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Secondary Metabolism as a Measurement of Efficacy of Botanical Extracts: The Use of *Azadirachta indica* (Neem) as a Model

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1. Introduction

This chapter is primarily concerned with the concept and correct use of natural products as insecticides. All organisms need to transform and interconvert a vast number of organic compounds in order to be able to live, grow, and reproduce. They need to provide themselves with energy in the form of ATP and a supply of building blocks to construct their own tissues. Despite the extremely varied characteristics of living organisms, the pathways for generally modifying and synthesizing carbohydrates, proteins, fats, and nucleic acids are found to be essentially the same in all organisms, apart from minor variations. These processes demonstrate the fundamental unit of all ways of life, and are collectively described as primary metabolism. In contrast to these metabolic pathways, which synthesize, degrade, and generally interconvert compounds commonly encountered in all organisms, there is also an area of metabolism concerned with compounds which have a much more limited distribution in nature called secondary metabolism (Dewick, 2009).

Secondary metabolites or natural products are commonly reserved for organic compounds of natural origin that are unique to one organism, or common to a small number of closely related organisms (Mann, 2005). Secondary metabolites are not necessarily produced under all conditions, and in the vast majority of cases the function of these compounds and their benefit to the organism are not yet known. In fact, they are usually an expression of the individuality of species (Dewick, 2009). In most examples they appear to be non-essential to the plant, insect, or microorganism producing them. For instance, the morphine only occurs in two species of poppy, *Papaver somniferum* and *P. setigerum*, and although it is widely used and abused by man, it has no function which is known in these plants (Mann, 2005).

Plants produce a large diversity of natural products which are usually sub-divided in classes according to metabolic pathways at polyketides, lignans, coumarins, flavonoids, terpenoids, steroids, alkaloids, *etc.* These are of great importance for the plant for their interaction with the environment due to their roles as pollinator attractants, for symbiosis and for defense against attacks by microorganisms, other plants or animals. Moreover, they are economically important to man as a source of pharmaceuticals, flavours, fragrances, insecticides, dyes, food additives, toxins, *etc.* (Zarate et al., 2010). Structures of an estimated 200,000 natural products have been elucidated (Dixon & Strack, 2003). Nowadays,

researches for new insecticides are a promising area which is showing the growing demand for natural products. The diversity of secondary metabolites from plants may be commercially explored as botanical extracts or pure compounds after extraction and isolation by phytochemical process.

2. Natural products as insecticides

Insecticides are the cornerstone upon which the pest management practices are based, and are likely to remain so as long as affective and inexpensive chemicals are available (Hayves, 1988). Natural products have been used as botanical pesticides since ancient times. Apparently, almost every plant species has developed a unique chemical complex that protects itself from pests (allelochemicals). Thus, plants offer us a diverse group of complex chemical structures and almost every imaginable biological activity. For thousands of years, agricultural practices relied heavily on crop rotation or mixed crop planting to optimize natural pest control (such as predation, parasitism, and competition). Therefore, the concept of 'natural pesticides' arose early along with the development of agriculture (Dayan et al., 2009). The medical compendium known as the Ebers Papyrus of *c.* 1600 B.C. includes both chemical and organic substances recommended as insecticides (Panagiotakopulu et al., 1995). In the first century AD, Pliny the Elder, the Greek philosopher, wrote ''Natural History'' in which he recorded all the known pest control methods. At the same time the Chinese recorded their use of powdered chrysanthemum as an insecticide. Methods such as mulching and burning, as well as the use of oils for pest control were mentioned. A survey of the Shengnong Ben Tsao Jing era (25–220 A.D.) shows that 267 plant species were known to have pesticidal activity (Dayan et al., 2009; Yang &Tang, 1988).

In fact, chemistry has always fulfilled an important role with the introduction of a lot of essential products to humanity. These improvements are easily seen in food production. In the last 50 years, farmers around the world have trusted substantially in the use of fertilizers and protecting organic-synthetic pesticides, therefore improving each year their yields of production and supplying the world demand of foods and natural fibers (Knowles, 2008). In this period, the synthetic pesticides have been the main insecticide tool (Saxena, 1989). Chemical industry has been assisting the demand of consumers, which increased the gains in crops through continuous development and introduction of new synthetic products. However, not only have the yield in crops increased, but also the world population, which causes a constant pressure to improve such a production (Knowles, 2008).

Although the use of synthetic insecticides have been efficient to control insect pests, their extensive and sometimes indiscriminate use have caused various problems of social and environmental repercussion (Alkofani et al., 1989). As negative consequences of using these products are countless and cumulative damages to environment. Among them there is both the contamination of soil, air, waters, fishes, animals, and the man himself (as much in fields as in consumption of contaminated products) and the reduction of biodiversity, population of natural enemies, pollinators and bees. Such reductions allow secondary pests to appear. Furthermore, the indiscriminate use of synthetic insecticides has allowed insects to develop resistance against them (Dayan et al., 2009; Jadeja et al., 2011). Concern about the adverse impacts of pesticides on the environment and on human health started to be voiced in the early 1960s (Carson, 1962). Since then, debate on the risks and benefits of pesticides has not ceased and a huge amount of research has been conducted into the impact of pesticides on the environment (van der Werf, 1996).

Along with the evolution of knowledge about environment damages, the natural-products concept has come back to the worldwide scenario as a proposal for alternative pest control agents but with reduced environmental consequences, which have been creating or causing an evolution in several research lines. In fact, prior to the discovery of the organochlorine and organophosphate insecticides in the late 1930s and early 1940s, botanical insecticides were important products for pest management (Isman, 1997).

Natural products represent a rich source of biologically active compounds and are an example of molecular diversity, with recognized potential in pesticides discovery and development. They are in general structurally more complex, selective and biodegradable, environmentally compatible and less toxic to non-target organisms than synthetic pesticides (Alkofani et al., 1989; Duke et al., 2000; Rattan, 2010). Each molecule present in the secondary metabolism of plants may be explored individually either as a model to the development of new insecticides or as an isolated molecule which is an active compound of new insecticides. Their chemical diversity, resulted from effects of evolutionary pressure to create biologically active molecules, is similarly structured to protein targets of many species, showing several biological activities (Harvey, 2007). Moreover, plants usually feature synergism, which is a combination of effects equal to the sum of those of individual components, or it takes place when combinations of bioactive substances exceed in effects that are greater than the sum of those of individual components (Schmidt, 2008). This last feature describes several important properties of the use of botanical extracts. The mixture of secondary metabolites may be deterrent to insects for a longer period than single compounds and, different physical properties may allow more deployment or longer persistence of defenses (Rattan, 2010). Thus, it is necessary that technical and economical studies evaluating if it is better to work by using a single molecule or botanical extracts are developed.

Compounds of botanical extracts, in many cases, have the function of protecting the active molecules against alterations, namely oxidations, hydrolyses, or other, and they are able to allow better absorption by target organisms facilitating the transport through membranes or inhibit enzymatic systems. Furthermore, insects tend to acquire resistance against formulated active compounds, which is harder to occur by using botanical extracts. For instance, Feng & Isman (1995) show that *Myzus persicae* acquired in few generations resistance against azadirachtin, an isolated secondary metabolite. However, this species did not show any resistance during forty generations by using botanical extracts of *Azadirachta indica*, the main source of azadirachtin. Finally, botanical extracts are cheaper than both the development of new synthetic compounds and isolation processes of secondary metabolites (Duke et al., 2000).

