Influence of Operating Parameters on the Use of the Microwave-Assisted Process (MAP) for the Extraction of Azadirachtin-Related Limonoids from Neem (*Azadirachta indica*) under Atmospheric Pressure Conditions

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The use of the microwave-assisted process (MAP) for the extraction of azadirachtin-related limonoids (AZRL) from various parts of the neem tree was investigated under different operating conditions. The influence of microwave power, solvent, and irradiation time on the recovery of AZRL was studied. 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 of conventional extraction methods. The content of AZRL in the extracts was estimated with a vanillin-based colorimetric assay and a multivariate calibration technique. The results showed that the MAE technique can enhance the extraction of AZRL from different parts of neem possessing microstructures. Investigation of the influence of the solvent also indicted that the solvent used not only influences the efficiency but also affects the selectivity of the MAE.

Keywords: *Azadirachtin; neem; vanillin assay; MAP; microwave-assisted extraction*

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 (*1*) (limonoids). One of these limonoids, azadirachtin A (commonly referred to as azadirachtin), is considered to be the most important active principle, due to its various effects on insects (*2*, *3*). Azadirachtin (AZ) content in neem extracts or in commercially available neem-based pesticides can be estimated by HPLC (*4*−*6*) or by supercritical fluid chromatography (*7*). However, AZ may not be the only active component. According to Verkerk and Wright (*8*), neem extracts containing equivalent amounts of azadirachtin have 3−4-fold greater activity than the synthetic azadirachtin. This might be due to synergistic effects or the presence of other active limonoids in the extract. Dai et al. (*9*) developed a colorimetric method, based on a vanillin assay, that allows direct and rapid measurement of the total azadirachtin-related limonoids (AZRL) in the crude extracts of neem. A multivariate calibration technique (*10*) was also developed using AZ, limonene, and tannic acid as standards, to eliminate possible interference caused by the presence of simple terpenoids (ST) and phenolics during the vanillin assay. Knowledge of the total AZRL in the extracts can help to predict the relative activity of different neem species. The neem seed is the main source of these limonoids, but some were also isolated from other parts of the neem tree such as leaf, twig, root, or bark (*1*, *8*, *11*, *12*). The extraction and purification of AZ involves repeated partitioning between different solvents after the removal of lipids followed by column chromatography or HPLC. Recently, supercritical fluid (*13*) has been also used to optimize the extraction of AZ. However, extraction times can possibly be shortened through the application of the microwave-assisted extraction (MAE) technique. MAE is an example of an microwave-assisted process (MAP) that uses microwave energy (*14*) and solvents that are fully or partially transparent to microwaves to extract target compounds from various matrices (plants, animals, soils, etc.). Application of microwave energy as a heat source causes selective heating of the plant matrix over the extractant (*14*−*17*). The highly localized temperature and pressure can cause selective migration of target compounds from the material to the surroundings at a more rapid rate and with similar or better recoveries compared with conventional extractions. In addition to versatility, speed, and selectivity, it offers also the advantages of minimal solvent consumption, reduced byproduct formation, and lower energy expenditure (*18*). Various publications (*9*) have placed the azadirachtin content of neem kernel in the range of 0.056−0.6% w/w. The recovery of AZ

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extracted from the various parts of *A. indica* was reported to be in the following order: seed > seed shell > leaf > leaf stem.

MATERIALS AND METHODS

General Experimental Procedures. Synthewave 402 (focused MAP system at atmospheric pressure) was obtained from Prolabo (Fontenay-Sous-Bois, Cedex, France). It operates with an emission frequency of 2450 MHz and a 300 W full power. It is equipped with an IR temperature sensor, a 250 mL quartz extraction vessel, and a Graham type condenser. Conical flasks (50 or 250 mL capacity) with magnetic stirring were used for conventional extractions. The solvents were evaporated using a Büchi Rotovapor R114 (Fischer Scientific, Montreal, Canada).

