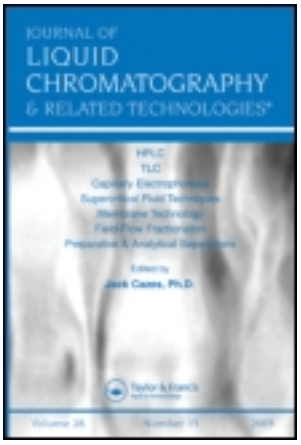

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PURIFICATION OF AZADIRACHTIN VIA SILICA GEL COLUMN CHROMATOGRAPHY

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□ *Separation efficiency for purifying azadirachtin from the mixture of neem limonoids was investigated by silica gel column chromatography in this study. The reliability of this method was confirmed by using limonoid powders A and B with initial azadirachtin purities of 18% and 7%, respectively. The silica gel chromatography employed in this study was capable of increasing the azadirachtin purity up to 4-fold with an azadirachtin recovery of approximately 50%. Powders with an azadirachtin content of approximately 50% and 28% were produced from limonoid powders A and B, respectively. Meanwhile, low separation efficiency was produced when starting material contained 50% azadirachtin. Therefore, this method is effective in increasing azadirachtin purity using starting materials with azadirachtin content below 20%.*

Keywords azadirachtin, column chromatography, limonoids, neem, separation efficiency, silica gel
ABBREVIATION EtOAc, ethyl acetate

INTRODUCTION

Bioactive compounds in neem oil have been known to possess many important properties such as anti-virus, anti-bacterial, and anti-feedant. Some compounds have been investigated as potential pesticide or insecticide to fight numerous plants diseases.[1–5] These compounds belong to triterpenoids. The major compounds as reported in literature are azadirachtin (azadirachtin A), salanin, nimbin, 3-tigloylazadirachtol

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(azadirachtin B), desacetylnimbin, and desacetylsalanin (azadirachtin D).[1–8] Many other compounds are present in smaller quantities in neem seeds.[9–13] The most prominent compound is azadirachtin, also named azadirachtin A to differentiate it from other azadirachtin related compounds, namely, compounds that are structurally related to azadirachtin. Azadirachtin and other structurally related compounds are classified as limonoids.

Limonoids from neem possess medium polarity. They can be dissolved in solvents with medium to high polarity such as methanol, ethanol, diethyl ether, dichloromethane, and ethyl acetate. Meanwhile, their solubility in water is very low and they are not soluble in nonpolar solvents such as hexane and petroleum ether. Limonoids have polarities that are very close to each other due to their close similarity in structure. This characteristic produces significant difficulties in the separation of individual limonoids.

Purification of azadirachtin from limonoids mixture is commonly carried out using column chromatography in either reverse phase or normal phase mode. Several column materials have been investigated to purify azadirachtin from neem sources such as silica gel, attapulgite clay, octadecylsilane (ODS), and phenyl. The mobile phase usually consists of a mixture of medium polarity solvent such as ethyl acetate or dichloromethane and low polarity solvent such as hexane or petroleum ether for normal phase chromatography. For reverse phase chromatography, the mobile phase usually is a mixture of water with methanol or acetonitrile. Crude mixtures of limonoids obtained by conventional pretreatment are usually used as the starting material. Combination of several column chromatography methods is required to produce a high purity azadirachtin. In most combinations, high purity (>70%) azadirachtin can be obtained after the application of HPLC method as the final step.[14–21]

A simple method to produce a mixture of neem limonoids or limonoid powder has been developed by Melwita et al.[22] Further investigation is required to develop limonoid powders as the potential starting material for neem-based product. In this work, limonoid powder was employed as the starting material for column chromatography purification of azadirachtin. Silica gel was selected as the column stationary phase. Silica gel chromatography itself is a common method in azadirachtin purification. However, information regarding separation efficiency of azadirachtin using silica gel chromatography has been insufficiently reported. Considering the important role of silica gel chromatography in azadirachtin purification, this work aimed to provide more concise information on separation efficiency of this method. Parameters such as the ratio of silica gel to sample and the mobile phase composition that affect separation efficiency were systematically investigated.

