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Determination of ametoctradin fungicide residues in grapes using high performance liquid chromatography

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Abstract
A simple and inexpensive method was developed using liquid - liquid extraction, together with high performance chromatographic method for determination of ametoctradin residues in grapes. The evaluated parameters include the extracts by distilled water and acetonitrile solvents. The method was validated using grapes samples spiked with ametoctradin at different fortification levels (0.05, 0.1 and 0.5 µg/g). Average recoveries (using each concentration three replicates) ranged 87-96%, with relative standard deviations less than 5%, calibration solutions concentration in the range 0.05-5.0 µg/mL and limit of detection (LOD) and limit of quantification (LOQ) were 0.02µg/g and 0.05µg/g respectively. Finally the grapes residue samples were analyzed by HPLC.

Keywords: HPLC, ametoctradin, grapes, LOD and LOQ

1. Introduction
Fungicides are widely used in agriculture to protect crops, fruits and vegetables in the field and during the storage process. Therefore, the concentration of pesticide residues in many products, including fruits and vegetables, must be monitored. Their regulations have been developed [1] for food safety. Ametoctradin, 5-ethyl-6 octyl[1, 2, 4]triazolo[1,5-a]pyrimidin-7-amine belongs to a new class of chemistry called triazolopyrimidine in FRAC group 45, which was discovered and developed by BASF in 2010.
It is well known that grape and apple are among the most important horticultural fruit crops in the world. Tomato, cumber and cabbage are widely distributed in the world and considered important vegetables. Unfortunately, all cultivars are susceptible to several diseases; fungi and oomycetes are the major pathogens that compromise the cultivation and economic profit from these plants [2, 3, 4]. Ametoctradin is a mitochondrial respiration inhibitor that interferes with complex III (complex bc1) in the electron transport chain of the pathogen; thus, ATP synthesis in the fungal cells is inhibited, and ametoctradin controls all major oomycete pathogens, e.g., Plasmopara viticola in grapes, Phytophthora infestans in potatoes and tomatoes, and *Pseudoperonospora cubensis* in cucurbits [5]. Some studies have demonstrated that ametoctradin does not show cross-resistance to fungicide classes such as Qo inhibitors, phenylamides, and carboxylic acid amides [6]. Hence, this fungicide offers a significant improvement for growers in controlling downy mildew and late blight in potato, grape, cucumber, apple, tomato and other crops. In addition; it is in the process of being registered on a global scale for use against diseases of fruit and vegetable crops [7].

2. Experimental
2.1 Standards, Reagents and samples
The analytical standard of ametoctradin (99.8%) was obtained from Sigma Aldrich. HPLC grade acetonitrile and water was purchased from rankem and grapes were purchased from local market.

2.2 Standard stock solutions
The ametoctradin stock solutions was individually prepared in acetonitrile at a concentration level 500 µg/g and stored in a freezer at -18 °C. The stock standard solutions were used for up

to 3 months. Suitable concentrations of working standards were prepared from the stock solutions by dilution using acetonitrile, immediately prior to sample preparation.

2.3 Sample preparation

Representative 500.0 gram portions of grapes fortified with 0.5 mL of working standard stock solution. The sample was allowed to stand at room temperature for one hour, before it was kept at refrigerator condition, until analysis.

2.4 Extraction procedure of grapes

The representative homogenized sample (50g of grapes and 50g of juice) was taken in a 500 ml stoppered conical flask. To this added 100 ml acetonitrile, water (80:20) and extracted using an end-over end mechanical shaker for about 30 minutes and filtered. Extraction was repeated with 50 ml of same solvent. Combined the extract and dried over sodium sulphate. Reduced the volume using vacuum rotary evaporator. Made up to suitable volume with acetonitrile for HPLC analysis.

2.5 HPLC-PDA Separation parameters

Instrument : Shimadzu High Performance Liquid Chromatograph system equipped with LC-20AT pump and SPD-20A PDA detector, SIL -20AC interfaced with LC-solution software system.

Column used : Agilent C_{18} column (250 mm x 4.6 mm i.d. x 5 µm particle size)

Volume injected : 20 µl

Time	Acetonitrile (A%)	0.1% v/v Triethyl amine, in Milli-Q water) (pH = 3.0 adjusted by using acetic acid) (B%)
0.01	45	55%
11.00	45	55%
16.00	65	35%
17.00	45	55%

Flow rate (ml/min) : 2.0
Wave length (nm) : 245 nm
Retention time : (approximately)
Ametoctradin : 11.8 minutes

2.6 Method validation

Method validation ensures analysis credibility. In this study, the parameters accuracy, precision, linearity and limits of detection (LOD) and quantification (LOQ) were considered [8, 9]. The accuracy of the method was determined by recovery tests, using samples spiked at concentration levels of 0.05, 0.1 and 0.5 µg/g. Linearity was determined by different known concentrations (0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 µg/mL) were prepared by diluting the stock solution. The limit of detection (LOD µg/g) was determined as the lowest concentration giving a response of 3 times the baseline noise defined from the analysis of control (untreated) sample. The limit of quantification (LOQ µg/g) was determined as the lowest concentration of a given fungicide giving a response of 10 times the baseline noise.

