A Summary of Analytical Methods Used for the Analyses of Pesticide Residues in Bees and Hive Matrices

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DISCLAIMER

This document represents EPA Region 5's summary of analytical methods used primarily by USDA, EPA and Region 5 State Lead Agencies for the analyses of bees and hive matrices. It does not change or substitute for any legal requirements. It is intended for informational purposes only. In the event of a conflict between the discussion in this document and any statute or regulation, this document would not be controlling. Deviations from this document on the part of any duly authorized official, inspector, or agent to follow its contents shall not be a defense in any enforcement action; nor shall deviation from this document constitute grounds for rendering the evidence obtained thereby inadmissible in a court of law.

This document contains summary information for several analytical methods currently in use by State Lead Agencies in Region 5 or EPA/USDA laboratories that perform analyses of pesticide residues in bees and hive matrices. Included in **Table 1** is information that describes the type of laboratory instrumentation needed to perform sample analyses; instrument-specific settings, minimum sample weights/sizes, and detailed information pertinent to sample extraction and clean-up procedures associated with each example method.

Each of the summarized methods (with the exception of "Example 2") shown in **Table 1** is a variation of the original QuEChERS approach. QuEChERS stands for "Quick, Easy, Cheap, Effective, Rugged and Safe". The QuEChERS approach can be used to analyze residues of hundreds of different pesticides and their degradates/metabolites, 121 of which have been found in bees and hive matrices (Mullin *et al*. 2010[1]). Extracts of wax, beebread (honey/pollen mixture), and adult bee carcases or brood (larvae/pupae) carcases can also be analyzed for select degradates/metabolites of pesticides using the QuEChERS approach. For example, the methods identified as Examples #1 & 4 are methods used by the EPA Office of Pesticide Programs Biological and Economic Assessment Division Analytical Chemistry Branch to analyze neonicotinoids and their metabolites in bee carcases and hive matrices.

The modified QuEChERS variations shown in **Table 1** are used by State or EPA/USDA laboratories when: 1) a particular pesticide(s) is/are suspected; 2) selecting a method for pesticide analysis in an atypical matrix (*e.g*., wax, pollen, nectar or honey); 3) sample weight is limited, 4) a laboratory's instrumentation is limited; and 5) when a laboratory cannot achieve the method detection levels pertinent to the data quality objectives of investigation with their own methods.

When States/Tribes perform analyses of environmental samples through a cooperative agreement or performance partnership agreement with EPA, such work is routinely done in accordance with State/Tribe regulations and procedures and under an EPA approved quality management plan and/or quality assurance project plan. This summary anticipates that States/Tribes will continue to use their own standard operating procedures (SOPs) and analytical methodologies, as appropriate, for collecting and analyzing environmental samples related to cases of pesticide-related bee mortality. If a laboratory cannot otherwise achieve the objectives of such an investigation, then the methods described in **Table 1** may be considered for use.

It is recommended that prior to investigating an incident involving the loss of bees that the state/tribal inspector contacts the laboratory that will analyze samples. This is intended to ensure that the equipment used for sample collection and preservation are consistent with any data quality objectives associated with the investigation and are consistent with laboratory SOPs.

Laboratories have used general screening on bee matrix extracts to help determine the presence of pesticides when it was not apparent that a specific pesticide product(s), pesticide degradate(s) or products containing pesticides (*e.g*., treated seeds) were clearly related to the bee kill. In a general screening, detected organic compounds can be identified by comparing the mass spectra of the unknown sample with the spectra of several different classes of compounds in the National Institute of Standards and Technology (NIST) mass spectra library. The NIST library contains a collection of nearly

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[1] Mullin, C. A., M. Frazier, J. L. Frazier, S. Ashcraft, R. Simonds, D. vanEngelsdorp and J. S. Pettis. 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoSone 5(3): e9754. doi:10.1371/journal.pone.0009754

240,000 organic mass spectra and chemical identification and structure information. Some labs may also utilize an Automated Mass Spectral Deconvolution and Identification System (AMDIS) program in addition to the NIST library search. The AMDIS program extracts spectra for individual components in a gas chromatography/mass spectroscopy (GC/MS) data file and identifies target compounds by matching these spectra against a reference library. It was developed at NIST with support from the United States Department of Defense and is available without charge.

