Microwave-Assisted Extraction (MAE) of Neem and the Development of a Colorimetric Method for the Determination of Azadirachtin Related Limonoids (AZRL)

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Extraction and Colorimetric Determination of Azadirachtin Related Limonoids

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ABSTRACT

Jianming Dai M. Sc. (Agr. & Biosystems Eng.)

Microwave-Assisted Extraction (MAE) of Neem and the Development of a Colorimetric Method for the Determination of Azadirachtin Related Limonoids (AZRL)

A colorimetric method was developed to determine the quantity of total azadirachtin related limonoids (AZRL) in neem extracts. A mathematical model was also developed to aid in the multivariate calibration technique for the analysis of the spectra. With this model and the multivariate calibration technique, the colorimetric method can be used directly to analyse the purified neem seed kernel extracts and to eliminate interferences from other absorbing species. The AZRL and simple terpenoids (ST) content in the neem seed kernel, the seed shell, the leaf and the leaf stem was determined with conventional extraction method and the newly developed quantification technique. The results showed that the AZRL content in these parts of neem decreases in the order of: seed kernel > leaf > seed shell > leaf stem. With the HPLC quantification technique, the content of azadirachtin in the neem seed kernel was determined, and the comparison of the azadirachtin content and the AZRL content suggested that azadirachtin accounts for around 58% of the total AZRL. Microwave-assisted extraction (MAE) of AZRL and ST from various parts of neem was also investigated. Various parameters affecting the extraction such as the power and the microwave irradiation time were studied. The comparison of the MAE with two conventional extraction methods, viz., room temperature extraction (RTE) and reflux temperature extraction (RFX) revealed that the property of sample matrix affected the special accelerating effect of the MAE. The study on the influence of solvents on the MAE showed that the solubility of the solvent to the target components and the ability of the solvent to absorb microwave energy played an important role in MAE.

RÉSUMÉ

Jianming Dai M.Sc. (Génie Agricole et des Biosystèmes)

L'Extraction Assistée par Micro-onde de Lilas des Indes et le Développement d'une Méthode Calorimétrique pour la Détermination Quantitative des Extraits de Terpénoïdes (Azadirachtine, Limonoïdes)

Une méthode colorimétrique en deux étapes et deux phases a été développée pour la détermination quantitative des extraits de terpénoïdes (azadirachtine, et de triterpènes) de graines du Lilas des Indes (Margousier ou Neem *Azadirachta indica*). Une méthode basée sur un modèle mathématique a été développée afin de faciliter le calibrage, à variables multiples, de l'analyse fondée sur la mesure des couleurs. De ce fait, la méthode colorimétrique peut être directement utilisée dans l'analyse des extraits purifiés de graines du lilas des Indes, de même que dans l'analyse des extraits obtenus de l'écorce, de la feuille et de la tige du lilas des Indes. La composition en azadirachtine et terpénoïdes simples des graines, de l'écorce, des feuilles et de la tige du lilas des Indes a été déterminée à l'aide d'une méthode traditionnelle d'extraction, et à l'aide de la nouvelle méthode de colorimétrie. Les résultats ont démontré que la composition, du lilas des Indes, en azadirachtine et terpénoïdes simples est décroissante dans cet ordre: graine > feuille > écorce > tige. La concentration en azadirachtine a été mesurée avec la méthode quantitative HPLC, et cette concentration suggère que l'azadirachtine représente 58% du total des terpénoïdes contenus. L'extraction assistée par micro-onde de l'azadirachtine et des terpénoïdes simples de différentes parties du lilas des Indes à fait l'objet d'une étude. Plusieurs paramètres tels l'intensité micro-onde et le temps d'exposition ont été étudiés. L'extraction assistée par micro-onde a été comparée à deux méthodes d'extraction traditionnelles, soit l'extraction à température ambiante et l'extraction en phase vapeur. Cette comparaison a révélé que la propriété de la matrice de l'échantillon influençait directement l'effet accélérateur de l'extraction assistée par micro-onde. Une étude de l'effet des solvants sur l'extraction assistée par micro-onde a démontré que la solubilité du composé cible dans le solvant et la capacité du solvant à absorber l'énergie micro-onde, ont un rôle important à jouer dans l'extraction assistée par micro-onde.

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THESIS FORMAT

This thesis is prepared in manuscript format in accordance with the Part C of the "Guidelines for Thesis Preparation." Here I quoted the entire text that applies to this format:

> *"C. **MANUSCRIPT-BASED THESIS:** Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.) The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.*

In accordance with the above statement, this thesis is in the following structure:

The thesis started with a general introduction in Chapter I to state the background of this project. In Chapter II, a literature review is provided. Chapters III to VI are manuscripts. The manuscripts are linked via connecting statements. A general conclusion and

recommendation for future work is presented in Chapter VII. All the references cited are listed in the References Section of the thesis.

CONTRIBUTION OF AUTHORS

Chapter II in combination with Chapter V has been accepted by Journal of Agricultural and Food Chemistry and coauthored by Jianming Dai, V. Yaylayan, G.S.V. Raghavan and J.R.J. Paré. Most of the work is done by Jianming Dai, author of the thesis. Professors Yaylayan and Raghavan are co-supervisors and Dr. Paré is a collaborator in this paper.

CHAPTER I

GENERAL INTRODUCTION

1.1. Introduction

The neem tree, *azadirachta indica*, is a plant that is widely distributed through the tropics and subtropics. From the time immemorial, the medicinal properties of neem have been recognized by the people of India. The Sanskrit name of neem is *Arishtha*, reliever of all sickness, and the earliest Sanskrit medical writings have described the medical properties of all parts of neem, the leaf, twig, bark, seed, root and the flower (Champagne *et al.*, 1992). The pesticidal values of neem have also been recognized by the farmers for a long time. Traditionally, farmers in India mix the neem leaves with stored grains to protect the grain from insect pests. The "neem tea", produced by soaking crushed neem seed in water, was used by farmers to protect crops from various pests.

Even though, the pesticidal properties of neem has been known from the ancient times, it was not until 1927 that the repellent properties of neem was reported by Mann and Burns (1927), who observed that neem leaves were not eaten by locust during the locust cycle in 1926-1927. The first demonstration on the antifeedant property of neem was made in 1962 by Praghan *et al.*, (1962) who observed that as low as 0.1% aqueous neem kernel suspension was able to provide complete protection to treated foliage against desert locust S. *grearia* and migratory locust *Locusta migratoria*. This discovery attracted the attention of biologists, chemists, entomologists from all over the world, resulting in a detailed investigation of neem as a pest control agent.

Studies revealed that the neem seed is abundant in limonoids which are responsible for most of the pesticidal properties and part of the medicinal properties of neem. To date, around two hundred limonoids have already been isolated and identified from neem. Azadirachtin, one of the limonoids, is believed to be the most important ingredient in the neem seed due to its abundance and high pesticidal properties. For this reason, azadirachtin

content was widely accepted as the standard for the determination of the quality of the neem seeds and for the determination of the grade of the neem-based pesticides. The azadirachtin content in the neem seed extracts or in the commercial neem-based pesticides can be estimated by HPLC quantification method with commercial azadirachtin (95 % purity) as the standard (Warthen *et al*, 1984; Yamasaki *et al*, 1986).

1.2. Identification of the problems

1.2.1. The standard for determining the quality of commercial neem based pesticides

As azadirachtin is not the only active principle in the seeds or in the commercial neem-based pesticides, it might be too arbitrary to use one of the active principles as the standard for the determination of the quality. Since most of the limonoids were reported to be pesticidally active, it might be more reasonable to use the content of total azadirachtin related limonoids as standards.

1.2.2. Quantification of the total limonoids in the neem extract

The HPLC technique is a very powerful quantification method. With appropriate separation condition and the standards for each component, this method can not only be used to determine the quantity of a single component in a mixture, but also to quantify individual components in a mixture. However, as far as the analysis of neem extracts is concerned, it is still impossible to use the HPLC method for determination of all the components in the extracts. Around 200 different limonoids in neem are known to exist and it is impossible to find a separation condition to separate all of these components by HPLC method. Thus, the quantification of all of the components becomes impossible. Furthermore, the lack of standards for most of the components makes the quantification even more difficult. As a result, if one or a few of the components of the neem extract are to be quantified, the HPLC technique can be a good choice. However, if all the component are to be analysed or even the amount of total limonoids are to be determined, a new method need to be developed.

1.2.3. Possible solution

Colorimetric method followed by visible spectroscopic technique is one of the earliest analytical techniques used for the determination of a group of components in a mixture. This method is especially useful for the analysis of the natural products which are very complicated mixtures. Therefore, this method may provide a solution for the quantification of the total limonoids in the neem extracts.

1.2.4. The status of neem-based pesticides

The synthetic pesticides have played an important role in pest control, but at the same time it appears that they are also causing more and more serious ecological and environmental problems. For this reason, people are turning to the biological world for the solution to control the pest problems. Neem is one of the most attractive plant-based pesticides.

1.2.5. The production of neem-based pesticides

The neem-based pesticides are produced today by the manufactures all over the world. The most commonly used method for the production of the neem-based pesticides are based on a one-step extraction of the neem seed with water or a solvent.

1.2.6. Microwave-assisted extraction

Microwave-assisted extraction is a newly developed technique for the solid-liquid extraction with microwave as the energy source. Microwave-assisted extraction method was reported to be an efficient extraction method in terms of selectivity, yield, and speed. Paré and Belanger (1997) suggested that MAE method is especially useful for the extraction of the samples with plant origin. If the MAE method can be used for selective extraction of AZRL from the neem seed, it might be of great economical value.

1.3. Objectives

The objectives of this project are:

a) To develop a method for the determination of the AZRL in neem extracts.

b) To determine the azadirachtin related limonoids content in various parts of the neem tree.

c) To investigate the microwave-assisted extraction of AZRL from neem seed kernel and other parts of neem.

1.4. Scope

In Chapter II, a thorough literature review on neem, microwave extraction, and the current colorimetric method for the estimation of the AZRL content in the neem extracts has been provided. The development of a new colorimetric method for the determination of the AZRL in the neem extracts is presented in Chapter III. The development of a mathematical model for simplifying the multi-calibration method for the determination of AZRL and simple terpenoids (ST) in the extracts is presented in Chapter IV. After setting up of the analytical method, the investigation of the content of AZRL and ST in various parts of neem and the investigation of microwave-assisted extraction of neem were undertaken and presented in Chapter V and VI respectively.

CHAPTER II

LITERATURE REVIEW

2.1. Abstract

The neem tree is described highlighting its typical medicinal and pesticidal properties. The chemistry of bio-active components in the neem tree, the extraction and quantification of azadirachtin, and azadirachtin in commercial neem-based pesticides were reviewed to show the importance and the status of the neem tree and its product and more importantly to have a clear view on the chemical composition of the neem extracts. Microwave-assisted extraction method was also reviewed to show the advantage of this technique and to decide the possibility of applying it in extracting neem. The visible spectroscopic quantification technique and the related vanillin assay colorimetric method was also reviewed in order to establish the experimental design for the development of the colorimetric method to determine the total azadirachtin related limonoids (AZRL) in neem extracts.

2.2. Review on neem

2.2.1. The neem tree– general description

Adrien Henri Laurent de Jussieu described in 1830 the neem tree as *Azadirachta indica*. Its taxonomic position is as follows (Schmutterer, 1995):

Order: Rutales
Suborder: Rutineae
Family: Meliaceae (mahogany family)
Subfamily: Melioideae
Tribe: Melieae
Genus: *Azadirachta*
Species: *Azadirachta indica*

The neem tree is an evergreen, or deciduous, fast-growing plant which may reach a height of 25 meters, with branches widely spread to form an oval crown. The trunk is relatively short, straight and may reach a girth of 1.5-3.5 m. The bark of neem is composed of a moderately thick, fissured, gray outer bark and a reddish brown inner one. The unpaired, pinnate leaves are 20 to 40 cm long and medium to dark green leaflets number up to 31 and approximately 3-8 cm long (Schmutterer, 1990). In India, neem flowers from January through April, and the fruits mature from May to August. The fruits are oval in shape, 1.4-2.4 cm long and have, when ripe, a yellowish sweet pulp that encloses a brown seed kernel, embedded in a hard white shell. In India, a 15-20 year-old neem tree can yield around 13 kg of fruits and in West Africa an average fruit yield of about 20.5kg/tree was obtained. The weight of the seed kernel accounts for about 10% of that of the whole fruit (Koul *et al.*, 1990; Schmutterer, 1990).

Neem adapts to a wide range of climate and soil conditions. It is normally found at elevations between sea level and 700m. But it can grow at an altitude up to 1500m, as long as the temperature remains moderate. It can tolerate extremely high temperatures, but its normal range is about 9.5 - 37 °C. It is also high drought tolerant, and once established, it can survive 7-8 months' dry seasons. The root system of neem can access ground water within 9-12m of the surface so that it can survive in areas with rain fall of 130mm per year, but it performs best in zones with an average annual rain fall of 450-1200mm.

Neem is native to the Indo-Pakistan subcontinent, but it is now distributed throughout southeast Asia, East and sub-Sahelian Africa, Fuji, Mauritius, parts of Central America, the Carribean and Puerto Rico. Some planting have started in the United States. During the last 20 years neem has been introduced in many countries mainly for afforestation and fuel wood production in dry areas, bot also for other purpose, including use as an avenue or shade tree and as a producer of natural pesticides (Schmutterer, 1990).

2.2.2. Medical properties of neem

The neem tree is believed to have its origin on the Indian subcontinent. The medical properties have been known among Indians for thousands of years. The Sanskrit

name of the neem tree is Arishtha, reliever of sickness. According to Ayurvada, an ancient Hindu system of medicine, neem leaves have many advantages. It can cure all types of eye troubles, intestinal worms, biliousness, lack of appetite, heal boils and skin ulcers. Young twigs can provide relief for cough, asthma, and piles. The seed kernel relieves leprosy and intenstinal worms, and the bark can be used to cure fever. Neem also was used in other ancient systems of medicine in India such as Unani Tibb system and the Homeopathic system. Modern research has also proven some claims on the medical properties of neem. The details are discussed in the next few sections.

2.2.2.1. Dental Care

Fresh neem twigs are used daily by millions of people in India. The benefit of neem on teeth has been proven by modern medical research. It was proven that it is effective in preventing periodontal diseases (Elvin-Lewis, 1980; Henkes, 1986). Neem products were also reported to produce remarkable healing effect of gum inflammations and paradontosia.

2.2.2.2. Immunomodulatory effects

Neem, especially neem bark, is recognized for its immunomodulatory polysaccharide compounds. These compounds appear to increase antibody production (Chiaki *et al.*, 1987; Kroes *et al.*, 1993). Other compounds in neem enhance the immune system via a different mechanism: the cell-mediated immune response (Upadhyay *et al.*, 1990,1993; Sen *et al.*, 1993) or the body's first form of defense. Neem oil acts as a non-specific immunostimulant that activates the cell mediated immune response. This then creates an enhanced response to any future challenges by disease organisms. When neem oil was injected under skin there was a significant increase in leukocytic cells and perioneal macrophages showed enhanced phagocytic activity and expression of MHC class II antigens. Production of gamma interferon was also induced by the injection. Spleen cells showed higher lymphocyte reaction to infection but did not augment anti-TT antibody response. (Upadhyay *et al.*, 1992). In studies on the birth control effects of neem, the major factor in that effect appears to be an increase in the immune response where neem has been applied that causes the body to reject the fetus as a

foreign body (Upadhyay *et al.*, 1993; Tewari *et al.*, 1989; Garg *et al.*, 1994). Thus by enhancing the cellular immune response most pathogens can be eliminated before they cause the ill feeling associated with the disease. This mechanism could also help in diseases that involve the immune system, like AIDS. Taking neem leaf or bark powder every other day or drinking a mild neem tea will enhance antibody production and the body's cell-mediated immune response, helping to prevent infections.

2.2.2.3. Anti-inflammatory Activity

Taking neem leaf orally or applying a cream containing neem oil topically has been used for centuries to reduce inflammation. A compound called sodium nimbinate found in neem leaves has been shown to provide significant relief to inflamed tissues (Okpanyi, 1981; Lorenz, 1976). Other compounds such as nimbin, nimbinin and nimbidol are comparable to cortisone acetate in reducing inflammation (Wali *et al.*, 1993; Tandan *et al.*, 1990).

2.2.2.4. Antimalaria

Abatan and Makinde (1986) obtained solvent-free extracts from the leaves of neem and Pisum sativum and screened for their antimalarial action using Plasmodium berghei in mice. Four days of oral dosing with 500 mg/kg and 125 mg/kg of the methanol extract showed a parasite suppression. A 50 mg/kg oral dose of the aqueous extract of P. sativum was found to have significant prophylactic activity by producing a parasite suppression of 31.9 percent.

2.2.2.5. Dermatological Effect

One of the most significant medicinal properties of neem extracts is its dermatological effect. It was also known that the neem product can provide relief from various skin diseases without side effects. Rao *et al.* (1969) reported that 10 percent aqueous extracts of neem leaves prevent viral skin infections in rabbits and monkeys. The neem oil is a useful remedy in some chronic skin diseases and ulcers and has a common external application for rheumatism, Leprosy, and sprain.

2.2.2.6. Other effects

According to Vijayalakshmi *et al.* (1995), other medical properties of neem include: antiseptic, antiviral, antipyretic, and antifungal uses, and can be used to treat dental diseases, blood disorders, hepatitis, eye diseases, cancers, ulcers, constipation, diabetes, indigestion, sleeplessness, stomachache, boils, burns, cholera, gingivitis, malaria, measles, nausea, snakebite, rheumatism and syphilis.

2.2.3. Pesticidal properties

Interestingly, the neem tree not only has very good medical properties, but also it is famous for its pesticidal properties. Research on the chemical composition of neem led to the isolation and identification of more than three hundred compounds from various parts of neem, some of them show pesticidal activities which will be discussed in more detail in the next section. Intensive investigations have been made on the neem products, from simply leaf or seed kernel powder and their extracts, oil, cake, commercial pesticides, or even pure active ingredients and on the pests from storage, household pests to various crop pests. The entire species of pests which are sensitive to the neem products are reviewed by the Schmutterer and Singh *et al.* (1995) and the number of these species are presented in Table 2.1. For more details on the species and the action modes of neem products, please refer to the entire list in Schmuterer and Singh *et al.* (1995).

Beside its wide spectrum for pest control, some other properties are more promising as far as the effect and the side effects are concerned. Unlike most synthesized pesticides which have a "knock down" effect on pests, neem pesticides control pests through the combination of many different modes of actions (Schmutterer, 1990). By repelling the pests, it protects the crops or stored grains from being damaged even being touched; by affecting the feeding behavior, the growth, or activity of pests, it can minimize the damage made by the pests and kill the pests in a longer cycle run; by affecting the reproduction of the pests, it controls the pests right from the beginning of the cycle. Through this multi-mode of action, it seems impossible that the pests can develop resistance to neem pesticides.

Table 2.1. Number of neem-sensitive insect pest species, arranged by order (adapted from Schmutterer and Singh, 1995)

Order	Number of Species/Subspecies Tested
Blattodea (Roaches or Cockroaches)	6
Dermaptera (Earwigs)	1
Caelifera (Short-horned Grasshoppers and Locusts)	21
Ensifera (Long-horned Grasshoppers and Crickets)	3
Phasmida (Walkingsticks)	1
Isoptera (Termites)	6
Thysanoptera (Thrips)	13
Phthiraptera (Lice)	4
Heteroptera (True Bugs)	32
Homoptera (Leaf- and planthoppers, Aphids, Psyllids, Whiteflies, Scale Insects)	50
Hymenoptera (Sawflies and Wasps)	8
Coleoptera (Beetles)	79
Lepidoptera (Butterflies and Moths)	136
Diptera (Midges and Flies)	49
Siphonaptera (Fleas)	4
Total	413

Furthermore, investigations showed that the neem products have almost no effect on the natural enemies of pests, such as birds, animals, and some insects which feed on the pests (Schmutterer, 1990). These properties of neem pesticide makes it one of the most promising alternatives to the synthesized pesticides for eco-friendly pest control in the future.

2.2.4. Active compounds from neem and their bioactivity

The medical properties and the pesticidal properties of neem attracted the interest of biologists, chemists, entomologist, pharmaceutical scientists, etc. Up to date, more than 300 compounds have been isolated and identified from all parts of neem. Among these compounds, organic sulfuric compounds, polysaccharide compounds, and especially limonoids are the main contributors to its biological activities.

2.2.4.1. Limonoids

Limonoids are a class of highly oxidized triterpenoids and constitutes one third of all the compounds isolated and identified from the neem tree. Most of the pesticidal, antibacterial, antifungal properties, part of the medicinal properties are due to the limonoids. The main source of limonoids is the seed which is also the most important source for neem pesticidal properties. Some limonoids are also isolated from leaves, bark, twig, and the fruit coat of neem.

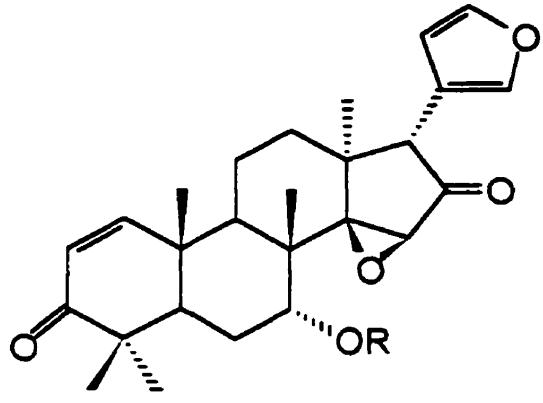
Based on the structure, limonoids from neem can be classified into nine groups: azadirone group, amoorastatin group, vepinin group, vilasinin group, gedunin group, nimbin group, nimbolinin group, salannin group and azadirachtin group (Kraus, 1995). Among these groups, the most important ones are azadirachtin group, and salannin group. In some groups such as Amoorastatin group and Vepinin group, even though there are compounds isolated, due to the small trace amount present, no biological activities are tested.

Azadirone group. A few compounds isolated from the seed or the oil of neem showed low to moderate biological activity, mainly antifeedant activity to some species of pests.

Nimbinin **(1)**, Azadirone **(2)**, azadiradione **(3)** are the three main types of compounds in this group. Nimbinin **(1)** was isolated as early as 1942 (Siddiqui,1942) from the neem oil and was proved later to have antifeedant activity to *E. varivestis* and bactericidal activity to four bacterial species (Siddiqui, 1990). Cohen *et al.* (1996) reported that nimbinin has cytotoxic activity to N1E-115 neuroblastoma (mouse), 143B.TK- osteosarcoma (human) and Sf9 (insect) cultured cell lines. Azadirone **(2)** and Azadiradione **(3)** were isolated by Lavie,

et al. (1971). Azadirone was shown to possess low antifeedant activity (Schwinger *et al.*, 1984), while azadiradione had better performance concerning its antifeedant activity. Among several species of insects tested, azadiradione showed antifeedant activity in the range of 42 - 5,500 ppm, depending on the nature of the tested insects (Kraus, 1995). This compound was also found effective against a number of bacterial species. Azadiradione and a series of its analogues have already been synthesized (Fernandez-Mateos and Barba, 1995; Fernandez-Mateos *et al.*, 1997).

Other active compounds are mainly derivatives of these three types of compounds. They are Nimbocinol **(4)**, 7-Benzoylnimbocinol **(5)**, 17-Epi-azadiradione **(6)**, 17b-Hydroxyazadiradione **(7)**, 17b-Hydroxynimbocinol **(8)**, 7-Deacetyl-7-benzoylnimbinin **(9)**, 1b,2b-Epoxynimbinin **(10)** from the neem seed. These compounds showed moderate antifeedant activity (Kraus *et al.*, 1981; Ishida *et al.*, 1992; Lee, *et al.*, 1988; Jeyabalan and Murugan, 1997).



