SPECIAL GUEST EDITOR SECTION

Determination of Triazines and Triazoles in Grapes Using Atmospheric Pressure Matrix-Assisted Laser Desorption/ Ionization High-Resolution Mass Spectrometry

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A chromatography-free atmospheric pressure matrix-assisted laser desorption/ionization high-resolution mass spectrometry (AP-MALDI HRMS) method is described for the simultaneous and quantitative detection of triazines and triazoles in grapes. The analytes were detected reproducibly with high mass accuracy (mass error within 5 ppm) and further confirmed by collision-induced dissociation fragmentation in tandem MS. The LODs and LOQs for all the analytes were found to be in the nanogram per gram level (15–20 ng/g LOQ). Internal standard–normalized high-resolution accurate mass–extracted (HR-AM) peak intensities of the detected ions were used to generate the concentration response curves. Linearity (with R^2 values around 0.99) was obtained for these curves within a concentration range of 20–200 ng/g of the individual analytes. The accuracy and precision of the method were further established using QC samples. Validation and performance comparison of the AP-MALDI HRMS method with an existing standard method using LC with triple quadrupole MS was carried out (evaluating sensitivity, accuracy, precision, and analysis time) using 20 table-grape field samples after QuEChERS extraction.

Pesticides are a vital part of modern agricultural practices. Their excessive application in the field results in the presence of pesticide residues in food and produce, and prolonged exposures affect the ecosystem and human health (1–3). Organizations such as the U.S. Food and Drug Administration, the U.S. Environmental Protection Agency, the European Commission, and the Food Safety and Standards Authority of India have established maximum residual levels (MRLs) for each pesticide residue (4, 5).

Triazines and triazoles are important classes of pesticides with widespread use. They are commonly used during the production of a diverse variety of crops including grapes, rice, sugar cane, corn, pulses, and other fruits and vegetables (6). Triazine and triazole pesticides are routinely monitored and controlled, with strict limitations on permissible residue levels. Several analytical methods for the determination of triazine and triazole pesticides exist in literature. These include TLC (7, 8), GC (9, 10), electrokinetic capillary chromatography (11), HPLC with diode-array detection (11), and LC-MS (12–15). Various LC-MS methods have been published, with successful application of sample preparation techniques using on-line SPE (16, 17), nanotube-based extraction (18), and molecularly imprinted polymer bead–based extraction (19). Analytical methods for the detection of various classes of pesticides in a variety of matrixes using multiple platforms have been reviewed previously (9, 20, 21). Chromatography-free MS-based direct analysis methods have also been explored for quantitative analysis of various pesticides (22–24). However, most of these have not been implemented or practiced in a routine testing laboratory.

The high-throughput screening achievable with the use of matrix-assisted laser desorption/ionization (MALDI) MS, along with the minimal sample preparation procedures involved, make it attractive, especially in resource-limited settings. MALDI MS was reported previously for the determination of melamine (derivative of 1,3,5-triazine) contamination in milk (24). Qualitative MALDI MS–based detection and characterization of triazine pesticides has been previously explored with different MALDI matrixes. Furthermore, a study illustrating

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the selection of appropriate matrixes for the detection of several classes of pesticides by MALDI MS is available (22). However, a comprehensive and quantitative MALDI MS method for the analysis of triazines and related pesticides, supplemented with field study, has not yet been described.

In this work, we report the detection and quantitation of triazines and triazoles in grape samples using AP-MALDI HRMS. Confirmation of analytes was based both on high-resolution data (high mass accuracy) and tandem MS (MS/MS) fragmentation. To assess the applicability of the high-throughput AP-MALDI HRMS method, 20 grape samples were used for cross-platform validation using LC with triple quadrupole (QQQ) MS, which is a method widely accepted by regulators.

Experimental

Chemicals and Reagents

Certified triazine and triazole reference standards (purity >98%) were procured from Dr Ehrenstorfer GmbH (Augsburg, Germany). LC-MS grade solvents (methanol and acetonitrile) were purchased from J.T. Baker (Phillipsburg, NJ). α-Cyano-4-hydroxycinnamic acid (CHCA), trifluoroacetic acid, and verapamil were purchased from Sigma-Aldrich. Ultra-pure water with specific resistivity of 18.2 megohm-cm^{-1} was obtained using a water purification unit from Millipore. For QuEChERS extraction, primary secondary amine (PSA) sorbent was procured from Agilent. Anhydrous sodium sulfate and anhydrous magnesium sulfate (analytical reagent grade) were purchased from Merck India Ltd.