Plant Material. Fresh neem seeds, leaves, leaf stems, and bark were collected from Bangalore, India, during May 1998 and transported by plane within 48 h. The seeds were removed from their shells, blended with a coffee bean blender, and stored at −5 °C.

Chemicals and Reagents. Azadirachtin (95% purity) was purchased from Sigma Chemical Co. Stock solution (0.1 mg/mL) in dichloromethane (DCM) was prepared and stored below 0 °C in the refrigerator. Limonene was obtained from Aldrich. HPLC grade methanol ($\epsilon' = 32.6$), DCM ($\epsilon' = 9.1$), and acetonitrile were purchased from Fisher Scientific; petroleum ether ($\epsilon' \sim 4$, bp 60−80 °C) was purchased from ACP Chemicals Inc. (Montreal, Canada). Vanillin and concentrated H_2SO_4 (98%) were obtained from Fisher Scientific.

Extraction Procedures. *Procedure 1. Extraction of Neem Seed, Seed Shell, Neem Leaf, and Leaf Stem Using MAE, Room Temperature Extraction (RTE), and Reflux (RFX) Methods.* All neem samples were extracted as is except the seed samples, which were defatted before the extraction. Blended neem seeds (1.0 g) were defatted by stirring overnight in petroleum ether (30 mL) at room temperature. After filtration, the defatted residue was used to study the efficiency of different extraction methods.

(a) MAE. The defatted seeds were placed in a 250 mL quartz extraction vessel of the Synthewave 402 microwave system. Methanol (30 mL) was then added. The vessel was inserted inside the microwave cavity and fitted with a condenser. The sample was irradiated with microwaves using the following irradiation sequence at 150 W: 30 s on, 30 off, for a total of 10 min of irradiation time and 20 min total of extraction time. At the end of the irradiation sequence the solution was left for 1 min before it was filtered and evaporated in a vacuum to yield an orange amorphous solid. The combined methanol extracts from triplicate experiments were redissolved in methanol (10 mL) followed by the addition of 10 mL of water and 1 mL of 5% NaCl solution. The aqueous methanol solution was then partitioned with petroleum ether (3 × 20 mL) to remove any remaining fat. The residue was then extracted with DCM (3 × 20 mL). The combined dichloromethane extracts were dried over anhydrous Na_2SO_4, and the solvent was evaporated under vacuum after filtration to obtain an amorphous light yellow solid. The product was redissolved in DCM for colorimetric determination of AZRL content. Extractions of the seed shell, the neem leaf, and the leaf stem were the same as 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 the extraction was carried out in refluxing methanol for 20 min. All experiments were performed in triplicates.

Procedure 2. Investigation of the Influence of Solvent on MAE of the Seed, Seed Shell, and Neem Leaf. Extraction procedure was the same as that of the seed as outlined above. However, three different solvents (methanol, dichloromethane, and petroleum ether) were used in this investigation. The partitioning procedure was also the same as that outlined in Procedure 1 except in the case of neem leaf the water/methanol ratio was increased to 2:1 (v/v).

Procedure 3. Investigation of Time Dependence of MAE of Neem Seed Kernel with Methanol as Solvent. Blended fresh neem seeds (1.0 g) were extracted with methanol (30 mL) under microwave irradiation without defatting, following the same steps as outlined in Procedure 1 except the irradiation times were varied as follows: 10 s, 30 s, 1 min, 2 min, 3 min, 5 min and 10 min. For irradiation times >30 s, a 30 s on and 30 s off sequence was used. The extractions were repeated twice, and the combined extracts were partitioned following the same procedure as described above.

Procedure 4. Investigation of Time and Power Dependence of MAE of Neem Leaf with Methanol as Solvent. The microwave extraction and partitioning of the extracts were the same as those of procedure 2 for neem leaf. Power dependence experiments were performed at two different irradiation times (3 and 10 min), and for each irradiation time four different power levels (30, 90, 150, and 240 W) were used. Time dependence experiments were performed at a fixed power (150 W) and at different irradiation times: 10 s, 30 s, 1 min, 2 min, 3 min, 5 min, and 10 min. For irradiation times >30 s, a 30 s on and 30 s off sequence was used. The extractions were repeated twice, and the combined extracts were partitioned as described above.