EXPERIMENTAL

Materials and Reagents

All solvents used were of analytical grade. Silica gel for column chromatography was procured from Silicycle (Quebec, Canada) with the following characteristics: particle size, 60–200 μm; specific surface area, 500 m^2/g; pore size, 60 Å; pH 7; and water content, 6%. The gel was activated by drying in an oven at 150°C for 1 hr to remove the adsorbed water. Water for chromatographic analysis was purified using a Nanopure purification system (Barnstead, USA). HPLC grade methanol, ethyl acetate, hexane, and toluene were purchased from Acros (USA). Standards of azadirachtin and salanin were obtained from Sigma Chemicals Co. (USA) and ChromaDex (USA), respectively. Limonoids powders with azadirachtin contents of 18% (A) and 7% (B) were prepared according to the method by Melwita et al.[22]

Column Chromatography

Column chromatography was performed in a glass column (L × i.d. 30 cm × 1.5 cm). Slurry of silica gel was loaded into the column at a predetermined silica gel to limonoid powder ratio. The column was washed with the mobile phase prior to sample loading. Isocratic solvent system consists of ethyl acetate-hexane was employed in chromatography process. The sample (200 mg limonoid powder) was dissolved in the mobile phase solvent and loaded into the column. The mobile phase was run through the column by gravity. Fractions of 10 mL each were collected. Each fraction was analyzed for its azadirachtin content using TLC, and fractions containing azadirachtin were pooled together. Into these fractions, hexane was added at a hexane to fraction ratio of 12 (v/v) to induce precipitation. The precipitate was separated using filter paper (Advantec no. 2) and then dried in an oven at 40°C for 30 min. A white powder was obtained as the final product. Data calculations were performed according to the following formulas:

$$\textit{Azadirachtin fractions } (\%) = \frac{\textit{Azadirachtin fractions } (g)}{\textit{Limonoids powder } (g)} \times 100\% \qquad (1)$$

$$\textit{Azadirachtin purity } (\%) = \frac{\textit{Azadirachtin } (g)}{\textit{Azadirachtin fractions } (g)} \times 100\% \qquad (2)$$

$$\textit{Azadirachtin recovery } (\%) = \frac{\textit{Azadirachtin content in azadirachtin fractions } (g)}{\textit{Azadirachtin content in limonoids powder } (g)} \times 100\% \qquad (3)$$

Azadirachtin Analysis

Azadirachtin content was analyzed according to the method of Melwita et al.[22] Analysis was performed using a Jasco HPLC PU-2089 (USA) with an UV-Vis detector (model UV-2077 Plus) equipped with a 20 μL Rheodyne injector. A Luna C18(2) column 250 mm × 4.6 mm (Phenomenex, USA) containing 5 μm particles was used as the stationary phase. Isocratic chromatography was performed using Methanol/Water (50:50 v/v) at 1 mL/min. Eluent was monitored at 215 nm. A standard solution of azadirachtin (1000 ppm) was prepared by dissolving 0.5 mg of azadirachtin in 5 mL HPLC grade methanol. This standard solution was diluted to prepare 10–50 ppm azadirachtin standards for the preparation of calibration curve. Samples were filtered through a 0.2 μm filter (Whatman) before injection.

TLC was performed on a silica gel plate (Merck, Germany) according the method of Jarvis et al.[18] The mobile phase used was toluene/methanol (4:1, v/v). After layer development, the plate was dried and dipped in a vanillin solution. The plate was heated with a hot air blower until the colored spots appeared. Azadirachtin was visualized as a green spot and other limonoids appeared as violet spots.

Morphology Examination

Scanning electron microscopy (FESEM JEOL JEM 2100F, USA) was used to examine the morphology of powder particles. The samples were prepared on the specimen stubs with two-sided carbon tape and gold coated prior to the imaging process.