The attached spreadsheet includes six tables containing information that is derived from approaches used by Region 5, EPA and USDA laboratories in planning and conducting pesticide residue analyses. As previously discussed, **Table 1** identifies six example methods that laboratories have used, depending upon the instrumentation available at the laboratory and the objectives of the investigation. **Tables 2** though **4** identify the pesticide analytes associated with each of the example methods. **Table 5** includes a list of analytes that have been reported in the open literature to have been found in bee-related samples, available median lethal doses to 50% of the adult bees tested (LD_{50} data) and limits of detection (LOD) that are possible with the methods listed. However, the LOD will be dependent on the quality of the samples, the methods used to prepare the samples for analysis and the instrumentation used to conduct the analysis. The LD_{50} values reported in **Table 5** are derived from open literature and their use in this summary should not be construed as an endorsement of their accuracy.

The LD_{50} data provided in **Table 5** is intended only to provide information that may be relevant to an estimate regarding whether an LOD is sufficiently low to reflect an effect level. When no other more useful benchmark is available, laboratories have sometimes used, as a “rule-of-thumb”, whether their method detection limit can achieve 10% of the LD_{50}.

Table 6 lists figures of 10% of LD_{50} values for pyrethroid insecticides and is included primarily for the benefit of laboratories that use GC/MS instrumentation. Experience indicates that detection limits for pyrethroids achievable by GC/MS are generally about 5-10 times higher than LODs for other pesticides. LOD levels achievable by using the QuEChERS methods shown in example methods 3, 5 and 6 of **Table 1** and widely used instrumentation, such as GC/MS, have been found to be around or lower than 10% of the LD_{50} values indicated.

Table 1: EPA Region 5 Bee Inves Investigation S

Sampling amout > 50g is desirable for all examples. Minimum sample amount for each example is shown in Row 6.

	Example #1	Example #4	Example #3		Example #5	Example # 6-Minnesota*	Example #2
Analyte	**see Tables sheet (corresponding lists of analytes are listed in the corresponding tables)**						
Min Sample weight	15 g	3 g	3 g		5 g	10 g	5-10 g - ASE
Extraction solvent	(H2O : 2% TEA/ACN) 12 mL : 15 mL	(H2O : 2% TEA/ACN) 12 mL : 15 mL	(H2O:ACN:CH3COOH) 27 mL (44:55:1)		(H2O:ACN) 30 mL (15:15)	(H2O:ACN) 20 mL (10:10)	
Extraction & equipment **	3min. Tissumizer	3min. Tissumizer or 2 min. Geno grinder	shake 1 min. high speed disperser		vortex & sonicate	vortex	soxhlet (EPA 3540C) or ASE (EPA 3545A)
Salt	6g MgSO4 1.5 g NaOAc	6g MgSO4 1.5 g NaOAc	6g MgSO4 1.5 g NaOAc		6g MgSO4 1.5 g NaOAc	4g MgSO4 1.0 g NaCl	
Clean up	a. 0.5g MgSO4 b. 12 mL to C18 SPE (1g) elute with 2% TEA/ACN	a. 0.5g MgSO4 b. 6 mL to C18 SPE (1g) elute with 2% TEA/ACN	SPE elute with acetone:tol = 7:3 500 mg PSA 250 mg GCB, 800 mg MgSO4	dispersive 150 mg MgSO4 50 mg PSA 50 mg C18	dispersive (for 6mL extarct) 1.5 g MgSO4 1g PSA 0.5 g C18 & 2mL toluene	dispersive 150 mg MgSO4 50 mg PSA 50 mg C18	GPC (EPA 3640A) Florisil (EPA 3620)
Final volume	1ml ISTD solution in H2O/MeOH (75:25) 0.7 um & 0.2 um glass filter	1ml ISTD solution in H2O/MeOH (75:25) 0.2 um glass filter	2 mL to 0.4 mL	27 mL H2O:ACN:CH3COOH	6mL to 1mL	10 mL exchange to tol	as low as possible (5 mL or less)
ISTD	add at Final volume d3-imidacloprid	add at Final volume d3-imidacloprid	add before extraction		add before extraction c13-alaclor d4-imidacloprid	not used	
Sample weight/mL	6.7 g/mL	1.3 g/mL	0.55 g /mL	0.11 g /mL	2g/mL	1g /mL	1-1.5 g/mL
Instrument	LCMSMS	Waters Xevo LCMSMS	GC/MS	LCMSMS	LCMSMS	GC/MS,GCMSMS	GC/MS
Column	Waters HSS-T3				3.5 um 2.1 x 150 mm Agilent Zorbax SB-C18	DB-5 ms capillary column 30m, 0.25 mm id, 0.25 µm film thickness 3.5 um 2.1 x 150 mm Agilent Zorbax SB-C18	
Best matrix	Honey and bee pollen	Bees, pollen,honey	Honey Bees,Wax or Pollen		Bee, pollen, wax, nectar, foliage honey, soil		Any homogenized matrix
Comments	MDL<1ppb, mainly for CCD research	Modified (Example1)for cleaner extract. MDL>1ppb	Recoveries of pH sensitive pesticides are better than original QuEChERS. Extracts may be dirtier than the original QuEChERS.		Recoveries of parent neonics are better than original QuEChERS.	MDLs vary from 10 to 50 ppb, but are less than corresponding LD50s	Not a QuEChERS method