Preparation of Standard Samples

Calibration standards were prepared by serial dilution of a pesticide standard mixture stock solution to achieve the working range of 20–200 ng/g (20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 ng/g) using methanol. Verapamil was used as an internal standard (IS) for signal normalization. A stock solution (2 mg/kg) of verapamil was prepared in acetonitrile–water (9 + 1, v/v), which was further diluted to 200 ng/g concentration. MALDI matrix (20 mg CHCA) was prepared in a 50% acetonitrile–water solvent system containing 100 ng/g verapamil. Equal volumes of the pesticide standard mixture and the CHCA solution premixed with the IS were added separately for each dilution level. The final concentration of analytes in the mixture ranged from 10 to 100 ng/g (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 ng/g), and the concentration of the IS on the MALDI target plate was 50 ng/g. Technical QC samples with concentrations intermittent to the calibration range were also prepared similarly. After vortex-mixing, 1 µL aliquots of the CHCA–analyte–IS mixtures were spotted on a stainless steel MALDI target plate and allowed to air-dry.

Grape Samples

The samples were obtained from the Indian Council of Agricultural Research, National Research Centre for Grapes (Pune, India). The 20 grape samples were collected from different parts of Maharashtra State, India, during the harvest season of 2015–2016. The sampling procedure for pesticide residue analysis in table grapes was followed as per the standardized procedure (25, 26). Grape samples devoid of pesticides were separately used for matrix-matching and estimating QC recoveries.

Sample Preparation

Extraction of grape samples was carried out using the QuEChERS method (27). Briefly, 10 mL acetonitrile was added to a 10 g portion of the homogenized grape sample, and the mixture was vigorously shaken for 1 min. Subsequently, 4 g magnesium sulfate ($MgSO_4$) and 1.7 g anhydrous sodium acetate (CH_3COONa) were added and shaken for 1 min. The extract was centrifuged at 5000 rpm for 5 min. A 5 mL aliquot of the supernatant was then transferred to a 15 mL graduated centrifuge tube. After the addition of 50 mg PSA, the solution was centrifuged (Kubota 6500; Kubota Corp., Tokyo, Japan) at 10 000 rpm for 5 min. Later, the supernatant was transferred through a 0.22 µm PTFE filter. The filtrate was then concentrated 10× using a vacuum concentrator (CentriVap Benchtop Vacuum Concentrator; Labconco, Kansas City, MO). The concentrated grape extract was used for analysis by AP-MALDI HRMS and LC-QQQ MS.

AP-MALDI HRMS

All experiments were performed on a Q Exactive benchtop mass spectrometer (Thermo Scientific) coupled to an AP-MALDI pulsed dynamic focusing (PDF)+ source (MassTech, Inc., Columbia, MD) with a 355 nm Nd: YAG laser source. MS and MS/MS spectra were acquired at a resolution of 35 000 FWHM (full width at half-maximum). For MS/MS, the isolation window was set at 0.5 *m/z*, and normalized collision energy of 40 units stepped by 50% was used to achieve efficient fragmentation. The AP-MALDI ion source parameters of ion source voltage (1000 V), ion source temperature (280°C), and optimal laser energy (80%) with 20 µs delay for Pulsed Dynamic Focusing (PDF) were maintained throughout the analysis.

LC-QQQ MS

An ultra-fast liquid chromatograph coupled with an API 5500 QTRAP mass spectrometer (Sciex, Toronto, Canada) was used for residue analysis. The chromatographic separation of the test compounds was achieved on an Ultra AQ C18 column (100 × 2.1 mm, 3 µm; Restek Corp., Bellefonte, PA). Mobile phase A composition was water–10 mM ammonium formate and mobile phase B composition was methanol–10 mM ammonium formate, both with 0.1% formic acid, at a flow rate of 0.5 mL/min with a gradient profile (28, 29). The column oven temperature was maintained at 40 ± 1°C, and the injection volume was 20 µL. Measurements were performed with electrospray ionization (ESI) in the positive polarity mode with optimized multiple reaction monitoring (MRM) transitions (28, 29). The source parameters of ion source voltage (5500 V), nebulizer gas (30 psi), heater gas (60 psi), ion source temperature (550°C), and curtain gas (40 psi) were maintained throughout the analysis. Residue estimation was performed by retention time–dependent scheduled MRM, with two mass transitions for each test molecule: one for quantification and the other for confirmation. The MRM detection window was 90 s, with the target scan

time of 1 s. The ion ratio for the two mass transitions was used for unambiguous identification of each pesticide as per the SANTE/11945/2015 guidelines (30).