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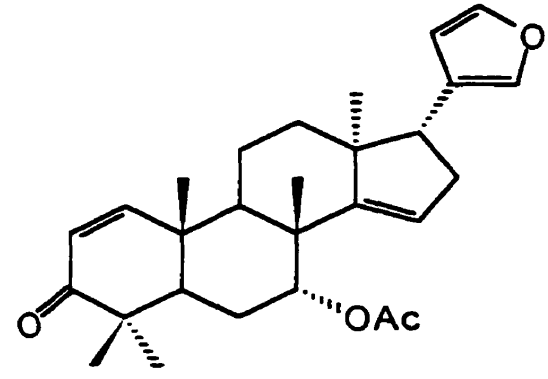

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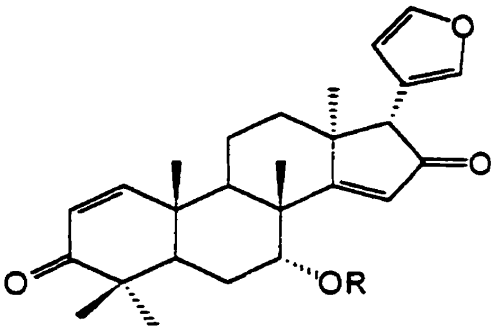
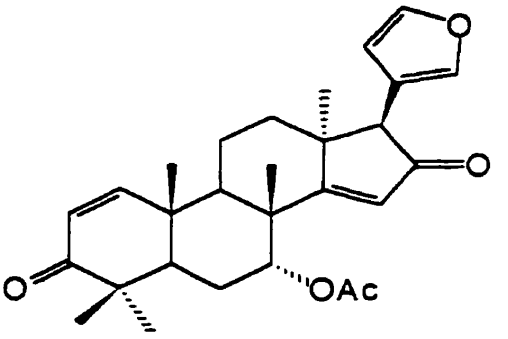


(3) R = Ac
(4) R = H
(5) R = Bz



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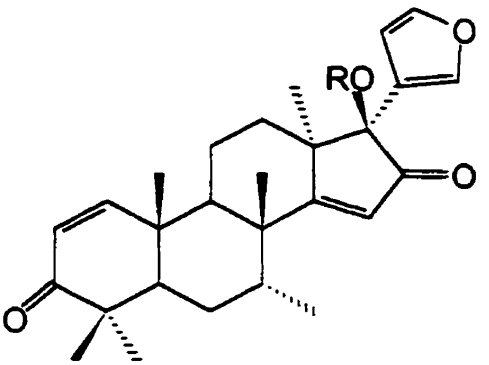
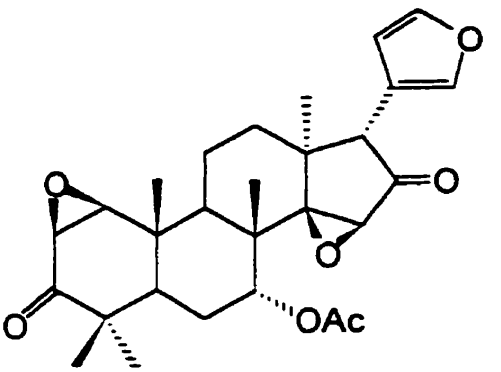
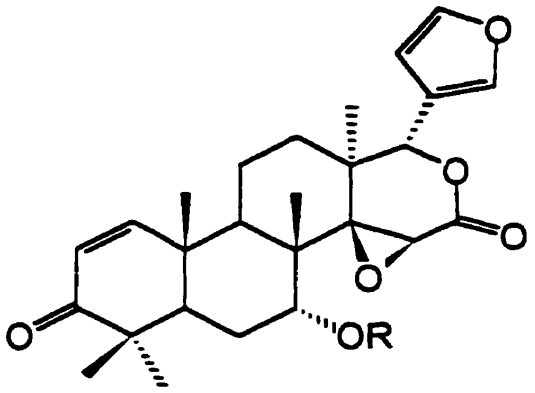


(7) R=Ac
(8) R=H

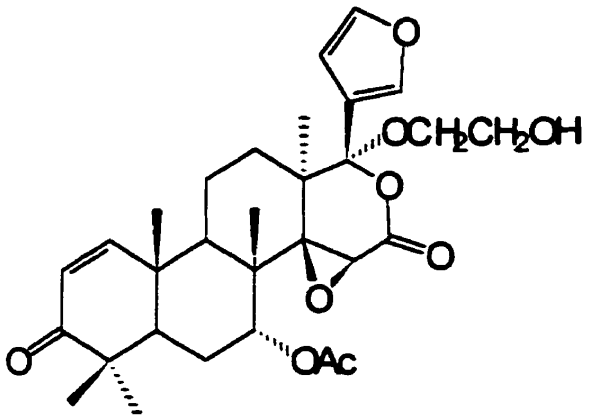


(10)

Gedunin Group. Gedunin **(11)**, and its derivatives 7-deacetylgedunin **(12)**, 7-benzoylgedunin **(13)**, mahmoodin **(14)** are mainly isolated from neem seeds, but **11** and **12** were also found in the leaf and the bark (Lavie and Jain, 1967; Kraus *et al.*, 1981; Siddiqui, 1992; MacKinnon, 1997). The first three showed antifeedant and growth inhibition effects on some species of pests (Ishida, 1992) and mahmoodin showed antibacterial activity (Siddiqui, *et al.*, 1992). Recently, Jeyabalan and Murugan (1997) reported that gedunin **(11)** and deacetylgedunin can also affect the development, reproduction of a polyphagous insects. MacKinnon (1997) studied the antimalaria activity of gedunin and its 9 derivatives; the results showed that only gedunin was active.



(11) R = Ac
(12) R = H
(13) R = Bz



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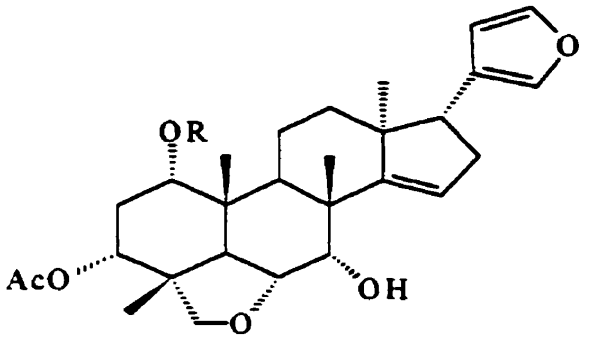
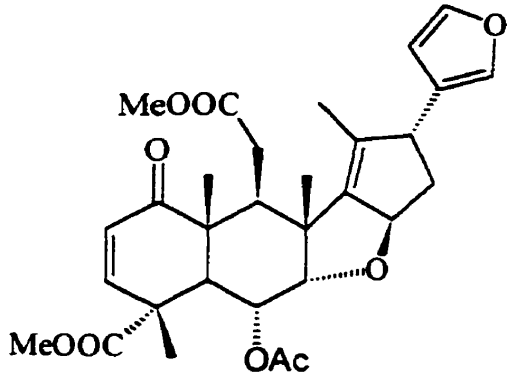


(15) R = Ac
(16) R = Tig
(17) R = Sen

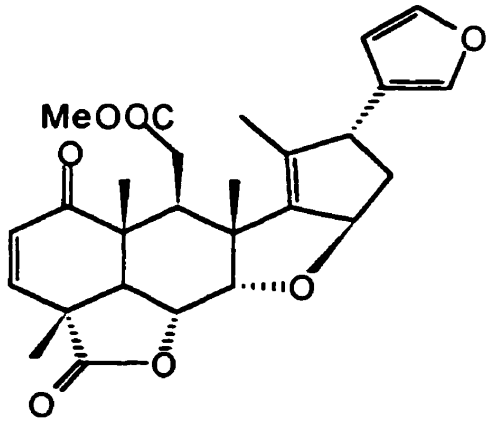
Vilasinin Group. Vilssinin itself was separated from the neem leaf, but some of its derives were isolated from the seed or the seed oil of neem. The vilasinin derives, 1,3-Diacetylvilasinin **(15)**, 1-Tigloyl-3-acetylvilasinin **(16)**, and 1-Senecioyl-3-acetylvilasinin **(17)** showed very good antifeedant activity (Kraus, 1995).

Nimbin Group. Nimbin **(18)** and some of its derivatives were separated from the neem seed, but more derivatives of this compound were isolated from other parts such as the leaves and bark of the neem tree and were the main contributors to some of its medical

properties. Nimbin was one of the first isolated compounds from the neem tree. As early as 1942, Siddiqui (1942) isolated nimbin from the neem seed and the test showed later to have antifeedant activity to *Epilachna varivestis* with EC50 50 ppm (Kraus, 1995).



(18)



(19)

Nimbolide **(19)** was isolated by Ekong (1967) from the fresh leaf of neem. As was proved later, this compound attributed to various medicinal properties especially the antitumor property of neem. Cohen *et al* (1996) reported that Nimbolide showed cytotoxic activity against N1E-115 neuroblastoma (mouse), 143B.TK- osteosarcoma (human) and Sf9 (insect) cultured cell lines, with an IC50 ranging from 4 to 10 mM and averaging 6 mM for the three cell lines. It was also found that at 10 mM it acts rapidly in the neuroblastoma cells to induce blebbing associated with disruption of plasma membranes almost instantaneously and 50 percent loss of cell viability within 30 min, and at a lower concentration 5 mM, and some irreversible cytological changes lead to cell death. However this compound showed low toxicity to mice (Glinsukon *et al.*, 1986).

A salt named sodium nimbinate found in neem leaves was shown to provide significant relief to inflamed tissue (Okpanyi and Ezenrkwa, 1981; Lorenz, 1976).

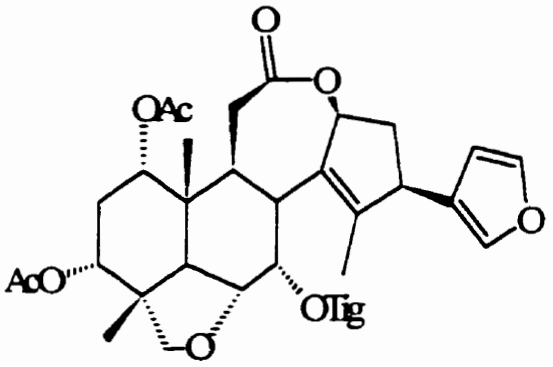
Nimbolinin Group. Two compounds from this group isolated from the neem seed showed biological activities. Ohchinolide B **(20)** was isolated by Govindachari *et al.* (1992) from the neem seed kernel. This compound showed higher antifeedant activity than that of

nimbin **(18)** with EC_{50} 20 ppm to the same species of pest, and it also showed moderate growth inhibition effect to some species. Another compound 21-Oxo-ohchinolide **(21)** also showed antifeedant activity (Kraus, 1995).

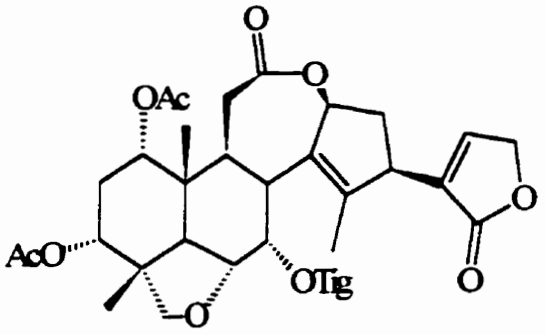
Table 2.2. Biological activity of salannin and its derives (Adapted from Kraus, 1995)

Compounds and Test Organisms	Biological Activities
Salannin (22)	
Earias insulana	A 0.01%
Epilachna varivestis	A EC_{50} = 14 ppm
Heliothis virescens	G EC_{50} = 170 ppm
Musca domestica	A 100% at 1,000ppm
Popillia japonica	A EC_{50} = ~ 280 ppm
Reticulitermes speratus	A EC_{50} = 19.5 µg/disc
Spodoptera frugiperrda	A ED_{50} = 13 µg/cm2
Spodaptera littoralis	A 0.01%
3-Deacetylsalannin (23)	
Epilachna varivestis	A EC_{50} = 20 ppm
Heliothis virescens	A EC_{50} = 170 ppm
Popillia japonica	G EC_{50} =~390 ppm
Reticuliterms speratus	A EC_{50} = 55.2 µg/disc
Salannol (24)	
Epilachna varivestis	A EC_{50} = 10 ppm
Salannolacetate (25)	
Epilachna varivestis	A EC_{50} = 9 ppm
Popillia japonica	A EC_{50} =~260 ppm
Salannolactame-21 (26)	
Epilachna varivestis	A 95% at 100 ppm
Salannolactame-23 (27)	
Epilachna varivestis	A 95% at 100 ppm

A = antifeedant activity; G = Growth disrupting activity; EC = Effective Concentration; ED = Effective Dose.

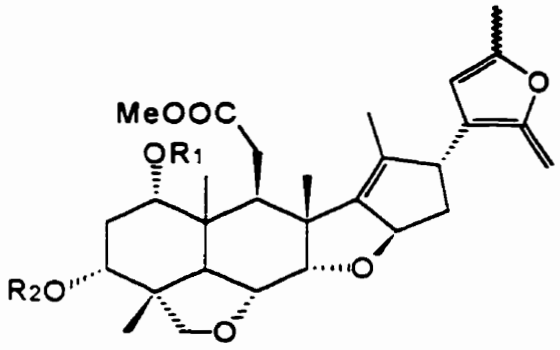


(20)

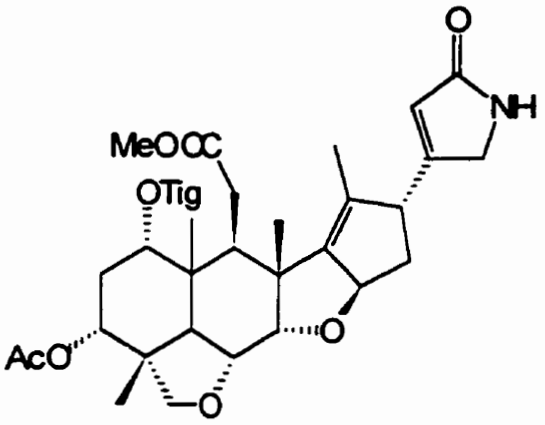


(21)

Salannin Group. Salannin **(22)** is one of the most abundant compounds in the neem oil and the biological test revealed that salannin and most of its derivatives showed higher antifeedant activity than the compounds belonging to azadirone, gedunin, or nimbin groups. The biological activity was reviewed by Kraus (1995) (Table 2.2).



(22) R ^{1} = Tig, R ^{2} = Ac
(23) R ^{1} = Tig, R ^{2} = H
(24) R ^{1} = i. - Val, R ^{2} = H
(25) R ^{1} = i. - val, R ^{2} = Ac
(26) R ^{1} = Sen, R ^{2} = H



(27)

Azadirachtin and its analogues. This is the most important group contributing to the pesticidal activity of neem. Most of the azadirachtins and their analogues are more active than most other limonoids (Table 2.3).

Table 2.3. Bioactivity of azadirachtins and their analogues (Adapt from Kraus, 1995)

Compounds			Biological activity
Azadirachtin A (28)			
Acricotopus lucidu		G	100% at 1µg/ml
Epilachna varivestis		A	EC_{50} = 13 ppm
	(F)	G	LC_{50} = 1.66 ppm
Helicoverpa zea		G	EC_{50} = 0.7 ppm
Heliothis virescens		G	ED_{50} = 0.7 ppm
		G	EC_{50} = 0.07 ppm
Oncopeltus fasciatus	(T)	G	ED_{50} = 3.5 ng
Peridroma saucia		A	EC_{50} = 8.0 ng/cm^2
	(F)	G	ED_{50} = 0.26 ppm
Schistocerca gregaria	(F)	A	100% at 0.07 ppm
Spodoptera frugiperda		G	EC_{50} = 0.08 ppm
		L	LC_{50} = 1.00 ppm
Spodoptera littoralis		A	EC_{50} = 0.07 ppm
Azadirachtin B (29)			
Epilachna varivestis	(T)	G	70% at 0.01 µg
	(F)	G	85% at 2 ppm
Heliothis virescens		L	LC_{50} = 0.80 ppm
		G	EC_{50} = 0.17 ppm
Spodoptera littoralis		FI	97% at 1 ppm
Azadirachtin E (33)			
Epilachna varivestis	(T)	G	EC_{50} = 0.57 ppm
Azadirachtin G (34)			
Epilachna varivestis		G	EC_{50} = 7.69 ppm
Azadirachtin L (35)			
Epilachna varivestis	(F)	G	EC_{50} = 0.25 ppm
3-Deacetylazadirachtin (36)			
Epilachna varivestis		G	EC_{50} = 0.38 ppm
Heliothis virescens		L	LC_{50} = 0.37 ppm
		G	EC_{50} = 0.09 ppm

Vepaol (37)			
Epilachna varivestis	(F)	G	100% at 1 ppm
Heliothis virescens		A	57% at 1 ppm
Spodoptera littoralis		A	EC_{50} = 2 ppm
Azadirachtol (38)			
Epilachna varivestis		G	EC_{50} = 0.08 ppm
2',3'-Dihydrotigloylazadirachtol (39)			
Epilachna varivestis		G	EC_{50} = 0.45 ppm
Spodoptera littoralis		FI	79% at 1 ppm

EC = Effective Concentration; (F) = Application by Feeding; FI = Feeding Inhibition; G = Growth Inhibition; L = Larvicidal Activity; (T) = Topical Application

Azadirachtin was one of the earliest separated compounds from the seed of neem. However, in 1984, Rembold *et al.*, (1984) found that azadirachtin from neem was actually composed of two major components, azadirachtin A **(28)** and B **(29)** and two minor components, azadirachtin C and D **(30)**. In 1991, Govindachari *et al.*, (1991) separated two other azadirachtin H **(31)** and I **(32)** from neem with HPLC. To date, azadirachtin A-L have already been isolated and identified. Among these azadirachtins, azadirachtin A consists of around 85 percent. Without specification, azadirachtin refers to azadirachtin A.

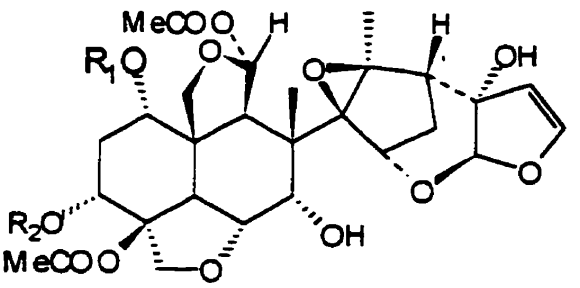
2.2.4.2. Non-limonoidal compounds

Besides limonoids, compounds isolated and identified from neem include other terpenoids and non-terpenoidal compounds, also showing biological activities.

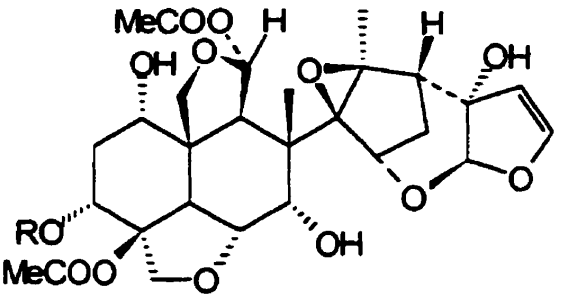
Other terpenoids. Some diterpenoids are also separated from the bark showing biological properties. Out of around 10 terpenoids separated mainly from stem bark and the root bark of neem (ARA, *et al.*, 1988, 1989, 1990; Majumder, 1987), three of them: margolone **(40)**, margolonone **(41)**, and isomargolonone **(42)** are reported to be active against some *Klebsiella*, *Staphylococcus*, and *Serratia* species (ARA, *et al.*, 1989).

Four pentatrortriterpenoid: nimbandiol **(43)**, 6-acetylnimbandiol **(44)**, nimbinene **(45)**,

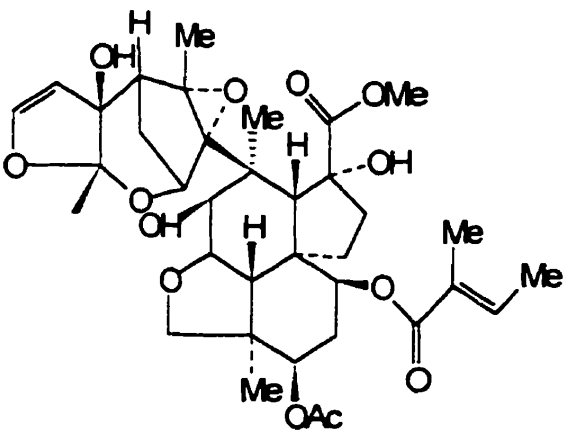
and 6-deacetylnimbinene **(46)** were found in neem seed oil which showed moderate antifeedant, growth inhibiting, and larvicidal effect to some species of pests (Kraus, 1995).



(28) R1 = Tig; R2 = Ac
(33) R1 = H ; R2 = Ac
(36) R1 = Tig; R2 = H



(29) R = Tigloyl
(38) R = H
(39) R = 2',3'-Dihydrotigloyl



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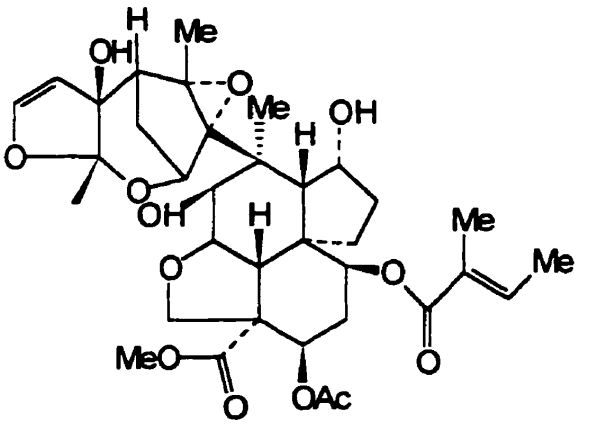

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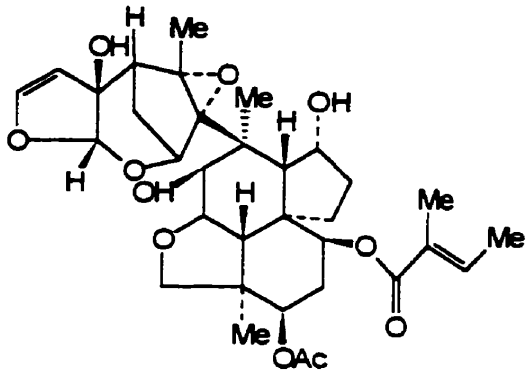

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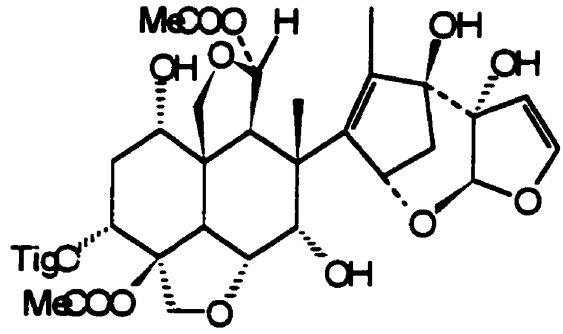

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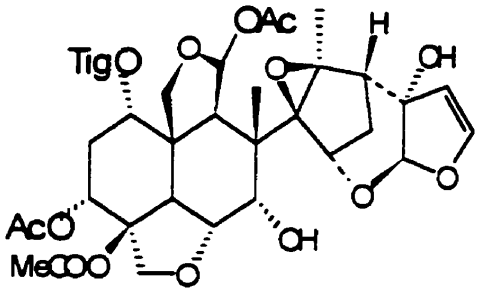

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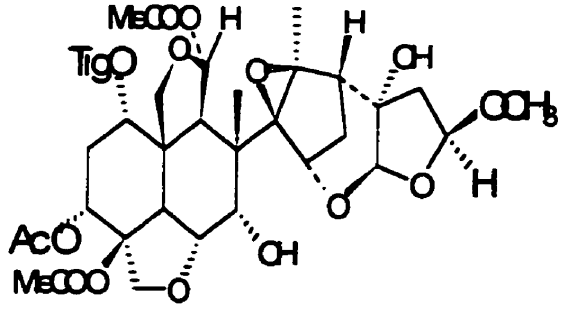

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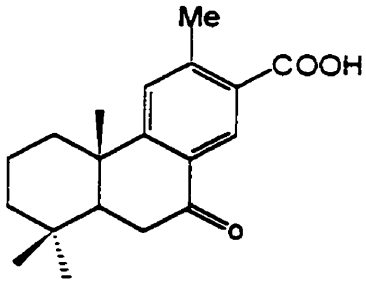

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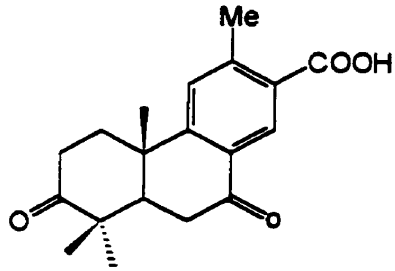

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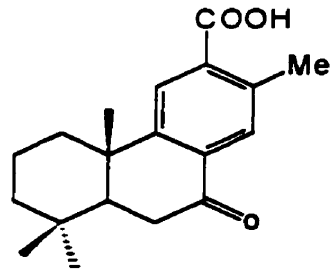

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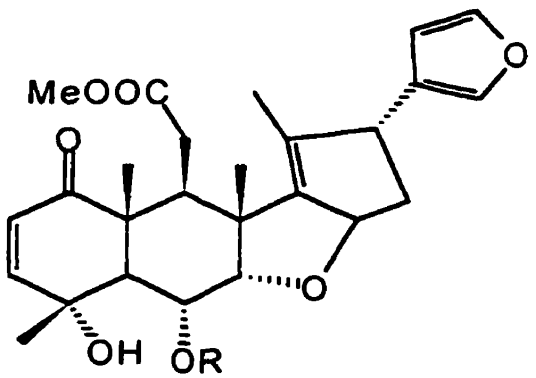
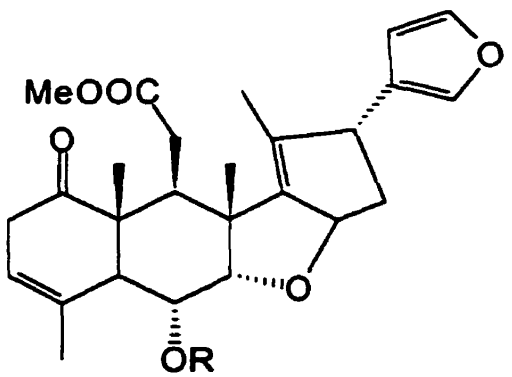


(43) R = H
(44) R = Ac

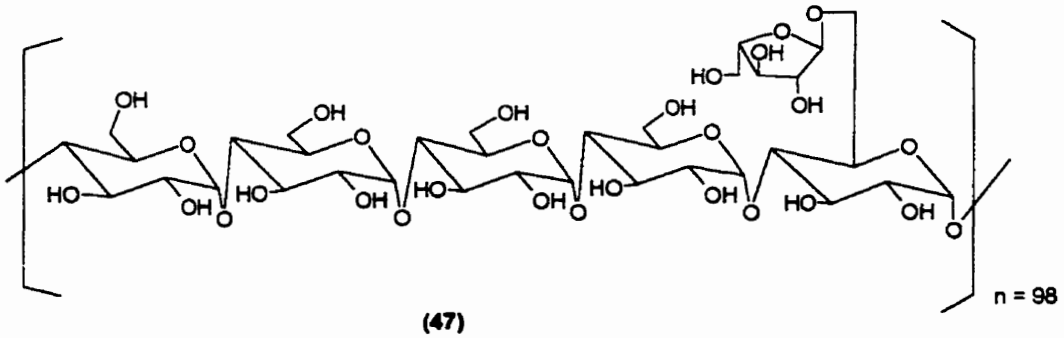


(45) R = Ac
(46) R = H

Organic sulfuric compounds. Sulfur containing compounds from neem seed are responsible for the typical smell of crushed neem seeds. With the aid of GC/MS, Balandrin, *et al.* (1988) studied the sulfur containing compounds from the freshly crushed neem seeds and found that these compounds are mainly cyclic tri- and tetrasulfides containing 2, 3, 5, 6, and 9 carbon atoms. One major component of these compounds di-n-propyldisulfide constitutes 75 percent of all the volatile from crushed neem seeds showing larvicidal activity.

