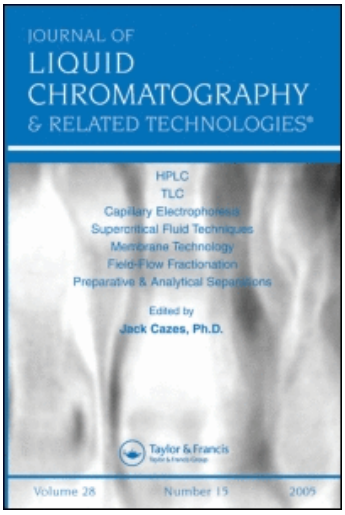

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QUANTITATIVE ANALYSIS OF BIOLOGICAL AND CHEMICAL INSECTICIDE MIXTURE BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

Diazinon has been used widely for eradication of harmful insects. However, due to the induction of insect resistance and considerable toxicity to humans and cattle, its usage has been restricted. In efforts to minimize these untoward side effects, investigators have observed a marked synergistic insecticidal activity when diazinon or its analogues were mixed with β-exotoxin, a bioinsecticide found in *Bacillus thuringiensis.* This compound is also known as thuringiensin. For developing the lowest effective dose of diazinon to exert the optimal synergistic action,

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a fast and accurate quantitative analytical method for both compounds is required for this process. Capillary electrophoresis (CE) is a new analytical technique, which provides simple and rapid analysis with high accuracy and high efficient separation. In addition, it can be used to analyze either hydrophilic or hydrophobic compounds by modifying its running buffer.

In this study, a quantitative analysis of the mixture of diazinon and β-exotoxin was demonstrated by using CE. In this study micellar electrokinetic capillary chromatographic (MEKC) mode was used because Diazinon has relatively higher hydrophobicity and is easier to be dissolved in MEKC running buffer and is not easily precipitated during the electrophoresis. The running buffer contained 20 mM disodium hydrogen phosphate, 20 mM boric acid, and 100 mM sodium dedocyl sulfate (SDS). The final mixture contained 20% acetonitrile. For quantitative analysis, a fixed amount of tryptophan was used as an internal standard. The standard linear regression equation was generated by using peak area ratio (diazinon to tryptophan) as the y-axis and the concentration of diazinon as the x-axis. From this equation, and similar equations previously established for quantitative analysis of β-exotoxin, the amounts of diazinon and β-exotoxin were calculated. With this method, the ratio of two insecticides can be monitored for making a minimized toxic and maximal effect formulation of combined chemical and biological insecticide.

Key Words: Diazinon; Thuringiensin; Capillary electrophoresis; Micellar electrokinetic capillary chromatography; Quantitative analysis of insecticides

INTRODUCTION

The β-exotoxin is a heat-stable, relatively water-soluble fly toxin produced by the bacterium *Bacillus thuringiensis*.[1–3] This exotoxin is effective against some orders of insects, such as Lepidoptera, Diptera, Hymenoptera, Coleoptera, and Orthoptera.[4] The mechanism of insecticidal action is the inhibition of the production of DNA-dependent RNA polymerase by competition with ATP.[5] The toxicity of β-exotoxin against warm-blooded animals and fish is extremely low when compared with chemical pesticides.[5] However, this biopesticide has generally lower pesticidal potency and costs more than chemical pesticides. In order to reduce the production costs of biopesticides and to minimize the animal toxicity of the chemical pesticides, the study of the possibility to combine

exotoxin with chemical pesticides has been investigated. Inoue, Dowke and Itoh[6] found a synergistic effect existed by combining exotoxin and diazinon.

