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# Insecticide Formulations Based on Nicotine Oleate Stabilized by Sodium Caseinate

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Organic farming and new trends toward the use of safer insecticides for crop protection have created new opportunities for botanical insecticides in the pesticide market. In this study, the botanical insecticide nicotine was formulated as a dispersion (20 vol %) stabilized by sodium caseinate, with nicotine oleate solutions used as the dispersed phase. The formulation showed a phase transition on increasing the nicotine oleate concentration, being an emulsion at 7.5−8.2 wt %, a suspo-emulsion at 8.2−9.7 wt %, and a suspension at 9.7−10.8 wt %. Biological activity, apparent viscosity, dispersion time, and protein surface coverage were dependent on nicotine oleate concentration. The emulsion with 8.2 wt % nicotine oleate and the suspo-emulsion with 8.7 wt % nicotine oleate were found to be the most appropriate formulations for insecticide purposes due to their high bioactivity, low viscosity, and low dispersion time. Nicotine oleate formulations showed good creaming and microbiological stability for at least 4 months without losing their biological activity.

**KEYWORDS: Nicotine oleate; insecticide formulations; sodium caseinate; nicotine bioactivity**

## INTRODUCTION

Botanical insecticides are considered, and promoted, as an alternative to conventional insecticides for crop protection due to their low mammalian toxicity, short environmental persistence, and target pest selectivity, characteristics that follow guidelines of the 1996 Food Quality Protection Act of the United States that pursue safe consumer products (*1*, *2*). Despite the considerable number of botanical insecticides reported in the literature, some of them with outstanding biological activity, only four have been used for crop protection: nicotine from tobacco leaves, rotenone from derris tree roots, pyrethrum from chrysanthemum flowers, and azadirachtin from neem tree (*3*). Today, pyrethrum and azadirachtin are the most important botanical insecticides, representing around 1% of the global insecticide market (*2*). At the beginning of the 20th century, nicotine was the main insecticide for crop protection, but it was practically displaced from the pesticide market after the appearance of parathion, the first synthetic insecticide, which showed higher performance in the field and lower mammalian toxicity (*1*). However, the end of the 20th century brought new requirements for botanical insecticides that could give nicotine a second chance.

The highest insecticidal activity of nicotine is observed on soft-body insects (e.g., aphids, whitefly, thrips, spider mites), and its performance, as for any insecticide, is dependent on the type of formulation used to deliver the active compound (*3*−*7*). Nicotine has been formulated for insecticidal purposes in different forms: pure compound, nicotine sulfate, tobacco dust, and soap (*4*, *6*, *8*). Its major insecticidal activity is observed in the pure compound and the soap formulation (*9*−*11*). Pure nicotine is considered toxic to mammals ($LD_{50}$ = 50 mg/kg) and classified as highly hazardous by the World Health Organization (WHO), representing a high risk during its handling and application as an insecticide (*8*, *12*, *13*). Therefore, use of pure nicotine as a botanical insecticide is restricted. Nicotine soap, prepared by neutralization of nicotine with a fatty acid, can be easily dispersed in water, behaving as an emulsifiable concentrate (*10*), and it is expected to have a lower mammalian toxicity (*14*).

Insecticides formulated as oil-in-water emulsions (O/W) can reduce dermal irritation and mammalian toxicity as compared with emulsifiable concentrates but show similar biological activity (*14*). Additionally, they have little or no flammability. The purpose of this work is to prepare a nicotine insecticide dispersion stabilized by sodium caseinate, an emulsifier widely used in foodstuffs (*15*), of high biological activity and good colloidal and microbiological stability.

## MATERIALS AND METHODS

**Materials.** Commercial tobacco dust was provided by the Colombian Tobacco Co., Coltabaco S.A., Medellin, Colombia. The material was ground and sieved through 60 mesh.

**Chemicals.** Commercial petroleum ether (50−70 °C), sodium hydroxide, and oleic acid were purchased from Protokimica Ltd.