Method Validation

The method was validated with a performance comparison of calibration curve statistics and recovery for standardized QC samples. Before calibration curve estimation, LOD and LOQ were determined for each pesticide by minimum S/N criteria of 3 and 10, respectively (30). The calibration curve was prepared with data acquired using the pesticide standard mixture serially diluted at six levels of concentration, with four replicates at each concentration level. Two concentration levels (30 and 60 ng/g) of the pesticide standard mixture were used for standard QC validation. Matrix-matched calibrations and QC samples were also separately used for comparison and estimation of matrix effects on recoveries.

Software and Data Processing

AP-MALDI HRMS data were acquired in full-scan mode using the standard Xcalibur 2.2 package (ThermoFisher Scientific). The qualitative data analysis was performed using mMass (open source) software. For quantitative data analysis, the in-house-built software "MQ" was used for data processing, the generation of the calibration curves, and the determination of unknowns. For generating calibration curves, IS-normalized peak intensities of the analytes within a narrow mass extraction window (MEW; around 10 ppm at the base of the peak) were used for the high-resolution accurate mass (HRAM) analysis. These calibration models were subsequently used to determine the concentration of pesticides in grape extracts.

Results and Discussion

Detection and Quantitation Using AP-MALDI HRMS

The AP-MALDI HRMS profile of a pesticide mixture of triazines and triazoles in the positive ion mode shows predominantly protonated adduct peaks, along with sodium and potassium adducts. Supplemental Figure 1 shows a representative HRMS spectrum obtained for thiabendazole. The *m/z* for the $[M + H]^+$ adducts observed for all the analytes under investigation are listed in Table 1. The mass accuracy of all the analytes detected was within 5 ppm. The signal intensities showed consistent behavior, as well as analyte-to-analyte variations that are generally expected from desorption ionization processes. Qualitative confirmation of all the analytes was carried out using MS/MS experiments on the observed individual precursor ions (supplemental Table 1). The observed AP-MALDI MS/MS product ions matched with the theoretical *m/z* values and were well within 5 ppm mass accuracy.

LOD and LOQ were subsequently determined using AP-MALDI HRMS. Minimum S/N of 3:1 and 10:1 were used for the LOD and LOQ estimations respectively. Automatic preprogramed data acquisition was performed in an unbiased fashion, and the HRAM detection within 10 ppm MEW was ensured by using the in-house-developed data processing algorithm MQ to minimize any human errors. The LOD and LOQ values observed for the analyte standards are summarized in supplemental Table 2. Low nanogram-per-gram level LOQs were reproducibly obtained for all the analytes over several days of analytical runs. These values met or far exceeded the stringent MRL criteria for grapes.

A calibration range was chosen based on the LOQs, and the calibrators were analyzed using AP-MALDI HRMS. Remarkably, all of the calibrators in multiple replicates, along with the QC samples, could typically be accommodated on a single AP-MALDI target plate, leaving room for unknowns as well. As above, both the data acquisition and processing using MQ were performed in an automatic mode with the HRAM criterion of 10 ppm MEW. All the pesticides showed linear responses over their respective calibration ranges of concentration for analysis (Table 1). The regression value R^2 was consistently observed to be >0.98 in most cases, with low to acceptable RSD values (Table 1). Of note, these values were consistent over the numerous technical replicates that can be meaningfully performed using the high-throughput AP-MALDI interface. Recovery for the technical QC samples was found to be within the acceptable error limits of 80–120%, with RSDs <20%. To further understand the matrix-induced ion suppression effects, matrix-matched standards were used as calibrators and QC samples. Excellent linearity and reproducibility were obtained (supplemental Table 3). A matrix-matched QC sample yielded recoveries comparable to the standard calibrators mentioned above for most of the analytes, with a few exceptions that showed some deviation from expected values. These results clearly establish AP-MALDI HRMS as a quantitative method for the analysis of triazines and triazoles, and the method was subsequently used for the analysis of field samples.

