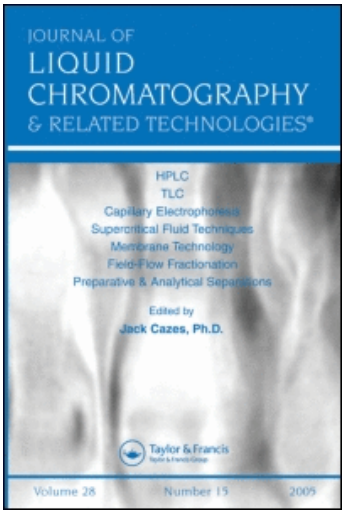

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AZADIRACHTIN IN CONIFER AND DECIDUOUS FOLIAGE, FOREST SOILS, LEAF LITTER AND STREAM WATER

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ABSTRACT

A high performance liquid chromatographic (HPLC) method for the determination of azadirachtin (AZ-A) in conifer and deciduous foliage, forest soil, litter and stream water is described. AZ-A was extracted from the solid matrices by aqueous methanol, concentrated, partitioned with hexane and re-extracted into dichloromethane layer. After evaporation of the dichloromethane layer, the residues were dissolved in ethyl acetate, purified on a Florisil® mini-column, eluted with ethyl acetate and analyzed using a reversed-phase C-18 column, with UV detection at 210 nm and acetonitrile/water gradient system. Analysis of AZ-A in the stream water required only the steps from dichloromethane extraction onwards. Limits of detection were 0.2 μg/g for foliage and soil, 0.25 μg/g for litter and 5.0 μg/L for water. Overall mean recoveries from terrestrial samples fortified with AZ-A at 0.50 μg/g to 10.0 μg/g and stream water at 10.0 μg/L to 200 μg/L were > 80% with good reproducibility. The applicability of the method was demonstrated by studying the recovery levels of AZ-A in samples fortified with standard Margosan-O.

INTRODUCTION

Azadirachtin-A (AZ-A) is the predominant compound among the structurally related and biologically active tetranortriterpenoids (1), extracted from the seeds of neem tree (*Azadirachta indica* A. Juss.). It causes feeding inhibition and growth disruption in various insect orders (2,3), and is especially effective against horticultural, agricultural and household insects (4,5). The material is registered in U.S.A. for the protection of non-food crops (6).

Recent laboratory studies (7) have shown that Margosan-O®, a neem preparation containing AZ-A, has potential to control the spruce budworm (*Choristoneura fumiferana* Clem.), a major destructive pest of fir and spruce forests of eastern North America, thus prompting an interest in the material for forestry use. Before a chemical is field tested, it is necessary to develop suitable analytical methods to study its persistence and degradation in various forestry matrices. This is necessary to evaluate its safety for non-target organisms, because most of the botanicals (pyrethrum, rotenone, sabadilla, ryania, nicotine, etc.) are toxic to some of these organisms (8).

A number of high performance liquid chromatographic (HPLC) methods using a variety of extraction and partition techniques have been reported to isolate and separate AZ-A from neem seed kernels (9,10,11,12). Stokes and Redfern (13) and Barnby *et al* (14) reported HPLC methods to screen the residual amounts of AZ-A after exposure to light. Other HPLC methods have been described for the determination of AZ-A in neem extracts and formulations (15,16,17). However, most of these methods are inadequate for the determination of AZ-A residues in complex forestry matrices, such as deciduous and conifer foliage, forest soils, litter and natural waters. The aim of this work was to develop a sensitive and reliable HPLC method for the determination of AZ-A in these matrices. Moreover the applicability of the method was demonstrated by determining the AZ-A concentrations in the forestry matrices after fortifying them with Margosan-O® containing known amounts of the analyte.

MATERIALS AND METHODS

Reagents

Azadirachtin (analytical standard > 95% purity) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HPLC analysis conducted (17) on this material confirmed that it contained mainly the AZ-A. Margosan-O® was a gift from W.R. Grace and Co. (Cambridge, MA, U.S.A.). Anhydrous sodium sulphate [purified (18) and dried for 8 hours at 300°C and stored in a desiccator] and sodium chloride were ACS grade and obtained from BDH (Toronto, Ont., Canada). Florisil® (calcined at 650°C, PR grade, 60-100 mesh) was from Floridin Co. (Pittsburgh, PA, U.S.A.) and used after purification (18). All organic solvents and water were HPLC grade obtained from BDH and filtered through Gelman 0.20 μm Nylaflo filter.

