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QuEChERS-Based Method for the Multiresidue Analysis of Pesticides in Beeswax by LC-MS/MS and GC×GC-TOF

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ABSTRACT: The validation of an analytical procedure for the determination of pesticide residues in beeswax, an interesting matrix for environmental pollution monitoring, is presented. Using the QuEChERS template, the impacts of wax particle size, sample amount, and cleanup procedure (water addition, dispersive solid phase extraction, freeze-out, and combinations thereof) on extraction yield and coextractive load were studied. Sample preparation through liquid−liquid partitioning between acetonitrile and melted wax (~80 °C), followed by freeze-out and primary−secondary amine dispersive cleanup, was performed on incurred and pesticide-free samples for 51 residues. Determinations were made through LC-MS/MS and GC×GC-TOF, and the whole procedure was validated. Matrix effects were evaluated, with recoveries between 70 and 120% and RSDs below 20% in almost all cases. LC-MS/MS LOQs ranged from 0.01 to 0.1 mg/kg for most pesticides, but for GC-amenable pesticides, GC×GC-TOF sensitivity was lower (0.1−0.2 mg/kg). This methodology can be applied for routine analysis of pesticide residues in beeswax.

KEYWORDS: *beeswax, QuEChERS, pesticides*

INTRODUCTION

Beeswax is a pharmaceutical and cosmetic commodity listed in the U.S. and EU Pharmacopeias. It is also widely used as a food additive (E 901), for example, for the coating of fresh fruits, dried fruits, sweets, and cheeses, as well as a component of different polishing waxes. Beeswax is a very complex mixture of lipophilic compounds. Mono-, di-, and triesters of long-chain aliphatic alcohols with fatty acids or hydroxy-fatty acids constitute the largest fraction representing ca. 65% of the total weight. Free fatty acids, mostly C26 and C30, and long-chain hydrocarbons represent ca. 12% each. Other components of beeswax are free hydroxy acids, free aliphatic alcohols, and carotenoids. In ancient times it was believed that beeswax was collected from flowers or made from pollen; it was not until 1744 that H. C. Hornbostel discovered that it is synthesized by four pairs of wax-secreting epidermal glands on the ventral side of worker bees' abdomens. Bees use wax mainly for building the honeycombs; they remove, reshape, mold, and use it over and over again. The combs are literally the nursery, walls, storage pantry, home, pharmacy, and dance floor for the colony.[1]

Pesticides can enter the hive either directly or indirectly.[2,3] Various acaricides, such as coumaphos, amitraz, and fluvalinate, are applied in beekeeping for *Varroa* mite control, but other environmental contaminants also find their way into the hive when bees fly around searching for nectar and pollen. For this reason honey bees and bee products have been used as bioindicators of environmental pollution in several countries.[4−9] An emerging problem for apiculture is caused by the fact that beeswax is widely recycled when establishing a new hive, thus leading to a progressive accumulation of pesticides in it. In several studies conducted in different countries, residues of numerous pesticides have been found in beeswax.[3,10−16]

There are only a few methods described in the literature for the analysis of pesticide residues in beeswax. The most reported methodology is the single-residue one.[14] There are some other methods for the quantitative analysis of particular groups of pesticides, such as acaricides[11,12] or lipophilic pesticides,[17] using either LC or GC techniques.[2,3,18] Mullin et al. analyzed 259 real samples from beehives in the United States covering a very broad scope of GC- and LC-amenable analytes. A total of 87 different pesticides and metabolites were identified using the QuEChERS approach as sample preparation method. Nevertheless, the evaluation of the analytical method employed was not published.[19] The methodologies employed in the studies mentioned above involved mainly beeswax dissolution followed by liquid−liquid extraction and solid phase extraction for cleanup.

A simple variation of the QuEChERS method[20] allowing the determination of 51 pesticides in beeswax by LC and GC is presented in this work. Thirteen GC-amenable and 38 LC-amenable pesticides employed in this study were selected on the basis of their relevance for beeswax as reflected by the frequency of residue findings in beeswax and other apiarian products according to the literature and the pesticides-online

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Table 1. Mass Spectrometry Settings for the LC-MS/MS System[a]