3. Phytochemical sources and insecticidal activity

The growing demand for natural products has been intensified in the past decades as they are extensively used as biologically active compounds and are being considered an important alternative strategy for sustainable insect pest management in agriculture, because they are biodegradable and potentially suitable for the use in integrated management programs (Rattan, 2010). A brief research into the literature reveals many investigations applied into the biological activity of many plant components against a large number of pathogens and arthropods. An old review with an agricultural focus by Roark (1947) described around 1200 plant species that have been listed in the literature as

having potential insecticidal value. These studies have exposed an array of botanical insecticides in several families such as Meliaceae, Agavaceae, Lamiaceae, Rutaceae, Cactaceae, Asteracae, Labiatae, *etc*., containing a wide spectrum of bioactive fungicides, nematicides, acaricides, insecticides and carcinogenic (Shaalan et al., 2005). Some of the botanical extracts of insecticidal interest are described in Table 1. It is possible to identify extracts prepared from roots, stems, branches, fruits, seeds, leaves, flowers, *etc*. of plants, which show different biological activities against insect pests (different ways of action), a large diversity of phytochemical techniques employed during the production of these materials, variation in the chemical profile and various compositions in formulations which were assayed.

Plant	Organs	Insect	Source
Aglaia odorata	leaves	*Spodoptera littoralis*	Nugroho et al., 1999
Yucca periculosa	barks	*Spodoptera frugiperda*	Torres et al., 2003
Ocimum gratissimum	oils	*Sitophilus oryzae* *Tribolium castaneum* *Oryzaephilus urinamensis* *Rhyzopertha dominica*	Ogendo et al., 2008
Evodia rutaecarpa	essential oil	*Callosobruchus chinensis* *Sitophilus zeamais* *Triboliumcastaneum*	Liu & Ho, 1999
Azadirachta indica	seeds, metabolites, oils	*Hypsipyla grandella* *Plodia interpunctella* *Callosobruchus maculatus* *Plutella xylostella*	Mancebo et al., 2002 Rharrabe et al., 2008 Lale & Abdulrahman, 1999
Artemisia scoparia	essential oils	*Callosobruchus maculatus* *Sitophilus oryzae* *Tribolium castaneum*	Negahban et al.,2006
Dysoxylum malabaricum	leaves	*Anopheles stephensi*	Nathan et al., 2006a
Melia azedarach	leaves, seeds	*Hyblaea puera,* *Plutella xylostella*	Nathan et al., 2006b
Ocimum basilicum	essential oils	*Callosobruchus maculatus*	Kéita et al., 2001
Roldana barba-johannis	aerial parts	*Spodoptera frugiperda*	Céspedes et al., 2004
Myrtillocactus geometrizans	roots, aerial parts	*Spodoptera frugiperda* *Tenebrio molitor*	Céspedes et al., 2005

Table 1. Botanical extracts and their potential use against insect pests.

In fact, it is not so hard to identify botanical extracts which show some kind of activity against insect pests. In the universe of plants, the Meliaceae family has drawn attention. This family comprises 50 genera and 1400 species, mostly distributed in the pantropical zone. Among the genera, the ones which show greater insecticidal activities are *Aglaia, Aphanamixis, Azadirachta, Garapa, Cedrela, Chukrasia, Dysoxylum, Guarea, Khaya, Melia, Soymida, Swietenia, Trichilia, etc*. Most of the plants of this family are trees and are well known for their quality timber (Benerji &Nigam, 1984).

Taking *Azadirachta indica* A. Juss (Meliaceae) (Neem tree) as a highlight, researches have depicted various mechanisms of action for its insect control. More than 100 compounds have been isolated from various parts of the Neem (Luo et al., 1999). It is common to find a range of biological activities, including insect anti-feedant and growth regulating properties, anti-bacterial, anti-fungal and anti-viral activities, anti-protozoal, and anti-sickling properties. In the recent past, chemical constituents of Neem seeds have been intensively explored since they have proved to be an excellent source of a wide variety of chemicals useful to the management of pestiferous insects (Kumar et al., 2003). More than 500 insect pest species are listed as sensitive to Neem seed extracts (Morgan, 2009). These biological proprieties are caused mainly by terpenoid compounds several papers are reported to contain bitter substances, popularly known as limonoids (Figure 1) (Luo et al., 1999; Siddiqui et al., 1999; Siddiqui et al., 2001). The main metabolite of Neem is a limonoid known as azadirachtin (Figure 1).

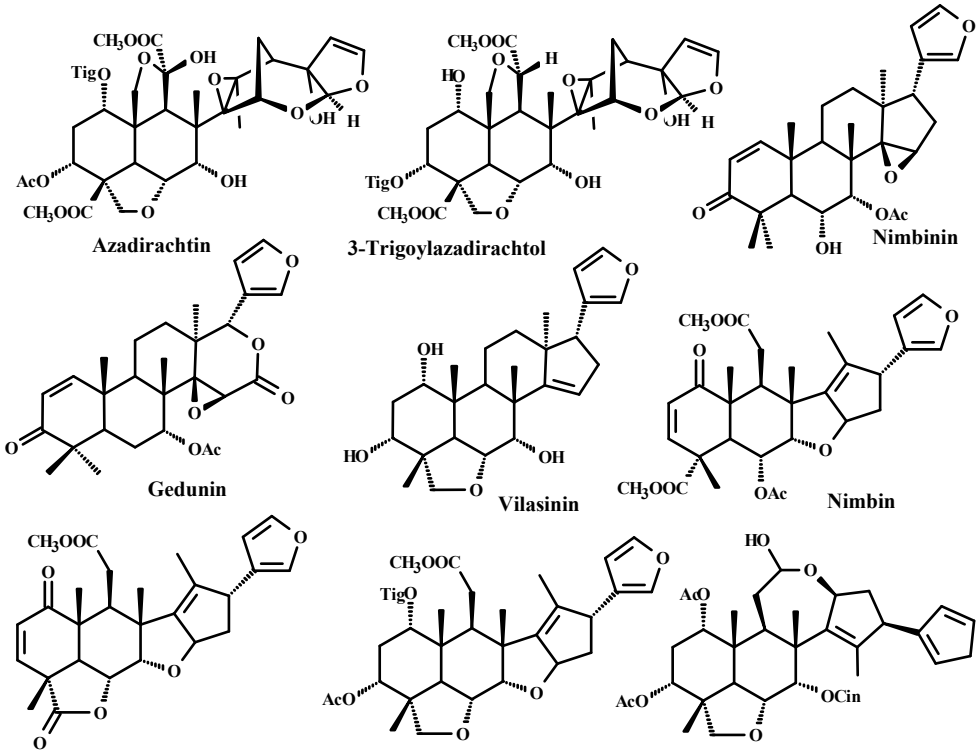

Fig. 1. Main limonoids isolated from species *Azadirachta indica*.

