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Review

Sample preparation and gas or liquid chromatography-mass spectrometry for analysis of pesticides in pollen, beebread, bees, and honey

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ABSTRACT

This article reviews the recent analytical methods used for the analysis of pesticides in bee health surveys which include bees, beebread, beeswax, honey, nectar and pollen. Limits of detection (LOD) and limits of quantitation (LOQ) for pesticide residue methods need to be <1/10 the acute toxicity (LD50) for bees which is more challenging for insecticide analysis. LD50's are expressed in units of micrograms/bee where mass of a bee is ~0.1 g. The most frequent multi-residue analysis method used was liquid chromatography-tandem mass spectrometry for pesticide classes including carbamates, conazole fungicides, neonicotinoid insecticides, and strobilurin fungicides. For improved LODs for organochlorines and pyrethroids, GC-MS or GC-MS/MS analysis was also required. Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) was predominately used in recent studies of pesticides in pollen, beebread, and bees which can be attributed to the ease and cost benefit of this approach with considerations of modifications of the method to account for removal of fats and phenolic compounds either in the extraction step or in the clean-up step requiring appropriate selection of the dispersive solid phase extraction (dSPE) sorbent used during clean-up. Sample preparation approaches were more varied for the analysis of pesticides in honey and beeswax and also included solid phase extraction (SPE), liquid-liquid extractions, and specialized miniaturized approaches. LODs varied for many individual target analytes over an order of magnitude which is attributed to the extraction and clean-up approach and chromatographic-mass spectrometry operating conditions. Future method development for pesticide residue analysis should consider further reductions in sample size requirements and combining sample preparation approaches to minimize interferences. Separation conditions in ultrahigh performance liquid-chromatography (UPLC) or nano-LC should be optimized to improve detection limits and further minimize impacts of interferences. This would allow for analysis of environmentally relevant concentrations of pesticides from individual beehives, thereby reducing the need to pool samples to obtain sufficient extract for a more complete analysis of a full range of pesticides in usage.

KEYWORDS: pesticide residue analysis, honeybees, beebread, pollen, liquid chromatography-tandem mass spectrometry.

1. Introduction

The use of pesticides in agriculture has been associated with decreased honeybee colony stability and survival in Americas, Asia, Australia, and Europe [1-16]. Of particular concern is the high amounts of pesticides used on crops including canola and maize, and the lack of diversity of landscapes for foraging in and around these crops within a 2-4 km radius. This is of particular concern for managed

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honeybees used in pollination leading to a higher risk of pesticide exposure in their diet [9, 12, 14, 17-20]. Worker larvae exposed to a diet containing insecticides have been shown to have increased concentrations with age or decreased survival [4, 11, 21-26]. Fungicides, such as pyraclostrobin, have also been associated with decreased longevity of older worker Africanized honeybees [27]. Insecticides and antibacterial substances are registered products in some regions for use by beekeepers for protection against honeybee death incidences caused by parasites of which the most common substances permitted for use for *Varroa* infestations, pending regulatory requirements in different counties, are amitraz, coumaphos (organophosphorus insecticide), fluvalinate-tau or permethrin (pyrethroid insecticides). Other treatments include formic acid and thymol as well as veterinary-prescribed antibiotics including Fumadil-B® or Terramycin® [6, 9, 28]. As a result of concerns particularly related to insecticides and fungicides, the number of surveys of pesticide residues in beebread, beeswax, honey, honeybees, nectar and pollen globally have increased since 2015 as the occurrence of pesticides in beehives is considered one of the critical factors influencing bee health and leading to the increase in incidence of bee mortality [1-6, 16, 24, 29-37]. Toxicity of selected pesticides of concern has been assessed for the most commonly analyzed insecticides and fungicides. Honeybees can be exposed to pesticides by a number of pathways including direct contact during foraging on flowering plants, through diet (pollen and beebread), beeswax stored in beehives, and from exposure to pesticides in air either in the gas and particle phase including dust formed from the sowing of seeds with coated (pesticide) treatments [1, 17, 18, 22, 38-40]. Evidence of occurrence and potential negative impacts on pollinator species has resulted in some pesticides being restricted or banned from usage with recent studies focusing on neonicotinoid insecticides [1, 38, 41, 42]. Although insecticides are the most toxic to bees, fungicides have received attention due to potential impact on the viability of the colony. Fungicides have been associated with an increased incidence of viruses or reduced brood production or survival [1]. Mixtures of insecticides and fungicides further increase the potential toxicity [1]. Herbicides can also lead to direct toxicity or reduced larvae or adult honey bee development although specific studies have been more limited [43].

Pesticide toxicity expressed as oral or contact exposure of bees often controls the requirements for method selection for pesticide residue analysis as the analysis method must be able to detect the pesticide at these low levels. It is necessary to have LODs < 1/10 LD_{50} to be able to detect the pesticide at a concentration relevant to impacts (note LD50s units with bee mass ~0.1 g). To complete analysis of a full range of pesticides both liquid and gas chromatography-mass spectrometric methods are required. Some target pesticides can only be analyzed by one approach (LC or GC) with often a preference to use LC-MS/MS when adequate LODs can be obtained for a variety of sample matrices including bees and bee products (pollen, beebread, honey). Organochlorines (OCs) and pyrethroids (PYR) are typically analyzed by GC-MS or GC-MS/MS, while neonicotinoid insecticides (NEON) and carbamates (CAR) are analyzed by LC-ESI^+-MS/MS. Other classes of pesticides such as OPs (organophosphorous pesticides), conazole fungicides (CZ) and strobilurin fungicides (STROB) can be analyzed by GC or LC approaches with selection often based on specific target analytes, and LC-ESI^+-MS/MS was generally selected for bee and bee product matrices [15, 44, 45]. Table 1 shows that there is a large range in detection limits (LODs) and limits of quantitation (LOQs) even for the most commonly analyzed target analytes in pollen with the greatest challenge in having LODs < 1/10 LD50s for insecticides particularly OPs and PYRs. This can greatly impact the reporting of occurrence of pesticides in the diet of honeybees [46]. Even with the lowest detection limits some target analytes still have LODs above the LD50 (see Table 1). The process of selection of analytical methods depends on the physicochemical properties of the pesticides such as polarity and their stability in solvents which is often a function of pH. This can limit recoveries of base-sensitive pesticides in the extraction and choices of sorbents used in the clean-up steps in sample preparation. In this review, various analytical challenges in sample preparation and chromatography-mass spectrometric analysis of matrices used specifically for bee health studies will be examined as highlighted in Figure 1. In studies with analysis of more than one matrix the LODs were often determined only in one matrix (bees) and these LODs were used to infer detection capability for other matrices such as pollen and beebread even though interferences were significantly different.

Table 1. Target analytes in pesticide residue analysis (pollen) and contact LD50.

Compound	Class	Contact LD50 (µg/bee)	Limit of detection (ng/g)	Limit of quantification (ng/g)	Reference
Fungicides and selected acaracides (A)					
Amitraz	A	50			[6]
Azoxystrobin	STROB	25	1250-25000		[46]
		200	0.05	0.16	[47]
		>200*	0.1	0.3	[27]**
			0.0062	0.0186	[48]
			0.25	2.5	[32]
Boscalid		200	35.5		[2]
		>200*	3.6	12.0	[27]**
		100	2.50	7.50	[32]
			0.12-8.20		[38]
			2.50	7.50	[32]
Carbendazim	BA	756 >50*	37800-756000		[46]
		>50*	0.08-24.00		[38]
		50	0.3	1	[6]
			0.1064	0.3191	[48]
Carboxin		181*	0.12		[38]
Chlorothalonil		111[23] 135*	84.00-1521.00		[38]
Cyflufenamid		100	0.97	3.21	[47]
Cyproconazole II	CZ	>100*	6.5	21.7	[27]**
Cyprodinil		100	0.48	1.57	[47]
		100	0.4		[2]
Difenoconazole	CZ	101	1.4	4.3	[47]*
		101	1.4		[2]
		>100*	0.3	1.1	[27]**
			0.25	2.50	[32]
Epiconazole	CZ	>100*	0.84		[38]
Etoxazole	CZ	>200[a],*		1	[29]
Fenbuconazole	CZ	290*	0.3	1	[27]**
		290	35.5		[2]
			1.00	5.00	[32]
Fluoxastrobin	STROB	>200*	0.01-0.27		[38]
Flusilazole	CZ	150*	0.24		[38]
			0.2	0.7	[27]**
			0.25	2.50	[32]
Fluxapyroxad		100	3.6		[2]

Table 1 continued..