pesticide	first transition $m/z \rightarrow m/z$	DP	CE	CXP	second transition $m/z \rightarrow m/z$	DP	CE	CXP
amitraz	294.3 → 121.9	51	43	8	294.3 → 163.2	51	21	14
atrazine	216 → 174	66	25	12	218.1 → 176.1	51	25	10
azoxystrobin	404.2 → 329	46	41	18	404.2 → 344.1	46	35	20
boscalid	343.1 → 139.9	76	29	12	343.1 → 306.8	76	29	18
carbaryl	202.2 → 127.1	46	39	8	202.2 → 145.1	46	13	10
carbendazim	192.2 → 132	56	43	22	192.2 → 160.1	51	25	10
carboxin	236.1 → 143.1	46	23	8	236.1 → 87	46	35	14
chlorpyrifos-ethyl	351.9 → 200	61	25	1	351.9 → 97.1	61	53	4
clodinafop-propargyl	350.1 → 238.2	41	31	4	350.1 → 266.1	41	23	4
coumaphos	363 → 227	81	37	12	363 → 306.9	81	25	18
cyhalothrin-λ	467.1 → 225.1	51	23	12	467.1 → 450.1	51	15	6
dimethoate	230.1 → 124.9	46	29	10	230.1 → 170.9	46	21	14
epoxiconazole	330.1 → 121.1	51	29	8	330.1 → 75	51	101	6
hexythiazox	353.1 → 168	66	37	14	353.1 → 227.7	61	23	14
fenpropathrin	367.2 → 125.1	41	23	6	367.2 → 350.1	41	11	4
imidacloprid	256.1 → 175	51	19	10	256.1 → 209	51	21	12
iprodione	330.1 → 244.9	61	21	14	330.1 → 287.9	61	19	16
linuron	249 → 159.9	61	25	10	249 → 182	61	23	12
metalxyl	280.2 → 192.1	61	25	10	280.2 → 220.1	61	21	12
methomyl	163.1 → 105.9	46	15	18	163.1 → 107.1	46	11	8
methoxyfenozide	369.3 → 149.1	21	23	4	369.3 → 313.2	21	15	6
metolachlor	284.1 → 176.1	46	35	10	284.1 → 252	51	21	16
metribuzin	215.2 → 187.1	66	25	12	215.2 → 74.1	66	49	12
myclobutanil	289.2 → 125	61	49	8	289.2 → 70	61	37	12
omethoate	214.1 → 124.9	51	31	8	214.1 → 154.8	51	23	14
pirimicarb	239.2 → 182.1	51	23	12	239.2 → 72	51	37	12
profenofos	373.1 → 128.1	41	55	4	373.1 → 302.9	41	25	4
pyraclostrobin	388.1 → 163	51	35	14	388.1 → 194	51	19	16
tebuconazole	308.1 → 125	51	55	4	310.1 → 70	51	43	4
tebufenozide	353.3 → 133	16	25	4	353.3 → 297.2	16	15	4
tetraconazole	371.9 → 159	61	49	10	371.9 → 70	61	51	4
thiacloprid	253.1 → 126	41	29	4	255.1 → 128	41	27	4
thiamethoxam	292 → 131.9	26	29	4	292 → 181.2	26	29	4
thiodicarb	355 → 107.9	61	21	20	355 → 87.9	56	33	14
thiophanate-methyl	343.1 → 151	76	23	14	343.1 → 310.8	76	17	18
triflumuron	359.1 → 139	31	47	4	359.1 → 156.2	31	23	4

[a]DP, declustering potential; CE, collision energy; CXP, cell exit potential.

database[21] as well as on the basis of recent data concerning the usage and import of pesticides in Uruguay. This paper focuses on the validation of an analytical procedure for the determination of pesticide residues in beeswax, a peculiar matrix often containing high levels of incurred pesticides, as well as on the strategies applied to overcome the analytical difficulties found.

EXPERIMENTAL PROCEDURES

Chemicals and Standards. Acetonitrile and methanol of HPLC quality were from Merck (Darmstadt, Germany). Water was deionized in the laboratory using a Millipore (Billerica, MA, USA) Direct-Q 3 water purification system. Ammonium formate was purchased from Sigma-Aldrich (Steinheim, Germany). Magnesium sulfate anhydrous, reagent grade, was from Sigma-Aldrich (St. Louis, MO, USA), and formic acid p.a. (98−100%) was purchased from Merck. A solution of 5% formic acid (v/v) was prepared in acetonitrile. The bulk amino sorbent (Bondesil-PSA, 40 μm) was from Varian (Palo Alto, CA, USA). Analytical standards, ≥95% purity, were from Dr. Ehrenstorfer (Augsburg, Germany), Riedel-de Haen (Seelze, Germany), and LGC Promochem (Wesel, Germany). Stock standard solutions of 1 mg/mL were prepared, considering standard purity, by weighing individual analytical standards into flasks and dissolving and diluting them to accurate volume with acetonitrile using an automatic dispenser.

Working standard mixtures were prepared by appropriately diluting multiple stock solutions with acetonitrile using an automatic dispenser. The stock solutions of PCB 138 and chlorpyrifos D_{10}, which were employed as internal standards (ISTDs), were prepared in the same way as the pesticide stock standard solutions. An ISTD working mixture containing 20 μg/mL of each ISTD was also prepared in acetonitrile. The ISTD working mixture was mainly used for spiking the matrix prior to sample preparation in recovery experiments. An appropriate dilution of this ISTD solution to 2 μg/mL with acetonitrile was also prepared and was mainly used for the preparation of matrix-matched calibration standards. All solutions, except the ISTD working solution, were stored in the dark at 4 °C.

Apparatus. The automatic dispenser was an OPUS model (20 or 50 mL) from Hirschmann Laborgeräte (Eberstadt, Germany). A Repro high-precision sample divider from Bürkle (Bad Bellingen, Germany) was employed to portion the solids needed for dSPE.