These substances are important enzymatic and metabolic inhibitors. Apart from efficacy and spectrum-of-action described to Neem, its biological criteria still include favorable toxicology and minimal environmental impacts (*i.e.*, vertebrate selectivity; selectivity favoring natural enemies and pollinators; and rapid environmental degradation) (Isman, 1997). Some biological activities by Neem are described in Table 2.

Activity	Botanical extracts	Target species	Source
Acaricidy	Extracts of neem oil	*Sarcoptes scabiei larvae*	Du et al., 2008
Parasiticide	Seed oil	*Muscidifurax raptor*	Ruiu et al., 2008
Antifeedant	Extracts of the seeds	*Schistocerca gregaria*	Butterworth & Morgan, 1971
Endocrine control	Azadirachtin	*Locusta migratoria*	Sieber & Rembol, 1983
Control of pests	Extracts of the leaves	*Spodoptera frugiperda* *Macrodactylus spp.* *Frankliniella spp.*	Montes-Molina et al., 2008
Nematicidy	Extracts of the leaves and cake	*Meloidogyne javanica*	Javed et al., 2008
Growth inhibitor	azadiradione	*Heliothis virescens*	Lee et al., 1988
Antifeedant, Disruptor of insect development, Effective sterilant	Azadirachtin, Azadirachtin-containing extracts	*Schistocerca gregária* *Phormia terrae-novae* *Leptinotarsa decemlineata* *Oncopeltus fnsciatus*	Schmutterer, 1988
Mortality, Control of weight	Extracts of seed kernels	*Nilaparvata lugens*	Nathan et al., 2007
Effects on survival, Fecundity, Development, Oviposition, Feeding	Seed oil	*Plutella xylostella*	Charleston et al., 2006
Insecticide	Seed kernels	*Cnaphalocrocis medinalis*	Nathan et al., 2006c
Oviposition, Larval development, Feeding	Seed oils	*Mamestra brassicae*	Seljasen et al., 2006
Antifeedant	Neem oil	*Hylobius abietis*	Thacker e tal., 2004

Table 2. Different activities identified of *Azadirachta indica* to control several insect pests.

4. Problems associated to the use of natural products

Despite the favorable characteristics of the use of botanical extracts above-mentioned, as well as the large availability of vegetal species, and the huge quantity of works carried out proving their biological efficacy, few products have been commercially available lately. It is possible to identify some limitations inherent to their success though. Problems as low production, regulation by Federal Institutions, difficulty during application, storage, stability, quali and quantitative reproducibility of secondary metabolites, repeatability of biological activity, *etc.*, need to be approached before the botanical extracts are acquired safely (Isman, 1997).

The comprehension of these problems perhaps helps in the understanding of why various authors have described different results of tested insecticidal activity by using botanical extracts of the same species, and at the same time, to learn their efficient manipulation and

correct use. How is it possible for botanical extracts prepared from the same vegetal species to show different results towards a target insect? For instance, Roel et al. (2010) describe a lethal action against the larvae of *Spodoptera frugiperda* by using formulations with 0.4% (w/v) of the Neem oil. However, Viana & Plates (2003) relate a dose of 1.0% (w/v) of aqueous extract of the Neem leaves to obtaining the same action, which was 2.5 times higher concentration than the one related by Roel et al.

Biological activities of botanical extracts come from secondary metabolites, which are present in these materials. Several factors may change the stability of products or active compounds of natural source. Each component, active or not, present in different quantities, may affect the stability of the products. Other factors known as extrinsic such as temperature, radiation, light, air (especially oxygen, carbon dioxide, and steam of water), humidity, seasonality, place and hour of the collection, storage, *etc*., may change the stability and quantity of an active compound or botanical extract (Gobbo-Neto & Lopes, 2007). For example, contend of hypericin and pseudo-hypericin in *Hypericum perforatum* nearly increase 30 times in the summer (Southwell & Bourke, 2001). Concentration of biflavones as ginkgetin in *Ginkgo biloba* leaves also shows seasonal changes (Lobstein et al., 1991). Wallaart et al. (2000) described the reduction of biosynthetic precursor dihydroartemisinic acid happening concomitantly with the production of artemisinin in *Artemisia annua* after metabolic stress caused by low temperature. In *Hypericum perforatum* flowers a significantly rise in concentration of flavonoids, hypericins and chlorogenic acid occur under hydric stress along with the reduction in hyperforin content (Gay et al., 2003). Higher production of phenolic compounds as flavonoids, tannins, anthocyanins, *etc*. are usually observed in plants under high solar radiation. Such a factor has also affected other secondary metabolic classes such as alkaloids and terpenoids (Gobbo-Neto & Lopes, 2007). Also, there are the intrinsic factors such as incompatibility, pH, hydrolyzes, racemization, and oxidation (Barrek et al., 2004).

For example, a rapid photodecomposition of azadirachtin, the main metabolite found in extracts of seeds from Neem, has been observed with spray applications onto conifer and deciduous foliage. Experimental studies showed a dissipation half-life (DT_{50}) to azadirachtin of about 20 h (Sundaram & Curry, 1994). Its short environmental persistence is due to the presence of sensitive moieties such as *p*-electrons, ester linkages, furan, and an epoxide ring (Gopalakrishnan et al., 2001; Wei-Hong & Zhan-qian, 2006). However, the major problem is its sensitivity to photodegradation, therefore it is rapidly lost in sunlight. Through a simple experiment carried out, a pure sample of azadirachtin was solubilized in a mixture of water:ethanol (4:1) and exposed to light, at ambient temperature, during seven days. In the end, a mixture of sub-products of degradation was obtained, considering that the three most abundant sub-products were identified by spectroscopy and spectrometric techniques. These sub-products are shown in Figure 2. Sub-product 3 was isolated and its structure determined in literature, by Kumar et al. (1996), as 1-tigloil-3-acetilazadirachtinin. It is important to highlight that this product was firstly identified as a natural product, however, it could solely have been a product of degradation. These examples show low stability for molecules of botanical extracts as azadirachtin. In this case, it is necessary that new formulations or systems of protection for the insurance and efficient use in field are developed.

Another situation is the degradation of extracts, which happens in commercial products, for instance in this case of Neem (oils of seed kernels). This degradation may occur even when it is stored under appropriate conditions, light shelter, temperature and humidity. In another

work, by using chromatography techniques, it was possible to observe the degradation of azadirachtin in commercial oil of Neem. Samples of Neem oil were left to rest in a dark chamber at 20°C. At specific times, the samples were homogenized and a fraction of those was analyzed. Figure 3 shows the relation between the quantity of azadirachtin and the time. It is easy to observe that the quantity of azadirachtin in these products was gradually decreased during the time of storage. These results are extremely alarming. Considering that azadirachtin has been the most potent secondary metabolite of Neem, its degradation may compromise the efficiency of commercial products (Nathan et al., 2006c).