3. Results and discussion

3.1 Specificity

Aliquots of ametoctradin, control sample solution, extracted solvents and mobile phase solvents were assayed to check the specificity. There were no matrix peaks in the chromatograms to interfere with the analysis of residues shown in (Fig - 1 and Fig - 2). Furthermore, the retention time of ametoctradin was 11.8 min (Approximately).

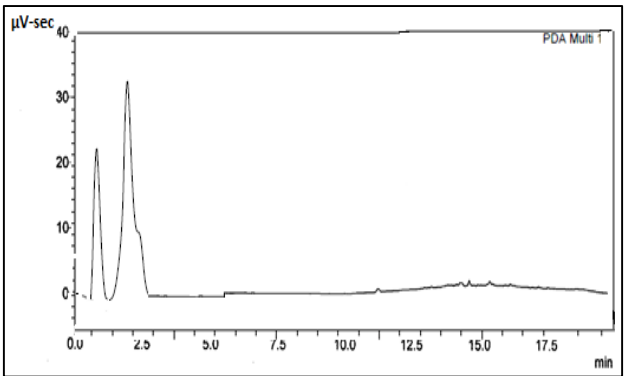

Fig 1: Representative Chromatogram at grapes control

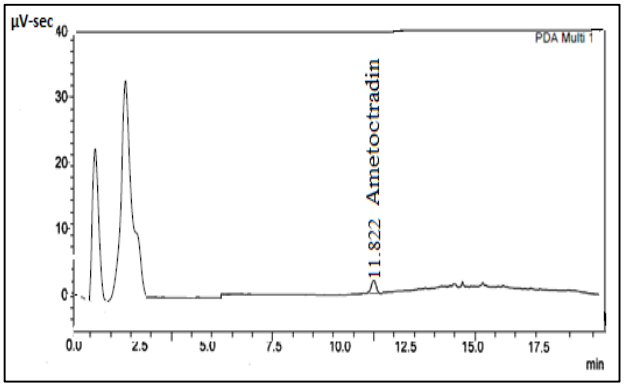

Fig 2: Representative Chromatogram at fortification level of 0.05 µg/g

3.2 Linearity

50.10 mg of ametoctradin reference standard was taken into 50 mL volumetric flask and dissolved in HPLC water, sonicated and made upto the mark with the same solvent. The concentration of the stock solution was 1000 µg/mL. From this stock solution prepared by different known concentrations of standard solutions (0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 µg/mL) were prepared into different 10 mL volumetric flasks and made upto the mark with acetonitrile. The serial dilution details were presented in Table- 1. These standard solutions were directly injected into a HPLC. A calibration curve has been plotted of concentration of the standards injected versus area observed and the linearity of method was evaluated by analyzing six solutions. The calibration details were given in Table- 2. The peak areas obtained from different concentrations of standards were used to calculate linear regression equation. This was Y=7123.00 X + 96.54 with correlation coefficient of 0.9999 respectively. A calibration curve showed in (Fig- 3).

Table 1: Serial dilutions of linearity standard solutions

Stock solution concentration (µg/mL)	Volume taken from stock solution (mL)	Final make up volume (mL)	Obtained concentration (µg/mL)
1000	0.100	10	10.0
10	5.00	10	5.0
10	2.00	10	2.0
10	1.00	10	1.0
10	0.5	10	0.5
10	0.1	10	0.1
1.0	0.5	10	0.05

