International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(2): 2907-2912

Received: 24-01-2018
Accepted: 25-02-2018

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Dissipation pattern of trifloxystrobin 25% + tebuconazole 50% WG (Nativo 75% WG) on cowpea and soil from Western Maharashtra

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Abstract
Field experiment was conducted during the year 2016 at the Pulse Improvement Project, MPKV, Rahuri, Dist.- Ahmednagar to determine the residues and dissipation of trifloxystrobin 25% + tebuconazole 50% WG (Nativo 75% WG) in/on cowpea and soil. Two sprays of Nativo 75 WG @ 87.5 + 175 g a. i./ha (350 g ha^{-1}) and 175+350 g a.i./ha (700 g ha^{-1}) were given at an interval of 10 days initiating first spray at pod formation stage. Samples of the immature pods were collected periodically at 0, 1, 3, 5, 7, 10, 15 and 20th day after last spray. The mature pods, mature seeds and soil samples were collected at harvest. The samples were analysed for residues by using validated QuEChERS method on Gas Chromatography Mass spectrometry at AINP on Pesticide Residues, MPKV, Rahuri. The results revealed that average initial residues of trifloxystrobin in immature pods were found to be 0.87 and 1.43 mg/kg and that of tebuconazole were 1.70 and 2.60 µg g^{-1} at single and double dose and reached BDL on 10th and 15th days, respectively. The half life values of trifloxystrobin were observed to be 1.89 and 2.01 days and those for tebuconazole were 2.00 and 2.00 days, respectively. The residues of trifloxystrobin, its metabolite CGA-321113 and tebuconazole in mature pods, mature seeds and in soil at harvest were found to be BQL. Present studies suggests 10 days pre harvest interval (PHI) for safe consumption of cowpea pods after the application of Nativo 75 WG at 350 g ha^{-1}.

Keywords: Cowpea, trifloxystrobin, tebuconazole, persistence and QuEChERS

1. Introduction
Cowpea (*Punica granatum* L.), an ancient and commercially important pod of both tropical and subtropical countries, belongs to the smallest botanical family punicaceae. It is a good source of carbohydrate and minerals such as calcium, iron and sulphur and rich in vitamin-C and citric acid. In India, it is regarded as a "vital cash crop", grown over an area of 1.5 lakh ha with a production of 11.0 lakh tons (Jadhav and Sharma, 2009) [6]. Among states growing cowpea, Maharashtra is the largest producer of cowpea occupying 2/3rd of total area in the country followed by Karnataka, Andhra Pradesh, Gujarat and Rajasthan (Anon., 2009) [1].
Diseases are the major constraints in production of good quality cowpea pods. The diseases are spread faster and become a major limiting factor in attaining high yield. Nativo 75 WG, a water dispersible granular (WDG) formulation containing 25% w/w trifloxystrobin and 50% w/w tebuconazole is a broad spectrum systemic fungicide with protectant and curative properties. Trifloxystrobin methyl (E)-methoxyimino-{(E)-α-[1-(α,α,α-trifluorom-tolyl)ethylideneamin-ooxy]-o-tolyl}acetate, a strobilurin fungicide, is a new class of substance that is included in the Quinone outside inhibitors (QoI) fungicide group. It showed outstanding biological activity for controlling diseases, like powdery midew, scabs, leaf spots and blight **(**Karaoglanidis and Karadimos [8], 2006., Reuveni, 2000) [11]. Tebuconazole (RS) -1- p - chlorophenyl - 4, 4 – dimethy l – 3 - (1H - 1, 2, 4 – triazol -1- ethyl) pentan-3-ol is a systemic fungicide of the triazole group which, like other triazole fungicides, interferes in the metabolism of fungal pathogens, mainly by inhibiting ergosterol biosynthesis. It was found effective against powdery mildews, loose smuts and rusts of legume and non-legume crops (Daniel *et al.* 2007 [2], Ilhe *et al.* 2008 [5], Mohapatra *et al.* 2010) [9]
The presence of residues in cowpea is a matter of serious concern especially when immature pods are consumed. It is necessary to evaluate Nativo 75 WG (25% w/w trifloxystrobin + 50% w/w tebuconazole) from residue point of view in cowpea for the safety of the consumers. At present, no information is available on persistence of trifloxystrobin and tebuconazole on cowpea.

