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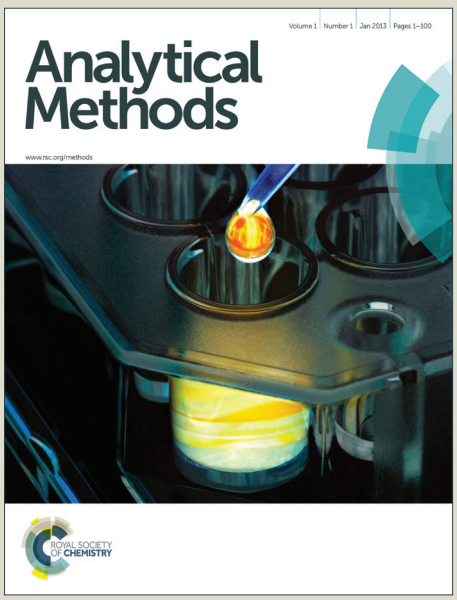

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Application of liquid chromatography/mass spectrometry in assessment of potential use of azadirachtins (Treeazin™) against Asian Longhorned beetle

E. Beltrán[a], M. Ibáñez[a], E. Gracia-Lor[a], , J. V. Sancho[a], F. Hernández*[a], D. G. Thompson[b]

Received (in XXX, XXX) XthXXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Azadirachtins are natural triterpenoid compounds derived from Neem tree extracts with potential for use as systemic insecticides against invasive wood-boring insect pests. In this work, a sensitive and selective analytical method has been developed for the simultaneous determination of azadirachtin A and azadirachtin B (3-tigloylazadirachtol) in foliage and twigs of various tree species. Samples were mixed with C18 and primary-secondary amine (PSA), and extracted with acetonitrile. Then, an aliquot of the raw extract was 10-fold diluted with water and directly analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The method was validated in foliage and twigs matrices of four different tree species (London Plane Tree, Red/Freemani maple, Norway maple and Sugar maple) that are known hosts of the exotic invasive insect pest – Asian Longhorn Beetle (ALB). Analytical results for replicate (N=5) samples, fortified at 0.01, 0.1 and 1 mg·kg^{-1}, showed good recoveries (86 - 119%) and precision (<20% RSD). The methodology was successfully applied to the analysis of 200 samples taken from a field experiment designed to investigate uptake, translocation and expression of azadirachtins in representative high-value urban trees following stem injection with TreeAzin™.

Introduction

Azadirachtins are a family of natural triterpenoid compounds derived from extracts of the Neem tree, *Azadiractha indica*[1]. A wide variety of extracts of the Neem tree have been developed and used both historically, and up until present day, for an equally wide variety of benefits including crop protection, veterinary and human health[2,3]. More recently, azadirachtins have been developed as natural, systemic insecticides for use in integrated pest management strategies against exotic invasive wood-boring insect pests such as Emerald Ash Borer[4-7].

Formulated products based on Neem seed extracts contain high levels of different azadirachtin analogues, being the major forms Azadirachtin A and B[8]. These compounds are considered the putative active ingredients for biological activity against insect pests including antifeedent, growth disruption, and reproductive effects as observed in various Lepidopteran and Dipteran pest species[1,9].

Relative to many conventional synthetic pesticides, azadirachtins have innately positive ecotoxicology and environmental fate profiles, exhibiting low toxicity to mammals[10], facile environmental degradation via photolysis, hydrolysis, and microbial degradation[7,11] and limited to no impacts on non-target organisms[12-14]. Under direct stem injection scenarios, as employed for control of wood boring insect pests, direct exposures are limited only to those organisms feeding within the treated trees further mitigating any potential risks to either humans or the environment.

In eastern North America, a number of invasive alien wood-boring insects including the emerald ash borer (*Agrilus planipennis*), Asian Long-horned Beetle (ALB) (*Anoplophora glabripennis*) and Brown Spruce Longhorn Beetle (*Tetropium fuscum*) have become major pest problems. Both individually and cumulatively, such pests represent a massive threat to forests in

[a]*Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat,s/n. E-12071 Castellón, Spain. Fax: 964 387368; Tel.: 964 387366;E-mail: Felix.hernandez@uji.es*

[b]*Natural Resources Canada ,Canadian Forest Service, Great Lakes Forestry Centre, 1219 Queen Street East, Sault Ste. Marie, Ontario, Canada P6A 2E5*

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the region, with significant potential for economic, ecological and aesthetic impacts.

To understand the uptake, translocation and expression of azadiracthins in twigs and foliage relative to potential magnitude and duration of specific insect pest exposures, sensitive and selective analytical methodologies are required[15]. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been shown as the most suitable technique for the wide majority of pesticides[16-19]. Usually, pesticides included in multiresidue methods are easily ionized in the atmospheric pressure sources commonly used in LC-MS/MS, and the protonated $[M+H]^+$ or deprotonated molecule $[M-H]^-$ use to be the most abundant ion, eventually selected as precursor ion in MS/MS methods. However, the absence of acidic or basic centers in some pesticides, as occurs for azadirachtin and related compounds, hampers their ionization, requiring the formation of appropriate adducts in order to be measured by LC-MS.

Most LC-MS methods for azadirachtin determination based on electrospray source monitor its sodium adduct[20-21], although some authors have selected the ammonium adduct[22-23], it seems that sodium adduct is the preferred option in terms of sensitivity. In fact, some authors after attempting to promote the ammonium adduct formation have finally chosen the sodium adduct for monitoring this compound[24-25].

In this paper, we have developed modern analytical methodology in order to be applied in quantifying the uptake, translocation and expression of azadirachtin residues in twigs and foliage of several tree species susceptible to attack by exotic invasive species – the Asian Longhorned Beetle (ALB)-following stem injection with the azadiracthin-based TreeAzin™ formulation specifically developed for use against wood boring insect pests. Foliage and twigs represent critical substrates required for maturation feeding and development of adult ALB. We report on the development and application of LC-MS/MS with triple quadrupole for quantification of azadirachtin A and B residues in samples taken from field experiments. LC-QTOF MS has also been used for confirmation of the identity of the compounds detected and to investigate the presence of other azadirachtin-related compounds in the samples.