Particle Size Measurement

Particle sizes of powders were measured by the laser diffraction method. The measurements were carried out using a Malvern Zetasizer Nano S90 (Malvern Instruments Ltd, UK) according to the practice guide of particle size characterization published by Jillavenkatesa et al.[23] The sample was dispersed in deionized water with the aid of an ultrasonic probe (Transonic 780/H, Elma) for 5 min. After that, the sample was filtered with a filter paper (Advantec no. 5C). Approximately 1 mL filtrate was put into the sample cell and placed in the measurement chamber.

Thermogravimetric (TGA) Analysis

TGA analysis was performed using Diamond TG/DTA (PerkinElmer, USA). Approximately 5–6 mg sample was placed in a platinum pan. The sample was heated to 800°C at 10°C/min. Air was used as the heating gas at a flow rate of 20 mL/min.

RESULTS AND DISCUSSION

Effect of Chromatographic Parameters on Separation Efficiency

In this work, separation efficiency of silica gel column chromatography is represented by azadirachtin purity and azadirachtin recovery. Two variables, that is, ratio of silica gel to sample and solvent composition, were investigated to produce optimum separation (Table 1). Apparently, the effects of varying parameters on separation efficiency are not very significant. Azadirachtin purity around 50% can be obtained at almost all combinations of parameters. Azadirachtin recoveries obtained are also not significantly different.

Normally, separation efficiency will improve by increasing adsorbent to sample ratio due to higher surface area available for interactions. However, this phenomenon was not observed distinctly in this chromatographic system. The reason may be due to compound characteristics. Limonoids' polarities are known to be very close to each other. Therefore, their separations are inherently difficult. In addition, limonoids can make strong interactions with silica gel due to the existence of polar functional groups. Increasing silica gel ratio will increase limonoids' adsorption on silica gel and reduce their contents in the eluent. Among the major limonoids, nimbin and salanin were eluted earlier than azadirachtin. This indicates the weaker affinity of these compounds to silica gel compared to that of azadirachtin. Among limonoids, azadirachtin B and azadirachtin H have the closest similarity to azadirachtin. As can be seen in Figure 1a, these three compounds appear closely in HPLC chromatogram. At a silica gel to sample ratio of 50 (Figure 1b), the chromatogram still shows these three compounds. However, as the ratio increases to 100 (Figure 1c), only azadirachtin appears in the chromatogram.

Varying solvent polarity only resulted in slightly different separation efficiency. The lower polarity solvent EtOAc/hexane (2:1, v/v) was not

TABLE 1 Effect of Parameters on Chromatographic Separation

Parameters	Solvent Composition (v/v)[a]	
	EtOAc/Hexane (2:1)	EtOAc/Hexane (3:1)
Azadirachtin fractions (%)	17.40 ± 0.64[b]	40.95 ± 0.64
	11.27 ± 0.25[c]	13.25 ± 0.25
Azadirachtin purity (%)	51.74 ± 2.31	30.13 ± 2.31
	54.75 ± 2.45	53.86 ± 2.06
Azadirachtin recovery (%)	50.06 ± 4.06	68.55 ± 4.06
	34.28 ± 2.10	39.63 ± 0.46

[a]Mean ± SD, data replication at least in duplicate.
[b]Ratio of silica gel to sample of 50.
[c]Ratio of silica gel to sample of 100.

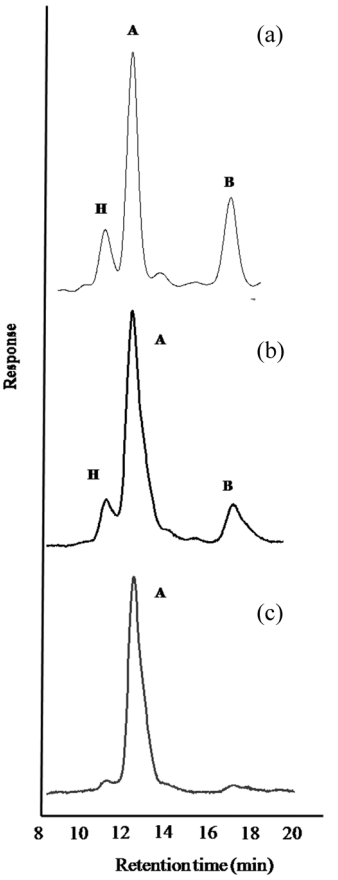

FIGURE 1 HPLC chromatogram of: (a) Limonoids powder, (b) Azadirachtin fractions at ratio silica gel to sample of 50, and (C) Azadirachtin fractions at a ratio of silica gel to sample of 100.