GC×GC-TOF analysis was performed with a Pegasus 4D GC×GC/TOF-MS from Leco Corp. (St. Joseph, MI, USA). Sample injection volume was 3 μL with helium as carrier gas at a flow rate of 2 mL/min. The initial inlet temperature was set at 40 °C and increased after 0.6 min to 300 °C at a rate of 720 °C/min with a hold for 2 min and then decreased to 280 °C at the same rate with a hold for 5 min. The split valve remained open during the first 30 s following injection to allow

solvent evaporation at a flow rate of 20 mL/min and a pressure of 0.73 psi (solvent vent mode). After this 30 s period, the split valve was closed again (to transfer the analytes to the analytical column) and reopened at 2 min after injection. The first-dimension column was a DB-5MS (30.3 m, 0.25 mm i.d., 0.25 μm df). The temperature profile started at 80 °C, which was kept for 2 min and then ramped at 30 °C/min to 220 °C, then ramped again at 8 °C/min to 280 °C, and finally ramped at 20 °C/min to 300 °C with a hold for 10 min. The second-dimension column was a VF-17 ms (1.8 m, 0.1 mm i.d., 0.1 μm df), and the temperature profile was exactly the same as the first dimension only with a temperature offset of +30 °C. The second-dimension separation time was set to 4 s and controlled with an internal thermomodulator creating a hot/cold pulse. This instrument utilizes electron impact ionization (EI). The ion source temperature was set at 200 °C. The detector voltage was set at 1600 V and the filament bias at −70 V. The mass range collected was 70−550 amu with an acquisition rate of 200 spectra/s.

LC-MS/MS was performed with a Waters (Milford, MA, USA) Acquity UPLC system coupled to an API 4000 Qtrap MS/MS from Applied Biosystems (Foster City, CA, USA) run in the MS/MS mode. LC separation was performed on a Phenomenex (Torrance, CA, USA) Aqua 150 mm × 2 mm, 3 μm particle size, C18 column. The operation of the LC gradient involved the following two eluent components: (A) water/ammonium formate 5 mM; (B) methanol/ammonium formate 5 mM (%). It was run at 100 μL min^{-1} starting with 100% component A at injection time and gradually changing to 30% A (70% B) over 3 min, then to 15% A (85% B) over 3 min at 300 μL min^{-1}, followed by a shift to 10% A (90% B) over 3 min. This eluent composition was kept for 3 min and then shifted back to the starting conditions (100% component A) and kept there until 22 min after injection. The injection volume was 5 μL. MS/MS detection was performed in the multiple-reaction monitoring (MRM) mode using an ESI interface in the positive ion mode. The ionization voltage was 4500 V, the nebulizer gas was synthetic air at 70 psi, and the curtain gas was nitrogen at 30 psi. The solvent evaporation in the source was assisted by a drying gas (heated synthetic air at 425 °C/50 psi). Numerous experiments using solutions of the individual analytes were performed to determine the optimal MRM transitions, collision energies (CE), and declustering potentials (DP) for each individual compound. For this, the standard solutions were infused directly to the instrument by a syringe at constant flow. The MS/MS settings used in this study are listed in Table 1.

Methodology. Wax (2 ± 0.1 g) was weighed into a 50 mL centrifuge tube, 10 mL of acetonitrile was added, and the tube was closed and placed in a water bath at ∼80 °C. After the wax had melted, the tube was shaken vigorously for 10−15 s and placed back into the water bath to melt again. The procedure was repeated four times. Then, the tube was left to cool to room temperature and put into the freezer (−18 °C, for at least 2 h, e.g., overnight) for precipitation of the wax. An aliquot of the extract was transferred into a PP single-use centrifugation tube, which contained 25 mg of PSA primary−secondary amine (PSA) and 25 mg of C18 sorbent per milliliter of extract; the internal standard was added and the tube shaken vigorously for 30 s and centrifuged for 5 min at 3000 U/min. Finally, the cleaned extract was transferred into a screw-cap vial, and the pH was quickly lowered to ca. 5 by adding a 5% formic acid solution in acetonitrile (v/v) (10 μL/mL extract) and injected in LC-MS/MS and/or GC×GC-TOF.

Matrix-Matched Calibration and Recovery Calculations. Five-level matrix-matched calibration curves were constructed for matrix effects evaluation. Two-level calibrations within the dynamic range were performed for the evaluation of each recovery level: calibrant 1 at 60% and calibrant 2 at 120% of the aimed concentration of each level (0.01, 0.1, and 0.2 mg/kg). Calibration levels were prepared by adding the desired volume of appropriate dilution of working standard solutions to 1 mL of blank extract and equaling all of the final volumes within a batch (recoveries, blanks, and calibration vials) with acetonitrile. Recoveries were calculated as the percentage from the known added concentration of pesticides of the calculated concentration of the extract by interpolation.

