Extraction of Azadirachtin A from Neem Seed Kernels by Supercritical Fluid and Its Evaluation by HPLC and LC/MS

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A new supercritical extraction methodology was applied to extract azadirachtin A (AZA-A) from neem seed kernels. Supercritical and liquid carbon dioxide (CO_2) were used as extractive agents in a three-separation-stage supercritical pilot plant. Subcritical conditions were tested too. Comparisons were carried out by calculating the efficiency of the pilot plant with respect to the milligrams per kilogram of seeds (ms/mo) of AZA-A extracted. The most convenient extraction was gained using an ms/mo ratio of 119 rather than 64. For supercritical extraction, a separation of cuticular waxes from oil was set up in the pilot plant. HPLC and electrospray mass spectroscopy were used to monitor the yield of AZA-A extraction.

Keywords: *Supercritical fluid extraction; azadirachtin A; HPLC; LC/MS; neem seed kernels*

INTRODUCTION

Synthetic pesticides possess quick knockdown, but they are often toxic to mammals and nontarget organisms (Klassen et al., 1986; Marquis, 1986); for these reasons safe ecological pesticides that do not leach residues into the environment have great importance (Cook and Baker, 1983).

In this framework, compounds from the evergreen neem tree (*Azadirachta indica*), a plant belonging to the Meliaceae family and native to the arid regions of India, Pakistan, and Africa (Schmutterer, 1990), are of special interest (Cernia et al., 1997), making its oval fruits and leaves important sources of insecticides used for pest control (Chattopadhyay et al., 1992). The ripe fruit is yellow with a sweet pulp and a brown seed kernel that accounts for 10% of the whole fruit.

Azadirachtins are important active principles contained in neem seed kernels (Saxena et al., 1989), and several active compounds were isolated from neem seed kernels, such as salannin, genudin, and nimbin (Jones et al., 1989). The amount of azadirachtins may vary considerably depending on environmental and genetic factors. They are steroid-like tetranortriterpenoids formed by a group of closely related isomers, called azadirachtin A (AZA-A) to azadirachtin G (Rembold et al., 1984, 1987). The chemical structure of AZA-A is shown in Figure 1. It has been demonstrated that azadirachtins have deterrent, antiovipositional, antifeedant, growth-disrupting, growth-regulating, fecundity, and fitness-reducing properties on insects (Broughton et al., 1986; Kraus et al., 1985). Neem-based products are medium- to broad-spectrum pesticides for phytophagous insects (Schmutterer, 1990; Jacobson, 1986).

Neem biopesticides are commercially manufactured, and they are now used in India on cotton, vegetables, fruit trees, coffee, tea, and rice (Chary et al., 1984). They are usually extracted by organic solvents, which can cause oxidative degradation of the neem biopesticides and require proper disposal due to their toxicity (Schroeder and Nakanishi, 1987). To solve these problems, supercritical fluid extraction (SFE) using carbon dioxide (CO_2) has been proposed (Djarmati et al., 1991; Muehlnikel, 1992).

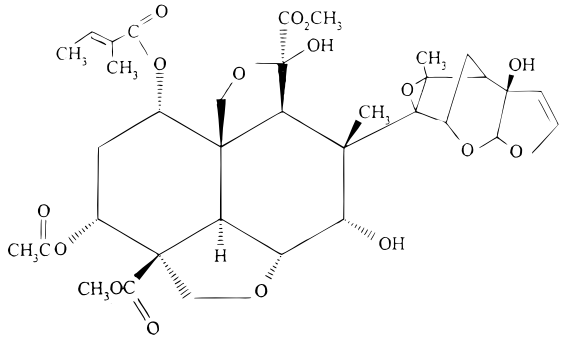

Figure 1. Chemical structure of azadirachtin A.

SFE-CO_2 has been widely applied in the agricultural and food fields during the past 20 years and is of increasing interest for new separation processes in foodstuffs and cosmetic and pharmaceutical fields (Spanos et al., 1993). SFE-CO_2 can be used like any other classical solvent used in maceration and percolation processes. The only restriction is that because high pressure is required to perform CO_2 extraction, the whole apparatus needs to be handled with care (Sharpe and Crabb, 1980). The gaslike qualities of supercritical fluids provide ideal conditions for extracting analytes with a high degree of recovery in a very short time (Greibrokk, 1992).