Materials and Methods

Reagents and chemicals

Azadirachtin A (purity 96.1%) and azadirachtin B (98.5%) reference standards were purchased from EID Parry (Tamil Nadu, India). Individual stock solutions of azadirachtins A and B were prepared by dissolving 5 mg of the powdered material in 10 mL of acetone to yield a final concentration of 500 $\mu g \cdot mL^{-1}$. Stock solutions were stored at -20ºC. Standars were considered stable up to 3 months when storage at -18ºC according to the stability study of these analytes performed by Grimalt et al.[15].

A 5 $\mu g \cdot mL^{-1}$ mixed standard of azadirachtin A and azadirachtin B was prepared by mixing appropriate volumes of individual stock solutions and diluting with acetonitrile. Working solutions, prepared by diluting the reference standard mixture, were used for spiking samples in the validation study and also for calibration standards. Working solutions were stored in amber glass bottles under refrigerated conditions (5 ºC).

Sodium acetate (NaAc), ammonium acetate (NH_4Ac), spherical C18 bonded flash silica 40-60 µm, and primary-secondary amine (PSA) bonded silica were purchased from Scharlau (Barcelona, Spain). HPLC-grade methanol and HPLC-grade acetonitrile were also purchased from Scharlau. HPLC-grade water was obtained by purifying demineralised water in a Milli-Q Gradient A10 (Millipore, Molsheim, Germany).

Instrumentation

UHPLC-MS/MS

Determination was performed by means of an UPLC™ system (Acquity, Waters, Mildford, MA, USA) interfaced to a triple quadrupole mass spectrometer (TQS, Waters Micromass, Manchester, UK) equipped with an electrospray ionization source (ESI). Chromatographic separation was achieved with a Discovery C18 analytical column (50 × 2.1 mm, 5µm) (Supelco, Bellefonte, PA, USA) employing as mobile phase water (A) and methanol (B) both containing 10 µM NaAc, at a 0.2 mL min^{-1} flow rate. A linear gradient program was set up as follows: min 0, 30% B; min 4, 90% B and maintained during 1 min. Finally, the gradient was held to initial conditions (30% B) to re-equilibrate the column. The total run time was 7.5 min. Temperature of the column was set to 40ºC. The injection volume was 10 µL.

ESI experiments were performed in positive ionization mode. Cone as well as desolvation gas were nitrogen (Praxair, Valencia, Spain) set at 250 $L \cdot h^{-1}$ and 1200 $L \cdot h^{-1}$, respectively. Source temperature was set to 150 ºC. For operating in MS/MS mode, collision gas was argon (99.995%; Praxair) with a pressure of approximately $4 \cdot 10^{-3}$ mbar in the collision cell (0.15 $mL \cdot min^{-1}$). For electrospray ionization, desolvation gas temperature and capillary voltage were set at 650 ºC and 3.5 kV, respectively. Dwell time (0.038 s) was automatically selected by the software (MassLynx 4.1, Manchester, UK).

TargetLynx application manager (MassLynx v. 4.1, Waters, Manchester, UK) was used to process the quantitative data obtained from calibration standards and samples.

UHPLC-QTOF MS

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK) using an orthogonal Z-spray-ESI interface operating in positive ion mode.

The UHPLC separation was performed using an Acquity UPLC BEH C18 1.7 mm particle size analytical column 100 x 2.1 mm (Waters) at a flow rate of 0.3 mL min^{-1}. The mobile phases used were A = H_2O with 10 µM NaAc and B = MeOH with 10 µM NaAc. The initial percentage of B was 10%, which was linearly increased to 90% in 14 min, followed by a 2-min isocratic period and, then, returned to initial conditions during 2 min in total run duration of 18 min. Nitrogen was used as drying and nebulizing gas. The gas flow was set at 1200 L h^{-1}. TOF-MS resolution was approximately 18000 at full width half maximum at *m/z* 556. MS data were acquired over an *m/z* range of 50-1200. A capillary voltage of 0.7 kV and cone voltage of 20V were used. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The desolvation temperature was set to 600ºC, and the source temperature to 120ºC. The column temperature was set to 40ºC.

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Calibrations were automatically conducted from *m/z* 50 to 1200 with a 1:1 mixture of 0.05M NaOH:5% HCOOH, 25-fold diluted with ACN:H_2O (80:20). For automated accurate mass measurement, a solution of leucine enkephalin (2µg/ml) in ACN:H_2O (50:50) with 0.1% HCOOH was used as lock mass and pumped at a flow rate of 20 µl/min. The protonated molecule and a fragment ion of leucine enkephalin (*m/z* 556.2771 and *m/z* 278.1141, respectively) were used for recalibrating the mass axis and ensuring a robust accurate mass measurement at any time. For MSE experiments, two acquisition functions were created: the low energy function (LE), selecting a collision energy of 4 eV, and the high energy (HE) function, with a collision energy ramp ranging from 30 to 80 eV[26,27].

QTOF MS data were processed using MetaboLynx XS application manager (Micromass v 4.1).

Field Study-Experimental design

Semi-operational stem injections of TreeAzin™ (5% azadirachtins; ~76:24% ratio of AzaA:AzaB) were made to (N=5) individual trees of four different tree species (Norway Maple (*Acer platanoides*), Sugar Maple (*Acer saccharm*), Red/Freemani Maple (*Acer x freemanii*) and London Planetree (*Platanus x acerifolia*)) all of which are considered susceptible to attack by ALB. All experimental trees were growing in boulevards within the Town of Oakville, Ontario, Canada, and were uniquely identified based on the municipal tree inventory system. The experimental trees were considered typical of the type of high value individuals that might potentially receive systemic injections for protection from ALB attack under urban forest scenarios.

