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Analytical Study of Azadirachtin and 3-Tigloylazadirachtol Residues in Foliage and Phloem of Hardwood Tree Species by Liquid Chromatography−Electrospray Mass Spectrometry

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Supporting Information

ABSTRACT: A rapid and sensitive LC-ESI-MS method has been developed and validated for the quantitation of azadirachtin and 3-tigloylazadirachtol in deciduous tree matrices. The method involves automated extraction and simultaneous cleanup using an accelerated solvent technique with the matrix dispersed in solid phase over a layer of primary−secondary amine silica. The limits of quantification were 0.02 mg/kg for all matrices with the exception of Norway maple foliage (0.05 mg/kg). Validation at three levels (0.02, 0.1, and 1 mg/kg), demonstrated satisfactory recoveries (71−103%) with relative standard deviation <20%. Two in-source fragment ions were used for confirmation at levels above 0.1 mg/kg. Over a period of several months, quality control analyses showed the technique to be robust and effective in tracking the fate of these natural botanical insecticides following systemic injection into various tree species for control of invasive insect pest species such as the emerald ash borer and Asian longhorned beetle.

KEYWORDS: azadirachtin, 3-tigloylazadirachtol, neem, foliage, phloem, liquid chromatography, mass spectrometry, stability study

INTRODUCTION

Invasive alien insect pests such as the emerald ash borer (EAB) (*Agrilus planipennis*) and Asian longhorned beetle (ALB) (*Anoplophora glabripennis*) represent unique pest problems in North America. These pests cause widespread tree mortality and may result in significant economic and ecological impacts. One alternative for controlling these pests is through the use of natural insecticides, such as azadirachtin, **1** (commonly referred to as azadirachtin A), and 3-tigloylazadirachtol, **2** (commonly referred to as azadirachtin B) (Figure 1). These compounds belong to the limonoid group of natural products derived from the neem tree (*Azadirachta indica*). Both compounds, azadirachtins A and B, demonstrate substantial antifeedent, growth disruption, and sterility effects in Lepidoptera and Diptera.[1] They are also characterized by relatively low toxicity to mammals[2] and are quite susceptible to photolysis, hydrolysis, and microbial degradation and are thus nonpersistent in the environment.[3] When applied via direct systemic injection into trees, they provide a control option for invasive wood-boring insect species that is effective and carries minimal environmental risk. Through systemic injections, both adults, which mature and feed on foliage and twigs, as well as the more destructive larvae, which feed at the cambium−phloem interface, can be targeted. Our initial studies have demonstrated substantial effectiveness against EAB following systemic injection of a combination of those natural compounds,[4] and ongoing studies are focused on extrapolating this success for potential control of ALB.

A complete understanding of the efficacy and environmental behavior of systematically injected insecticides requires studies of uptake, distribution, and ultimate fate of the active ingredients within the tree. Therefore, simple and accurate analytical methods with sufficient sensitivity, specificity, and applicability to multiple species and matrix types are required. Several analytical methods based on liquid chromatography (LC) have been published for the determination of azadirachtin A in environmental matrices[5−7] and vegetable samples[8] primarily using the photodiode array detector (DAD). These methods demonstrate good recovery, precision, and sensitivity at least in simple matrices such as water (e.g., 0.003 mg/L).[5] Owing to its inherently greater specificity, mass spectrometry (MS) techniques have been the focus of more recent methods developed for complex plant matrices.[9−14] The high specificity achievable by LC-MS, especially tandem MS (MS/MS), allows limits of quantification (LOQ) as low as 0.01 mg/kg in matrices such as orange fruit.[13] However, attaining such a low LOQ in leafy samples may present a significant analytical challenge, as exemplified in the case of azadirachtin analysis in cabbage by ultraperformance LC-MS/MS,[14] for which recoveries were <70%. Many of those MS-based analytical techniques were focused on the detection and quantitation of the sodium adduct ions of azadirachtin A[10,13,14] and azadirachtin B.[10]

An additional problem in the analysis of azadirachtin and related compounds is their instability or degradation when in contact

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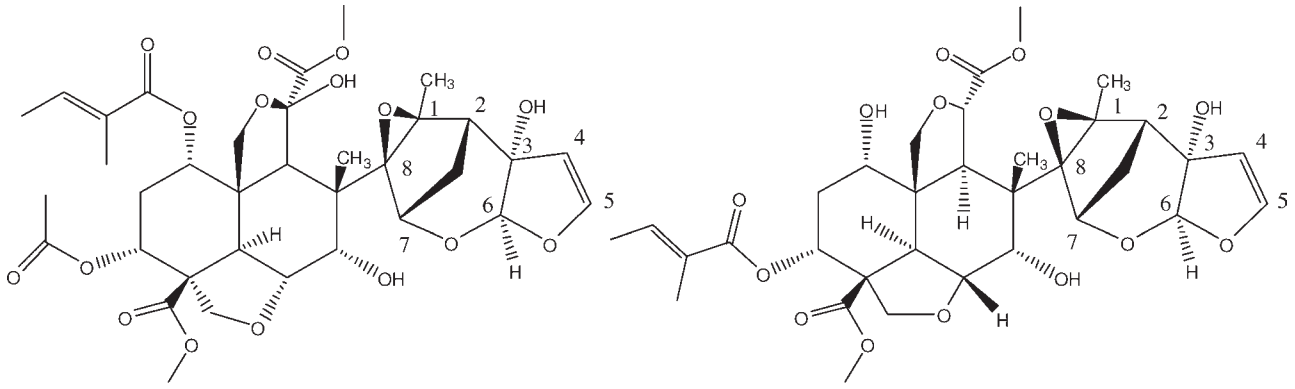