Imazalil		35.1	1755-35100		[46]
		39*	2.2	7.2	[27]**
		39	1	3	[6]
			0.25	2.50	[32]
Iprodione		400	355.3		[2]
		400*	1.1	3.7	[27]**
			10		[5]
			4.10-75.00		[38]
Kresoxim-methyl	STROB	22*	10		[5]
Metalaxyl		141*	0.50		[38]
			1.00	5.00	[32]
Metconazole	CZ	>100*	0.50		[38]
			0.1	0.4	[27]**
Myclobutanil	CZ	33.9	1695-33900		[46]
		39.6	35.5		[2]
Paclobutrazol	CZ		10		[5]
Penconazole	CZ	12	1.9	6.29	[47]
		12*	2	6.8	[27]**
			10		[5]
Penthiopyrad		312	1.4		[2]
Prochloraz		312*	0.33-0.60		[38]
Propamocarb		100	0.19	0.63	[47]
Propiconazole	CZ	67.5[23]	2.9	9.8	[27]**
		50*	10		[5]
Pyraclostrobin	STROB	100	0.05	0.16	[47]
		>100*	1.7	5.8	[27]**
			0.24-12.00		[38]
Pyrimethanil		>100	10		[5]
			0.0145	0.0435	[48]
Spirodiclofen	A	256-252[a]	2		[29]
Tebuconazole	CZ	83	4150-83000		[46]
		>200*	0.19-3.50		[38]
			1.6	5.2	[27]**
			10		[5]
			0.3	1	[6]
		>83.5	0.25	2.50	[32]
			2.8	9.2	[27]**
Thiabendazole	CZ	4	200-4000		[2, 46]
			0.6	0.8	[27]**
			2.50	7.50	[32]

Table 1 continued..

Thiophanate-methyl		100	0.05	0.16	[47]
		100	1.4		[2]
Triadimefon	CZ		10		[5]
			0.0029	0.0088	[48]
Triadimenol	CZ	50*	10		[5]
Trifloxystrobin	STROB	200	0.4	1.1	[47]*
		>110[32]	0.24-4.80		[38]
			8.6	28.7	[27]**
		200	0.4		[2]
			10		[5]
			0.25	2.50	[32]
Triticonazole	CZ	49*	0.24		[38]
Insecticides or insecticide growth regulators					
Acephate	OP	1.78	10		[5]
		1.8*	0.2691	0.8072	[48]
Acetochlor	OC		10	25	[1]
			10		[5]
			1.7	5	[6]
Alachlor	OC		10	25	[1]
			1	3	[6]
Acetamiprid	NEON	7.9*	0.3	0.97	[47]
		7.9	1.4		[2]
		14.53	726.5-14530		[46]
			0.02-0.84		[38]
		7.9-14[a]		1	[29]
			0.3	1.1	[27]**
		0.044	0.6		[5]
			0.3	1	[6]
			0.0114	0.0343	[48]
			0.021	0.070	[49]***
Acrinathrin	PYR	0.17*	1	3	[6]
Azinphos-ethyl	OP		0.3	1	[6]
Azinphos-methyl	OP		0.3	1	[6]
			1.00	5.00	[32]
Biallethrin	PYR		10	25	[1]
Bifenthrin	PYR	0.1	5-100		[46]
		5.35*	50	100	[1]
			0.3	1	[6]
			2.50	7.50	[32]

Table 1 continued..

Carbaryl	CAR	0.84	3.6	10.8	[47]
		0.21	10.5-210		[46]
		0.84	3.6		[2]
		0.84*	0.1087	0.3261	[48]
		0.210	0.25	2.50	[32]
Carbofuran	CAR	0.05	50		[46]
		0.036	10		[5]
		0.16*	20.00		[38]
			0.4	1.4	[27]**
		0.036	10		[5]
		0.3	1		[6]
		0.0060	0.0179		[48]
Chloroantraniliprole		4	0.1	0.31	[47]
Chlorpyrifos methyl	OP	0.28*	10	25	[1]
			1.00	5.00	[32]
Chlorpyrifos	OP	0.25	12.5		[46]
		0.07-0.24[a] 0.072*	21.00-377.00, 1		[38] [29]
		0.059	10		[5]
		0.072, 0.0762[23]	0.3	1	[6]
			1.00	5.00	[32]
Chlorfenvinphos	OP	4.1			[6]
		0.550	0.25	2.5	[32]
Clothianidin	NEON	0.004 (oral)	0.2-5		[46]
		0.0379[5]	0.12-0.72		[38]
		0.044	35.5		[2]
		8.09	0.6		[5]
		0.039*	1	3	[6]
			2.50	7.50	[32]
Coumaphos	NEON	4.6	230-4600		[46]
		20*	4	13.1	[27]**
		24	10		[5]
		20	0.3	1	[6]
			1.00	5.00	[32]
Cyfluthrin	PYR	0.037	35.5		[2]
		0.001	10		[5]
		0.019*	1.00	5.00	[32]
Cyhalothrin (lambda)	PYR	0.022*	11.00-202.00		[38]

Table 1 continued..

Cypermethrin	PYR	0.034*	24.00-430.00		[38]
Deltamethrin	PYR	0.024*	3.5-70		[46]
		0.015	10		[5]
Diazinon	OP	0.09 0.38*	4.5-90		[46]
			3.4	11.3	[27]**
			0.3	1	[6]
			1.00	5.00	[32]
Dichlorvos	OP	0.46*	10		[5]
			0.2483	0.7450	[48]
			2.50	7.50	[32]
Dimethoate	OP	0.1	5-100		[46]
		0.12*	0.3	1	[6]
			0.50		[38]
			0.0366	0.1098	[48]
			0.25	5.00	[32]
Dinotefuran	NEON	0.049*	0.20		[38]
		0.023	1.5		[5]
Disulfoton	OP	3.7*	25	60	[1]
Endosulfan (alpha or beta)	OC	6.3*	25	50	[1]
Fenthion	OP	0.308	15.4-308		[46]
		0.22*	10		[5]
			0.3	1	[6]
			2.50	7.50	[32]
Fenitrothion	OP	0.16	10		[5]
		0.52*	2.50	7.50	[32]
Fenpropathrin	PYR	0.05[23]	10		[5]
Fenvalerate	PYR	0.23	10		[5]
Fipronil		0.0042	0.21-4.2		[46]
		0.007*	0.3	1	[6]
		0.0417[5]			[27]
Fipronil sulfone		0.064[5]			[27]
Flumethrin	PYR	0.05	1	3	[6]
Fluvalinate (tau)	PYR	12.6	630-12600		[46]
			2.50	7.50	[32]
		8.7	0.3	1	[6]
Imidacloprid	NEON	0.0037	0.185-3.7		[46]
		0.044	0.69	2.26	[47]
		0.037[5]	0.05-10.00		[38]
		0.044	3.6		[2]
		0.08	1		[5]

Table 1 continued..

		0.061*	0.3	1	[6]
			0.05	0.13	[7]
			0.0809	0.2427	[48]
			0.25	2.50	[32]
			0.021	0.070	[49]***
Indoxacarb		0.118	35.5, 36*	108*	[2, 47]*
Malathion	OP	0.4	20-400		[46]
		0.47*	10	25	[5]
			0.0610	0.1829	[48]
			1.00	5.00	[32]
Methomyl	CARB	0.28	14-280		[46]
Nitenpyram	NEON	0.138	2.02	6.66	[47]
O-methoate	OP		10		[5]
			1.7	5	[6]
			0.1383	0.4149	[48]
Parathion methyl	OP	2.7*	50	100	[1]
			2.50	7.50	[32]
Phorate	OP	0.32	10		[5]
Phosmet	OP	0.62	355	1077	[47]
		0.62	355.3		[7, 2]
			10		[5]
			0.25	2.50	[32]
Pyridaben		0.024	10		[5]
Pyriproxyfen		100	0.3	1	[6]
			10		[5]
Spinetoram J		0.024	1.4	4.3	[47]
Spinetoram		0.024	1.4		[2]
Spinosad		0.003*	1.4		[2]
		0.003*	1.00		[38]
		0.003-0.057[a]	2		[29]
Spinosad A			0.2	0.6	[27]**
Spinosad A and D			0.3	1	[6]
]Spirotetramat		242-195[a]	1		[29]
Tebrufos	OP	4.1*	15.1	50.4	[27]**
Tetramethrin	PYR	0.16*	10		[5]
Thiacloprid	NEON	37.83	3.6	10.8	[47]
		37.83	3.6		[2]
			0.04-0.91		[38]
			0.1	0.4	[27]**
			0.027	0.090	[49]***

Table 1 continued..