Analytical procedure

Sample treatment was based, with some modifications, on the previous work of *Grimalt et al.*[15], who used acetonitrile for sample extraction, a solvent also used for AZAs extraction from strawberries[28]. A representative macerated and homogenized sample (1g) was weighed directly in a 100 mL beaker. Then, 2 g of octadecylsilane (C18) and 1 g of primary-secondary amine (PSA) were added to the analytical subsample, and it was extracted with 40 mL of acetonitrile in the Ultraturrax® homogenizer-extractor at 8000 rpm for three minutes at room temperature. After cleaning the blender rod with acetonitrile, the entire extract was filtered through a filter paper and washed with acetonitrile (ca. 5 mL). The final volume was adjusted to 50 mL with acetonitrile in a volumetric flask. An aliquot of the raw extract was 10-fold diluted with water. Finally, 10 µL of the diluted extract was injected into the LC-MS/MS system. All results reported in this work are expressed as wet mass.

Validation study

The linearity of the method was studied at six concentrations ranging from 0.005 to 5 mg·kg^{-1} (equivalent to 0.01 to 10 µg·L^{-1} in the extract) injecting extracted matrix-matched standards in triplicate at each level. Satisfactory linearity was assumed when the correlation coefficient (r) was higher than 0.99 with residuals lower than 20%. Method accuracy (expressed as recovery percentage) and precision (expressed as repeatability in terms of relative standard deviation (RSD)) were evaluated by means of recovery experiments with blank samples fortified at three concentrations (0.01, 0.1 and 1 mg·kg^{-1}) for both azadirachtin A and B in the four different types of tree foliage and twigs. All experiments were performed in quintuplicate (N=5).

The limit of quantification (LOQ) objective was established as the lowest concentration level tested and validated, from spiked samples subjected to the overall analytical procedure, with satisfactory recovery (70-120%) and acceptable RSD (<20%). The limit of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was estimated for a signal-to-noise ratio of three (S/N = 3) from the chromatograms of samples spiked at the lowest analyte concentration tested, i.e. 0.01 mg·kg^{-1}.

Results and Discussion

MS and LC optimization

Full-scan MS and MS/MS spectra of azadirachtin A were obtained from infusion of 1 mg·L^{-1}azadirachtin A reference standard in acetonitrile/water (50:50, v/v), at a flow rate of 5 µL·min^{-1}. The full-scan spectrum showed an abundant peak at *m/z* 743.3 corresponding to the sodium adduct $[M+Na]^+$. Similarly, the full-scan spectrum of azadirachtin B was obtained, showing an abundant $[M+Na]^+$ peak at *m/z* 685.3.

The addition of ammonium acetate (1 mM) into the infusion vial was tested trying to minimize the $[M+Na]^+$ adduct formation and to favor the formation of the protonated molecule and/or the ammonium adduct. A peak corresponding to $[M+NH_4-NH_3-H_2O]^+$ was observed at *m/z* 703.1 and 645.2 for azadirachtins A and B, respectively. However, the sensitivity resulting for the product ions obtained under these conditions was lower than from the sodium adducts. These results are in agreement with other authors who have tried to promote the ammonium adducts by adding different concentrations of ammonium salts into the mobile phase. In general, the selection of the ammoniated molecules has been the preferred option in multiresidual methods[22,23]. However, in individual or compound-related methodologies, the sodium adduct has been chosen as the best option in terms of sensitivity[24,25]. Thus, sodium adducts were finally selected as precursor ions for subsequent experiments.

In relation to azadirachtin A, fragments at *m/z* 725.3 (corresponding to the loss of H_2O from $[M+Na]^+$), 665.2 (corresponding to the loss of H_2O and CH_3COOH) and 565.2 (due to the loss of $C_5H_8O_2$ from 665.2) were optimized at collision energies of 30, 35 and 40 eV, respectively. In this way, three transitions were finally selected for the MS/MS method for azadirachtin A: 743.3 > 725.3 used for quantification; 743.3 > 665.2 and 743.3 > 565.2 used for confirmation.

Several abundant product ions were obtained in the MS/MS spectra of azadirachtin B. The *m/z* 667.2 ion was optimized at a collision energy of 30 eV, and corresponded to the loss of H_2O from $[M+Na]^+$. Fragments at *m/z* 639.3 and *m/z* 567.2 (corresponding to $[M+Na-H_2O-C_5H_8O_2]^+$) were optimized at collision energies of 30 and 35 eV, respectively. Three transitions were selected for the LC-MS/MS method for azadirachtin B: 685.3 >667.2 used for quantification; 685.3 > 639.3 and 685.3 > 567.2 used for confirmation.

Regarding the chromatographic behaviour, different additives (ammonium acetate and sodium acetate, at different

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concentrations) were tested. The addition of ammonium acetate to the mobile phase led to the formation of $[M+NH_4-NH_3-H_2O]^+$, similarly to infusion experiments described above when ammonium acetate was added directly into the vial. Again, sensitivity was lower than that obtained with sodium adducts. Therefore, in order to promote the formation of the $[M+Na]^+$ adducts and improve the reproducibility, 10 µM sodium acetate was used in both water and methanol obtaining good sensitivity and peak shape for both analytes[20,21,29].

Sample treatment optimization

Initially, extraction was based on a previous work dealing with azadirachtin determination in foliage and phloem by using accelerated solvent extraction (ASE)[15]. However, as ASE system was not available at our laboratory, an alternative approach based on the use of a homogenizer Ultraturrax extractor was developed for the simultaneous extraction of both azadirachtins. We performed a preliminary study comparing ASE (kindly offered by the Laboratory of Public Health from Valencia) and Ultraturrax. No significant differences were found in recoveries between the two extraction systems, however, the results in terms of precision were significantly better when Ultraturrax extraction was employed, especially for AZA A (see Table 1). Furthermore, the use of the latter was also preferred based on direct availability, its simplicity and common use in many analytical laboratories.