Analysis of Field Table-Grape Samples and Performance Comparison of AP-MALDI HRMS and LC-QQQ MS Methods

LC-QQQ MS has been considered a robust and sensitive analytical method for triazine and triazole analysis in food matrixes (31). To compare the sensitivity and accuracy of the AP-MALDI HRMS method, 20 grape samples were selected from different geographical locations of the Maharashtra region in India. These samples were subjected to extraction using the established QuEChERS method (27), and two aliquots were drawn from the processed samples for further analysis. These aliquots, matching calibrants, and technical QCs were subsequently analyzed using both analytical platforms (AP-MALDI HRMS and LC-QQQ MS).

Of the 20 samples, azoxystrobin and myclobutanil were positively detected in three and five grape samples, respectively, using both analytical platforms. In the case of AP-MALDI HRMS, the ions were detected within 5 ppm mass error. Representative AP-MALDI HRMS profiles of two grape samples with these detected analytes are shown in Figure 1. LC-QQQ MS showed the expected confirmatory ion fragments for these pesticides as well. In addition, naturally occurring abundant isotopic *m/z* peaks of the detected analytes were observed using AP-MALDI HRMS, further confirming the presence of the detected analytes (Figure 2).

IS-normalized peak area–based calibration curves (obtained with 10 ppm MEW) were generated from the calibrants on the same target plate and used for the quantitation of the detected residues. Figure 3 shows the calibration curves obtained with

Table 1. LOQ, linear regression statistics, and standard QC recoveries obtained for triazines and triazoles subsequent to AP-MALDI HRMS analysis

Analyte	MW	Observed *m/z* $[M + H]^+$	LOQ, ng/g	Calibration range, ng/g	R^2	QC recovery (RSD), %	
						30 ng/g level	60 ng/g level
Atrazine-desethyl-desisopropyl	145.0150	146.0228	10	10–90	0.99	75.8 (1.1)	99.9 (9.0)
Atrazine-desisopropyl-2	155.0802	156.0880	30	10–90	0.99	74.8 (6.7)	64.1 (16.6)
Atrazine-desethyl	187.0619	188.0698	5	10–90	0.99	75.3 (8.7)	98.2 (12.8)
Atrazine-desethyl-2-hydroxy	169.0958	170.1036	10	10–90	0.99	84.5 (4.7)	105.7 (15.0)
Azoxystrobin	403.1163	404.1241	20	10–90	0.99	87.6 (8.8)	91.3 (8.9)
Bitertanol	337.1785	338.1863	25	10–90	0.98	83.8 (7.8)	83.3(14.4)
Cyantraniliprole	472.0045	473.0123	30	10–90	0.98	86.6 (10.6)	89.4 (12.4)
Cyprazine	227.0932	228.1011	10	10–90	0.97	96.2 (7.4)	105.4 (7.4)
Difenoconazole	405.0641	406.0720	10	10–90	0.99	88.7(3.4)	102.5 (3.5)
Diniconazole	325.0743	326.0821	10	10–90	0.99	85.5 (10.5)	104.3 (8.7)
Fenarimol	330.0321	331.0399	30	20–90	0.96	76.6 (15.9)	81.1 (16.3)
Flubendazole	313.0857	314.0936	10	10–90	0.98	96.5 (9.8)	98.5 (2.9)
Flusilazole	315.0998	316.1076	10	10–90	0.98	91.8 (5.0)	110.3 (5.6)
Myclobutanil	288.1136	289.1215	20	10–90	0.98	75.7 (2.0)	102.4 (13.3)
Paclobutrazol	293.1289	294.1368	5	10–90	0.99	81.7 (11.7)	105.3 (11.0)
Penconazole	283.0638	284.0716	10	10–90	0.99	78.1 (6.2)	103.8 (14.6)
Propiconazole	341.0692	342.0771	10	10–90	0.98	87.4 (9.1)	106.7 (6.7)
Pyraclostrobin	387.0980	388.1059	20	10–90	0.99	81.7 (10.8)	104.6 (14.8)
Simazine	201.0776	202.0854	4	10–90	0.99	83.7 (6.9)	107.4 (7.1)
Tebuconazole	307.1446	308.1524	5	10–90	0.99	85.0 (6.7)	105.2 (6.5)
Tetraconazole	371.0210	372.0288	20	10–90	0.99	76.7 (5.2)	103.7 (14.7)
Thiabendazole	201.0355	202.0433	0.5	10–90	0.98	96.0 (7.0)	95.9 (15.8)
Triadimenol	295.1082	296.1160	25	10–90	0.98	84.0 (14.4)	96.9 (17.0)
Triflumizole	345.0850	346.0929	10	10–90	0.99	87.5 (6.5)	91.7 (10.0)
Trifloxystrobin	408.1291	409.1370	20	10–90	0.99	83.3 (9.3)	98.2 (17.2)
Triticonazole	317.1289	318.1368	10	10–90	0.98	92.4 (4.0)	100.5 (4.0)