Figure 1. Chemical structures of (1) azadirachtin A and (2) azadirachtin B.

with the matrix as noted by Caboni et al.[11,12] Given the matrix-dependent issues associated with coextractive interference, rapid and effective cleanup procedures are required for complex plant matrices. Although liquid−liquid extraction[8] and solid phase extraction (SPE)[6,7] have been used in previous methods, automated methods such as accelerated solvent extraction (ASE) are preferable for fast and easy extraction of pesticides in environmental[15,16] and food samples.[17,18] Generally, the extraction step is followed by a cleanup step involving either SPE [7] or dispersive solid phase extraction (D-SPE).[17] Most recently, simultaneous extraction and cleanup approaches, such as matrix solid phase dispersion (MSPD)[19,20] and the method known as QuEChERS (quick, easy, cheap, effective, rugged and safe),[20,21] are being used to reduce overall sample handling and processing time for the analysis of pesticides in fruit and vegetable samples.

The aim of this study was to develop and validate a rapid, sensitive, and selective analytical method suitable for the quantitation and confirmation of azadirachtins A and B in foliage and phloem samples from different hardwood tree species. Once developed and validated, the analytical technique would have direct applicability in studies on the uptake, translocation, and environmental behavior of these compounds following systemic injection into trees for control of invasive wood-boring insects.

MATERIALS AND METHODS

Reagents and Chemicals. LC-MS Optima grade acetonitrile, acetone, and methanol were purchased from a commercial supplier (Fisher Scientific Ltd., Ottawa, ON, Canada). LC grade water was obtained by purifying demineralized water in a Milli-Q system (Millipore, Billerica, MA). Spherical C_{18} bonded flash silica 45−75 μm, Supelclean Envi-Carb 120/400, primary−secondary amine (PSA) bonded silica (Supelco, Bellefonte, PA), and diatomaceous earth (DE) (Dionex, Sunnyvale, CA) were also purchased from commercial suppliers.

Reference standards of azadirachtins A and B were purchased from EID Parry (Tamil Nadu, India). Stock analytical standard solutions of azadirachtin A (purity = 96.1%) and azadirachtin B (purity = 98.5%) were prepared by dissolving 5 mg of the powdered material in 10 mL of acetone to yield a final concentration of 500 μg/mL. The stock solution was stored in the dark at −20 °C. Working solutions, used for LC-MS analysis and for test sample fortification, were obtained by diluting stock solutions with acetonitrile. Calibration standards of various lower concentrations were prepared by further dilution of working solutions with appropriate volumes of 50:50 acetonitrile/water (v/v). Working and calibration solutions were stored in the dark under refrigerated conditions (1−5 °C).

Instrumentation. A Waters Alliance 2690 HPLC system (Waters, Milford, MA) was interfaced with a ZMD single-quadrupole mass spectrometer (Waters) via an orthogonal Z-spray electrospray interface (ESI). The LC separation was performed by injecting 25 μL sample volumes. The column used was a 50 × 2.1 mm i.d., 2.6 μm, Kinetex C_{18} (Phenomenex, Torrance, CA) with a 10 × 2.1 mm i.d., 2.5 μm, SunFire guard column of the same material (Waters). The mobile phase was a 10 μM sodium acetate water−10 μM sodium acetate methanol gradient, where the percentage of methanol was changed linearly as follows: 0.0 min, 30%; 1.0 min, 30%; 3.0 min, 60%; 4.0 min, 90%; 9.8 min, 90%; 9.9 min, 30%; and, 15.0 min, 30%. The flow rate was 200 μL/min. Nitrogen generated from pressurized air in a high-purity nitrogen generator model 75-72 Gas Station from Parker Balston (Haverhill, MA) was used as both the drying and nebulizing gas. The desolvation gas and cone gas flows were set as 600 and 60 L/h, respectively. Infusion experiments conducted to optimize instrumental parameters were performed using the built-in syringe pump, directly connected to the interface.

Capillary voltages of 3.5 kV were used in positive ionization mode. The interface temperature was set to 350 °C and the source temperature to 120 °C. Dwell times of 0.2 s/scan were chosen for each transition. A solvent delay of 5.0 min was selected to give an additional cleanup using the built-in divert valve controlled by the Masslynx NT v.3.5 software. Three fragment ions in-source were selected for each compound and acquired by single ion monitoring (SIM) mode. The most abundant was selected as the quantitation ion (Q) (743.2 and 685.2 for azadirachtins A and B, respectively) and the other two as confirmation ions (q) (725.2 and 665.2 for azadirachtin A; 667.2 and 567.2 for azadirachtin B). Data processing and quantitation were performed using the quantify application module in Masslynx v 3.5.