Sample extraction efficiency was studied by comparing blank extracts spiked before and after the extraction procedure. Results showed that azadirachtins, especially azadirachtin A, were not completely extracted from the selected matrices (recoveries around 70%), even when the extraction time was increased or samples were re-extracted twice. Therefore, samples were quantified against matrix-matched standards that were subjected to the entire analytical procedure, including the extraction step. As control samples were available for every matrix from field experiments, these blank samples were used to prepare matrix-matched standards. Following this approach, extraction losses and/or possible matrix effects were fully compensated for.

Validation results

Extracted matrix-matched standards calibration showed excellent linearity in the studied range (0.005 to 5 mg·kg^{-1}) with correlation coefficients ≥ 0.995 in all studied matrices for both compounds. Linear calibration was applied in all cases.

The method was validated in four different foliage matrices (i.e. London Plane Tree, Red/Freemani Maple, Norway Maple and Sugar Maple) and in their corresponding twigs matrices, at three fortification levels (0.01, 0.1 and 1 mg·kg^{-1}). Table 2 shows the mean recoveries and RSDs for azadirachtin A and B in the matrices tested. As can be seen, the method showed satisfactory recoveries in all analyte/matrix combinations tested, within the range of 70-120%, and good precision, with RSD values below 20%. No significant differences in mean recovery or precision were observed either for azadirachtin A and B, or for foliage and twigs matrices.

Table 1. Recoveries (%) obtained for Azadirachtins A and B in *platanus* foliage using homogenizer extraction (UT) and Accelerated Solvent Extractor (ASE) for 3 replicates. Average and RSD (both in %)

	0.01 mg·kg^{-1}				0.1 mg·kg^{-1}			
	AZA A		AZA B		AZA A		AZA B	
	UTX	ASE	UTX	ASE	UTX	ASE	UTX	ASE
F1	121	60	117	120	107	81	96	117
F2	115	100	114	130	104	114	110	94
F3	109	100	105	130	95	91	92	103
Average (%)	112.0	86.7	112.0	126.7	102.0	95.3	99.3	104.7
RSD (%)	3.8%	26.6%	5.6%	4.6%	6.1%	17.7%	9.5%	11.1%

Table 2. Validation of the developed HPLC-MS/MS method for azadirachtin A and B in studied matrices. Mean recoveries (%) and relative standard deviation (%, in brackets) of the overall procedure (n=5). LOD: estimated limit of detection.

		Spiked level						LOD (mg·kg^{-1})	
		0.01 mg·kg^{-1}		0.1 mg·kg^{-1}		1 mg·kg^{-1}			
		AZA A	AZA B	AZA A	AZA B	AZA A	AZA B	AZA A	AZA B
London Planetree	**Foliage**	99 (20)	105 (12)	103 (17)	95 (14)	102 (2)	105 (1)	0.002	0.002
	Twigs	87 (3)	97 (5)	98 (5)	96 (5)	115 (2)	115 (2)	0.001	0.001
Red/Freemani Maple	**Foliage**	111 (18)	98 (16)	107 (5)	110 (3)	96 (1)	97(1)	0.001	0.001
	Twigs	107 (5)	106 (5)	101 (6)	106 (5)	100 (5)	102 (4)	0.002	0.001
Norway Maple	**Foliage**	113 (4)	117 (6)	100 (8)	98 (4)	95 (2)	96 (2)	0.003	0.002
	Twigs	101 (2)	103 (11)	104 (14)	101 (4)	103 (3)	101 (3)	0.003	0.003
Sugar Maple	**Foliage**	119 (9)	104 (15)	86 (9)	92 (5)	92 (6)	114 (4)	0.002	0.002
	Twigs	103 (5)	107 (4)	98 (3)	101 (4)	101 (4)	101 (3)	0.001	0.001

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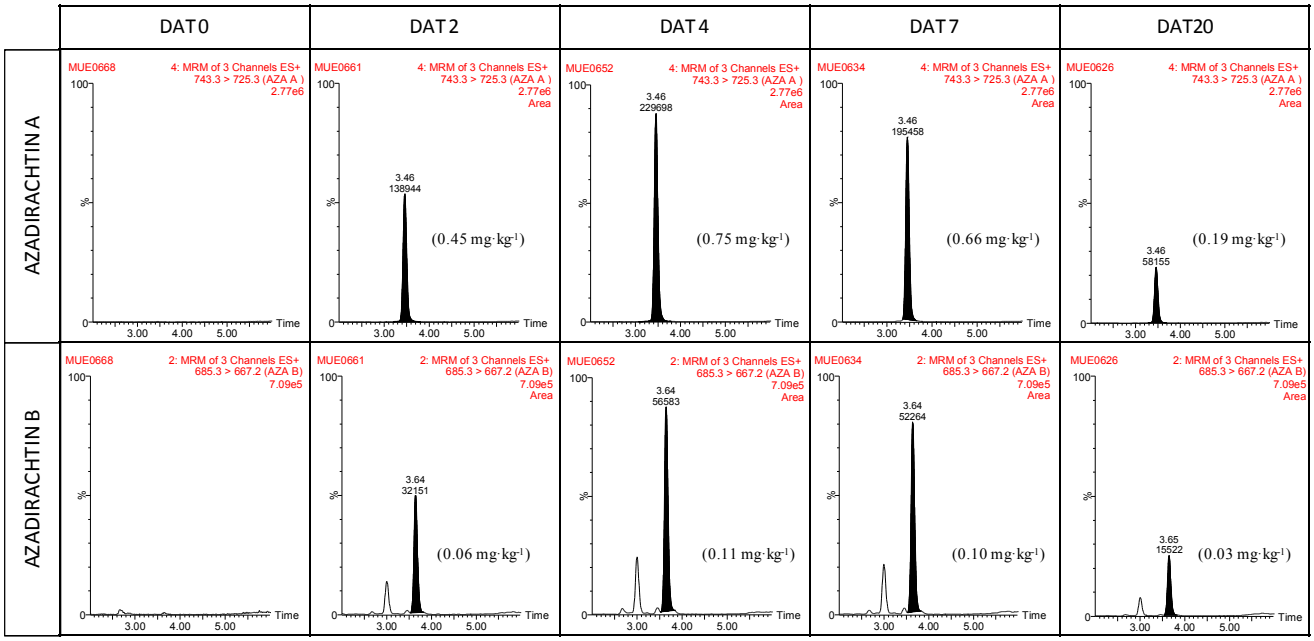

Fig.1. LC-MS/MS chromatograms (only Q transition is shown) for azadirachtin A and B in a Red/Freemani Maple Twigs sample collected at different days after application. Detected concentrations expressed in mg·kg^{-1} are shown in brackets.