Keeping this in view, the present study was carried out to investigate the dissipation pattern of trifloxystrobin and tebuconazole residues in cowpea.

2. Materials and Methods

2.1 Field Experiment

Study was initiated on cowpea (var. Phule Pandhari) at the Pulse Improvement Project, MPKV, Rahuri, Dist: - Ahmednagar (M.S.) during 2015. The experiment was laid on cowpea plants (3.66 X 3.05 m) in randomized block design (RBD). The first application of Nativo 75WG (trifloxystrobin 25% + tebuconazole 50%) @ 350 g ha^{-1} and 700 g ha^{-1} was made at pod formation stage using knapsack sprayer. Subsequently two applications were made at 10 day interval. Each treatment was replicated thrice and control plots were sprayed with water only.

Samples of immature pods (1 kg) were collected at random from each treatment plot at 0 (2 hr after spray), 1, 3, 5, 7, 10, 15 and 20 days after last application. Mature pods, mature seeds and soil samples 1 kg each were collected at harvest. Samples were brought to the Pesticide Residues Laboratory and extracted immediately. The samples were homogenized and analysed for respective fungicides. The soil samples were air dried in shade, sieved and analysed for residues. Meteorological conditions *viz.* temperature (°C), relative humidity (%) and rainfall (mm) were recorded during the experimental period i.e. from first day till completion of experiment (Table1).

Table 1: Meteorological observations during experimental period

Month	Met. week	Date	Temp (°C)		Humidity (%)		Rain Fall (mm)
			Max	Min	I	II	
July'2016	27	02-08	29.6	23.6	79	69	90
	28	09-15	28.4	22.9	82	74	22.6
	29	16-22	29.6	29.8	76	67	2.00
	30	23-29	29.2	22.7	84	66	127.8
August'2016	31	30-05	28.3	22.7	82	72	50.6
	32	06-12	30.1	23.0	70	65	1.00
	33	13-19	30.6	22.0	72	59	0.00
	34	20-26	31.8	22.2	72	55	0.00
	35	27-02	31.7	22.8	74	56	1.6
Sept.'2016	36	03-09	30.8	20.3	69	53	0.00
	37	10-16	31.4	21.9	75	58	62.0
	38	17-23	28.8	22.2	86	77	163.6
	39	24-30	30.1	21.1	82	66	60.8
Oct.'2016	40	01-07	28.6	21.7	87	71	110.6
	41	08-14	31.6	20.6	80	53	0.00
	42	15-21	31.7	17.2	66	37	0.00
	43	22-28	31.3	16.4	66	35	0.00
Nov.'2016	44	29-04	30.1	13.4	51	40	0.00
	45	05-11	29.6	11.6	53	25	0.00
	46	12-18	29.4	12.6	64	41	0.00
	47	19-25	29.1	10.1	56	27	0.00
	48	26-02	30.9	10.5	62	26	0.00

2.2 Chemicals and Reagents

The certified reference material of trifloxystrobin, its metabolite CGA321113 and tebuconazole were obtained with purity of 99.60, 93.40 and 95.60 per cent, respectively. The commercial formulations were supplied by M/s Bayer Crop Science India Ltd., Mumbai. All the solvents used were of analytical grade. The solvents, ethyl acetate (HPLC grade), acetonitrile (HPLC grade), sodium chloride (AR grade), magnesium sulphate (AR grade), sodium sulphate anhydrous (AR grade) were obtained M/s. Rankem Fine Chemicals, Ltd., New Delhi. (India). PSA was procured from Agilent Technology, Bangalore.

2.3 Standard Preparation

The stock solutions of trifloxystrobin, CGA 321113 and tebuconazole were prepared at 1000 mg/kg in Toluene. These solutions were diluted further to obtain concentrations of 0.05, 0.1, 0.25, 0.4, 1.0 µg g^{-1}.

