EURL-SRM - Analytical Method Report

Concerning the following…

- **Compound(s)**: Bifenazate, Bifenazate-diazene
- **Commodities**: Fruit and vegetables, cereals
- **Extraction Method(s)**: QuEChERS modified
- **Instrumental analysis**: LC-MS/MS

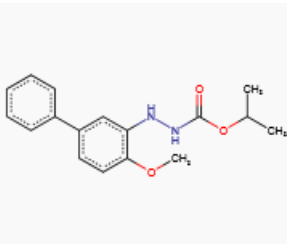
Analysis of Bifenazate (sum) by the QuEChERS Method using LC-MS/MS

Version 1 (last update: 17.03.2017)

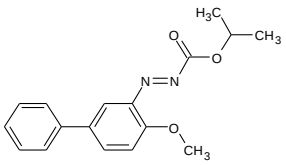
Short Description:

A method is presented for the analysis of Bifenazate (sum) in fruits and vegetables. Bifenazate and Bifenazate-diazene are extracted using the CEN-QuEChERS method. The raw or cleaned-up extract is then treated with ascorbic acid to protect Bifenazate from oxidation and to convert Bifenazate-diazene to Bifenazate. Bifenazate is then analyzed via LC-MS/MS in the ESI (pos.) mode against a Bifenazate calibration solution that is also stabilized by ascorbic acid or against a Bifenazate-diazene solution transformed to Bifenazate by ascorbic acid.

Compound details

Bifenazate (CAS: 149877-41-8), IUPAC: propan-2-yl N-(2-methoxy-5-phenylanilino)carbamate			
Parameter	**Value**	**Notes**	
Molecular Mass	300.358 g/mol		
Pka	12.94 (±0.06) at 23 °C (very weakly acidic)		
LogPow	3,4	at 20°C, pH independent up to ca. pH 11	
Water solubility	1.66 mg/L 3.76 mg/L	at 20°C; pH 5 (JMPR report 2006[1]) Tomlin, C.D.S. (ed.). The Pesticide Manual - 11 th ed., 1997	
Stability	Contradictive information on hydrolysis stability: 1) *"In a study simulating food processing conditions, Bifenazate was hydrolytically stable under all the conditions tested in this study, with Bifenazate-diazene less than 2% applied radioactivity (AR)." [EFSA Peer Review Report 2017[2]].* 2) *Decomposes in sterile water solution in the dark. It first oxidizes producing Bifenazate-diazene, which then hydrolyses to methoxy- and hydroxy- biphenyls (e.g. 3,4-dihydroxybiphenyl and 3-hydroxy-4-methoxybiphenyl) [JMPR report 2006].*		
Hydrolysis rates in water (DT50)	9.1 days	25°C; pH 4	According to JMPR report 2006[4]
	5.4 days 22 hours	25°C; pH 5 pH 5; under simulated sunlight irradiation	
	20 hours	25°C; pH 7	
	1.6 hours	25°C; pH 9	
Residue definition EU	Bifenazate (sum of Bifenazate plus Bifenazate-diazene expressed as Bifenazate) (F)[3]		
Use	Non-systemic acaricide used on various cultures such as citrus, tree nuts, pome fruit, stone fruit, berries, peppers, tomatoes, aubergines, cucurbits, legume vegetables and herbs		
Approved in…	AT, BE, BG, CY, CZ, DE, DK, EL, ES, FI, FR, HU, IE, IT, MT, NL, PL, PT, RO, SE, UK		
ADI / ARfD	0.01 mg/kg bw/day [JMPR2006] / 0.1 mg/kg bw [EFSA Peer Review Report 2017]		

[1] http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation06/Bifenazate06.pdf
[2] http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2017.4693/epdf
[3] Reg. (EU) 2016/1, valid since 25/01/2016

Bifenazate diazene (CAS: 149878-40-0), IUPAC: diazenecarboxylic acid, 2-(4-methoxy-[1,1'-biphenyl], 1-methylethyl ester			
Parameter	**Value**	**Notes**	
Molecular Mass	298,34 g/mol		
Pka	Not ionizable		
LogPow	3.8 4.48	BVL[4] pH independent (calculated by Chemicalize)	
Water solubility	Very low		
Stability	Decomposed by alkaline media to produce methoxy- and hydroxy- biphenyls. (e.g. 3-hydroxy-4-methoxybiphenyl).		
Hydrolysis rates in water (DT_{50})	58 hours	pH4	According to JMPR report 2006
	50 hours	pH5	
	18 hours	pH7	
	0.28 hours	pH9	
Residue definition EU	Bifenazate (sum of Bifenazate plus Bifenazate-diazene expressed as Bifenazate) (F)		
Approved in…	AT, BE, BG, CY, CZ, DE, DK, EL, ES, FI, FR, HU, IE, IT, MT, NL, PL, PT, RO, SE, UK		
ADI / ARfD	The finalisation of the toxicological assessment of Bifenazate-diazene is pending[5].		