Diazinon(O,O-Diethyl-O-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphoro thioate) is a non-systemic organophosphous insecticide used in home gardens and farms to control a wide variety of sucking and leaf eating insects. However, the disadvantages of using diazinon include the development of resistance, the high toxicity to humans and cattle, and the risk to natural enemies. The major mode of action of diazinon is the inhibition of acetylcholinesterase. In this report, a fast and effective method, capillary electrophoresis (CE), was used to analyze the mixture of β-exotoxin and diazinon. Capillary electrophoresis is a new analytical technique, which provides simple and rapid analysis with high efficient separation.[7] Use of HPLC or CE to quantify the amount of thuringiensin in solution or fermentation broth was demonstrated in the previous report.[8,9] In this study, we demonstrated a novel method in analyzing the mixture of diazinon and thuringiensin in solution by "micellar electrokinetic capillary chromatography" (MEKC) mode of CE. From the results of this study, the combination of these two insecticides can be monitored and the optimal ratio can be used as the formulation of the final product.

EXPERIMENTAL

Materials

Diazinon was kindly supplied by Dr. S.C. Wang, Director of Department of Toxicology, Taiwan Agricultural Chemicals and Toxic Substances Research Institute. The purity is over 99.5% (analytical grade), and was manufactured by Sinon Co., Ltd. (Taiwan). Thuringiensin broth was obtained from Dr. W.T. Wu, National Tsing Hua University. The broth was partially purified by high speed centrifugation ($7500 \times g$ for 30 min), and passed through a 0.45 μm membrane filter. The purification of thuringiensin has been reported elsewhere.[10] In brief, the thuringiensin in fermentation broth was absorbed by calcium silicate and the liquid phase was removed by centrifugation. The precipitation was dissolved by adding disodium hydrogen phosphate solution, and the dissociated thuringiensin was then collected. The dissociated thuringiensin was subsequently purified by preparative HPLC column and electrodialysis; the purity of the final product was analyzed by CE and mass spectrometry.[11]

Chemicals

Boric acid (99.5%), sodium hydroxide (96%), disodium hydrogen phosphate (85%) were purchased from Katayama Chemical Co. (Osaka, Japan).

β-Mercaptoethanol (>98%) was purchased from Sigma Chemical Company (St. Louise, MO). Sodium dodecyl sulfate (>99%) was purchased from Merck (Darmstadt, Germany). Acetonitrile (>99.5%) was purchased from Mallinckrodt (St. Louis, MO). Tryptophan (98%) was purchased from Laborat GMBH (Berlin, Germany).

Apparatus

Capillary zone electrophoresis (CZE) was performed on a P/ACE System Model 2100 (Beckman Instruments, Fullerton, CA). UV absorbance was monitored with a fixed-wavelength detector at 254 nm. Due to the low water solubility of diazinon and thuringiensin, the MEKC mode was used for this study.[10] Capillary was purchased from Polymicro Technologies (Phoenix, AZ). The dimensions of capillary were 47 cm × 50 μm I.D. without coating.

Methods

Diazinon (192 mg) or purified thuringiensin crystal (25 mg) were weighed and dissolved in 10 mL borate buffer as the stock solutions. Further dilution or mixing was decided based on the experimental design. The solubility of diazinon in water is approximately 60 mg/L (data from EXTOXNET). In this experiment, the running buffer contained surfactant SDS and acetonitrile was used to increase the solubility of diazinon. The running buffer was prepared by adding 1.24 g boric acid and 7.16 g sodium hydrogen phosphate into 850 mL of deionized distilled water. After dissolving completely, 28.84 g of SDS was added, and pH was then adjusted to 9.2 by 1 M sodium hydroxide. The final solution was then made up to a total volume of 900 mL with deionized water in a 1000 mL volumetric cylinder. Acetonitrile 100 mL was added and mixed completely. The final buffer contained 20 mM boric acid, 50 mM dibasic sodium phosphate, 100 mM SDS, with final acetonitrile concentration of 10%.

