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A Simple and Selective Method for the Measurement of Azadirachtin and Related Azadirachtoid Levels in Fruits and Vegetables Using Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry

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Neem-based insecticides containing azadirachtin and related azadirachtoids are widely used in agriculture. Here, we report an analytical method for the rapid and accurate quantification of the insecticide azadirachtin A and B and other azadirachtoids such as salannin, nimbin, and their deacetylated analogues on tomatoes and peaches. Azadirachtoids were extracted from fruits and vegetables with acetonitrile. Using high-performance liquid chromatography/electrospray ionization tandem mass spectrometer, azadirachtoids were selectively detected monitoring the multiple reaction transitions of sodium adduct precursor ions. For azadirachtin A, calibration was linear over a working range of 1−1000 μg/L with $r > 0.996$. The limit of detection and limit of quantification for azadirachtin A were 0.4 and 0.8 μg/kg, respectively. The presence of interfering compounds in the peach and tomato extracts was evaluated and found to be minimal. Because of the linear behavior, it was concluded that the multiple reaction transitions of sodium adduct ions can be used for analytical purposes, that is, for the identification and quantification of azadirachtin A and B and related azadirachtoids in fruit and vegetable extracts at trace levels.

KEYWORDS: HPLC/ESI-MS/MS; azadirachtin A; azadirachtin B; deacetylnimbin; deacetylsalannin; nimbin; salannin; tomato; peach

INTRODUCTION

The methanolic extract of the seeds of the neem tree (*Azadirachta indica*) contains the biologically active tetranortriterpenoid azadirachtin A (AZA) as well as other insecticidally active ingredients, namely, azadirachtin B (AZB), deacetylnimbin (DNI), deacetylsalannin (DSA), nimbin (NI), and salannin (SA) (*1, 2*). This extract is biologically active, to varying extents, against different target organisms under both laboratory as well as field conditions (*3, 4*).

Commercial formulations of neem are labeled against aphids, thrips, fungus gnats, caterpillars, beetles, mushroom flies, mealybugs, leafminers, and gypsy moths, acting both by contact and ingestion with an insect growth regulator and antifeedant and oviposition (egg-laying) deterrent properties. AZA is used throughout the European community, and up to now, it is not yet inserted in the Annex I of 91/414/EEC with a decision pending, while in Italy it is registered on different cultivations with a maximum residue level (MRL) of 0.5 mg/kg and a preharvest interval (PHI) of 3 days (*5*).

Few chromatographic methods are reported for the determination of AZA in fruits and vegetables. Caboni et al. reported the analysis of AZA in olives while Thompson et al. described the analysis in water sediments with a high-performance liquid chromatography/diode array detection (HPLC/DAD) technique (*6, 7*). Furthermore, an HPLC/DAD-MS method in the single ion mode was reported for the simultaneous determination of azadirachtoids levels in fruits and vegetables with a limit of quantitation (LOQ) of 10 μg/kg (*8*). HPLC/tandem mass spectrometry (MS-MS) is the standard technique for the determination of polar and low volatile pesticides at trace levels in fruit and vegetable samples (*9, 10*). It has been used on the analysis of insecticides because its high sensitivity enables quantification of pesticides at μg/kg levels in food extracts and because it can provide unambiguous compound identification. Recently, Sannino reports the determination of AZA using a LC/MS-MS method in apple and tomato purées as well as in lemon juices and canned peas with a LOQ of 5 μg/kg (*11*), while Pozo et al. report the analysis of abamectin and azadirachtin residues in orange samples by electrospray tandem mass spectrometry with a detection limit of 7 μg/kg (*12*).

