin Toxicity to Last Stadium Larvae of the Tobacco Budworm.

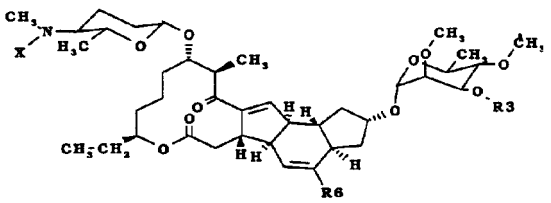
	LD_{50} (µg/larva)			Ratio	Top/Inj
Compound	24 hr	48 hr	72 hr	24 hr	72 hr
Topical					
Spinosyn A	6.13	7.27	4.08	7.30	5.44
Spinosyn B	83.5	22.2	5.81	18.0	4.61
Spinosyn D	11.8	5.87	3.21	6.86	4.65
Spinosyn J	>50	>50	>50	--	--
Cypermethrin	1.06	1.51	0.87	0.82	1.04
Ratio A / Cyp	5.78	4.81	4.69		
Injection					
Spinosyn A	0.84	1.17	0.75		
Spinosyn B	4.69	4.77	1.26		
Spinosyn D	1.72	1.14	0.69		
Spinosyn J	>50	>50	>50		
Cypermethrin	1.29	1.27	0.84		
Ratio A / Cyp	0.65	0.92	0.89		

Table 3. Effect of Piperonyl Butoxide (PB)[1] on Spinosyn A Toxicity

Treatment	LD_{50}	95% Fiducial Limits	Synergist ratio[2]
Adult House Fly (ng/fly)			
Permethrin	9.8	8.1 - 11.9	
Permethrin + PB	1.6	1.3 - 2.1	6.13
Spinosyn A	63.9	46.8 - 87.3	
Spinosyn A + PB	68.8	52.5 - 90.1	0.92
H. virescnes larvae[3] (ug/larva)			
Spinosyn A	2.2	1.8 - 3.1	
Spinosyn A + PB	3.1	2.8 - 3.8	0.73

1- 10 ug/insect 2- Synergist ratio: LD_{50} compound / LD_{50} compound + PB

3- 3rd instar



spinosyn A	**X = Me,**	**R6 = H,**	**R3' = Me**
spinosyn B	**X = H,**	**R6 = H,**	**R3' = Me**
spinosyn D	**X = Me,**	**R6 = Me,**	**R3' = Me**
spinosyn J	**X = Me,**	**R6 = H ,**	**R3' = H**

Figure 1. Structure spinosyns A, B, D and J. Tracer® (common name - spinosad) = 85% spinosyn A, 15% spinosyn D.

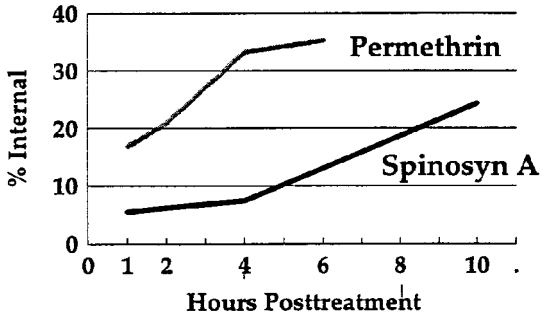


Figure 2. Penetration of topically applied spinosyn A and permethrin into last stadium (day 3) *T. ni* larvae.

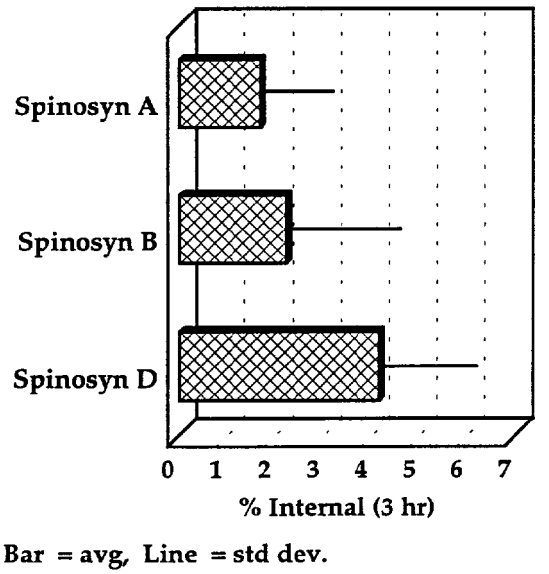


Figure 3. Penetration of topically applied spinosyns into last stadium (day 3) *T. ni* larval hemolymph. 1 ug/larva, 3 hr posttreatment.