Sample Preparation. Seven different tree matrices were examined, including the foliage of green ash (*Fraxinus pennsylvanica*), white ash (*Fraxinus americana*), London planetree (*Platanus acerifolia*), sugar maple (*Acer saccharum*), red maple (*Acer rubrum*), and Norway maple (*Acer platanoides*), as well as phloem from green ash. Premaceration preparation involved manually separating leaves from twigs (twigs were discarded) and cutting phloem into small pieces. Both sample types were macerated with a grinder, Grindomix GM200 (Glen Mills Inc., Clifton, NJ), at 7000 rpm for a period of 0.2 min. A small amount (0.5 g) of the homogenized sample was accurately weighed (precision = 1 mg) and mixed with 1 g of C_{18} silica and 0.75 g of DE for approximately 2 min. The mixture was quantitatively transferred into an 11 mL stainless steel ASE extraction cell containing a layer of PSA solid phase (0.5 g). The PSA layer was used to retain some of the potential coextractive interferences.

All extractions were performed on an ASE 200 Accelerated Solvent Extractor (Dionex Corp., Oakville, ON, Canada) using 100% acetonitrile as the extraction solvent, a pressure of 2000 psi, and 5 cycles with a static time of 2 min at room temperature and a total flush volume of 90%. In ASE, the static time is defined as the length of time the sample is held in contact with the extraction solvent prior to flushing. The raw extract was adjusted to a constant volume of 25 mL with acetonitrile, and a 5 mL aliquot was further treated by shaking for 1 min with 0.1 g of PSA. The cleaned-up extract was evaporated to dryness under a stream of nitrogen in a Meyer N-EVAP apparatus (Oganomation, Berlin, MA) at 45 °C and then reconstituted with 1 mL of HPLC grade acetonitrile/water 50:50 (v/v). To remove solid microparticles, the preconcentrated extract was passed through an Acrodisc 0.20 μm nylon syringe filter (Pall Canada Ltd., Ville St Laurent, PQ, Canada). An aliquot of 25 μL of the final sample was directly injected in the LC-MS system.

For method validation studies, homogenized matrix samples were fortified by delivering 1 mL of the appropriate mixed standard solutions (0.5, 0.05, or 0.01 μg/mL of azadirachtins A and B in acetonitrile) as required to obtain concentrations equivalent to 1, 0.1, and 0.02 mg/kg of matrix fresh weight (fw) respectively.

Matrix Effect Study. To detect a matrix effect, the response areas for the analytes azadirachtins A and B were observed in pure solvent solution and compared to response areas derived from an equivalent concentration spiked into a particular matrix extract. The matrix effect was calculated as the ratio of the two areas expressed as a percent.[22]

In this study, the matrix effect was determined on the basis of a comparison of the area resulting from a 0.05 mg/kg concentration level, for each of the seven different matrices, with that derived from a standard solution in solvent at level of 5 ng/mL (i.e., equivalent to the expected concentration in the final volume of the matrix sample).

Validation Study. Validation data were obtained for each sample matrix following standard guidelines for validation of analytical procedures used in pesticide residue analysis.[23] The calibration curve was obtained by analyzing 10 matrix-matched standard solutions at concentrations between 1 and 1000 ng/mL. Acceptance criteria required correlation coefficients of >0.99 and linearity residuals of <30%. The accuracy and precision were obtained by quintuplicate analysis of azadirachtins A and B in blank matrix samples fortified at three different concentration levels (0.02, 0.1, and 1 mg/kg). Acceptance criteria were established as recovery efficiency between 70 and 110% and relative standard deviation (RSD) of <20%.[23]

The limit of detection (LOD), defined as the lowest concentration for which the detector response can be reliably differentiated from background noise levels, was estimated based on a signal-to-noise ratio of 3 for those chromatograms derived from the lowest fortified matrix samples. The LOQ was set as the lowest concentration in fortified test matrix samples meeting acceptance criteria for recoveries and precision as described above.

The specificity of the analytical procedure was determined by analysis of a procedural blank, a sample blank, and a blank sample fortified at the LOQ level. The acceptance criteria for method specificity was established as a blank response that did not exceed 30% that of the LOQ.

Confirmation Study. Confirmation of analyte presence, as well as improved specificity, was achieved by selection and acquisition of in-source fragment ions for azadirachtins A and B. Confirmation could be evaluated using identification points (IPs),[24] with 1 IP for each ion acquired for a given analyte. As noted above, confirmation was performed by acquisition of a total of two ions (q_1 and q_2). A ratio between quantitation ion (Q) and q_i was calculated on the basis of relative abundance. The average Q/q calculated for all standards injected in a sequence was taken as the theoretical ion ratio (IR), and the IRs calculated for three concentration levels (0.02, 0.1, and 1 mg/kg) were compared with the theoretical value. Confirmation was considered reliable if the difference between standard and sample IR was within 20% of the theoretical IR values between 1 and 2, <25% for theoretical IRs between 2 and 5, <30% for IRs between 5 and 10, and <50% for IR values >10.[24] Additionally, the limit of confirmation (LOC) was calculated for these two confirmative ions, which was estimated for a signal-to-noise ratio of 3 from the chromatograms at the lowest analyte concentration tested for each matrix.