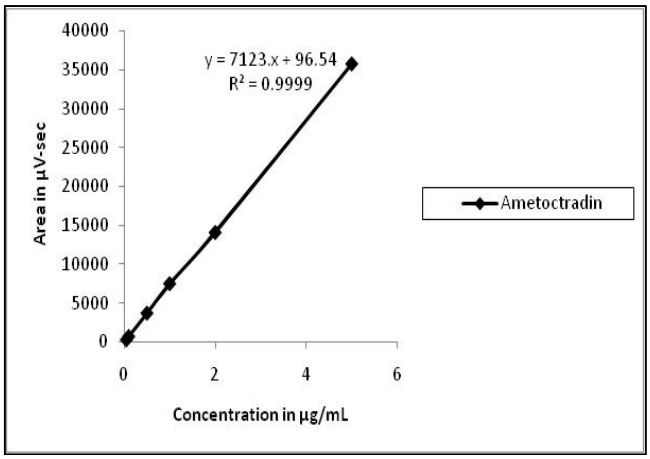

Fig 3: Representative Calibration curve of ametoctradin

3.3 Accuracy and Precision

Recovery studies were carried out at 0.05, 0.1 and 0.5 µg/g fortification levels for ametoctradin in grapes. The recovery data and relative standard deviation values obtained by this method are summarized in Table- 2.

Table 2: Recoveries of the ametoctradin from fortified grapes control sample (n=3)

Fortified Concentration (mg/kg)	Replication	Recovery (%) Grapes
0.05	R1	87.79
	R2	87.49
	R3	86.92
	Mean ± S.d.	87.40 ± 0.44
0.1	R1	90.56
	R2	93.87
	R3	92.47
	Mean ± S.d.	92.30 ± 1.66
0.5	R1	96.12
	R2	95.89
	R3	96.62
	Mean ± S.d.	96.21 ± 0.37

These numbers were calculated from four (3) replicate analyses of given sample (ametoctradin) made by a single analyst on one day. The repeatability of method satisfactory (RSDs<5%).

3.4 Detection and Quantification Limits

The limit of quantification was determined to be 0.05 µg/g. The quantitation limit was defined as the lowest fortification level evaluated at which acceptable average recoveries (87-96%, RSD<5%) were achieved. This quantitation limit also reflects the fortification level at which an analyte peak is consistently generated at approximately 10 times the baseline noise in the chromatogram. The limit of detection was determined to be 0.05 µg/g at a level of approximately three times the back ground of control injection around the retention time of the peak of interest.

3.5 Storage Stability

A storage stability study was conducted at refrigerator condition (5 ± 3 °C) and Ambient temperature (25 ± 5 °C) of 0.5 µg/g level fortified fruit samples were stored for a period of 30 days at this temperature. Analysed for the content of ametoctradin before storing and at the end of storage period. The percentage dissipation observed for the above storage period was only less than 3% for ametoctradin showing no significant loss of residues on storage. The results are presented in Table- 3 and Table- 4.

Table 3: Storage stability Details at refrigerator condition (5 ± 3 °C)

Fortification Concentration in µg/g	Storage Period in Days	Recovery in %
		98
		97
		96
		94
	0	95
		96
	Average	96.00
	STDEV	1.41
	RSD in%	1.47
0.5		92
		94
		94
	30	93
		94
		95
	Average	93.67
	STDEV	1.03
	RSD in%	1.10

Table 4: Storage stability Details at ambient Temperature (25 ± 2 °C)

Fortification Concentration in µg/g	Storage Period in Days	Recovery in%
		95
		93
		94
		92
	0	95
		93
	Average	93.67
	STDEV	1.21
	RSD in%	1.29
0.5		90
		92
		93
	30	92
		94
		93
	Average	92.33
	STDEV	1.37
	RSD in%	1.48

3.6 Calculations

The concentration of acetaminophen in the samples analyzed by HPLC was determined directly from the standard curve.

$Y = mx + c$

Where,

Y = peak area of standard (µV*sec)

m = the slope of the line from the calibration curve

x = concentration of injected sample (mg/L)

c = 'y' intercept of the calibration curve

The recovered concentration or Dose concentration was calculated by using the formula:

$$\text{Recovered concentration or Dose concentration} = \frac{(x-c)\ X\ D\ X\ 100}{m\ X\ P}$$

Where,
m = the slope of the line from the calibration curve
x = sample area of injected sample (μV*sec)
c = 'y' intercept of the calibration curve
D = Dilution Factor
P = Purity of Test item

$$\% \text{ Recovery} = \frac{\text{Recovered Concentration}}{\text{Fortified Concentration}} \times 100$$

4. Conclusions

This paper describes a fast, simple sensitive analytical method based on HPLC-PDA to determine the ametoctradin residues in grapes. The LLE extraction procedure is very simple and inexpensive method for determination of ametoctradin residues in grapes. Satisfactory validation parameters such as linearity, recovery, precision and LOQ were established by following South African National Civic Organization (SANCO) guidelines [10]. Therefore, the proposed analytical procedure could be useful for regular monitoring, residue labs and research scholars to determine the ametoctradin residues in different commodities (fruit, juice, seed, oil, and water and soil samples).

5. Acknowledgement

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