Forest Matrices

Red oak (*Quercus rubra* L.) and balsam fir [*Abies balsamea* (L.) Mill.] foliage were collected from a mixed forest north of Sault Ste. Marie (Ont., Canada). Forest litter (pH 5.4, organic matter, OM, > 88%) and soils (sandy loam, pH 5.6, OM 5.4%, sand 56%, clay 6% and silt 38%; clay loam, pH 5.1, OM 11.7%, sand 38%, clay 16% and silt 46%) were collected from forested areas around Timmins (Ont., Canada). Water [pH 6.1, alkalinity and hardness (mg/L of $CaCO_3$) respectively 16.2 and 14.3, turbidity 0.31 JTU] was collected in 1-L Teflon® bottles from a slow-flowing stream near Searchmont (Ont., Canada) and stored at 4°C in the dark. Detailed procedures used in the collection of various matrices, their transportation, storage and processing them prior to fortification with a specific analyte are described elsewhere (18,19).

HPLC Equipment

The HPLC system consisted of a Hewlett Packard (HP) (HP Chromatographic Division, Mississauga, Ont., Canada) model 1084B instrument,

consisting of automatic degassing system, dual solvent and pumpheads and fitted with a variable wavelength (190-600 nm) detector (model 79875A), variable volume injector and autosampler (model 79842), microprocessor and electronic integrator (model 79859B) linked to an HP LC terminal (model 79850B) to provide the necessary chromatographic parameters (area, area %, retention time, etc.) for each peak. An HP column oven set at 80°C permitted temperature regulation. Quantification was done at 210 nm with a detector setting of 1.6 x 10^{-3} AU/cm. A flow-rate of 1.0 mL/min and an injection volume of 50 µL were used. The analytical column (15 cm x 4.6 mm i.d. stainless steel; Regis, Morton Grove, IL, U.S.A.) was prepacked with 5 µm Spherisorb C-18 ODS-2 and operated in a degassed acetonitrile/water (v/v) gradient system. The chromatographic run time was 32.5 minutes. The elution was started with the mobile phase consisting of 20% acetonitrile and increased linearly to 35% acetonitrile for 22.5 minutes. The column was then flushed with pure acetonitrile for an additional 10 minutes to remove any late eluting impurities. Under these conditions the retention time (RT) of AZ-A was 20.6 minutes.

Standard Solutions

A stock standard solution of AZ-A in methanol containing 0.50 mg/mL was prepared in amber-coloured volumetric flasks and stored at -4°C. Aliquots of the solution were diluted with mobile phase immediately before use, to give working solutions containing AZ-A in the range of 0.5 to 25 µg/mL. The stock solution was used for fortification and recovery tests.

Fortification of AZ-A

Five gram aliquots of processed (18) fir needles, oak foliage strips, litter, sandy and clay loam soils were taken in separate screw-capped 250 mL Erlenmeyer flasks and each sample was fortified in quadruplicate with 5, 10, 50 and 100 µL of AZ-A stock solution to produce respectively, samples containing

0.50, 1.0, 5.0 and 10.0 μg/g of the analyte. One hundred mL aliquots of filtered (5 μm PTFE membrane filter under suction) stream water, in 250 mL Teflon separatory funnels, were fortified separately in quadruplicate with 2.0, 4.0, 20.0 and 40.0 μL of AZ-A stock solution to produce samples containing 10, 20, 100 and 200 μg/L of the analyte respectively. A 5.0 g sample of each matrix and 100 mL of water with no AZ-A added served as control.

Margosan-O containing 0.31% by mass of AZ-A (17) was diluted with methanol to prepare a stock solution containing 0.50 mg/mL of the analyte and used in the fortification of the matrices, according to the above described procedure.