Polysaccharide. Polysaccharides are mainly from the bark of neem and are responsible for the anti-cancer, anti-tumor activities of neem. Polysaccharides GIa **(47)** and GIb were separated from the neem bark in 1982; these two polysaccharides were shown to have antitumor effect against Sarcoma-180 in mice (Fujiwara *et al.*, 1982). Subsequently, some other polysaccharides were sepreated, GIIa **(48)**, GIIIa, CSP-I, II, III, N9GI etc. showing anti-inflammatory and antitumor activities (Shimizu and Nomura, 1984). Some antitumor, anti-inflammatory polysaccharides products were patented by Terumo Corporation of Japan (Shimizu *et al.*, 1985, 1988, and 1990; Yamamoto *et al.*, 1988). Although the mechanism of the anti-inflammatory and antitumor effect is not quite clear, the immune

stimulating effect, according to some reports, should be one of the most possible reasons (Upadhyay *et al.*, 1990).



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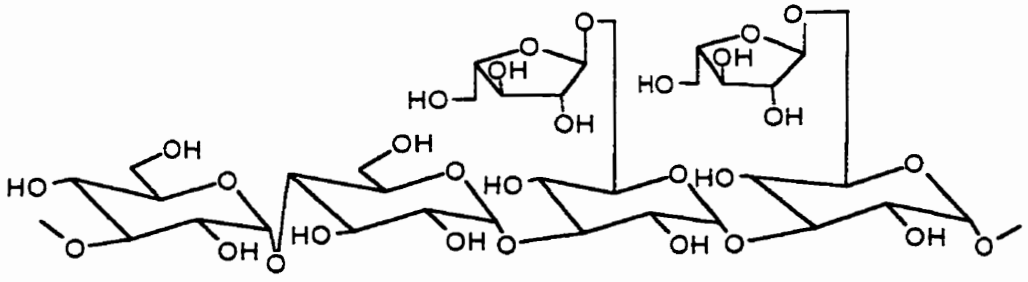

(48)

2.2.5. Azadirachtin

Azadirachtin is one of the most important limonoids from neem. Due to its high content in the seeds and due to its high activity as pesticides, this compound became the focus of many research topics on neem.

2.2.5.1. Azadirachtin content of neem seed kernel

The azadirachtin content in neem seed kernel varies greatly with the different regions of the world. Kraus (1995) reviewed the azadirachtin content of the neem seed kernels from 27 countries (Table 2.4). It was found that the highest content of azadirachtin are those samples from the south and southeast Asia such as India, Myanmar, and Thailand. Samples from countries with extremely high temperatures during the summer time such as Sudan, Somalia, Mali, Niger, have lower azadirachtin content. It was suggested by Ermel *et al.*

(1987) that the combination of high temperature and high relative humidity had the strongest negative influence on the quality of neem seed as far as the azadirachtin content is concerned.

Table 2.4. Azadirachtin content in the neem seed from different countries. (Adapted from Kraus, 1995)

Country	No. of samples	Azadirachtin content (mg/g ± S.E)
Dominican Rep.	44	3.43 ± 0.74
Haiti	16	3.05 ± 0.59
Honduras	1	4.20
Ecuador	26	3.99 ± 1.19
Guinea Bissau	1	2.40
Mali	2	2.05
Senegal	22	3.30 ± 0.63
Gambia	6	2.98 ± 0.88
Niger	15	3.40 ± 0.67
Togo	3	5.40
Benin	57	3.75 ± 0.94
Sudan	9	2.53 ± 0.60
Somalia	19	2.90 ± 0.88
Zanzibar	1	4.80
Madagascar	1	2.20
Iran	4	2.75 ± 1.65
Yemen	7	4.44 ± 0.90
India	9	5.14 ± 1.80
Sri Lanka	3	3.40 ● 0.34
Myanmar (Burma)	3	6.10 ● 0.70
Thailand	6	5.20 ± 1.10
Australia	1	4.90

Even within one country, due to different locations and different climates, the azadirachtin content in the neem seed can still be different. Rengasamy *et al.*(1993) reported

the azadirachtin content from some parts of India and found a great variety between the different locations. The lowest content was found in the northern plain and the central highland with the azadirachtin content (0.28± 0.20) percent for seven samples, and the highest content was found in the Deccan plateau and Western ghats where the azadirachtin content of neem seed kernel was found to be 0.99 percent and 1.52 percent respectively.

Different season can affect not only the azadirachtin content, but also the composition of the azadirachtins. Sidhu and Behl (1996) studied the variation of azadirachtin content and the variation of the content of different type of azadirachtin in different seasons and found that normal season seeds yield higher (1.53%) azadirachtin-rich fractions as compared to the winter season seeds (1.26%). They also found that in the winter season seeds the azadirachtins B and F content increases a lot; and the total amount of Azadirachtins A, B, and F increases also.

Within the neem tree, the azadirachtin is not evenly distributed in every parts of neem. Sundaram (1996) studied the azadirachtin content in the seed kernel, bark, leaf, root, and stem of neem and found that the azadirachtin content is in the order of:

Seed kernel >> leaf > bark > root > stem

The details are listed in Table 2.5.

Table 2.5. Azadirachtin concentration in the samples of seed kernels , bark, leaves, roots, and stem parts obtained from Kanthayapalayam, South India (Sundaram, 1996)

plant parts	Moisture content (% m/m)	AZ-A concn. (mg/100g mass with moisture
Seed kernels	25	24.8
Bark	17	0.42
Leaves	35	0.59
Root	15	0.24
Stem	20	0.15

The information about the variety of the azadirachtin content with the difference in location, climate and other conditions, as well as the information about the azadirachtin

content in various parts of neem is important for the selection of materials for the producing of commercial neem-based pesticides.

2.2.5.2. Extraction and separation of azadirachtin

The pioneering work for the isolation of azadirachtin was carried out by Butterworth and Morgan (1971). They used solvent partition followed by preparative thin layer chromatography to obtain 1.5 g extracts with 90 percent purity of azadirachtin from 2 kg neem seeds. In 1972, the same author reported the partial structure of this compound. However, it was not until much later that the final acceptable structure of azadirachtin was established (Kraus, *et al.*, 1985, 1986). The advent of preparative HPLC technique made it possible to obtain azadirachtin with higher purity. Lee and Klocke (1987) isolated 364.8 mg azadirachtin with more than 99 percent purity from 1.5 kg neem seed by combining Florisil column displacement chromatography and droplet countercurrent chromatography followed by preparative chromatography.

Extraction and partitioning are commonly used methods for the analysis of azadirachtin or for producing azadirachtin enriched pesticides. Since oil constitutes around 40% of the seed kernel, a defatting procedure by extracting with non-polar solvent like petroleum ether or hexane or de-oil with mechanical expression was ordinarily carried out before the extraction. The extraction of azadirachtin was ordinarily carried out with polar alcohols like methanol or ethanol, and the extract was subjected to partitioning between different solvent or solvent mixtures to enrich the azadirachtin before further chromatographical purification method (Yamasaki *et al.*, 1986; Azam *et al.*, 1995; O'Shea *et al.*, 1999). The method used for partitioning included further removal of oil from the extract and further enrichment of azadirachtin through extraction. Partitioning was usually carried out by first dissolving the extract into aqueous methanol or water then extracting with PE or hexane to remove remaining oil. This procedure retained azadirachtin in the aqueous methanol layer or the aqueous layer. In order to obtain a powder form, azadirachtin soluble but not aqueous methanol soluble solvent had to be chosen to extract the azadirachtin into its layer and leave the polysacchrides in the aqueous layer. Dichloromethane or ethyl acetate

was usually chosen for this purpose. After this partitioning, the azadirachtin content ordinarily reached 20-30 percent (Govindachari, *et al.*, 1991; Sankaram, *et al.*, 1998). If further purification was needed, separation through a column chromatography and preparative HPLC is required. However, for the quantification of the azadirachtin in the extracts, the partitioning process was sufficient.

2.2.5.3. Quantification of azadirachtin in the extracts

Azadirachtin is a non-volatile and highly polar substance and gas chromatography is not suitable for the analysis, therefore, HPLC was the analytical method of choice. Several HPLC quantification methods for azadirachtin were published and generally the reverse phase HPLC systems were used for the analysis. Warthen *et al.* (1984) developed an HPLC method for the estimation of the azadirachtin content in crude extracts and formulations. The column used was a reverse phase column (Radial-Pak μ Bondapak C_{18}, 10 μm) and the mobile phase was: methanol/water (50/50, v/v). A wavelength of 214 nm was used for the detection. The quantification was carried out with commercial azadirachtin standard 1 ppm as external standard. Yamasaki, *et al* (1986) reported the use of phenyl column (Phenomenex phenyl 250 X 0.46 cm i.d., 5 μm) with acetonitrile/water (7/3, v/v) as mobile phase. The detection was carried out at 214 nm. Azam *et al.* (1995) also applied a phenyl column (Shimpack CLC-phenyl column, 15cm x 6 mm i.d.) in combination with methanol/water (65/35) as mobile phase at wavelengths of 214 nm. The quantification was carried out with calibration curve (concentration versus peak area) and the linearity was found in the range of ppm and 250 ppm. Yakkundi *et al.* (1995) reported a quantification method with anisole as internal standard. The conditions used for the analysis was a Waters Novapsk C_{18} column (4.6 mm x 15 cm, 4 μm) with the mobile phase acetonitrile/water (40/60) flow rate 1 mL/min for 5 minutes and a gradient to 100% acetonitrile for 5 minutes at 1 mL/min to clean the column.

Besides the application of HPLC, supercritical-fluid chromatography (SFC) was also used by Huang and Morgan (1990) for quantitative determination of azadirachtin. An aminopropyl silica column (15 x 0.46 cm, 7.5 μm particle size) was used and 7.5% methanol

in supercritical CO_2 was used as the mobile phase. The process was carried out at 55 ^{0}C. Huang and Morgan (1990) suggested that SFC was a better method for the quantification of azadirachtin due to the fact that most solvents used for HPLC absorbs UV at short wavelengths that is required for the detection while supercritical CO_2 is transparent to short-wavelength UV. With this SFC method the amount of azadirachtin detectable was as low as 10 ng at 212 nm.

Table 2.6. Azadirachtin in some commercial neem-based pesticides

Pesticides	Description	Azadirachtin content	Producer
Margosan-O	EC formulation	0.1%	W.R. Grace and Co. Columbia, MD
Azatin®	EC	3%	Agridyne Technologies Inc., Salt Lake City
Align™	EC	3%	
RH 999	Wettable powder	20%	Rohm and Haas Co. (Philadelphia, PA)
Neem-EC	EC	4%	Phero Tech Inc. (BC, Canada)
Godrej Achook	/	0.3%	Bahar Agrochem & Feeds Pvt. Ltd., Bombay (marketed by Godrej Agrovet Ltd., Bombay)
NeemAzal Technical	Powder	25%	EID Parry Ltd., India
NeemAzal T/S	EC	1%	Trifolio-M Company (Lahnau, Germany)
NeemAzal-F	EC	5%	
Green Gold*	/	0.3-90%	Australia

2.2.5.4. Azadirachtin in the commercial neem products

Due to the excellent properties of neem as pesticides, the neem pesticides were developed quickly during the last decade. With the development of the neem-based pesticides,

the grading of the pesticides became a problem. As we can see from the previous sections, there are many pesticidally active components in the neem seed extracts. It is impossible to use all the individual components to indicate the grade of the pesticides, therefore, the most important component, azadirachtin , was widely accepted as the standard to determine the grade of the neem-based pesticides. Table 2.6 lists some of the neem-based pesticides and their azadirachtin content.

2.3. Review on microwave assisted extraction

2.3.1. Microwave

The microwave region of the electromagnetic spectrum lies between infrared and radio frequencies with frequencies between 300 MHz and 30 GHz. Besides the application of microwave for heating purposes, this region of the electromagnetic waves are used extensively for RADAR transmission and telecommunications. In order not to interfere with these uses, regulations were made to limit the frequencies that can be used for industry, scientific, and medical purpose (ISM frequencies). The frequencies of 2450 MHz and 915 Hz are generally used frequencies in industry and 2450 MHz is used for most domestic microwave ovens.

2.3.2. Microwave and its applications in chemistry

A reliable device for generating fixed frequency microwave was designed by Randall and Booth at the university of Birmingham as part of the development of RADAR during World War II. Even in its early days of application, microwave energy was found to be able to heat water in a dramatic way. Since the 1950s, domestic and commercial applications of microwave in heating and cooking food began to appear in the United States. As a result of effective Japanese technology transfer and global marketing, the widespread domestic uses of microwave oven occurred in the 1970s and 1980s.

The application of microwave in chemistry as a sample preparation method occurred in the 1970s. Abu-Samra *et al.* (1975) first applied microwave as a wet-ashing method for digesting biological sample for trace element analysis. As a result of its various advantages, this method developed quickly and its application extended to the acid digestion of biological, geological, and soil samples. Due to the essential problem for the introduction of organic solvents which is flammable into the microwave field, it was not until 1986 when Ganzler *et al.*, (1986) first applied microwave on the extraction of various types of compounds from soil, seeds, food, and feed with organic solvents. He found that the microwave-assisted extraction is more effective than the conventional extraction methods. Several patents on the Microwave-Assisted Process of liquid phase extraction, gas phase extraction, organic reaction (Paré *et al.*, 1991; Paré and Belanger, 1994; Paré, 1995a, 1995b, 1996), systematically interpreted the application in various aspects of chemistry. The appearance of the commercial microwave equipment for extraction, digestion, or synthesis have successfully solved the problem of direct exposure of flammable organic solvent in the environment of microwave. Furthermore, the single mode, focused microwave makes it more efficient for either sample preparation or organic synthesis; the high pressure teflon vessel in the microwave environment makes full use of microwave fast heating effect to reach much higher temperatures then the boiling points of the solvents and as a result accelerating the digestion process enormously.

2.3.3. Microwave-matter interaction

The microwave-matter interaction is mainly caused by the interaction of the microwave with the polar molecules. In a polar molecule, due to the difference of the electronegativity of different atoms and the specific structure of the molecule, the whole molecule exhibits a partial positive charge and a partial negative charge and forms a dipole. When the microwave, which consists of an alternative electric field and magnetic field, is applied on the matter with polar molecules in it, the alternative electric field causes the dipoles fast oscillating. At the frequency of 2450 MHz, the dipoles will change their directions 2.45×10^9 times per second. The friction caused by the fast oscillations results in the rapid heating

of the material. Since microwave can penetrate into the materials, the heating effect happens throughout the material which is called volumetric heating. This special heating effect makes microwave a very efficient method for heating, which also contributes to the various applications including the chemical applications.

Two parameters, namely dielectric constant (ε') and loss factor (ε") define the dielectric properties of materials and are important parameters for the microwave heating. The dielectric constant describes the ability of a molecule to be polarized by the electric field, while the loss factor measures the efficiency with which the microwave energy can be converted into heat. These two parameters change with the increase of microwave frequency. Take the dielectric properties of water as an example (Figure 2.1), at lower frequencies, the dielectric constant will reach a maximum as the maximum energy can be stored in the material, while the loss factor is low due to the low rotation rate. As the frequency increases to a certain value, the dipole can no longer align efficiently with the directional change of the electric field, causing the dielectric constant to drop, while the loss factor keeps increasing to a maximum value before it drops. In practical uses, the loss tangent which is defined as the ratio of the dielectric loss and the dielectric constant is often used. It describes the ability of a material to convert the electromagnetic energy into heat energy at a given frequency and temperature.

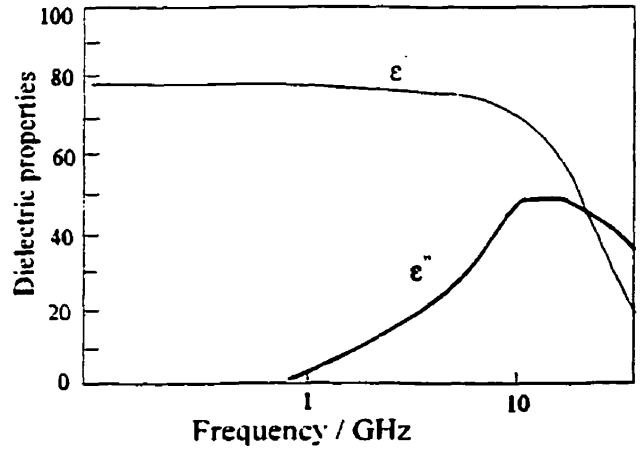

Figure 2.1. Dielectric properties of water as a function of frequency (Adapted from Michael *et al.*, 1995).

Molecules with different dielectric properties, when exposed to microwave radiation, will have different response to it. Ordinarily, the higher the dielectric constant, the more efficient the molecule absorbs the microwave energy and be heated more efficiently. Molecules with very low dielectric constants and loss factors cannot couple with microwave oscillation efficiently, therefore will not absorb microwave energy. We call this type of molecule transparent to microwave energy.

If the material in question is a good conductor, another mechanism, namely conduction loss, becomes significant. It is important for most food process, inorganic acid digestion, ceramic process *etc*. For most organic reactions, extractions, this part of heating effect is negligible.

2.3.4. Microwave-assisted solvent extraction (MAE) of plant materials

Microwave-assisted extraction is a process of applying the microwave energy to a liquid-solid system and partition compounds of interest from the solid sample into the surrounding solvents. The special heating mechanism of microwave to materials and the fact different chemical substances absorb microwave to different levels make microwave-assisted extraction an efficient method for extraction, and more importantly, make selective extraction of target compounds possible.

The microwave-assisted extraction process is a combination of different effects. When a polar solvent with a relatively high dielectric constant and loss factor is used, the solvent will be heated by the microwave energy through dipole rotation. The heated solvents will accelerate the process of the desorption of matrix-solvent interface and the diffusion of the target compounds into the solvent (Hawthorne *et al.*, 1995). In this case the microwave serves mainly as an energy supplier to heat the system. The special extraction mechanism of microwave-assisted extraction can be better interpreted when a non-polar solvent is used in extracting fresh plant materials. It need to be noted here, water, a polar molecule which can very efficiently absorb microwave energy, plays an important role in the MAE process.

Paré and Belanger (1994) studied the extraction of fresh mint leaf with a non-polar solvent under microwave irradiation. Fresh plant materials such as mint leaves are made of a multitude of pocket-like cavities that are defined by the cells, glands, vascular vessels, and the like, all of which contain different chemical species and more importantly different level of water content. When the system of fresh mint leaf and a nonpolar solvent is exposed to microwave radiation, microwave will travel freely through the solvent which is transparent to microwave energy and reach the sample. A significant fraction of microwave rays is absorbed by the sample, mainly the water in the glandular and vascular systems, which results in a sudden temperature rise in temperature inside the sample. The rise of temperature causes gasification of the water in the glandular and vascular systems; The gasification causes a dramatic expansion in volume and thus creates an explosion at the cell level. The substances located in the cells are then free to flow out of the cell to the surrounding solvent. The scanning electron micrographs of the extracted fresh pepper mint reveals that after 40 s of irradiation the gland system of the pepper mint leaf was totally disrupted, while an two-hour distillation causing only the shrinkage of them (Figure 2.2). Even though not all samples have the same micro-structure as the fresh leaves, selective and localized fast heating effects play an important role in the high efficiency of extracting target components into the solvents.

A B C

Figure 2.2. Scanning electron micrograph of (A) Untreated fresh mint gland; (B) Soxhlet extraction for 6 hrs; (C) Microwave irradiation for 20 s (adapted from Paré and Belanger, 1994).

From the above description, it is quite understandable that microwave assisted extraction is a very efficient method for extraction of key components from plant materials. Ganzler *et al.*, (1986) reported that for the extraction of crude fat, vicine, convicine, and gossypol from oil seeds, duration with MAE method is almost 100 times less than those for traditional methods. Paré (1995b)compared the MAE extraction of apiole and oil from sea parsley with distillation method. The results showed that with 50 s irradiation of microwave at 625W with hexane as solvent, the yield of the apiole is almost the same as that with microwave and the oil extracted a little bit lower than that obtained by 90 min distillation. Paré (1995b), in his patent listed the application of microwave-assisted extraction on various plant materials, the results showed that 40 s to around two minutes microwave irradiation treatment to sea parsley, pepper mint leaves, cedar, garlic obtained extracts of interest compared to that obtained by 90 min to several hrs of steam distillation or similar standard methods.

It is suggested by Paré (1995b) that the microwave is better in extracting heat sensitive component than distillation method. In the MAE with non-polar solvent, the extraction is obtained through the mechanisms described above, while the heat sensitive components are subjected to the higher temperature for a relatively short period of time. Table 2.7 shows the extraction of garlic which contains high heat sensitive components. The results showed that with MAE for 30 s with CH_2Cl_2 as solvent, it obtained the components B and C which have already been destructed in steam distillation.

Even though the selective extraction is the case when the non-polar solvent as solvent, the extraction can not be carried out with non-polar solvents for all extraction. The solubility of the target compounds are important factors that need to be taken into consideration. When polar solvent are used, the super heating effect generated by MW heating will accelerate the destruction of the heat sensitive compounds.

Table 2.7. Comparison of the components by MAE and steam distillation method (Adapted from Paré, 1995b).

Composition of Garlic Extracts (%)										
Microwave Irradiation (30 s; in CH_2Cl_2)			Steam Distillation (2 hrs)							
A*	B	C	A*	D	E	F	G	H	I	J
22.2	28.4	49.4	14.7	5.80	45.9	9.92	8.96	4.84	5.96	3.94

* Component A is the only component that is common to both extracts

From the mechanism, it can be seen that the selective extraction is possible through the microwave assisted extraction method. However, the advantage of this selectivity is present only when the target component is located in the glandular and vascular systems which have high level of water and where the localized heating will happen. On the other hand, if the target compounds are located in the places without high content of water, the short period of extraction time will not allow these compounds to access the solvent environment resulting in the low yield of these compounds. In such cases, the MAE method is not suggested.

Although Paré and Belanger (1997) suggested that non-polar solvents are preferred when the MAE method was used, it is not always the case. When the target compounds are non-polar and if these components are located in the glandular or vascular systems with high content of water, by selectively heating, this may be the case. However, the solubility of the target compounds in the solvent selected and the polarity of the solvent need to be taken into consideration in most cases. Mattina *et al.*, (1997) use methanol, 95 percent ethanol and chloroform to extract a terpene from the plant material, even with 55 percent moisture content of the materials, the recovery with chloroform is far lower than that obtained by extraction performed with polar solvent methanol or 95 percent ethanol. Table 2.8 shows that with steam distillation the extracts obtained are mainly non-polar volatile; the hexane extracts have similar components and similar content of corresponding component to those obtained

through steam distillation method. However, the extracts obtained with MAE with ethanol as solvent have completely different components from that obtained by either steam distillation or MAE with hexane as solvent. This means that the solubility of the solvents to the target compounds is an important factor when the solvents are to be selected.

Table 2.8. Influence of solvent on the extraction components obtained by MAE (Adapted from Paré, 1995b).

	10 most Important Components of Cedar Essential oils (%)									
Extraction Conditions	1	2	3	4	5	6	7	8	9	10
Steam Distillation	2.02	15.9	61.3	10.9	3.05	1.86	1.93	0.92	0.97	1.26
Microwave:										
Ethanolic Extract	0	0	3.15	0	0	0	0	0	39.6	54.3
Hexane Extract	2.63	14.1	59.7	11.1	3.68	0	5.03	3.85	0	0

2.4. Review on UV/VIS spectroscopy

UV/VIS spectroscopy is an old technique. Long before the advent of HPLC, this technique has already been widely used by analytical chemists and biologists to assay materials. Earlier UV spectroscopy was generally based on a single wavelength measurement and its specificity was very limited and highly application dependent. Ordinarily, the analytes need to be chemically or physically separated from the sample matrix to allow the use of simple spectroscopy. These methods were rapidly overtaken by the chromatographical techniques which have a powerful combination of separative and quantitative capacities. However, some unique characters make the UV/VIS spectroscopy method still in use even today. Firstly, this technique is a very simple method, no complicated instrument is needed and fast analysis becomes possible with this technique. Secondly, with the chromatographical techniques, it is quite common to take a long time to find a separation condition, while with

the existing colorimetric method the analysis is quite easy and fast. Thirdly, in situations where a group of components need to be quantified instead of a single compound, the UV/VIS spectroscopy method have more advantages over the HPLC technique. For these reasons, this technique is still in use as the official method for the analysis of many analytes such as the tannic acid, the total phenolic compounds. And some methods such as vanillin assay colorimetric method is still widely used as a standard colorimetric method for flavanols (Swain and Hillis, 1959; Broadhurst and Jone, 1978; Price, *et al.*,1978; Sun, *et al.*, 1998).

2.4.1. Principle of UV/VIS spectroscopy

Radiation energy that is visible to the human eyes is called light and covers the spectral region from 400 to 750 nm. Together with the UV region of the spectra, the energy of the photon in the region of 200-800 nm permits the excitation of the outer valence electrons and the inner shell, d-d transitions with associated vibrational levels. The absorbance of the solution to the light can be described by the Beer-Lambert's law:

$$A = \varepsilon lC \qquad (2.1)$$

Where: A = absorbance

ε = molar absorptivity

l = the path length of the light through the sample

C = concentration of the solute in mol/*l*

The Beer-lambert's law is the base of the UV/VIS spectroscopy quanitfication method. For the quantitative analysis, a spectrum of the sample needs to be obtained to determine at which wavelength the measurement need to be carried out. Commonly, the maximum absorbance wavelength is the first choice. However, sometimes, a wavelength other than the maximum absorbance wavelength can also be used in order to avoid interference. After the working wavelength is determined, a calibration curve of absorbance versus the concentration at the

selected wavelength is done and the molar absorptivity can thus be determined. Therefore, by obtaining the absorbance from the solution, the concentration of the analytes can be calculated by the following equation: $C = A/\varepsilon l$.

2.4.2. Colorimetric method

For the components having color by themselves, quantitative analysis can be carried out directly by VIS spectroscopy . However, in most cases, the analytes have no color by themselves, therefore, some physical or chemical methods need to be taken to colorize the analytes before the quantification is undertaken. Many colorimetric methods are available for the analysis of different compounds or different classes of compounds; some of these method are official methods for the analysis of a certain class of compounds and can be found in the handbooks.