capable of significantly improving azadirachtin separation than EtOAc/hexane (3:1, v/v). This illustrates the difficulty in separating azadirachtin from other limonoids due to their small differences in polarity. The advantage of using EtOAc/hexane (2:1, v/v) over EtOAc/hexane (3:1, v/v) is that by using the higher amount of hexane, azadirachtin can be separated from the solvent via precipitation. This step is crucial to avoid heating to remove solvent, which will cause degradation of the thermally unstable azadirachtin. A higher ratio of hexane to EtOAc is beneficial for the precipitation of azadirachtin from EtOAc. Therefore, EtOAc/hexane (2:1, v/v) is more suitable as the mobile phase.

A second chromatography was employed using limonoid powders obtained from the first silica gel column chromatography, with an azadirachtin content of about 50%, as the starting material in order to increase the azadirachtin purity further. After investigating the effects of silica gel to

limonoid powder ratio and mobile phase composition on the azadirachtin purity and recovery in the product, it was found that the second silica gel column chromatography failed to significantly improve the purity and recovery of azadirachtin in the product (data not shown).

Results of chromatography separation indicated that increasing azadirachtin content in sample (limonoids powder) resulted in decreasing separation efficiency. This phenomenon may be caused by the difference in compositions of impurities in samples used in silica gel column chromatography. At low azadirachtin purity (below 20%), the sample still contains significant amount of impurities such as salanin which possess large differences in polarity from azadirachtin as shown in Figure 1a. Such impurities can be separated from azadirachtin easily. Thus, azadirachtin content can be raised considerably by using silica gel column chromatography when the initial azadirachtin content in the sample is below 20%. In samples with initial azadirachtin content considerably higher than 20%, the major impurities in the sample (such as azadirachtin H and azadirachtin N shown in Figure 1b) have polarities close to that of azadirachtin. When a sample with high azadirachtin content was used, it was very difficult to separate such impurities from azadirachtin by silica gel column chromatography. Hence, poor separation efficiency was obtained.

Characteristic of Chromatography Product

Fractions produced in the first step chromatography were characterized to determine the chemical and physical properties of compounds in those fractions that contain around 50% azadirachtin. Azadirachtin and other limonoids in the fractions were precipitated from solvents using hexane. The white powder obtained from the precipitation process was characterized using HPLC to determine its chemical characteristics. Morphology examinations were carried out using SEM and laser diffraction and thermal properties were examined using TGA.

HPLC analysis of azadirachtin fractions (Figure 1c) shows the presence of azadirachtin as a single peak. Other compounds cannot be detected within the range of UV wavelength 215–280 nm employed in HPLC analysis. Precipitation of azadirachtin and other limonoids produced fine powders with irregular particle shapes (Figure 2). The average volume diameter of particles is 280 nm as measured by laser diffraction.

TGA curve of azadirachtin fractions (Figure 3) shows that the maximum degradation temperature of compounds in the fraction is around 600°C. The majority of compounds begin to degrade at 300°C as indicated by the highest degradation peak. The second degradation peak can be observed at around 500°C. Considering the fact that azadirachtin content in the powder is around 50%, apparently, azadirachtin belongs to the group of