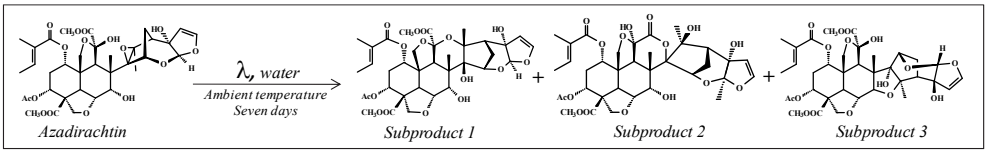

Fig. 2. Identified products of degraded azadirachtin.

Hypothetically, when farmers buy a natural pesticide as Neem oil, in fact they might be buying a product with approximately 1,000 µg kg^{-1} or with 200 µg kg^{-1} containing azadirachtin as standard. Obviously, the results and efficiency in field will be very different. In this case, the efficiency depends on the quantitative and manufacturing data of both examples of Neem oil.

These problems may create a generalized discredit about the use of natural products such as Neem. A not very well-informed consumer may use Neem oil sold after a long period of storage and not have the expected action in field. This farmer will probably not trust in the use of this kind of product anymore. As in any other commercial product, botanical extracts need to be subjected to and monitored by control quality programs.

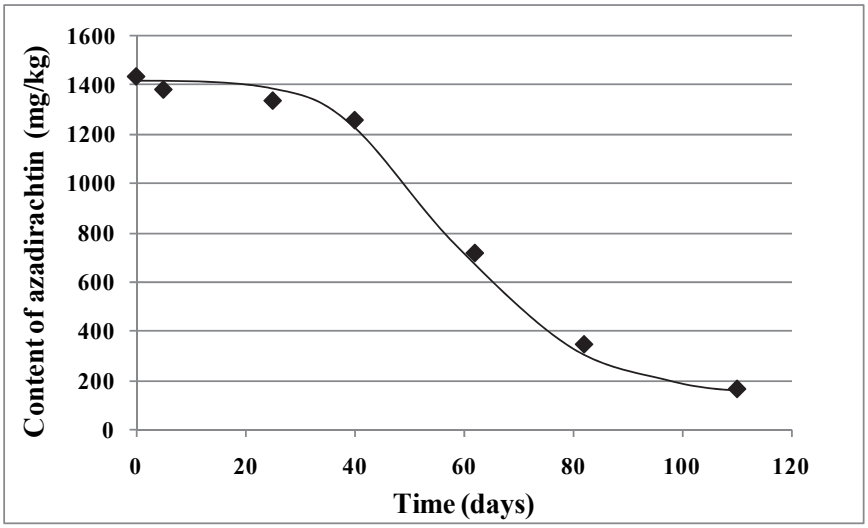

Fig. 3. Degradation curve of azadirachtin to commercial Neem oil in storage.

5. Quality control on natural products

The potential of natural products as agents to control insect pests is clear in several papers which describe their biological activities (Céspedes et al., 2004, 2005; Charleston et al., 2006; Nathan et al., 2006a, 2006b; Negahban et al.,2006; Rharrabe et al., 2008; Torres et al., 2003). However, in order for the use of natural products to thrive, as an alternative to traditional synthetic pesticides, some parameters of production and quality control should be observed, especially a) seasonal variation; b) formulation and stability and c) development of methods to quality control. These concerns introduce the importance of quality control to botanical extracts, which are desirable to be used as the new source of natural pesticides.

Naturally, the quali and quantitative composition of secondary metabolites in a botanical extracts vary according to how plants are affected by genetics, ontogenesis and environment factors in which they are cultivated (Arimura et al., 2005). In studies carried out by Forim et al. (2010a) using seed kernels of Neem from various Brazilian regions analyzing the content of azadirachtin e 3-tigloylazadirachtol by HPLC, two of the main limonoids responsible for the insecticidal activity of Neem (Sidhu et al., 2003), it varies from 1,516.4 to 5,117.1 mg kg^{-1} and from 224.7 to 1,116.5 mg kg^{-1}, respectively (Forim et al. 2010a).

Genetics and seasonal variations are not easy to control. However, the content of quality markers to botanical extracts, such as azadirachtin (Figure 1) to Neem extracts should be monitored. The quantitative knowledge of these markers allows commercial products to be prepared by reproducing the amount of such markers and, consequently, the biological efficacy of these products.

In general, one or two markers of the active components in botanical extracts are currently employed to evaluate their quality and authenticity. This kind of analysis, however, does not give a complete profile of a botanical product because multiple constituents are usually responsible for its pesticide effects. These multiple constituents may work "synergistically" and can hardly be separated into active parts. Thus, it should be necessary to determine most of the phytochemical constituents in order to ensure the reliability and repeatability of the botanical pesticide effect.

Considering the large diversity of compounds present in one botanical extract, several, slow and expensive chromatographic techniques, such as high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), *etc*. should be applied to this kind of documentation (Liang et al., 2004). In addition to these techniques, the following technological revolution which had a tremendous impact upon the analysis of natural products was the development of detectors such as electrospray ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR). These detectors may be utilized online combined with chromatography techniques as a powerful complement to HPLC detection system (Strege, 1999). Through the use of these techniques, along with appropriate procedures of sample preparation, it is possible to develop methods of high-throughput screening identifying and quantifying botanical extracts. However, these methods are expensive for routine use.

On the other hand, through the use of markers it is possible to simplify the demand of equipment, time and the cost of analyses, which make possible the use of simple analytical methods during the development of new botanical products. As a matter of fact, this procedure should be a routine practice. In general, simple HPLC equipment is necessary. When coupled with phododiode-array UV-Vis absorbence detection, HPLC serves as a powerful tool for the rapid characterization of natural product extracts (Strege, 1999).

The step of sample preparation for the analysis of constituents present in botanical extracts is as important as choosing the analytical instrumentation. This procedure may be selective or specific to botanical markers, or not, when the aim is a full characterization of the extract. This step involves two parts: extraction and pre-treatment phases. The main methods of botanical extracts preparation are sonication, soxlet extraction, microwave assisted extraction, supercritical fluid extraction, accelerated solvent extraction, pressurized hot water extractions, *etc.*, by using solvents as methanol, ethanol, water, a mixture of them, carbon dioxide, *etc.* (Ong, 2004). Pre-treatment steps may be performed by using solid-phase extraction, solid-phase microextraction, matrix solid-phase dispersion, filtration, dilution techniques, *etc.* (Rijke et al., 2006). It is very important to be sure that in extracting procedures the extraction efficiency is as high as possible, and that during the pre-treatment steps there is nether loss nor degradation of analytes.

6. Methods of Neem quality control

In the case of Neem, azadirachtin, previously known as azadirachtin A, is a good quality marker. The amount of this limonoid may be easily determined in commercial products, organic extracts, seed kernels and cakes by using HPLC (Forim et al., 2010a). In our laboratory, we have been using a HPLC of Agilent 1200 Series Liquid Chromatography apparatus (Agilent Technologies, Santa Clara, USA), configured with a degasser G1322A, quaternary pump G1311A, autosampler G1329A, column oven G1316A and a simple UV detector G1314B. The degasser and quaternary pump are optional, which may be replaced by a simple pump simplifying the instrumental demand. Chromatography run is a reversed-phase procedure utilizing a stainless steel Zorbax Eclipse XDB®C18 column (150x4,6 mm i.d., 5µm particle size, Agilent, USA) fitted with a Phenomenex® C18 (4x3mm i.d., 5µm particle size, Torrance, CA, USA) security guard cartridge. The control of the HPLC system, acquisition and processing of the data collection are realized by Agilent Technologies EZCrom SI software (G6702AA).