For quantitative analysis of both diazinon and thuringiensin, an internal standard solution of tryptophan was prepared by adding 102.1 mg of tryptophan (MW 204.2 daltons) with 100 mL of running buffer, which yielded a final concentration of 5 mM (1.02 mg/mL). The mixture from above was then subjected to CZE analysis. The sample was injected by IO-second positive pressure injection and the electrophoretic field strength was 319 V/cm (15 kV on total capillary length of 47 cm) for 30 min. The detection wavelength used was 254 nm, which is the maximal absorption for diazinon and the moderate

absorption for thuringiensin (Fig. 1). It is well known that tryptophan is a heteroaromatic compounds, the maximal absorption range is located in between 220 nm to 262 nm. Figure 2(B) showed the distinct tryptophan peak in the concentration of 5 mM. The test compounds were injected individually, or injected after mixing each other, to verify the interference. The peaks were integrated by "System Gold" (San Ramon, CA) and the electropherograms were modified by using "Origin" (Microcal, Northampton, MA) and Excel (Microsoft, Seattle, USA). A linear regression analysis was used to determine the suitability

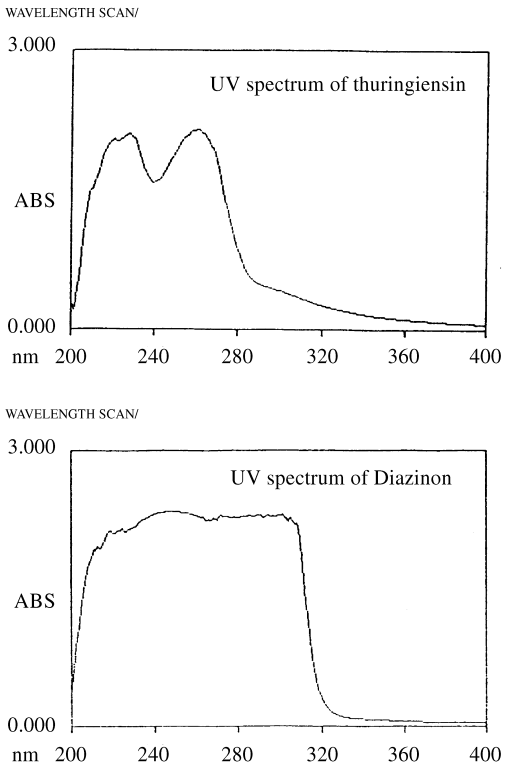

Figure 1. The upper panel shows the UV spectrum (200–400 nm) of the purified thuringiensin (1 mM). The sample was diluted with running buffer (20 mM boric acid, 50 mM dibasic sodium phosphate, and 100 mM SDS with 10% acetonitrile, pH 9.2). The reference cuvette was filled with running buffer only. The lower panel shows the spectrum of the diazinon (1 mM). The scanning process was the same as in upper panel.