Procedure 5. 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 the same as in procedure 1 except the programs used for the MAE were different. In this section, the irradiation times used were fixed while the microwave power was varied. Two different irradiation times, 3 and 10 min, were used; for each irradiation time four power levels, 30, 90, 150, and 240 W, were investigated. The partitioning was performed as in procedure 1.

Analytical Methods. Vanillin assay (*9*) with multivariate calibration (*10*) was used for the quantification of the AZRL. A summary of the published procedure is presented here. To a DCM solution (0.7 mL) of standard AZ or neem seed extract was added a methanol solution (0.2 mL) of vanillin (0.02 mg/mL). After manual shaking, the mixture was left at room temperature for 2 min. Concentrated sulfuric acid (0.3 mL, 98 %) was then added in three portions (0.1 mL each), and the mixture was stirred for 10 s 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 homogeneous solution that instantly developed a blue-green color. The solution was left at room temperature for 5 min before the absorbance was measured at 577 nm for AZ, at 625 nm for limonene, at 577 and 625 nm for neem seed kernel extracts, and at 625, 577, 550, and 499 nm for the extracts from other parts of neem using a spectrophotometer equipped with a 10 mm quartz cell. The blank solution was obtained by substituting the test solution with an equal volume of DCM in the above procedure. The spectra were analyzed using a multivariate calibration method (*10*) for the determination of AZRL in neem extracts.

RESULTS AND DISCUSSION

To investigate the advantages of using MAP over other conventional techniques, various parts of the neem tree were extracted using (i) MAE under atmospheric pressure, (ii) RTE, and (iii) RFX methods using the same extraction time and solvent (see procedure 1). The percent recovery of AZRL was used as a measure of extraction efficiency (see Figure 1). The percent recoveries were calculated by considering the yields obtained through 24 h room temperature extraction with methanol to be optimal (*9*). Inspection of Figure 1 indicates that there was a considerable enhancement of extraction efficiency due to microwave irradiation of the seed shell, leaf, and leaf stem. The recovery of AZRL from these

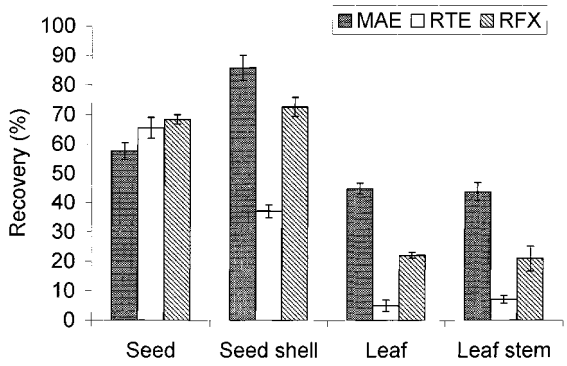

Figure 1. Comparison of percent recovery of AZRL by microwave-assisted extraction (MAE), room temperature (RTE), and reflux (RFX) methods (extraction time = 20 min, solvent = methanol).

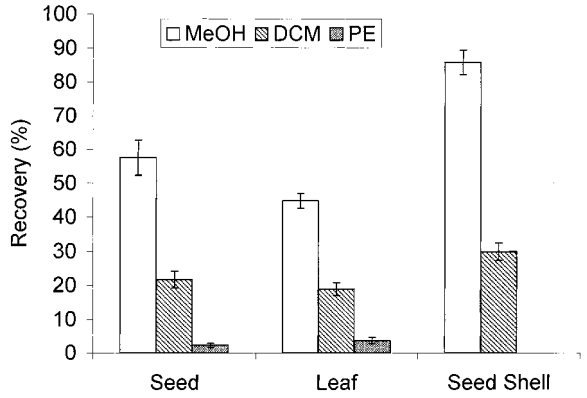

Figure 2. Influence of solvent on the percent recovery of AZRL by MAE (150 W, 20 min).Recovery of AZRL using PE from seed shell was zero.