Materials[6]:

- Bifenazate (purity 98%) was purchased from Dr. Ehrenstorfer GmbH, (Cat #: C10579500)
- Bifenazate-diazene (purity 99.9%) was a kind gift by the applicant company, an acetonitrile solution (100 µg/mL) may be purchased from HPC Standards GmbH; (Cat #:676338)
- Chloryrifos (diethyl D10) (purity 97%, isotopic purity 99%) was purchased from LGC (Cat #: DRE-C11600100)
- Stock solutions of native Bifenazate, Bifenazate diazene and Chlorpyrifos-D10 at 1 mg/ml was prepared by dissolving 15mg of the compound in 1 mL acetonitrile and filling it up to 15 mL with acetonitrile
- Working solutions were prepared by appropriately diluting stock solutions with acetonitrile
- Ascorbic acid reagent grade, crystalline was purchased from Sigma-Aldrich (Cat #: A7506 Sigma)
- Ascorbic acid solution 30% (w/w) was prepared by dissolving 3 g of ascorbic acid in 10 mL water. The solution is shaken until ascorbic acid is completely dissolved.
- All other materials and chemicals used as listed in EN 15662

Apparatus and Consumables[6]:

Use materials described in the QuEChERS standard procedure (EN15662). As a mechanical shaker you can use a horizontally or vertically reciprocating shaker or a rotatory shaker (e.g. HS260 by IKA or GenoGrinder by Spex or SSL1 Labscale Orbital Shaker by Stuart). To filter the extract use e.g. polyester disposable syringe filters of 0.45 µm pore size.

[4] http://www.bvl.bund.de/SharedDocs/Downloads/04_Pflanzenschutzmittel/01_zulassungsberichte/006823-00-00.pdf?__blob=publicationFile&v=3

[5] **http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2017.4693/epdf**

[6] **Disclaimer**: Names of companies are given for the convenience of the reader and do not indicate any preference by the EURL-SRM towards these companies and their products

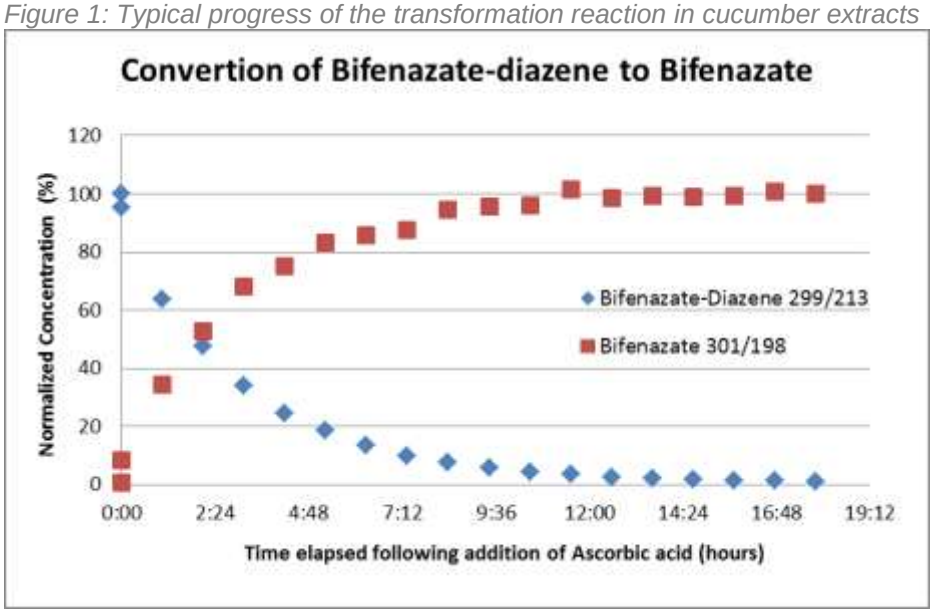
Analytical Procedure

Extraction:
Apply the **citrate buffered QuEChERS (EN 15662)**. Weigh 10 g of frozen fruit or vegetable homogenate or 5 g of cereals; adjust water content to 10 mL where necessary, add 10 mL acetonitrile and internal standard (e.g. 100 µL of an appropriately concentrated solution of Chlorpyrifos D10). Shake 15 min using a mechanical shaker. Add a mixture of 4 g $MgSO_4$, 1 g NaCl, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogen citrate sesquihydrate, shake 1 min and centrifuge.