Thiamethoxam	NEON	0.005	0.25-14.5		[46]
		0.050[5]	0.10-1.80		[38]
		0.024	3.6		[2]
		0.3	1		[6]
		0.14	0.36		[7]
		0.0028	0.0084		[48]
		0.005	2.50		[32]
Selected Herbicides					
Atrazine	TRIAZ		10		[5]
			0.3	1	[6]
Bromacil			10		[5]
Fluazifop			50	100	[1]
Metolachlor		110	10		[5]
Pendimthalin			10	25	[1]
Simazine			1	3	[6]
Terbuthylazine	TRIAZ		10		[5]

LD50 taken from reference noted or *Pesticide Manual (2009), ECOTOX and AGRITOX databases; Major pesticide classes: BA, benzimidazole; CAR, carbamates; CZ, conazole fungicides; NEON, neonicotinoid insecticide, OP, organophosphorus pesticide; PYR, pyrethroid insecticide; STROB, strobilurin fungicide; TRIAZ, triazine; **bee matrix; ***honey matrix; LODs unless noted for pollen matrix.

In general, LODs for pesticides are lower in the bee matrix than pollen or beebread due to the greater complexity of the sample matrix such that selection of an appropriate sample preparation method for each sample matrix is critical and needs to be considered along with the final instrumental analysis approach [3, 31, 45, 46, 50]. Pesticides in bees and bee products need to be extracted from solid or liquid matrices followed by removal of residual co-extracts prior to GC-MS or LC-MS/MS analysis in order to minimize occurrence of false positive or negative results [3, 31, 46, 50]. For example, beebread has high levels of carbohydrates >> proteins > fats >ash and has been observed to have higher LODs than pollen [35, 51]. In some cases where several matrices were studied using the same sample preparation method, such as a modified Quick, Easy, Effective, Cheap, Rugged and Safe (QuEChERS) procedure, the LODs of pesticides were influenced by matrix. LODs for pesticides in beeswax were greater than those obtained in honey. LODs of pesticides in both of these matrices were generally higher than reported in beebread or pollen [36, 37]. Most studies only assessed LODs in one matrix or used past validated methods, although there can be significant differences in co-extracts with location of sample collection or new target analytes being included for analysis. Co-extracts if not adequately removed during sample preparation steps can lead to significant mass spectrometric (MS) signal suppression and interferences in both GC and LC separation and MS detection reducing the reliability of the identification and quantitation (see Figure 1). Among the most challenging analytes are chlorpyrifos, o-methoate (and other OPs), clofentezine, cyhalothrin, fenpyroximate, pirprofixen, spinosad, and thiabendazole [6, 29].

2. Discussion

2.1. Challenges associated with modified QuEChERS methods

As shown in Table 2, for studies conducted in the last 6 years pesticides in beebread, honeybees, and pollen are almost exclusively extracted with further clean-up using modified QuEChERS methods [1-3, 5-8, 16, 24, 29, 30, 34, 35, 37, 47, 48, 54-57] although other methods such as accelerated solvent

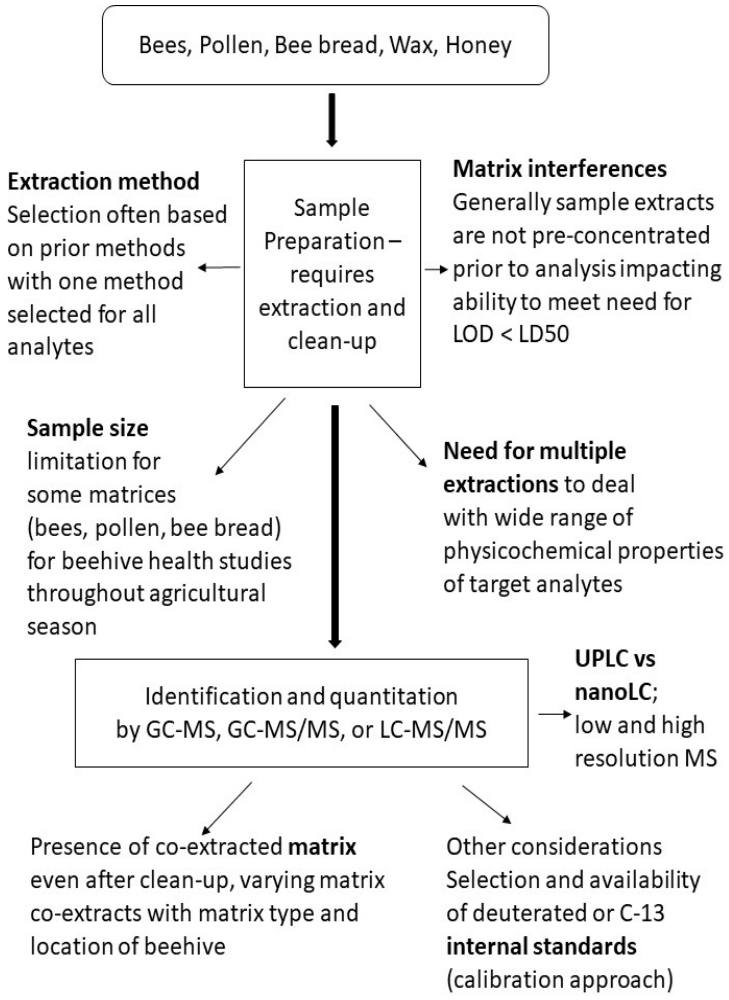

Figure 1. Analytical challenges in the sample preparation and chromatography-mass spectrometry analysis of pesticides in bees and bee products from beehives.

extraction have been previously used [58]. This is mainly due to the ease and low cost of QuEChERS methods, and the ability of acetonitrile salt-out extraction to provide acceptable recoveries for a wide range of moderate polarity to relatively polar pesticides (excluding highly polar herbicides) analyzed most frequently with LC-ESI$^+$-MS/MS. Modified QuEChERS methods were initially developed for fruits, vegetables, and other food matrices with further application to the extraction of pesticides from bees, beeswax, pollen, beebread, wax or honey. The first sample preparation methods for extraction of pesticides from bees or bee product matrices were applications of QuEChERS and these modified QuEChERS methods continue to be the basis of recent methods. Muller *et al*. (2010) developed the first modifications of QuEChERS focusing on reducing sample size requirements for pollen to 3 g and further changing the clean-up step to include solid phase extraction (SPE) using primary-secondary amine (PSA) and graphitized carbon black (GCB) rather than dispersive SPE (dSPE) to improve the capacity of the sorbent used in SPE for removal of interfering matrix components. This method focused on GC-MS amenable pesticides [15]. Wiest *et al*. (2011) also reduced sample sizes using 2 g of pollen or 5 g of honeybees with citrate buffer rather than acetate buffer [50]. The most significant change to the QuEChERS method was the addition of hexane in the salt-out extraction step for additional removal of lipids [50]. When dSPE was used for clean-up

Table 2. Sample preparation methods used in bee health survey studies.