R^2 values of 0.9 in both cases. Quantitation with LC-QQQ MS used the standard protocols, with calibrations from the extracted ion chromatograms from the monitored reactions. Table 2 summarizes the averaged estimated concentration for pesticides using the AP-MALDI HRMS and LC-QQQ MS analysis platforms. Sample results in which estimations were below the LOQ levels were not considered for the comparisons. Figure 4 shows the concentration estimations of pesticide residues in grape samples for both methods. Assuming the LC-QQQ MS values to be true, five of the total eight positive samples were within 15% deviation. Deviations for the remaining samples were slightly higher (21–27%). Significantly, the results using LC-QQQ MS and AP-MALDI HRMS are in excellent agreement with each other and unequivocally validate the latter method.

Both platforms have shown comparable accuracy and sensitivity for the analysis. Overall, the chromatography-

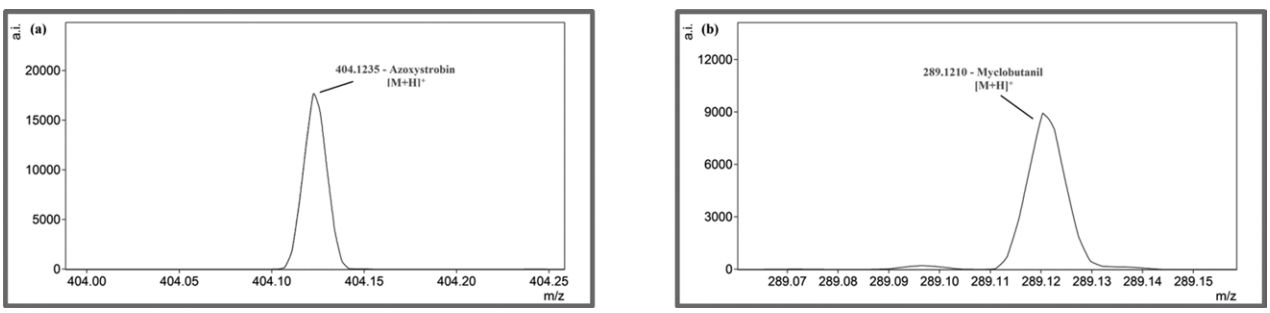

Figure 1. Representative AP-MALDI HRMS profiles for the detected pesticides in field grape samples showing the $[M + H]^+$ ion peak for (a) azoxystrobin in Sample 9 and (b) myclobutanil in Sample 11.