These findings suggested the need to develop a specific, unequivocal, rapid, and repeatable chromatographic method to

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Table 1. HPLC/ESI-MS/MS Characteristics of Azadirachtoids

compound	t_R (min)	transition (*m*/*z*) MW	parent	product	CE (V)
AZA	8.46	720.7	743.5 $[M + Na]^+$	725.0 $[M + Na - H_2O]^+$	−26
AZB	8.89	662.7	685.6 $[M + Na]^+$	667.0 $[M + Na - H_2O]^+$	−34
DNI[a]	11.22	498.6	521.4 $[M + Na]^+$		
DSA	11.29	554.7	577.5 $[M + Na]^+$	477.0 unknown	−30
NI	12.05	540.6	563.7 $[M + Na]^+$	504.0 $[M - Na - 2H_2O]^+$	−26
SA	12.54	596.7	619.4 $[M + Na]^+$	519.0 $[M + H - 2H_2O - COCH_3]^+$	−30

[a] Levels of DNI were monitored in the SIM mode.

Table 2. LC/MS (ESI+) Characteristics of Azadirachtin A and Their Related Limonoids

compound	formula	MW	LC/MS (ESI) *m*/*z* (% relative abundance)
AZA	$C_{35}H_{44}O_{16}$	720	743.7 $[M + Na]^+$ 100; 759.6 $[M + K]^+$ 73; 703 $[M + H - H_2O]^+$ 96; 685.8 $[M + H - 2H_2O]^+$ 22; 585.5 $[M - 2H_2O - TFA]^+$ 15; 567.9 $[M - 3H_2O - TFA]^+$ 15; 722.2 $[M + H]^+$ 10
AZB	$C_{33}H_{42}O_{14}$	662	685.9 $[M + Na]^+$ 100; 627.5 $[M + H - 2H_2O]^+$ 20; 701.6 $[M + K]^+$ 15; 645.9 $[M + H - H_2O]^+$ 5
DNI	$C_{28}H_{34}O_8$	498	521.3 $[M + Na]^+$ 100; 413.4 [unknown] 70; 621.4 [unknown] 70; 467.4 [unknown]$^+$ 50; 537.4 $[M + K]^+$ 47; 499.4 $[M + H]^+$ 30
DSA	$C_{32}H_{42}O_8$	554	577.7 $[M + Na]^+$ 100; 491.6 [unknown] 43; 599.4 [unknown] 30; 555.8 $[M + H]^+$ 25
NI	$C_{30}H_{36}O_9$	540	563.5 $[M + Na]^+$ 100; 616.2 [unknown] 30; 516.9 [unknown] 30; 541.4 $[M + H]^+$ 20
SA	$C_{34}H_{44}O_9$	596	619.7 $[M + Na]^+$ 100; 413.8 [unknown] 60; 597.6 $[M + H]^+$ 50; 635.4 $[M + K]^+$ 25; 521.6 [unknown] 20

Table 3. LODs and LOQs (μg/kg) Values of Azadirachtoids

compound	μg/kg LOD	LOQ
AZA	0.4	0.8
AZB	0.4	0.8
DNI	8	24
DSA	0.8	4
NI	0.8	4
SA	0.8	4

examine levels of AZA and AZB as well as other potentially active tetranortriterpenoids in fruits and vegetables. In this paper, for the first time, we report the development of a method for the determination of AZA, AZB, DNI, DSA, NI, and SA levels in peaches and tomatoes with the use of HPLC/ESI-MS/MS.

MATERIAL AND METHODS

Chemicals. Acetonitrile and methanol were of HPLC grade (Baker, Milan, Italy); sodium acetate and 99% formic acid were from Sigma Aldrich (Steinheim, Germany). Water was distilled and filtered through a Milli-Q apparatus (Millipore, Milan, Italy) before use. Standards of azadirachtoids AZA, AZB, DNI, DSA, NI, and SA were previously isolated in our laboratory with a purity greater than 95% using a vacuum liquid chromatography method (*8*).