The chromatographic analyses were performed in isocratic mode. The mobile phase consists of acetonitrile and water (35:65, v/v). The column temperature is maintained at 30°C. The flow rate is 1.0 ml min^{-1} with an injection volume of 10 µl. All experiments are performed at 217 nm. This wavelength was selected because it is a UV maximum and provides the sensitivity needed for the quantification of the low markers concentration in the samples (Forim et al., 2010a). Other analytical methods applied to the quality control of botanical extracts of Neem have been described by Sharma et al. (2003) by using HPLC-PDA under isocratic conditions; Shidu et al. (2003) by using HPLC-UV under gradient conditions; Thejavathi et al. (1995) by using HPLC-UV under gradient conditions and work with internal standard, *etc.* These methods have been successively well employed in quality control of Neem products.

It is important to highlight that all analytical methods needed to be previously validated in order to be reliable. HPLC methods usually use parameters of validation such as linearity, specificity, accuracy, precision, robustness, recovery, limits of quantification (LOQ) and detection (LOD), and repeatability (ICH, 1996).

A good linearity to the analysis of Neem products was found from 1 to 70 µg ml^{-1}; the limits of detection and quantification were smaller than 1.0 and 0.3 µg ml^{-1}, respectively; the precision and accuracy were inferior to 3%. Azadirachtin and 3-tigloylazadirachtol

showed a separation factor (α) of 1.10 and the resolution (R_s) was 2.09 between themselves (Forim et al., 2010a). Figure 4 shows a chromatogram of the analysis of azadirachtin and its selectivity between 3-tigloylazadirachtol in methanolic extract of seed kernels from *Azadirachta indica*. As important as developing and validating a new analytical method is to identify an efficient procedure to prepare the samples. Extracts of seed kernels or Neem oil undergo a process of clean up by using cyano solid extraction phases columns before HPLC analyses.

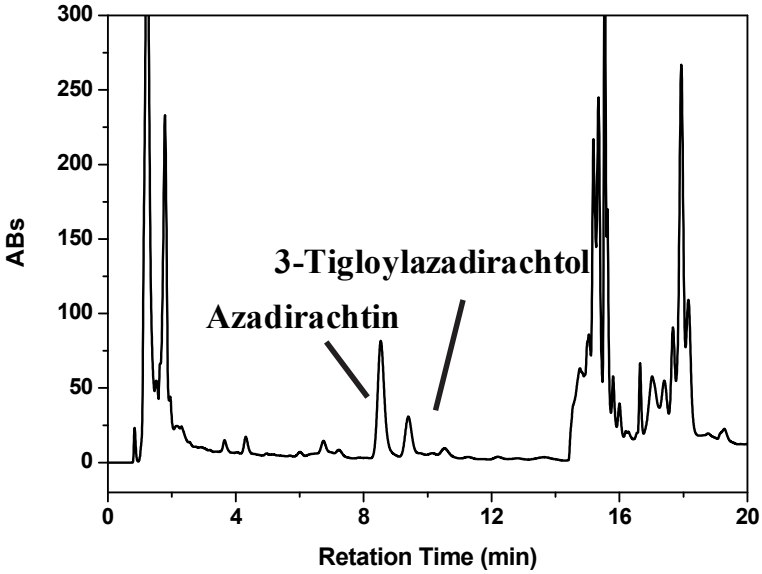

Fig. 4. Standard chromatogram of analysis of *Azadirachta indica* products.

7. Biological activity of Neem and analytical methods

Methods as above described are useful to control the quality of botanical extracts and help in the connection of these products with their botanical assays and the desired insecticidal activity. Forim et al. (2010b) published a work in which they use this HPLC method to evaluate the biological activity of Neem extracts. In this work several Neem extracts were obtained from different ways and assayed against *Spodoptera frugiperda,* an important maize pest. All botanical extracts were prepared by using the same seed kernels (lot). In this case, changes in genetics, ontogenesis and environment factors did not occur among Neem extracts prepared. The only change was in the way of preparing the extracts.

Techniques of extraction were: a) maceration of mill seed kernels by using two solvents at ambient temperature: firstly, maceration by using *n*-hexane (5 x 12 hours) which created a non-polar extract and a cake, and secondly by using ethanol (5 x 12 hours) to extract more polar compounds of the cake (**MHE**); b) maceration by using methanol (5 x 12 hours) at ambient temperature (**MM**); c) maceration by using ethanol (5 x 12 hours) at ambient temperature (**ME**); d) maceration by using ethanol under constant agitation (5 x 12 hours) at ambient temperature (**MEA**); e) extraction by ethanol using ultra-son (5 x 10 minutes) at

ambient temperature (**EU**); f) extraction by ethanol using centrifugation (5 x 10 minutes) at ambient temperature (**EC**); and g) maceration by ethanol under simple agitation (5 x 10 minutes) at ambient temperature (**MV**). At the end, all solvents were evaporated by using rota-evaporator, producing dry botanical extracts which were assayed against *Spodoptera frugiperda.*

Through the HPLC analytical method for Neem previously described, it was possible to determine the quantity of this marker in the seed kernels utilized in the extraction processes, and in the final Neem extracts, which made the calculations of extraction efficiency possible. The amount of azadirachtin in each extract and the extraction efficiency of each process are described in Table 3.

Extract	Extraction Efficiency (%)	Amount of azadirachtin (mg kg^{-1})
MHE	100.1	32,480.3
MM	54.3	12,070.8
ME	100.1	21,046.9
MEA	99.1	19,534.8
EU	56.6	29,464.6
EC	45.1	1,385.0
MV	58.0	18,459.2

[a] Relative Standard Deviations among extracts were smaller than 12% (n = 3); [b] Initial amount of azadirachtin in seed kernels was 2,348.5 mg kg^{-1}.

Table 3. Amount of azadirachtin in Neem extracts, which were assayed against *S. frugiperda*.

Naturally, each process showed individual yield in both the extraction mass and extraction efficiency. It is important to observe that different extraction processes produced singular botanical extracts, which means that each process was able to withdraw a specific quantity of secondary metabolites. Similarly to the stability problem previously described, in which 1 kg of commercial Neem oil may present different biological activities from another one in an advanced degradation stage, botanical extracts will certainly show a distinct action in insect control.

As a matter of fact, each botanical extract of Table 1 was assayed against *S.frugiperda* in concentrations of 100, 250 and 1000 mg of extract to 1,000 g of artificial diet (Forim et al., 2010b). The diet prepared at a concentration of 100 mg kg^{-1} by using the **MHE** extract shows the best results having 100% of mortality of *S.frugiperda* larvae. **EU** extract displayed a tendency to prolong the larval phase to 10 days, a reduction in 35% of the pupal weight, and presenting 70 % of mortality, approximately. The **EC** extract itself did not show biological activity. Results of this experiment are described in Table 4. In these experiments, it is easy to observe the importance of the composition of metabolites as azadirachtin. The higher the quantity of azadirachtin, the better were the results.