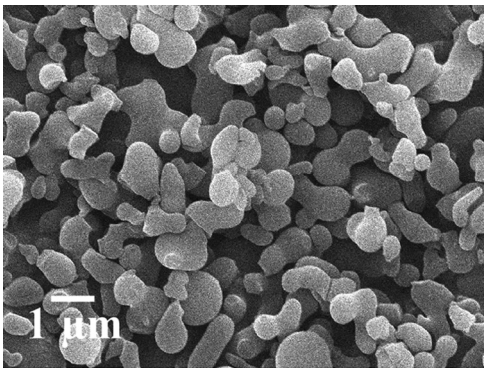

FIGURE 2 MORPHOLOGY of azadirachtin fractions.

compounds degraded at around 300°C. Thermal properties of other limonoids are unknown. However, close similarity in the structures of limonoids suggests that they may also degrade at temperatures close to that of azadirachtin. Compounds that degraded at around 500°C probably consist of other terpenoids.

Comparison of Chromatographic Purification of Azadirachtin

Comparison of separation efficiency in chromatographic purification of azadirachtin is a difficult task. Most literatures did not report the efficiency of their chromatographic method comprehensively. Therefore, the separation efficiency of this work was compared only with results in literatures that provided detailed separation efficiency (Table 2). This chromatographic method can produce a similar separation efficiency compared to

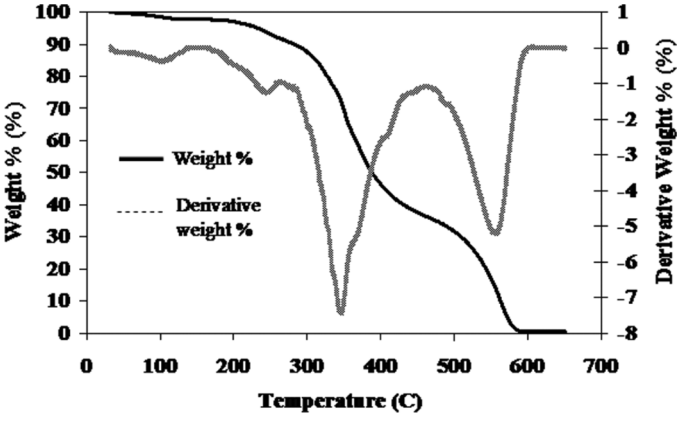

FIGURE 3 TGA curve of azadirachtin fractions.

TABLE 2 Comparison of Chromatographic Purification of Azadirachtin

Ref	Stationary Phase	Mobile phase	Chromatography Mode	Initial Azadirachtin Purity (%)	Azadirachtin Purity (%)	Azadirachtin Recovery (%)
[16]	Silica gel	EtOAc/Hexane (3:1, v/v)	Vacuum liquid chromatography	Not reported	Not reported	Not reported
[17]	Silica gel	Chloroform/Methyl cyanide (3:1, v/v)	Flash chromatography	Not reported	Not reported	Not reported
[17]	ODS	Methanol/water (60:40, v/v)	Preparative HPLC	Not reported	Pure	Not reported
[18]	Attapulgite clay	Petroleum ether/EtOAc (gradient elution from 7:3 to 2:8, v/v)	Flash chromatography	Not reported	Not reported	Not reported
[18]	Attapulgite clay	Petroleum ether/EtOAc (5:5, v/v)	Flash chromatography	Not reported	Pure	Not reported
[19]	Silica gel	Diethyl ether-methanol (49:1, v/v)	Flash chromatography	Not reported	7.3	95
[19]	ODS	Methanol/water (3:2, v/v)	Flash chromatography	7.3	26	61
[19]	Silica gel	Isopropanol-hexane (1:3, v/v)	Preparative HPLC	26	70	60
[19]	Phenyl	Acetonitrile-water (3:7, v/v)	Preparative HPLC	70	>99	29
[21]	C18	Methanol/water (60:40, v/v)	Preparative HPLC	9.14	90	88.62
This work	Silica gel	EtOAc/hexane (2:1, v/v/)	Gravity flow chromatography	7	28	61
This work	Silica gel	EtOAc/hexane (2:1, v/v/)	Gravity flow chromatography	18	50	50
This work	Silica gel	EtOAc/hexane (1:1, v/v/)	Gravity flow chromatography	50	55	92

flash chromatography method using ODS column.[19] Azadirachtin purity can be increased around 4-fold of its initial content with a recovery of around 60%. This method was more advantageous because it uses ethyl acetate and hexane as solvents. Azadirachtin can be precipitated easily from solvents. On the other hand, a mixture of methanol and water was used as the mobile phase for chromatographic separation using ODS column. Separation of azadirachtin from this mixture was more difficult due to the presence of water.