Vanillin assay is a colorimetric method for quantitative analysis of condensed tannin in sorghum, wood, and the estimation of the proanthocyanidins (PA) in food products (Price *et al.*, 1978; Scalbert *et al.*, 1989; Deshpande *et al.*, 1986; Sun *et al.*, 1998). The colorimetric process was described as follows: a 2-mL portion of freshly prepared solution of vanillin (1 g / 100 mL) in 70% sulfuric acid is added to 1 mL of aqueous extract, then allow the reaction to proceed in a water bath at 20 +/- 0.5 °C for 15 minutes before the measurement was carried out at the maximum absorbance wavelength of 500 nm (Scalbert, *et al.*, 1989). Under the selected conditions, the vanillin assay method was reported to be very specific to a narrow range of flavanols (monomers and polymers) and dihydrochalcones that have a single bond at 2,3-position and free meta-oriented hydroxy groups on the B ring (Sarkar and Howarth, 1976). Besides vanillin assay as a colorimetric method for the quantitative analysis, the vanillin sprayer is also used for the visualization of terpenoids on the TLC. Yamaski *et al.* (1986) reported that the azadirachtin can also be visualized on TLC by the vanillin sprayer.

2.4.3 Multivariate calibration technique

A multivariate calibration technique can be used for simultaneous determination of more than one components in a mixture, if these components have different absorbance behavior under the same colorimetric method. This method is also quite useful for the elimination of the interferences if the spectrum of the interfering compound is known.

The principle of this technique is still based on the Beer-Lambert's law. For each component, at a given wavelength, the absorbance can be described as:

$$A_{i\lambda} = e_{i\lambda} C_i l \tag{2.2}$$

Where: $A_{i\lambda}$ = the absorbance of the component i at wavelength λ.

$e_{i\lambda}$ = the molar absorptivity of component i at wavelength λ.

l = the path length of light through the sample.

Assuming there is no interactions between the components, the total measured absorbance at any wavelength will be the sum of the individual absorbance:

$$A_{net\lambda} = \sum_{i=1}^{n} e_{i\lambda} C_i l \tag{2.3}$$

Where: $A_{net\,\lambda}$ = the net absorbance of the mixture at wavelength λ.

Through a multi-wavelength-multi-standard calibration, the molar absorptivity of the used standard at necessary wavelength can therefore be obtained. Through the multi-wavelength measurement, the concentration of different components can be determined simultaneously. If one or some of the components are interferences, a relative absorptivity can be used without the need to calibrate and thereafter remove the interferences and at the same time calculate the concentration of the target components.

2.5. Summary

In this chapter, various aspects of neem, the microwave-assisted extraction, the vanillin assay and the visible spectroscopic technique were reviewed. The chemical composition of the bioactive components in neem showed that the limonoids and some simple terpenoids (ST) such as diterpenoids and triterpenoids are the active ingredients contributing to its pesticidal properties and part of the medicinal property. The great diversity of these limonoids and the bioactivity of each compounds indicated that the use of azadirachtin as the standard for determining either the quality of the neem seeds or the grade of neem-based pesticides has great limitation; the use of the total limonoids as the standard is more reasonable. The literature review on the colorimetric method and the vanillin assay method provide some information about the need for the development of a completely new analytical method for the quantification of total azadirachtin related limonoids in the neem extracts. The extraction and the quanitfication technique with HPLC reported in the literature as well as the review on microwave-assisted extraction technique provide a guide for the experimental design for the investigation of the total AZRL content in various parts of neem and for the investigation of the microwave-assisted extraction of various parts of neem.

CONNECTING STATEMENT 1

After the identification of the problem in Chapter I and the literature review in Chapter II, the development of a colorimetric method for determining the amount of total AZRL and simple terpenoids (ST) will be presented in Chapter III.

CHAPTER III

DEVELOPMENT OF A COLORIMETRIC METHOD FOR THE ESTIMATION OF THE AZRL AND ST CONTENT IN NEEM

3.1. Abstract

Driven by the need to quantify the AZRL in the neem extracts, inspired by a phenomenon on a related analytical method, borrowing a concept from ordinary chemical reactions, a two-phase-two-step colorimetric method was developed. The detailed developmental process, from the trials to the investigation of various factors influencing the colorimetric reaction are described.

3.2. Introduction

The neem tree, *Azadirachta indica* A. Juss, has been increasingly attracting the interest of researchers from various fields. More than 300 compounds have been isolated and characterized from neem seed, one third of which are tetranortriterpenoids (limonoids) (Kumar *et al.*, 1996). One of these limonoids, azadirachtin (AZ), is considered to be the most important active principle in neem due to its various effects on insects (Schmutterer, 1990; Govidachari *et al.*, 1995). Therefore, the azadirachtin content is widely accepted as the standard for determining the quality of the neem seeds and some commercial neem-based pesticides. However, as we can see from Chapter II that azadirachtin is not the only active component. Most of the limonoids existing in neem are pesticidally active and some of them are even more active than azadirachtin. Furthermore, in the presence of all these active principles, a synergetic effect may make the mixture more active than any of the individual component including azadirachtin. This was demonstrated by Verkerk and Wright (1993)

who found that neem extracts containing equivalent amounts of azadirachtin have 3- to 4-fold greater activity than the synthetic azadirachtin. Therefore, it might be more reasonable to use the total azadirachtin related limonoids (AZRL) as a standard for the determination of quality of the neem seeds and to predict the relative activity of the commercial neem-based products.

Azadirachtin content in neem extracts or in commercially available neem-based pesticides can be estimated by HPLC (Sundaram and Curry, 1993; Azam *et al.*, 1995; Yamaski *et al.*, 1986), or by supercritical-fluid chromatography (Huang and Morgan, 1990). However, due to the lack of the standards for all of the components existing in the extracts, these method are not appropriate to estimate the content of each individual components or the AZRL in the extracts. Actually, to our knowledge, there is no such method that exists until to date. Based on a visualization method on TLC for terpenoids and for the azadirachtin by vanillin sprayer (Eweig and Shermer, 1972; Yamasaki *et al.*, 1986; Allan *et al.*, 1994), we developed a fast colorimetric method for the estimation of the AZRL content in neem extracts with commercial azadirachtin (95% purity) as standard. A two-phase-two-step colorization process was employed to increase the sensitivity of the vanillin assay for the determination of AZRL. Furthermore, due to the fact that the simpler terpenoids (ST) exhibit similar absorbance behavior to limonene, a two-standard colorimetric method was used to estimate both the AZRL and the ST in the extracts which is especially useful for the investigation of the AZRL and the ST content in the leaf, leaf stem, and the shell of neem. A mathematical model was introduced in CHAPTER IV to simplify the calculation and to obtain further information about the absorbance-structural relationship.

3.3. Development of the new colorimetric method

Vanillin assay is a widely used colorimetric method quantifying tannins and other polyphenolic compounds in various samples (Price *et al.*, 1978; Scalbert *et al.*, 1989; Sun *et al.*, 1998). In our trial, the extracts subjected to the same colorimetric conditions as

described by Price *et al.*, (1978) for tannins did not develop any color. This indicates that the same method cannot be used in our method for the colorization of standard azadirachtin or neem seed extracts for the quantification. In order to avoid the unnecessary high cost associated with the commercial azadirachtin , a simple terpene, limonene was selected to develop the procedure.

3.3.1. Trials with limonene

The condition for the colorization of azadirachtin on the TLC is to spray the vanillin reagent followed by heating with a hot air gun. The relative concentrations of azadirachtin and the sulfuric acid might be crucial for the color development. The addition of the sulfuric acid after mixing limonene methanol solution with the vanillin methanol solution, caused a greenish blue color to develop. We further investigated the various conditions for this colorization. However even under optimum condition, an absorbance of around 0.25 can be obtained at maximum wavelength of 630 nm for the limonene methanol solution with a concentration 0.1 mg/mL. In addition, a calibration curve can be obtained with the limonene methanol solution between the concentrations of 0.1 and 0.6 mg/mL. By taking the molecular weight into consideration, under the same condition the concentration required for azadirachtin might be still higher and a calibration with commercial azadirachtin was still unaffordable; therefore further investigation was carried out.

3.3.2. Development of a two-phase-two-step colorimetric method

From the previous trial, we noticed that the relative concentration of sulfuric acid is one of the critical factors affecting the sensitivity of the colorization. Therefore, if a method allowing the colorization reaction to happen at a relatively high concentration, the sensitivity will increase. Based on a concept from chemical reactions, one can achieve this by a two-phase colorimetric method.

A two-phase system can be easily created with one phase being concentrated sulfuric acid. During the process, the sulfuric acid layer can extract the reactants and the colorization reaction occurs in the environment of concentrated sulfuric acid. After the reaction, in order

to measure the absorbance, another solvent was added to combine the two-layer into one homogenous colorized solution which can be subjected to the spectrometric measurement. This concept turned out to be functional when limonene diechloromathane (DCM) solution was used. Into limonene DCM solution, the vanillin methanol solution was first added, with the addition of the sulfuric acid a two-phase system was formed. After shaking adequately, the reactants are extracted into the sulfuric acid layer where the colorization reaction happen. After the colorization reaction, a certain amount of methanol addition converted the two-phase system into a homogeneous solution and a greenish blue color was developed instantly after the addition of the methanol. The colorized solution was therefore ready for the spectrophotometric measurement.

Through the optimization of the factors such as the concentration of the vanillin methanol solution, the amount of the vanillin methanol solution, the amount of concentrated sulfuric acid, time used for each step of reaction, a colorization procedure was selected. The condition is described below:

To a limonene dichloromethane solution (0.7 mL), a methanol solution (0.2 mL) of vanillin (0.02 mg/mL) was added; after shaking manually, the mixture was left at room temperature for two minutes; concentrated sulfuric acid (0.3 mL, 98 %) was then added in three portions (0.1 mL each) and the mixture was stirred for 10s after each addition; after the addition of sulfuric acid was completed, methanol (0.7 mL) was added to convert the two layered mixture into a homogenous solution that instantly developed a blue green color. Under this condition, the scan of absorbance versus wavelength shows that the maximum absorbance occurs at around 625 nm (Figure 3.1). Therefore, 625 nm was selected for the measurement. A calibration curve of the absorbance at 625 nm versus the concentration of the limonene DCM solution was obtained between the concentrations of 2 and 20 µg/mL (Figure 3.2). Linear regression shows that the R^2 is 0.9999 and a slope of 52.145. Therefore, a relationship between the absorbance and the concentration of the limonene DCM solution was obtained:

$$A_{625nm} = 52.145C_{limonene} \quad (3.1)$$

Where: $A_{625\ nm}$ — the absorbance at the wavelength 625 nm,

$C_{limonene}$ — the concentration of the limonene dichloromethane solution.

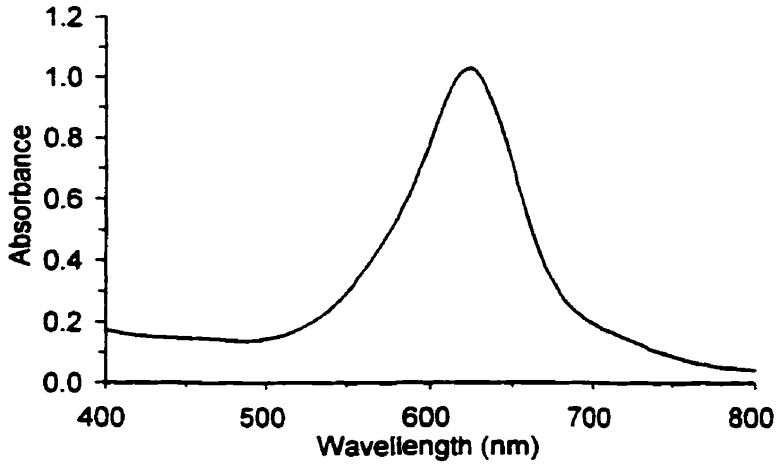

Figure 3.1. Visible spectrum (800 - 400 nm) of limonene DCM solution (0.02 mg/mL) after subjecting it to the colorimertic method.

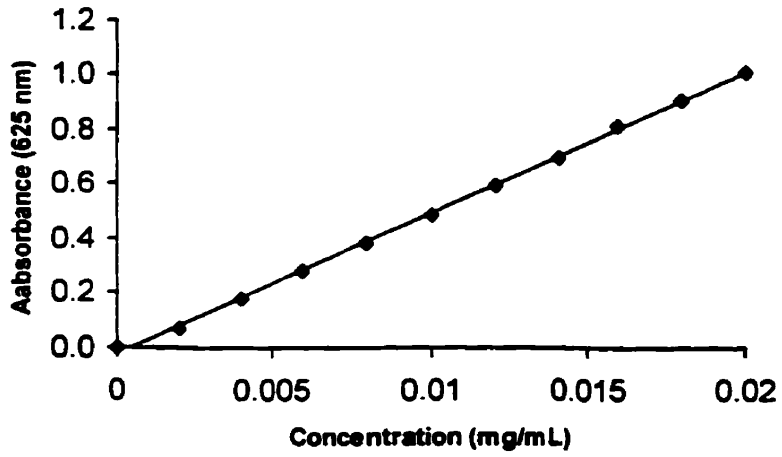

Figure 3.2. Calibration curve with limonene as standard; DCM solutions 0.002 - 0.02 mg/mL were used; Absorbance was measured at 625 nm.

Due to the high sensitivity obtained and the good linear relationship for the calibration curve, it is possible to use commercial azadirachtin (95%) to perform the calibration.

3.4. Investigation with commercial azadirachtin

The purpose of our work is to develop colorimetric method that can be used to estimate the content of the azadirachtin related limonoids (AZRL). Therefore it must be confirmed that the calibration curve is applicable for the analysis of the extracts.

The color developed under a colorimetric method is due to more than one group in the molecule. In a complicated molecule it is more likely that the color is due to the combination of many functional groups; therefore, if the sample undergoing colorimetric reaction exhibits the same typical absorbance behavior as that of the standard, it is likely that these components have similar structures as that of the standard. Any deviation from the absorbance behavior of the standard might mean that there are different groups in the structure of the components or there are interferences from other components which have different absorbance behavior due to the presence of other classes of compounds. Information regarding the composition of the sample to be analyzed is an important aid to minimize these interferences. In our case, it is that AZRL are the main components in the neem seed extracts, and that these compounds are structurally related to azadirachtin. If the absorbance behavior is the same or almost the same as that of azadirachtin, then the calibration with azadirachtin can be used for the analysis of the extracts.

Following the conditions used for the colorization of the limonene DCM solution, purified neem seed extract, crude DCM extract (See CHAPTER IV for detailed description of extraction and purification) and the commercial azadirachtin (95% purity) were used for the investigation. As shown in Figure 3.3, both azadirachtin (0.1 mg/mL) and purified neem seed extract (0.18 mg/mL) exhibit similar absorbance behavior in the wavelength range of 400 to 700 nm and maximum absorbance wavelength for both are around 577 nm. For the crude extracts, although it has similar absorbance behavior as that of azadirachtin and has maximum

absorbance at 577 nm too, the absorbance peak is a little wider than the other two; this means there might be interference from other components that can also be colorized under the same condition but exhibit different absorbance behavior from that of azadirachtin. Therefore, purified neem seed extract can be directly subjected to the colorimetric analysis and this method is workable with azadirachtin calibration.

3.4.1. Factors influencing the colorimetric method for azadirachtin

Although there were similarities between the colorization reactions of the azadirachtin and limonene, a more sensitive colorization condition is required so that we can avoid any factors that might affect the accuracy of the test. Since it has been observed that the colorization behavior of the purified neem seed kernel extract is similar to that of the standard azadirachtin, one could use the extracts instead of the azadirachtin.

The initial conditions used to optimize the vanillin assay to determine AZRL, were chosen based on the results of the earlier investigation with limonene. Figure 3.3 shows the absorbance, in the visible range, of a commercial azadirachtin solution and crude neem seed extracts and purified one through partitioning between different solvents. Both the extracts and azadirachtin showed a similar absorption bands centered at 577 nm. Consequently, this wavelength was chosen to study the effect of time and concentrations of vanillin and sulfuric acid on the sensitivity. Color development with time (Figure 3.4) was investigated with standard azadirachtin solutions of 0.04, 0.06, 0.08 mg/mL under the conditions obtained from the study of limonene. It was found that the color production was stabilized after around 5 minutes as shown in Figure 3.4. The effect of vanillin concentration (Figure 3.5) and the amount of conc. sulfuric acid (Figure 3.6) on the intensity of the absorbance at 577 nm was also investigated with purified neem seed kernel extracts. The results indicated that the absorbance increases with the concentration of vanillin solution up to 0.02 g/mL. At higher concentrations, the blank solution exhibited stronger absorbance than the samples. Accordingly, a concentration of 0.02 g/mL of vanillin was selected as the optimum concentration to study the influence of sulfuric acid. As shown in Figure 3.6, the absorbance of the sample at 577 nm increases sharply as the volume of conc. sulfuric acid (98 %) increases from 0.1 to 0.3 mL. Similar to vanillin concentration, at volumes higher than 0.3

mL, the absorbance of the blank solution was higher than the samples.

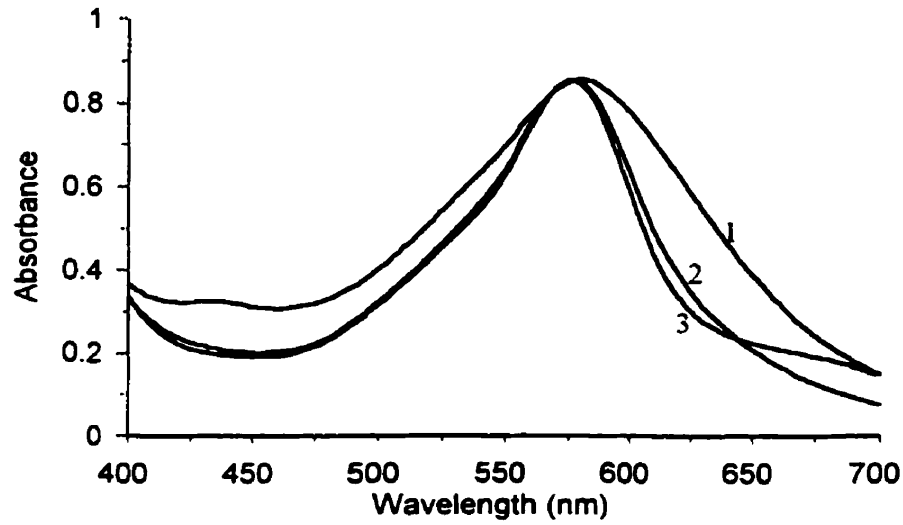

Figure 3.3. VIS spectra (700-400 nm) of azadirachtin and neem seed extracts; 1 — crude neem seed extract, 2 — purified neem seed methanol extract, 3 — azadirachtin (0.1 mg/mL)

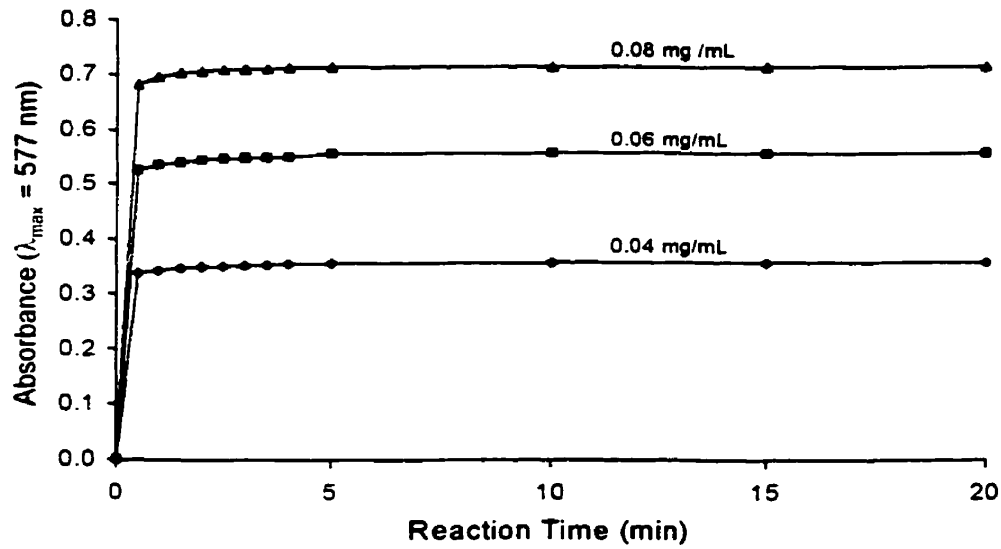

Figure 3.4. Absorbance vs. time (min) of azadirachtin DCM solution at different concentrations subjected to vanillin assay.

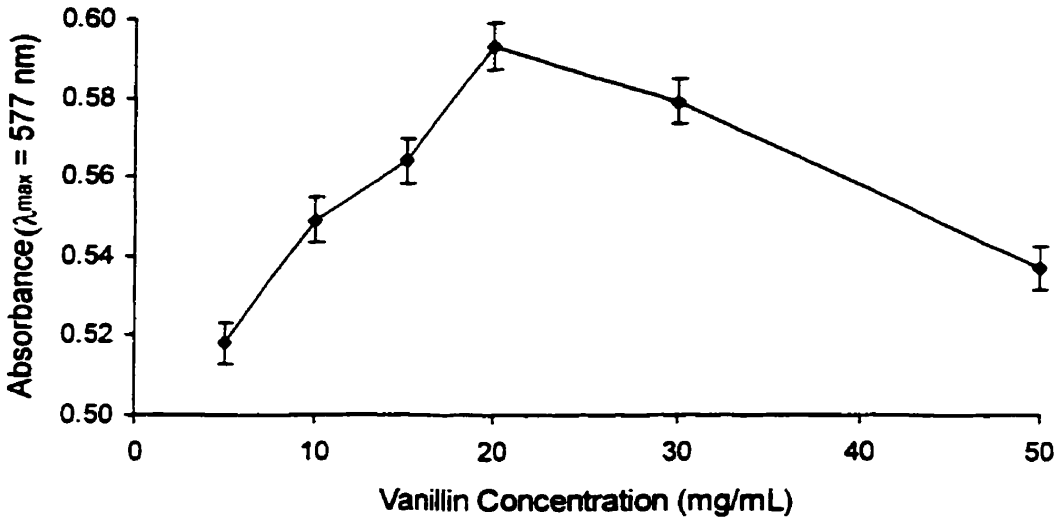

Figure 3.5. Absorbance vs. vanillin concentration

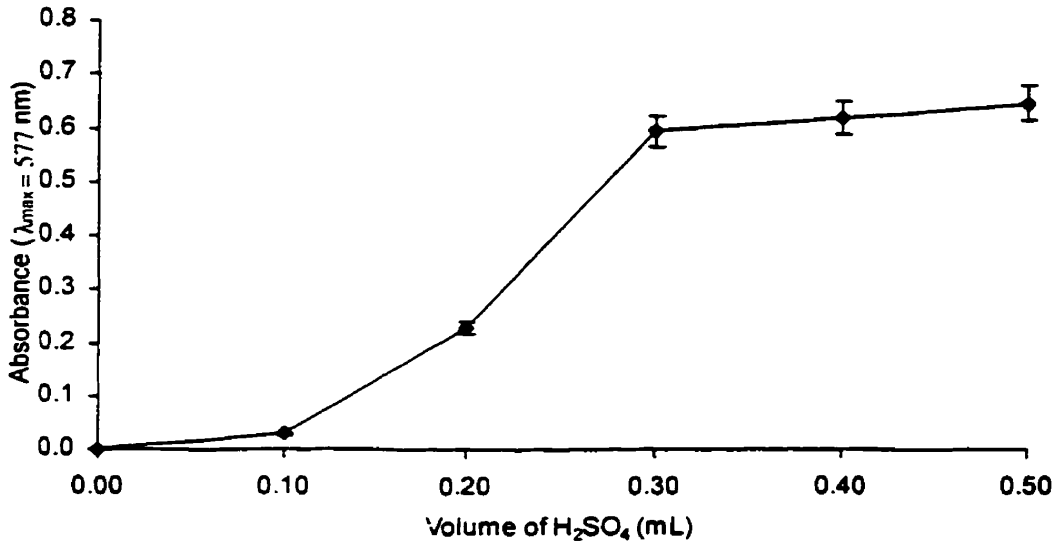

Figure 3.6. Absorbance vs. mL of H_2SO_4 (98%)

3.4.2. Calibration curve with azadirachtin as the standard

A calibration curve (Figure 3.7) with standard azadirachtin DCM solution was obtained in the concentration range of 0.01 to 0.10 mg/mL. Linear regression shows R^2 as 0.9995. By taking into consideration the purity of the azadirachtin used for the calibration, the absorbance-concentration equation can be expressed as:

$$A_{577nm} = 9.0024 * C_{AZ} / 0.95 = 9.4752 * C_{AZ} \quad (3.2)$$

Where: $A_{577\,nm}$ — absorbance at 577 nm

C_{AZ} — Concentration of the 95% purity standard azadirachtin DCM solution in mg/mL

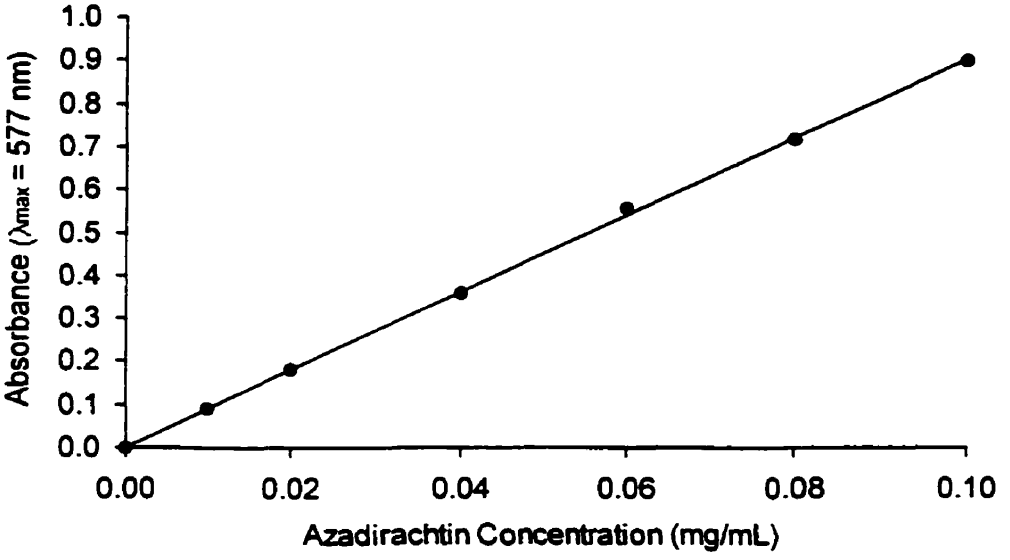

Figure 3.7. Absorbance vs. concentration (mg/mL) of standard azadirachtin solution subjected to vanillin assay.