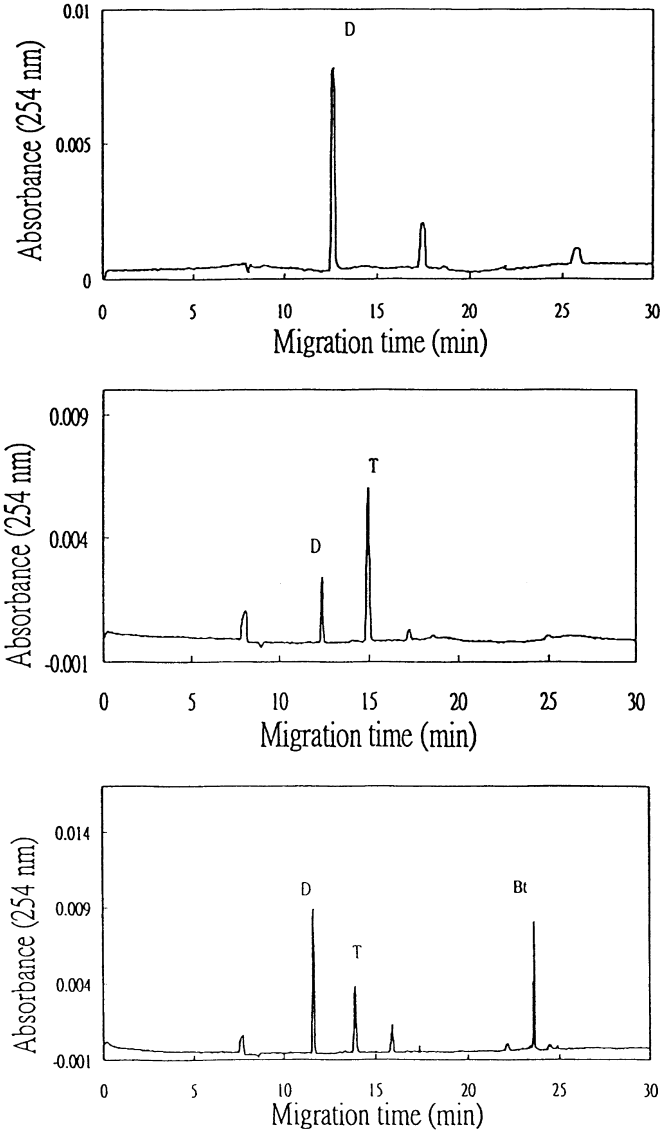

Figure 2. Electropherograms of diazinon (top panel, "D" represent the diazinon peak), and diazinon mixed with tryptophan (mid-panel, "T" represent tryptophan peak), and diazinon mixed with tryptophan and thuringiensin (bottom panel, "Bt" represent thuringiensin peak). The running buffer was the same as described in Fig. 1.

of the range selected. The *x*-axis is the concentration of diazinon and the *y*-axis is the peak ratio of diazinon to tryptophan (Fig. 4).

RESULTS AND DISCUSSION

In the initial experimental results we found the solubility of diazinon is very high in water or some aqueous buffers, such as borate or phosphate buffer. However, when MEKC buffer (20 mM boric acid, 50 mM dibasic sodium phosphate, 100 mM SDS, with final acetonitrile concentrations of 10%) was used as a solvent, the solubility increased at least 300 fold. One milliliter of MEKC buffer can dissolve 19.2 mg completely. The effective dose of diazinon is in the range from 0.4 mg/mL to 1.5 mg/mL. The concentration of diazinon in the mixtures are from 2.4 mg/mL to 19.2 mg/mL, which is close to the dose range for formulation before the additives were added. Diazinon is a hydrophobic compound, therefore, the running buffer containing SDS and acetonitrile was used to increase the solubility in this experiment. It not only solved the solubility problem but also minimized the sample solvent interference.

In the initial experiment, 19.2 mg of diazinon was completely dissolved in 1 mL running buffer without any precipitation. The solubility was increased at least 300 fold than that of water. It may be due to SDS or acetonitrile, the former is surfactant and the latter is organic solvent, which increased the solubility tremendously. The absorption spectra of diazinon and thuringiensin were scanned by UV spectrophotometer. The maximal absorbance of thuringiensin is located on 220 nm and 260 nm and diazinon is located from 210 nm to 310 nm. In this study the 254 nm UV absorptive detection was performed for capillary electrophoresis (Fig. 1). Sodium dedocyl sulfate is an anionic surfactant, when added to water in concentration above its critical micelle concentration (cmc), the micelles may be formed. The micelles which formed by SDS are possessed with highly polar surface and negatively charged. A solute which is not solubilized by micelles at all should migrate with the same velocity as the electroosmotic flow and be eluted first. On the other hand, a solute which is completely solubilized with miscelles should migrate with the same velocity as that of a micelle and be eluted last.[10] Figure 2 showed that 5 mg of diazinon dissolved in 1 mL of running buffer, which was then subjected to MEKC analysis. The electropherogram showed that diazinon peak appeared approximately from 12.4 to 12.8 min and the peaks at 17.2 and 25.7 min were unknown compounds (Fig. 2, top panel).