Study location (matrices)	**Extraction method Note: Solvents and salts used for salt-out extraction and special conditions**	**Clean-up approach after extraction/volume supernatant used Note: dSPE salts or other clean-up approach**	**Sample size**	**Analysis technique Note: Unless specified GC-ionization is EI**	**Pesticide targeted**	**Ref.**
QuEChERS or modified QuEChERS methods						
Brazil (pollen)	15 mL MeCN, 4 g $MgSO_4$, 1 g NaCl, citrate buffer	95 mg PSA, 750 mg $MgSO_4$/ 1mL	2 g	GC-MS/MS with selected analysis by LC-FLD	MR (OC, OP, PYR)	[1]
China (pollen and nectar)	10 mL H_2O, 10 mL MeCN, 4 g $MgSO_4$, 1 g NaCl, citrate buffer (QuEChERS EN 15662)	400 mg PSA, 200 mg C18, 1200 mg $MgSO_4$/ all	5 g	GC-MS and LC-ESI^+-MS/MS	MR (48 pesticides OC, OP, NEON, STROB and CZ)	[5]
Spain (pollen)	2 mL H_2O, 4 mL MeCN, 0.8 g $MgSO_4$, 0.2 g NaCl, 0.2 g Na_3citrate, 0.1 g Na_2Hcitrate sesquihydrate , fat-freeze	50 mg PSA, 50 mg C18, 300 mg $MgSO_4$/2 mL. subsequent 1 mL extract SPE 40 mg Z-Sep	2 g	GC-MS/MS (solvent exchange) and LC-ESI^+-MS/MS)	MR	[54]
Spain, (pollen)	8 mL H_2O:MeCN 25:75 v:v, 1 g $MgSO_4$, 0.5 g NaCl, 0.8 g Na_3citrate, fat-freeze	10 mg PSA, 150 mg $MgSO_4$/ 2 mL, then dried with final volume 1 mL, 0.45 µm nylon filter	1 g	LC-ESI^+-MS/MS	Spinetoram J and L	[55]
Spain (pollen)	15 mL MeCN:H_2O 70:30 v/v, 1 g NaCl; fat freeze	1000 mg EMR-Lipid/all, dried final volume 2 mL, filter 0.45µm nylon filter	0.5 g	LC-ESI^+-MS/MS	Flubendiamide (insecticide)	[56]
Spain (beebread, honeybee, beewax)	7.5 mL H_2O, 10 mL MeCN; 6 g $MgSO_4$, 1 g NaCl; for beeswax 10 mL MeCN heated to 80 °C, then fat freeze.	50 mg PSA, 50 mg C18, 150 mg $MgSO_4$/ 1 mL (2 mL for wax), in-line filter vial 0.22 µm PTFE	5 g beebread honeybee; 2 g for wax	LC-ESI^+-MS/MS	MR (63 pesticides OP, PYR, CAB, TRIAZ, NEON)	[16]
China (pollen and nectar)	10 mL MeCN, 3 mL hexane, 4 g $MgSO_4$, 1 g NaCl	50 mg PSA, 150 mg $MgSO_4$ /2 mL, dried diluted to 0.2 mL, filtered 0.22 µm nylon	2 g	LC-ESI^+-MS/MS	NEON (IMI and THM)	[7]
France (beebread)	5 mL H_2O, 5 mL heptane, 10 mL MeCN with 2 % trimethylamine, 1.5g NaOAc, 6 g $MgSO_4$; fat-freeze -18 °C	150 mg PSA, 900 mg $MgSO_4$/6mL, 4 mL dried with final volume 560 µL	2g	UPLC-ESI^+-MS/MS	NEON, PYR	[57]

Table 2 continued..

China (beebread and pollen)	3 mL H_2O; 10 mL MeCN with acetate buffer; 2 g glass beads; 0.5 g $MgSO_4$	1.25 g of 1:1:3:0.15 w:w:w:w PSA:C18: $MgSO_4$:GCB)/5 mL, 2.5 mL dried with final volume 0.5 mL, 0.22 µm filter	2 g	LC-ESI^+-MS/MS (UPLC , 3 µL injection); GC-MS	MR includes PYR; OP; STROB; CZ; NEON	[34]
Greece (honeybee, pollen, honey)	7 mL MeCN, 2 mL hexane, 3 mL H_2O, 500 mg $MgSO_4$, 200 mg NaOAc, 200 mg PSA; *2% trimethylamine in MeCN for selected NEONs	500 mg $MgSO_4$, 200 mg PSA/7 mL; subsequently ExtraBond C18 cartridge 200 mg; preconcentration to 1 mL and PTFE filtered	1 g	LC-ESI^+-MS/MS	MR (115 analytes including NEON, OP, CZ, CAR, dicarboximides, dintroanilines,	[3]
USA (honeybee, pollen, and wax)	10 mL (20 mL for wax) 7:3 EtAc: hexane with 0.2% HOAc	500 mg Z-Sep /3 mL, dried with final volume 0.5 mL	1 g (honey bees or pollen), 2 g wax	GC-MS (NCI for PYR, OPs, and chlorothalonil) eI for atrazine	MR (11 pesticides) PYR, OP, selected others	[8]
Egypt (wax)	20 mL MeCN:EtAc (1:3), water bath 60 °C, fat-freeze -20 °C	200 mg PSA, 1000 mg $MgSO_4$/ 2mL, 0.45 PTFE filter	2 g	GC-MS/MS and LC-ESI^+-MS/MS	MR (>300 pesticides)	[59]
USA (beebread, pollen)	15 mL H_2O, 15 mL MeCN, 150 µL HOAc, 1.5 g NaOAc, 6 g $MgSO_4$	4 mL MeCN, 1.5 g MgSO4, 0.5 g PSA, 0.5 g C18/8 mL, 7 mL preconcentrated to 1 mL, filter 0.21 µCostar spin-filter	3g (beebread combined from 4 hives at 0.75 g/hive)	LC-ESI^+-MS/MS	MR (NEON, CAR, PYR; CAZ, selected others)	[2, 47]
Spain (honeybee, pollen)	4 mL H_2O, homogenized, 5 mL 1% HOAc in MeCN ultrasonic assisted; AOAC method with 4 g $MgSO_4$:NaOAc (4:1); or EN method: citrate buffer, $MgSO_4$, NaCl	dSPE with final selection 200 mg alumina, compared to PSA, C18, or 100 mg PSA+100 mg C18	1 g	LC-ESI^+-MS/MS	10 pesticides of varying class	[29]
China (pollen)	4 mL H_2O, 2 g glass beads, 10 mL MeCN with acetate buffer, 0.5 g $MgSO_4$	50 mg PSA, 50 mg C18, 3.75 mg GCB, 150 mg $MgSO_4$/ 5 mL, 2.5 mL dried with final volume 0.5 mL, 0.22 µm membrane filter	1 g	UPLC-ESI^+-MS/MS	MR (54 pesticides including OPs, NEON, CARB)	[48]

Table 2 continued..

Australia (beebread, honey, pollen, and bees wax)	27 mL of 45:55 v/v H_2O:MeCN with 6 g $MgSO_4$, acetate buffer based on modified QuEChERS[41]	For LC: dSPE 50 mg PSA, 50 mg C18, 150 mg MgSO4; for GC SPE with GCB/PSA 250mg/500 mg and 80 mg $MgSO_4$ added to top with 2 mL supernatant loaded, drying and final volume 0.4 mL –no differences with matrix specified	3 g	GC-MS and LC-ESI^+-MS/MS	MR 174 pesticides	[35]
Thailand (pollen)	27 mL of 45:55 v/v H_2O:MeCN with 16 g $MgSO_4$, acetate buffer based on modified QuEChERS[41]	For LC: dSPE 50 mg PSA, 50 mg C18, 150 mg MgSO4; for GC SPE with GCB/PSA 250mg/500 mg and 80 mg $MgSO_4$ added to top with 2 mL loaded, drying and final volume 0.4 mL –no differences with matrix specified	3 g	GC-MS and LC-ESI^+-MS/MS	MR 174 pesticides	[23]
Mexico (beeswax and honey)	6 g $MgSO_4$, 1.5 g NaOAc with sample, then 15 mL MeCN,	400 mg PSA, 400 mg C18, 1200 mg $MgSO_4$/8 mL	15 g	GC-MS/MS or LC-ESI^+-MS/MS (Q-TOF)	MR 93 pesticides	[37]
(beebread, beebrood, beeswax)	5 mL H_2O, 5 mL MeCN, ultrasonic assisted, 2 g $MgSO_4$, 0.5 g NaCl, 0.5 g Na_3 citrate $2H_2O$, 0.25 g Na_2Hcitrate sesquihydrate	SPE 40 mg Z-Sep/1 mL with elution with MeCN giving 2.5 fold dilution; 750 mg $MgSO_4$ and 125 mg PSA for wax/5 mL	2 g beebread or brood, 10 g wax	GC-MS/MS and LC-ESI^+-MS/MS	MR (30 pesticides)	[24]
Poland (honeybee)	10 mL H_2O, 1% acetic acid in 10 mL MeCN, 4 g $MgSO_4$, 1 g NaCl, 1 g Na_3citrate $2H_2O$, 0.5 g Na_2Hcitrate sesquihydrate, fat-freeze -60°C,	100 mg EMR-lipid/ 8 mL, 0.2 µm PTFE filter	2, 5, 10 g	LC-ESI^+-MS/MS	MR (52 pesticides (OP, NEON, CAR)	[52]
(pollen)	2 mL H_2O, 2 mL MeCN, 0.4 g $MgSO_4$, 0.2 g NaCl	Disposable pipette tip with 125 mg PSA with 100 µL extract with MeCN for condition and elution	1 g	LC-ESI^+-MS/MS	NEON only (THM, ACE, THC)	[60]
Spain (beeswax, honeybees, pollen)	7.5 mL H_2O, 10 mL MeCN, 6 g $MgSO_4$, 1 g NaCl	50 mg C18, 50 mg PSA, 150 mg $MgSO_4$/ 1mL	5 g	LC-ESI^+-MS/MS	MR (63 pesticides) including OP, PYR, NEON	[6]

Table 2 continued..