This calibration curve can be used directly for the estimation of the AZRL in the purified neem seed extract. However, when this method is used for the analysis of the

extracts from other part of neem such as the leaf, the leaf stem and the seed shell, even after purification, severe interference occurred. In order to make this method more accurate and applicable to the analysis of other parts of neem, a mathematical method for eliminating the interference was developed and the details are discussed in the next chapter.

3.5 Summary

In this chapter the development of a new colorimetric method for the estimation of the total AZRL in neem extracts was described. Driven by the need to quantify a class of compound which can not be done with existing method, inspired by a phenomenon on a related analytical method -visualization on HPLC, borrowing concepts from ordinary chemical reactions, a two-phase-two-step colorimetric method was developed. After the development of this method, it can be concluded that it can be used for the estimation of the content of a class of compounds in the extract which can not be obtained with existing methods. Most importantly, this method makes it possible to build a new, more reasonable standard for determining the quality of the neem seeds and the commercial neem-based pesticides.

CONNECTING STATEMENT 2

Although we have a sound colorimetric method, due to the complicated nature of the natural products, the method can not be used directly for the analysis of the neem extracts, especially the extracts from the seed shell, the leaf and the leaf stem of neem. In order to eliminate the interferences caused by other components coexisting in the extracts, a multivariate calibration technique was employed. A brand new mathematical modeling method was introduced in CHAPTER IV in order to aid in the multivariate calibration technique and also to provide a brand new method for analysing visible spectra not only for this application but also for other types of spectroscopic methods.

CHAPTER IV

MULTIVARIATE CALIBRATION TECHNIQUE FOR INTERFERENCE ELIMINATION AND THE DEVELOPMENT OF A MATHEMATICAL MODEL

4.1. Abstract

Through the analysis of the visible spectra of the standards and the extracts subjected to the colorimetric method, the interferences were identified. With a mathematical modeling technique, the models for the absorbance peaks of the standards and the interferences were developed. These models were used to aid in the elimination of interferences and in the simultaneous calculation of the AZRL and ST content in the extracts with the multivariate technique. With the information obtained from the models of the spectra of azadirachtin and limonene, the mechanism of the colorimetric reaction was studied.

4.2. Introduction

One of the most important shortcomings of colorimetric methods for the analysis of natural products is the difficulty in avoiding interferences from undesired components. Extracts of plant origin contain different components which may or may not belong to the same class. Ideally, a colorimetric method should undergo color reaction with target components only. However, in reality this is not the case. It is more likely that compounds from other classes may also undergo the same color reactions under the same experimental condition. Even though the absorbance behavior of the interference may be different from that of the targets, an interference is likely to occur. Therefore, in order to make the colorimetric method workable for the analysis, some method must be developed to eliminate such interferences.

Several methods can be used for the elimination of interferences. One of the most commonly used technique is to purify the extracts to remove the interfering components (Sun *et al.*, 1998). However, in most cases the interfering components are not easy to remove through simple purification methods. Another method used employs "purification" of the spectrum. Here, information about the spectrum of the interfering components is needed. Generally, through subtracting the interference spectrum, a "purified"spectrum can be obtained. In this chapter a multivariate technique as reviewed in CHAPTER II, is used to determine the amount of interference. Based on the observation of the spectra of standards and the extracts, a mathematical modeling method was developed in this chapter to aid in the multivariate calibration technique. With the mathematical modeling and the multivariate calibration technique, the interference can be eliminated and the amount of AZRL and simple terpenoids (ST) can be determined simultaneously.

4.3. Analysis of spectra

The behavior of absorbance is a reflection of the structure of the compounds undergoing the colorimetric reaction. Through close observation of the spectra, it is possible to obtain useful information about the target components and the interferences.

4.3.1. Analysis of the spectra of neem seed extracts

As was demonstrated in the previous chapters, purified neem seed extracts after undergoing colorization process exhibit similar absorbance behavior to that of azadirachtin. However, a closer look shows that it is actually not a perfect fit. As shown in Figure 4.1, the spectrum of the extract fits well at the wavelengths range of 400-577 nm with that of azadirachtin, but the distribution becomes wider for the extract at higher wavelengths which indicates the presence of interfering components. By assuming that the interference does not affect the intensity of absorbance at 577 nm, we subtracted the azadirachtin spectrum from that of the extracts. What was left after this operation had a maximum absorbance at around

625 nm and the shape of the spectrum was similar to that of limonene (Figure 3.1). With the knowledge that simple terpenoids (ST) such as diterpenoids or triterpenoids are also important components of the neem seed extracts, it is reasonable to assume that the interference at higher wavelengths are due to the ST. Therefore, it is possible to use limonene as a standard to determine the amount of ST in the extracts. With the two-component calibration, the amount of AZRL as equivalent to that of azadirachtin and the amount of ST as equivalent to limonene can be determined simultaneously.

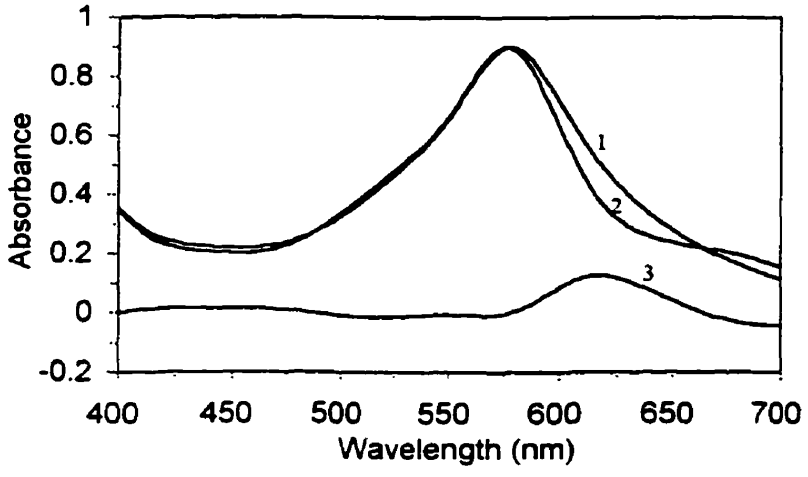

Figure 4.1. Visible spectra of standard azadirachtin, purified neem seed extracts, and the subtraction of them after vanillin assay; 1 — purified neem seed methanol extract, 2 — standard azadirachtin (0.1 mg/mL DCM solution), 3 — 1 minus 2

4.3.2. Analysis of the spectra of the extracts from neem leaf, the leaf stem and the seed shell

The spectra of the extracts from the neem leaf, the leaf stem, and the seed shell are quite different from that of the seed extracts, even after subjecting them to the same purification procedure. As shown in Figures 4.2, 4.3, and 4.4, even though the maximum absorbance still falls in around 577 nm, the absorbance is much wider in distribution than that

that of azadirachtin. This indicates severe interferences even though AZRL are still the main components in the extracts.

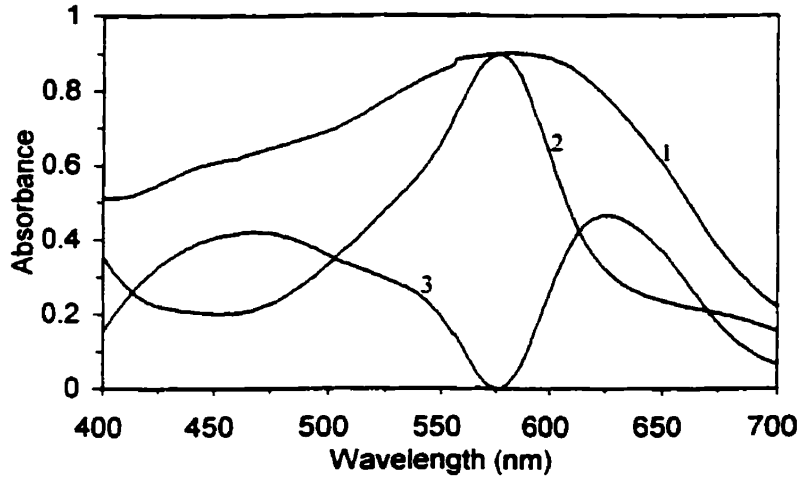

Figure 4.2. Visible spectra of purified neem seed shell extract, standard azadirachtin, and the subtraction of them; 1 — purified seed shell extract, 2 — standard azadirachtin, 3 — 1 minus 2

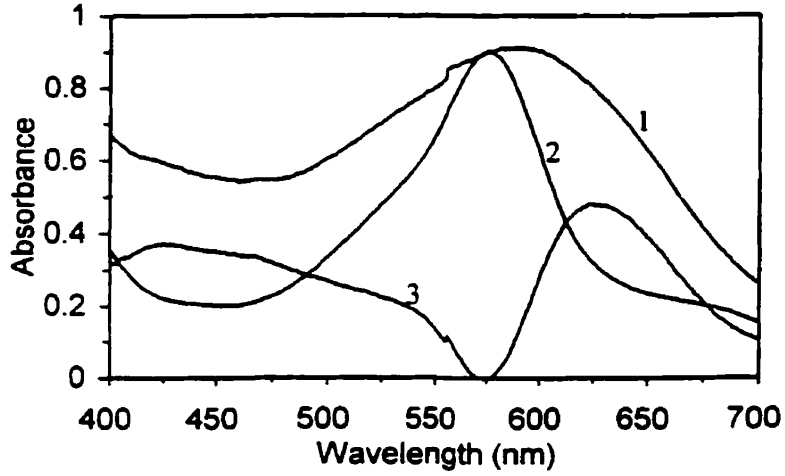

Figure 4.3. Visible spectra of purified neem leaf extract, standard azadirachtin, and the subtraction of them; 1 — purified seed shell extract, 2 — standard azadirachtin, 3 — 1 minus 2

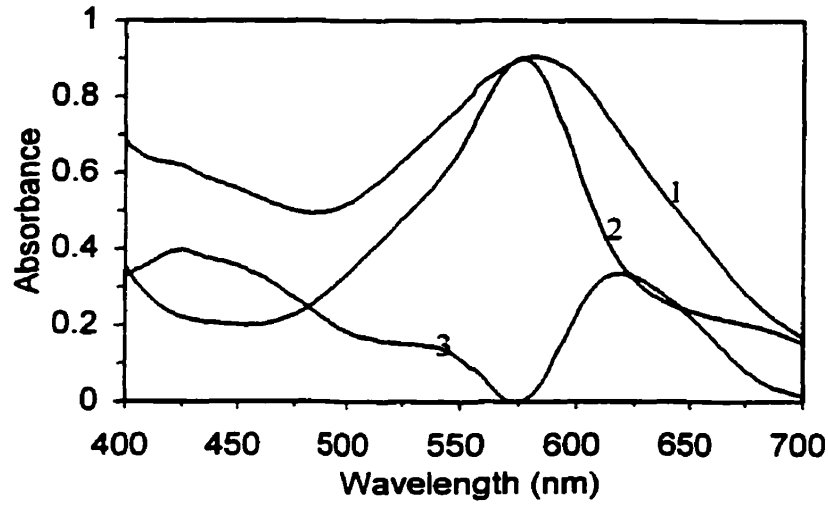

Figure 4.4. Visible spectra of purified neem leaf stem extract, standard azadirachtin, and the subtraction of them; 1 — purified seed shell extract, 2 — standard azadirachtin, 3 — 1 minus 2

By closely analysing the absorbance, we found out that the maximum absorbance is still centered around 577 nm, but instead of a sharp peak in the case of azadirachtin, the extracts have a much wider peaks (Figures 4.2, 4.3, 4.4). This indicates interference at around 577 nm which have a widening effect on the absorbance peaks. By subtracting the spectrum of azadirachtin from that of the extract, we obtained the curve 3 in Figures 4.2, 4.3, 4.4 indicating the interfering compounds absorb at around 625 nm and 500 nm. As was described in CHAPTER II, ST are even more important in the extracts of the leaf, the leaf stem and the seed shell than in the seed kernel extracts. Therefore, the interference at 625 nm is likely to be due to the existence of ST. The interference at 500 nm is quite likely to be due to the phenolic compounds which are common components of samples of plant origin. The phenolic compounds after subjecting to the vanillin assay exhibit maximum absorbance at around 500 nm (Sun *et al.*, 1998). In order to find out the interference existing at around 577 nm which had a widening effect to the spectrum, the subtraction method was employed

again. By subtracting the spectra of azadirachtin, limonene, and tannic acid (phenolic compound) from the that of the extracts, we obtained the curve 2 in Figure 4.5. The shape of this curve is similar to the spectrum of the neem seed kernel extract as shown in Figure 4.5 (curve 1). Therefore, we believe that the compounds belonging to phenolic and ST families are responsible for the interference at around 577 nm. With azadirachtin, limonene, tannic acid, and a PE layer extract of neem seed kernel as standard, a multivariate technique can be used to eliminate the interferences and to determine the amount of AZRL and ST in the extracts.

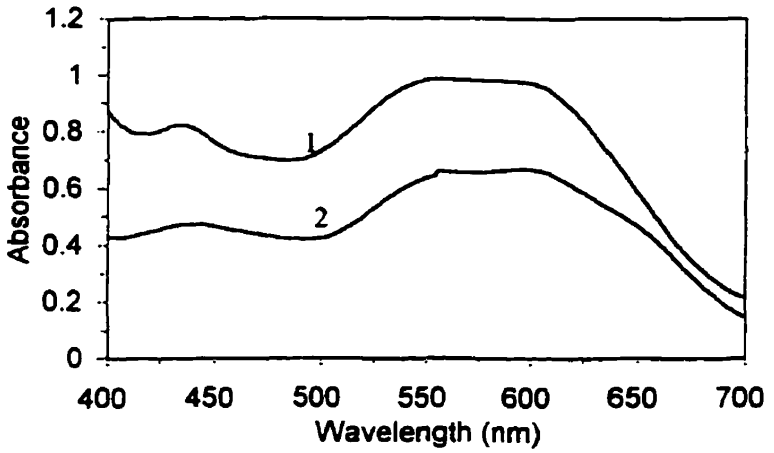

Figure 4.5. Visible spectrum of the PE layer of the neem seed kernel extract and the spectra of interferences for the leaf, the leaf stem, and the seed shell at around 577 nm: 1 — spectrum of the PE layer of the neem seed kernel extract; 2 — interference obtained by subtracting the spectra of azadirachtin, tannic acid, and limonene from that of the neem leaf extract.

4.4 Mathematical modeling of spectra

Through close examination, we observed that in most spectra peaks obeyed a Gaussian distribution. However, in some cases a shoulder peak or an unsymmetrical

distribution occurred. We can assume here that if there is only one functional group that is responsible for the color development or the absorbance, the absorbance over the wavelengths will still obey a Gaussian distribution, but if there are more than one group that contribute to the spectrum, a shoulder peak or an unsymmetrical distribution may occur.

Based on these observations, we can develop a mathematical model for each absorbance peak by Gaussian regression or linear regression. With this procedure, we do not need to calibrate one standard at many wavelengths if the multivariate calibration technique is applied. Furthermore, through the model, information can be obtained about the absorbance behavior and the absorbance-structural relationship.

4.4.1. Mathematical modeling of azadirachtin and limonene

Close analysis of the absorbance behavior of azadirachtin shows that it is not symmetrical. However, the right side of the peak can be a perfect fit to a Gaussian distribution. The following model was used for the right side of the absorbance:

$$y = y_0 + ae^{-\left[\frac{(x-577)^2}{b}\right]} \tag{4.1}$$

Through linear transformation, the following form was obtained:

$$p = \ln a - \frac{1}{b}q \tag{4.2}$$

Where:

$P = \ln(y-y_0)$

$q = (x-577)^2$

y_0 was obtained from the figure

Linear regression shows that R^2 is 0.9992 and all the parameters are also obtained through this linear regression. Therefore the spectra from 577 nm to 650 nm can be expressed as:

$$A = 0.23 + 0.61e^{-(\lambda-577)^2/1036.5} \tag{4.3}$$

Where:

A — absorbance

λ — wavelength

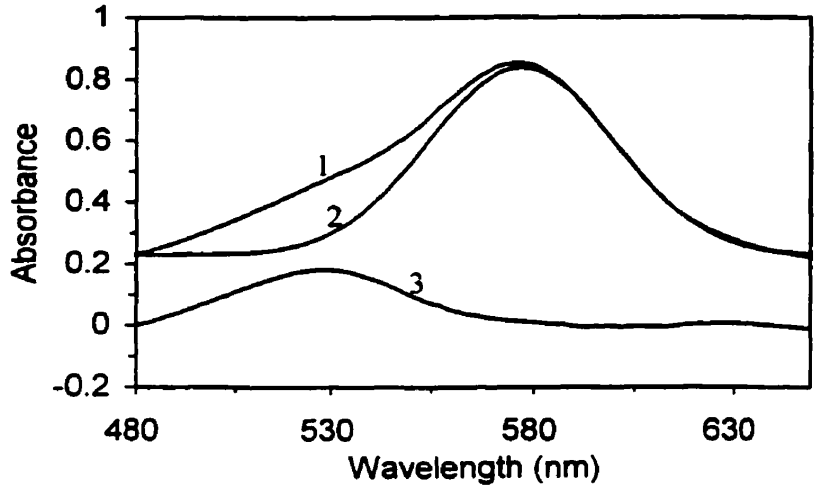

Figure 4.6. Composition of the spectra of azadirachtin following the vanillin assay: 1 — spectrum of azadirachtin; 2 — a Gaussian distribution curve obtained based on the linear regression; 3 — 1 minus 2

By applying the model to the whole wavelength range for azadirachtin and plotted together with that of the azadirachtin, we found that on the left side it did not fit. By subtracting the model from the original one, we obtained another Gaussian distribution

(Figure 4.6). Gaussian regression showed that the R^2 is 0.9963 and the equation was obtained as follows:

$$A = -0.0067 + 0.1885e^{\left(-(\lambda-526.85)^2/955.07\right)} \quad (4.4)$$

Where:

A — absorbance

λ — wavelength

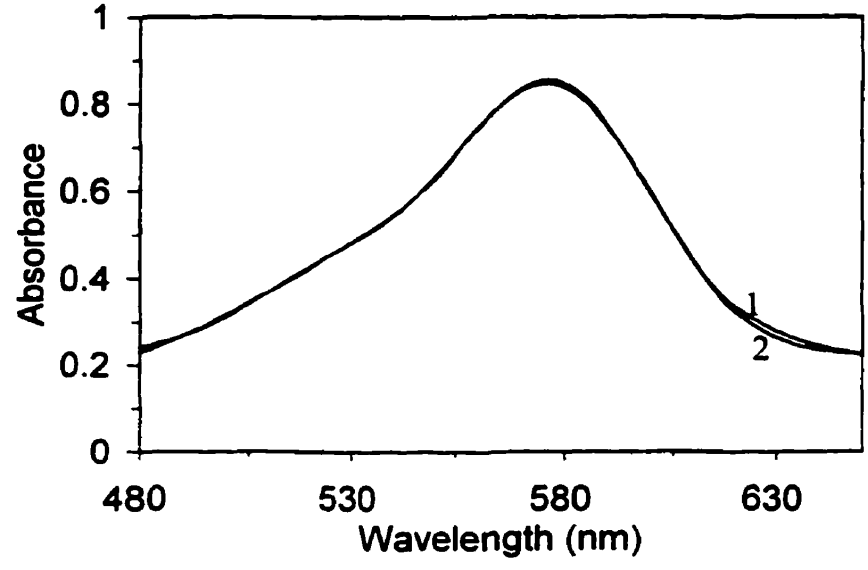

Figure 4.7. Simulation of the spectra of azadirachtin subjected to vanillin assay: 1 — standard azadirachtin; 2 — simulation curve

By Combining both of these models, we obtained a mathematical model for the absorbance of azadirachtin in the whole range of wavelengths (Equation 4.5). From Figure 4.7 we can see that the simulation curve obtained with the model, fits very well with the

experimental spectrum of azadirachtin. Therefore, we can use the following mathematical model to perform all the calculations.

$$A = 0.223 + 0.1885e^{-(\lambda-526.85)^2/955.07} + 0.61e^{-(\lambda-577)^2/1336.5} \quad (4.5)$$

Where:

A — absorbance

λ — wavelength

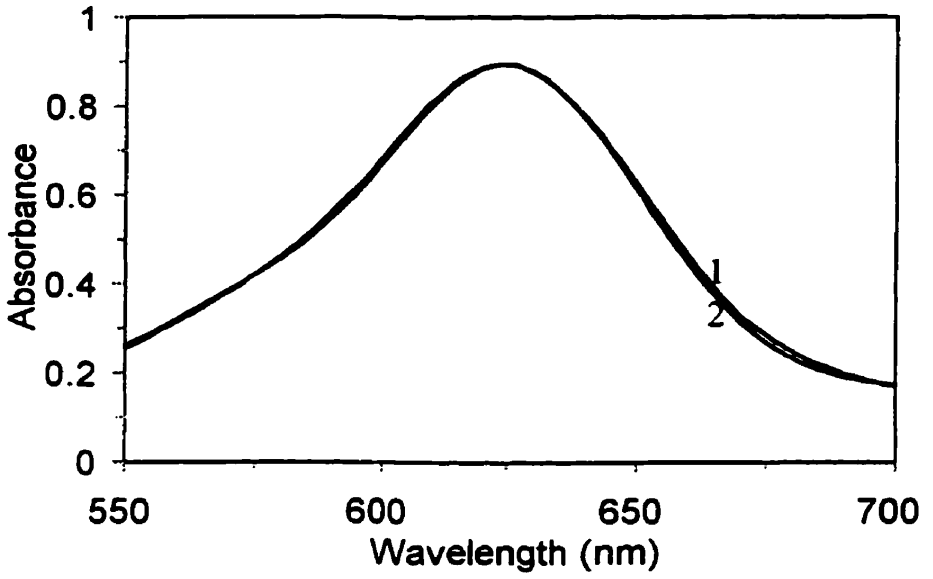

Figure 4.8. Simulation of the spectra of limonene subjected to vanillin assay: 1 — limonene; 2 — simulation curve

Following the same linear regression and Gaussian regression method, the mathematical model for limonene absorbance was also obtained:

$$A = 0.165 + 0.7243e^{-(\lambda-625)^2/1300.4} + 0.1522e^{-(\lambda-572)^2/867.5} \quad (4\text{-}6)$$

Where:

A — absorabnce

λ — wavelength

The R^2 for the linear regression and the Gaussian regression were 0.9998 and 0.997 respectively. The simulation curve was plotted together with the original limonene absorbance curve (Figure 4-8) and showed very good fit.

Through the R^2 obtained for the regressions and through the visible comparison of the simulated and original spectra, it can be concluded that the model can be used for the elimination of interferences without causing significant errors. The following models were obtained by using the calibration curve developed in the previous chapter to calibrate the model at 577 nm for azadirachtin and at 625 nm for limonene.

$$A_{AZ}^{mg/mL} = 2.496 + 6.826e^{-(\lambda-577)^2/1036.5} + 2.109e^{-(\lambda-527)^2/955} \quad (4.7)$$

$$A_{Limonene}^{mg/mL} = 9.46 + 41.56e^{-(\lambda-625)^2/1300.4} + 8.74e^{-(\lambda-572)^2/867.5} \quad (4.8)$$

Where:

$A_{AZ}^{mg/mL}$ — absorbance for azadirachtin at the concentration of 1 mg/mL

$A_{Limonene}^{mg/mL}$ — absorbance for limonene at the concentration of 1 mg/mL

4.4.2. A two-component model

By assuming that there are no interactions among the absorbance of different species, we can obtain the model for the absorbance of a mixture of components as follows:

$$A^{C}_{Extract} = C_{AZRL} * A^{mg/mL}_{AZ} + C_{ST} * A^{mg/mL}_{Limonene} \quad (4\text{-}9)$$

Where:

$A^{C}_{Extract}$ — absorbance for the extract with the concentration in mg/mL

C_{AZRL} — the concetration of azadirachtin related limonoids as equivalent to that of azadirachtin in mg/mL

C_{ST} — the concentration of simple terpenoids at equivalent to that of limonene in mg/mL

With this model, after obtaining absorbances at two different wavelengths, the concentration of each component can thus be determined. This model was tested with the mixture composed of azadirachtin and limonene with known concentrations. The concentration calculated with the two-component model agree well with the known values. The predicted and the experimental spectra are shown in Figure 4-9 a, b.

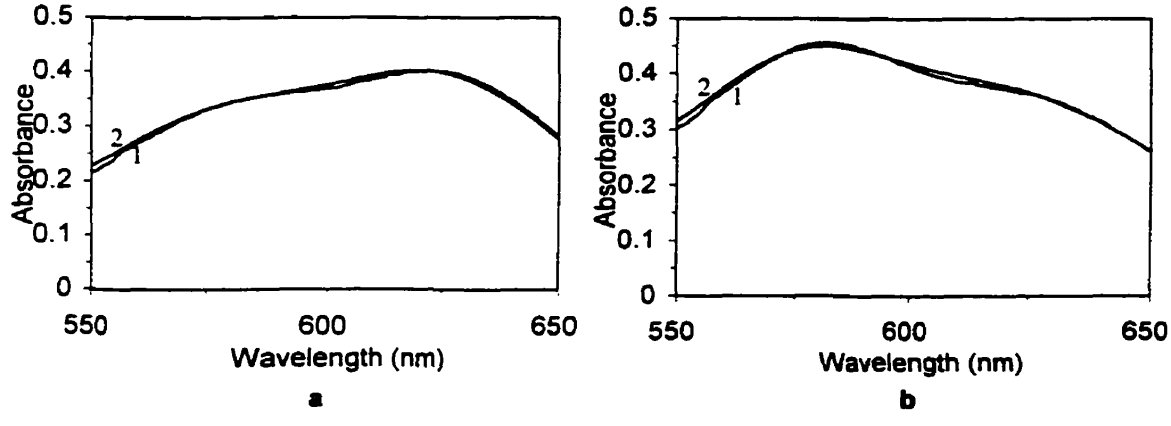

Figure 4.9. Spectra and the simulation curve of a two-components system: 1 — simulation curve; 2 — experimental spectra. a — $C_{Limonene}$ =0.013 mg/mL , $C_{Azadirachtin}$= 0.020 mg/mL; b — $C_{Limonene}$ = 0.010 mg/mL , $C_{Azadirachtin}$= 0.040 mg/mL.