Due to its characteristics, SFE-CO_2 presents many benefits for the extraction of products from natural materials. The fluid is stable and is environmentally safe because no waste is produced. In addition, SFE-CO_2 might be performed at temperatures ranging from

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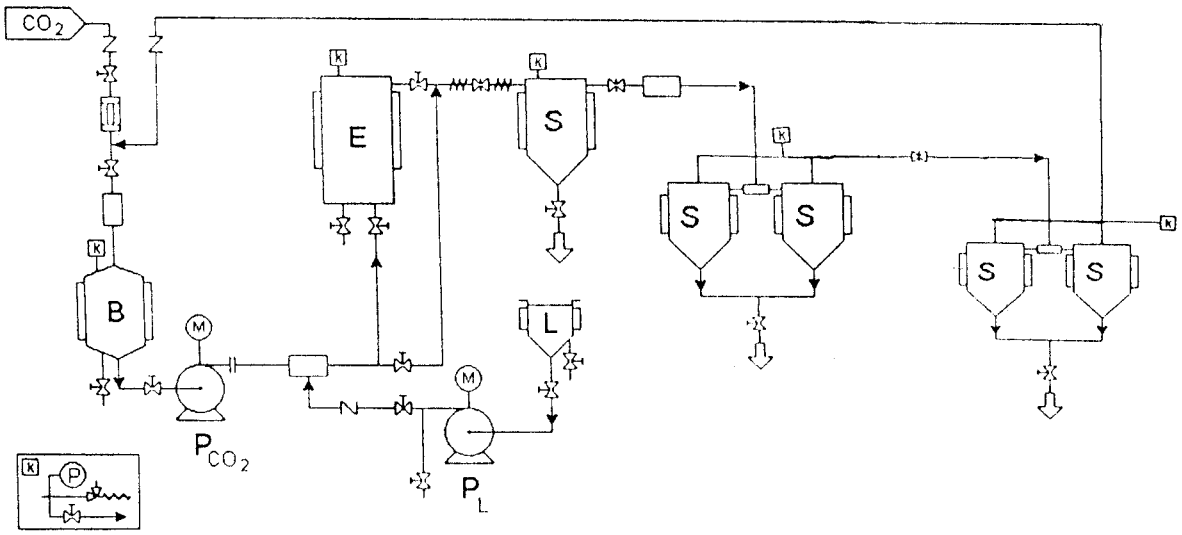

Figure 2. SFE pilot plant with three separators operating in series. P, CO_2 pump; E, extractor; S, separator; PL, coeluent pump; L, coeluent tank; B, CO_2 liquid tank.

20 to 40 °C, making it suitable for heat-sensitive compounds (Reverchon et al., 1993, 1994a). Bacteriostatic conditions are reached during the process, with exclusion of atmospheric oxygen and limitation of oxidative reactions. CO_2 is a solvent generally recognized as safe (GRAS status) for the production of food ingredients by the U.S. Food and Drug Administration (FDA), readily available, and cheap at high purity.

SFE-CO_2 offers the possibility to change the solvent power within a wide range by adjusting the gas density. This can be done by changing temperature and pressure parameters. By modulating the solvent power, extraction selectivity can be adjusted; the extracted products can be separated in different fractions and recovered at various stages in the same process (Reverchon et al., 1995).

The extraction solvent CO_2 has a strong lipophilic selectivity, and polar substances such as organic and inorganic salts, sugars, glycosides, amino acids, saponins, tannins, and phospholipids are completely insoluble, as well as polymers such as proteins, polysaccharides, and polyterpenes. This offers the advantage of obtaining an extract that is virtually free of these substances and, hence, selectivity in extraction (Reverchon et al., 1994b).

The aim of this study was to optimize AZA-A extraction by the SFE-CO_2 technique by investigating the effects of pretreatments of neem seed kernels and modifying the extraction temperature. HPLC and LC/MS analyses were used as tools to set up and optimize SFE-CO_2 conditions to enhance the yield extraction of AZA-A.

MATERIALS AND METHODS

Sample. Neem kernel seeds (20 kg) harvested in 1997 were obtained from India. Kernels were opened and seeds milled in a Waring blender (Waring product division, New Hartford, CT) before extraction. Conventional pressure was performed on a hydraulic forging press working at 300 bar.