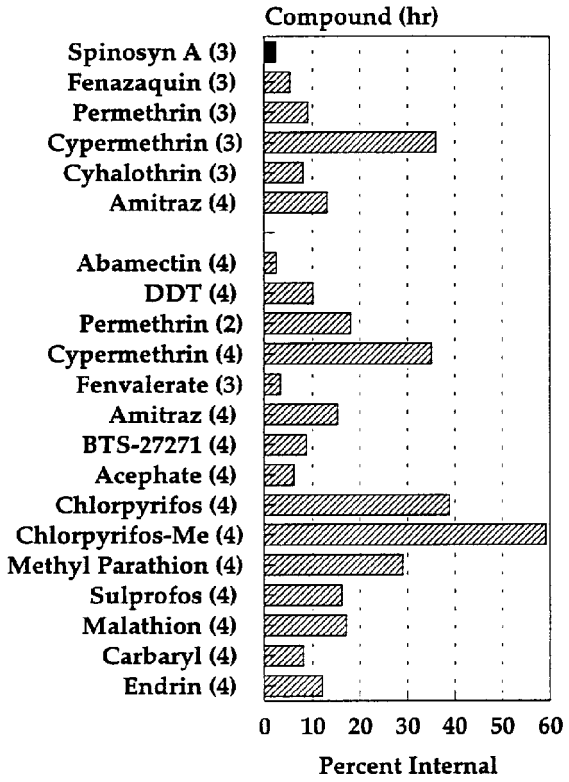


Figure 4. Penetration of topically applied insect control agents into third instar *H. virescens* larvae. Data from DowElanco (top group) and the literature (bottom group). Numbers in ( ) are time of assay (hours posttreatment).

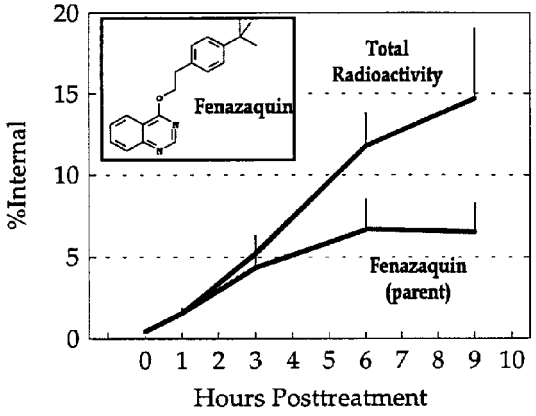


Figure 5. Penetration and turnover of topically applied fenazaquin in third instar *H. virescens* larvae. Data points are means (SD).

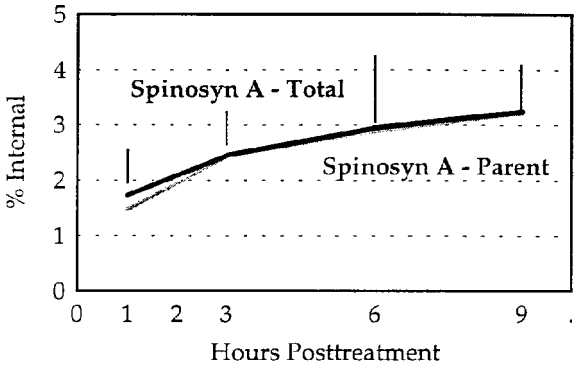


Figure 6. Penetration and turnover of topically applied spinosyn A (1 ug/larva) in last stadium (day 2; 200 mg) *H. virescens* larvae. Data points are means (SD).

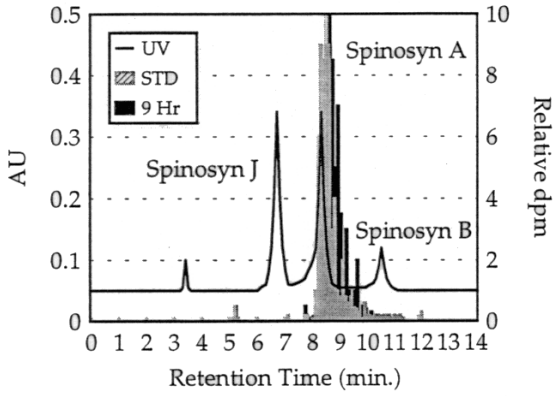


Figure 7. Radiochromatogram of internal extract (black - 9 hr after treatment) from last stadium *H. virescens* larvae topically treated with C14-Spinosyn A. UV trace shows unlabeled standards; Gray - radiolabeled spinosyn A standard.

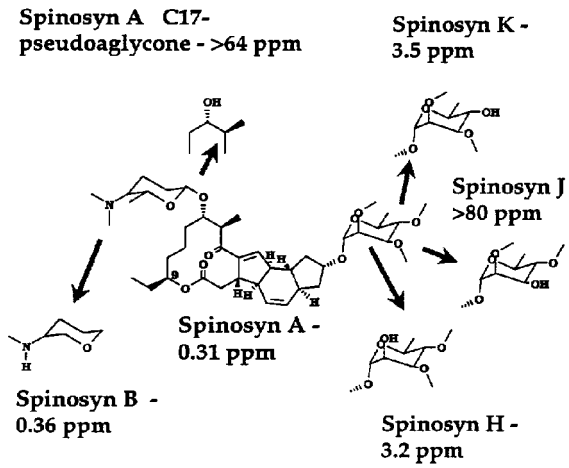


Figure 8. Potential spinosyn A metabolites and their LC50s to neonate *H. virescens* larvae. Toxicity data from Sparks *et al.* 1996.