2.4 Method Validation

Prior to analysis of samples, linearity of trifloxystrobin, its metabolite; CGA321113 and tebuconazole was established on GCMS. Accuracy and precision of the method was determined by per cent mean recovery and per cent relative standard deviation. Linearity was studied by injecting standard solution of fungicides under study at five linear concentrations i.e. 0.05, 0.10, 0.25, 0.40 and 0.50 µg g^{-1}. The linearity curve was established with concentration of the standard and corresponding peak area. Recovery study was conducted in order to establish the reliability of the method of analysis. The cowpea samples from control plots were used for recovery studies. Ten g homogenized sample was taken in 15 ml polypropylene tube. The samples were spiked with three different concentrations viz. 0.05 µg g^{-1} (LOQ), 0.25 µg g^{-1} (5×LOQ) and 0.5 µg g^{-1} (10×LOQ) in triplicate. The extraction and clean up were performed as described hereunder.

Per cent recovery was calculated by using following formula.

$$\text{Per cent recovery} = \frac{\text{Quantity of pesticide recovered}}{\text{Quantity of pesticide added}} \times 100$$

2.5 Extraction and clean up

2.5.1 Immature Pods

Immature pods were extracted by QuEChERS method (Sharma, 2013) [15]. The entire laboratory sample (1 Kg) was crushed thoroughly in a mixer cum grinder and weighed 10 g homogenized sample in a 50 ml polypropylene tube and the tube was kept in deep freezer for 10 min. To this, added 10 ml

ethyl acetate and 10 g anhydrous sodium sulphate, hand shaken vigorously and centrifuged the content at 3500 rpm for 5 min. Transferred 2 ml supernatant to 15 ml tube containing 50 mg PSA. The content was vortexed for 30 Sec and then centrifuged for 2 min at 2500 rpm. The supernatant was filtered through 0.2 micron filter in GC vials and performed GC-MS analysis.

2.5.2 Mature pods and mature seeds

Mature pods and mature seed samples were extracted by QuEChERS method (Sharma, 2013) [15]. The entire laboratory sample (1 Kg) was crushed thoroughly in a mixer cum grinder and weighed 5 g homogenized sample in a 50 ml polypropylene tube and the tube was kept in deep freezer for 10 min. To this, added 5 ml distilled water and 10 ml ethyl acetate and 10 g anhydrous sodium sulphate, hand shaken vigorously and centrifuged the content at 3500 rpm for 5 min. Transferred 2 ml supernatant to 15 ml tube containing 50 mg PSA. The content was vortexed for 30 Sec and then centrifuged for 2 min at 2500 rpm. The supernatant was filtered through 0.2 micron filter in GC vials and performed GC-MS analysis.

2.5.3 Soil:

Weighed 10 g homogenized soil sample in a 50 ml polypropylene tube. To this, added 20 ml acetonitrile and shaken vigoursly. To this, added 4 g magnesium sulphate and 1 g sodium chloride and hand shaken vigorously and centrifuged the content at 3300 rpm for 5 min. Transferred 10 ml supernatant to 15 ml tube containing 250 mg PSA and 1.5 g $MgSO_4$. The content was sonicated for 1 min and centrifuged for 10 min at 4400 rpm. Collected 4 ml supernatant from the above centrifuge tube and evaporated to dryness using nitrogen concentrator at 40^0C (water bath temperature). Reconstituted the dry residues in 2 ml of ethyl acetate and filtered through 0.2 micron filter in GC vials and performed GC-MS analysis.

2.6 Chromatographic conditions:

Trifloxystrobin and tebuconazole residues were estimated on Gas Chromatograph-Mass Spectrometry (Model GCMS-QP 2010 Plus) equipped with GC Real-time analysis data software.

Column : VF 5 MS 30m x 0.25 µ x 0.25 µ
Column Temperature : 110 ^{0}C to 185 ^{0}C @ 10^{o}C/min1, min hold, 185 ^{0}C
min to 280^0 C@ 6^{0}C/min, 3 min hold
Injector Temperature : 250^{0}C
Interface Temperature : 285^{0}C
Ion source Temperature : 250^{0}C
Injection volume : 1 µl
Column flow : 1.46 ml/min
Retention time Approx. : CGA : 15.80 min.
Trifloxystrobin: 19.44 min
Tebuconazole : 20.28 min