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(Medellin, Colombia). Perchloric acid, acetic acid, chloroform, and crystal violet indicator were provided by Sigma Chemical Co. (St. Louis, MO). Celite 545 was purchased from J. T. Baker (Phillipsburg, NJ). Sodium caseinate (>82 wt % protein, <6 wt % moisture) was from DMV International (Veghel, The Netherlands).

**Nicotine Extraction Procedure.** Nicotine dust was mixed with a 4 wt % sodium hydroxide solution in a 1:1.25 weight ratio and digested for 2 h. Afterward, 3 L of petroleum ether was added per kilogram of digested nicotine dust and left 24 h for percolation extraction. During that time, the percolation system was agitated manually every 6 h to guarantee a homogeneous extraction. After percolation, the nicotine extract was separated from the depleted nicotine dust by filtration. Petroleum ether was evaporated from nicotine extract using a Büchi Rotavapor R-114 (Fisher Scientific, Montreal, Canada) to obtain a dark brown pastelike material. Soxhlet extraction was also implemented in order to have nicotine extracts with higher alkaloid content than those obtained by percolation extraction. In that case, the Soxhlet was filled with digested nicotine dust, and five washing procedures were carried out with petroleum ether; the nicotine extract was evaporated until removal of the extraction solvent.

**Alkaloids Quantification.** The alkaloids content (as nicotine) of the extracts was determined by using AOAC method 960.08 (*16*). Total alkaloids are referred to as nicotine content in the present document.

**Dispersed Phase Preparation.** Percolation, Soxhlet, and mixtures of percolation and Soxhlet nicotine extracts were neutralized with oleic acid to produce nicotine oleate solutions to be used as the dispersed phase. A first set of insecticide formulations was prepared with nicotine oleate solutions obtained from the neutralization of a nicotine extract (25 wt % nicotine) with oleic acid at base-to-acid molar ratios (alkaloids as nicotine/oleic acid) of 1.0:0.6, 1.0:0.8, 1.0:1.0, 1.0:1.2, and 1.0:1.5. A second set of nicotine insecticide formulations used nicotine oleate solutions at a 1.0:1.0 base-to-acid molar ratio, where percolation extract (20.0 wt % nicotine) and Soxhlet nicotine extract (33.7 wt % nicotine) were mixed in different proportions to produce dispersed systems with nicotine oleate concentrations from 7.5 to 10.8 wt %.

**Preparation and Characterization of Insecticide Formulation.** Nicotine dispersions (3 wt % sodium caseinate, 20 vol % nicotine oleate) were prepared at room temperature using a high-pessure homogenizer working at 300 bar, as described by Casanova and Dickinson (*17*). Droplet size distributions were carried out using a Malvern Mastersizer 2000. Control stress rheometry was determined using a Hakke Rheostress RS-150 with a Z20 DIN TI concentric cylinder measuring cell at 20 °C. Apparent viscosity was obtained for applied shear stress in the range 0.1−1000 Pa. Optical microscopy was carried out at magnification ×40 using an interface contrast Nikon TMS biological microscope.

**Formulation Stability to Creaming.** The creaming behavior was determined by visual observation of the height of a discrete layer formed in the dispersed system, thermostated at 25 °C, at regular time intervals during a 4 month time period.

**Dispersion Test.** The formulation dispersibility in water was measured as the time required for a drop to fully disperse into 50 mL of distilled water in quiescent conditions. The procedure was repeated five times for each sample to obtain a mean value of dispersion time. The estimated error for the test is ±7% of the reported value.

**Protein Surface Coverage Quantification.** The total protein surface coverage ($\Gamma$) at the oil−water interface was determined by the depletion method. Before centrifugation, the dispersed systems were diluted 2:1 (by weight) with distilled water. Diluted systems were centrifuged at $12 \times 10^4$ *g* for 40 min at 10 °C, and the resulting serum layer was removed with a syringe. A second centrifugation procedure was carried out with the serum layer to ensure the complete removal of dispersed particles. Protein content was determined by the Biuret method, measuring absorbance at 560 nm and interpolating the reading value on a calibration curve of sodium caseinate standard solutions. The protein surface coverage was calculated from the difference between the amount of protein detected in the aqueous phase and the known amount of protein used to make the dispersion.