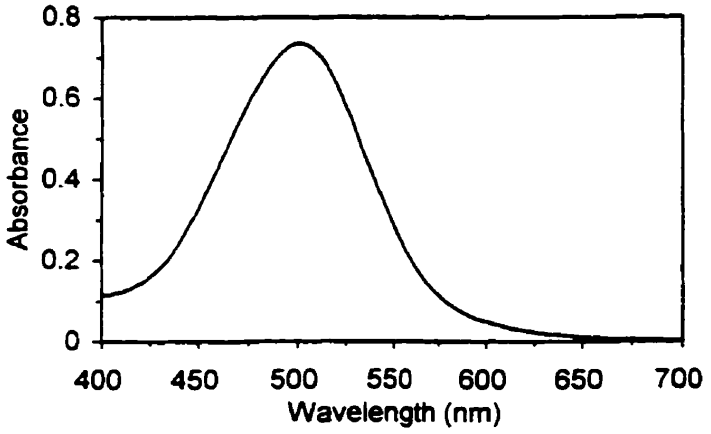

Figure 4.10. Spectra of tannic acid subjected to vanillin assay (interference for the leaf. leaf stem and seed shell extracts at around 500 nm)

4.4.3. Mathematical models for the interferences

As determined in section 4.2.2, the interferences for the colorimetric method in the extracts of the neem leaf, the leaf stem, and the seed shell are due to the ST at 625 nm, the phenolic compounds at 500 nm. The interferences at both sides of 577 nm peak had similar spectrum to that of the PE layer extract of neem seed kernel. The same was true for the neem seed extracts. Limonene was used as the standard for the determination of the amount of ST in these parts of neem. The interference from the phenolic components can be eliminated with tannic acid as the standard (Figure 4.10) and the spectrum of that fraction of the neem seed kernel extract was used for eliminating the interference at around 577 nm. Following the same regression method, the spectrum of the tannic acid over the wavelength range of 450-600 nm and the PE layer extract of neem seed kernel over the wavelength range of 550-650 nm can be expressed as:

$$A_{Tannic-acid} = 0.0732 + 1.3965e^{-0.5*\left(\frac{\lambda-499}{36.1591}\right)^2} \quad (4.10)$$

$$A_{Unknown} = 0.4697 + 0.5155e^{-0.5*\left(\frac{\lambda-573.12}{58.7282}\right)^{3.8}} \quad (4.11)$$

where:

$A_{tannic\text{-}acid}$ — absorbance of the tannic acid subjected to vannilin assay

$A_{unknown}$ — absorbance of the petroleum ether layer extract of neem seed kernel subjected to vannilin assay

The R^2 for these two Gaussian regressions were 0.9994 and 0.9993 for PE layer and for tannic acid respectively.

4.5. Application of the model

As described above, the mathematical equations for the standards and the interferences have been obtained through linear or Gaussian regressions with good fits as reflected by the R^2 values. Therefore, it is possible to use the mathematical models instead of the experimental data to make the analysis of the extracts simpler with minimum error. With the mathematical models, it is easier for the analysis to be carried out with the aid of a computer. Furthermore, the mathematical modeling can provide additional information that can not be obtained by the existing methods.

4.5.1. Analysis of the neem seed extracts with the two-component model

As shown in the previous sections, the spectra of purified neem seed extracts can be simulated with the two-component model. With this model (Equation 4.9), after taking two absorbance data in the range, the concentrations of both AZRL and ST can be determined as

equivalent to azadirachtin and to limonene srespectively. In order to make the calculation more convenient, the maximum absorbance for each component was used. Simulation curves with and without considering the influence of ST and the original absorbance curve are shown in Figure 4.11. It was found that even after partition, simple terpenoids are still present in the extracts and they still influence the results of the AZRL content. Therefore, all the calculations in the following chapters for the estimation of the content of AZRL are carried with the two-component model.

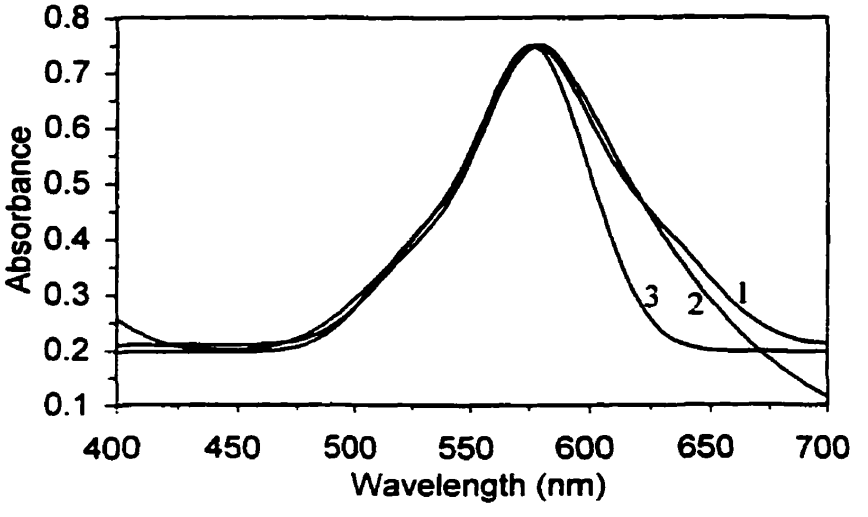

Figure 4.11. Simulation of the spectra of purified neem seed extract subjected to vanillin assay with two-component model and one-component model: 1 — two-component model simulation curve; 2 — neem seed extract; 3 — one-component model simulation curve.

4.5.2. Elimination of the interferences and quantification of the AZRL and ST in the leaf, leaf stem, and the seed shell of neem

The visible spectra of the extracts from the neem leaf, the leaf stem and the seed shell have several contributors as discussed previously. As far as the interfering components are concerned, such as the phenolic compounds and the interference at around 577 nm, we do

not need to calibrate these two components with standards. For the target components, the AZRL and the ST, we used azadirachtin and limonene as standards to calibrate the mathematical model so that we can obtain their concentrations relative to these standards. After obtaining four readings on the absorbance curve, which can represent the typical absorbance of each component, a matrix was used to calculate the concentration of each component:

$$A = EC \tag{4.12}$$

Where:

$$A = \begin{vmatrix} A_{\lambda_1}^{AZ} * C_{AZ} + A_{\lambda_1}^{Limonene} * C_{ST} + A_{\lambda_1}^{Tannic-acid} * C_{Phenolic} + A_{\lambda_1}^{PE-layer} * C_{Unknown} \\ A_{\lambda_2}^{AZ} * C_{AZ} + A_{\lambda_2}^{Limonene} * C_{ST} + A_{\lambda_2}^{Tannic-acid} * C_{Phenolic} + A_{\lambda_2}^{PE-layer} * C_{Unknown} \\ A_{\lambda_3}^{AZ} * C_{AZ} + A_{\lambda_3}^{Limonene} * C_{ST} + A_{\lambda_3}^{Tannic-acid} * C_{Phenolic} + A_{\lambda_3}^{PE-layer} * C_{Unknown} \\ A_{\lambda_4}^{AZ} * C_{AZ} + A_{\lambda_4}^{Limonene} * C_{ST} + A_{\lambda_4}^{Tannic-acid} * C_{Phenolic} + A_{\lambda_4}^{PE-layer} * C_{Unknown} \end{vmatrix}$$

$$= \begin{vmatrix} A_{\lambda_1}^{Total} \\ A_{\lambda_2}^{Total} \\ A_{\lambda_3}^{Total} \\ A_{\lambda_4}^{Total} \end{vmatrix}$$

$$E = \begin{vmatrix} A_{\lambda_1}^{AZ} & A_{\lambda_1}^{Limonene} & A_{\lambda_1}^{Tannic-acid} & A_{\lambda_1}^{PE-layer} \\ A_{\lambda_2}^{AZ} & A_{\lambda_2}^{Limonene} & A_{\lambda_2}^{Tannic-acid} & A_{\lambda_2}^{PE-layer} \\ A_{\lambda_3}^{AZ} & A_{\lambda_3}^{Limonene} & A_{\lambda_3}^{Tannic-acid} & A_{\lambda_3}^{PE-layer} \\ A_{\lambda_4}^{AZ} & A_{\lambda_4}^{Limonene} & A_{\lambda_4}^{Tannic-acid} & A_{\lambda_4}^{PE-layer} \end{vmatrix}$$

$$C = \begin{vmatrix} C_{AZ} \\ C_{ST} \\ C_{Phenolic} \\ C_{Unknown} \end{vmatrix}$$

The values in the matrix E can be obtained through Equations 4.7, 4.8, 4.10, and 4.11. Through this calculation, the concentration of each component can be obtained. By subtracting the interferences from the phenolic compounds and the unknown components with the obtained concentration values, the absorbance spectra of the AZRL and the ST were left. As shown in Figures 4.12, 4.13, 4.14, after the elimination of the interferences, it is obvious that the remaining components fit the two-component model quite well. This fact indicated that through this calculation, the interferences can be eliminated and the content of both AZRL and ST can be reliably obtained. In the following chapters the calculation of the AZRL and the ST content of neem leaf, leaf stem, and the shell were carried out with this method.

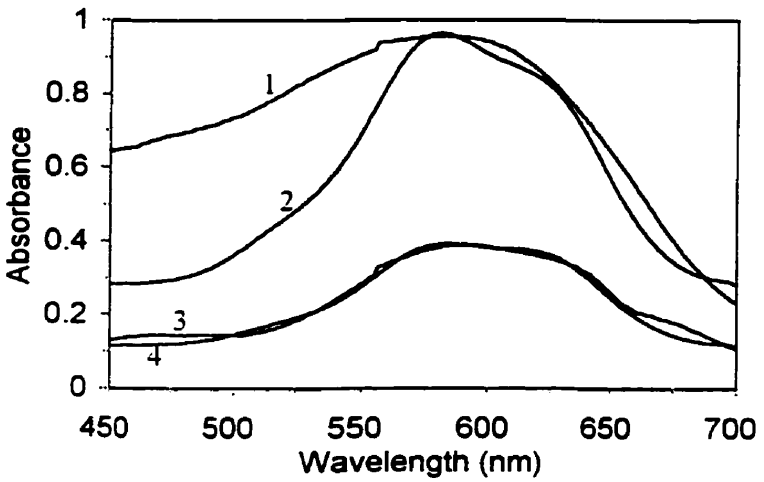

Figure 4.12. Simulation of the neem seed shell extract subjected to vanillin assay with the two-component model before and after removal of the interferences: 1 — neem seed shell extract subjected to vanillin assay; 2 — simulation curve before the removal of the interferences; 3 — spectra after the removal of interferences; 4 — simulation curve after the removal of the interferences

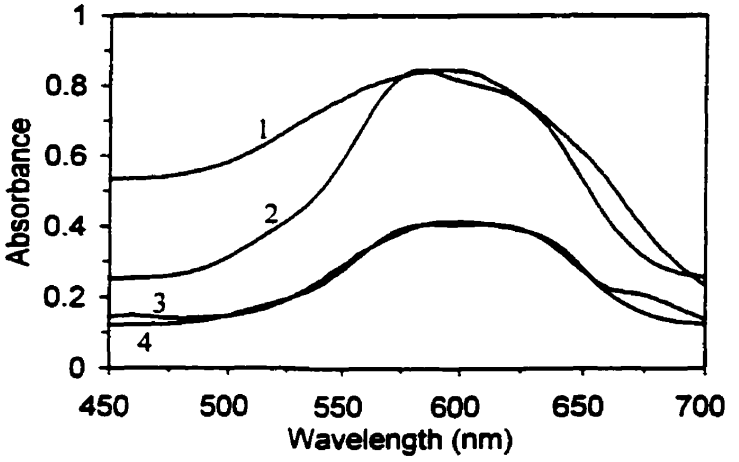

Figure 4.13. Simulation of the neem leaf extract subjected to vanillin assay with the two-component model before and after removal of interferences: 1 — neem leaf extract subjected to vanillin assay; 2 — simulation curve before the removal of the interferences; 3 — spectra after the removal of interferences; 4 — simulation curve after the removal of the interferences

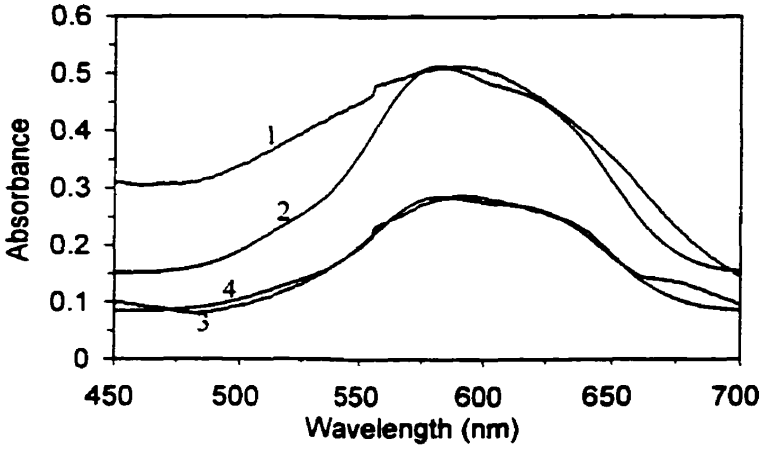

Figure 4.14. Simulation of the neem leaf stem extract subjected to vanillin assay with the two-component model before and after removal of interferences: 1 — neem leaf stem extract; 2 — simulating curve before the removal of the interferences; 3 — spectrum after removing interferences; 4 — simulation curve after removing interferences

4.5.3. Information from the mathematical models

Simplification of the analysis of the extracts is not the only application of the mathematical modeling method, it can lead to information that can not be obtained with other existing methods.

The maximum absorbance wavelength and the shape of the spectrum are closely related to the functional groups that are responsible for the absorbance. The maximum absorbance wavelength is easy to obtain, but it is not always easy to characterize the shape of the absorbance peak. Traditionally, the information of the peak is mainly obtained though plotting the spectrum together with a standard. However, with the newly developed modeling method, the shape of the absorbance peak can easily be quantified. The maximum absorbance peak can be determined with the parameter λ_o in the models. The shape of the absorbance can also be quantified by the parameters b and c. Through the comparison of the b and c in the model, it is possible to obtain more quantifiable information of how the two absorbances fit. Furthermore, the quantification of the spectra with these parameters makes it easier to computerize the analysis of the typical absorbance behavior of each component.

4.6 Summary

In this chapter, we introduced a mathematical modeling method for the analysis of absorbance behavior of the standard compound and the extracts. With the aid of the mathematical modeling, the elimination of interferences for the analysis of leaf, leaf stem, and the seed shell extracts, and the simultaneous determination of AZRL and ST content in all parts of neem are simplified. Most importantly, the mathematical modeling can provide information that can possibly be used to characterize the absorbance of individual compound or functional group. From the information on the mathematical model provided, we suggest

that the colorimetric reactions for azadirachtin and limonene are related. The application of this mathematical modeling method can possibly be extended to many areas of spectroscopic technique.

CONNECTING STATEMENT 3

After the development of the colorimetric method in Chapter III and the development of the analysis method of the spectra in Chapter IV, a new analytical technique was developed. With this technique, and the HPLC quantification, the content of azadirachtin in the neem seed kernel and the content of the total AZRL and ST of various parts of neem was investigated in Chapter V. To our knowledge, this is the first time that such an investigation has been performed.

CHAPTER V

INVESTIGATION OF AZADIRACHTIN, AZRL, AND ST CONTENT IN VARIOUS PARTS OF NEEM

5.1. Abstract

The azadirachtin content of the neem seed kernel was determined with the HPLC quantification technique. The results showed that the azadirachtin content of the sample we obtained from near Bangalore, India was among the highest compared with the reported values from other parts of the world. The AZRL and ST content in the seed kernel, the seed shell, the leaf, and the leaf stem of neem was determined with the newly developed colorimetric method and the multivariate calibration technique with the aid of a newly developed mathematical modelling method. From the comparison of the azadirachtin and the AZRL content in the same neem seed kernel extract we determined that azadirachtin accounted for around 58% of all the AZRL in the neem seed kernel extract. The AZRL and ST content in various parts of neem showed that the neem seed kernel was the most abundant source for the AZRL followed by the leaf. The Leaf stem and the seed shell contained much lower levels of AZRL. As compared to AZRL, the ST were less important for the neem seed kernel as compared to the other parts of neem as indicated by the ST to AZRL ratio. The neem bark and the commercial neem seed oil were also tested for their AZRL content and found no AZRL in the neem bark and only negligible amount of AZRL was detected in the commercial neem seed oil.

5.2. Introduction

Neem tree is famous for both pesticidal and medicinal properties. Studies have shown that the existence of limonoids and other terpenoids are responsible for most of its pesticidal and part of its medicinal properties. Azadirachtin is believed to be the most important limonoid due to its high activity as pesticide and its high content in the neem seed. For this reason, azadirachtin is widely accepted as the standard for determining the quality of the neem seed or the grade of the commercial neem-based pesticides. However, as we know from previous chapters, azadirachtin is not the only active component in the neem extract. Most other limonoids are also pesticidally active and some are even more active than azadirachtin. Therefore, it is too arbitrary to use the azadirachtin content alone as the standard. It might be more reasonable to use the total azadirachtin related limonoids as the standard for the determination of either the quality of the neem seed or the standard of the commercial neem-based pesticides. In this chapter, an HPLC method was used for the estimation of the azadirachtin content in the neem seed and with the newly developed colorimetric method and the mathematical modelling, the contents of the total azadirachtin related limonoids (AZRL) and simpler terpenoids (ST) in the seed, leaf, leaf stem, and the seed shell of neem are determined.

5.3. Materials and Methods

5.3.1. Materials

Fresh neem seeds, leaf, leaf stem, and the old bark were collected from Bangalore, India during May, 1998. The seed kernels were removed from their shells and blended and blended with a coffee bean blender. The blended neem seed kernel was stored at below 0°C. Commercial neem oil was obtained from Medinova Chemicals, Chamarajpet, Bangalore, India. Conical flasks with volumes 100 and 250 mL were used for the extraction and magnetic stirrer was used to stir the samples at each conditions. Separatory funnels were used

for the partition of the extracts between different solvents or solvent mixtures. The solvents were evaporated using Büchi Rotovapor R114 (Fischer Scientific, Montreal, Canada). Test tubes, pipettes were used for the colorimetric method.

5.3.2 Chemicals

Azadirachtin (aprox. 95 % purity) was purchased from Sigma Chemical Company. Two stock solutions were prepared: 0.1 mg/mL in dichloromethane and 0.1 mg/mL in methanol, stored below 0°C in the refrigerator. HPLC grade methanol, dichloromethane and acetonitrile were purchased from Fisher Scientific; petroleum ether (60-80°C) was purchased from ACP chemicals Inc (Montreal, Canada). Vanillin and conc. H_2SO_4 (98 %) were obtained from Fisher Scientific.

5.3.3 Extraction procedures

Procedure 1. Extraction of neem seed kernel. A suspension of blended neem seed kernel (2.0 g) in petroleum ether (60 mL) was stirred at room temperature for 12 hrs. The defatted sample was extracted with methanol (3 x 20 mL) by stirring at room temperature for 12 hrs. The extract was evaporated under vacuum to obtain a yellow oil. The methanol extract was redissolved in methanol (10 mL) and water (10 mL) followed by the addition of 5 % sodium chloride solution (1.0 mL). This mixture was extracted with petrolium ether (6 x 20 mL) to further remove any remaining fat. The residue was then extracted with dichloromethane (3 x 20 mL). The combined dichloromethane layers were dried over Na_2SO_4 and the solvent was evaporated under vacuum to obtain a light yellow solid. The product was dissolved in dichloromethane and in methanol for further analysis. The process was repeated to obtain three replicates.

Procedure 2. Extraction of seed shell. A suspension of neem seed kernel (2.0 g) in methanol (2 x 20 mL) for 12 hours. The solution was filtered to a flask and every time 10 ml methanol was used to wash the filter paper and the combined solution was vacuum evaporated to dryness. The partition method was almost the same as procedure 1 except that the amount of solvents used were different. For the redisolving into aqueous methanol, 5 mL

methanol and 5 mL distilled water were used instead of 10 mL. For the de-emulation, only 0.5 mL 5 % NaCl solution was needed. The PE and DCM partitioning, 10 mL of PE or DCM were used each time instead of 20 mL. The DCM layer was vacuum evaporated, weighed, and was made into DCM solution for further analysis. The process was repeated to obtain three replicates.

Procedure 3. Extraction of the neem leaf and the leaf stem. The extraction procedures of the neem leaf and the leaf stem were almost the same as the extraction of the seed shell except 5.0 g of sample was used instead of 2.0 g for the extraction of the leaf stem. After extraction and the evaporation, the partitioning was almost the same as that in the procedure 1, but this time the partitioning is to remove the chlorophyll from the extracts and the water to methanol ratio was different from that for the partitioning of the seed or seed shell extract. Into the evaporated methanol extracts, 5 mL methanol and 10 mL of distilled water were added and 1 mL 5% NaCl solution was used for the de-emulation of the system. The rest of the procedure was same as in the above extractions.

Procedure 4. Extraction of the neem bark. The old bark of neem 5.0 g was cut into small pieces and placed in a 50 mL conical flask. Methanol 50 mL was added and a magnetic stirrer was used to stirr the sample under room temperature for 24 hrs. The extract was then filtered to a flask and was evaporated with a rotary evaporator under reduced pressure. A brown extract with part of crystal structure was then obtained. The extract was redissolved in DCM for further analysis.

Procedure 5. Extraction of the neem oil. Commercial neem oil (Medinova Chemicals, Chamarajpet, Bangalore, India, 2 g) was dissolved into 10 mL methanol followed by the addition of 10 mL distilled water. After the addition of 2 ml 5% NaCl solution, the aqueous methanol solution was first extracted with PE (6 x 20 mL) to remove the fats. The aqueous methanol layer was then extracted with DCM (3 x 20 mL) to enrich the AZRL into the DCM layer. The DCM layer was then evaporated of its solvents to obtain a dry extract.

5.3.4. Determination of azadirachtin content in neem seed by HPLC

An HPLC quantification method was developed for the estimation of the azadirachtin content in the neem seed. The quantification was based on a calibration curve of peak area versus concentration of commercial azadirachtin (95% purity). The HPLC Beckman system Gold consisted of a programmable variable wavelength UV detector model 166 and a solvent delivery module 110B was used with a Rheodyne injector equipped with a 20 μL loop. The system was controlled by Beckman Gold software. The separation was performed on a Waters Spherisorb ODS-25 column (4.6mm x 25 cm I.D., 5 μm) equipped with a Waters ODS guard column. The mobile phase was acetonitrile-water (4:6) and the flow rate was 1 mL/min. The detector was set at 214 nm. A calibration curve was generated by injecting standard azadirachtin solutions in methanol with concentrations ranging between 2 and 20 μg/mL. The concentration of azadirachtin in the extracts was calculated using the area of the peak and the calibration curve.

5.3.5. Determination of AZRL and simple terpenoids (ST) in various parts of neem

The newly developed colorimetric method and the mathematical modeling were used for the determination of the content of AZRL and ST simultaneously in various parts of neem. The colorization procedure is described as follows: into an dichloromethane solution (0.7 mL), a methanol solution of extract (0.2 mL) and vanillin (0.02 mg/mL) were added; after shaking manually, the mixture was left at room temperature for two minutes; concentrated sulfuric acid (0.3 mL, 98 %) was then added in three portions (0.1 mL each) and the mixture was stirred for 10s after each addition; after the addition of sulfuric acid was completed, methanol (0.7 mL) was added to convert the two layered mixture into a homogenous solution that instantly developed a blue green color. The colorized solution was left at room temperature for 5 minutes before the scan of the absorbance was carried out from the wavelength 700 to 400 nm using a Beckman DU-64 spectrophotometer equipped with a 10 mm quartz cell. The blank solution was obtained by substituting the test solution with an equal volume of dichloromethane in the above procedure. Absorbances at the wavelengths of 625, 577, 550, and 499 nm were read. Total AZRL and ST concentrations were calculated

using the mathematical modeling method as described in CHAPTER IV.

5.4. Results and Discussion

Limonoids are the most important principles in neem which are responsible for its excellent pesticidal and medicinal properties; one of these limonoids, azadirachtin is believed to be the most important one due to its high pesticidal activity and its high content in the neem seed. With the aid of HPLC quantification technique, the azadirachtin content in the neem seed kernel was determined. With the newly developed colorimetric method, mathematical modelling, and the multivariate calibration technique, the total AZRL and ST were determined in the neem seed kernel, the seed shell, the leaf, and the leaf stem. The azadirachtin content in the total AZRL was also determined through the comparison of the azadirachtin and the AZRL content in the neem seed.

