International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2017; 5(2): 237-241

Received: 10-01-2017
Accepted: 11-02-2017

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Determination of Indaziflam and its metabolite residues in black grapes

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Abstract
A simple and inexpensive method was developed using liquid - liquid extraction, together with high performance liquid chromatographic method with UV detection for determination of indaziflam and its metabolite (diamino triazine) residues in black grapes. The method was validated using fruit samples spiked with indaziflam and its metabolite (diamino triazine) at different fortification levels (0.05 and 0.5 µg/g). Average recoveries (using each concentration six replicates) ranged 84-97%, with relative standard deviations less than 3%, calibration solutions concentration in the range 0.05-5.0 µg/mL and limit of detection (LOD) and limit of quantification (LOQ) were 0.05µg/g and 0.02µg/g respectively. Finally the fruit residue samples were analyzed by HPLC.

Keywords: HPLC, indaziflam, metabolite (diamino triazine) and black grapes

1. Introduction
Indaziflam belongs to the chemical class of alkylazines and acts in susceptible plants by inhibiting cell wall biosynthesis [1, 2]. Indaziflam acts only where cellulose synthesis is occuring such as in actively growing meristematic tissues, dividing cells, expanding cells, and growing roots. Indaziflam is currently registered or being registered for use in perennial crops (e.g., citrus, tree nut, grapes, pome and stone fruit), residential and commercial areas (e.g., turfgrass, landscape ornamentals, Christmas trees, hardscapes), non-residential and non-crop areas (e.g., railroad and rail yards, roadsides, fence rows, industrial sites), and forestry sites [3, 4].
This study has been undertaken to develop an improved method for analysis of indaziflam and its metabolite diamino to determine residue retention in black grapes.

2. Experimental
2.1 Standards, Reagents and samples
The analytical standards of indaziflam (98.24%) and diamino triazine (97.85%) were obtained from Sigma Aldrich. Acetonitrile and HPLC water were purchased from rankem, New Delhi and black grape fruits were purchased from local market.

2.2 Standard stock solutions
The indaziflam and metabolite- diamino triazine standard stock solutions were individually prepared in acetonitrile at a concentration level 1000 µg/mL 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 20.0 g portions of grape fortified with 0.1 mL of working standard solutions. 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
Weighed 50 grams of grape samples and 20 gm of grape samples were taken in different 250 ml glass jars with a screw cap lid. Added 100 ml of 90:10 (v/v) acetonitrile: water to each jar cap and vigorously shaken for 30 minutes.

Removed the cap and placed a disposable magnetic stir bar into each glass jar. Loosely attach the lid to the glass jar. Placed the jars in the microwave oven, evenly speed around the centre of the carousel insert the thermo-well into the untreated control sample and insert the fibre optic temperature probe into the thermo-well with the microwave door open, turn on the manual magnetic stirrer control and check to see that the stir bars are turning but without splashing the samples. The temperature was raised upto 50 °C for 15 minutes. On completion of the extraction cycle, allowed the glass jars to cool. Added 25 ml of 90:10 (v/v) acetonitrile: water and vigourously extracted. The sample solutions was then filtered into different round bottom flask and the filterate was evaporated to near dryness and made up to 20 ml using 90:10 acetonitrile: water (v/v) and injected into the HPLC system.

2.5 Chromatographic separation parameters

The HPLC-UV system used, consisted shimadzu high performance liquid chromatography with LC- 20AT pump and SPD-20A interfaced with LC solution software, equipped with a reversed phase C18 analytical column of 150 mm x 4.6 mm and particle size 5 µm (Agilent Eclipse -C18) Column temperature was maintained at 35 °C. The injected sample volume was 20µL. Mobile Phases A and B was acetonitrile and 1 L of HPLC water containing 0.1 ml of orthophoric acid (95:5 (v/v)) with program. The flow- rate used was kept at 0.8 mL/min. A detector wavelength was 220 nm. The calibration curve method was used for determination of indaziflam and metabolite- diamino triazine residues in grapes.

Mobile phase composition program:

Time (min)	A Conc (%)	B Conc-(%)
0.0	95	5
10.0	50	50
20.0	40	60
25.0	50	50
30.0	95	5

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. The accuracy of the method was determined by recovery tests, using samples spiked at concentration levels of 0.05 and 0.5 mg/kg. 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/mL) 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/mL) was determined as the lowest concentration of a given a response of 10 times the baseline noise [5, 6, 7].