Analyte Stability Study. To determine the stability of azadirachtins A and B, four compound leaves composed of seven to nine leaflets per leaf and total weight of approximately 15 g were allowed to take up azadirachtin compounds from 40 mL of a dilute aqueous solution (5 μg/mL each of azadirachtins A and B). One day after absorption, the entire foliar sample was macerated and a subsample was analyzed using the validated method as described. Additional subsamples were stored frozen (< −18 °C) and analyzed 7, 14, 21, 28, and 84 days after treatment. Three aliquots of these samples were analyzed in duplicate for each storage period, and the average concentration was calculated as a percentage relative to the initial analysis conducted 1 day after treatment. For this stability experiment, a blank sample fortified at 0.1 mg/kg was used as quality control (QC) and analyzed together with the stability samples. Results were accepted if QC analyses gave acceptable recoveries (70−110%). Analytes were considered to be stable if the observed concentrations did not differ from the initial concentration by more than 20%.

Field Sample Analyses. Routine analyses of azadirachtins A and B in field samples were conducted using an external matrix-matched standard calibration curve. To ensure the quality of the analyses, at least two fortified QC samples (0.1 and 1 mg/kg) were included with each batch of samples. Batch sample analyses were considered to be acceptable if QC recoveries were between 70 and 110% and the RSD lower than 20%.

RESULTS AND DISCUSSION

MS and LC Optimization Experiments. The full-scan mass spectra of azadirachtins A and B are shown in Figure 2. These examples were obtained from the chromatographic peak of 5 μg/mL standard solution, using scan mode acquisition at different cone voltages for positive ESI. To study the effect on proton or adduct ionization, low concentrations of various additives were incorporated in the mobile phase, including formic acid or sodium, potassium, lithium, and ammonium acetate. The sodium adduct ($[M + Na]^+$) was selected as optimal, due to its relatively higher abundance and the number of fragment ions obtained. Using a different cone voltage resulted in significant fragmentation of $[M + Na]^+$ in-source as shown in Figure 2B,D for azadirachtins A and B, respectively.

Both compounds, azadirachtins A and B, easily lost a molecule of H_2O (m/z 725.2 and 667.2, respectively), resulting in abundant fragment ions at high cone voltage in-source. In the case of azadirachtin A, at m/z 665 a neutral loss of acetic acid was considered following the loss of H_2O. However, the same loss was not observed for azadirachtin B, probably due to the lack of an acetoxyl group in the left-hand cyclohexane ring. The m/z at 625.2 and 567.2 for azadirachtins A (Figure 2B) and B (Figure 2D), respectively, came from a neutral loss of 2-methyl-2-butenoic acid from their respective dehydrated ions (725−100, 667−100). Additionally, both molecules showed the neutral loss of $C_{10}H_{10}O_4$ corresponding to the nucleus of heterocycloheptane and heterocyclopentene rings, with hydrogen migration to C-8 (m/z 531 and 473 for azadirachtins A and B, respectively). Finally, for azadirachtin A, a 565.2 fragment ion was obtained in-source after the loss of H_2O and the two carboxylic acids (acetic acid and 2-methyl-2-butenoic acid). These main fragment ions were studied

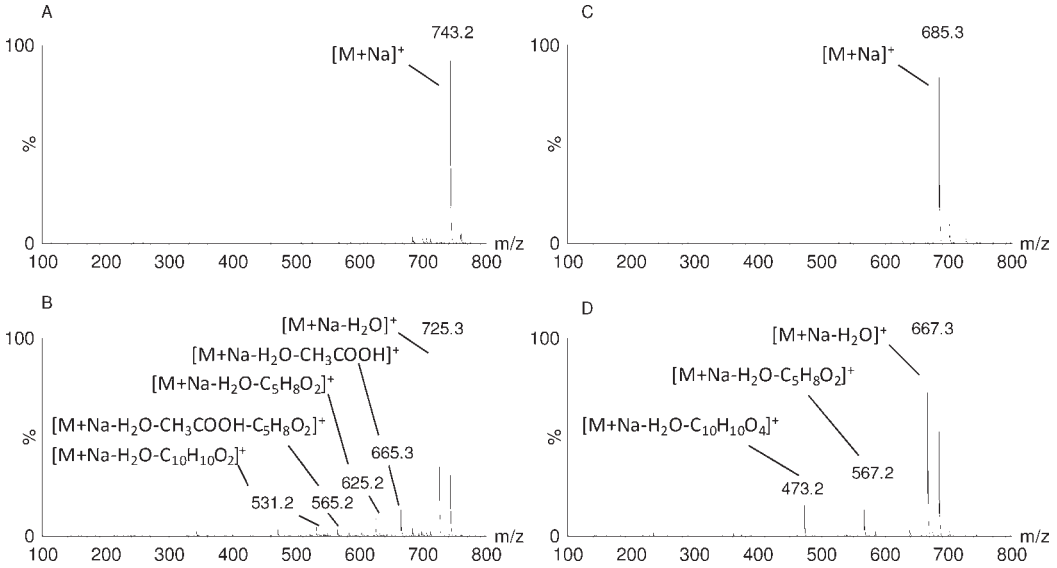

Figure 2. Full scan mass spectra acquired in positive ion electrospray obtained from combined spectra from the chromatographic peak of a 5 μg/mL standard solution of azadirachtin at (A) 30 V and (B) 70 V and of azadirachtin B at (C) 30 V and (D) 70 V. The chemical formulas of the main ion and its fragment ions are indicated.