Cleanup: Cleanup via dispersive SPE using PSA-Sorbent as described in EN15662 is optional for fruits and vegetables.

Transformation of Bifenazate-diazene to Bifenazate and Bifenazate stabilization: Transfer 1 mL of raw or cleaned-up extract into an auto-sampler vial, add 25 µL aqueous ascorbic acid solution (30 % w/w) and leave the vial standing for >15 hours (e.g. overnight) before LC-MS/MS analysis.

Figure 1: Typical progress of the transformation reaction in cucumber extracts



Preparation of calibration standards: Matrix-matched calibration standards are prepared using an extract of a blank commodity produced as described above, however without addition of an internal standard. Spike the blank extract with appropriate amounts of Bifenazate and internal standard and add 25 µL of the aqueous ascorbic acid solution (30 % w/w)to each vial to stabilize Bifenazate. If the sample contains mainly Bifenazate-diazene it is advisable to prepare the calibration solution with Bifenazate-diazene and to conduct the transformation to Bifenazate as described above.
Note: If the sample extract is found to exclusively contain Bifenazate-diazene you may quantify by calibrating with Bifenazate-diazene (without conducting the conversion step with ascorbic acid).

LC-MS/MS analysis: Measurement is conducted by LC-MS/MS (ESI-positive mode). Exemplary measurement conditions are given in Table 1 and Table 2.
For screening purposes Bifenazate and Bifenazate-diazene may be analyzed via LC-MS/MS directly from QuEChERS raw or cleaned-up extracts. In case of positive findings add 25µL ascorbic acid solution (30 % w/w) to both sample extract and calibration standard solution(s), wait for the reaction to complete and re-analyze. See exemplary measurement conditions in Table 1 and Table 2.

Table 1: Instrumentation details

LC/GC	**UPLC Acquity Waters**		
MS/MS	API 4000 Q		
Mode	ESI pos		
MRMs	Bifenazate: 301/152, 301/170, 301/198 Bifenazate-Diazene: 299/213, 299/197, 299/184		
Column	**Waters BEH C18 2.1x100 mm, 1.7 um**		
Pre-column	**Waters BEH C18 2.1x5 mm, 1.7 um**		
Mobile Phase	(A) 5mmol NH4 formiat in water/ methanol 95/5 (B) 5mmol NH4 formiat in methanol		
Gradient	**Time** min	**Mobile Phase A** %	**Mobile Phase B** %
	0	95	5
	0.5	60	40
	2	10	90
	5	10	90
	5.1	95	5
	9	95	5
Flow	0.4 ml min^{-1}		
Injection volume	2 µL		
Column temperature	40°C		

Tab. 2: MRM Details for Bifenazate and Bifenazate-diazene (ESI-neg. mode using ABSciex API 4000 QTrap):

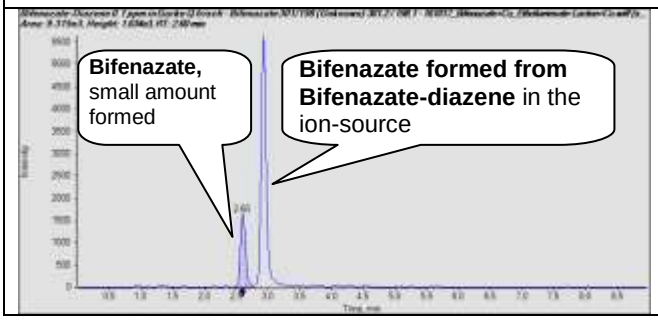
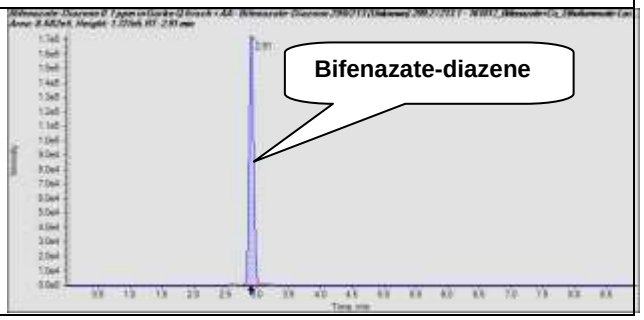
Name of Transition	Rel. Sensitivity	Parent mass *	Daughter mass	DP	CE	CXP	Mode
Bifenazate	1	301.2	152.1	61	63	10	ESI pos.
Bifenazate	2	301.2	170.2	61	29	10	ESI pos.
Bifenazate	3	301.2	198.1	61	15	12	ESI pos.
Bifenazate-diazene	1	299.2	213.1	41	17	2	ESI pos.
Bifenazate-diazene	2	299.2	197.0.	41	29	10	ESI pos.
Bifenazate-diazene	3	299.2	184.0	41	27	10	ESI pos.
Internal Standard (option)							
Chlorpyrifos D10		360.1	199.0	66	23	12	ESI pos.