Samples prepared with 250 mg of extracts in 1,000 g of diet assayed against *S. frugiperda* also showed a relation between the content of azadirachtin and the insecticidal action. It is possible to observe in Figure 5 that the biological action increased along with the quantity of extracts in diets, *i.e.* the increase which occurred was in the content of azadirachtin, thus making the mortality of *S. frugiperda* larvae rise. Again, the **EC** extract showed the worst results against the target insect. Diets with 1,000 mg of extract in each 1,000 g (0,1% w/w)

showed 100% of mortality, except when prepared by using the **EC** extract (90 % of mortality after 15 days).

Extract	Larval phase (days)	Pupal phase (days)	Mean weight gained (mg)	Mortality (%)
EU	27.67	11.50	171.67	70.0
MV	17.80	11.00	251.60	60.0
MEA	19.12	10.62	217.12	20.0
ME	15.89	10.11	276.78	10.0
MM	16.10	10.00	267.90	10.0
Controle	17.56	9.56	267.89	10.0

Temperature: 25±2 °C; UR: 70 ± 5%; Fotophase of 12 h; After 10 days of incubation, mean of ten replicates (n = 10).

Table 4. Comparison of the efficacy of the diets prepared against larvae of *S. frugiperda* by using 100 mg kg^{-1} of different Neem extracts.

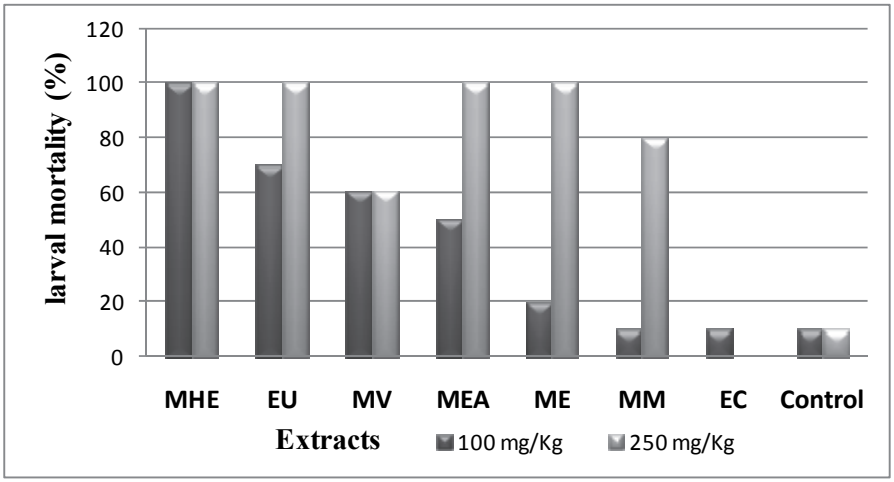

Fig. 5. Average mortality of *S. frugiperda* larvae fed by artificial diet prepared by using extracts of Neem at 100 and 250 mg kg^{-1}.

In these experiments, there is a strong relationship between the biological activity and the content of azadirachtin. Simple calculations of the amount of azadirachtin in diets showed that 100% of mortality was obtained just when this molecule was higher than 3 mg kg^{-1} (Table 5). These data establish a minimal limit of azadirachtin which needs to be applied to crops in order to reach the success on *S. frugiperda* control. Furthermore, these data may also be used in industrial.

Obviously, the relations above described could only be observed through the use of monitoring methods such as HPLC. At the same time, such relations reinforce the concern on natural products traditionally sold without any programs or processes of quality control.

Extract	Concentration of Azadiractin (mg kg^{-1})		
	100 mg kg^{-1} (Extract/Diet)	250 mg kg^{-1} (Extract/Diet)	1,000 mg kg^{-1} (Extract/Diet)
MHE	3,2	8,1	32,5
MM	1,2	3,0	12,1
ME	2,1	5,3	21,0
MEA	2,0	4,9	19,5
EU	2,9	7,4	29,5
EC	0,1	0,3	1,4
MV	1,8	4,6	18,5

Table 5. Concentration of azadirachtin in diets used in assays against *S. frugiperda*.

8. Formulations of botanical insecticides

Problems such as seasonal change, genetics, ontogenesis and production techniques may compromise the biological efficacy of natural products. Simple formulation methods and incorporation processes of botanical extracts into commercial products may minimize these problems. The commercial Neem oil is usually extracted from seed kernels of the Neem tree by pressing the seed kernels which are crushed and squeezed. This releases and separates the oil and the cake. Some of the active ingredients in Neem oil are susceptible to heat, therefore oil cold pressing is recommended. Completing the process, the remaining Neem seed cake is extracted with hexane. Obviously, for reasons already discussed before, the azadirachtin content in these commercial products may change constantly.

As it also occurs to synthetic pesticides, commercial products of botanical extracts need to be properly formulated. This development has led to a need for a wide range of product formulation types, additives and technological processes to prepare formulation of botanical extracts with various physical and chemical properties (Knowles, 2008).

Commercial Neem oil containing emulsifier agents may be formulated by using botanical extracts with a higher concentration of azadirachtin. Neem oils usually show a range of azadirachtin amounts from 300 to 1,500 mg kg^{-1}. In order for the product to be successful and viable to agricultural crops, it is necessary to always prepare the commercial formulations with the same content of this marker. Neem oils containing 1,000, 2,000 or 4,000 mg kg^{-1} of azadirachtin may be prepared by either the dilution process by using another poor Neem oil or Neem extracts containing a higher quantity of azadirachtin. For instance, specific quantities of Neem ethanolic extracts, easily prepared by phytochemical techniques with amounts of azadirachtin higher than 5%, may be incorporated in oil matrixes resulting in commercial products with reproducible contents of active compounds. Figure 6 shows chromatograms of enriched Neem oils which were prepared with different concentration of azadirachtin. In these formulations, specific quantities of extract and Neem oil were utilized, which had 61,698 mg kg^{-1} and 738 mg kg^{-1} of azadirachtin, respectively.

Again, these formulations and planning are only possible through the use of monitoring analytical methods, which also help in self-life studies. Otherwise, the commercial products would just be simple botanical extracts without any guarantee of biological activity reproducibility.

However, the ability of reproducing the content of a marker, many times, is not enough to guarantee the stability and quality of botanical extracts. By using the Neem as an example,

the azadirachtin is extremely labile in the presence of sunlight decreasing its biological and residual efficiency in field. In this case, knowing the chemical profile of Neem oil and/or extracts is not enough. For purposes of effective use, the azadirachtin molecule must be stabilized.