RESULTS AND DISCUSSION

Impact of Sample Amount on Pesticide Residue Recoveries. The distribution of pesticides between the added acetonitrile and the liquefied beeswax phase depends on the volume ratio of the phases and the lipophilicity of the pesticides as reflected by their Log K_{OW} values. Increasing the beeswax phase volume will affect the partitioning equilibrium of the pesticides and consequently their recoveries, with lipophilic pesticides experiencing the highest losses. Influence of wax sample size was studied as follows: Different sample amounts of wax (0.5, 1, 1.5, 2, 3, 4, and 5 g) were spiked with 200 μL of a pesticide mixture containing 1 μg/mL of each pesticide dissolved in acetonitrile (0.2 μg of each pesticide) and immediately extracted according to the described procedure.

Figure 1 shows how the variation of the sample size affected the recoveries for some of the most lipophilic pesticides. As

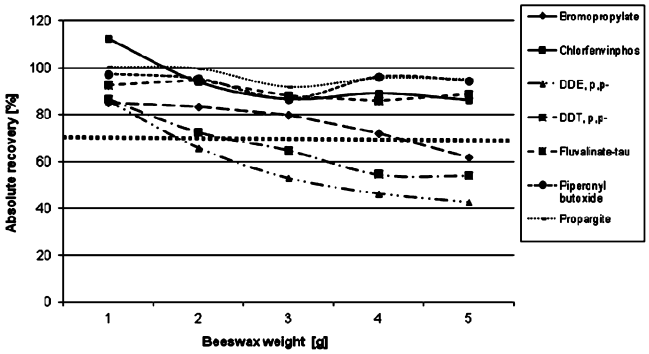

Figure 1. Influence of sample weight on pesticide recovery.

expected, pesticide recoveries decreased as the volume of the wax phase increased. The recovery drop was most pronounced for pesticides with strong lipophilic character such as DDT, DDE, and bromopropylate (Log K_{OW} values of 6.91, 6.36, and 5.4, respectively) that have a strong tendency to remain in the liquefied wax rather than to partition into the acetonitrile phase. On the basis of the results of this experiment, a ratio of 2 g of wax sample to 10 mL of acetonitrile was considered as a good compromise giving acceptable recoveries (70−120%) for almost all tested wax-relevant pesticides and a tolerable load of coextractives in the final extract of ca. 0.7 mg/mL, which corresponds to ca. 0.35% of the initial matrix weight employed for extraction (200 mg/mL). The same sample to acetonitrile ratio has been employed for the extraction of pesticide residues from vegetable oils.[20] The authors reported similar observations regarding the matrix load in the final extracts. It should be noted that when the ratio between wax and acetonitrile is kept constant, the recoveries achieved should be in principle highly reproducible, thus allowing for recovery-based result corrections with the help of suitable recovery factors derived from validation experiments.

Extractability of Incurred Residues Depending on Particle Size. The poor ability of acetonitrile to dissolve highly lipophilic compounds is, on the one hand, beneficial because it limits the amount of lipids (including waxes) dissolved in the raw and cleaned-up extract; on the other hand, it also considerably limits the accessibility of apolar pesticides incorporated in these lipids, which is the key to achieving good extraction yields and a broad scope method.

To demonstrate the importance of melting the wax to obtain optimal accessibility of residues enclosed in wax and to check