**Bioassay for Insecticidal Activity.** The bioassay for insecticidal activity against adults of *Drosophila melanogaster* was carried out following the procedure of Granados et al. (*18*): a filter paper disk (diameter 4 cm) was impregnated with 0.15 mL of diluted insecticide formulation (2 wt % nicotine oleate) and placed inside glass vials (5 cm wide × 6 cm deep). A total of 30 individuals were introduced into a glass vial, and the number of dead individuals was recorded every minute, or every 5 min, depending on the insecticide activity of the sample. Data were recorded until all individuals were dead or the completion of 3 h observation time. Five replicates for each dispersion sample were carried out. Lethal time 50 ($LT_{50}$) values and regression analysis data were calculated with the software Statgraphics Plus 4.1. A time-dependent bioactivity test was carried out for the 8.7 wt % nicotine oleate dispersion. In that case, the sample was kept in a dark glass vial for 4 months, and dispersion aliquots were used for monthly bioassays.

**Microbiological Analysis of Concentrated Emulsion Samples.** The counting of mesophile formation units was carried out following a heterotrophic counting procedure for samples diluted at volume ratios of $1:10^{-1}$, $1:10^{-2}$, and $1:10^{-3}$ according to APHA method 9610 (*19*).

## RESULTS AND DISCUSSION

High-pressure homogenization of a nicotine extract (25 wt % nicotine) with a sodium caseinate solution (3.69 wt %) produced a highly heterogeneous dispersion composed of emulsion droplets ($d_{32} = 2.05$ $\mu$m) and macroscopic solid aggregates (diameter > 1 mm), indicating a poor emulsification process. Additional floc formation was observed on dilution of the concentrated formulation, showing the instability of the nicotine colloidal system. Therefore, that nicotine formulation is inappropriate for insecticide application, since on dilution it could show stratification of the insecticide principle on the spray tank, leading to inhomogeneous delivery or spray nozzle obstruction by the aggregates (*20*, *21*).

A set of formulations was prepared by high-pressure homogenization of a sodium caseinate solution (3.69 wt %) with nicotine oleate solutions obtained from the neutralization of nicotine extract (25 wt % nicotine) with oleic acid at base-to-acid molar ratios of 1.0:0.6, 1.0:0.8, 1.0:1.0, 1.0:1.2, and 1.0:1.5. Nicotine oleate formulations prepared at molar ratios of 1.0:0.6 and 1.0:0.8 showed aggregate formation similar to those observed for the non-neutralized nicotine formulation. However, a homogeneous system of dispersed oil droplets (i.e., an emulsion) was obtained after high-pressure homogenization of the nicotine oleate solution with a 1:1 base-to-acid ratio. The resulting emulsion (9 wt % nicotine oleate, 3 wt % protein) showed a volume-surface average diameter $d_{32}$ of 8.06 $\mu$m and a viscosity of 3800 Pa·s (measured at an applied stress of 1 Pa), with a creaming stability of at least 4 months and without any significant change in its average droplet size. Formulations with molar ratios of 1.0:1.2 and 1.0:1.5 produced homogeneous dispersed systems but with relatively high viscosity (10 000 Pa·s at a shear stress of 1 Pa) and high dispersion time (>5 min), therefore making them inappropriate for pesticide application.

The nicotine oleate formulation at a 1:1 base-to-acid molar ratio resembles nicotine soaps, as first reported by Moore in 1918 (*9*) and developed as a way of nicotine fixation. In Moore's work, nicotine soaps were obtained by mixing pure nicotine with aliphatic monocarboxylic acids at a base-to-acid ratio of 1:1 at room temperature. The addition of the nicotine soap to water produced a turbid system ready for use for insecticidal purposes. Such types of pesticide formulation could be classified as an emulsifiable concentrate due to the formation of an emulsion on dispersion of the nicotine soap in water at the moment of application. Our nicotine system differs from nicotine soap in that it is already formulated as an emulsion stabilized by sodium caseinate.