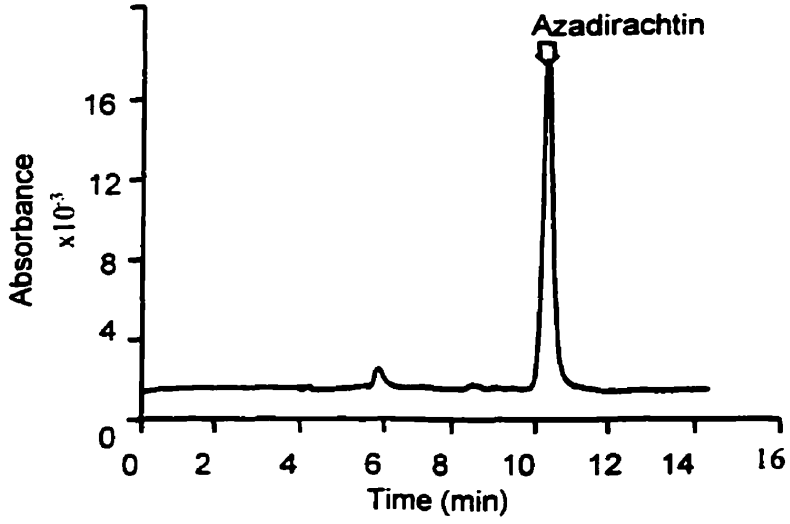

Figure 5.1. HPLC chromatogram of Azadirachtin (95% purity, 20 μg/mL)

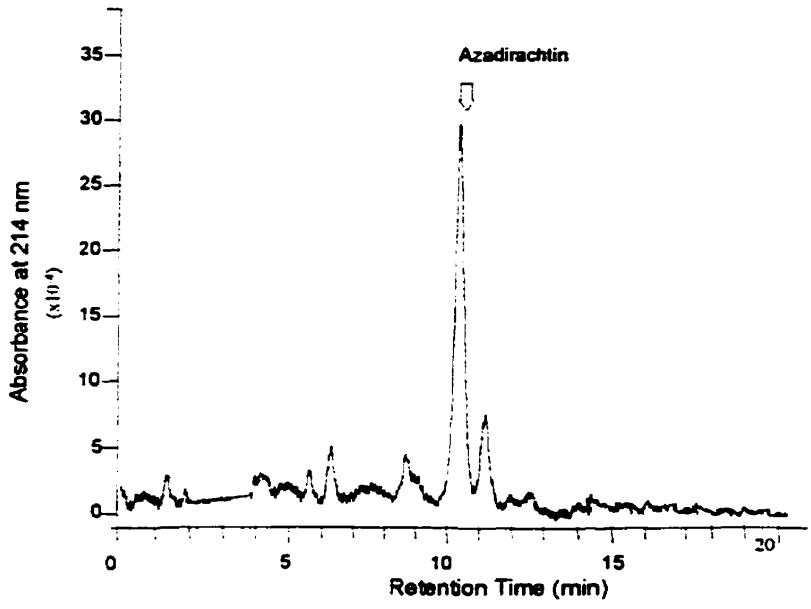

Figure 5.2. HPLC chromatogram of purified neem seed kernel extract (aprox. 15% azadirachtin 0.029 mg/mL)

5.4.1. Determination of azadirachtin content in neem seeds with HPLC quantification technique

The chromatogram of azadirachtin (95% purity) gave a distinctive peak at 10.2 minutes corresponding to azadirachtin (Figure 5.1). The smaller peak at 5.8 minute might be due to the 5% impurity in the commercial azadirachtin used. The chromatogram of the purified neem seed kernel extract is shown in Figure 5.2. As can be seen, the peak corresponding to azadirachtin separates well from the adjacent peaks which means this condition for HPLC can be used for the quantification.

A calibration curve of the peak area versus concentration with commercial azadirachtin as standard with the concentrations ranging from 2 to 20 μg/mL is shown in Figure 5.3. Linear regression showed an R^2 of 0.9993 and the peak area-concentration relationship is:

$$Area = 0.2038 * (C_{AZ} * 0.95) \mu g / mL \quad (5.1)$$

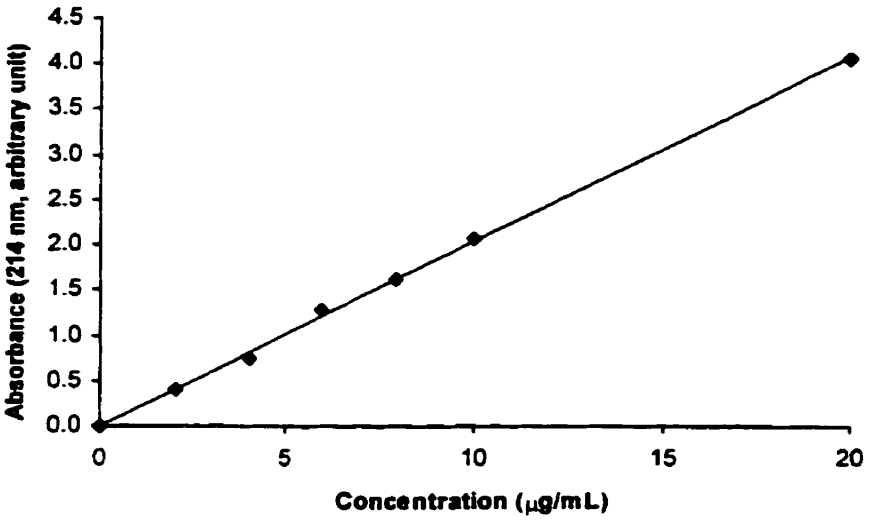

Figure 5.3. Calibration curve for HPLC quantification with commercial azadirachtin (95% purity) as standard.

Based on this calibration equation, the azadirachtin content in the extracts and in the neem seed kernels can be calculated by the following equations:

$$AZ\% \text{ in the extract} = 100 * \text{Area} / (226 * C_t^{HPLC}) \quad (5.2)$$

$$AZ \text{ content in the kernel (mg/g)} = W_e \text{ (mg)} * \text{Azadirachtin content} / W_{sample} \text{ (g)} \quad (5.3)$$

Where:

Area — the area of the chromatogram peak related to azadirachtin

C_{AZ} — Concentration of azadirachtin in μg/mL

C_t^{HPLC} — concentration of test solution for HPLC in mg/mL

W_e --- Mass of the extracts obtained by extraction

W_{sample} --- Mass of the sample for extraction

Azadirachtin content of the neem seed along with those reported in literature are shown in Table 5.1. It can be seen that the content determined here agrees with that reported in the literature for the seed from India. Compared to the values shown in Table 2.4 of CHATER II, the azadirachtin content is among the highest. This result agrees with that reported by Ermel (1995) that the highest azadirachtin A content were determined in samples coming from south or southeast of Asia. The climatic condition in Bangalore area of India is appropriate for the production of high quality neem seeds in terms of azadirachtin content.

Table 5.1. Azadirachtin content in the neem seeds and the comparison with the neem seeds from other parts of India.

Location	Bases	% AZ	References
Bangalore, India	Fresh neem seed kernel	0.57	Current study
Pune, India	Neem seed kernel	0.29	Azam (1995)
Madras, India	Neem seed kernel	0.6	Govindachari (1992)
/	Dry neem seeds	0.38	Yakkundi (1995)

5.4.2. Percentage of azadirachtin in the total AZRL

With the newly developed colorimetric method and the mathematical modeling, the content of total AZRL in the neem seed kernel is obtained (Table 5.2). Azadirachtin is one of the components of AZRL and the mount of which has also been determined with the HPLC method. Therefore, by simply comparing these two contents, we found that azadirachtin accounts for around 58% of the total AZRL in the neem seed kernel (Table 5.2). From this result we also know that other limonoids still accounts for around half of the total AZRL, and since most of them are pesticidally active, it might be more reasonable to use the total AZRL as a standard than arbitrarily using azadirachtin.

Table 5.2. Percentage of azadirachtin in the total AZRL in the neem seed kernel.

AZ% in the extract	AZRL%	AZ% in total AZRL
0.57	0.987	57.75

5.4.3. AZRL and ST content in the seed kernel, the seed shell, the leaf, the leaf stem of neem

The contents of AZRL and ST in various parts of neem were determined by the colorimetric method. The mathematical modelling and the multivariate calibration technique were used for the calculations. The results are shown in Table 5.3. It can be seen that the seed kernel has the highest AZRL content which accounts for almost 1% of the wet weight of seed. From Chapter II we know that most of these AZRL are pesticidally active and the high content of them is the main reason why the neem seed extracts are used traditionally as pesticides and commercially as the material for producing neem-based pesticides. The AZRL contents in other parts of neem decreased in the order Seed kernel > leaf > seed shell > leaf stem. Even though Sundaram (1996) reported that the azadirachtin content in the seed kernel of neem was more than 40 times of that in the leaf, the AZRL content we obtained is only around 6 times of that of the leaf. This indicates that in the leaf, the amount of different limonoids are in high diversity, while in the seed kernel, azadirachtin is the dominant principle. The AZRL in the leaf stem and the seed shell are much lower which may be due to their structure.

Table 5.3. AZRL and ST contents in various parts of neem

Part	AZRL %	% Simpler terpenoids	ST / AZRL
Seed	0.987	0.0147	0.0149
Seed shell	0.0687	0.0133	0.194
leaf	0.158	0.0197	0.125
leaf stem	0.0265	0.0050	0.188

As far as ST is concerned, the highest content was found in the leaf followed by the seed kernel and the seed shell. The lowest content was found in the leaf stem which is much lower than the other three parts. As compared with the contents of AZRL in terms of mass percentage, the ST contents were lower in all parts of neem and the lowest ST to AZRL ratio was found in the seed kernel (Table 5.3). The higher ST to AZRL ratio found in the leaf stem and the seed shell are mainly due to the extremely low AZRL content in these two parts. The information about the AZRL and ST content reported here may provide an important clue to understanding the biosynthesis of the AZRL in the neem tree. As we know limonoids are a class of highly oxidized terpenoids which have very complicated structures. ST may be the raw material for the biosynthesis of these limonoids, and the process is mainly carried out in the leaf which was shown to contain higher ST content and much higher ST to AZRL ratio than that in the seed kernel. After the AZRL are synthesized, they are stored mainly in the seed kernel.

The old bark of neem are also extracted with methanol as solvent. The extracts when subjected to the same colorimetric method showed the maximum absorbance at around 500 nm. The spectrum is similar to that of the tannic acid subjected to the same colorimetric method. This agrees with the reports that the bark of neem contains a high content of tannin (Tewari, *et al.*, 1992). As compared with the report that azadirachtin content is around 0.042% in the bark (Sundaram *et al.*, 1996), the results obtained in this study showed the absence of AZRL. We suggest that the AZRL exist in the fresh bark only. From the evolution point of view, the existence of these pesticidally active components has a function to protect the tree from the damage by the pests. When the bark of neem becomes older, it is no longer exposed to the damage by pests, therefore, the existence of these pesticidally active components are no longer necessary.

Commercial neem oil was also analysed with the colorimetric method, only negligible amount of AZRL was detected. The oil is produced by expression, based on the result obtained we suggest that AZRL can not be obtained along with the expressed oil. Therefore, the cake after the expression becomes an ideal material for the production of AZRL without the need of defatting the materials prior to the extraction.

5.5. Conclusions

In this chapter, the content of azadirachtin was determined with the HPLC quantification method. The results showed that the azadirachtin content in the sample used is among the highest content compared to the neem seeds from all over the world. The AZRL and ST contents in various parts of neem were determined with the newly developed colorimetric method, the mathematical modelling method and the multivariate calibration technique. The distribution of the AZRL and the ST in various parts of neem revealed that the leaf is the "factory" to assemble the AZRL with the ST as raw materials, and the assembled AZRL were subsequently stored in the seed kernel. Through the comparison of the content of azadirachtin and the AZRL, it was found that azadirachtin accounts for around 55% of the total AZRL in the neem seed kernel. To our knowledge, this is the first time that this quantification is done.

The newly developed colorimetric method, the mathematical modelling method and the multivariate calibration technique works well throughout this investigation. It was proved that these methods are fast and convenient to use and more importantly, this is the first method to date to be able to determine the total azadirachtin related limonoids in the neem extracts. Hopefully, this new method can be used for the quality determination for either commercial neem-based pesticides or the neem seeds.

CONNECTING STATEMENT 4

With the newly developed colorimetric method and multivariate calibration technique, and the mathematical modelling method, the AZRL and ST content in various parts of neem was investigated. In the next chapter, the microwave-assisted extraction method was investigated for extracting AZRL and ST from various parts of neem, and the newly developed quantification technique was used for the evaluation of the extraction efficiency.

CHAPTER VI

MICROWAVE-ASSISTED EXTRACTION OF AZADIRACHTIN RELATED LIMONOIDS (AZRL) FROM NEEM

6.1. Abstract

Microwave-assisted extraction of azadirachtin related limonoids (AZRL) from various parts of the neem tree was conducted in this chapter. The AZRL content in the extract was estimated with the newly developed colorimetric method and multi-variate calibration technique. A mathematical modeling method was also used to aid in the calculation. The influence of microwave power and irradiation time on the extraction yields were also investigated. The efficiency of the microwave-assisted extraction (MAE) of the seed kernel, the seed shell, the leaf and the leaf stem was compared to that with conventional extraction methods. The results showed that MAE technique can accelerate the extraction process except for the extraction of the seed kernel and also revealed that the increase in the temperature was one of the factors for this acceleration. The investigation on the influence of the solvent on MAE of the seed kernel, the seed shell and the leaf showed that the solvent used for MAE can not only affect the efficiency of the extraction, but also affect the components of the extracts.

6.2. Introduction

The neem tree is famous for its pesticidal property. Guided by the traditional practices such as mixing the neem leaf with the stored grains to protect from pests, modern research revealed a group of compounds, namely limonoids to be the main contributors to the pesticidal properties of the neem tree (Schmutter, 1990). Although neem seed is the main source of these limonoids, some of them were also isolated from other parts of the neem tree

such as leaf, twig, root or bark (Verkerk and Wright, 1993; Kumar *et al.*, 1996; Ragasa *et al.*, 1997; Ara *et al.*, 1988). The extraction of these limonoids is ordinarily carried out by soaking the seeds or other parts of neem in a solvent which is a time consuming process. However, this processing time can possibly be shortened by applying the microwave-assisted extraction (MAE) technique.

Extraction is a separation technique involving the transfer of the target components from the solid sample into a solvent. Traditional extraction methods are based mainly on the diffusion of target components in the sample matrices into the surrounding solvent. However the introduction of microwave irradiation to the extraction system brings some new characters to the extraction technique. The fast heating effect of the microwave energy to the solvents can shorten the time used for heating the solvents to a desired temperature and consequently accelerate the extraction process. More importantly, the fast and selective heating effect creates a new extraction mechanism rather than simply the diffusion of target components into the solvents (Paré and Belanger, 1997; Spiro and Chen, 1995; Chen and Spiro, 1995). Under this new mechanism, the extraction can be accelerated to an unbelievable speed (Paré, 1995b). Furthermore, it was also suggested the MAE technique can lower the solvent consumption, increase the extraction yield, and lower the pollution caused by the use of hazardous solvents (Paré *et al.* 1991; Paré, 1995b). Due to these attractive properties of the MAE technique, an investigation of the MAE of AZRL from various parts of neem was conducted.

6.3. Materials and Methods

6.3.1. Materials and Chemicals

The materials and the chemicals were the same as that in Chapter V, except that the sample neem bark and commercial neem oil, were no longer needed and the solvents and equipment related to the HPLC were not required.

6.3.2. Experimental procedures

Procedure 1. Investigation on time dependence of MAE of neem seed kernel with methanol as solvent: blended fresh neem seed kernel (1.0 g) was placed in a 250 mL quartz extraction vessel of the Synthewave 402 microwave system. Then methanol (30 mL) was added. The vessel was inserted inside the microwave cavity and fitted with a condenser. The sample then was irradiated with microwave (2450 MHz) for periods of 10 s, 30 s, 1 minute, 2 minutes, 3 minutes, 5 minutes and 10 minutes of total irradiation time. For the times longer than 30 s, a 30 s on, 30 off pattern sequence was used, and the power was set at 50 % (150 W). After the extraction, the solution was filtered to a flask and evaporated under vacuum to dryness. The extraction was repeated for another two times and the three extracts were redissolved in 10 mL methanol followed by the addition of 10 mL of water and 1 mL of 5% NaCl solution. The extract's aqueous methanol solution was partitioned with PE (3 X 20 mL) to further remove any remaining fat. The residue was then extracted with dichloromethane (3 x 20 mL). The combined dichloromethane extracts were dried over Na_2SO_4 and the solvent was evaporated under vacuum to obtain an amorphous light yellow solid. The product was dissolved in dichloromethane for further analysis.

Procedure 2. Investigation of power dependence of MAE of neem seed kernel with methanol as solvent. The extraction and partitioning method for the investigation of the power dependence of MAE for seed kernel was essentially the same as the procedure 1 except the programs used for the MAE were different. In this section, the irradiation times used were fixed while varying the microwave power. Two different irradiation times of 3 minutes and 10 minutes were used; for each irradiation time four power levels 30, 90, 150, and 240 W were used. The partitioning were the same as that of *Procedure 1* and the DCM layer was made into DCM solution for further colorimetric analysis.

Procedure 3. Investigation of time dependence of MAE for neem leaf with methanol as solvent. The extraction and the partitioning of the extract were the same as that of *procedure 1* except in the partitioning procedure, the water to methanol ratio increased to 2/1 (v/v). The DCM layer after partition was dissolved in DCM for further colorimetric analysis.

Procedure 4. Investigation of power dependence of MAE for neem leaf with methanol

as solvent. The extraction and the partitioning procedure were the same as that for *procedure 3.* The DCM layer after partition was dissolved in DCM for further colorimetric analysis.

Procedure 5. Comparison of MAE, RTE, and RFX for the neem seed, seed shell, neem leaf, and the leaf stem. Blended neem seed (1.0 g) was stirred overnight in petroleum ether (30 mL) at room temperature. After filtration, the defatted residues were used to study of the efficiency of different extraction methods. All the other samples used were not required to be defatted with PE prior to investigation of the different extraction methods. Three different extraction methods were investigated: (a) MAE. The defatted seeds were extracted with methanol (30 mL) using the following irradiation sequence at 150 W: 30 s on, 30 off for a total of 10 min irradiation time. At the end of the irradiation sequence the solution was left for around 1 min before it was filtered and evaporated in vacuum to yield an orange amorphous solid. Triplicated extractions were made and the combined methanol extracts were partitioned following the same method as that used in *procedure 1* and the DCM layer after partitioning was dissolved in dichloromethane for further colorimetric measurement. The extraction of the seed shell, the neem leaf and the leaf stem was the same as that for the seed kernel except that a sample of 2.5 g instead of 1.0 g was used for the leaf stem. (b) RTE. The procedure was essentially the same as that of MAE except that the extraction step was performed with stirring at room temperature for 20 min. (c) RFX. The same procedure was used except that the extraction was carried out in refluxing methanol for 20 min. All experiments were performed in triplicates.

Procedure 6. Investigation of the influence of solvent on MAE efficiency for the seed, seed shell and neem leaf. Three solvents methanol, dichloromethane, and petroleum ether were used for this investigation. The extraction of the seed were the same as the one with 10 minutes irradiation time in *procedure 1.* For the extraction with other solvents, all the procedures were the same as that with methanol except that the solvent used are different. All the extracts followed the corresponding partitioning method as described previously and the DCM layers of each extract were dissolved in DCM for further colorimetric investigation.

6.3.3. Quantification methods

The quantification method was the same as that described in Section 5.3.5 of CHAPTER V.

6.4 Results and Discussion

6.4.1. Investigation of the power and irradiation time dependence of MAE efficiency for the extraction of the seed kernel and the leaf

Investigation of time and dependence of MAE of neem seed kernel and the leaf are presented in Figures 6.1 and 6.2. The AZRL yields presented in the figure were based on the value obtained through 24 hrs conventional extraction as shown in Table 5.3 in CHAPTER V. These yields were believed to be 100%. And this conventional extraction-based yield was used as the basis to calculate the yield throughout the discussion of this paper.

As can be seen from Figure 6.1 a, the amount of crude extracts obtained through MAE increases with the irradiation time; the increase is fast in the first few minutes and then becomes constant after around 5 minutes of total irradiation time. The AZRL yield versus irradiation time has a similar trend to the crude extracts (Figure 6.1 c). However, due to the decrease of the AZRL content in the crude extracts with the increase of irradiation time when the irradiation is longer than one minute (Figure 6.1 b), the curve becomes flatter and the yields became almost constant at around 50 %. The time dependence of the amount of crude extracts and the AZRL yield showed a similar trend to the investigation on seed (Figures 6.2 a, b, c) but the yields were less than 50 %. This investigation showed that after around 10 minutes of irradiation time, the amount of crude extract or the AZRL yield reaches more than 50%. Thus, further increase of the extraction time is not suggested for the extraction from the energy consumption point of view and it appears that the MAE method is not favorable for the extraction of AZRL from neem.

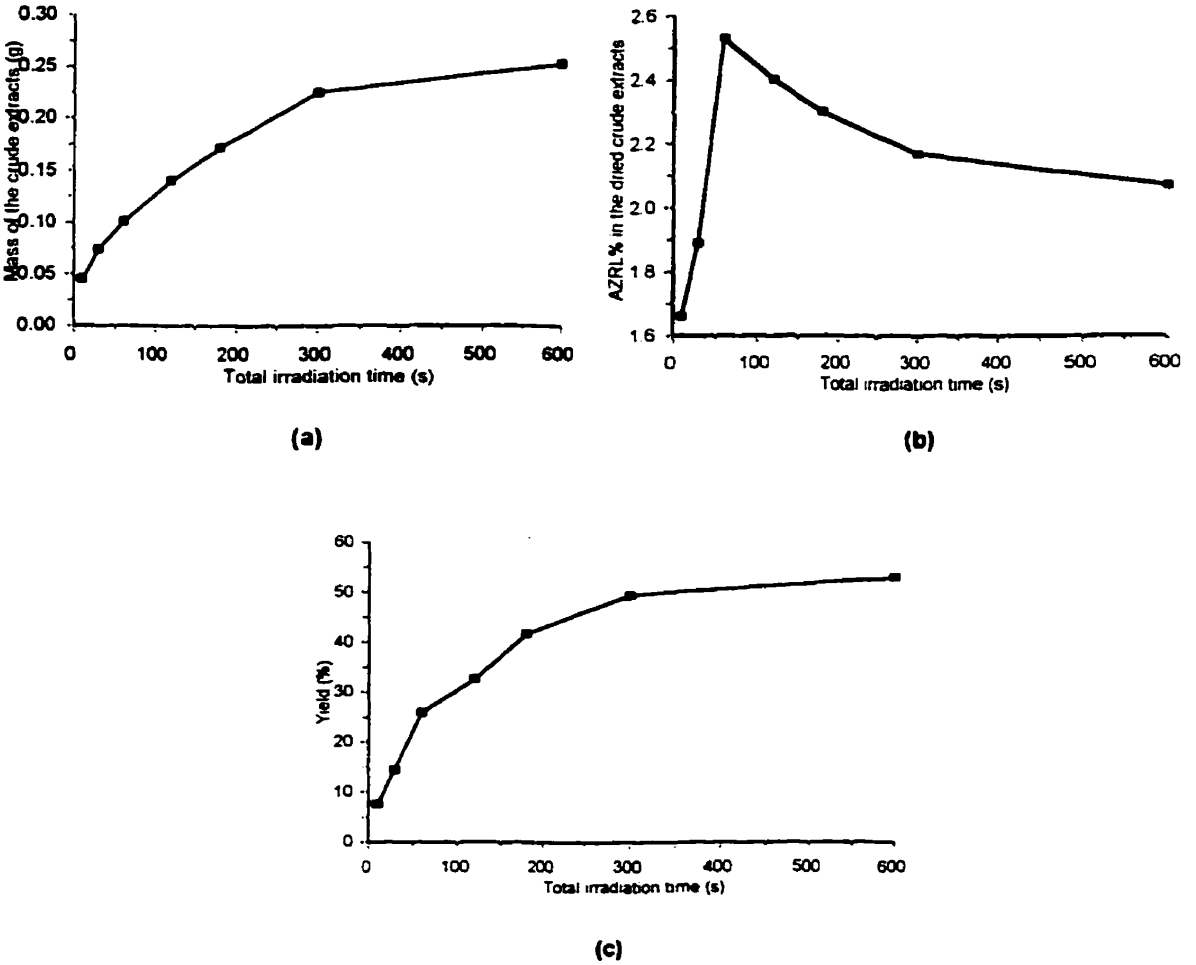

Figure 6.1. Time dependence of MAE of neem seed kernel: (a) Mass of crude extract versus irradiation time; (b) AZRL% in the crude extract versus irradiation time; (c) AZRL yield versus irradiation time

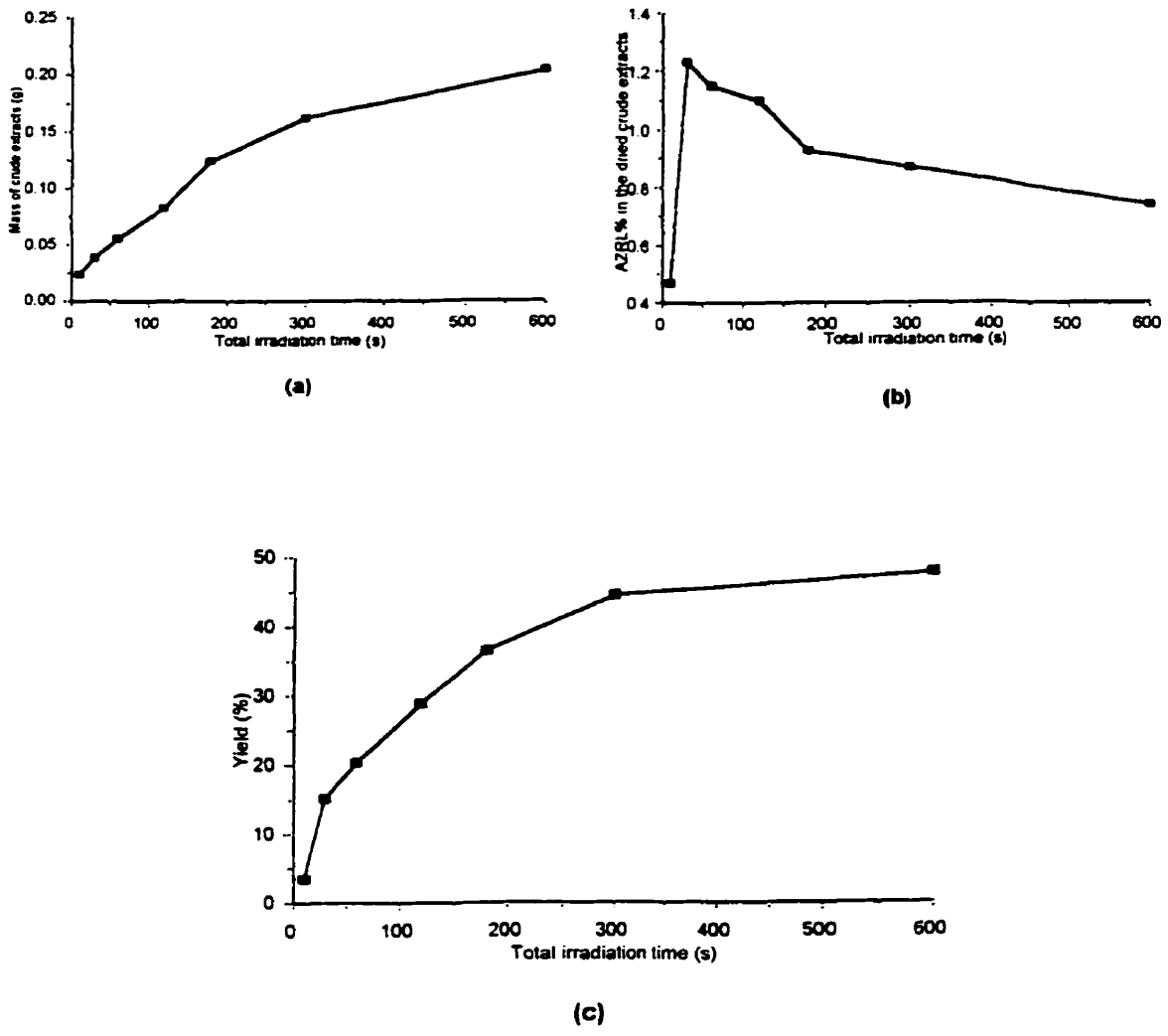

Figure 6.2. Time dependence of MAE of neem leaf: (a) Mass of crude extract versus irradiation time; (b) AZRL% in the crude extract versus irradiation time; (c) AZRL yield versus irradiation time.