3. Results and Discussion

3.1 Specificity

Aliquots of indaziflam and its metabolite diamino triazine standard solutions, spiking sample solution, fruit control, 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 (Figure 1 and 2). Furthermore, the retention times of indaziflam and metabolite of diamino triazine were constant at 19.5 ± 0.2 and 4.3 ± 0.2, minutes.

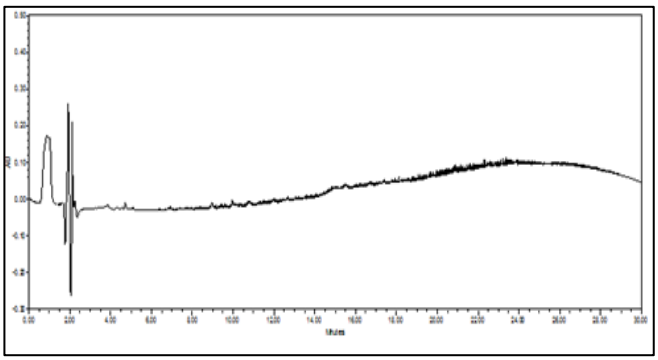


Fig 1: Representative Chromatogram of grape fruit control

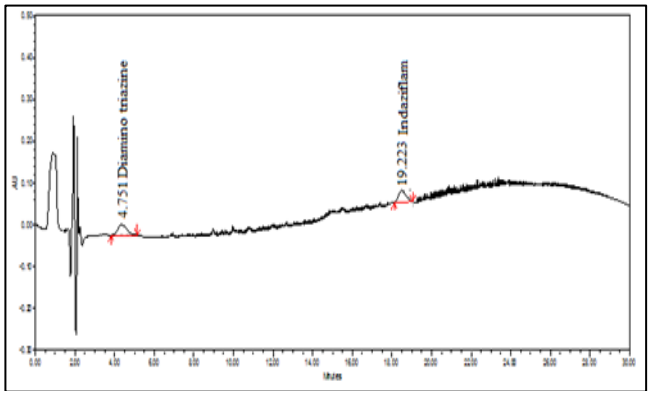


Fig 2: Representative Chromatogram at fortification level of 0.05 µg/g

3.2 Linearity

3.2.1 Preparation of indaziflam standard stock solution

Accurately weighed 10.18 mg of reference standard of indaziflam and its metabolite diamino triazine (Purity 98.24%) in 10 mL volumetric flask and dissolved in acetonitrile, sonicated and made upto the mark with the same solvent. The concentration of the stock solution was 1000 µg/mL.

3.2.2 Preparation of metabolite of diamino tri

Accurately weighed 10.35 mg of reference standard of metabolite diamino triazine (Purity 97.85%) in 10 mL volumetric flask and dissolved in acetonitrile, sonicated and made upto the mark with the same solvent. The concentration of the stock solution was 1000 µg/mL.

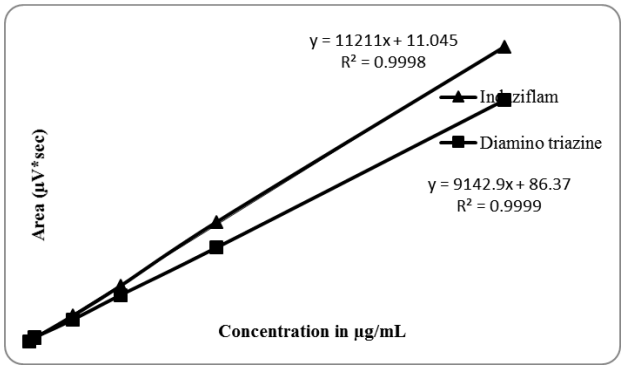
3.2.3 Preparation of Calibration solutions

Different known concentrations of standard solutions (0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 µg/mL) were prepared in acetonitrile by diluting the above stock solutions. 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 [8]. The peak areas obtained from different concentrations of standards were used to calculate linear regression equations. These were Y=11211X + 11.04 and Y=9142X + 86.37 with correlation coefficients of 0.9999 and 0.9999 for indaziflam and metabolite of diamino triazine respectively. A calibration curve showed in (Figure 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	1.000	10	100
100	0.500	10	5
100	0.200	10	2
100	0.100	10	1
10	0.5	10	0.5
10	0.1	10	0.1
1	0.15	10	0.015

Fig 3: Representative Calibration curve of indaziflam and metabolite (diamino triazine)



3.2.4 Accuracy and Precision

Recovery studies were carried out at 0.05 and 0.5 µg/mL fortification levels for indaziflam and metabolite- diamino triazine in fruit. The recovery data and relative standard deviation values obtained by this method are summarized in

Table 2.