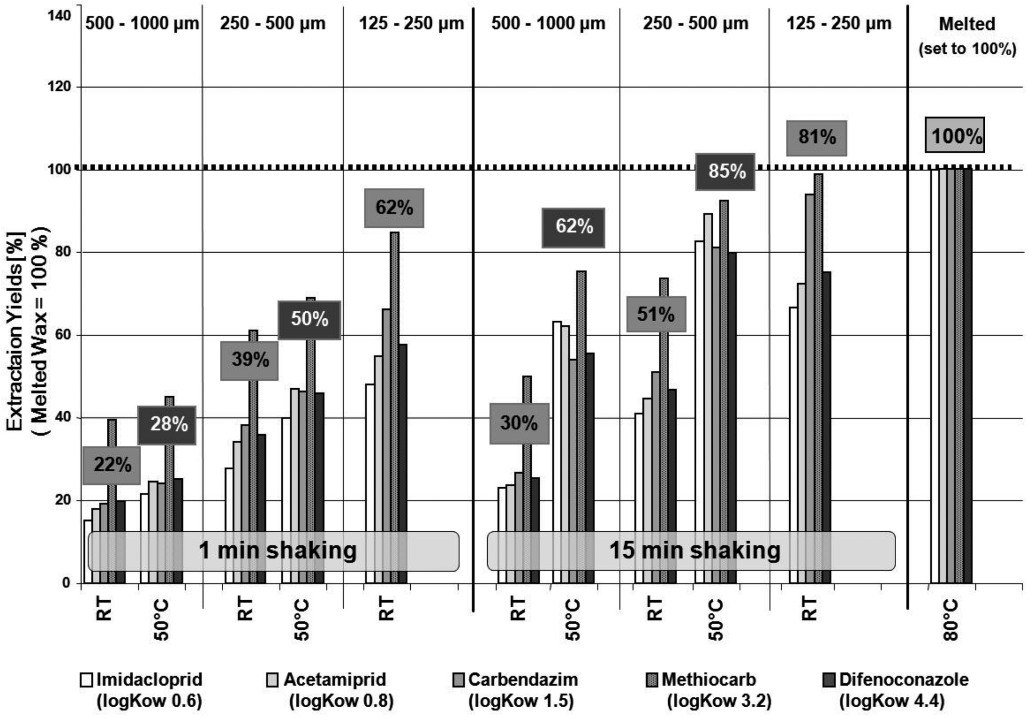

Figure 2. Impact of particle size (fractions 125−250, 250−500, and 500−1000 μm), temperature (room temperature, 50 and 80 °C), and shaking time (1 and 15 min) on the extraction yields of pesticides incorporated in beeswax. Results obtained when the wax was extracted at 80 °C (using the procedure described here) were set to 100%, n = 3, in each case. The percentage values in boxes indicate the average relative extraction yields of five wax-incorporated pesticides (imidacloprid, acetamiprid, carbendazim, methiocarb, and difenconazole).

how the comminution grade of wax affects extractability, a certain amount of beeswax was melted and spiked with a mixture of pesticides covering a broad polarity range at a level of ca. 0.1 mg/kg. By spiking the melted beeswax, a homogeneous residue distribution within it was ensured. Then, the spiked wax was deep frozen (−80 °C), intensively milled with a knife mill, adding some dry ice to keep it cold, and finally sieved into fractions (125−250, 250−500, and 500−1000 μm). Afterward, 2 g of each beeswax fraction was extracted for 1 or 15 min with acetonitrile without the addition of water. The extractions were conducted in triplicate at two different extraction temperatures: ambient (room temperature) and 50 °C. The results obtained for each pesticide as well as for all pesticides together were averaged. The results obtained when the wax was extracted at 80 °C (in melted condition as described under Methodology) were set to 100%. Figure 2 shows the results of this experiment, whic simultaneously demonstrates the positive effect of a good comminution grade (larger surface area) and of higher temperatures (better extraction kinetics and soaking of the wax) on the pesticide yields. Nevertheless, even for the finest wax fraction (125−250 μm) the average relative extraction yield at ambient temperature was only 81% compared to the yield at 80 °C, although the full volume of wax was available for potential partitioning losses at 80 °C as oposed to only the surface of the wax at ambient temperature. This experiment nicely demonstrates the difficulty of the extraction solvent to access residues incorporated in the wax and that decreasing particle size (i.e., increasing wax surface area) improves residue accessibility and thus extraction yields. Melting the wax ensured optimal accessibility to the residues via liquid−liquid partitioning.

Similar observations were made when a commercial beeswax containing incurred residues of τ-fluvalinate, piperonil butoxide, propargite, and coumaphos was extracted (results not shown here).

Comparison of Different Cleanup Possibilities. Beeswax is composed of a variety of highly lipophilic compounds such as esters of long-chain aliphatic alcohols with fatty acids or hydroxy-fatty acids, long-chain hydrocarbons, and trace levels of carotenoids. On the basis of their low solubility in acetonitrile, such highly lipophilic compounds are not expected to be present at remarkable levels in the final extracts. It is also expected that these compounds can be additionally removed to a large extent either by freezing-out (which further reduces their solubility, forcing them to precipitate) or via dispersive solid phase extraction (dSPE) using RP-C18 sorbents. Beeswax also contains a relatively more polar fraction of compounds that contains free long-chain fatty acids such as melissic and cerotic acid (that make ca. 12% of the beeswax), free hydroxy-fatty acids, and free aliphatic fatty alcohols. These compounds are still lipophilic enough to be removed to a certain degree via freeze-out or via dSPE using C18 sorbents but, in particular for the fatty acids, the cleanup approach of choice is dSPE with amino sorbents (such as PSA). Some of the polar pigments originated from the flowers visited by bees (e.g., anthocyanidines) can also be removed via dSPE.

Various experiments were conducted to study the extent to which beeswax coextractives can be removed via freeze-out and/or dSPE using C18 and/or PSA. The efficiency of cleanup was assessed on the basis of the remaining dry residue following extract evaporation but also by observing GC×GC-TOF chromatograms. Practical aspects, that is, the simplicity of the cleanup procedure, were also taken into account.

Following the first extraction step (2 g of wax + 10 mL of acetonitrile at 80 °C in a water bath), aliquots were taken from the initial extract and subjected to different cleanup procedures