Extraction of AZ-A from the Matrices

The fortified samples were shaken well for 5 min. to improve the sample/AZ-A contact and allowed to equilibrate for another 15 min. Fifty mL of aqueous methanol (1:1, v/v) was added to each sample of solid matrix, vortexed, shaken (Magni-Whirl® shaker at control setting 20) for 30 min. and filtered under gentle aspiration through a Buchner funnel (43 mm I.D.) using methanol-washed Whatman #1 filter paper. The filter-cake was reextracted twice with aqueous methanol (50 mL each time) and filtered through the same funnel. The extract was concentrated under low pressure to about 80 mL, transferred quantitatively to a 250 mL Teflon separatory funnel, 5 mL of 5% aqueous sodium chloride were added and partitioned twice with 30 mL of hexane each time to remove nonpolar and lipid impurities. After the hexane was discarded, the aqueous layer was extracted thrice, each time with 60 mL of dichloromethane. The lower organic phase was drained through a column of anhydrous sodium sulphate (3 cm diam. x 3 cm length). The column was rinsed with 2 x 10 mL of dichloromethane, the rinses were added to the extract and it was flash evaporated to dryness at 40°C. The residue was dissolved in 1.0 mL of ethyl acetate for Florisil minicolumn cleanup.

Aqueous sodium chloride was added to the water samples, which were then extracted with 3 x 60 mL of dichloromethane. The above-described procedure was then followed.

The matrix samples fortified with aliquots of the stock solution prepared from Margosan-O, were also extracted and processed according to the procedures described above.

Column Cleanup

The chromatographic minicolumn (disposable pasteur pipet, 15 cm x 0.8 cm I.D.) was packed from bottom to top with a small wad of glasswool, 5 cm of activated Florisil and 2 cm of anhydrous sodium sulphate. After prewashing the column successively with hexane (10 mL) and ethyl acetate (10 mL), the crude extract was added and eluted with 15 mL of ethyl acetate without allowing the column to run dry. The eluate was concentrated under a stream of dry nitrogen at 35°C and analyzed by HPLC after adjusting the volume of each sample to the predetermined linear range of the UV detector.

RESULTS AND DISCUSSION

Chromatograms

A typical chromatogram of the AZ-A standard obtained by injecting 0.475 μg in 50 μL onto the HPLC column is shown in Fig. 1. The peak with RT 20.6 min is symmetrical, with the baseline separated and well removed from the solvent front. Deviation in RT for each injection was negligible. Figures 2A and 2B show chromatograms of extracts from blank and fortified (1.0 μg of AZ-A/g) conifer needles, respectively. The AZ-A peak is well separated from the other peaks derived from the endogenous materials present in the needle and they did not interfere with the determination of the analyte. Similar patterns were also observed in the chromatograms obtained respectively for blank and fortified samples of oak foliage (Figs 3A and 3B), forest soils (Figs

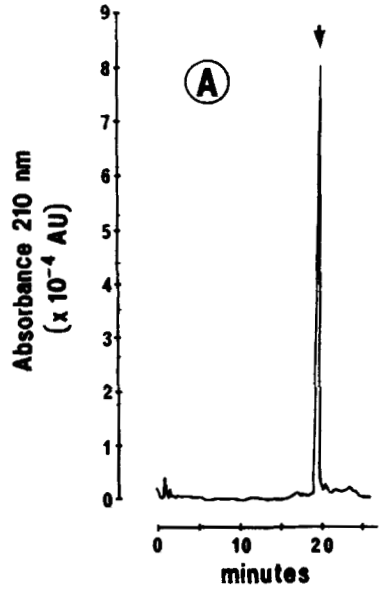

Figure 1. Chromatographic trace of azadirachtin standard, 0.475 μg in 50 μL injection

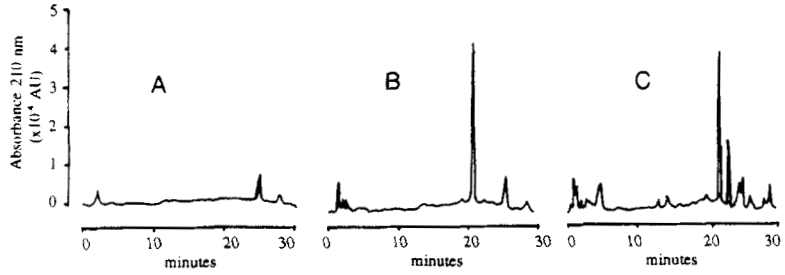

Figure 2. Chromatographic trace of conifer needle extracts:
A) blank foliage
B) fortified (1.0 μg of AZ-A/g) foliage
C) fortified (Margosan-O) foliage