parts of the plant showed the following order: MAE > RFX > RTE. In the seed, however, no particular enhancement of extraction efficiency was observed. These results suggest that enhancement of extraction efficiency by microwave irradiation, under the operating conditions used herein, can be achieved only in plant materials possessing relatively weak microstructures. Localized superheating during microwave irradiation can cause explosion of these structures inside the sample, releasing the components to the environment (*14*, *18*). The recoveries of AZRL obtained with MAE of leaf and leaf stem were more than double that obtained by the RFX method, although the time and bulk temperature of the extractions were the same in both cases.

Influence of Solvent on the Selectivity and Efficiency of MAE. To study the influence of solvent on the extraction efficiency of AZRL, three solvents having different physical properties were selected. Methanol is a relatively good absorber ($\epsilon' = 32.6$) of microwave energy and a good solvent for AZRL; DCM is a good solvent but not a good absorber of microwave energy ($\epsilon' = 9.1$); petroleum ether (PE) is neither a good absorber ($\epsilon' \sim 4$) nor a good solvent. In addition to their differences in solvation abilities and dielectric properties, these solvents have comparable boiling points except for DCM. The same extraction conditions were used for the three solvents (see procedure 2). Figure 2 indicates that both solvation ability and dielectric properties (which also control the rate of temperature increase) of the medium play an important role in the efficiency of MAE. The data also indicated that the

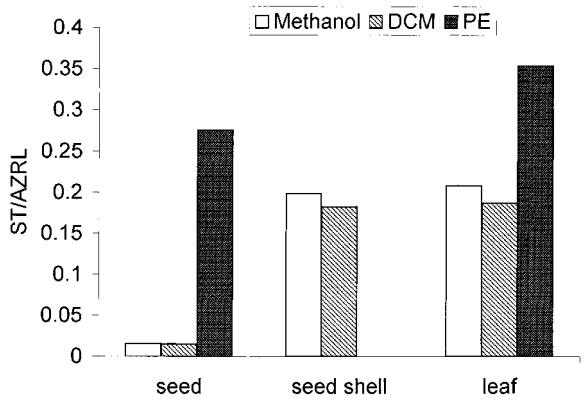

Figure 3. Influence of solvent on the ST to AZRL ratio by MAE (150 W, 20 min). Recovery of AZRL using PE from seed shell was zero.

temperature of the extraction (boiling point of the solvent) plays a less significant role than the dielectric properties of the solvent. Selectivity of the solvent to extract AZRL relative to simple terpenoids (ST) was also investigated. The solubility of the ST in PE is known to be higher than that of AZRL, whereas in methanol and DCM the differences in solubility are not significant. Figure 3 indicates the importance of solvent in enhancing the selectivity during MAE. The ratio of ST to AZRL was not affected when the extraction was performed with methanol or DCM as both are good solvents for AZRL. However, as Figure 3 indicates, there was a large increase in this ratio when PE was used, which is known to dissolve ST more than AZRL.

Influence of Irradiation Time on the Efficiency of MAE. The effect of irradiation time on the efficiency of MAE of AZRL from neem seed kernel (see procedure 3) and leaf (see procedure 4) was investigated at 150 W power of the microwave. The samples were extracted with methanol and the crude mass, percent AZRL in the crude mass, and percent recovery were plotted as a function of irradiation time as shown in Figure 4. The mass of crude extract obtained increased with the irradiation time for both the seed and the leaf. However, the percent AZRL in the crude extract showed a different behavior. In the case of the seed, the AZRL content increased from ~1.65% after 10 s of irradiation to a maximum of 2.55% after 60 s and then decreased gradually to 2.3% at the end of irradiation time (10 min). However, the maximum content of AZRL (1.23%) in the neem leaf extract was reached only after 30 s of irradiation and decreased further to 0.93%. The increase of the crude mass of the extract over time was mainly attributed to the coextraction of chlorophyll in the leaf and fats in the seed. The decrease in the percent AZRL extracted over time can be due to either its thermal destruction or an increase in the content of coextractants relative to AZRL. The percent recovery of AZRL as a function of irradiation time showed a similar logarithmic increase for both the seed and the leaf (see Figure 4c). The recovery of AZRL did not improve much after ~5 min of irradiation time (10 min of extraction time). These results suggest a competitive process over materials inside cells (being released quickly when the cells rupture) and materials randomly distributed within the plant microstructure (*18*).