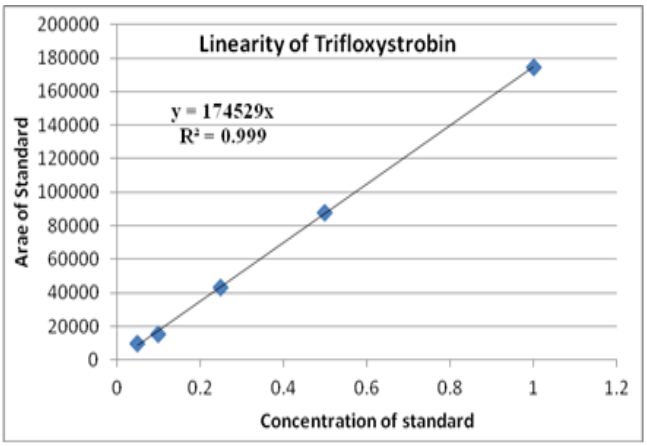
The amount of fungicide was calculated according to the equation based on the ratio of the peak areas of the respective external standards and sample (Sharma *et al.*, 2007) [14]

3. Results and Discussion

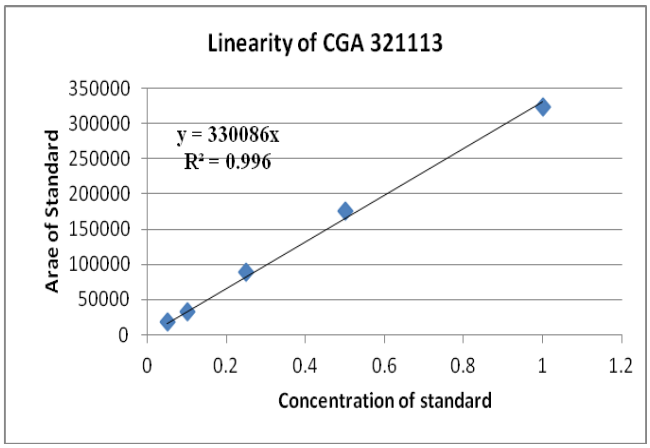
3.1 Method Validation

The detector response to the neat standards of the insecticides was studied by injecting five linear concentrations of above fungicides. The graph was plotted with detector response against respective concentrations and linearity line was drawn. The response of the instrument was linear over the range tested and R^2 value was 0.99 for above fungicides (Fig.1). These results indicated that the GCMS analysis is a valid method for residue determination of the tested fungicides in cowpea pods. Accuracy of the analytical method was determined by recovery studies. The per cent recovery was within acceptable range of 70-120 per cent prescribed by SANCO (2011) [13] as mentioned in Table 2.

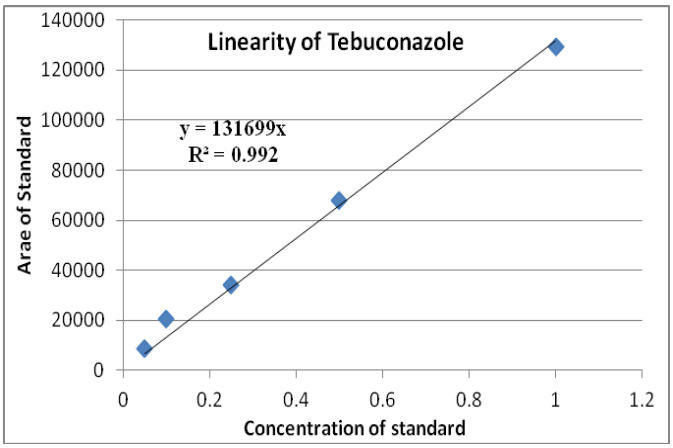
Residues were estimated by comparison of peak area of the standards with that of the unknown or spiked samples run under identical conditions. The persistence of trifloxystrobin and tebuconazole has been expressed in terms of DT_{50} i.e. time for disappearance of pesticide to 50% of its initial concentration.



a. Trifloxystrobin



b. CGA-32113



c. Tebuconazole

Fig 1: Linearity of trifloxystrobin, CGA321113 and Tebuconazole standard

Table 2: Recovery of trifloxystrobin, metabolite CGA321113 and tebuconazole on cowpea and soil