The LOQ objective was set at 0.01 mg·kg^{-1}, as the method was satisfactorily validated for all analyte/matrix combinations at this level. LODs between 0.001-0.003 mg·kg^{-1} were estimated for the different foliage and twigs matrices. Similar detection limits were obtained in comparison with previous works dedicated to the study of azadirachtins[21,24,25]. However, the present method shows the advantage of minimizing the sample treatment compared with other methods reported where a pre-concentration step was necessary to achieve the desired levels. This is surely due to the higher sensitivity affordable with the last-generation triple quadrupole analyzer used in this work.

The specificity of the method was also tested. No peaks were observed in any of the matrices at the same retention time as target compounds. However, for azadirachtin B, which eluted at 3.66 minutes, most samples showed a peak at 3.02 minutes sharing the same SRM transitions. Subsequently, these samples were analyzed by QTOF in an attempt to identify this possible related-compound.

Application to field samples

The developed method was applied to more than 200 foliage and twig samples resulting from a field experiment conducted in Canada. The field study involved systemic injection of TreeAzin™ at a rate of 5 mL/cm of tree diameter at breast height (equivalent to approximately 250 mg of azadirachtins A+B per tree) to N=5 replicates of four different ALB-susceptible tree species (Norway Maple, Sugar Maple, Red/Freemani Maple, and London Plane Tree). Samples were collected at different days after treatment (DAT). In this paper, we report for illustration mean maximum residue concentrations observed in foliage and twigs. Details of residue dynamics will be the subject of a separate paper.

Quantitative analysis was performed against matrix-matched standards subjected to the entire analytical procedure. Moreover, quality control samples (QCs) were included in every sample sequence. QCs consisted on blank samples spiked at three different concentrations level (0.1, 1 and 5 mg·kg^{-1}) subjected to the overall analytical procedure and processed as the same time as the samples. At 5 mg·kg^{-1} the detector was near the saturation level; therefore, when samples show concentrations higher than this level, extracts were diluted.

The highest mean concentration of azadirachtin A (6.23 mg·kg^{-1}) in foliage was observed in Norway Maple two DATs (Table 3). This pattern of rapid and significant uptake parallels that documented following systemic injections into ash, as previously described[5]. Overall, mean maximal levels of azadirachtins in foliage exceeded 1 mg·kg^{-1} in 3 of 4 test species. As an exception, the maximum mean residue levels of azadirachtins in both foliage and twigs of Sugar Maple were approximately an order of magnitude lower than those observed in the other three test species. Maximal concentrations of total azadirachtins in twig samples were substantially lower than those in foliage in all cases. Fig. 1 shows, as illustrative example, the LC-MS/MS chromatograms for azadirachtin A and B in a Red/Freemani Maple twig samples at different DATs.

Table 3. Maximal mean residues found in foliage and twigs in the four tree species studied. Concentrations in mg·kg^{-1}.

	FOLIAGE		TWIGS	
	AZA A	AZA B	AZA A	AZA B
London Planetree	4.33	1.04	0.38	0.11
Red/Freemani Maple	1.89	0.32	0.56	0.09
Norway Maple	6.23	1.17	2.07	0.33
Sugar Maple	0.19	0.02	0.20	0.03

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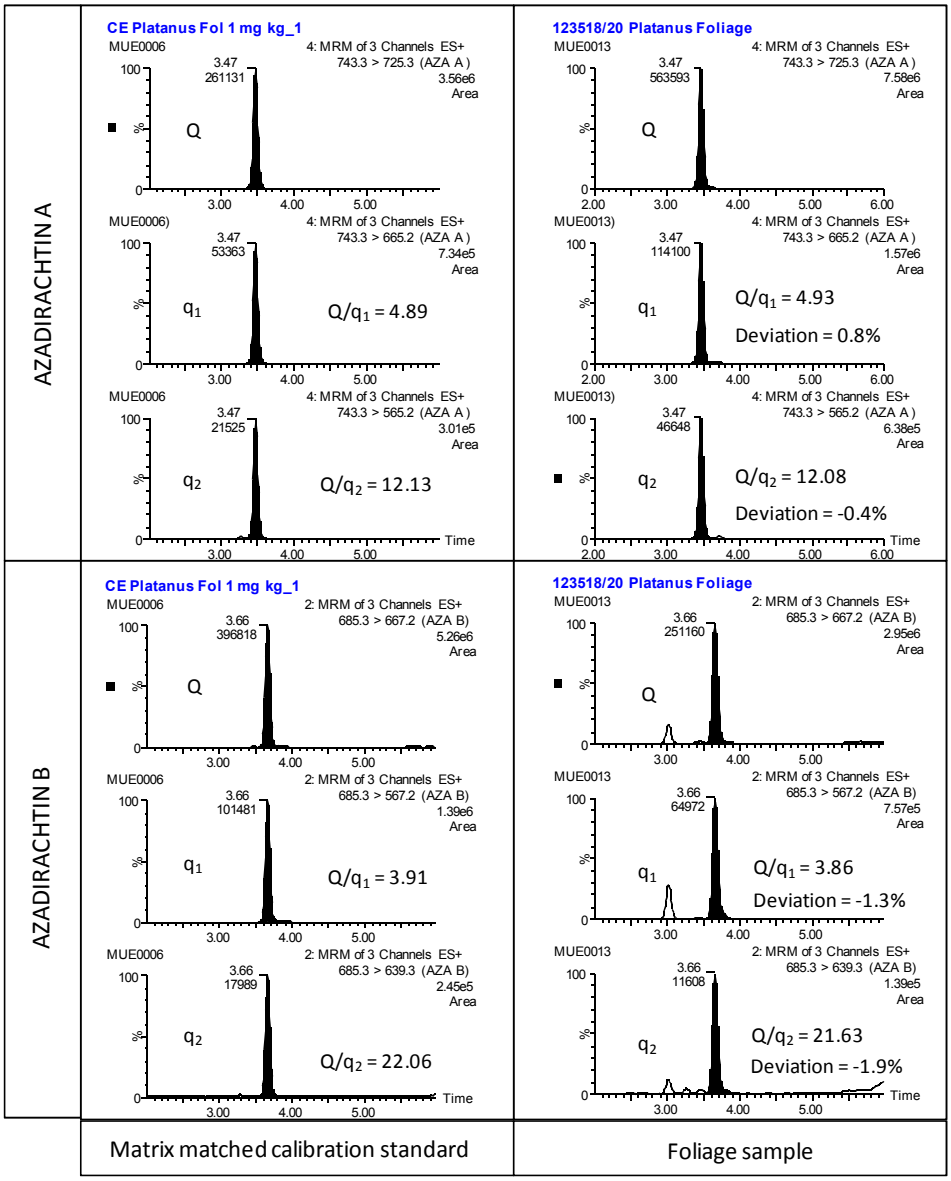