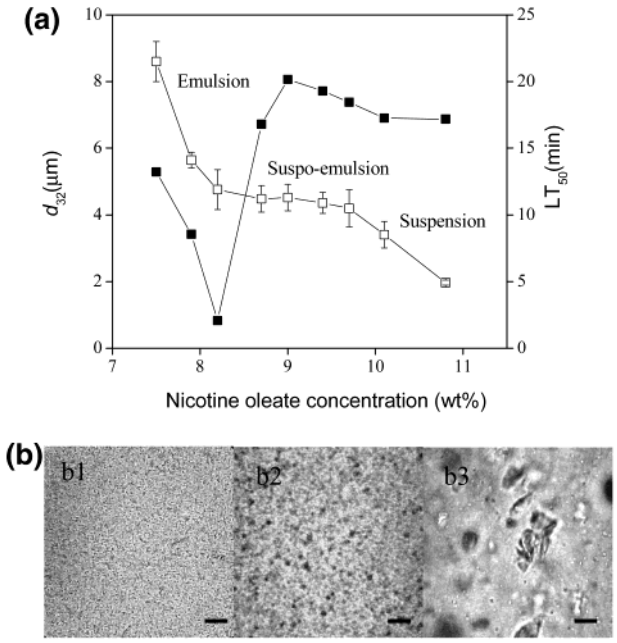


**Figure 1.** (a) Effect of nicotine oleate concentration present on insecticide formulations on volume-surface average diameter ($d_{32}$) (■) and lethal time 50 ($LT_{50}$) (□). Error bars for particle diameter are ±1% of reported data. (b) Photomicrographs of nicotine formulations at (b1) 7.9, (b2) 9.0, and (b3) 10.8 wt % nicotine oleate. Scale bar = 20 μm.

Structural studies of nicotine salts by Perfetti (*22*) showed that aliphatic monocarboxylic acid reacted with nicotine at a base-to-acid ratio of 1:3, while 1:2 salts are formed with dicarboxylic acids, and 1:1 salts are produced with aromatic acids. The nicotine dispersed system, stabilized with sodium caseinate, was prepared by mixing the nicotine with oleic acid in a molar ratio of 1:1, which differs from the 1:3 base-to-acid ratio suggested in Perfetti's work for aliphatic monocarboxylic acids. The nicotine extract is a very heterogeneous system, where nicotine makes up only about one-fourth of its composition, with the other fraction composed of tobacco resins, low-molecular-weight carboxylic acids, and colored material (*23*, *24*). This complex matrix might not allow the protonation of the pyridinic nitrogen and the formation of dimers held by hydrogen bonding on the pure nicotine salt systems, as suggested by Perfetti (*22*). In the nicotine percolation extract, probably only the pyrrolidine nitrogen undergoes protonation, since that nitrogen has the strongest base character in the nicotine molecule, producing the nicotine oleate at a 1:1 base-to-acid molar ratio.

To obtain an easy way to disperse insecticide at the time of application with the highest possible concentration of insecticide principle (i.e., nicotine oleate), a set of formulations was prepared at nicotine oleate concentrations from 7.5 to 10.8 wt %. Figure 1a shows the average particle size ($d_{32}$) and lethal time 50 ($LT_{50}$) data for the set of formulations. The pH value of these nicotine dispersions was in the range 7.9−8.1. The increase in nicotine oleate concentration from 7.5 to 8.2 wt % produced a significant decrease in the $d_{32}$ values, from 5.89 to 0.84 μm. At the same time, the $LT_{50}$ associated with these formulations dropped from 22 to 11 min, indicating a 2-fold increase in the bioactivity, since $LT_{50}$ and bioactivity have an inverse relationship. At 8.7 wt % nicotine oleate, the average particle size increased to 6.72 μm, and the highest $d_{32}$ value (i.e., 8.06 μm) was obtained at 9 wt % nicotine oleate. The average particle size decreased smoothly with the increase in nicotine oleate concentration over 9 wt %, showing a value of