Reagents. All organic solvents (HPLC grade) used for chromatography were purchased from Merck (Darmstadt, Germany). Water for HPLC mobile phase was purified in a Milli-Q system (Millipore, Bedford, MA). Authentic standard AZA-A was kindly supplied by Dr. D'Andrea (ENEA, C.R.E. Casaccia, Department Innovation, Division BIOAG, Rome, Italy).

SFE Apparatus. SFE experiments were performed on a pilot plant schematically shown in Figure 2. It consists of an extraction vessel that can be equipped with internal baskets of different volumes (5, 10, 15, and 20 dm^3). A thermostatic jacket allows the control of the extraction temperature, ranging between a minimum of 20 °C and a maximum of 70 °C. A high-pressure pump (Lewa, Germany) delivers liquid CO_2 flow rates from 20 to 100 kg/h. The last separator is designed to perform operations up to 320 bar, allowing full recycle of CO_2 downstream.

The apparatus was arranged with three separation stages operating in series. The first one had an internal volume of 3 dm^3 and operated with cool thermal services. The second and third separation stages were provided by two cyclonic chambers of 0.2 dm^3 internal volume, working in parallel. The cyclonic separators allow the periodical discharge of extracts during the extraction process. A differential pressure transducer measured the pressure drop along the extraction vessel. A calibrated Coriolis mass flow meter positioned after the pumping unit was used to measure the liquid CO_2 flow rate during the extraction process.

HPLC. HPLC analysis was carried out using LC-10AD pumps and a diode array detector (DAD) from Shimadzu (Japan). The oil SFE extracted samples were mixed for 2 min by vortex (test tube shaker/mixer, Model TK3S, Kartell Labware Division, Milan, Italy) and diluted 1:40 in MeOH before HPLC analyses. Analytical separation was achieved using a 5 μm particle size Phenomenex column C_8 (250 × 4.6 mm i.d.). Samples were filtered through a 0.22 μm syringe filter (Millipore, Yonezawa, Japan), and 20 μL was injected into the column.

To evaluate the amount of AZA-A in cuticular waxes, the following protocol was applied. Cuticular waxes (100 mg) were dissolved in diethyl ether (500 μL) and then purified by thin-layer chromatography with silica gel as stationary phase and chloroform/methanol 95:5 as eluent phase; detection was carried out using iodine vapors. All compounds with R_f values <0.95 were scraped off and dissolved in 100 mL of MeOH. The organic phase was reduced to 5 mL under vacuum at 35 °C on a rotary evaporator. The residue was filtered through a 0.22 μm filter and resuspended in 1 mL of HPLC phase; 20 μL was injected into the column. HPLC conditions were as described by D'Andrea et al. (1991). Raw data were collected from 200 to 600 nm.

LC/MS. A Perkin-Elmer LC series 200 connected to a 785A UV−vis detector and coupled with an API-100 single-quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Canada) was used. A flow of 20 μL min^{-1} was split from the LC eluent after chromatographic separation into the ion spray source. A probed voltage of 5300 V and a declustering potential