Table 2. Extract Dry Residue Derived with Different Cleanup Procedures

	dry residue (mg) per mL of extract	compared to dry residue in raw extract (=100%)	compared to initial sample amount (200 mg/mL = 1000‰)
without cleanup (raw extract)	3.12	100	15.6
freeze-out 1 h	2.80	90	14.0
freeze-out 2 h	2.72	87	13.6
freeze-out 4 h	2.60	83	13.0
+3% H_2O freeze-out 1 h	2.75	88	13.7
+3% H_2O freeze-out 2 h	2.78	89	13.9
+6% H_2O freeze-out 2 h	2.60	83	13.0
+10% H_2O freeze-out 2 h	2.40	77	12.0
PSA (25 mg/mL)	1.16	37	4.3
PSA (50 mg/mL)	1.15	37	4.1
+ 3% H_2O PSA/$MgSO_4$ (25/150 mg/mL)	1.00	32	5.0
+ 6% H_2O PSA/$MgSO_4$ (25/150 mg/mL)	0.97	31	4.9
C18 (25 mg/mL)	2.65	85	13.3
C18 (50 mg/mL)	2.55	82	12.7
+ 3% H_2O C18/$MgSO_4$ (25/150 mg/mL)	2.40	77	12.0
+ 6% H_2O C18/$MgSO_4$ (25/150 mg/mL)	2.32	74	11.6
+ 10% H_2O C18/$MgSO_4$ (25/150 mg/mL)	2.17	70	10.9
+ 3% H_2O freeze-out 2 h/filtration/PSA/$MgSO_4$ (25/150 mg/mL)	0.80	26	4.0
PSA/C18 (25/25 mg/mL)	0.85	27	4.3
freeze-out/PSA (25 mg/mL)	0.77	25	3.8
freeze-out/PSA/C18 (25/25 mg/mL)	0.71	23	3.5

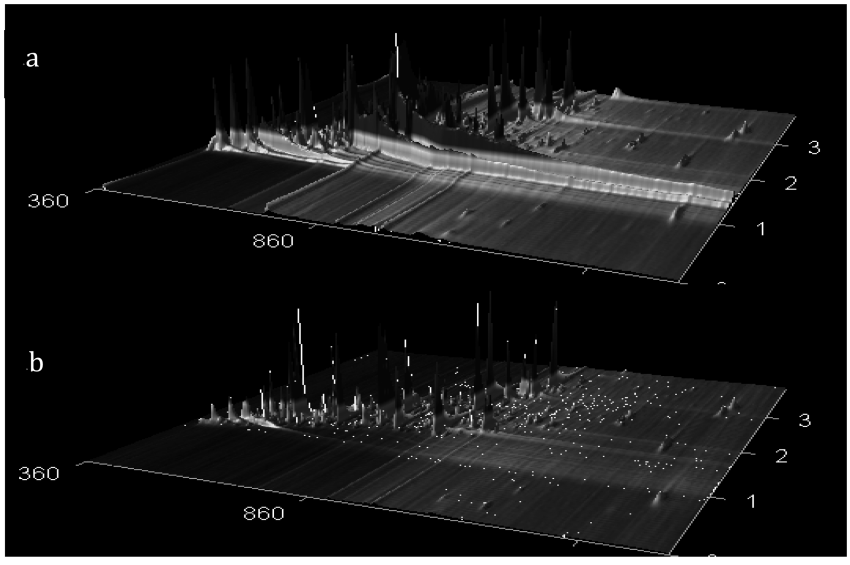

Figure 3. GC×GC-TOF plots of (a) raw extract (without cleanup) and (b) extract following dSPE with PSA.

as follows: (a) freeze-out (leave the aliquot for 1, 2, and 4 h in a freezer (−20 °C)); (b) dSPE with PSA (25 and 50 mg/mL); (c) dSPE with C18 (25 and 50 mg/mL); (d) freeze-out followed by dSPE with PSA (25 mg/mL); and (e) dSPE with PSA plus C18 (25 mg/mL each).

The influence of water addition to the raw extracts on the removal of coextractives during cleanup was also studied. A positive effect was expected in the removal of lipophilic compounds due to an increased polarity of the solvent and a negative effect regarding the efficiency of dSPE with PSA due to water interference with hydrogen bond formation between the amino groups of the sorbent and polar moieties of fatty acids or other coextracted matrix components. The following additional experiments involving water addition to the initial extract were conducted: (f) water addition (3, 6, and 10%) followed by freeze-out (1 and 2 h); (g) water addition (3, 6, and 10%) followed by dSPE with C18 (25 and 50 mg/mL); and (h) water addition (3 and 6%) followed by dSPE with PSA (25 mg/mL).

As can be seen in Table 2, dSPE using PSA removed the largest fraction of coextractives from the initial extracts (ca. 70−

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Table 3. Recoveries, RSDs, and LOQs of the Pesticides Analyzed by GC×GC-TOF (n = 5 Replicates)

	0.2 mg/kg		0.1 mg/kg					
	recovery (%)	RSD (%)	recovery (%)	RSD (%)	LOQ (mg/kg)	linear range (mg/kg)	linear correlation coefficient (r^2)	matrix effect (%)
bromopropylate[a]	83	4	102	9	0.1	0.025−1	0.992	139
chlorfenvinphos	91	10	80	6	0.1	0.25−1	0.998	15
p,p-DDE	61	21	73	4	0.1	0.025−1	0.98	144
p,p-DDT	103	3	108	17	0.1	0.025−1	0.995	127
diazinon	81	4	94	12	0.1	0.25−1	0.999	155
endosulfan α	120	10	158	18	0.2	0.25−1	0.999	245
endosulfan β	88	12	104	40	0.2	0.25−1	0.997	89
endosulfan sulfate	71	11	84	8	0.1	0.25−1	0.997	61
lindane	93	5	101	7	0.1	0.05−1	0.999	185
phorate	106	5	112	9	0.1	0.25−1	0.999	283
piperonyl butoxide[a]	95	1	102	10	0.1	0.025−1	0.999	79
propargite[a]	76	13	284	28	0.2	0.25−1	0.998	−32
trifluralin	79	13	85	16	0.1	0.25−1	0.999	188
τ-fluvalinate[a]						0.25−1	0.999	23

[a]Pesticide present as incurred residue in the beeswax sample used for spiking.