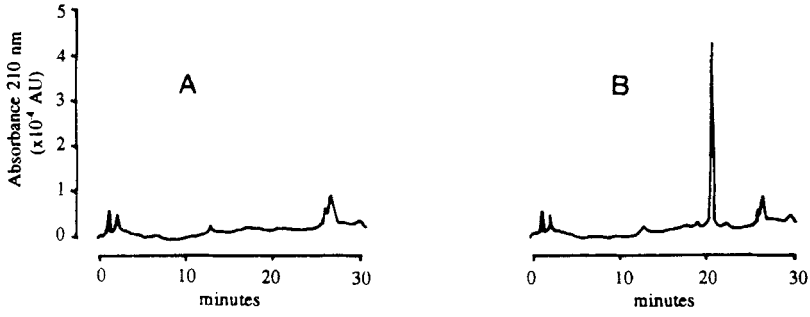

Figure 3. Chromatographic trace of oak foliage extracts:
A) blank foliage
B) fortified (1.0 μg of AZ-A/g) foliage

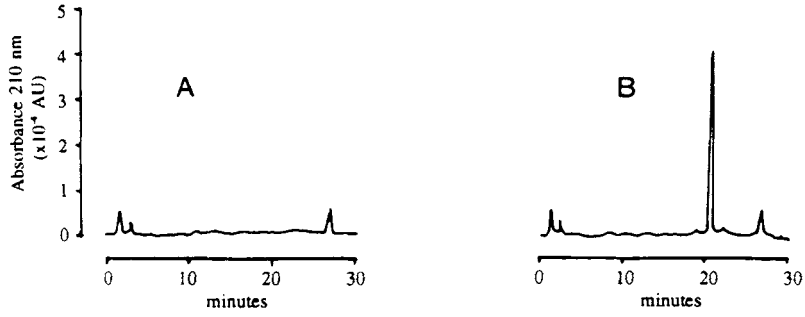

Figure 4. Chromatographic trace of sandy loam soil extracts:
A) blank soil
B) fortified (1.0 μg of AZ-A/g) soil

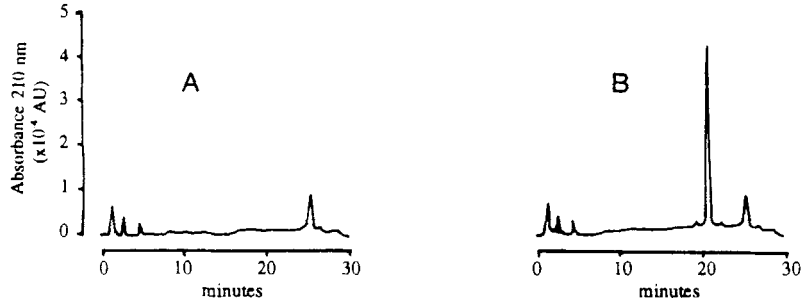

Figure 5. Chromatographic trace of clay loam soil extracts:
A) blank soil
B) fortified (1.0 μg of AZ-A/g) soil

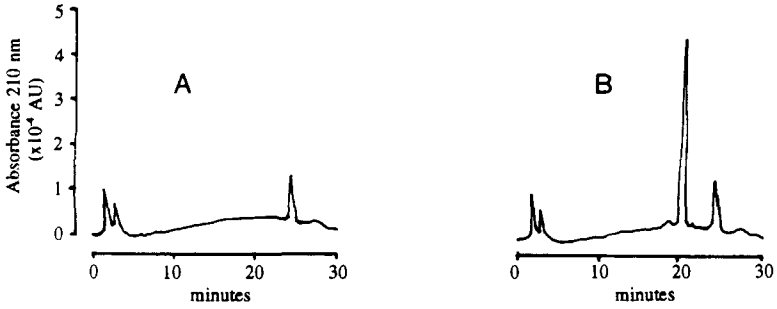

Figure 6. Chromatographic trace of litter extracts:
A) blank litter
B) fortified (1.0 μg of AZ-A/g) litter