Influence of Microwave Power on the Efficiency of MAE. The effect of microwave power (30−240 W) on

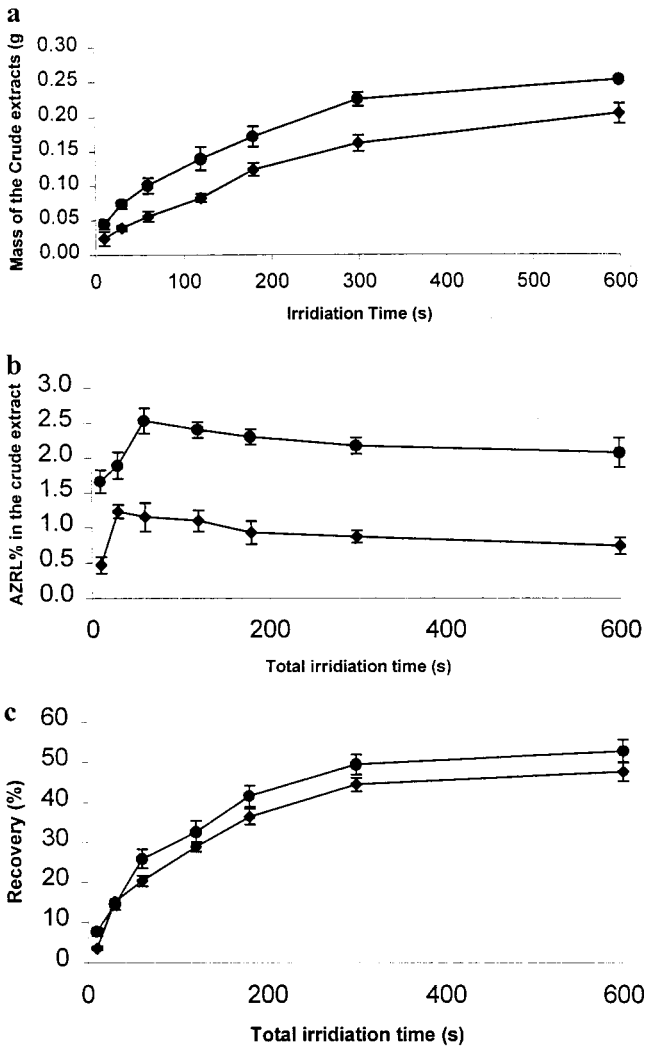

Figure 4. Influence of total irradiation time (seconds) of MAE of neem seed kernel (●) and leaf (◆) on (a) mass of crude extract, (b) percent AZRL in the crude extract, and (c) percent recovery of AZRL (10 min of total irradiation time = 20 min of total extraction time).

the efficiency of extraction of AZRL from neem seed kernel and leaf (see procedures 4 and 5) was investigated at two irradiation time intervals (3 and 10 min). The samples were extracted with methanol, and the crude mass of the extract, percent AZRL in the crude mass, and percent recovery of AZRL were plotted as a function of microwave power as shown in Figure 5. The mass of the crude extract and percent recovery obtained from the leaf increased with increasing microwave power for both time intervals as shown in Figure 5a,c. However, the percent recovery and mass of extract obtained from the seed more or less reached a plateau at 150 W power (Figure 5a,c). The difference in the power dependence between the seed and the leaf was more pronounced when percent AZRL in the crude extracts was compared (see Figure 5b). For both irradiation times, the percent AZRL extracted from the seed decreased as the power increased (except at 150 W for 3 min). The reverse of this trend was observed in the case of the leaf, where the percent ARZL increased with increasing power. However, the decreasing trend in the percent AZRL extracted from the seed became more pronounced than the increasing trend in percent AZRL extracted from the leaf as the microwave power increased (see Figure 5b). Again, the observed differ-