Fig.2. Confirmation of azadirachtin A and B detected in a selected London Planetree foliage sample by the accomplishment of Q/q ratios.

Confirmation of the analytes' identity

Three SRM transitions were selected for each compound to assure the reliable identification of the compounds detected. The most sensitive transition was used for quantification (Q) whereas the other two were used for confirmation (q1 and q2). Confirmation of positive findings was carried out calculating the peak area ratios between the quantification (Q) and confirmation (q1 and q2) transitions. The finding was considered as positive when the experimental ion-ratios and the retention time were in agreement with a reference standard, according to the European Guideline SANCO 12571/2013[30].

As an example, Fig. 2 shows the confirmation of positives in the samples in a London Planetree foliage sample. The deviation between the experimental and the theoretical ion ratios was below 2%, which allowed to confirm the identity of azadirachtin A and azadirachtin B in the sample.

QTOF MS experiments

In addition to quantitative analyses performed by UHPLC-MS/MS (QqQ), it was also investigated the potential presence of other azadirachtins or azadirachtin-related compounds in the samples. We observed the presence of a chromatographic peak sharing the three transitions of azadirachtin B, but eluting at a different retention time, in most foliage and twigs samples. This compound might be a related compound, *i.e*, another azadirachtin or a possible metabolite of azadirachtins A or B. Different foliage extracts were then injected in the UHPLC-QTOF MS system

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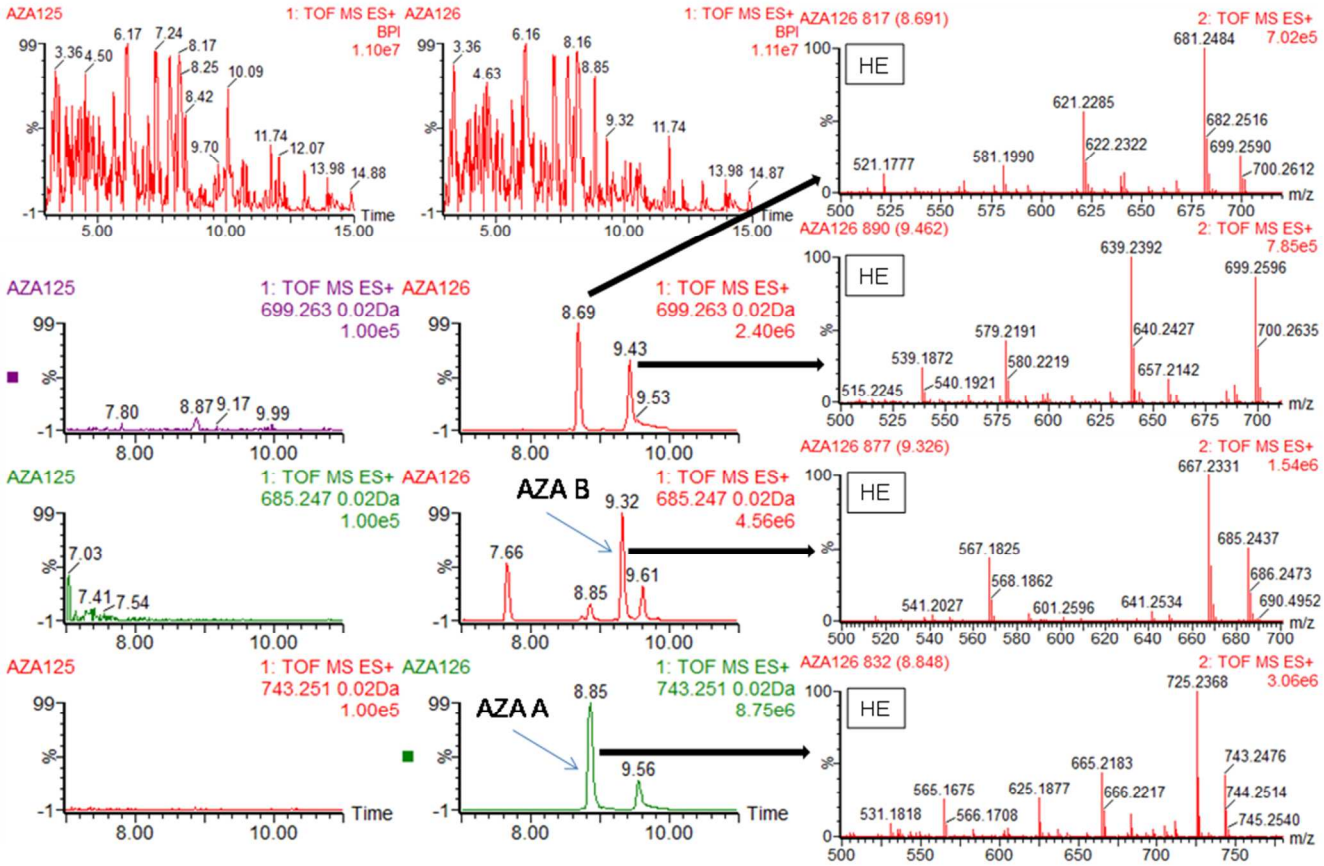