Substrate	Level of fortification µg g^{-1}	Per cent Recovery		
		Trifloxystrobin	CGA321113	Tebuconazole
Immature cowpea pods	0.05	84.52(±1.84)	92.36(±1.11)	95.40(±0.87)
	0.25	97.16(±2.72)	96.48(±1.88)	91.28(±1.33)
	0.50	97.79(±2.96)	98.29(±3.13)	91.92(±3.70)
Mature cowpea pods	0.05	89.15(±0.72)	91.09(±6.26)	93.37(±1.39)
	0.25	72.01(±1.12)	72.40(±0.81)	96.32(±3.46)
	0.50	87.00(±1.35)	93.45±1.81	75.43(±1.82)
Mature cowpea seeds	0.05	86.65(±1.96)	91.97(±5.66)	92.04(±5.14)
	0.25	74.48(±0.55)	73.27(±1.05)	93.35(±2.93)
	0.50	95.60±1.15	95.89(±1.68)	85.17(±0.59)
Soil	0.05	74.67(±2.51)	81.41(±1.49)	99.88(±0.75)
	0.25	91.21(±2.38)	92.85(±1.58)	90.38(±5.61)
	0.50	93.52(±4.99)	88.52(±4.52)	82.50(±3.95)

* Mean of three replications Figures in parenthesis are standard deviation values

3.2 Dissipation of trifloxystrobin, CGA321113 and Tebuconazole

The average initial residues of trifloxystrobin on cowpea pods were observed to be 0.87 and 1.43 µg g^{-1}, respectively, following two applications of trifloxystrobin 25% + tebuconazole 50% at recommended and double dose, respectively (Table 3). Residues of trifloxystrobin dissipated below quantification level (BQL) of 0.05 µg g^{-1} in 10 and 15 days at single and double dose, respectively. Half life (DT_{50}) values of trifloxystrobin on cowpea pods were observed to be 1.89 and 2.01 days, respectively. Cowpea pods did not record any residues of metabolite, CGA 321113. Mature pods, seeds and soil samples collected at harvest did not record any residues of trifloxystrobin and its metabolite CGA 321113 at quantification limit of 0.05 µg g^{-1}.

Table 3: Residues of trifloxystrobin and tebuconazole on immature pods, mature pods, seeds and soil at different time intervals

Days after treatment	Untreated control	Residues (µg g^{-1})					
		Trifloxystrobin 25%+ Tebuconazole 50% WG @ 350 g ha^{-1}			Trifloxystrobin 25%+ Tebuconazole 50% WG @ 700 g ha^{-1}		
		Trifloxystrobin *Mean±SD	CGA 321113 Mean±SD	Tebuconazole Mean±SD	Trifloxystrobin Mean±SD	CGA 321113 Mean±SD	Tebuconazole Mean±SD
Immature pods							
0	ND	0.87±0.07	BQL	1.70±0.11	1.43±0.18	BQL	2.60±0.25
1	ND	0.68±0.06	BQL	1.32±0.36	1.12±0.13	BQL	2.25±0.11
3	ND	0.39±0.03	BQL	0.91±0.05	0.78±0.06	BQL	1.67±0.06
5	ND	0.15±0.01	BQL	0.41±0.03	0.31±0.02	BQL	0.68±0.02
7	ND	0.07±0.01	BQL	0.14±0.01	0.14±0.02	BQL	0.29±0.02
10	ND	BQL	BQL	BQL	BQL	BQL	0.09±0.01
15	ND	BQL	BQL	BQL	BQL	BQL	BQL
20	ND	BQL	BQL	BQL	BQL	BQL	BQL
At harvest							
Mature pods	ND	BQL	BQL	BQL	BQL	BQL	BQL
Mature seeds	ND	BQL	BQL	BQL	BQL	BQL	BQL
Soil	ND	BQL	BQL	BQL	BQL	BQL	BQL
DT_{50} (days)	-	1.89	-	2.00	2.01	-	2.00

Mean of three replicates ND- Not Detected, SD – Standard deviation, BQL= Below Quantification Limit LOQ-0.05 mg/kg