These numbers were calculated from four (6) replicate analyses of given sample (indaziflam and diamino triazine) made by a single analyst on one day. The repeatability of method satisfactory (RSDs<3%).

Table 2: Recoveries of the indaziflam and metabolite (diamino triazine) from fortified Grape sample (n=6)

Fortification Concentration in µg/mL	Replication	Recovery (%)	
		indaziflam	diamino triazine
0.05	R1	82.36	84.23
	R2	85.23	84.36
	R3	84.66	85.19
	R4	85.29	84.09
	R5	86.96	85.02
	R6	84.52	84.05
	Mean	84.84	84.49
	STDV	1.49	0.49
	RSD	1.76	0.58
0.5	R1	97.41	96.23
	R2	96.32	95.68
	R3	92.69	95.89
	R4	93.56	97.25
	R5	98.39	98.69
	R6	97.25	98.23
	Mean	95.94	97.00
	STDV	2.29	1.26
	RSD	2.39	1.30

3.2.5 Detection and Quantification Limits

The limit of quantification was determined to be 0.05 µg/mL. The quantitation limit was defined as the lowest fortification level evaluated at which acceptable average recoveries (84-97%, RSD<3%) 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/mL at a level of approximately three times the back ground of control injection around the retention time of the peak of interest.

3.2.6 Storage Stability

A storage stability study was conducted at refrigerator condition (5 ± 3 °C) and Ambient temperature (25 ± 5 °C) of 0.1 µg/g level fortified fruit samples were stored for a period of 30 days. Analysed for the contents of indaziflam and metabolite diamino triazine before storing and at the end of storage period [5, 6]. The percentage dissipation observed for the above storage period was only less than 3% for indaziflam and metabolite diamino triazine showing no significant loss of residues on storage. The results are presented in Table 3 and 4.

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

Fortification Concentration in µg/mL	Storage Period in Days	Recovery in %	
		indaziflam	diamino triazine
		93.27	93.65
		95.29	92.69
		96.05	94.23
		94.85	93.87
	0	93.96	92.59
		95.78	93.25
	Average	**94.87**	**93.38**
	STDEV	**1.08**	**0.66**
	RSD in %	**1.13**	**0.70**
0.1		91.05	92.62
		90.36	90.36
		91.25	90.56
	30	92.74	90.78
		92.13	91.42
		90.89	92.17
	Average	**91.40**	**91.32**
	STDEV	**0.87**	**0.92**
	RSD in %	**0.96**	**1.01**

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

Fortification Concentration in µg/mL	Storage Period in Days	Recovery in %	
		indaziflam	diamino triazine
		94.25	96.12
		95.63	95.26
		94.12	94.98
		95.98	95.29
	0	95.36	95.74
		95.23	95.41
	Average	**95.10**	**95.47**
	STDEV	**0.75**	**0.40**
	RSD in %	**0.79**	**0.42**
0.1		92.36	93.21
		93.65	93.58
		93.28	93.11
	30	94.12	92.99
		94.57	92.59
		93.25	93.71
	Average	**93.54**	**93.20**
	STDEV	**0.77**	**0.41**
	RSD in %	**0.82**	**0.44**

3.2.7 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 (mAU*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:

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

Where,

m = the slope of the line from the calibration curve

x = sample area of injected sample (mAU*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 LLE-HPLC-UV simultaneous determination of indaziflam and metabolite of diamino triazine residues in grapes. The LLE extraction procedure is very simple and inexpensive method for simultaneous determination of indaziflam and metabolite of diamino triazine residues in grapes. The mobile phase composition was showed good separation and resolution and the analysis time required for the chromatographic determination of the indaziflam and metabolite diamino triazine were very short (around 30 min for a chromatographic run). Satisfactory validation parameters such as linearity, recovery, precision and very low limits were obtained and according to the SANCO guidelines [9]. Therefore, the proposed analytical procedure could satisfactorily be useful for regular monitoring of indaziflam and metabolite diamino triazine residues on a large number of leaf, seed, oil, fruit, water and soil samples.

5. Acknowledgement
The authors are thankful to the Dr. Gowtham Prasad, S.V.V University, Hyderabad for his keen interest and help.

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