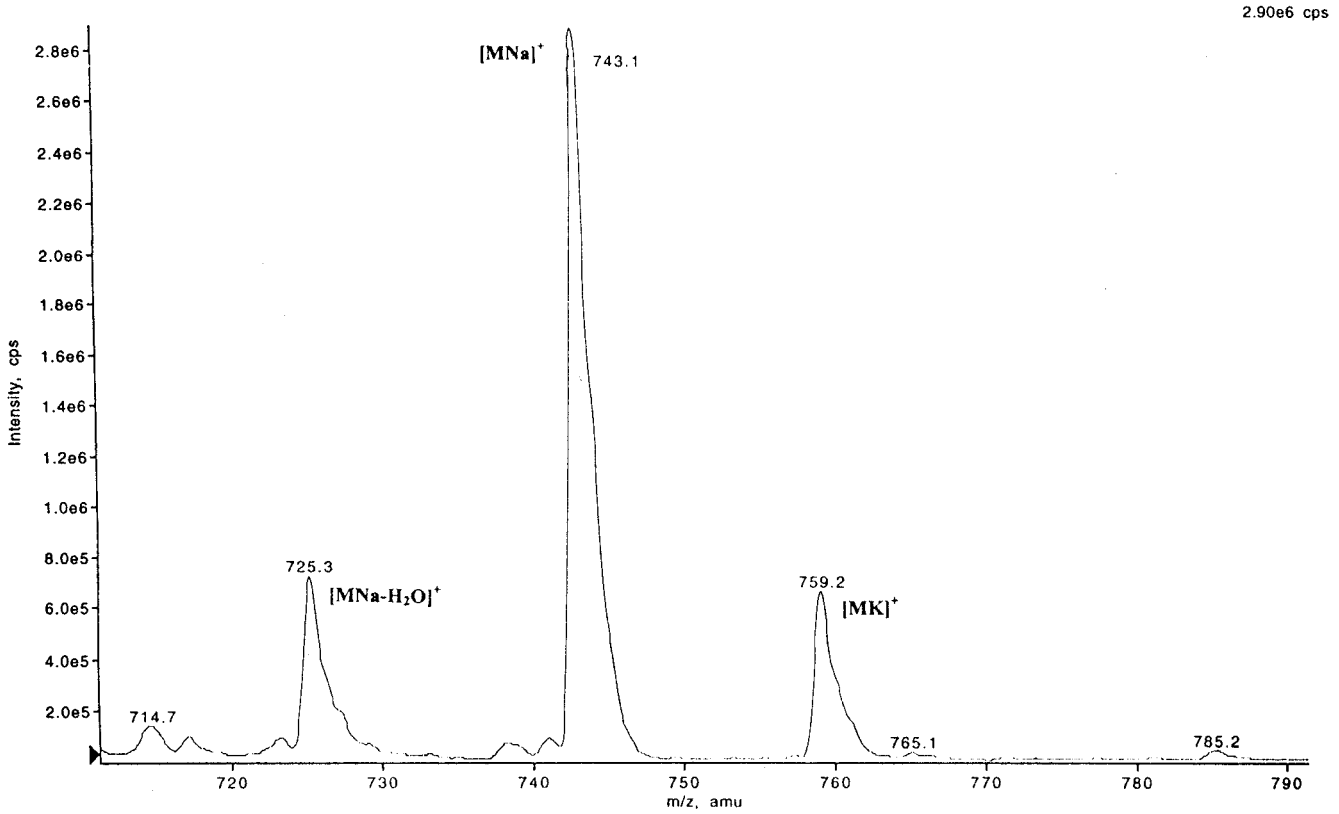

Figure 3. ES mass spectrum of AZA-A. Peak identity is discussed in the text.

of 50 V were used. Full-scan spectra were acquired from 500 to 800 amu using a step size of 0.1 amu and a dwell time of 1.0 ms. The same HPLC conditions described for the analytical separations were applied.

RESULTS AND DISCUSSION

The calibration curve obtained using an authentic standard of AZA-A showed an $R^2 = 99\%$. AZA-A was detected at 215 nm (D'Andrea et al., 1991). In these conditions linearity ranged from 12.5 to 400 mg/L, and the lowest detectable concentration was 0.100 mg/L.

The HPLC chromatogram obtained by SFE-CO_2 extraction is comparable with that reported in the literature (D'Andrea et al., 1991). By co-injection with pure standard, the peak eluted at 18.35 min was identified as AZA-A. LC/MS was performed on the neem oil extract. LC/MS was performed with a soft ionization technique such as atmospheric pressure ionization without heating of sample. This feature permits structural information on heat-sensitive organic compounds in complex matrixes to be obtained.

The mass spectrum of AZA-A is reported in Figure 3. The molecular ion $[MH]^+$ at 720 uma corresponding to AZA-A is not evident, $[MNa]^+$ and $[MK]^+$ being the main peaks at 743 and 759 uma, respectively. The molecular ion at 725 uma corresponds to $[MNa - H_2O]^+$.

Project design for liquid or SFE-CO_2 extractions required different conditions of pressure and temperature, using three separation stages. The extraction fractionation technique allowed a separation of different classes of compounds, such as waxes and oils. In fact, cuticular waxes, being located on the surface of seeds, were obtained in the first separator where operative conditions were set for their precipitation. On the other hand, oil compounds located in the internal part of the seeds were extracted in the second and third separators, their extractions requiring more time. In fact, time is required to allow the penetration of solvent, the dissolution of oils, and their transport toward the outside. For waxes, the optimized temperature to gain a selective precipitation is 0 °C, a temperature at which CO_2 is in subcritical state.