*Original QuEChERS

** during state laboratory review of this table, one laboratory suggested to "leach" bees in preference to extraction after homogenization

Method Notes and References

Example 1	*J. Agric Food Chem.* **2010,** *58* (10), 5926-5931. - EPA Maryland Lab
Example 3	PLoS ONE, 2010, 5 (3), e9754. - USDA Gastonia Lab
Example 5	PLoS ONE, 2012, 7 (6), e39114. - CT Agricultural Experiment Station
The original QuEChERS	*J. AOAC Int.* **2003**, *86* (2), 412-431.
Buffered QuEChERS	*J. AOAC Int.* **2007**, *90* (2), 485-520.

Method examples Nos.1 ,4 and 2 are used by EPA

Example 6 is used by the Minnesota Department of Agriculture laboratory

Analyte Tables

Tables 2 through 4 below describe different pesticide residues, best analyzed by corresponding example methods, shown in Table 1.
Some of the pesticides shown in Table 3 may not be registered in the United States.

Table 2 (for examples 1 and 4) **neonicotinoids and their metabolites**
imidacloprid
imidacloprid olefin
imidacloprid, 5-hydroxy
imidacloprid urea
imidacloprid, desnitro HCL
6-chloronicotinoic acid
dinotefuran
dinotefuran UF
dinotefuran DN phosphate
thiamethoxam
clothianidin

*DMPF – dimethylphenyl formamide
**DMPMF – methyl-dimehylphenil formamidine

Table 3 (for example 6 "Minnesota")	
Acephate	Malathion
Acetochlor	Metazachlor
Alachlor	Methamidophos
Allethrin	Methidathion
Amitraz DMPF*	Methyl Parathion
Amitraz DMPMF**	Metolachlor
Atrazine	Metribuzin
Azinphos Methyl	Mevinphos
Bifenthrin	Monocrotophos
Bromophos	Pendimethalin
Chlorfenvinphos	Permethrin cis & trans
Chlorpyrifos	Phenothrin
Chlorthalonil	Phorate
Clomazone	Phosphamidon
Coumaphos	Pirimiphos-methyl, ethyl
Cyanazine	Prallethrin
Cyfluthrin	Prometon
Cyhalothrin	Propachlor
Cypermethrin	Propazine
Cyphenothrin	Pyrethrins
Deltamethrin	Cinerin I
Desethylatrazine	Cinerin II
Desisopropylatrazine	Jasmolin I
Diazinon	Jasmolin II
Dichlorvos	Pyrethrin I
Dimethenamid	Pyrethrin II
EPTC	Resmethrin
Esfenvalerate	Simazine
Ethalfluralin	Sulfotep
Fenpropathrin	Tefluthrin
Fenthion	Terbufos
Flumethrin	Tetramethrin
Fluvalinate (tau)	Tralomethrin
Fonofos	Triallate
Imiprothrin	Trifluralin
	Vinclozolin

Table 4 (for examples 2,3,5 and 6)
A wide range of pesticides such as OPs, carbamates, pyrethroids, OCls including imidacloprid, dinotefuran, thiamethoxam and clothianidin, but not their metabolites

Table 5 - Pesticide Analytes and corresponding LD 50 information, where available (1)