to get optimum cone voltage by scan acquisition mode, ranging between 10 and 120 V with 10 V step increments. On the basis of this information, the most sensitive ions for quantitative purposes, as well as two additional ions for confirmative purposes, were selected. For azadirachtin A, 743.2 (Q), 725.2 (q_1), and 665.2 (q_2) were selected with optimized cone voltages of 30, 70, and 80 V, respectively. Similarly, 685.2 (Q), 667.2 (q_1), and 567.2 (q_2) were selected for azadirachtin B, with optimized cone voltages of 40, 70, and 80 V, respectively.

After optimization of the MS, liquid chromatographic parameters were set up. This is particularly important in single MS, as compared to tandem MS, due to the relatively lower specificity of the single MS analyzer. With single MS, good chromatographic resolution must be obtained to avoid coelution of potential interferences having the same m/z as the analytes of interest.

Sodium adducts are commonly observed for some analytes in ESI,[25] even without the addition of additives. In general, to improve the reproducibility of sodium adduct formation, addition of a sodium salt into the mobile phase is advisible. However, the presence of salts in the mobile phase can also result in ion suppression, drastically reducing the sensitivity of the method.[25] In this work, the influence of different sodium acetate (CH_3COONa) concentrations (from 1 to 50 μM) was tested. On the basis of comparative analysis of replicate (n = 3) matrix-matched standards (100 ng/mL) as compared to test samples with no additive, addition of CH_3COONa at low concentrations such as 5 or 10 μM resulted in an approximate doubling of the response, with improved RSD. Ultimately, 10 μM CH_3COONa was considered to be optimal in terms of sensitivity and robustness (RSD < 2%).

The influence of injection volume (ranging from 10 to 50 μL) and chromatographic column conditions were also examined, with 25 μL considered as the optimal volume. Retention times observed under the chromatographic conditions described above were 7.7 and 7.9 min for azadirachtins A and B, respectively.

Sample Treatment Optimization. Sample preparation choices may depend upon the amount of sample available, specificity of the analytical method, and ease and efficiency of sample throughput, as well as safety and cost of the reagents. In our case, experimental requirements resulted in only small amounts of sample, and only a single-quadrupole MS instrument was available. Under these constraints, optimal extraction and cleanup steps were required, and these were developed using the green ash foliage matrix as a test case, with subsequent extension to other matrices.

Given the advantages of automation and lower total solvent use, sample extraction was carried out by ASE. Due to the small amount of sample, extraction cells of 11 mL were selected, and diatomaceous earth was introduced with the sample to improve solvent−matrix interactions and to ensure consistent small extract volumes. The extraction procedure was performed at room temperature as a precaution given the thermal instability of azadirachtin. Finally, various combinations of static time (from 2 to 8 min) and number of cycles (from three to five cycles) were tested. Optimal extraction of the analytes was observed with a 2 min static time and a total of five cycles.

Preliminary experiments involving different types of SPE columns (C_{18}, PSA and Envi-carb) indicated that both C_{18} and PSA resulted in reduction of chromatographic interferences, as well as increased sensitivity for quantitative determination of both studied analytes. In contrast, Envi-Carb showed a potential loss of azadirachtin as a consequence of adsorption to the active carbon. Considering these results and given prior reports of simultaneous extraction and cleanup by MSPD using C_{18} with other pesticides in plant matrices,[19] we examined the potential to include C_{18} and PSA directly in the extraction cell. Thus, 1 g of macerated green ash foliage was mixed with different amounts of C_{18} (between 1.5 and 0.5 g). The mixture was gently blended using a mortar and pestle, and the homogenized mixture was introduced into the ASE cell and extracted under the optimized conditions noted above. The same process was also tested by mixing 1 g of sample with different proportions of PSA (1−0.2 g) and C_{18} (1−0.5 g). Ultimately, the optimal sample treatment was achieved using 1 g of C_{18} mixed with the sample and a 0.5 g of PSA inside the cell, with the different materials separated by a cellulose filter. This approach significantly reduced processing time and resulted in reproducible extraction and cleanup simultaneously in the ASE cell. When applied to the variety of other

matrices involved in this study, the extraction method was readjusted to utilize only 0.5 g of the sample, with the same proportions of C_{18} and PSA inside the cell.