In the third separator conditions were set up to minimize the amount of compounds that could saturate the recycling stream, such as oil compounds dissolved in the gaseous CO_2. The pressure at this stage depends on the pressure value in the suction section of the pump to recycle the process fluid. A special design for the internal device of this separator allowed minimal trapping of the precipitated extracts in the gaseous stream.

In the extractor vessel, a constant pressure of 300 bar was chosen and two different temperature conditions such as 20 and 40 °C were assayed; the density for each extracted sample was calculated and is given in Table 1. Samples are divided into subsamples according to the ratio of the amount of CO_2 used (ms) to that of neem seed kernels (mo).

Sample 1 was obtained through conventional pressure on raw seed. Samples 2, 3A, 3B, and 5 were extracted at 40 °C using CO_2 in its supercritical fluid state, whereas samples 4A and 4B were extracted at 20 °C using CO_2 in its subcritical liquid state.

Sample 1 was exposed to conventional pressure for oil extraction. These seeds were then used for SFE extraction (sample 2). Trials on samples 3A, 3B, 4A, 4B, and 5 were carried out using raw seeds. Samples were treated using an ms/mo ratio of 64 or 119, according to the amount of CO_2 used. The AZA-A present in the oil samples is reported in Table 1. The AZA-A concentration allowed calculation of the efficiency of SFE related to the sample composition and to supercritical and subcritical extraction conditions. To compare the AZA-A

Table 1. Conditions of SFE and Yield in AZA-A Extractions According to Amount of Starting Material, Temperature of SFE, and ms/mo Ratio

sample	conditions of SFE and status of starting matrix	seeds (kg)	*T* (°C) of SFE	ms/mo	density d^{25}_4	oil yield (g/100 g)	AZA-A (mg/kg of oil)	AZA-A (mg/kg of seeds)
1	conventional pressure on raw seeds	4.2			0.945	8	550	44
2	supercritical on pressed seeds	3.9	40	64	0.906	14	4112	576
3A	supercritical on raw seeds	2.5	40	64	0.924	18	3650	639
3B	supercritical on raw seeds	2.5	40	119	0.907	26	8810	2291
4A	subcritical on raw seeds	2.5	20	64	0.916	18	1600	288
4B	subcritical on raw seeds	2.5	20	119	0.880	24	1502	358
5	supercritical on raw seeds	8.1	40	44	0.915	7	5450	382