USA (pollen)	~10 mL H_2O, 15 mL MeCN, 6 g $MgSO_4$, 1.5 g NaOAc, 150 µL HOAc	500 mg PSA/500 mg C18, 2 mL toluene/ 10 mL, then 6 mL extract dried to 1 mL	5 g	LC-ESI^+-MS/MS	MR	[30, 53]
Italy (honey)	11 mL MeCN, 4 g $MgSO_4$, 1 g NaCl, citrate buffer	400 mg PSA, 400 mg C18, 1200 mg $MgSO_4$/ 8 mL	5 g	GC-MS/MS	MR (66 pesticides varying classes including PYR, OP, NEON, STROB, CZ, CAR	[61]
Poland (honey)	10 mL H2O, 10 mL of 1% formic acid in MeCN, 4 g $MgSO_4$, 1 g NaCl, citrate buffer	200 mg Chitosan, 200 mg aluminum oxide, 200 mg C8	2, 5, 10 g	LC-ESI+-MS/MS	MR(132 insecticides including NEO, OP, CAR, PYR)	[62]
(beeswax)	10 mL MeCN; 4 g $MgSO_4$, 1 g NaCl and citrate buffer (QuEChERS, EURL-FV-2010)	150 mg PSA; 900 ,g $MgSO_4$/ 6 mL, then 1 mL diluted with 0.22 mL MeCN; filter	10 g	LC-ESI^+-MS/MS	MR	[33]
Other sample preparation methods						
Italy (pollen)	H_2O/ MeCN, liquid/liquid purification with hexane;	MSPD with PSA, salts (amounts not specified); preconcentration to 0.1 mL	10 g	LC-ESI^+-MS/MS	MR (66 pesticides different classes)	[32]
China (nectar, pollen)	Eppendorf tube 0.5 mL H_2O, 1 mL MeCN, 50 mg $MgSO_4$, 50 mg NaCl	20 mg PSA, 20 mg C18, 50 mg $MgSO_4$/0.8 mL; 0.45 µm PTFE filter	0.1 g (pollen) (0.02 g nectar)	nanoLC-high res MS	NEON (ACE, CLO, IMI, NIT, THM), coumaphos, fluvalinate	[63]
China (honey)	4 mL MeCN/H_2O (60/40 v/v)	No clean-up	2 g	LC-ESI^+-MS/MS	NEON (ACE, IMI, THC)	[49]
(honey)	Diluted with 10 mL H_2O, half taken for anion-exchange-disposable pipette extraction (DPX) column	No clean-up	5 g	LC-ESI^+-MS/MS	NEON and selected 9 other pesticides	[64]
(honey)	10% (w/v) Na_2SO_4 in sample volume, 100 µL octanol, in-couple syringe transfer 4 times	No clean-up	10 mL	LC-photodiode array	NEON	[35]

Table 2 continued..

Spain (beeswax)	10 mL MeOH/EtAc (70/30 v/v), freeze dry ice	1 g EMR-Lipid/all supernatant, dried with final volume 1 mL, 0.45 µm nylon filter	1 g	LC-ESI$^+$-MS/MS (QTOF)	NEON	[66]
(honey and royal jelly)	Honey diluted in two stages with 20 mL H_2O; 5 mL diluted in 15 mL H_2O. Royal jelly 10 mL H_2O followed by 5 mL MeOH; 5 mL for subsequent dilution with 25 mL H_2O	SPE Oasis HLB (500 mg, 6 cc) followed by preconcentration and solvent exchange with final volume 10 mL, 0.22 µm nylon filter	2 g	LC-ESI$^+$-MS/MS	NEON	[66]
Italy (honey)	Accelerated solvent extraction with 34 mL cell (in-line filter 0.2 µm, 2 g PSA with MeCN	In-line (during extraction), dried and final volume 1 mL	5 g	GC-MS/MS	MR (53 pesticides including PYR, OP, NEON, STROB, AZ, Carbamates	[61]

Notes: Acetate buffer: 1% HOAc (acetic acid) in acetonitrile and 1.5-2 g NaOAc added; Citrate buffer: 1 g trisodium citrate dehydrate and 0.5 g disodium hydrogencitrate sesquihydrate; dried refers to drying step to dryness and reconstitution in solvent for analysis with final volume noted; all refers to total volume supernatant taken for clean-up; acetonitrile - MeCN; ethyl acetate - EtAc; methanol - MeOH; $MgSO_4$ specified is anhydrous $MgSO_4$; MR – multiresidue.

following the salt-out extraction the most common sorbents continue to be primary-secondary amine (PSA) and C18 as shown in Table 2. The majority of methods used 1 to 4 mL of water per gram of pollen or bee or other bee products during the wetting stage with centrifugation or homogenization and optionally added ceramic chips or glass beads or used an ultrasonic bath with or without shaking and vortexing of the sample to improve recoveries [29, 54, 48]. Acetonitrile remains the most commonly used organic solvent for extractions particularly for bee, beebread, and pollen matrices. When acetonitrile or acetonitrile/water mixtures are used in the salt-out extraction, precipitation of proteins and low solubility of fats in acetonitrile results in their removal such that the supernatant taken for subsequent clean-up already contained a few interferences [48, 54]. Multiresidue methods commonly used only LC-ESI$^+$-MS/MS with one sample preparation approach such as a modified QuEChERS with 45% of methods for pollen or beebread matrices using acetate buffer and 25% of methods using citric acid buffer to minimize potential degradation of base sensitive pesticides. When OCs, OPs, and pyrethroids were analyzed using GC-MS there was no strong preference for buffered salt-out extractions. Acidification of the acetonitrile at 1% with either acetic acid or formic acid is used to improve the stability of pesticides that are prone to degradation at basic pH and improve recoveries for analytes such as OPs, NEONs, selected methylcarbamates, and spinosad [29, 52]. Other analytes including λ-cyhalothrin, o-methoate, and thiabendazole also observed low recoveries without acidification [6]. Citrate buffers (European Method EN 15662) or acetate buffer (AOAC Official 2007.01 method) have also been used particularly for certain classes of pesticides [22]. In some cases primarily when GC-MS analysis was used such as for pyrethroids and OCs, pollen may be extracted directly into acetonitrile with salt-out extraction; however matrix effects were generally severe although LODs in the 5-50 ng/g and LOQs in the 10-100 ng/g can still be obtained [1]. When water was not added to the sample prior to extraction or if water was not added to the extraction solvent (acetonitrile), it was necessary to add hexane to the extraction solvent in order that lipids from the sample matrix could be precipitated out during the salt-out extraction step as dSPE was not capable of removing the levels of lipid interferences typically present in bee product matrices [7].

Honeybees and pollen are considered a complex sample matrix and even after salt-out extraction and the dSPE or SPE clean-up step interferences may still be present in the sample extract used for subsequent analysis by GC-MS or LC-MS/MS. It is common that matrix interferences from bee product matrices co-elute with pesticides of interest in the separation or cause chromatographic disturbances or mass spectrometric signal suppression or enhancement. For example, the most common interferences remaining after salt-out extraction of pesticides and subsequent clean-up of pollen samples is phenolic compounds. In this case the sample extract also has a yellow coloration. Advancements in extraction and clean-up methods have focused on modifying existing QuEChERS methods to reduce co-extracts either during the initial extraction stage or often during the dispersive solid phase extraction (dSPE) clean-up of all or a fraction of the supernatant (acetonitrile layer). The use of ultrasonic-assisted extraction can improve the homogenization of samples, but for some analytes such as spinosad or fenpyroximate that bind strongly to bee or bee product matrices, recoveries of pesticides are lower or no significant improvements were observed [29]. Ultrasonic-assisted extraction has been used as a modification to QuEChERS when citrate buffer is also used, and has been applied to the extraction of pesticides from a variety of matrices including beebread, larvae and beeswax. Typically a fat-freeze step is also used as a further modification of QuEChERS in the salt-out extraction when the sample matrix is beebread due to the need for removal greater levels of lipids [24]. For most analytes citrate and acetate buffers along with dSPE either using $MgSO_4$, PSA and C18 or GCB, or EMR-lipid or Z-Sep+ often resulted in recoveries that are within the acceptable range of 70-110% with acceptable relative standard deviation of replicated recovery tests (10-20% of each other). As a number of different dSPE sorbents can remove common interferences the selection of the sorbent used in the dSPE clean-up step depends more on the target list of priority analytes in the subsequent GC-MS or LC-MS/MS analysis to ensure that the

dSPE sorbent does not strongly bind to a desired analyte leading to low recoveries. Anhydrous magnesium sulfate is used to remove residual water in the extract during clean-up. PSA was the most common dSPE sorbent which is used to remove organic acids, sugars and polar pigments commonly encountered in pollen and beebread samples, while C18, Z-Sep, Z-Sep+, EMR-Lipid are used to remove residual lipids and sterols with the choice often depending on the fat content remaining in the supernatant and pesticides analyzed [8, 24, 34, 35, 48, 54, 56]. PSA with C18 was the most frequently used materials for dSPE clean-up (see Table 2).