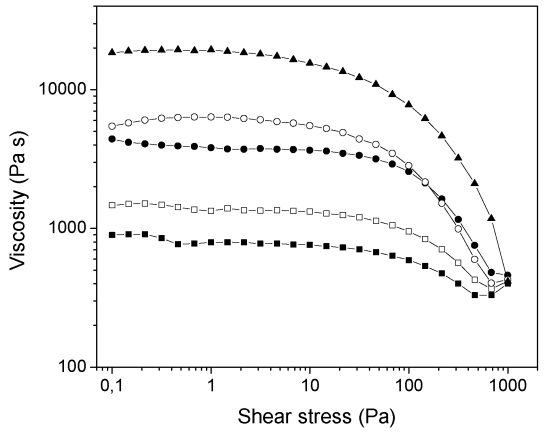


**Figure 2.** Controlled stress viscometry of nicotine insecticide formulations. Apparent shear viscosity is plotted against shear stress for formulations with dispersed phase at different concentrations of nicotine oleate: ■, 7.5; □, 8.2; ●, 9.0; ○, 9.4; and ▲, 10.8 wt %.

6.9 μm at 10.8 wt %. The $LT_{50}$ remains at a relatively constant value (i.e., 11 min) at nicotine oleate concentrations from 8.7 to 9.7 wt %, showing a decrease at higher concentrations, with the lowest value at 10.8 wt %. It is important to mention that the variation in bioactivity is not due to the change in nicotine oleate concentration on bioassays, since the bioassays were carried out at the same nicotine oleate concentration (i.e., 2 wt %) by adjusting the dilution factor for each formulation. Consequently, the variation in bioactivity should be explained in another way.

Microscopic analysis of nicotine oleate formulations showed a significant change in the physical state of the dispersed particles (Figure 1b). Between 7.5 and 8.2 wt % nicotine oleate, all particles were in a liquid state, forming an emulsion. On increasing the concentration of nicotine oleate over 8.2 wt %, solid particles were formed together with oil droplets, producing a suspo-emulsion. The rise in nicotine oleate concentration to 9.7 wt % increased the proportion of solid to liquid particles in the system. Indeed, above 9.7 wt %, all particles were solid, forming a suspension. The information from microscopic analysis suggests that the decrease in $LT_{50}$ values on increasing nicotine oleate concentration could be associated with the change in the physical state of the dispersed system, with nicotine oleate emulsions showing the highest $LT_{50}$ values (i.e., the lowest insecticidal activity), followed by suspo-emulsions and suspensions. Only the emulsion at 8.2 wt % nicotine oleate showed an insecticidal activity comparable to that of the suspo-emulsions, probably due to its very low particle diameter. While the system remains as a suspo-emulsion, its bioactivity does not change significantly, and only when all the dispersed phase turned fully into the solid state, forming the suspension, does the insecticidal activity increase again. Apparently, bioavailability of nicotine oleate is higher in the suspension system than in the suspo-emulsion and emulsion systems.

Viscometry of nicotine oleate dispersions is shown in Figure 2. The emulsions with 7.5 and 8.2 wt % nicotine oleate show themselves to be fairly Newtonian systems with relatively low apparent viscosity, 800 and 1300 Pa·s, respectively. Only at a shear stress of over 10 Pa do the emulsions behave as pseudoplastic fluids, decreasing the viscosity on increasing the shear stress. The suspo-emulsions with 9 and 9.4 wt % nicotine oleate showed higher low-shear stress viscosities than the emulsion systems (3800 and 4200 Pa·s, respectively) and higher pseudoplastic behavior with a more significant decrease in viscosity at a shear stress of over 10 Pa. The suspension with

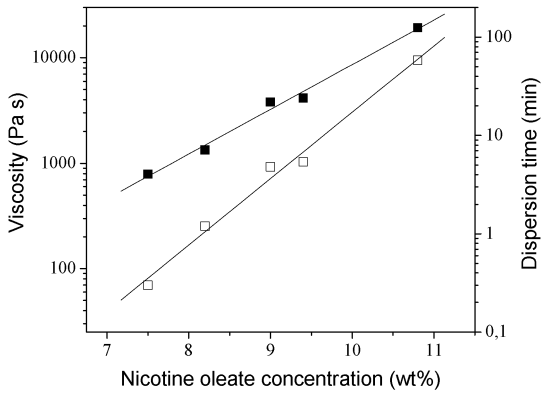


**Figure 3.** Effect of nicotine oleate concentration present on insecticide formulations on apparent shear viscosity (1 Pa applied stress) (■) and dispersion time (□).