An additional postextraction cleanup step was employed by shaking 5 mL of the extract with 0.1 g of PSA. This volume was evaporated under a stream of nitrogen to concentrate analytes and decrease the amount of organic solvent in the final sample. Extracts were taken to dryness and then reconstituted with different mixtures of the solvents water/methanol or acetonitrile. The temperature of the water bath was adjusted to 45 °C to avoid potential thermal degradation of azadirachtin. We observed that the most suitable solvent mixture for the final samples was 50:50 CH_3CN/H_2O, and a final volume of 1 mL was used as a standard protocol.

Matrix Effect Evaluation. Widely varying matrix effects were observed among the matrices examined in this study, as shown in Figure 3. For azadirachtin A, responses ranged from 19% for red maple foliage to 247% for phloem of green ash. Similarly, results for azadirachtin B ranged from 38% for red maple foliage to 106% for green ash foliage. These results indicated the unequivocal need for matrix-matched calibration standards to achieve accurate quantitation.

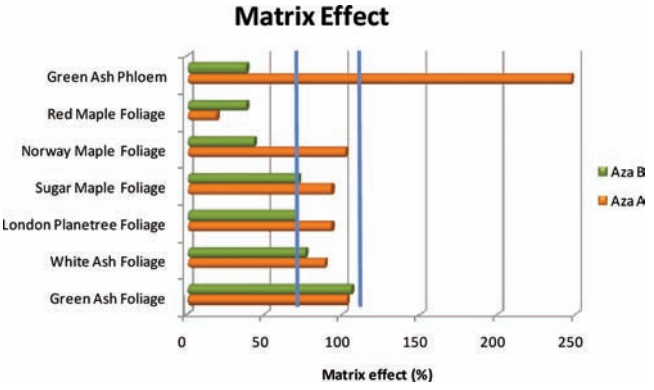

Figure 3. Graphic representation of matrix effects observed for seven different tree matrices. Matrix suppression and matrix enhancement were considered for values lower and higher than 100%, respectively.

Most matrices presented a suppression of expected response (around 70%), with greater suppression observed for red maple and Norway maple, resulting in reduced sensitivity, particularly for the Norway maple foliage (Figure 3). In general, azadirachtin A suffered less suppression than azadirachtin B for all types of matrices, with the exception being red maple foliage.

Validation Results. Calibration curves showed good fit to a second-order equations between 1 and 1000 ng/mL, with correlation coefficients higher than 0.995 in all cases and residuals lower than 30%. The specificity of the procedure was particularly good for azadirachtin B, for which no peaks were observed in procedural or sample blanks at the retention time for this compound. In the case of azadirachtin A, whereas no peaks were detected for the blank sample of green ash foliage and phloem matrices, other matrices showed a very small peak (<27% of LOQ response). Figure 4 provides examples of LC-MS chromatograms for azadirachtins A and B, respectively, as derived from matrix-matched standard solutions and blank sample extracts for the case of sugar maple foliage.

As detailed in Table 1, the final analytical method was characterized by satisfactory recoveries (71−103%) and good precision (RSD < 20%) at all three fortification levels. A general LOQ of 0.02 mg/kg was established. In the exceptional case of Norway maple foliage, a slightly higher variation of response was obtained at the LOQ level (RSD of 24%), and an LOQ of 0.05 mg/kg (recovery of 71% and RSD of 11%) was therefore established for this particular matrix to meet the European SANCO guidelines.[23] LODs ranged from 0.0003 mg/kg in green ash and sugar maple foliage to 0.0012 mg/kg in red maple foliage for azadirachtin A and from over 0.0001 mg/kg in green ash foliage to 0.0031 mg/kg in Norway maple foliage and green ash phloem for azadirachtin B.