For removal of additional fat from extracts, graphitized carbon black (GCB) or Z-Sep, Z-Sep+, and EMR-Lipid are often recommended. However, as with other sample matrices the use of GCB for clean-up of pollen can lead to recovery losses for some analytes including chlorpyrifos, clofentezine, dichlrovos, pyrimethanil, spirodiclofen, and spirotetramat that can strongly bind to GCB [29, 48], and Z-Sep+ (zirconia oxide and C18 bound on silica) has also been observed to cause signal enhancement for chlorpyrifos and pirimiphos-ethyl and low recoveries (<70%) for clothianidin, formentanate, o-methoate, monocrotophos, thiodicarb [52]. GCB can be used with PSA, C18, and $MgSO_4$ if the amount of GCB is reduced to 36 mg/5 mL supernatant and has been shown to provide acceptable recoveries for 66 target analytes in beebread and pollen [34]. OPs and NEON such as chlorpyrifos, dicrotophos, clothianidin, dinotefuran, imidaclorpid and monocrotophos are prone to losses in recoveries when Z-Sep+, EMR-lipid, or chitin are used with recoveries still in acceptable range for chlorpyrifos and clothianidin [52]. Enhanced matrix removal lipid (EMR-lipid) has been shown to have promise for removal of lipids from high-fat content samples and provided the greatest % of analytes with soft or moderate matrix effects as compared with other dSPE sorbents particularly when combined with fat-freezing after an acetate buffer salt-out extraction for multiresidue analysis [52] or targeted analysis for a less commonly analyzed insecticide (flubendiamide) [56]. Fat-freezing step improved recoveries by 5-10% for some analytes when either Z-Sep, Z-Sep+ or EMR-lipid was used, but some analytes exhibited lower recoveries such that this approach is still not widely used [52, 54]. In most cases fat-freezing step is used in the salt-out extraction (acetonitrile with citrate buffer) as a means of homogenizing the fat with the protein during the precipitation of solids to reduce requirements for lipid removal in the subsequent dSPE clean-up [54, 55]. Acetate buffer in the acetonitrile salt-out extract was used for the salt-extraction of beebread with fat-freezing used to improve recoveries and reduce matrix effects observed for neonicotinoids [57]. Citrate salts without the use of ultrasonic-assisted extraction was found to give the best recoveries for spinosad and required only the use of alumina or PSA (alone) to obtain acceptable recoveries, while good recoveries for spirodiclofen and spirotetramat were obtained with ultrasonic-assisted extraction and commonly used dSPE sorbents [29]. The use of alumina was found to be a low cost alternative to the widely used PSA for selected pesticides of different chemical classes, but has not been widely assessed for multiresidue methods [29]. A selective method for spinetram J and L also took advantage of fat-freezing following citrate-buffered acetonitrile salt-out extraction to obtain acceptable recoveries with PSA and C18 as dSPE sorbents [55].

Another approach of removing the fat in the sample was to perform a triple solvent extraction in the initial step with the addition of hexane to a miniaturized QuEChERS, which builds on the initial method developed by Weiss *et al.* 2011 [50]. In addition, advancements in sample preparation methods have focused on further reduction in sample size requirements with 1 g pollen or honey sample used along with a volume ratio of 3 mL: 7 mL:2 mL for water:acetonitrile:hexane [3]. With this approach, trimethylamine is added to acetonitrile along with PSA during extraction followed by a C18 SPE clean-up which allowed for a pre-concentration step with final volume of 1 mL. LODs improved and reduced matrix effects for neonicotinoids (clothianidin, imidacloprid, thiamethoxam) and carbendazim were observed [3]. EMR-lipid also provided similar or better recoveries for OPs, NEONs and spinosad from the honeybee matrix as compared to other dSPE sorbents when fat-freezing was used [52]. Modified QuEChERS with hexane without wetting of pollen

also resulted in improvements in recoveries and LOD and LOQ for imidacloprid and thiamethoxam [7]. Another alternative miniaturized QuEChERS method (1 g pollen) for selected NEONs was able to achieve acceptable recoveries using a salt-out extraction with no acidification with subsequent clean-up using only PSA loaded in a disposable pipette tip with reduced volume of extract undergoing clean-up to eliminate phenolic compound interferences [60]. A salt-out extraction with acetate buffer in acetonitrile and toluene was completed followed by dSPE clean-up with PSA and C18. The use of toluene removed more matrix such that extracts could be preconcentrated (6 mL to 1 mL) with good recoveries for most analytes with the exception of pyrethroids at concentrations near LODs [30, 53].

2.2. Extraction of pesticides from wax and honey sample matrices

Sample preparation methods for wax and honey samples had the largest diversity of approach. Modified QuEChERS methods with acetate buffer were less commonly used for honey and beeswax matrices than beebread and pollen [35, 36] with improvements in LODs for GC-amenable pesticides when SPE rather than dSPE was used [15, 35]. Most commonly dSPE with PSA and C18 were used when LC-ESI^+-MS/MS analysis was completed; however other sorbents including aluminum oxide, C8 and Z-Sep were also used when the extraction was citrate buffered [8, 33, 62]. Modified QuEChERS methods often required dilution of honey (e.g. 10 g/10 mL) with water and addition of ammonium hydroxide (aimed at improving recoveries particularly for amitraz) with magnesium sulfate used for phase separation. For analysis of pesticides (NEONs, amitraz and fipronil) in honey using atmospheric pressure chemical ionization positive ion mode (LC-$APCI^+$)-MS/MS rather than LC-ESI^+-MS/MS, clean-up of the supernatant from QuEChERS was not required if the sample of honey was diluted with water [67]. Due to the greater complexity of sample matrices and expected higher amounts of co-extracts a number of methods (modified QuEChERS and other methods) also utilized SPE clean-up, and pesticides from diluted honey were also directly extracted using SPE [3, 65]. Pesticides with low recoveries in wax using QuEChERS with acetonitrile included 2,4-D, cyromazone, ethirimol, fipronil, pyretrozine, but a large range of pesticides observed acceptable recoveries with only dSPE using PSA and C18 [33]. As honey samples can be diluted with water or directly undergo extraction into an organic solvent the reduced cost of using QuEChERS methods relative to other extraction and clean-up methods is not significant and consequently there is a greater variety of approaches for the honey matrix.

For more direct extraction approaches, honey (~70% glucose and fructose) requires matrix-induced sugaring-out to remove the sugars from the sample matrix. Mixtures of acetonitrile-water are used to remove the sugars with 60:40 v/v (acetonitrile/H_2O) optimal composition to obtain acceptable recoveries for neonicotinoid insecticides. [49]. Further improvements were made using temperature-assisted liquid-liquid extraction and salt-out extraction with acetonitrile [49]. Optionally, sugars in honey can be removed using a disposable pipette anion-exchange extraction after the honey is diluted with water to provide acceptable recoveries of neonicotinoid insecticides [64]. Dilution of honey or royal jelly prior to SPE (Oasis HLB) has also been used to obtain acceptable recoveries for neonicotinoid insecticides particularly for nitenpyram which is prone to lower recoveries with other extraction methods [65]. A small scale extraction of neonicotinoid insecticides from honey diluted with water was achieved using transfer between two in-coupled syringes [66]. This allowed for the partitioning of the neonicotinoid insecticides into 100 µL of octanal for subsequent analysis [66]. Some advantages of using accelerated solvent extraction for extraction of pesticides from honey are that it did not require dilution of the honey samples and in-line clean-up during extraction with PSA could be used with a preference to use acetonitrile as the extraction solvent over a mixture of hexane/ethyl acetate for better recoveries of pesticides [61].