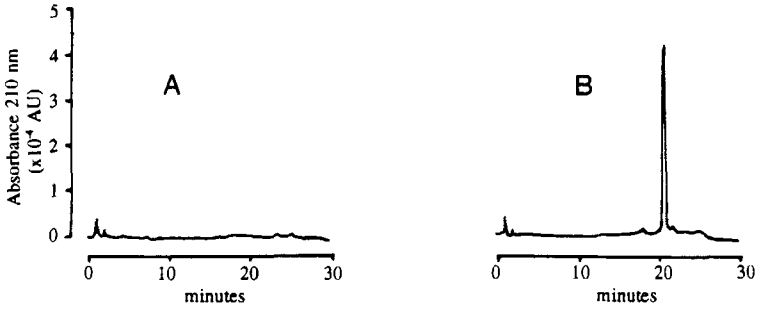

Figure 7. Chromatographic trace of water extracts:
A) blank water
B) fortified (20.0 μg of AZ-A/L) water

4A to 5B) and forest litter (Figs 6A and 6B). The optimal analytical and HPLC conditions described in materials and methods were found to be sufficiently adequate and effective in quantifying AZ-A from forest foliage, soil and litter. The chromatograms from the water extracts (Figs 7A and 7B) are cleaner, without as many extraneous peaks as were found in the other matrices. However, the chromatograms of all matrices fortified with Margosan-O did contain additional unknown peaks (Fig. 2C - conifer needles) probably arising from the additives present in the formulation, but they did not interfere with the determination of

TABLE 1

Determination of Recovery and Intra-assay Precision for the Analysis of AZ-A in Forestry Matrices (n=4)

Matrix type	Fortification level (μg/g)	Mean concn. ± S.D. (μg/g)	C. V. (%)	Recovery (%) (mean ± S.D.)
Fir Needles	0.50	0.41 ± 0.06	14.6	82 ± 12
	1.00	0.93 ± 0.08	8.6	93 ± 8
	5.00	4.88 ± 0.33	6.8	98 ± 7
	10.00	10.05 ± 0.81	8.1	101 ± 8
			Mean recovery	94 ± 9
Oak Foliage	0.50	0.39 ± 0.05	12.8	78 ± 10
	1.00	0.85 ± 0.09	10.6	85 ± 9
	5.00	4.91 ± 0.47	9.6	98 ± 9
	10.00	9.78 ± 1.02	10.4	98 ± 10
			Mean recovery	90 ± 10
Soil (sandy loam)	0.50	0.37 ± 0.05	13.5	74 ± 10
	1.00	0.83 ± 0.08	9.6	83 ± 8
	5.00	4.92 ± 0.33	6.7	98 ± 7
	10.00	9.88 ± 0.87	8.8	99 ± 9
			Mean recovery	89 ± 9
Soil (clay loam)	0.50	0.41 ± 0.06	14.6	82 ± 12
	1.00	0.82 ± 0.10	12.2	82 ± 10
	5.00	4.81 ± 0.49	10.2	96 ± 10
	10.00	9.89 ± 0.93	9.4	99 ± 9
			Mean recovery	90 ± 10

TABLE 1 (Contd.)

Determination of Recovery and Intra-assay Precision for the Analysis of AZ-A in Forestry Matrices (n=4)

Matrix type	Fortification level (μg/g)	Mean concn. ± S.D. (μg/g)	C. V. (%)	Recovery (%) (mean ± S.D.)
Litter	0.50	0.33 ± 0.06	18.2	66 ± 12
	1.00	0.79 ± 0.11	13.9	79 ± 11
	5.00	4.54 ± 0.53	11.7	91 ± 11
	10.00	9.32 ± 0.89	9.6	93 ± 9
			Mean recovery	82 ± 11
Stream water	10.0*	9.4 ± 0.8	8.5	94 ± 8
	20.0	22.2 ± 1.6	7.2	111 ± 9
	100.0	98.6 ± 7.8	7.9	99 ± 8
	200.0	202.4 ± 16.2	8.0	101 ± 8
			Mean recovery	101 ± 8

* concn. μg/L

AZ-A. Attempts to remove them by alumina or silica column chromatography gave lower recoveries for the analyte and no improvement in the chromatogram.