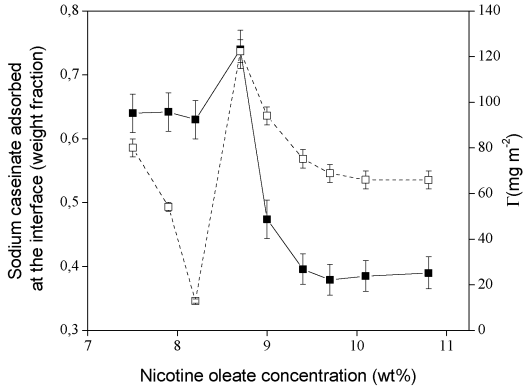


**Figure 4.** Effect of nicotine oleate concentration present on insecticide formulations on the weight fraction of sodium caseinate adsorbed at the interface (■) and protein surface coverage Γ (□).

**Table 1.** Regression Analysis Output for a Linear Model of Low-Shear Stress Viscosity and Dispersion Time Data against Nicotine Oleate Concentration

| dependent variable | fitted model | correl coeff | *p*-value |
|---|---|---|---|
| viscosity ($\eta$) | $\log(\eta) = -0.30 + 0.42$(nicotine oleate wt %) | 0.995 | 0.0004 |
| dispersion time (DT) | $\log(DT) = -5.5 + 0.67$(nicotine oleate wt %) | 0.994 | 0.0005 |

10.8 wt % nicotine oleate presented a very high low-shear viscosity (i.e., 19 300 Pa·s).

According to Stokes law, colloidal systems with high viscosity values present good creaming stability, a desirable characteristic for an insecticide formulated as a dispersed system. However, high viscosity values could increase the time required for dispersion of the concentrated insecticide to produce the low-concentration system that would be used for application in the field; therefore, consumer acceptance would be diminished. Figure 3 shows the change in low-shear stress viscosity and dispersion time required for dilution in quiescent conditions with the variation of nicotine oleate concentration in the insecticide system. The semilogarithmic plot presents a fairly good linear trend for both dependent variables against nicotine oleate concentration, whose regression analysis is summarized in Table 1. The increase in nicotine oleate concentration produces the rise in viscosity and dispersion time, with the latter being more affected, as is shown by its higher slope value. Formulations with nicotine oleate concentration up to 9.4 wt % showed dispersion times below 5 min, which are still acceptable for insecticide purposes. That behavior could be related to the reduction of sodium caseinate present at the interface, as seen in Figure 4. Here, it is observed that the weight fraction of protein at the interface changed from around 0.65 (nicotine oleate concentration $\leq$8.2 wt %) to approximately 0.4 at nicotine oleate concentrations above 9.0 wt %. Such a decrease could be related to the change in affinity of the emulsifier sodium caseinate with the particle interface when this changes from a liquid to a solid state, or a competitive adsorption process between the sodium caseinate and the nicotine oleate molecules at the particle interface. However, the increase of protein on the serum layer could just induce a small rise of insecticide formulation viscosity, but not the 2 orders of magnitude increase observed when nicotine oleate concentration changed from 7.5 to 10.8 wt %. Therefore, it is possible that sodium caseinate at the serum layer interacts with the nicotine oleate at the interface, promoting the formation of a gel-like system.

Although the total amount of protein present at the interface decreases with the rise in nicotine oleate concentration, the protein surface coverage (Γ) at nicotine oleate concentrations over 8.2 wt % increases (Figure 4), following a trend similar to that observed for average droplet size (see Figure 1). Therefore, protein surface coverage of nicotine oleate dispersions is mainly affected by particle size.