In the sample preparation of beeswax, wax must be solubilized in an organic solvent which requires heating, and a liquid-liquid extraction with acetonitrile would require heating near its boiling point which is not feasible. Consequently rather than using 100% acetonitrile, a mixture of

ethyl acetate: acetonitrile (1:3 v/v) was used such that a temperature of only 60 °C was required [59]. This results in lower LODs particularly for more nonpolar pesticides (pyrethroids) [59]. Prior methods for beeswax have also used hexane/isopropanol (8:2 v/v) with heating to 50 °C followed by addition of water for liquid-liquid extraction. Clean-up of the supernatant was achieved using diatomaceous earth in SPE format where the eluted fraction from SPE was pre-concentrated prior to analysis of neonicotinoid insecticides [68]. Methanol has also been used for large-scale (1 kg) beeswax extraction at 62-65 °C with a second extraction capable of removing OPs and pyrethroids from the wax [69]. In some cases solubilisation of the wax in acetonitrile was accomplished with subsequent salt-out extraction after addition of water and citrate buffer with good recoveries of flumethrin using magnesium sulfate and PSA in the clean-up [69].

2.3. Miniaturizing sample size in order to reach LODs of environmentally relevant concentrations relative to LD50s

In general, improvements in sample preparation methods which are still ongoing have focused on further reducing sample size requirements with many studies still using 2 to 10 g amounts. These miniaturization approaches should also consider the use of ultrahigh performance liquid chromatography-MS/MS which is a common laboratory upgrade to LC systems. NanoLC-high resolution MS shows promise as less matrix enters into the instrument with lower injection amounts needed. Miniaturized extraction methods are aimed at reducing amounts of sample required to 0.5-1 g or less. Further reduction in this sample size was made to assess the levels of pesticides in parts of bees without the need for a QuEChERS method. The pesticides in samples of homogenized bee parts (5.8-40 mg) were directly extracted into 300 µL acetonitrile followed by centrifugation to remove solids. This extract was then diluted at 1:5 (v/v) with water to reduce the amount of matrix injected [70]. The final extract was filtered prior to injection with a 0.45 µm nylon filter and pesticides were analyzed by nanoflow LC-high resolution MS using a 1 µL injection and flow rate of 200 nL/min to minimize matrix effects [70]. UPLC has also been used to improve LODs and LOQs for multiresidue analysis following an acetate-buffered acetonitrile salt-out extraction with flow rate of 0.45 mL/min and 3 µL sample injection bringing most LODs to <0.15 ng/g and LOQs to < 1 ng/g [48]. LOQs <0.5 ng/g and recoveries >90% were obtained for neonicotinoid insecticides and coumaphos with a miniaturized QuEChERS using an Eppendorf tube with only 0.1 g pollen required and PSA/C18 for clean-up followed by dilution (1:5) and analysis by nano-LC-high resolution MS [63].

Pre-concentration of extracts was another approach used to obtain lower LODs if co-extracts particularly fats were adequately removed during clean-up and this was used with methods that utilized SPE clean-up, fat-freeze, dSPE with EMR-lipid, or heptane or hexane in the salt-out acetonitrile extraction or liquid/liquid extraction [3, 7, 33-35, 37, 48, 55, 57, 66]. With LC-ESI$^+$-MS/MS most often used only a few methods optimized mobile phase conditions to minimize matrix interferences or to obtain the lowest LOQs for their target analytes included in the pesticide monitoring surveys of different sample matrices and this can also lead to a significant variation in LODs and LOQs reported [48, 63, 70]. Pesticides that were preferentially analyzed by GC-MS or GC-MS/MS methods included pyrethroids (such as lambda-cyhalothrin, beta-cypermethrin, deltamethrin, fenvalerate) and organochlorines which generally have low response with LC-ESI$^+$-MS/MS [34]. Conazole fungicides, strobilurin fungicides, some neonicotinoids, chlorthalonil, quizalofop-p-ethyl and boscalid can be analyzed by GC-MS, but LC-ESI$^+$-MS/MS methods were more commonly used for multiresidue analysis of these sample matrices [34].

3. Conclusions and future outlooks

Current methods for extraction, clean-up, and GC-MS or LC-MS/MS analyses of pesticides in bees, beebread, beeswax, nectar and pollen provide adequate detection for >90% of commonly analyzed pesticides. The two greatest challenges for pesticide analyses are obtaining LODs <1/10 LD50s and obtaining adequate recoveries particularly for insecticides. Greater consistency in method selection for specific analytes with low recoveries would aid in lowering LODs and LOQs below LD50s,

improving stability, and reducing matrix interferences. One area of further direction should include further efforts to miniaturize sample preparation methods along with increased access to analysis using UPLC or nano-LC. This would minimize the need to pool samples from different beehives and allow for assessments of differences in the occurrence of pesticides at individual beehives as well as the impact of pesticides on the phenotype of pollen origin and reduce sample size requirements for collection from beekeepers.

CONFLICT OF INTEREST STATEMENT

The author declares that there are no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

1. Cabrera de Oliveira, R., Queiroz S. C. N., Luz, C. F. P., Porto R. S. and Rath, S. 2016, Chemosphere, 163, 525.
2. McArt, S. H., Fersch, A. A., Milano, N. J., Truitt, L. L. and Boroczky, K. 2017, Scientific Reports, 7, 46554.
3. Kasiotis, K. M., Anagnostopoulos, C., Anatasiadou, P. and Machera, K. 2014, Sci. Total. Environ., 485-486, 633.
4. Chaimanee, V., Chantawannakul, P., Khongphinitbunjong, K., Kamyo, T. and Pettis, J. S. 2019, J. Apicultural Res., 58, 720.
5. Wen, X., Ma, C., Sun, M., Wang, Y., Xue, X., Chen, J., Song, W., Li-Byarlay, H. and Luo, S. 2021, Sci. Total Environ., 76, 147443.
6. Calatayud-Vernich, P. Calatayud, F., Simo, E. and Pico, Y. 2018, Environ. Pollut., 241, 106.
7. Jiang, J. Ma, D., Zou, N., Yu, X., Zhang, Z., Liu, F. and Mu, W. 2018, Chemosphere, 201, 159.
8. Fulton, C. A., Hartz, K. E. H., Fell, R. D., Brewster, C. C., Reeve, J. D. and Lydy, M. J. 2019, Chemosphere, 222, 489.
9. Gierer, F., Vaughan, S., Slater, M., Thompson, H. M., Elmore, J. S. and Girling, R. D. 2019, Environ. Pollut., 249, 236.
10. Traynor, K. S., vanEngelsdorp, D. and Lamas, Z. S. 2021, Ecotoxic. Environ. Safety, 214, 112105.
11. Mokkapati, J. S., Bednarska, A. J. and Laskowski, R. 2021, Sci. Total Environ., 775, 145588.
12. Iwasaki, J. M. and Hogendoom, K. 2021, Agriculture, Ecosystems and Environ., 314, 107423.
13. Agrebi, N. E., Wilmart, O., Urbain, B., Danneels, E. L., de Graaf, D. C. and Saegerman, C. 2019, Sci. Total Environ., 687, 712.
14. Hrynko, I., Lozowicka, B. and Kaczynski, P. 2019, Sci. Total Environ., 657, 16.
15. Mullin, C. A., Frazier, M., Frasier, J. L., Ashcraft, S., Simonds, R., vanEngelsdorp, D. and Pettis, J. S. 2010, PLoS One, 5, e9754.
16. Calatayud-Vernich, P., Calatayud, F., Simo, E., Aguilar, J. A. P. and Pico, Y. 2019, Chemosphere, 232, 471.
17. Abou-Shaara, H. F. 2014, Veterianarni Medicina, 59, 1.
18. Barraud, A., Vanderplanck, S. Nadarajah, S. and Michez, D. 2020, Acta Oecologica, 105, 103552.
19. Pohorecka, K., Sczena, T., Witek, M., Miszczak, A. and Sikorski, P. 2017, Comput. Struct. Biotechnol. J., 61, 105.
20. Goulson, D., Nicholls, E., Botias, C. and Rotheray, E. L. 2015, Science, 347, 1255957.
21. Milone, J. P., Rinkevich, F. D., McAfee, A. and Foster, L. J. 2020, Exotox. Environ. Safety, 206, 111213.
22. Bohme, F., Bischoff, G., Zebitz, C. P. W., Rosenkranz, P. and Wallner, K. 2019, J. Apicultural Res., 58, 542.
23. Traynor, K. S., vanEngelsdorp, D. and Lamas, Z. S. 2021, Ecotoxic. Environ. Safety, 214, 112105.
24. Morales, M. M., Ramos, M. J. G., Vazquez, P. P., Galiano, F. J. D., Valverde, M. G., Lopez, V. G., Flores, J. M. and Fernandez-Alba, A. R. 2020, Sci. Total Environ., 710, 136288.
25. Milone, J. P. and Tarpy, D. 2021, Scientific Reports, 11, 1020.
26. Zhu, W., Schmehl, D. R., Mullin, C. A. and Frzier, J. L. 2014, PLoS One, 9, e77547.
27. Domingues, C. E. C., Inoue, L. V. B., Silva-Zacarin, E. C. M. and Malaspina, O. 2020, Environ. Pollut., 266, 115267.