Apparatus and Chromatography. *HPLC/ESI-MS/MS Analysis.* A Varian tandem mass spectrometer (Palo Alto, CA) consisting of a ProStar 410 autosampler, two ProStar 210 pumps, and a 1200 L triple quadrupole mass spectrometer equipped with an electrospray ionization source was used. Varian MS workstation version 6.7 software was used for data acquisition and processing. The chromatographic separation was performed on a Waters XTerra RP-18 column (4.6 mm × 250 mm i.d., 5 μm). The mobile phase consisted of (A) acetonitrile and (B) bidistilled water containing 0.1% formic acid and 0.01% sodium acetate. The solvent gradient started at 65% A and 35% B, reached 90% A at 10 min, and was kept in this condition for up to 15 min. The mobile phase, previously degassed with high-purity helium, was pumped at a flow rate of 0.4 mL/min, and the injection volume was 10 μL. The electrospray ionization-mass spectrometer was operated in the positive ion mode. The electrospray capillary potential was set to 65 V, while the shield was at 725 V. Nitrogen at 49 mTorr was used as a drying gas for solvent evaporation. The atmospheric pressure ionization (API) housing and drying gas temperatures were kept at 54 and 375 °C, respectively. Sodium adducts of the parent compounds were subjected to collision-induced dissociation using argon at 3.80 mTorr in the multiple reaction monitoring (MRM) mode. **Table 1** reports the observed mass transitions and collision energy used for the quantitation of different azadirachtoids. The scan time was 1 s, and the detector multiplier voltage was set to 2000 V, with an isolation width of *m*/*z* 1.2 for the quadrupole 1 and *m*/*z* 2.0 for the quadrupole 3.

Standard and Working Solutions. Six stock standard solutions of AZA, AZB, DNI, DSA, NI, and SA (1000 mg/L) were prepared in methanol by weighing 0.01 g of the pure analyte into a 10 mL volumetric flask and diluting to volume. For the MS/MS analysis, an intermediary mixed standard solution was prepared daily by diluting the stock solutions with the mobile phase as listed above. Standard working solutions were prepared diluting the mixed standard solution with the extract obtained from the untreated matrix of peaches or tomatoes. Levels of calibration for all compounds, including a blank, were 1, 5, 10, 20, 50, and 100 μg/L, respectively. All standard solutions were stored in the dark at −20 °C until usage.

Standard Curves and Linearity. A five-point standard curve for each azadirachtoid was prepared. Standard solutions were prepared in triplicate containing all six azadirachtoids at 1, 5, 10, 20, 50, and 100 μg/L. Calibration curves were created by plotting the concentration of each compound against the standard peak area of the monitored transition. Simple linear regression analysis was performed to calculate the slope and intercept. The correlation coefficient (*r*) for each azadirachtoid was also determined.

Repeatability. To evaluate precision, the repeatability of the instrument used and the analytical method proposed were determined. Between-day repeatability was calculated performing six injections of the same standard at 10, 50, 100, 500, and 1000 μg/L for 6 consecutive days.

Extraction of Azadirachtoids from Peaches and Tomatoes. Fresh and uncontaminated fruits and vegetables were purchased at local markets in Cagliari, Italy. Samples were analyzed, unwashed, and in a