Sahoo *et al.*, (2012) reported initial deposits of trifloxystrobin on chilli as 0.31 and 0.59 µg g^{-1}, following two applications of Nativo 75 WG @ 250 and 500 g ha^{-1}, and residues dissipated with half life of 1.81 and 1.58 days, respectively. Similarly, 0.30 and 0.87 µg g^{-1} initial deposits of trifloxystrobin were reported on grapes at single (175 g ha^{-1}) and double dose (350 g ha^{-1}) of Nativo 75 WG, respectively (Daniel *et al.* 2007). However, higher initial deposits of 7.76 and 15.53 mg kg-1 were reported, following the application of trifloxystrobin 25%+ tebuconazole 50% (Nativo 75 WG) @ 43.75 and 87.5 g a.i.ha^{-1} on grapes, (Jyot *et al.* 2010) [7]. They reported the half life values 2.92 and 3.48 days for above doses. The initial deposits of trifloxystrobin in apple at four locations were found to in the range of 0.33 to 0.38 µg g^{-1} and 0.51 to 0.71 µg g^{-1} at the application rate of 100 and 200 g.a.i/ha. The half lives were found to be 19.38 - 24.93 and 19.84-28.86 days at these respective doses (Patyal *et al.*, 2013) [10].

As regards tebuconazole, the average initial deposits of tebuconazole on cowpea pods were found to be 1.70 and 2.60 µg g^{-1}, respectively (Table 3). Tebuconazole Residues dissipated below quantification limit (BQL) of 0.05 µg g^{-1} after 10 and 15 days for the respective doses. Half life of tebuconazole in cowpea pods was calculated to be 2.00 and 2.00 days at recommended and double the recommended doses, respectively. Mature pods and soil samples collected at harvest did not record the presence of tebuconazole at the quantification limit of 0.05 µg g^{-1} (Table 3).

Average initial deposit of tebuconazole were estimated to be 0.53 and 1.22 µg g^{-1}, in grapes following four application of Nativo 75 WG (trifloxystrobin 25%+ tebuconazole 50%) at 175 and 350 g ha^{-1}, respectively as reported by Mohapatra *et. al*, (2010) [9]. In case of apple, initial deposits of tebuconazole were below MRL of 0.5 µg g^{-1} (FAO/WHO, 2000) [3] at single dose. However, at double dose, the initial deposits were 0.95-1.05 and required 18.78-23.47 days to dissipate to below MRL. Half life values ranged between 19.38-25.99 days and 19.84-28.86 days at the application rate of 200 g.a.i. ha^{-1} and 400 g.a.i.ha^{-1}, respectively (Patyal *et al.*, 2013) [10]. Similarly, initial deposits of tebuconazole on chilli were found to be 0.95 and 1.88 µg g^{-1}, respectively, following two application of Nativo 75WG (trifloxystrobin 25%+ tebuconazole 50%) @ 250 and 500 g ha^{-1}, which dissipated with half life values of 1.37 and 1.41 days, respectively (Sahoo *et al.*, 2012) [12]. However, the dissipation studies of tebuconazole in peppermint revealed that three applications of tebuconazole @ 125 and 250 g a.i. ha^{-1} resulted in detection of residues at 0.26 and 0.80 µg g^{-1} at harvest (64 days) after the last application. Tebuconazole residues were also detected in peppermint oil to the range of 0.011 and 0.041 µg g^{-1} (Garland *et al.*, 1999) [4]. According to Jyot *et al.*, (2010) [7] the initial deposits of tebuconazole were recorded as 13.84 and 26.55 µg g^{-1} on grapes, following the application of tebuconazole @ 87.5 and 175 g a.i. ha^{-1}, respectively on grapes.

4. Conclusion

In the present investigation, the half life values for trifloxystrobin were observed to be 1.89 and 2.00 days for the recommended and double the recommended dose, respectively. For tebuconazole, the half life values determined were 2.01 and 2.00 days, respectively. Residue levels of both trifloxystrobin and tebuconazole were below quantification limit when immature cowpea pods were harvested 10 and 15 days after the last application under Rahuri condition. Present studies suggests 10 days pre harvest interval for safe consumption of immature pods after application of Nativo 70 WG (trifloxystobin 25% + tebuconazole 50%) at recommended dose of 350 g ha^{-1}.

5. Acknowledgement

Authors are thankful to the Coordinator, AINP on Pesticide Residues, ICAR, New Delhi for providing the financial support to the undertake the studies. Further Thanks are also due to the Pulse Breeder, Pulse Improvement Project, MPKV, Rahuri for sparing experimental site.

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