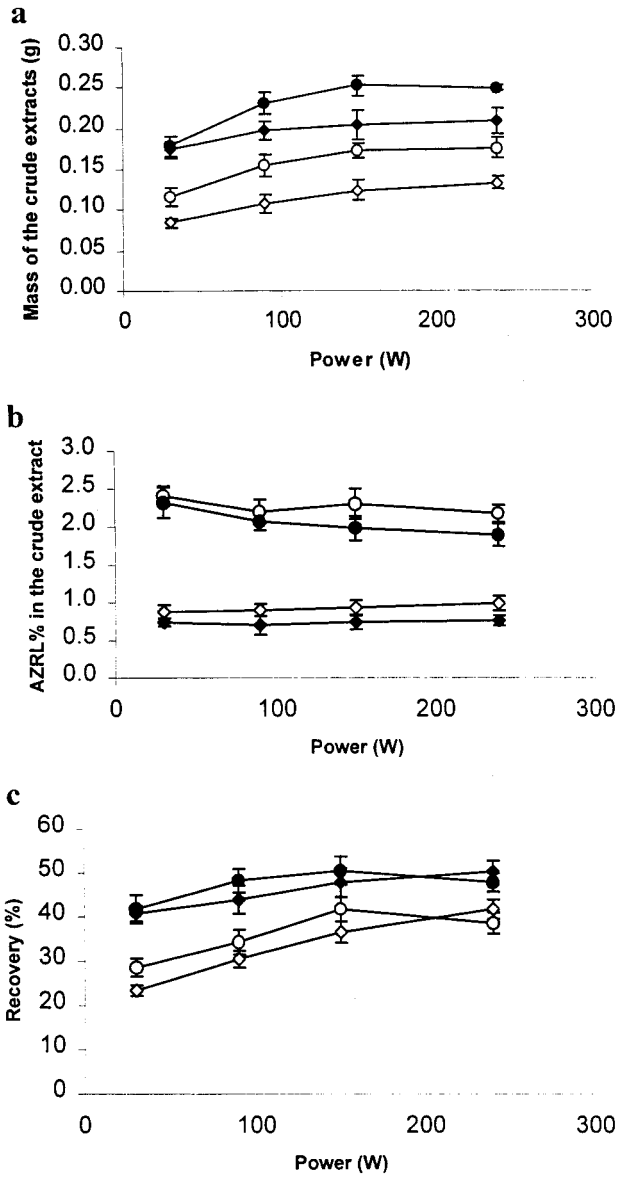

Figure 5. Influence of microwave power (watts) during MAE of neem seed kernel (○, 3 min; ●, 10 min) and leaf (◇, 3 min; ◆, 10 min) on (a) mass of crude extract, (b) percent AZRL in the crude extract, and (c) percent recovery of AZRL.

ences in the power dependence of percent AZRL in the crude extracts can be explained on the basis of the differences in their matrices and in the coextracting species. The main component in the seed that can be easily extracted relative to the AZRL is the fat, whereas in the leaf, AZRL can be extracted more easily relative to the chlorophyll. The composition of the extract will be richer in the component that can be easily extracted.

Conclusion. Operating parameters such as solvent, irradiation time, and microwave power have an influence on the efficiency of the MAP for the extraction of neem. In the specific case of AZRL significant enhancement of extraction efficiency over other traditional techniques was observed (except for seed), which makes MAE of neem for AZRL-based pesticides a convenient tool.

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