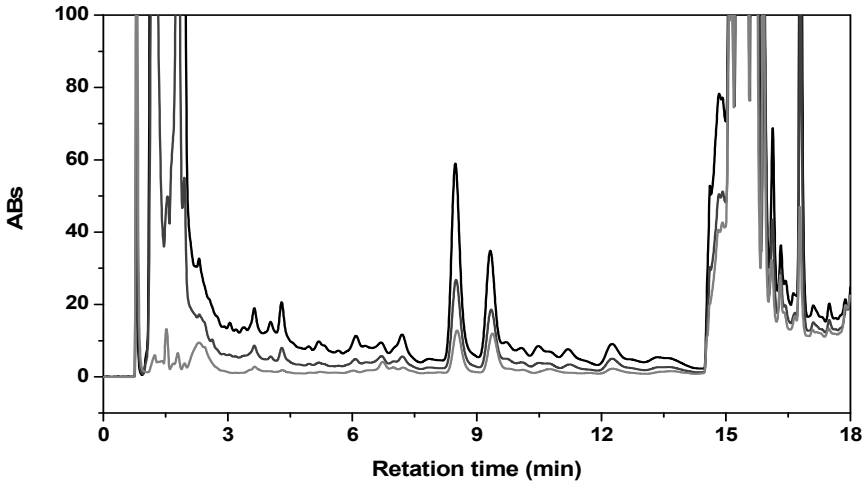

Fig. 6. Chromatograms of enriched Neem oil containing 1,000, 2,000 and 4,000 mg kg^{-1} of azadirachtin.

The addition of UV absorbing compounds to the formulations were found to protect photolabile pesticides, thereby extending their environmental life (Hussain, et al., 1990; Fowler et al., 1994). The UV absorbers can either absorb light preferably and prevent photo-excitation of the pesticide or accept the excess energy from the already excited pesticide molecules by different energy transfer or charge transfer mechanisms, thus extending the life-span of the molecule. Sundaram & Curry (1996) published a work by using UV absorbing compounds to stabilize the azadirachtin. Photostabilization of neem-based azadirachtin insecticide applied onto glass surfaces was studied in the presence of three UV absorbers, 2.4-dihydroxybenzophenone (Uvinul M-400, UM), 4-aminobenzoic acid (PABA), and fluorescent brightener-28 (FB-2X), a stilbene disulfonic acid derivative. The UV absorber UM, provided excellent protection, increasing the dissipation of half-life (DT_{50}) of pure azadirachtin from 3.87 to 22.54 days. On the other hand, the photostabilization due to PABA was marginal. The UV absorber, FB-2X acted as an effective photosensitizer, reducing the DT_{50} of azadirachtin from 3.87 to 0.31 days.

Kumar & Parmar (1999) formulated the azadirachtin employing either anthraquinone or epichlorohydrin as stabilizers in clay-based powders or Neem oil. Such compounds reduced the degradation rate by 26-60% compared to Neem oil, during the 14-day heat storage studies at 54 ± 1 °C in the laboratory. Other products used to control the azadirachtin stability were the addition of antioxidants such as ferulic acid, gallic acid, and rutin resulting in a moderate degree of photostabilization (Wei-Hong & Zhan-Qian, 2006). These examples show how the good laboratory practices may help during the development of commercial products of botanical extracts. They also highlight the importance of analytical methods to observe the associated phenomena.

The stability of Neem products have also been investigated through techniques of encapsulation in which several matrixes are used. Riyajan & Sakdapipanich (2009a) prepared capsules containing Neem extracts. Controlling the release of the biopesticide was achieved by the use of glutaraldehyde-alginate gel capsules modified by coating with a natural rubber layer. This work shows that the degree of release of azadirachtin (marker) from capsules into an aqueous environment was controlled by their formulation condition. In another work, Riyajan & Sakdapipanich (2009b) encapsulated Neem extracts into microcapsules by using hydrolyzed poly(vinyl acetate) crosslinked with glutaraldehyde by Spray-Drying technique to control its release and photodegradation stability. Sreenivasa et al. (2006) report an improved granular formulation of Neem seed extract having enhanced the storage stability, and the ability of a gradual release of azadirachtin for application to the plant rhizosphere. The formulations consist of an inert particle compound as a carrier, at least one lipophilic substance as a deactivator/binder, colorant and Neem extracts.

Nowadays, a recent technology has been revolutionizing the agribusiness: it is the nanotechnology. It has been presented in several areas of research such as material engineering (polymers, ceramic, metals), semiconductors, health and medicine, pharmaceutical, textiles, cosmetics, pesticides, *etc.* Among different techniques and concepts of nanoparticles, the polymeric nanoparticles have been a sophisticated approach towards agrochemical formulations, which may be applied into the search of new proprieties and efficient use of botanical extracts as Neem.

Nanoparticles are defined as colloidal polymeric particles containing an active compound including nanocapsules and nanospheres. Nanocapsules are carries composed of an oil core surrounded by a polymeric wall, whereas nanospheres consist of a polymeric matrix. Both colloids are stabilized by surfactants at the particle/water interface (Schaffazick et al., 2006). Both systems, nanocapsules and nanospheres, may be employed as support to delivery-controlled biopesticides programs or as stabilizer agents with several molecules such as azadirachtin. These systems can be prepared by methods based on the polymerization of dispersed monomers or the dispersion of a preformed polymer (Mora-Huertas et al., 2010).

In our laboratory, we have developed polymeric nanoparticles containing Neem oil and extracts. The main technique which has been employed is the modified interfacial deposition of a preformed polymer (nanoprecipitation) as described by Fessy et al. (1989). This method is based on the interfacial deposition of a polymer following the displacement of a semi-polar solvent miscible with water from a lipophilic solution. In these works, nanoparticles, nanocapsules and nanospheres, in colloidal suspension and in powders have been produced. The powders have been produced through Spray-drying techniques and by using drying inorganic support. The aims have been to enhance storage and UV stability for azadirachtin, to improve its dispersion in aqueous phases and to control the ability of azadirachtin release. The polymers employed are usually biopolymers, *i.e.* polymers which are biodegradable in a brief time. Whenever, a botanical extract is formulated, it is very important to employ materials which are compatible with desired features of natural products such as being environment-friendly. Figure 7 shows nanoparticles of Neem prepared by this technique.

Through the use of different quantities of Neem extracts in nanoparticles formulations, which have higher azadirachtin concentration, it was possible to prepare colloidal suspensions containing a larger range of this marker. The values of absolute recovery and entrapment efficiency for three nanoparticles formulations containing different azadirachtin concentrations are described in Table 6. Nanoencapsulated agrochemicals should be

designed in such a way that they possess all necessary properties such as effective concentration (with high solubility, stability and effectiveness), time controlled release in response to certain stimuli, enhance targeted activity and less ecotocity with safe and easy mode of delivery thus avoiding repeated application (Nair et al., 2010).

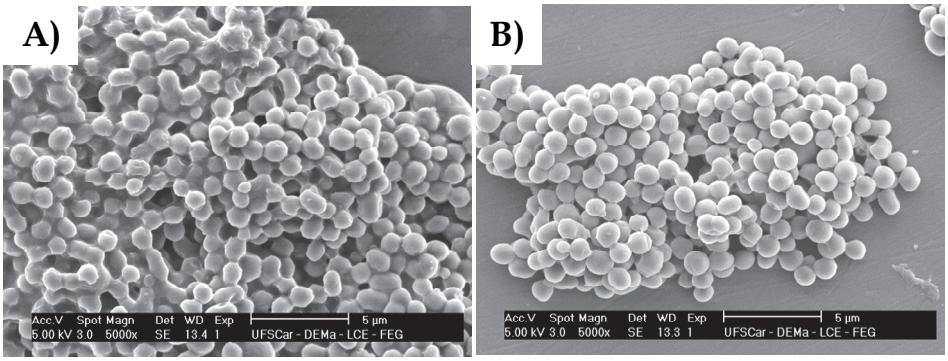

Fig. 7. Photomicroscopy of A) nanocapsules and B) nanospheres of PCL loaded with Neem extracts.