Thus far, high purity azadirachtin (>70%) can be produced only by an HPLC method. Silica gel, phenyl or C18 can be used as the stationary phase. A chromatographic system as employed by Deota et al.[21] gave the best result. Azadirachtin with high purity and recovery can be obtained using starting material with low azadirachtin content. Apparently, C18 can give better separation of limonoids compared to other stationary phases. However, reverse phase chromatography requires the use of mobile phases which consist of protic solvents such as methanol and water. Azadirachtin is known to be unstable in these solvents.[24] From this point of view, normal phase chromatography has advantages. Further studies are needed to improve the separation efficiency of this method to the value that is comparable to that of the reverse phase chromatography using C18.

CONCLUSION

Gravity flow silica gel column chromatography using EtOAc/hexane (2:1, v/v) as the mobile phase can increase azadirachtin purity 3- to 4-fold with a recovery of around 50%. The first step chromatography using limonoid powder A (azadirachtin content 18%) and limonoid powder B (azadirachtin content 7%) can produced enriched limonoid powders with azadirachtin content around 50% and 28%, respectively. Further purification of these products via a second step chromatography was not capable of increasing azadirachtin purity significantly. Close polarities of neem limonoids are believed to be the reason for the difficulty in their separation using this chromatography system.

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REFERENCES

1. Mordue, J.; Blackwell, A. Azadirachtin: An Update. *J. Insect Physiol.* **1993**, *39*, 903–924.
2. Morgan, E. D.; Azadirachtin, A. Scientific Gold Mine. *Bioorgan. Med. Chem.* **2009**, *17*, 4096–4105.
3. Isman, M. B. Botanical Insecticides, Deterrents, and Repellents in Modern Agriculture and an Increasingly Regulated World. *Annu. Rev. Entomo.* **2006**, *51*, 5145–5166.