75% of the dry residue was removed). PSA effectively removed fatty acids that give large tailing peaks in GC as shown in Figure 3. Dispersive SPE using C18 sorbent and freeze-out proved to act similarly, both removing ca. 15−20% of the dry residue when no water was added and between 25 and 30% when water was added. Nevertheless, GC-MS/MS chromatograms looked similar for both treatments. Freeze-out is more difficult to handle than dSPE with C18 sorbent as it requires a filtration step through cotton, during which part of the precipitated lipids redissolve due to temperature increase. However, as it was observed that when the extract was allowed to cool to room temperature on the bench, wax precipitation was slowed, an additional freeze-out step to speed precipitation and increase repeatability was included. This is followed by dSPE with PSA sorbent and C18 sorbent, the latter serving the further reduction of lipids in the extract but not being absolutely necessary. For simplicity the freeze-out step was conducted in the extraction tube with the aliquot employed for dSPE being directly withdrawn from the cold vessel without any need for a tedious filtration step.

Cleanup with a combination of PSA and C18 (or freeze-out) is highly recommended to reduce the matrix coextractives introduced into the GC and LC system. Introduction of non-cleaned-up extracts would require shorter maintenance intervals and reduce the overall productivity, especially for GC systems.

Water addition was slightly beneficial in all experiments, helping to remove additional lipids, but the benefits did not justify the additional workload introduced by water addition and its subsequent removal with $MgSO_4$. Within this context the conditions described under Experimental Procedures were preferred. Two grams of wax was melted at 80 °C in a water bath and extracted with acetonitrile. The extracting solution was left to cool to room temperature and frozen out for at least 2 h (e.g., overnight). From the cold solution, an aliquot was subjected to dSPE cleanup using C18 and PSA sorbents (25 mg/mL). Following acidification with 5% formic acid in acetonitrile (10 μL/mL) and ISTD addition, the extracts were analyzed by GC×GC-TOF and LC-MS/MS.

Method Validation. After the optimal sample amount, particle size, and the best cleanup approach had been chosen, five replicates of spiked blanks of beeswax at different levels (0.1 and 0.2 mg/kg for GC×GC-TOF analysis and 0.01, 0.1, and 0.2 mg/kg for LC-QTrap analysis) were analyzed to assess accuracy (% recovery) and repeatability (% RSD) of the procedure.

In agreement with the analytical quality control procedures document by DG-SANCO,[22] the limits of quantification (LOQs) were considered as the lowest successfully validated levels, that is, the levels at which acceptable recoveries (70−120%) and RSDs (<20%) were achieved. LOQs would have been surely different if other spiking levels were chosen. Of course, LOQs will also depend on other factors such as the type of instrumentation used and its condition.

For GC×GC-TOF matrix-matched calibration was used, whereas for LC-MS/MS calibration curves were constructed using both solvent-based and matrix-matched standards at five concentration levels (0.001, 0.002, 0.01, 0.02, and 0.05 μg/mL, corresponding to 0.005, 0.01, 0.05, 0.1, and 0.25 mg/kg beeswax). GC×GC-TOF analysis was conducted using an ISTD, whereas no ISTD was used in LC-MS/MS.

The matrix effects were calculated as the percentage difference in the best-fit slopes of the matrix-matched calibration curves versus the respective best-fit slopes of the solvent calibration curves. The peak areas rather than the relative peak areas against the ISTD were used to avoid matrix effects on the ISTD that potentially affect the calculation.[23]

In GC×GC-TOF large and positive matrix effects were observed (Table 3) except for propargite, which presented a negative effect.

In LC-MS/MS matrix effects were quite pronounced and variable from analyte to analyte as shown in Table 4. This is explained by the fact that matrix effects depend on whether a target analyte coelutes with coextracted matrix components, affecting the yield of free analyte ions generated within the ESI-ion source and thus the detection signals. A dSPE cleanup of the raw wax extracts with PSA contributed in reducing to some extent the matrix effects of certain compounds (boscalid from −10 to −1; imidacloprid from −50 to 3; tebuconazole from −33 to 9; dimethoate from 20 to −2; carbaryl from −20 to 3; thiacloprid from −25 to 1). Similar observations were also previously made using extracts of other "difficult" commodities such as oranges.[24] When matrix effects remain strong, the use of matrix-matched calibration standards (prepared from extracts of blank matrix so far available) or the method of standard

Table 4. Percentage Recoveries, RSDs, and LOQs of the Pesticides Analyzed by LC-MS/MS (n = 5 Replicates)