* $[M+H^+]^-$ in all cases

Note1: The transitions of Bifenazate-diazene can be of use for monitoring its presence in a first routine screening and to verify its absence following reduction with ascorbic acid.

Note2: Bifenazate-diazene partly transforms to Bifenazate in the ESI ion-source. As a result Bifenazate-diazene shows signals not only in its own MRM traces but also at the MRM-traces belonging to Bifenazate (see Figure 2).

Figure 2: Exemplary chromatograms of bifenazate-diazene immediately after addition of ascorbic acid

m/z 301/198 (belongs to Bifenazate)	m/z 299/213 (belongs to Bifenazate-diazene)
	

Validation:

Validation was conducted separately for Bifenazate and Bifenazate-diazene at 0.01 mg/kg in cucumber, orange juice and wheat flour. Calibration was done using two approaches: a) using Bifenazate standard solution and b) using Bifenazate-diazene standard solution. All calibration solutions were treated the same way as the sample extracts (adding ascorbic acid and leaving for 20 h at room temperature before measurement). **Bifenazate was measured in all cases**. Chlorpyrifos-D10 was used as internal standard. Calibration was matrix-matched. The measurement conditions were as described above.

The results of the validation experiments are shown in Tables 3 and 4. Table 3 shows the recovery rates obtained when using Bifenazate standard for calibration and Table 4 when using Bifenazate-diazene. The determined recovery rates were in all cases within the acceptable range (80-107%) with good precision (RSD≤5). When calibrating with Bifenazate-diazene the recovery rates included a procedural “correction” for the reaction yield from Bifenazate-diazene to Bifenazate, and are thus higher by 5% on average.

It should be noted that even when Bifenazate was spiked to the matrix, the raw-extracts of cucumber and wheat flour contained almost entirely Bifenazate-diazene. In such cases calibrating with Bifenazate-diazene is more reasonable. In the case of orange juice the situation was reverse with the extracts containing almost entirely Bifenazate even if Bifenazate-diazene was spiked.

In Tables 3 and 4 the recovery rates obtained using the more suitable approaches (i.e. calibrating with Bifenazate in the case of orange and with Bifenazate-diazene in case of cucumber and wheat) are highlighted in black.

*Tab. 3: Recovery rates of Bifenazate and Bifenazate-diazene determined as Bifenazate using **Bifenazate for calibration (which was stabilized with ascorbic acid)**.*

Commodity	Spiked with	Determ. as	Recovery rates in % (determined as Bifenazate and calculated as diazene)						
			1	2	3	4	5	Avg	RSD
Cucumber	**Bifenazate-diazene**	Bifenazate	91	90	90	94	89	**91**	**2**
Orange juice			100	98	98	100	102	**99**	**2**
Wheat flour			96	90	87	95	99	**93**	**5**
Cucumber	**Bifenazate**		74	82	81	85	80	**80**	**5**
Orange juice			97	96	103	103	106	**101**	**4**
Wheat flour			*92*	*85*	*96*	*96*	*91*	***92***	***5***

*Tab. 4: Recovery rates of Bifenazate and Bifenazate-diazene determined as Bifenazate using **Bifenazate-diazene for calibration (following transformation to bifenezate with ascorbic acid)**.*

Commodity	Spiked with	Determ. as	Recovery rates in % (determined as Bifenazate and calculated as diazene)						
			1	2	3	4	5	Avg	RSD
Cucumber	**Bifenazate-diazene**	Bifenazate	100	99	98	103	97	**99**	**2**
Orange juice			104	102	101	103	106	**103**	**2**
Wheat flour			98	92	90	98	101	**96**	**5**
Cucumber	**Bifenazate**		79	87	87	91	85	**86**	**5**
Orange juice			103	102	110	110	113	**107**	**4**
Wheat flour			94	87	98	98	93	**94**	**4**

A direct parallel quantification of Bifenazate and Bifenazate-diazene without prior conversion/stabilization by ascorbic acid is typically associated with poor precision and a higher variation and is thus not recommendable. Depending on the matrix a progressing transformation of Bifenazate-diazene to Bifenazate or vice versa is observed both in sample extracts and matrix-based calibration standards containing Bifenazate or Bifenazate-diazene.

Experiment and Document History

Action	When	Document Version
Experiments	March 2016-March 2017	
Observation document placed on-line	March 2017	V1