Tryptophan was used as an internal standard for quantitative analysis of thuringiensin[8] Tryptophan is an aromatic amino acid with strong UV absorption at 254 nm and the peak of tryptophan (1.02 mg/mL) appeared at

approximately in between 14.0 to 16.0 min (Fig. 2, mid panel), which is 2 to 3 min later than that of diazinon (2 mg). Figure 2, bottom panel showed the electropherogram of the mixture of diazinon (5 mg) and thuringiensin (1.25 mg) into 1 mL buffer with 5 mM (1.02 mg) tryptophan. In MEKC, the migration time of a specific analyte is determined by the combination of charge/mass ratios, hydrophobicity, and charge interactions at the surface to the micelles. In these experimental conditions, the charge/mass ratios are assumed as a dominant factor for the speed of migration. The phosphate group of thuringiensin as expected, has more negative charge in high pH solvent, thus it passed a detection window later than tryptophan and diazinon. Diazinon is a relatively low polarity compound, it has less negative charges compared with either tryptophan or thuringiensin and moves faster than these two compounds.

A standard correlation curve was generated based on the peak area ratio of diazinon (19.2, 9.6, 4.8 to 2.4 mg/mL) and the fixed amount of tryptophan (1.02 mg/mL). Each mixture was run four times for statistical analysis. The resultant electropherograms were analyzed by integrating the peak of each compound. The resultant electropherograms were shown in Fig. 3 (from top to bottom; 19.2 mg/mL; 9.6 mg/mL; 4.8 mg/mL, and 2.4 mg/mL). The peaks labeled with "D" and "T" are the abbreviation of diazinon and tryptophan, respectively. The peak appearing at 7.6 to 8.0 min was an impurity related to the tryptophan, and the peak at approximately 16.8 to 17.3 min was an impurity related to the diazinon. We used the main peaks to calculate peak area ratio of diazinon and tryptophan. Figure 4 showed a linear regression analysis between concentration of diazinon (x-axis) vs. the ratio of diazinon peak area/tryptophan peak area ratio (y-axis). A good correlation coefficient with $r = 0.9994$, $y = 0.1236x + 0.0345$ was found in this analysis, and the vertical bar represented the standard deviation of the mean from replication. The amount of diazinon could be calculated by integrating the peak area ratio of diazinon to tryptophan.

In the previous experiment, the formula for calculating thuringiensin by MEKC method has been established.[8] The amount of thuringiensin in the solution can be assessed by integrating the peak area ratio of thuringiensin peak area divided by tryptophan peak area first. Then, the concentration of thuringiensin (mg/mL) is equal to the ratio dividing by 0.7854 and plus 0.0119 ($x = y/0.7854 + 0.0119$, $x =$ concentration of thuringiensin, $y =$ the peak area ratio).

In this report, a method for simultaneous analysis of various amounts of diazinon and thuringiensin was demonstrated. The mixture of thuringiensin, diazinon and tryptophan was injected to CE with running buffer. The electropherogram showed that the thuringiensin peak appeared at approximately 23.5 to 24.0 min (Fig. 5). The diazinon and tryptophan remained at the same