Fig.3. UHPLC-QTOF MS experiments. Base-peak ion chromatograms (BPI) and extracted ion chromatograms(XIC) at 20 mDa mass window for m/z 743.251, 685.247 and 699.263, for control (left) and analyte (middle) Norway Maple foliage samples. High energy (HE) spectra for selected analytes (right).

under MSE mode, which allowed to get information on the accurate-mass of the molecule and of potential fragment ions of this compound[27], as well as to investigate the presence of possible metabolites. For the last purpose, extracts of samples taken before azadirachtin treatment were also injected and used as control/blank samples.

Fig. 3 shows the BPI (Base-Peak Ion) chromatogram as well as the low energy (LE) extracted ion chromatograms (20 mDa mass window) for the azadirachtin A exact mass ($[M+Na]^+$ *m/z* 743.2449) and azadirachtin B ($[M+Na]^+$ *m/z* 685.2418) in a Norway Maple foliage sample. In addition to the analyte peaks (8.85 min, azadirachtin A; 9.32 min, azadirachtin B), additional peaks were observed in the chromatogram corresponding to azadirachtin A (unknown at 9.56 min) and azadirachtin B (unknown at 7.66 and 9.61 min). Note that the peak at 8.85 corresponds to an in-source fragment of the azadirachtin A, which was not present in the blank sample).

Regarding the high energy (HE) spectra, both azadiracthins presented fragment ions corresponding to losses of H_2O (18.0106 Da), CH_3COOH (60.0211 Da) and CH_3-CH=$C_2H_4O_2$ (100.0524 Da). The same characteristic losses were observed in the HE spectra of the unknown compounds, although with different intensities, supporting the possibility of being chemically related compounds/metabolites.

On the basis of the information reported for azadirachtin H on fragmentation (common to azadirachtin B) and retention time (azadiracthin H elutes earlier than azadirachtin B), we might assume that the peak at 7.66 min corresponded to azadirachtin H, an isomer closely related to azadirachtin B[31]. However, it would be necessary to inject a reference standard for an unequivocal confirmation. For the rest of compounds, more information would be required for a reliable identification.

Selected sample extracts were also processed using MetaboLynx XS. This software compares extracted ion chromatograms of a control sample (before treatment) versus a positive/degraded sample with the objective of detecting, identifying and reporting differential ions/chromatographic peaks which would correspond, in principle, to transformation products/metabolites[32,33]. In addition to the peaks observed at *m/z* 743 and 685, two chromatographic peaks at *m/z* 699.2629 (8.69 and 9.43 min) were also observed (Fig. 3). From their HE spectra, losses of 18, 60 and 100 mDa were also obtained. One of these compounds might be azadirachtin D ($[M+Na]^+$ $C_{34}H_{44}O_{14}Na$, *m/z* 699.2629), already reported in the literature[31]. However, the little information available in the literature on the fragmentation of azadirachtin D was not enough to support its tentative identification in the samples.

In addition to the analysis of foliage and twig samples, the formulated product applied in the field experiments was also analyzed by LC-QTOF MS. With these analyses, we confirmed

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that the five unknown peaks detected in samples (peaks at *m/z* 699 at 8.69 and 9.43; *m/z* 743 at 9.56 min; and *m/z* 685 at 7.66 and 9.61 min) were also present in the technical product. After comparing the relative intensities of these unknown compounds in the sample and in the formulated product, no significant differences were observed for unknowns at *m/z* 699 at 8.69 min and *m/z* 685 at 7.66 min. This indicates that these two compounds could be azadirachtin-related compounds co-occurring with azadirachtins A and B in the technical product. However, relative intensities for the three remaining unknowns (*m/z* 699 at 9.43 min, *m/z* 685 at 9.61 min and *m/z* 743 at 9.56 min) in the sample extracts were significantly higher than in the formulated, suggesting that they could correspond to degradates and/or metabolites formed after application of the product into the field.

Conclusions

A rapid and sensitive analytical methodology has been developed for the quantification and confirmation of azadirachtin A and B in foliage and twigs matrices from four tree species susceptible to the invasive wood-boring insect pest Asian Long Horned Beetle. The methodology, based on extraction with acetonitrile, quantitation by extracted matrix-matched calibration and measurement by LC-MS/MS with triple quadrupole, has allowed accurate quantitation of residues as low as 0.01 mg kg^{-1} in all matrices tested with limits of detection estimated from 0.001 to 0.003 mg kg^{-1}. Analytical results for replicate (N=5) samples fortified at both 0.01 and 0.1 mg·kg^{-1}, showed good recovery (86-119%) and precision (<20% RSD). Application to approximately 200 field samples, collected following systemic injection of the TreeAzin™ formulation, showed general applicability of the method for rapid and facile quantitation of azadirachtin A and B residues in foliage and twigs. Maximal concentrations in both sample matrices were well in excess of limits of quantitation, indicating the method may be reliably used to track residue dynamics through time.

Further research involving the use of LC-MS/MS and LC-QTOF MS is currently being planned to enhance our understanding of azadirachtin residue dynamics and potential biological effects on ALB and other wood boring insect species. Components of planned field research include efficacy trials in woodlot and urban trees under light to moderate ALB pressure and further examination of residue dynamics in sugar maple, particularly under commercial sugar bush scenarios.