28. Frazier, M. T., Mullin, C. A., Frasier, J. L., Ashcraft, S. A., Leslie, T. W., Mussen, E. C. and Drummond, F. A. 2015, J. Econ. Entomol., 108, 2141.
29. Garcia-Valcarcel, A. I., Martinez-Ferrer, M. T., Campos-Rivela, J. M. and Guil, M. D. H. 2019, Talanta, 204, 153.
30. Drummond, F. A., Ballman, E. S., Eitzer, B. D., Clos, B. D. and Dill, J. 201, Environ. Entomology, 47, 378.
31. Niell, S., Jesus, F., Perez, N., Perez, C., Pareja, L., Abbate, S., Carrasco-Letelier, L., Diaz, S., Mendoza, Y., Cesio, V. and Heinzen, H. 2017, Sci. Total Environ., 581-582, 25.
32. Tosi, S., Costa, C., Vesco, U., Quaglia, G. and Guido, G. 2018, Sci. Total Environ., 615, 208.
33. Lopez, S. H., Lozano, A., sosa, A., Hernando, M. D. and Fernandez-Alba, R. 2016, Chemosphere, 163, 44.
34. Tong, Z. Duan, J., Wu, Y., Liu, Q., He, Q., Shi, Y., Yu, L. and Cao, H. 2018, Sci. Total Environ., 640-641, 1578.
35. Vichapong, J., Burakham, R. and Srijaranai, S. 2015, Talanta, 139, 21.
36. Manning, R. 201, J. Apicultural Res., 57, 696.
37. Valdovinos-Flores, C., Alcantar-Rosales, V. M., Gaspar-Ramirez, O., Saldana-Loza, L. M. and Dorantes-Ugalde, J. A. 2017, J. Apricultural Res., 56, 667.
38. Zioga, E., Kelly, R., White, B. and Stout, J. C. 2020, Environ. Res., 19, 109873.
39. Raina-Fulton, R. 2015, J. Agric. Food Chem., 63, 5152.
40. Stoner, K. A., Cowles, R. S., Nurse, A. and Eitzer, B. D., 2019, Environ. Entomology, 48, 351.
41. Sgolastra, F., Medrzycki, P., Bortolotti, L., Mainia, S., Porrini, C., Simon-delso, N. and Bosch, J. 2020, Biological Conservation, 241, 108356.
42. Auteri, D., Arena, M., Barmaz, S., Ippolito, A., Linguadoca, A., Molnar, T., Sharp, R., Szentes, C., Vagenende, B. and Verani, A. 2017, Sci. Total Environ., 579, 966.
43. Macri, I. N., Vazquez, D. E., Pagano, E. A., Zavala, J. A. and Farina, W. M. 2021, Insects, 12, 163.
44. Raina, R. 2011, Chemical analysis of pesticides using GC/MS/MS, GC/MS/MS, and LC/MS/MS. Pesticide Strategies for Pesticide Analysis, Prof Margarita Stoycheva (Ed.), ISB:978-953-307-460-3, Intech, 105-130.
45. Stachniuk, A. and Fornal, E. 2016, Food Anal. Methods, 9, 1654.
46. Toseli, G. and Sgolastra, F. 2020, Chemosphere, 258, 127358.
47. Urbanowicz, C., Baert, N., Bluher, S. E., Boroczky, K., Ramos, M. and McArt, S. H. 2019, Apidologie, 50, 379.
48. Tong, Z., Wu, Y., Liu, Q., Shi, Y., Zhou, L., Liu, Z., Yu, L. and Cao, H. 2016, Molecules, 21, 1652.
49. Chen, W., Wu, S., Zhang, J., Yu, F., Hou, J., Miao, Z. and Tu, X. 2019, Molecules, 24, 2761.
50. Weist, L., Bulete, A., Giroud, B., Fratta, C., Amic, S., Lambert, O., Pouliquen, H. and Arnaudguilhem, C. 2011, J. Chromatogr. A, 121, 5743.
51. Dramca, F., Ursachi, F. and Oroian, M. 2020, Foods, 9, 1357.
52. Kaczynski, P., Hrynko, I. and Lozowicka, B. 2017, Ecotox. Enirvon. Safety, 139, 124.
53. Stoner, K. A. and Eitzer, B. D. 2013, PLoS One, 8, e77550.
54. Vazquez, P. P., Lozano, A., Ucles, S., Ramos, M. M. G. and Fernandez-Alba, A. R. 2015, J. Chromatogr. A, 1426, 161.
55. Ruiz, P., Ares, A. M., Nozal, M. J., Martin, M. T. and Bernal, J. 2020, Microchem. J., 154, 104546.
56. Bernal, J., Nozal, M. J., Martin, M. T., Bernal, J. L. and Ares, A. M. 2019, Microchem. J., 148, 541.
57. Giroud, B., Vauchez, A., Vulliet, E., Wiest, L. and Bulete, A. 2013, J. Chromatogr. A, 1316, 53.
58. United States Environmental Protection Agency (EPA), 2013, A summary of analytical methods used for the analysis of pesticides in bees and hive matrices. U.S. Environmental Protection Agency Region 5, Land and Chemicals Division, Chemicals Management Branch.
59. Issa, M. M., Taha, S. M., El-Marsafy, A. M., Khalil, M. M. H. and Ismail, E. H. 2020, J. Chromatogr. B, 1145, 122106.
60. Tu, X. and Chen, W. 2020, Molecules, 25, 5703.

61. Chiesa, L. M., Labella, G. F., Panseri, S., Britti, D., Galbiati, F., Villa, R. and Arioli, F. 2017, Food Additives Contam. Part A, 34, 809.
62. Hrynko, I., Lozowicka, B. and Kaczynski, P. 2018, Food Anal. Methods, 11, 2307.
63. Moreno-Gonzalez, D., Cutillas, V., Hernando, M. D., Alcantara-duran, J., Garcia-Reyes, J. F. and Molina-Diaz, A. 2020, Sci. Total Environ., 715, 137005.
64. Song, S., Zhang, C., Chen, Z., He, F., Wei, J., Tan, H. and Li. X. 2018, J. Chromatogr. A, 1557, 51.
65. Hou, J., Xie, W., Hong, D., Zhang, W., Li, F., Qian, Y. and Han, C. 2019, Food Chem., 270, 204.
66. Valverde, S., Ares, A. M., Bernal, J. L., Nozal, M. J. and Bernal, J. 2018, Microchemical Journal, 142, 70.
67. Tomasini, D., Sampaio, R. F., Caldas, S. S., Buffon, J. G., Duarte, F. A. and Primel, E. G. 2012, Talanta, 99, 380.
68. Yanez, K. P., Bernal, J. L., Nozal, M. J., Martin, M. T. and Bernal, J. 2013, J. Chromatogr. A, 1285, 110.
69. Luna, A., Alonso, R., Cutillas, V. M., Ferrer, C. M., Gomez-Ramos, M. J., Hernando, D., Valverde, A., Flores, J. M., Fernandez-Alba, A. R. and Fernandez-Alba, A. R. 2021, Environ. Technol. Innov., in press https://doi.org./10.1016/j.eti.2021.101606.
70. Moreno-Gonzalez, D., Alcantara-duran, J., Gilbert-Lope, B., Beneito-Cambra, M., Cutillas, V. M., Rajski, L., Molina-Diaz, A. and Garcia-Reyes, J. F. 2018, J. AOAC International, 101, 367.