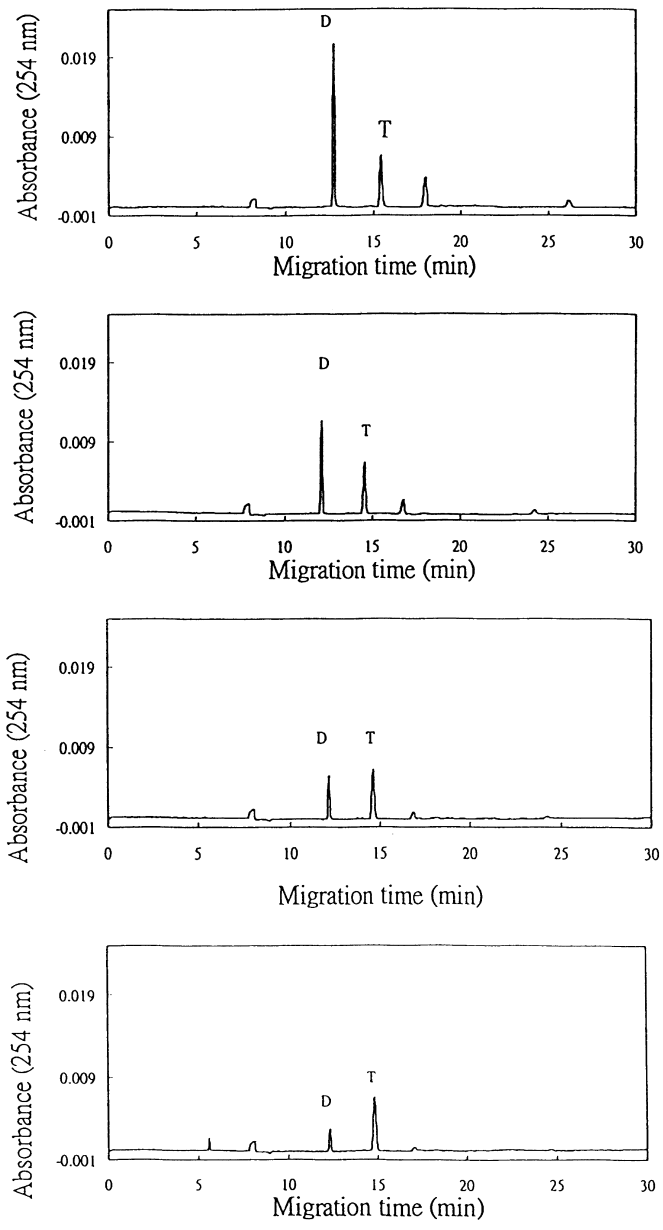

Figure 3. Electropherograms of diazinon from four concentrations (from top to bottom. 19.2, 9.6, 4.8, and 2.4 mg/mL) in tryptophan solution (1.02 mg/mL). "D" represents diazinon peak, "T" represents tryptophan peak.

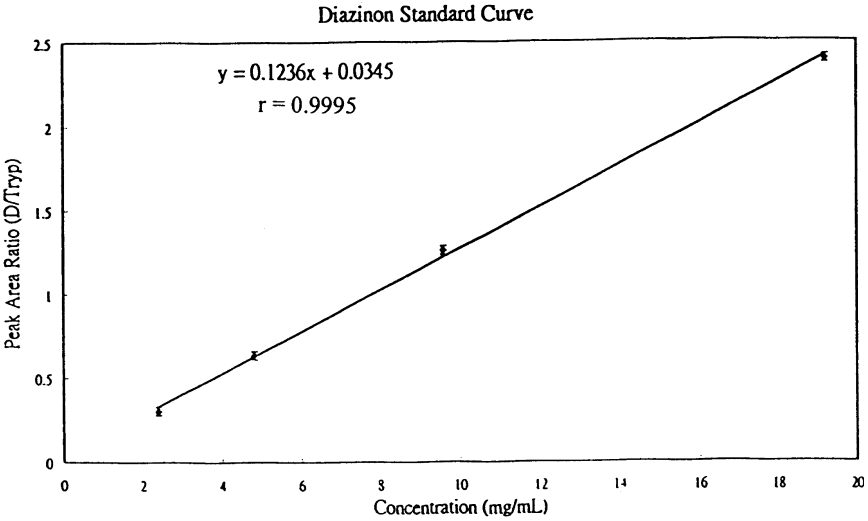

Figure 4. Linear relationship between the diazinon concentration and the peak area ratio of diazinon to tryptophan was shown in this regression analysis. The vertical bars represented the standard deviation of mean from each quadruplicate run.