Figure 6.1 b shows the content of AZRL in the crude extract as a function of irradiation time. In the first minute of irradiation time, the AZRL content increased from around 1.65 % to 2.55 % and then decreased with the increase of irradiation time. At the beginning of the extraction, those components that are very easy to extract or the methanol soluble components on the surface of the sample entered the solvents first, therefore the AZRL were lower in the crude extract. With the increase of extraction time, most of the easy-to-be-extracted components were extracted into solvents, therefore, the increase of the AZRL resulted in the increase of its percentage in the extract. With longer extraction time, other components which were more time dependent entered the solvent gradually causing the decrease in the percentage of AZRL in the crude extract. In case of MAE of neem leaf (Figure 6.2 b), the maximum content of AZRL in the extract was reached after 30 s of irradiation time and further decrease of the AZRL content in the crude extracts was mainly due to the increase of the amount of chlorophyll from the leaf. The appearance of the maximum content of AZRL in the crude extracts after half to one minute for seed kernels and for leaves indicated that the AZRL are the components that are easy to be extracted.

Microwave power used for the extraction also influences the amount of crude extracts or the yield of AZRL for both MAE of neem seed kernel and neem leaf. As presented in Figure 6.3 a, for both extraction with irradiation time of 3 minutes and 10 minutes, similar trends were observed. From 30 W to 90 W, a big increase was observed and then the increase slowed down. From 150 W to 240 W, the increase for the 3 minute irradiation was negligible and for 10 minute irradiation time a small decrease was noticed. Similar behavior of power dependence of the amount of crude extracts was observed for the MAE of neem leaf (Figure 6.4 a).

As for as the AZRL yield is concerned, 150 W is the optimum power level for the extraction of AZRL from neem seed kernel for either 3 minutes or 10 minutes irradiation time (see Figure 6.3 c). However, for the extraction of neem leaf, the yield of AZRL increased almost constantly with the power to a level of 90 % (Figure 6.4 c). Comparison of the 3 minute irradiation and 10 minute irradiation times showed that the influence of microwave power on the yield of AZRL became less significant with the increase of the extraction time.

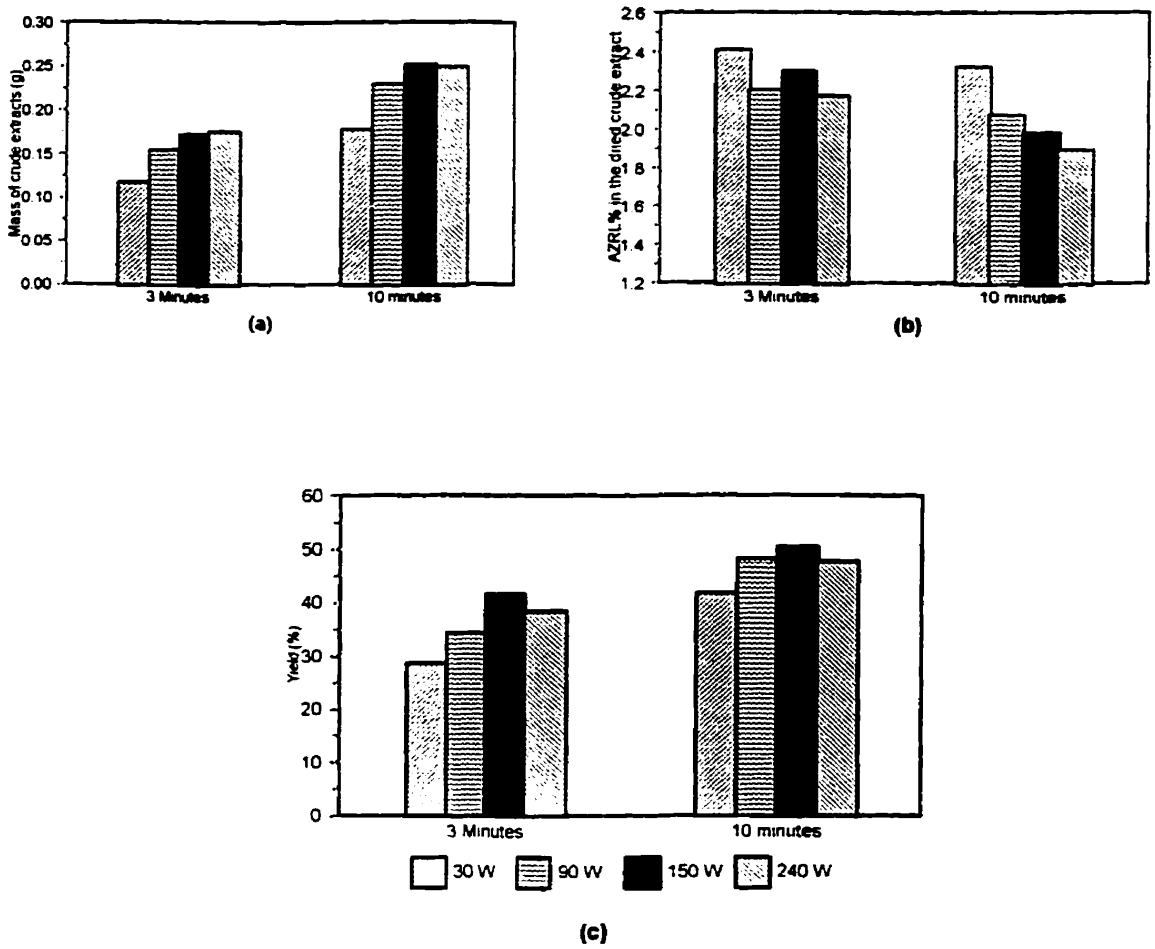

Figure 6.3. Power dependence of MAE of neem seed kernel: (a) Mass of crude extract versus irradiation time; (b) AZRL% in the crude extract versus irradiation time; (c) AZRL yield versus irradiation time

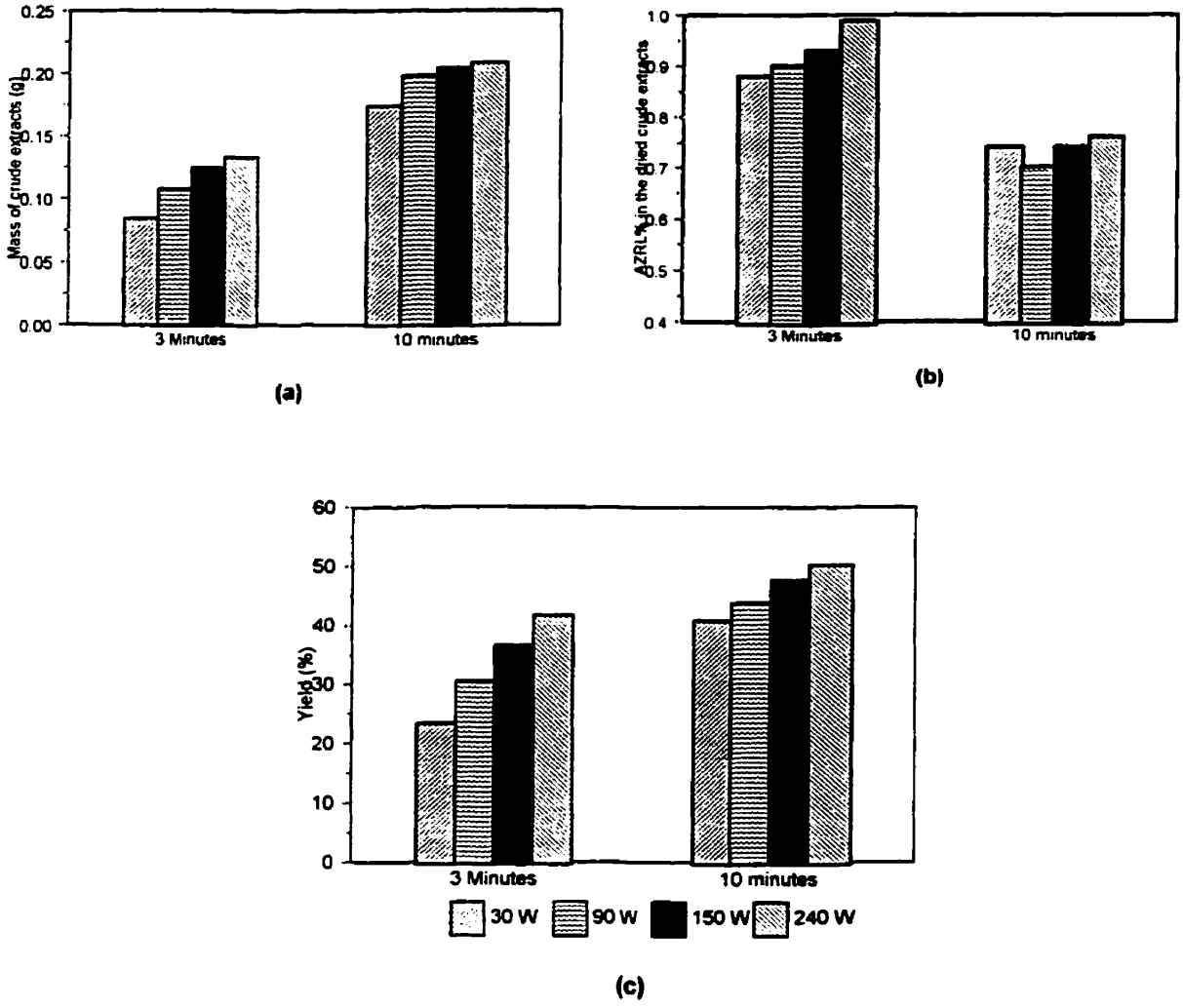

Figure 6.4. Power dependence of MAE of neem leaf: (a) Mass of crude extract versus irradiation time; (b) AZRL% in the crude extract versus irradiation time; (c) AZRL yield versus irradiation time

Figures 6.3 b and 6.4 b show the AZRL percentages in the crude extract for the MAE of neem seed kernel and leaf respectively. For the MAE of seed kernel, a general decrease of AZRL % in the crude extracts for both 3 minute and 10 minute irradiation time was observed, except at the 150 W for the 3 minute extraction time. However, it was interesting to note

that the AZRL percentages increased with the increase in power for both 3 and 10 minutes irradiation times. It was also interesting to observe that the decreasing trend with the increase of irradiation time becomes more apparent in the case of MAE of seed kernel, but the increasing trend became less significant or even reversed in the case of MAE of leaf. The decrease in the AZRL % in the crude extract for the MAE of neem seed kernel was mainly due to the amount of fatty acid extracted. With the increase of irradiation power, the amount of fat extracted increased. As observed from the time dependence investigation, the extraction of the fat was more time dependent and the longer the extraction time, the influence of the MAE power on the amount extracted became more significant. In the case of MAE of neem leaf, the influence on the AZRL % was mainly due to the amount of chlorophyll, the extraction of which was more time dependent than AZRL. And hence the power dependence of the extraction of AZRL was higher than that of chlorophyll.

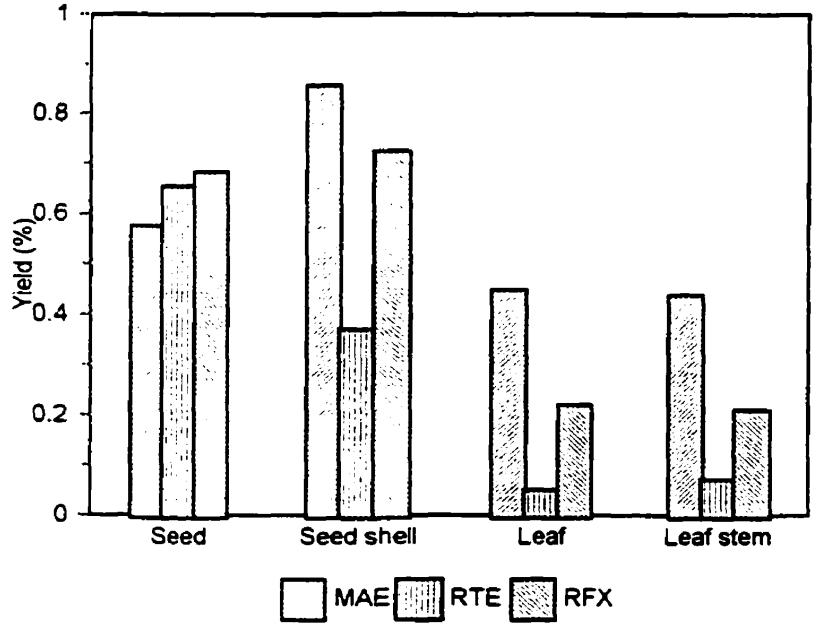

Figure 6.5. Comparison of the extraction efficiency of MAE, RTE, and RFX.

6.4.2 Comparison of extraction efficiency of MAE, RTE, and RFX methods

In order to get an idea about the advantage of the MAE over the conventional methods for the extraction of various parts of neem, a comparative experiment was designed for the extraction of seed kernel, seed shell, neem leaf, and leaf stem with MAE, room temperature extraction (RTE), and reflux temperature extraction (RFX) methods. To make the results comparable, the same extraction time was used for these three methods: 10 minutes irradiation time with 30 s on, 30 s off sequence for a total of 20 minutes for MAE, and 20 minutes extraction time for the other two methods. The results are presented in Figure 6.5. From Figure 6.5, it is surprising to find that for the extraction of neem seed kernel, the AZRL yield obtained by MAE was the lowest among the three methods used, even lower than the one by RTE. The reason for this disadvantage of the MAE method in this case might be due to the decomposition of the AZRL by the superheating effect created by the microwave heating. As reported, the MAE method could accelerate the extraction in many cases, and it worked especially well for the samples having plant origins and had very good selectivity (Paré and Belanger, 1994). However, the selectivity can be positive, but it can also be negative which means the target components are not the ones selectively extracted. In this case the lowest yield might also be due to the negative selectivity of the MAE method to the extraction of the AZRL from the seed kernel. By studying the yields obtained by these three methods, it was observed that with only 20 minutes extraction time, the yields had already reached around 60 %; this revealed that the AZRL in the seed kernel were very easy to be extracted, and it also suggested that the MAE method was not recommended as the method for the extraction of the neem seed to produce neem-based pesticides.

The extraction of the seed shell, the neem leaf, and the leaf stem showed a common order of the AZRL yield by these three methods: MAE > RFX > RTE. The fact that the highest yield was obtained by the MAE method revealed the accelerating effect of this method. However, it was also clear from the Figure 6.5 that the yield with RFX method is much higher than that obtained by RTE and only a little lower than that of MAE for the extraction of seed shell; this suggested that the temperature played a major role in the acceleration mechanism of extracting AZRL from the seed shell. It is possible that the higher

yield with the MAE method than RFX was caused by the super heating effect of the microwave heating which created a higher temperature environment for MAE than that for RFX.

For the extraction of neem leaf and the leaf stem, the accelerating effects were quite obvious. The yields obtained with MAE were more than double to that of RFX for both the extraction of leaf and leaf stem. This might be explained by the mechanism suggested by Paré *et al.*, (1997). The localized superheating on the micro-structural level of the leaf or the leaf stem caused explosion of the micro structures inside the sample causing the components flowing freely to the environment solvent or making it easier for the environmental solvents to contact with the target components. The temperature effect still exist in this case as indicated by the comparison of the RTE and RFX methods.

6.4.3 Influence of solvents on the extraction efficiency MAE

Paré *et al.*, (1997) suggested that for the extraction with MAE, microwave transparent solvent, ordinarily non-polar solvents were recommended. In this paper we designed an experiment to test the influence of solvents on the extraction efficiency of AZRL from various parts of neem. Three solvents were selected: methanol, both a good absorber to microwave energy and a good solvent to dissolve AZRL; DCM, a good solvent to dissolve the AZRL but not a good absorber of microwave energy; PE, neither a good absorber to microwave energy nor a solvent that can dissolve the AZRL. The same conditions were selected for all of these three solvents. The results are presented in Figure 6.6. An order of: methanol > DCM > PE is quite clear and the difference is so high that it suggested the solvents played a very important role in the MAE process. The comparison of the DCM and the PE, both of which are not good absorber to microwave energy suggested that the solubility of the solvent to the target components is a very important factor to consider. It is not suggested that the non-polar solvent be used for the extraction of any components with MAE method. The comparison of the MAE with methanol and DCM as solvents indicated again the temperature dependence of the extraction of the AZRL from various parts of neem. And the smaller difference in the extraction of leaf indicated a less temperature dependence

and a more MAE accelerating effect dependence of the extraction.

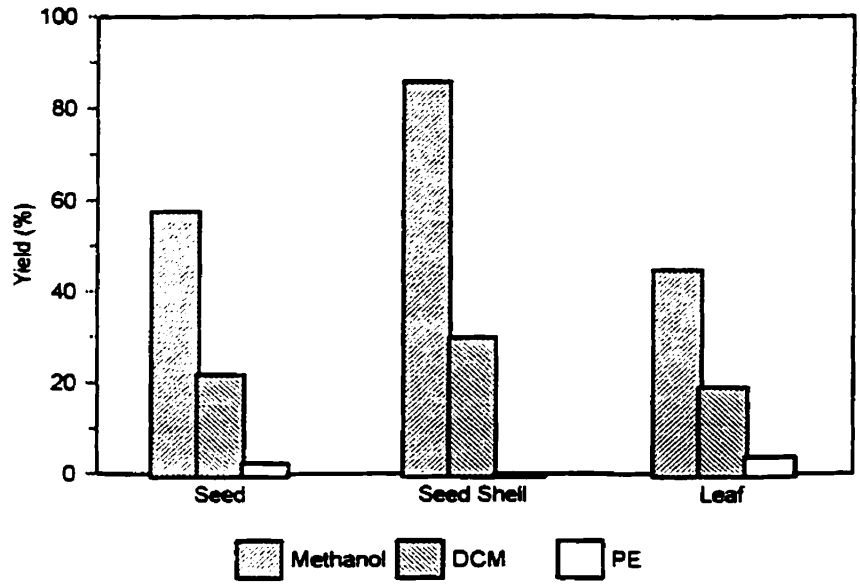

Figure 6.6. Influence of solvent on the MAE efficiency

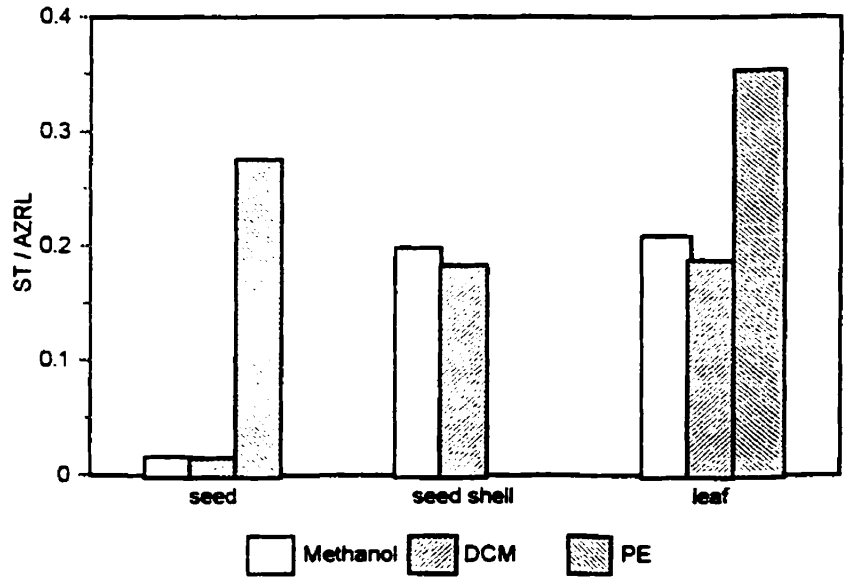

Figure 6.7. Influence of the solvents used on the ST to AZRL ratios

The components extracted with these three solvents were also investigated with the simple terpenoids (ST) to AZRL ratio as an indication (Figure 6.7). As we know, the solubility of the ST is much higher than that of AZRL for the solvent PE, while for methanol and DCM the difference is not that significant. From Figure 6.7, it is quite clear that the extraction with PE as solvent had the highest ST to AZRL ratio which indicated that with PE as solvent, more ST was extracted than with methanol or DCM as solvent. For MAE with methanol and DCM as solvents, there was no obvious difference between them. This again suggested the importance of the selection of the solvents on the MAE.

6.5. Conclusions

From the above discussion, it can be concluded that the crude extracts and the yield of AZRL were influenced by the irradiation time and the power used for MAE. The comparison of the three extraction methods MAE, RTE, RFX revealed an order of the extraction efficiency in terms of AZRL yield: MAE > RFX > RTE for the extraction of neem seed shell, the neem leaf, and the leaf stem. However, for the extraction of neem seed kernel, the MAE method had the lowest efficiency and is not recommended to be used in the production of neem-based pesticides commercially. A temperature dependent effect was found for all the extractions and the MAE accelerating effect was found for the extraction of neem leaf and the leaf stem. Solvent plays an important role in the MAE. The solubility of the solvents to the target components is an extremely important factor that affect the extraction efficiency and the selection of the solvents also affects the components extracted.

CHAPTER VII

GENERAL CONCLUSION AND RECOMMENDATIONS

Driven by the need to determine the quantity of the total azadirachtin related limonoids, inspired by the visualization method for azadirachtin and other terpenoids on TLC, borrowing a concept from the phase transfer reactions, a two-phase-two-step colorimetric method was developed for the determination of AZRL in neem extract. A multi-component calibration method was used with both commercial azadirachtin and limonene as standards so that the quantity of both azadirachtin related limonoids and simpler terpenoids can be determined simultaneously. A mathematical modeling method was developed to simplify the elimination of the interferences for the quantification of AZRL and ST in the extract of neem seed shell, leaf, and leaf stem. Also, this mathematical modeling method can provide useful information about the structure-absorbance relationship. The parameters of the models can possibly be used to analyze the structure of the principles or the functional groups of these principles. Furthermore, the application of this mathematical modeling method can possibly be extended to the analysis of the spectra of other spectroscopic methods.

An investigation of the azadirachtin content in the neem seed kernel by HPLC method and the AZRL and simpler terpenoids in the seed kernel, seed shell, leaf, and leaf stem by the new colorimetric method were undertaken. The azadirachtin content in the sample seed kernel was among the highest in its various locations and climatic conditions as compared to the content reported in the literature. Comparison showed that the azadirachtin accounted for around 58 % of the total azadirachtin related limonoids in the neem seed kernel; this conceded the report that azadirachtin is the most abundant limonoids in the seed kernel. Although azadirachtin as a component is in the highest content, other limonoids still accounts for around half of the total AZRL. Most of these AZRL are pesticidally active and some are even more active than azadiarachtin. Therefore, it might be more reasonable to use the content of AZRL as a standard for the determination of the quality of the seed and the grade of the commercial neem-based pesticides.

Through their applications in Chapter V and VI for quantifying the AZRL and ST in

the neem extracts, the newly developed colorimetric method, the multivariate calibration and the mathematical modeling were evaluated. For the analysis of purified neem seed kernel extracts, the two-components model proved to be efficient in quantifying the content of both AZRL and ST simultaneously. For the extracts from the neem seed shell, the leaf, and the leaf stem, the mathematical modeling and the multi-variate calibration technique eliminated the interference from the identified interferences and estimated the amount of AZRL and ST at the same time. With this quantifying technique, the problem for the lack of a reasonable standard for the determination of the grade of neem-based pesticides and for determining the quality of the neem seeds can be solved.

Microwave-assisted extraction method was investigated for the extraction of AZRL from various part of neem. The investigation of the extraction efficiency time-dependence and power-dependence suggested that not too long a irradiation time is necessary for either extraction of AZRL from either neem seed kernel or from the neem leaf, and a 50% power level is the best for the extraction of the seed while a higher power level is favorable for the extraction of the leaf. Comparison of the extraction of the AZRL from various parts of neem by three methods: MAE, room temperature extraction (RTE), and reflux temperature extraction (RFX) showed that the MAE is not favorable for extracting AZRL from the seed kernel as compared to the other two methods; therefore the MAE method is not suggested for the application in the production of the neem-based pesticides. For the extraction of the other parts of neem, a temperature dependence effect of extraction efficiency was observed and the microwave accelerating effect was found for the extraction of the leaf and the leaf stem. Study on the influence of the solvents on the extraction efficiency revealed that the selection of appropriate solvent is important for the MAE. The solubility of the solvent to the target components is one of the key factors to consider for the selection of a solvent. The study also showed that the components in the extract are quite different, when different solvents are used for the MAE.

Due to the limitation of time, many other aspects of this project are not investigated yet. As a development to this project, the following work is recommended for further investigation:

(1) Investigation of the mechanism of the colorimetric method.

(2) Investigation of the AZRL content-activity relationship to make this method a standard method for the determination of the quality of the neem seed or the grade of the commercial neem-based pesticides.

(3) Investigation of the AZRL content in the neem seed from a various locations to determine the quality of the neem seeds and to correlate the AZRL content with the location, and the climatic conditions.

(4) Investigation of the AZRL content in various commercially available neem-based pesticides and determine the grade with the new standard and compare that with azadirachtin as standard.

(5) Investigation of the interference absorabances in the neem leaf, seed shell, leaf stem extracts to make the multi-calibration method more reliable for the elimination of the interferences and for determination of the AZRL and ST content simultaneously.

(6) Investigate the relationship between the parameter of the mathematical models of the absorbances and the structure of the principles or the functional groups in the principles.

(7) Extend the application of the mathematical modeling method to the area of a variety of visible spectroscopy and other spectroscopic method.

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