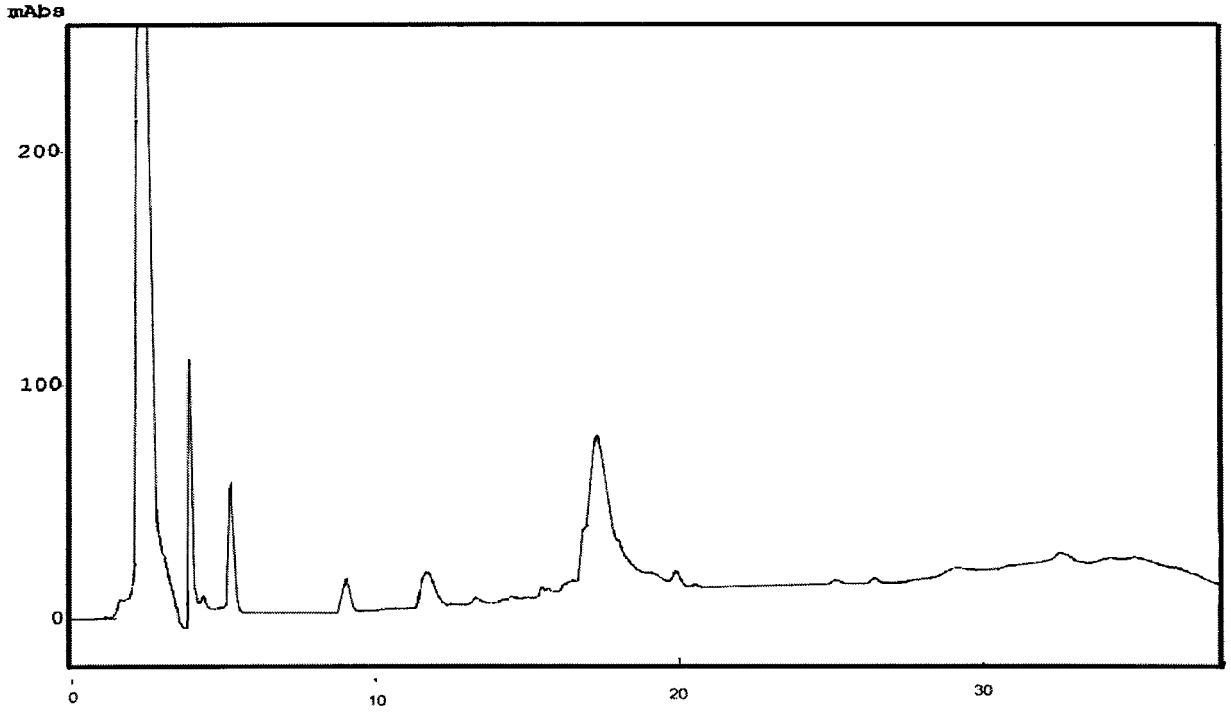

Figure 4. HPLC profile of extracts from cuticular waxes obtained by SFE at 215 nm on a Phenomenex column C_8/5 μm. Peak at retention time of 18.35 min is AZA-A.

extraction efficiency, a concentration in milligrams per kilogram of seeds was calculated.

Conventional pressure extraction on raw seeds (sample 1) led to a low yield in oil (8%) together with the lowest concentration of AZA-A per kilogram of oil and also to the lowest concentration of AZA-A per kilogram of seeds (44 mg/kg of seeds). Compared to conventional pressure extraction, both supercritical and subcritical pressures gave rise to a greater enrichment in AZA-A. On the other hand, significant differences are evident by comparison of supercritical and subcritical extractions at ms/mo = 64 and 119. In fact, samples 3A and 3B gave rise to an oil yield comparable to those of samples 4A and 4B but showed a greater extraction of AZA-A per kilogram of seeds (639 versus 288 mg/kg of seeds and 2291 versus 358 mg/kg of seeds, respectively). In particular, an ms/mo of 119 (sample 3B) is more effective than an ms/mo of 64 (sample 3A). Extraction on raw seeds is more efficient than SFE on pressed seeds for oil yield and recovery of AZA-A. In fact, although seed pressing removes part of the oil, allowing a greater enrichment of AZA-A in the SFE extract (4112 versus 3650 mg/kg of oil), it results in a lower oil yield, leading to a lower final concentration of AZA-A (576 versus 639 mg/kg of seeds).

It is worth noting that a significant quantity of pure cuticular waxes can be extracted at supercritical conditions in the first separator plate fixing the temperature at 0 °C. As shown in Figure 4 this procedure allowed a waxy sample rich in AZA-A to be obtained. The HPLC chromatogram of the extracted waxes displays a major peak at a retention time of 18.35 min corresponding to AZA-A without other major components.

An ms/mo ratio of 44 is shown to be not convenient in SFE. In fact, as shown in Table 1, although an oil very enriched in AZA-A is gained, the final amount of AZA-A per kilogram of seeds is not consistent (382 mg/kg of seeds) due to the low oil yield (7%). Although less CO_2 is used in this case, the amount of AZA-A extracted from seeds is too low, and this leads to an unfavorable increase in the cost of production.

CONCLUSION

HPLC coupled with electrospray mass spectrometry proved to be a powerful approach to quantify AZA-A from SFE extracts of neem seed kernels. It allows a screening of samples with a rapid identification and quantification of AZA-A content. Results of SFE demonstrated the efficiency of this technology, which can modulate the quality and the quantity of the final product. CO_2 is nontoxic, and its use reduces costs and adverse health effects usually associated with solvent disposal and long-term exposure to potential toxic vapors. It is worth noting that rather pure amounts of AZA-A can be obtained from extracted cuticular waxes

using supercritical conditions at subcritical transition temperatures due to a fall in solubility of AZA-A into the cuticular waxes. An increased use of waxy fractions is suggested for therapeutical and agricultural applications. Subcritical extraction is hardly efficient, leading to a low recovery of AZA-A in milligrams per kilogram of seeds.

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