Acknowlegments

The authors acknowledge the financial support from the Generalitat Valenciana, as research group of excellence PROMETEO/2009/054. The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of the TQS triple quadrupole mass spectrometer. Financial support to the field research aspects of this study were provided through a collaborative agreement between the Invasive Species Centre and Natural Resources Canada, Canadian Forest Service, Sault Ste. Marie, Ontario, Canada. We would like also to acknowledge the support of John McNeil (Town of Oakville, Manager of Forestry Services, Parks and Open Space), Joe and Steve Meating (BioForest Technologies Inc.) and staff of the Maple Hill Tree Services Company who assisted in semi-operational stem injection treatments.

References

1 R.J. Aerts, *J. Chem. Ecol*. 23, **1997**, 2117.
2 N.S. Randhawa Parmar, B.S., Neem: Research and Development, Soc. Pestic. Sci. New Delhi, **1993**, 225.
3 O. Koul, Neem: a global perspective. In Neem: today and in the new millennium., **2004**, 1.
4 B. V Helson, D.B. Lyons, K.W. Wanner, T.A. Scarr, *Can. Entomol.* 133, **2001**, 729.
5 N. McKenzie, B. Helson, D. Thompson, C. Otis, J. McFarlane, T. Buscarini, J. Meating, *J. Econ. Entomol*. 103, **2010**, 708.
6 S. Grimalt, D. Thompson, D. Chartrand, J. Mcfarlane, B. Helson, B. Lyons, J. Meating, T. Scarr, *Pest Manag. Sci*. 67, **2011**, 1277.
7 D.G. Thompson, D.P. Kreutrweiser, *ACS Symp. Ser.* 947, **2007**, 245.
8 M.B. Isman, *Annu. Rev. Entomol*. 51, **2006**, 45.
9 M. Kostic, Z. Popovic, D. Brkic, S. Milanovic, I. Sivcev, S. Stankovic, *Bioresour. Technol*. 99, **2008**, 7897.
10 R.B. Raizada, M.K. Srivastava, R.A. Kaushal, R.P. Singh, *Food Chem. Toxicol*. 39, **2001**, 477.
11 P. Caboni, G. Sarais, A. Angioni, F. Lai, F. Dedola, P. Cabras, *J. Environ. Sci. Heal. - Part B Pestic. Food Contam. Agric. Wastes*. 44, **2009**, 598.
12 J.D. Stark, *ACS Symp. Ser*. 947, **2007**, 275.
13 D. Kreutzweiser, D. Thompson, S. Grimalt, D. Chartrand, K. Good, T. Scarr, *Ecotoxicol. Environ. Saf.* 74, **2011**, 1734.
14 I. Goktepe, R. Portier, M. Ahmedna, *J. Environ. Sci. Heal. - Part B Pestic. Food Contam. Agric. Wastes*. 39, **2004**, 311.
15 S. Grimalt, D.G. Thompson, M. Coppens, D.T. Chartrand, T. Shorney, J. Meating, T. Scarr, *J. Agric. Food Chem*. 59, **2011**, 8070.
16 Y. Picó, C. Blasco, G. Font, *Mass Spectrom. Rev*. 23, **2004**, 45.
17 L. Alder, K. Greulich, G. Kempe, B. Vieth, *Mass Spectrom. Rev*. 25, **2006**, 838.
18 G. Martínez-Domínguez, P. Plaza-Bolaños, R. Romero-González, A. Garrido-Frenich, *Talanta*. 118, **2014**, 277.
19 F. Hernández, J. V Sancho, O.J. Pozo, *Anal. Bioanal. Chem*. 382, **2005**, 934.
20 O.J. Pozo, J.M. Marin, J. V Sancho, F. Hernández, *J. Chromatogr. A*. 992, **2003**, 133.
21 G. Sarais, P. Caboni, E. Sarritzu, M. Russo, P. Cabras, *J. Agric. Food Chem.* 56, **2008**, 2939.
22 R. Romero-González, A. Garrido Frenich, J.L. Martínez Vidal, O.D. Prestes, S.L. Grio, *J. Chromatogr. A*. 1218, **2011**, 1477.
23 A. Sannino, *Rapid Commun. Mass Spectrom*. 21, **2007**, 2079.
24 D. Drozdzynski, J. Kowalska, *Anal. Bioanal. Chem.* 394, **2009**, 2241.
25 O.D. Prestes, J.A. Padilla-Sánchez, R. Romero-González, S.L. Grio, A.G. Frenich, J.L. Martínez-Vidal, *J. Sep. Sci*. 35, **2012**, 861.
26 M. Ibáñez, L. Bijlsma, A.L.N. Van Nuijs, J. V Sancho, G. Haro, A. Covaci, F. Hernandez, *J. Mass Spectrom*. 48, **2013**, 685.
27 F. Hernández, L. Bijlsma, J. V Sancho, R. Díaz, M. Ibáñez, *Anal. Chim. Acta*. 684, **2011**, 96.
28 P. Caboni, G. Sarais, A. Angioni, A.J. García, F. Lai, F. Dedola, P. Cabras, *J. Agr. Food Chem* 54, **2006**, 10026
29 S. Grimalt, Ó.J. Pozo, J.M. Marín, J. V Sancho, F. Hernández, *J. Am. Soc. Mass Spectrom*. 16, **2005**, 1619.
30 European Commission, **2013**. Method validation and quality control procedures for pesticide residues analysis in food and feed. Document No. SANCO/12571/2013
31 S. Barrek, O. Paisse, M.-F. Grenier-Loustalot, *Anal. Bioanal. Chem*. 378, **2004**, 753.
32 M. Ibáñez, J. V Sancho, Ó.J. Pozo, F. Hernández, *Anal. Chem*. 76, **2004**, 1328.
33 J. V Sancho, Ó.J. Pozo, M. Ibáñez, F. Hernández, *Anal. Bioanal. Chem*. 386, **2006**, 987.2

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