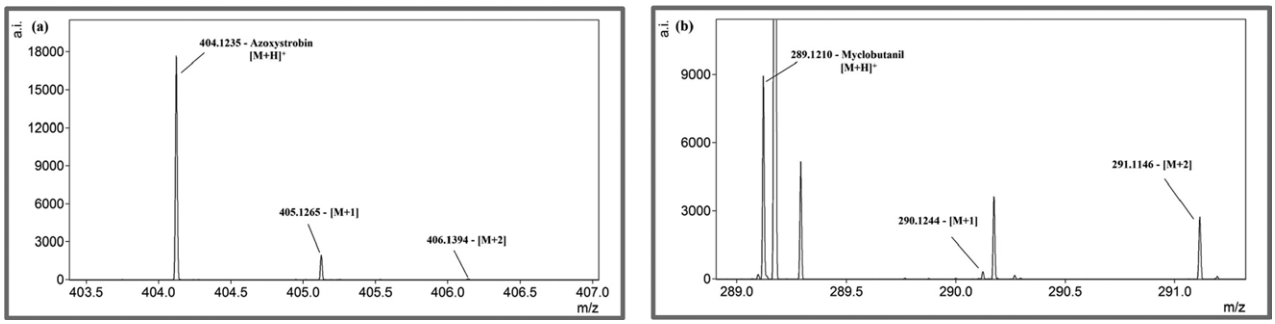

Figure 2. Profiles of the natural isotopic abundance pattern of (a) azoxystrobin in field Sample 9 and (b) myclobutanil in field Sample 11 used for qualitative confirmation of AP-MALDI HRMS data in addition to the MS/MS confirmation.

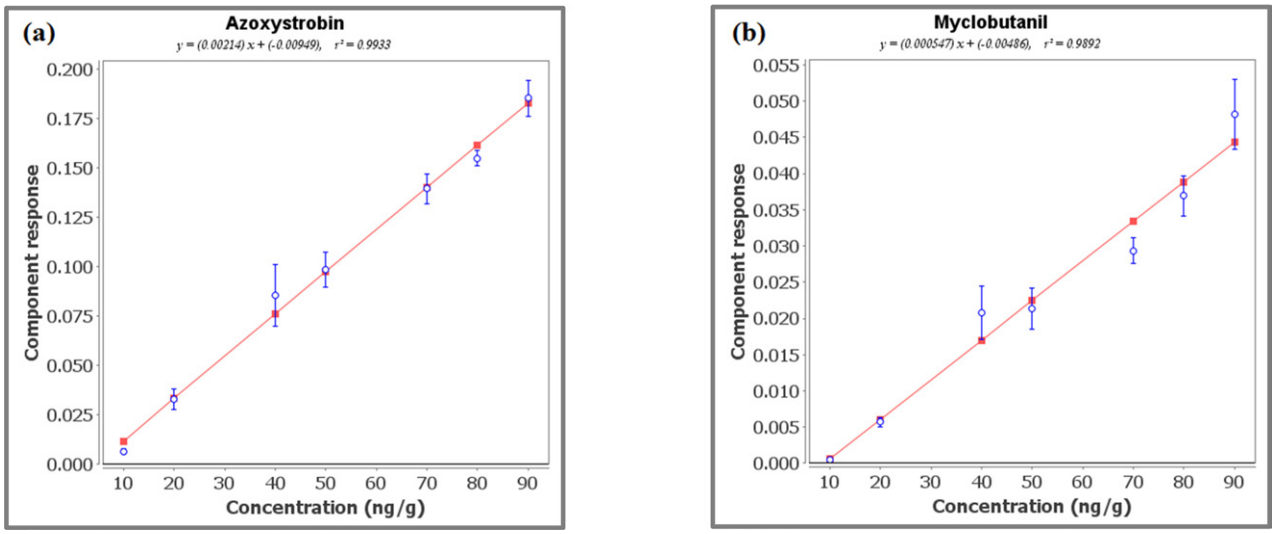

Figure 3. Linear regression fitted calibration curves for the reference standards for (a) azoxystrobin and (b) myclobutanil obtained using AP-MALDI HRMS.

free method of AP-MALDI HRMS showcases an analytical approach with improved throughput. Head-to-head comparison of the analysis run time for both platforms in the context of the current investigation is illustrated in supplemental Table 3. AP-MALDI HRMS achieved a fourfold increase in sample throughput (compared to LC-QQQ MS) while maintaining the high sensitivity, precision, and accuracy required for regulatory compliance. To our knowledge, this is the first report of its kind to describe residue analysis using AP-MALDI HRMS.