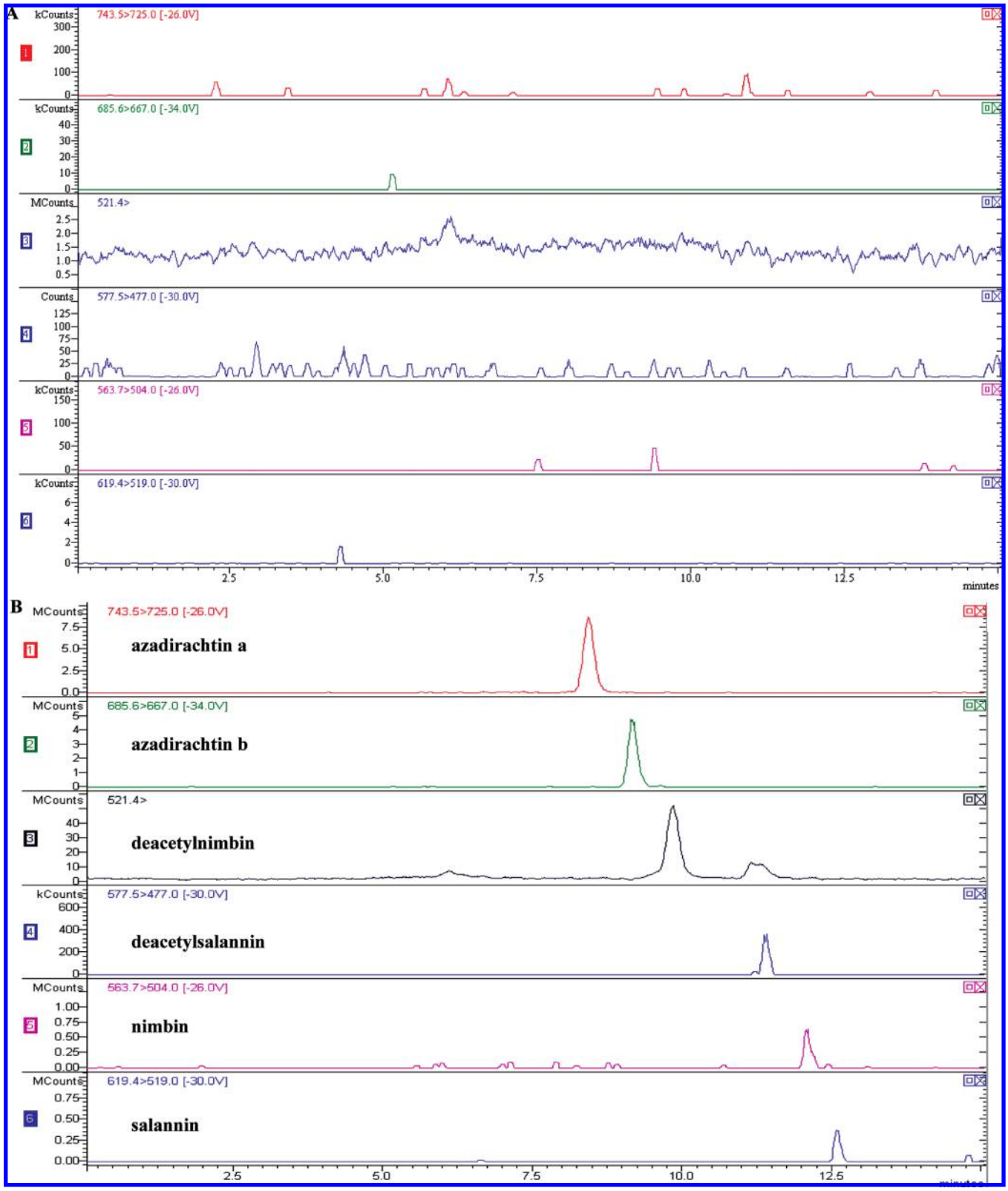

Figure 1. Chromatograms of (**A**) a blank of tomatoes and (**B**) a tomatoes extract spiked at 10 μg/L azadirachtoids.

raw state. Samples of fruits or vegetables were placed in a blender/cutter (Malavasi, Bologna, Italy) and chopped for 30 s.

A portion (5 g) of well-homogenized chopped tomatoes or peaches was weighed in a 40 mL screw-capped glass tube, and 2 g of sodium chloride and 20 mL of acetonitrile were added. The tube was agitated for 15 min in a rotary shaker at 9 rpm (FALC Instrumentals, Bergamo, Italy) at room temperature, and 1 mL of the mixture was evaporated to dryness under a gentle nitrogen stream. The residue was dissolved with 200 μL of the mobile phase previously reported and submitted to the chromatographic analysis in the MRM mode. The amount of sample in the final extract was 1.25 g/mL.