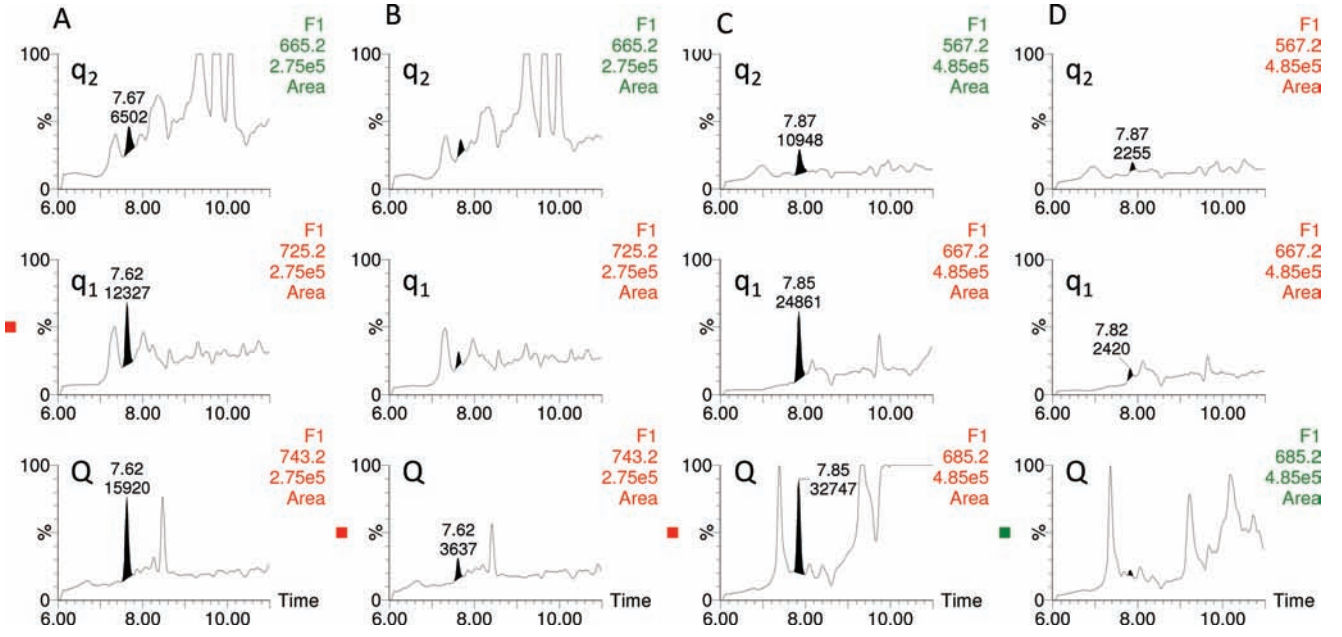

Figure 4. LC-ESI-MS chromatogram for sugar maple foliage (A) matrix-matched standard at 2.5 ng/mL of azadirachtin A, (B) blank foliage sample (bottom, quantitation ion (Q, 743.7); top, confirmation ions (q_1 725.7, q_2 665.7)); (C) matrix-matched standard at 2.5 ng/mL of azadirachtin B, (D) blank foliage sample (bottom, quantitation ion (Q, 685.7); top, confirmation ions (q_1 667.7, q_2 567.7)).

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Table 1. Mean Recoveries and Relative Standard Deviations (RSD) for Validation Test Samples Derived from Various Matrices Fortified with Azadirachtins A and B

	azadirachtin A recovery (%) (RSD (%))				azadirachtin B recovery (%) (RSD (%))			
matrix type (n = 5)	LOD (μg/kg)	0.02 mg/kg	0.1 mg/kg	1 mg/kg	LOD (μg/kg)	0.02 mg/kg	0.1 mg/kg	1 mg/kg
green ash foliage	0.3	81 (10)	101 (3)	91 (9)	0.1	71 (5)	91 (2)	90 (8)
London planetree foliage	0.6	99 (6)	79 (5)	71 (14)	1.5	92 (4)	87 (5)	73 (9)
sugar maple foliage	0.3	79 (20)	78 (6)	79 (4)	0.2	81 (6)	87 (6)	86 (2)
red maple foliage	1.2	82 (18)	80 (12)	94 (7)	1.9	82 (7)	85 (9)	89 (6)
Norway maple foliage	0.7	84 (24)	86 (19)	83 (5)	3.1	78 (10)	89 (6)	94 (4)
green ash phloem	0.4	79 (12)	74 (12)	73 (8)	3.1	103 (5)	86 (12)	86 (3)

Table 2. Confirmation Parameters for Azadirachtins A and B

matrix type (n = 5)	LOC_1 (μg/kg)	theor[a] IR_1	IR_1 at 0.02 mg/kg	IR_1 at 0.1 mg/kg	IR_1 at 1 mg/kg	LOC_2 (μg/kg)	theor[a] IR_2	IR_2 at 0.02 mg/kg	IR_2 at 0.1 mg/kg	IR_2 at 1 mg/kg
					Azadirachtin A					
green ash foliage	2.1	1.3	1.5 (15)[b]	1.2 (8)	1.2 (8)	2.9	3.6	3.2 (12)[b]	3.7 (1)	4.2 (16)
London planetree foliage	2.0	1.1	1.1 (1)	1.2 (9)	1.2 (9)	5.0	3.0	2.0 (33)	2.8 (7)	3.8 (27)
sugar maple foliage	0.9	1.3	1.3 (1)	1.1 (15)	1.2 (8)	7.5	3.7	1.8 (51)	3.6 (3)	4.2 (14)
red maple foliage	2.7	1.3	1.2 (8)	1.2 (8)	1.4 (8)	5.0	4.2	1.1 (74)	3.8 (10)	4.0 (5)
Norway maple foliage	1.0	1.4	1.2 (6)	1.2 (14)	1.4 (1)	2.4	4.3	1.4 (67)	4.0 (7)	4.9 (14)
green ash phloem	0.8	1.4	1.4 (3)	1.3 (7)	1.3 (4)	1.7	3.9	2.0 (49)	3.2 (17)	4.1 (6)
					Azadirachtin B					
green ash foliage	0.2	1.3	1.1 (18)	1.1 (18)	1.5 (15)	0.4	4.7	2.9 (38)	4.8 (2)	5.4 (15)
London planetree foliage	1.5	1.8	1.9 (6)	1.7 (6)	1.6 (11)	7.5	5.3	5.6 (6)	5.2 (2)	4.8 (9)
sugar maple foliage	0.7	1.5	1.3 (13)	1.5 (1)	1.6 (7)	1.0	4.4	2.3 (48)	3.7 (16)	4.6 (4)
red maple foliage	1.4	1.6	1.4 (12)	1.6 (1)	1.8 (12)	2.9	4.4	3.4 (23)	4.2 (4)	4.7 (7)
Norway maple foliage	6.3	1.5	1.6 (2)	1.5 (1)	1.6 (7)	10.1	4.0	2.9 (28)	3.1 (22)	4.2 (5)
green ash phloem	2.1	1.6	1.6 (1)	1.6 (4)	1.6 (2)	28.5	3.1	4.1 (10)	4.4 (4)	4.5 (3)