Pesticide	**Class #**	**GC/LC**	**LD*50 (ppb)***	**possible LOD (ppb)**
1-Naphthol (carbaryl)	Systemic Carbamades	LC	10,500	2
4,4-dibromobenzophenone	Miticides			2
Acephate	Systemic Organonophosphates	GC		35
Acetamiprid	Systemic Neoniccotinoids	LC	99,000	5
Aldicarb Sulfone	Systemic Carbamades	LC	3,730	10
Aldicarb sulfoxide	Systemic Carbamades	LC	3,730	20
Allethrin	Pyrethroids	GC	48,800	1
Amicarbazone	Herbicides	LC		30
Atrazine	Systemic Herbicides	GC	980,000	1
Azinphos methyl	Organophospates	GC	2,420	3
Azoxystrobin	Systemic Fungicides	LC	1,120,000	1
Bendiocarb	Systemic Carbamades	LC		2
Bifenthrin	Pyrethroids	GC	150	0.4
Boscalid	Systemic Fungicides	LC	1,550,000	1
Captan	Fungicides	GC	1,080,000	10
Carbaryl	Partial Systemic Carbamades	LC	10,500	5
Carbendazim	Systemic Fungicides	LC	500,000	1
Carbofuran	Systemic Carbamades	LC		5
Carbofuran, 3-hydroxy	Systemic Carbamades	LC		3
Carfentrazone ethyl	Partial Systemic Herbicides	LC		1
Chlorfenapyr	Partial Systemic Miticides			1
Chlorfenvinphos	Organophospates	GC		6
Chlorferone (coumaphos)	Organophospates	GC	46,300	25
Chlorothalonil	Fungicides	GC	1,110,000	1
Chlorpyrifos	Organophospates	GC	1,220	0.1
Coumaphos	Organophospates	GC	46,300	1
Coumaphos oxon	Organophospates	GC	46,300	5
Cyfluthrin	Pyrethroids	GC	220	1
Cyhalothrin	Pyrethroids	GC	790	0.1
Cypermethrin	Pyrethroids	GC	1,350	1
Cyprodinil	Systemic Fungicides	LC	3,320,000	5
DDD p,p'	Organochlorines	GC		4
DDE p,p'	Organochlorines	GC		3
DDT p,p'	Organochlorines	GC		2
Deltamethrin	Pyrethroids	GC	500	20
Diazinon	Organophospates	GC	2,220	1
Dicofol	Organochlorines	GC	370,000	0.1
Dieldrin	Cyclodienes	GC		4
Difenoconazole	Systemic Fungicides	LC		10
Diflubenzuron	Insect Growth Regulators			10
Dimethomorph	Systemic Fungicides	LC	308,000	15
Diphenamid	Systemic Fungicides			1

Diphenylamine	Fungicides			2
DMA (amitraz)	Formamidines	GC	750,000	50
DMPF (amitraz)	Formamidines	GC	750,000	4
Endosulfan I	Cyclodienes	GC	78,700	0.1
Endosulfan II	Cyclodienes	GC	78,700	0.1
Endosulfan sulfate	Cyclodienes	GC	78,700	0.1
Esfenvalerate	Pyrethroids	GC	2,240	0.5
Ethion	Organophospates	GC		2
Ethofumesate	Systemic Herbicides			5
Famoxadone	Fungicides			20
Fenamidone	Fungicides	LC		10
Fenbuconazole	Systemic Fungicides	LC	1,490,000	6
Fendpropathrin	Pyrethroids			0.4
Fenhexamid	Fungicides	LC	1,580,000	5
Fenoxaprop-ethyl	Systemic Herbicides	GC		6
Fenpropathrin	Pyrethroids		500	0.4
Fipronil	Insecticides		50	1
Fluoxastrobin	Systemic Fungicides	LC		4
Fluridone	Systemic Herbicides			5
Flutolanil	Systemic Fungicides	LC		4
Fluvalinate	Pyrethroids	GC	15,860	1
Heptachlor	Cyclodienes	GC		4
Heptachlor epoxide	Cyclodienes	GC		1
Hexachlorobenzene	Fungicides	GC		0.1
Imidacloprid	Systemic Neonicotinoids	LC	280	2
Imidacloprid olefin	Systemic Neonicotinoids	LC	280	25
Imidacloprid, 5-hydroxy	Systemic Neonicotinoids	LC	280	25
Indoxacarb	Insecticides		600,000	10
Iprodione	Fungicides		1,020,000	10
Malathion	Organophospates	GC	3,950	1
Metalaxyl	Systemic Fungicides	LC		1
Methidathion	Organophospates	GC	2,010	1
Methoxyfenozide	Insect Growth Regulators		1,000,000	0.4
Metolachlor	Partial Systemic Herbicides	GC	1,260,000	2
Metribuzin	Systemic Herbicides	GC	567,000	1
Myclobutanil	Systemic Fungicides	LC	1,870,000	2
Norflurazon	Systemic Herbicides		1,630,000	1
Oxamyl	Systemic Carbamades	LC		5
Oxyfluorfen	Herbicides		1,000,000	0.5
Parathion methyl	Organophospates	GC		1
p -Dichlorobenzene	Organochlorines	GC		6
Pendimethalin	Herbicides	GC	665,000	0.1
Permethrin	Pyrethroids	GC	1120	10
Phenothrin	Pyrethroids			10
Phosalone	Organophospates			10
Phosmet	Organophospates		8,030	2
Piperonyl butoxide	Insecticides	GC		6