Recovery Assays. A 50 μL aliquot of pesticide solution at the desired standard concentration was added to each 5 g sample of untreated tomatoes and peaches. Taking into account azadirachtoids LOQs, reported in **Table 3**, fortification levels were 1, 5, 10, 50, and 100 μg/kg for AZA and AZB, while the levels were 5, 10, 50, and 100 μg/kg for DSA, NI, and SA and finally were 30, 60, 120, and 200 μg/kg for DNI. The samples

Table 4. Recoveries (% ± RSD) of Azadirachtoids on Peaches and Tomatoes ($n = 4$)

	fortification level (μg/kg)	peaches	tomatoes
AZA	1	96.0 ± 4.1	98.1 ± 5.3
	5	94.0 ± 8.5	96.2 ± 1.2
	10	92.7 ± 11.6	93.8 ± 4.8
	50	110.1 ± 10.8	110.5 ± 12.1
	100	99.0 ± 5.1	101.0 ± 3.0
AZB	1	91.5 ± 3.5	90.8 ± 4.2
	5	97.7 ± 5.0	101.9 ± 6.2
	10	93.8 ± 3.6	96.9 ± 10.2
	50	89.7 ± 7.2	93.2 ± 7.9
	100	98.3 ± 2.6	94.2 ± 3.9
DNI	30	88.1 ± 6.1	91.8 ± 6.6
	60	90.1 ± 5.2	93.7 ± 2.9
	120	86.0 ± 0.9	89.4 ± 2.7
	200	94.6 ± 2.4	99.6 ± 4.3
DSA	5	111.3 ± 1.5	101.8 ± 1.6
	10	108.8 ± 3.1	92.3 ± 2.6
	50	98.2 ± 5.3	97.6 ± 3.1
	100	93.4 ± 1.8	92.4 ± 0.7
NI	5	91.0 ± 8.7	91.9 ± 8.8
	10	89.5 ± 1.9	93.1 ± 3.7
	50	108.8 ± 9.8	89.3 ± 0.1
	100	96.3 ± 1.7	95.8 ± 1.7
SA	5	86.4 ± 3.9	89.0 ± 3.8
	10	88.5 ± 9.7	90.7 ± 4.1
	50	96.4 ± 6.2	88.6 ± 1.4
	100	94.3 ± 1.5	103.7 ± 4.3

Table 5. Matrix Effect Calculated as Slope Ratios of the Calibration Curves

compound	$slope_{solv}/slope_{tom}$	$slope_{solv}/slope_{pea}$
AZA	1.27	1.02
AZB	2.15	5.49
DNI	0.56	0.47
DSA	0.62	1.53
NI	4.80	5.24
SA	1.48	3.82

were allowed to settle for 30 min prior to used extraction. They were later processed according to the above extraction procedure. A six-point calibration curve previously reported was used for the quantification of azadirachtoids in the extract. Four replicates for each level were analyzed by HPLC/ESI-MS/MS analysis.

RESULTS AND DISCUSSION

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were tested for the determination of azadirachtoids levels both in positive and in negative mode, respectively. ESI in the negative mode and APCI in the negative and positive mode did not give any signal when infusion at the rate of 0.6 mL/h of standard solutions of azadirachtoids at 1000 μg/L were recorded. Thus, ESI in the positive mode was chosen for the identification, quantification, and confirmation of azadirachtoids in tomatoes and peaches extracts for its most intense response.

Full scan spectra of azadirachtoids were acquired with a scan range of 350–850 amu, a scan time of 0.75 amu, a scan width of 0.70 amu, and detector at 1200 V (**Table 2**). The electrospray capillary potential as well as shield and needle voltages were optimized for each compound.

The formation of an even electron sodium adduct in the source was utilized to provide increased selectivity for all azadirachtoids if compared with the formation of the protonated adduct suggesting that azadirachtoids with a basic function can pick up alkali cations. Scanning for the sodium adducts ion as the precursor ion, mass fragments were produced by collision-induced dissociation (CID) using argon at 3.80 mTorr. For the reason that sodium is a nonvolatile element that can precipitate in the sampling cone at the end of the day, the HPLC/ESI-MS/MS interface was thoroughly washed with bidistillated water.