[a] Theoretical ion ratio (IR) average from matrix-matched calibration standard, IR for three concentration levels validated (0.02, 0.1, and 1 mg/kg (n = 5)), and its limit of confirmation (LOC). [b] Deviation values in percentage for IR_1 and IR_2 compared with respective theoretical IR. Maximum deviation suggested by guidelines[24] for IR_1 and IR_2 are 20 and 25%, respectively.

Table 3. Robustness and Reproducibility of the Method Based on QC Analyses for Two Matrices over a Period of 8 Months

	azadirachtin A % recovery (% RSD)			azadirachtin B % recovery (% RSD)		
matrix	0.02 mg/kg	0.1 mg/kg	1 mg/kg	0.02 mg/kg	0.1 mg/kg	1 mg/kg
green ash foliage (n = 52)	84 (10)	84 (8)	87 (4)	94 (17)	77 (12)	86 (7)
London planetree foliage (n = 6)	91 (46)	79 (15)	77 (15)	98 (29)	88 (16)	81 (8)

Confirmation Study Results. For confirmation purposes, ion ratio (IR) values were calculated based on the relative abundance of quantitation and confirmatory ions ($IR_1 = Q/q_1$ and $IR_2 = Q/q_2$) for both analytes in all matrices at 0.02, 0.1, and 1 mg/kg and compared with their theoretical values (Table 2). IR_1 for both analytes showed a high level of consistency among all three concentration levels assayed (deviation always lower than 18%). Higher deviations, but still <25%, were observed for IR_2 in most of matrices at the LOQ level. The result for IR_2 at the lowest concentration level assayed is a consequence of the low sensitivity of this ion by single ion monitoring (SIM) with a single-quadrupole MS. Therefore, confirmation with three IPs for these matrices only should be considered reliable at 0.1 mg/kg.

When LOCs were calculated as levels with signal-to-noise ratio of 3, their concentrations estimated for LOC_2 were lower than the LOQ level assayed. This fact was in discordance with the high deviation of IR_2 at LOQ for both analytes. In this sense, LOC values were estimated again on the basis of the lowest level of concentration assayed that presented an acceptable (<25%) IR deviation.[24] Considering the IR deviation average values in matrix-matched calibration standards from three replicate injections, LOC_1 was estimated as 0.01 mg/kg, whereas LOC_2 values were between 0.01 mg/kg for azadirachtin B in London planetree foliage and 0.5 mg/kg for azadirachtin A in red maple foliage (Supporting Information, Table 1).

Analyte Stability in Matrix. The average initial concentrations of azadirachtins A and B in green ash foliage fortified by systemic uptake of a dilute aqueous solution overnight were 0.335 and 0.110 mg/kg, respectively. Average concentrations of replicate subsamples stored frozen at −18 °C for up to 3 months

prior to analysis presented a relative deviation lower than 20% compared to this initial concentration (range from 2 to 19% for azadirachtin A and from 4 to 20% for azadirachtin B). This experiment demonstrated a satisfactory stability of these analytes within green ash foliage for at least 3 months of storage at −18 °C.

Application of the Method to Field Samples. To date, the validated analytical method has been applied to several hundred green ash (total 560) and London planetree (total 48) foliar samples derived from various field experiments over an 8 month period. For each set of samples (8−30 samples per set) at least two QC samples prepared by fortifying blank matrices with known amounts of azadirachtins A and B were used. As shown in Table 3, the average recoveries for QCs was from 77 to 98% for these two matrices with RSD < 17% in all cases except for London planetree at the lowest (0.02 mg/kg) level. These results demonstrate a suitable reproducibility and robustness at levels of 0.1 mg/kg of the analytical method as applied to two different tree matrices over long periods of operational use.

In summary, the validated LC-MS method allows for accurate and precise quantitation of azadirachtins A and B in foliage and phloem matrices from six tree species with an LOQ of 0.02 mg/kg. In-source fragmentation provides confirmation with a minimum of three IPs for the two analytes at levels between 0.02 and 0.5 mg/kg depending upon the analyte and matrix. Average recoveries from the validation study involving several different tree species were 83 and 86% for azadirachtins A and B, respectively, with excellent precision ranging from 9 to 10% RSD. Satisfactory stability of residues in foliar matrices for a period of up to 3 months under frozen storage was demonstrated. Thus, the method has direct utility in relation to field studies examining the uptake and translocation of these analytes in trees susceptible to infestation by alien wood-boring insect species and may be used as a basis for further research involving advanced MS techniques, including the use of a triple-quadrupole analyzer.

■ ASSOCIATED CONTENT

Supporting Information. LOC_2 corrections. This material is available free of charge via the Internet at http://pubs.acs.org.

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