4. Govindachari, T. R.; Suresh, G.; Gopalakrishnan, G.; Wesley, S. D. Insect Antifeedant and Growth-Regulating Activities of Neem Seed Oil: The Role of Major Tetranortriterpenoids. *J. Appl. Ent.* **2000**, *124*, 287–291.
5. Govindachari, T. R.; Narasimhan, N. S.; Suresh, G.; Partho, P. D.; Gopalakrishnan, G. Insect Antifeedant and Growth-Regulating Activities of Salannin and Other C-seco Limonoids from Neem Oil in Relation to Azadirachtin. *J. Chem. Ecol.* **1996**, *22*, 1453–1461.
6. Isman, M. B.; Koul, O.; Luczynski, A.; Kaminski, J. Insecticidal and Antifeedant Bioactivities of Neem Oils and Their Relationship to Azadirachtin Content. *J. Agric. Food Chem.* **1990**, *38*, 1406–1411.
7. Mitchell, M. J.; Smith, S. L.; Johnson, S.; Morgan, E. D. Effects of the Neem Tree Compounds Azadirachtin, Salannin, Nimbin, and 6-desacetylnimbin on Ecdysone 20-monooxygenase Activity. *Arch. Insect Biochem.* **1997**, *35*, 199–209.
8. Kumar, J.; Parmar, B. S. Physicochemical and Chemical Variation in Neem Oils and Some Bioactivity Leads Against *Spodoptera litura*. *F. J. Agric. Food Chem.* **1996**, *44*, 2137–2143.
9. Kumar, C. S. S. R.; Srinivas, M.; Yakkundi, S. Limonoids from the Seeds of *Azadirachta indica*. *Phytochemistry* **1996**, *43*, 451–455.
10. Ragasa, C. Y.; Nacpil, Z. D.; Natividad, G. M.; Tada, M.; Coll, J. C.; Rideout, J. A. Tetranortriterpenoids from *Azadirachta indica*. *Phytochemistry* **1997**, *46*, 555–558.
11. Hallur, G.; Sivramakrishnan, A.; Bhat, S. V. Three New Tetranortriterpenoids from Neem Seed Oil. *J. Nat. Prod.* **2002**, *65*, 1177–1179.
12. Govindachari, T. R.; Sandhya, G.; Ganeshraj, S. R. Isolation of Novel Azadirachtins H and I by High-Performance Liquid Chromatography. *Chromatographia* **1991**, *31*, 303–305.
13. Siddiqui, S.; Faizi, S.; Mahmood, T.; Siddiqui, B. S. Two New Insect Growth Regulator Meliacins from *Azadirachta indica* A. Juss (Meliaceae). *J. Chem. Soc. Perkin Trans.* **1996**, *1*, 1021.
14. Yamasaki, R. B.; Ritland, T. G..; Barnby, M. A.; Klocke, J. A. Isolation and Purification of Salannin from Neem Seeds and Its Quantification in Neem and Chinaberry Seeds and Leaves. *J. Chromatogr.* **1998**, *447*, 277–283.
15. Silva, J. C. T.; Jhama, G. N.; Oliveira, R. D. L.; Brown, L. Purification of the Seven Tetranortriterpenoids in Neem (*Azadirachta Indica*) Seed by Counter-Current Chromatography Sequentially Followed by Isocratic Preparative Reversed-Phase High-Performance Liquid Chromatography. *J. Chromatogr. A* **2007**, *1151*, 203–210.
16. Schroeder, D. R.; Nakanishi, K. A. Simplified Isolation Procedure for Azadirachtin. *J. Nat. Prod.* **1987**, *50*, 241–244.
17. Govindachari, T. R.; Sandhya, G.; Raj, S. P. G.. Simple Method for the Isolation of Azadirachtin by Preparative High-Performance Liquid Chromatography. *J. Chromatogr.* **1990**, *513*, 389–391.
18. Jarvis, A. P.; Morgan, E. D.; Edwards, C. Rapid Separation of Triterpenoids from Neem Seed Extracts. *Phytochem. Anal.* **1999**, *10*, 39–43.
19. Yamasaki, R. B.; Klocke, J. A.; Lee, S. M.; Stone, G. A.; Darlington, M. V. Isolation and Purification of Azadirachtin from Neem (*Azadirachta indica*) Seeds Using Flash Chromatography and High-Performance Liquid Chromatography. *J. Chromatogr.* **1986**, *356*, 220–226.
20. Sharma, V.; Walia, S.; Kumar, J.; Nair, M. G..; Parmar, B. S. An Efficient Method for the Purification and Characterization of Nematicidal Azadirachtins A, B, and H, Using MPLC and ESIMS. *J. Agric. Food Chem.* **2003**, *51*, 3966–3397.
21. Deota, P. T.; Upadhyay, P. R.; Patel, K. B.; Mehta, K. J.; Kamath, B. V.; Mehta, M. H. Estimation and Isolation of Azadirachtin-A from Neem (*Azadirachta indica A. Juss*) Seed Kernel Using High Performance Liquid Chromatography. *J. Liq. Chromatogr. R.T.* **2000**, *23* (14), 2225–2235.
22. Melwita, E.; Ju, Y. H. Separation of Azadirachtin and Other Limonoids from Crude Neem Oil via Solvent Precipitation. *Sep. Purif. Technology* **2010**, *74*, 219–224.
23. Jillavenkatesa, A.; Dapkunas, S. J.; Lum, L. H. *Particle Size Characterization*, National Institute of Standards and Technology: Washington, 2001.
24. Jarvis, A. P.; Johnson, S.; Morgan, E. D. Stability of the Natural Insecticide Azadirachtin in Aqueous and Organic Solvents. *Pestic. Sci.* **1998**, *53*, 217–222.