The collision energy was optimized to achieve the highest sensitivity. The CID resulted in the formation of ions unique to each compound. As seen in **Table 1**, a product ion scan of the individual compounds produces unique mass fragments for each azadirachtoids showing a common feature losing one or two water molecules. The most intense transition was chosen to provide selective detection of azadirachtoids in the extracts of peaches and tomatoes without the need for a further purification step. Selected reaction monitoring of the precursor−product ion transitions were as follows: m/z 743.5 → 725.0 for AZA, m/z 685.6 → 667.0 for AZB, m/z 577.5 → 477.0 for DSA, m/z 563.7 → 504.0 for NI, and m/z 619.4 → 519.0 for SA. The sodium adduct of DNI, m/z 521.4, was not dissociated in the conditions reported above, and for this reason, DNI was acquired in the single ion monitoring mode.

For the HPLC/ESI-MS/MS quantitation of azadirachtoids, an external standard method was used. Peak areas obtained from the MRM of azadirachtoids standards were used for the quantitative determination. The chromatographic separation of azadirachtoids was achieved with a gradient elution in the reverse phase mode, and the retention times of azadirachtoids were 8.46, 8.89, 11.22, 11.29, 12.05, and 12.54 min, respectively. Using a fruit and vegetable extract, which is usually a complex mixture, the selectivity of the MRM transitions for all azadirachtoids was determined. The result of the chromatographic separation indicates the presence of interfering extract components; however, the relative abundance of these compounds is minimal by comparison, representing less than 5% of the total peak area except for DNI, which was quantified in the SIM mode (**Figure 1**).

Standard solutions of azadirachtoids at a concentration ranging from 1 to 1000 μg/L were injected for the analysis in HPLC-ESI/MS-MS in a MRM experiment. For calibration curves, the correlation coefficients were 0.996 for AZA and 0.999 for AZB. The highest and the lowest coefficients of variation for peaches were 10.20 and 2.15% for interday and 9.78 and 2.53% for intraday instrument validation, respectively. Similarly for tomatoes, the highest and the lowest coefficients of variation were 11.27 and 3.77% for interday and 9.50 and 0.43% for intraday instrument validation, respectively.

The limit of detection (LOD) was determined as the sample concentration that produces a peak with a height three times the level of the baseline noise, and the limit of quantification (LOQ) was calculated as the sample concentration that produces a peak with 10 times the ratio of signal-to-noise. The stock standard solutions were diluted to a series of appropriate concentrations with the mobile phase, and an aliquot of the diluted solutions was injected into LC/MS-MS for analysis. LODs and LOQs for AZA and AZB were 0.4 and 0.8 μg/kg, respectively. LODs and LOQs of the other azadirachtoids expressed as μg/kg are reported in **Table 3**. Recovery data are listed in **Table 4**. Recoveries ranged from 86.0 to 111.3%, with coefficients of variation between 0.9 and 11.6% for peaches, and from 88.6 to 110.5%, with coefficients of variation between 0.1 and 12.1% for tomatoes.

The matrix effects (**Table 5**) for the two extracts were calculated as slope ratios of the calibration curve prepared in solvent with the slope of calibration curve prepared with the

corresponding extract. We observed the highest matrix effect for AZB and NI.

We report for the first time a LC/MS-MS method in the ESI positive mode for the simultaneous identification and quantitation of residues of AZA and AZB as well as of the other main constituents of the neem-based insecticides in tomatoes and peaches. The possibility of using the protonated sodium adduct of azadirachtoids for the quantification at residue levels has been investigated. The adduct fragmentation was stable and intense. The proposed LC/MS-MS analytical method for the determination of azadirachtoids has been demonstrated to be adequate, fast, precise, accurate, and robust and can be used for the determination of azadirachtoids in tomatoes and peaches.

Supporting Information Available: Positive ion electrospray full scan mass spectra of various compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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