Calibration Graph and Detection Limits

A calibration graph for AZ-A was constructed by triplicate injection (50 μL) of the standard solutions at concentrations ranging from 0.5 μg/mL to 25 μg/mL (0.025 μg to 1.25 μg) and plotting the concentration *vs* peak area. Good linearity was achieved with a correlation coefficient of 0.998. The limit of detection (LOD) defined as 2s/m, where s is the blank standard deviation (n=5) and m is the slope of the calibration graph (20) varied according to the type of matrix. The calculated values are 0.2 μg/g (0.05 μg) for foliage and soil, 0.25

μg/g (0.063 μg) for litter and 5.0 μg/L (0.025 μg) for water. The limit of quantification (LOQ) is arbitrarily fixed at twice the value of LOD. Recovery values of AZ-A in different matrices were computed by comparing the average peak area ratio of the clean extract of each matrix injected (50 μL) in triplicate with the average peak area ratio of the standard.

The data on the recovery levels of AZ-A and intra-assay precision of the method are given in Table 1. Higher mean percent recoveries with increased precision (lower S.D. and C.V.) are obtained for water at all the fortification levels compared to other substrates. Average recovery levels in litter at the four fortification levels ranged from 66 to 93 % and the mean recovery of 82 % was the lowest compared to the other matrices. The average recoveries in foliage and soil were around 90 %, ranging from 89 to 94 %, and the recovery in water was quantitative (101 %). Usually, the recovery levels at the lowest fortification level (0.50 μg/g) were rather low but satisfactory and ranged from 66 % in litter to 82 % in clay loam soil, correspondingly the intra-assay coefficient of variation was higher and varied from 18.2 % in litter to 12.8 % in oak foliage. However the overall high recovery levels of AZ-A obtained at 0.50 μg/g fortification level and higher and the corresponding intra-assay precision, demonstrate the applicability of the method for the determination of AZ-A residues in a wide range of forestry matrices.

Application

An attempt was made to determine whether the present method can be used to quantify AZ-A in the terrestrial matrices and stream water fortified respectively with a methanolic solution of standard Margosan-O containing 1.0 μg of AZ-A/g and 20.0 μg of AZ-A/L. Data in Table 2 show that the recoveries of the analyte from all the matrices and the inter-assay variabilities are not significantly different than the values reported in Table 1, indicating that the method is suitable and sensitive enough to quantify AZ-A in forestry matrices following the release of neem formulations in insect control programs. Further improvement in

TABLE 2

Analysis of Some Forestry Matrices and Stream Water Fortified with Margosan-O Containing 1.00 μg/g (Terrestrial) and 20.0 μg/L (Water) of AZ-A (n=4)

Matrix type	Mean concn. ± S.D. (μg/g)	C. V. (%)	Recovery (%) (mean ± S.D.)
Fir needles	0.94 ± 0.07	7.4	94 ± 7
Oak foliage	0.91 ± 0.04	4.4	91 ± 4
Soil (sandy loam)	0.88 ± 0.07	8.0	88 ± 7
Soil (clay loam)	0.84 ± 0.09	10.7	84 ± 9
Litter	0.81 ± 0.10	12.3	81 ± 10
Stream water	20.2 ± 1.0*	5.0	101 ± 5

* concn. μg/L

sensitivity using the present HPLC conditions is restricted because the analyte molecule has no strong UV absorbing chromaphores, unless it is derivatized and converted into a stable fluorescent product either at pre- or post-column levels and analyzed by using a fluorescent detector. Azadirachtin-A is a complex molecule and is relatively unstable (21). Whether it will remain intact or form stable fragments to react during derivatization should be explored before any fluorescence labelling is attempted.

CONCLUSIONS

This paper describes a high performance liquid chromatographic method for the determination of AZ-A in some typical forestry matrices. The method is reliable and has sufficient sensitivity to make it suitable for monitoring the residues of AZ-A in specialized forestry spray programs, such as forest nurseries, Christmas tree plantations, etc., where high dosage and volume rates are often

used. The method is currently used to study the fate, dissipation pattern and persistence of AZ-A in conifer and deciduous foliage following the release of Margosan-O at different dosage levels on potted trees using a laboratory spray chamber. With necessary modifications, the analytical scheme on which this method is based will be suitable to assay AZ-A in many other forestry matrices, including animal tissues.

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