Considering that the insecticide formulation has to have a low dispersion time (less than 5 min in quiescent conditions), the nicotine oleate suspensions, which have dispersion times above that limit, should not be considered as suitable formulations for insecticide purposes. Therefore, only emulsions and suspo-emulsions could be accepted for nicotine formulation. Taking that into account in conjunction with bioactivity and the droplet size values, we have to check the $LT_{50}$ and $d_{32}$ values (Figure 1) at nicotine oleate concentrations $\leq$9.0 wt %. The best combined values for the three variables is observed in the nicotine oleate emulsion with 8.2 wt %, which has a dispersion time of 1.2 min, a $LT_{50}$ of 11.9 min, and a $d_{32}$ of 0.83 $\mu$m. The suspo-emulsion with 8.7 wt % could also be considered as an alternative formulation, showing a dispersion time of 2.2 min, a $LT_{50}$ of 11.2 min, and a $d_{32}$ of 6.72 $\mu$m. The emulsion with 7.9 wt % nicotine oleate could also be considered as a formulation, but at this concentration bioactivity has already started to decrease due to the higher $LT_{50}$ value.

To determine the colloidal and microbiological stability of nicotine oleate dispersions (7.5−10.8 wt %), these formulations were monitored over a 4 month time period. Not one formulation showed any cream layer formation or any significant change in average droplet size $d_{32}$ during the evaluation period. The microbiological study of the nicotine oleate formulations showed an initial value of one mesophiles formation unit (MFU) (dilution 1:1000); that value did not change during the 4 months of monitoring. Consequently, the nicotine oleate formulations have a low and stable MFU numbers, indicating their good microbiological stability, despite the presence of protein (i.e., sodium caseinate) and water in the formulation, which could promote microorganism growth at the pH of the formulation (ca. 8.0). Therefore, the inhibition of microorganism growth could be associated with the presence of a compound in the nicotine oleate solution with bactericidal activity, which is still to be identified.

A time-dependent study of bioactivity was also carried out for the 8.7 wt % nicotine oleate suspo-emulsion in order to check

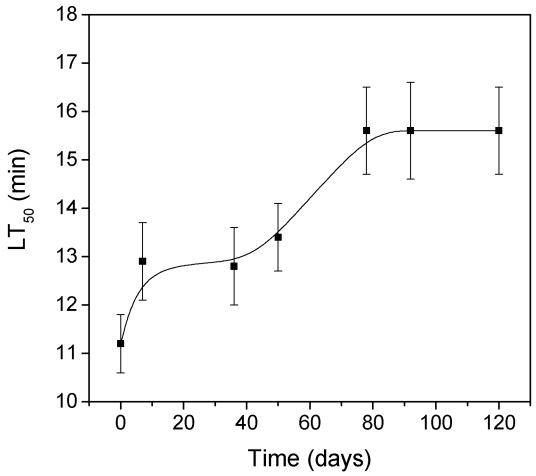


**Figure 5.** Time dependence plot of lethal time 50 for a nicotine insecticide formulation with 8.7 wt % nicotine oleate.

for any significant change in insecticidal performance. Figure 5 presents the $LT_{50}$ values obtained in an evaluation test over 4 months. The suspo-emulsion showed $LT_{50} = 11$ min at the beginning of the evaluation period. That figure increased to 13 min after 7 days and remained constant until day 35. Afterward, the $LT_{50}$ started rising again, reaching a value of 15.5 min on day 80, a figure that remained until the end of the bioactivity study. These data show a 40% increase in $LT_{50}$ values during the 4 month storage time, which is not considered a significant change for bioassay tests. The increase in the lethal time value could be associated with oxidation reactions that nicotine oleate undergoes in the presence of light and air; these oxidation reactions are still slower than those observed for the nicotine free base (*25*).

The nicotine oleate dispersions used as insecticide formulations have shown high bioactivity and high colloidal and microbiological stability. Changes in the physical state of the dispersed phase from liquid to solid apparently increased the bioactivity of the insecticide formulation, though an explanation for such behavior is still to be found. Further investigation is required to assess the mammalian toxicity of the nicotine oleate insecticide stabilized by sodium caseinate, in the pursuit of an environmentally friendlier and safer formulation to use in the field.

## ABBREVIATIONS USED

$LT_{50}$, lethal time 50; $LD_{50}$, lethal doses 50; $d_{32}$, volume-surface average diameter; Γ, protein surface coverage; DT, dispersion time.

## SAFETY

Nicotine is very toxic therefore it should be handled with extreme care while wearing gloves and protective masks.

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