Conclusions

A quantitative AP-MALDI HRMS method for the analysis of triazine and triazole pesticides in grape matrix was demonstrated with high accuracy and precision. The triazine and triazole pesticides were identified using exact mass criteria and characterized by MS/MS. Quantitation using a specified MEW for high-resolution data has been reported for LC-ESI HRMS analysis, and broad guidelines have also

Table 2. Performance evaluation results of the AP-MALDI HRMS method in comparison with the LC-QQQ MS method for the detection of pesticides using field grape samples

Analyte detected	Sample No.	*m/z* $[M + H]^+$	Averaged estimated concentration, ng/g		Deviation from LC-QQQ MS prediction, %
			LC-QQQ MS	AP-MALDI HRMS	
Azoxystrobin	6	404.1241	130	158	21.5
	9		127.2	132	3.8
	12		117.2	126	7.5
Myclobutanil	1	289.1215	37.6	42.2	12.2
	4		50.4	64	27.0
	8		91.6	116	26.6
	11		236.8	244	3.0
	18		84	85	1.2

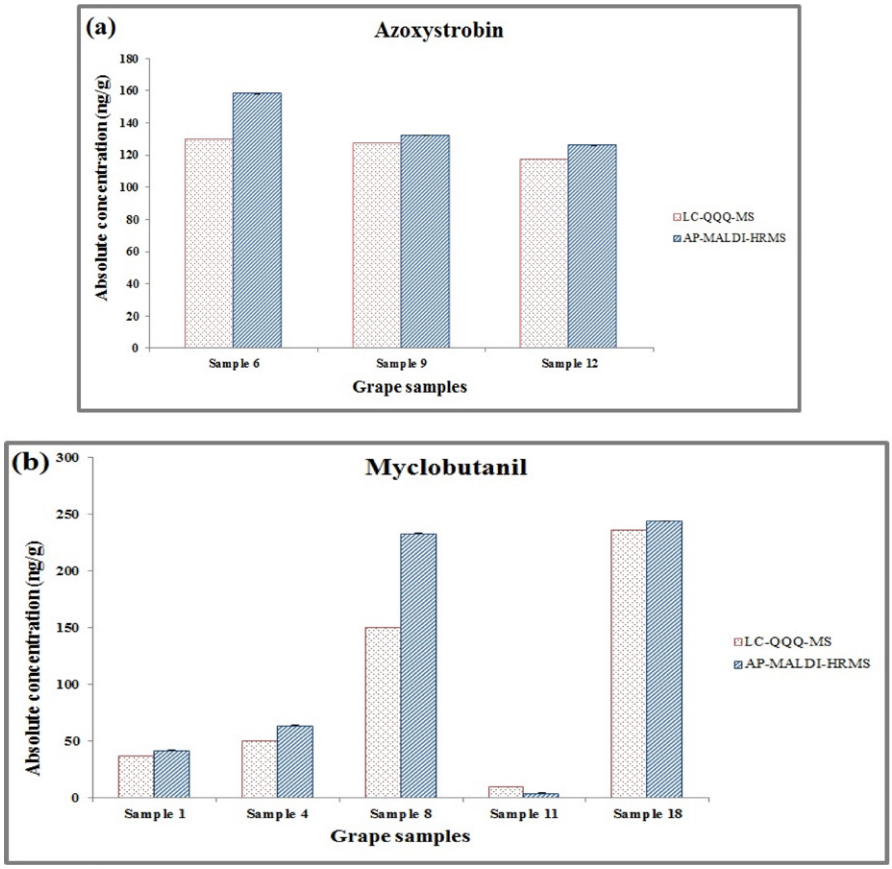

Figure 4. Comparison of concentration estimated in different grape samples using AP-MALDI HRMS and LC-QQQ-MS analysis platforms for (a) azoxystrobin and (b) myclobutanil.

been suggested for such an approach (32, 33). This work demonstrates a similar approach using AP-MALDI HRMS and does not require any chromatographic separation or elaborate sample preprocessing steps. Information regarding any further degradation products or additional analytes suspected in the sample can be readily verified because a full-scan MS spectrum is generated in this approach. Existing LC-QQQ MS reaction monitoring schemes, although sensitive and selective, lack this crucial additional feature. Unit resolution could also pose limitations in complex samples and in the case of compounds of similar chemical classes that are closely spaced. The total analysis time for AP-MALDI HRMS analysis was significantly shorter compared to the routinely followed HPLC-MS or GC-MS method, highlighting the potential of AP-MALDI HRMS for the rapid, accurate, and high-throughput analysis of residues and other contaminants in grape matrix.

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