Potasan (coumaphos)	Organophospates		46,300	10
Prallethrin	Pyrethroids			4
Pronamide	Systemic Herbicides		1,580,000	1
Propanil	Herbicides			10
Propiconazole	Systemic Fungicides	LC	625,000	3
Pyraclostrobin	Fungicides	LC	870,000	1
Pyrethrins	Pyrethroids	GC	1,480	20
Pyridaben	Miticides			1
Pyrimethanil	Fungicides	LC	1,000,000	2
Pyriproxyfen	Insect Growth Regulators			1
Quintozene = PCNB	Fungicides			1
Sethoxydim	Systemic Herbicides	LC		1
Simazine	Systemic Herbicides	GC	967,000	5
Spirodiclofen	Miticides			1
Spiromesifen	S INS			10
Tebuconazole	Systemic Fungicides	LC		20
Tebufenozide	Insect Growth Regulators		2,340,000	2
Tebuthiuron	Systemic Herbicides		650,000	1
Tefluthrin	Pyrethroids	GC		1
Tetradifon	Miticides			1
Tetramethrin	Pyrethroids	GC		6
Thiabendazole	Systemic Fungicides	LC	500,000	1
Thiacloprid	Systemic Neonicotinoids	LC	252,000	1
Thiamethoxam	Systemic Neonicotinoids	LC		5
THPI (captan)	Partial Systemic Fungicides	GC	10,800,000	30
Triadimefon	Systemic Fungicides	LC		2
Tribufos = DEF	Organophosphates	GC		2
Trifloxystrobin	Partial Systemic Fungicides	LC	1,750,000	0.5
Trifluralin	Herbicides	GC	685,000	1
Vincolzolin	Fungicides	GC	1,000,000	1
Amitraz parent	Formamidines	GC		

(1) The list of chemicals attributed to those found in bee-related samples and the LD 50 values shown in Table 5 have been reported in or derived from open literature and their use in this guidance should not be construed as an endorsement of their accuracy; rather the LD50 data provided in this table is intended only to give a laboratory benchmark values with which to estimate whether the Level of Detection of a specific analyte is sufficiently low to reflect an effect level.

Reference for LD50 data - J. AOAC Int. 2007, 90 (2), 485-520.

Possible LODs - reflects limits of detection that are possible using the methods shown. The LODs do not necessarily reflect those that might be achieved by a given laboratory or specific instument.

Acronyms

GC: Gas Chromatography
LC: Liquid Chromotography
LD50: Lethal Dose 50 - median lethal dose sufficient to kill 50% of a population of animals
LOD: Limits of Detection

Table 6: Pyrethroids and MDLs

Pesticide	GC/LC	LD50 (ppb)	MDL (1/10xLD50)
Allethrin	GC	48,800	4,880
Bifenthrin	GC	150	15
Cyfluthrin	GC	220	22
Cyhalothrin	GC	790	79
Cypermethrin	GC	1,350	135
Deltamethrin	GC	500	50
Esfenvalerate	GC	2,240	224
Fendpropathrin			0
Fenpropathrin		500	50
Fluvalinate	GC	15,860	1,586
Permethrin	GC	1120	112
Phenothrin			0
Prallethrin			0
Pyrethrins	GC	1,480	148
Tefluthrin	GC		0
Tetramethrin	GC		0

Acronyms
GC: Gas Chromatography
LC: Liquid Chromotography
LD50: Lethal Dose 50 - median lethal doses to 50% of the adult bees tested
MDL: Methods Detection Limit