position as aforementioned. The variable amount of diazinon (9.6 or 4.8 mg/mL) was mixed with a fixed amount of tryptophan (1.02 mg/mL) and thuringiensin (2.25 mg/mL). The electropherograms were shown in Fig. 5(A) and 5(B), respectively. Figure 5(C) showed the electropherograms of diazinon (4.80 mg/mL) mixed with tryptophan (1.02 mg/mL) and thuringiensin (1.12 mg/mL). The concentration of diazinon was assessed by using the linear regression equation. The estimated concentration of 9.72 mg/mL and 4.91 mg/mL were close to the original concentration, 9.60 mg/mL, and 4.80 mg/mL, respectively. Two different amounts of thuringiensin were mixed with a solvent of tryptophan (fixed amount) and diazinon (variable amount) (Fig. 5). The concentrations of thuringiensin were estimated by using the same method from linear regression equation which was established in previous report.[8] The estimated concentration of 2.25 and 1.20 mg/mL were close to the original concentration, 2.50 mg/mL and 1.25 mg/mL, respectively.

In conclusion, the quantitative analysis can be performed by CE with MEKC mode. The amounts of diazinon and thuringiensin are easily estimated from the mixture by using tryptophan as an internal standard. This rapid and simple method can help to optimize the doses and the ratio of these two insecticides. This is a critical technique for developing formulation for the final product of mixed insecticides.

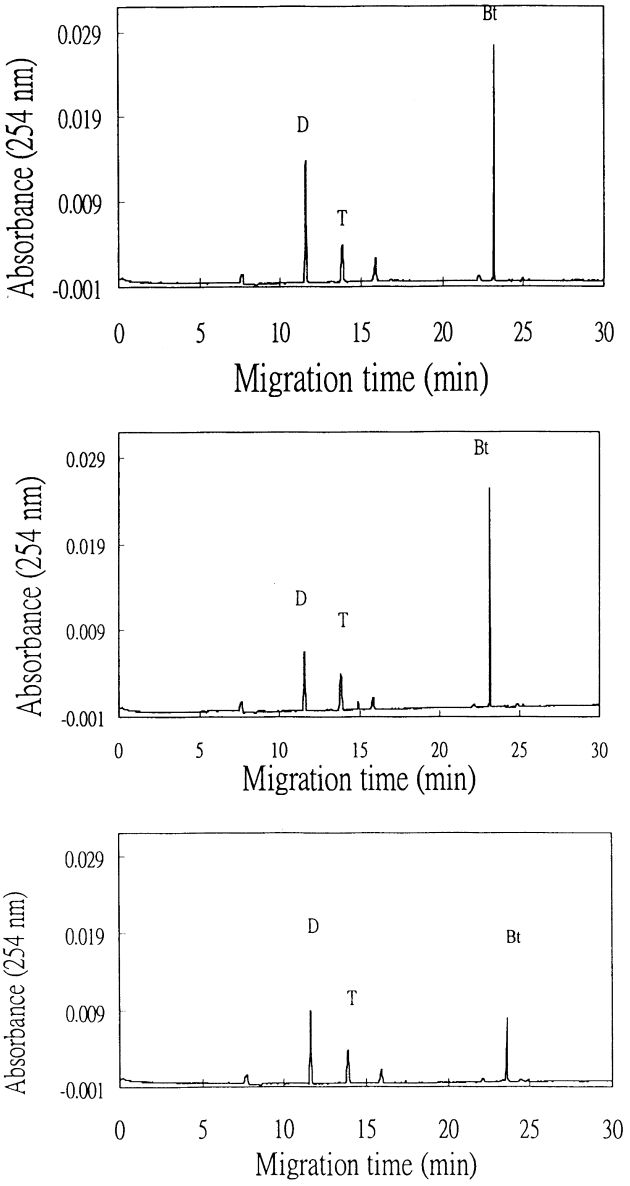

Figure 5. Electropherograms of two solutions with a fixed amount of thuringiensin (2.5 mg/mL) and variable amount of diazinon (9.6 mg/mL, B. 4.8 mg/mL; top panel and mid panel) were shown. The third electropherogram was for 1.25 mg/mL of thuringiensin and 4.8 mg/mL diazinon (bottom panel).

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