Formulation	Nominal concentration (µg ml^{-1})	Absolute recovery (%)	Entrapment efficiency (%)
01	2.200,0	102,2 ± 1,89	98,7 ± 0,01
02	2.800,0	99,2 ± 1,03	98,8 ± 0,04
03	3.400,0	95,8 ± 2,00	98,8 ± 0,01

The values were expressed as average result ± standard deviation (n = 3)

Table 6. Quantitative analysis of nanocapsules containing Neem extracts in colloidal suspension.

It is possible to observe that the nanoparticle production process did not affect the azadirachtin stability. Another important information in Table 4 is that the quantity of azadirachtin in formulation number 3 into a colloidal aqueous phase represents thirteen times the capacity of the azadirachtin to be solubilized in water. When nanocapsules and nanospheres were prepared, the average particle sizes were nearly 240 nm and 120 nm, respectively.

Through the Spray-drying technique nanoparticles containing Neem extract in powders were obtained. This process removes the water presence thus increasing the stability of the Neem product. Colloidal suspension and dried material were subjected to UV stability assays. This experiment was carried out in a mirror camera at 30 °C. The results are illustrated in Figure 8. This experiment shows that azadirachtin nanoencapsulated was more stable, confirming the importance of the use of an appropriate formulation. Furthermore, the products without water show a greater stability.

Obviously, more stable products will present a larger time of self-life and a higher residual action in fields. They will have ways to conserve the chemical profile and active molecules of botanical compounds causing direct impacts on the biological action and their efficacy in

field against insect pests. Thus, enriched or micro/nano-structured natural products are important tools to control the botanical action of these products in field.

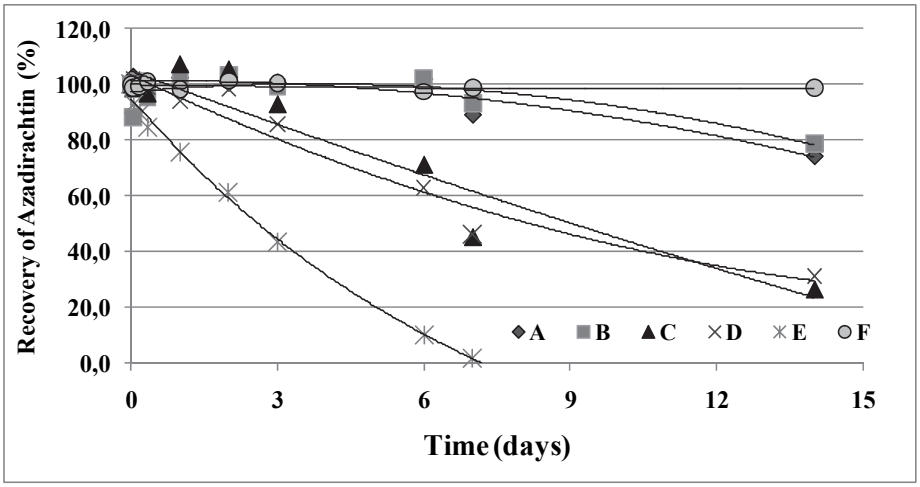

Fig. 8. Recovery of azadirachtin after UV radiation. A) Nanocapsules in powder without tensoactive; B) Nanocapsules in powder with tensoactive; C) Nanocapsules in colloidal suspension without tensoactive; D) Nanocapsules in colloidal suspension with tensoactive; E) Neem oil and F) Neem oil coated of UV radiation.

These results intensify the idea of the importance of correct formulations which need to be applied to obtain commercial botanicals. Moreover, these results also show the importance of the use of analytical methods during the development of botanical products. Through analytical methods, it is possible to monitor all the steps of development

9. Conclusions

Sustainable growth in agriculture is crucial for most of the developing countries to provide for the growing populations. Synthetic chemicals for crops protection are associated with pest resurgence, impact on non-target organisms, health and environment. Hence there is the need to develop safe alternative crop protectants, which should be more specific and cover a larger range of activities (Nathan et al., 2006c). A large number of different plant species representing different geographical areas around the world have shown to possess phytochemicals (secondary metabolites) that are capable of causing a range of insecticidal effects. Through the use of these plants it is possible to obtain botanical extracts which may be utilized in commercial bioinsecticidal formulations. Among these species, the *Azadirachta indica* has had an outstanding position. However, problems such as seasonal changes, genetics, ontogenesis and production techniques may compromise the reproducibility and biological efficacy of botanical extracts. These limitations have been easily observed through analysis in different Neem products. Such results enhance the importance of studies about preparation, manipulation, storage, formulation, and quality control of natural products before their agricultural application.

These problems may be minimized by using appropriate formulations. It is possible to use the techniques of enrichment, application of additives, or developing micro or nano formulations. Furthermore, real control in commercial natural products is just possible by using monitoring analytical methods. Chromatography methods of analysis have the potential to determine the quali and quantitative chemical profile. The method used is required to identify the active or marker compounds, composition analysis and fingerprinting purposes. The complex relation between the chromatographic analysis and efficacy of botanical extracts is the most important aspect for the quality control of botanical products. Chemical analysis of extracts from plant material will play a central role in the development and modernization of biopesticides. Chemical fingerprints or content of markers analysis might be linked to biological assays to provide assurance of efficacy and consistency of botanical extracts (Liang et al., 2004).
Thus, the researches concerning the relation between the chromatographic analysis and formulated extracts to efficacy of bioinsecticides are urgent requirements for the quality control of botanical products.

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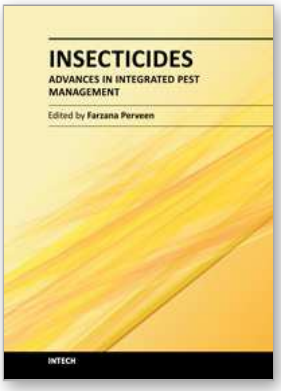

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This book contains 30 Chapters divided into 5 Sections. Section A covers integrated pest management, alternative insect control strategies, ecological impact of insecticides as well as pesticides and drugs of forensic interest. Section B is dedicated to chemical control and health risks, applications for insecticides, metabolism of pesticides by human cytochrome p450, etc. Section C provides biochemical analyses of action of chlorfluazuron, pest control effects on seed yield, chemical ecology, quality control, development of ideal insecticide, insecticide resistance, etc. Section D reviews current analytical methods, electroanalysis of insecticides, insecticide activity and secondary metabolites. Section E provides data contributing to better understanding of biological control through Bacillus sphaericus and B. thuringiensis, entomopathogenic nematodes insecticides, vector-borne disease, etc. The subject matter in this book should attract the reader's concern to support rational decisions regarding the use of pesticides.

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