	0.2 mg/kg		0.1 mg/kg		0.01 mg/kg					
	recovery (%)	RSD (%)	recovery (%)	RSD (%)	recovery (%)	RSD (%)	LOQ (mg/kg)	linear range (mg/kg)	linear correlation coefficient (r^2)	matrix effect (%)
amitraz	73	2	77	4	85	13	0.01	0.005−0.25	0.999	−2
atrazine	89	4	94	4	97	13	0.01	0.006−0.24	0.999	
azoxystrobin	110	3	111	4	112	14	0.01	0.005−0.25	0.997	24
boscalid	98	6	103	8	105	24	0.1	0.005−0.25	0.999	5
carbaryl[a]	104	4	111	5	165	6	0.1	0.005−0.25	0.995	4
carbaryl[b]					112	2				
carbendazim	72	4	78	6	100	15	0.01	0.005−0.25	0.999	32
carboxin	104	4	108	6	107	9	0.01	0.005−0.25	0.999	−6
chlorpyrifos-ethyl[a]	92	6	105	6	218	15	0.1	0.005−0.25	0.999	−13
clodinafop-propargyl	104	1	105	5	104	14	0.01	0.005−0.25	0.999	88
coumaphos[a]	144	3	229	4	1679	8		0.005−0.25	0.999	24
cyhalothrin-λ	92	16	100	9	not detectable		0.1	0.005−0.25	0.999	−23
dimethoate	102	6	106	6	107	7	0.01	0.005−0.25	0.997	2
epoxiconazole	94	4	106	7	not detectable		0.1	0.005−0.25	0.999	−33
fenpropathrin	95	7	102	11	159	15	0.1	0.006−0.24	0.997	
flutriafol	101	11	114	15	not detectable		0.1	0.005−0.25	0.998	−55
hexythiazox[a]	182	4	323	5	2800	3		0.005−0.25	0.999	−41
hexythiazox[b]					87	8				
imidacloprid	108	7	106	8	100	9	0.01	0.005−0.25	0.996	−0,1
iprodione	108	27	123	24	not detectable		0.2	0.005−0.25	0.997	−7
linuron	96	6	99	5	107	15	0.01	0.005−0.25	0.999	−41
metalaxyl	107	3	109	5	113	15	0.01	0.005−0.25	0.997	−20
methomyl	111	8	127	4	113	12	0.01	0.005−0.25	0.999	−21
methoxyfenozide[a]	107	4	111	5	146	7	0.1	0.005−0.25	0.999	−15
methoxyfenozide[b]					116	18				
metolachlor	96	3	103	2	120	17	0.01	0.005−0.25	0.999	−20
metribuzin	102	7	111	7	not detectable		0.1	0.005−0.25	0.999	−3
myclobutanil	106	5	102	10	123	14	0.1	0.005−0.25	0.998	−7
omethoate	96	11	104	8	111	6	0.01	0.005−0.25	0.992	−33
pendimethalin	81	19	83	19	not detectable		0.1	0.06−0.24	0.995	
pirimicarb	108	3	114	4	111	11	0.01	0.005−0.25	0.999	−10
profenofos	89	6	94	6	92	18	0.01	0.005−0.1	0.999	−32
pyraclostrobin	102	4	106	5	112	14	0.01	0.005−0.25	0.999	−33
tebuconazole	98	11	99	5	103	27	0.1	0.005−0.25	0.999	2
tebufenozide	96	4	99	5	104	15	0.01	0.005−0.25	0.998	−25
tetraconazole	103	7	104	6	111	23	0.1	0.005−0.25	0.996	−22
thiacloprid	113	7	120	5	108	18	0.01	0.005−0.25	0.999	−7
thiamethoxam	103	14	103	7	106	15	0.01	0.005−0.25	0.999	13
thiodicarb	90	5	85	7	107	12	0.01	0.005−0.25	0.999	−25
thiophanate-methyl	94	5	91	7	97	15	0.01	0.005−0.25	0.997	−22
triflumuron	91	5	98	10	104	12	0.01	0.005−0.25	0.999	−46

[a]Pesticides present as incurred residues in the beeswax sample used for spiking. [b]Recovery experiment at 0.01 in blank beeswax (Pharmacognosy collection).

additions is recommended to obtain reliable quantification.[22] Matrix coextractives in LC-MS/MS mainly cause signal suppression, whereas signal enhancement is typically observed in GC applications. This is due to the different mechanisms behind matrix effects in each case.[25,26] Matrix coextractives of beeswax behave as analyte protectants hampering pesticide degradation in the injector and along the chromatographic column, resulting in signal enhancement.

Recoveries of GC- and LC-amenable pesticides were in almost all cases between 70 and 120% with RSDs below 20% at least at one of the levels assayed (Tables 3 and 4). The exceptions were mainly due to the incurred residues found in the commercial beeswax that was used to conduct the recovery experiments. Some recovery experiments were repeated using as blank material all of the beeswax from the year 1932 that could be withdrawn from the Pharmacognosy collection of the Chemistry College of Universidad de la República, which did not contain pesticide residues. Recoveries could then be properly evaluated for carbaryl, hexythiazox, and methoxyfenozide at 0.01 mg/kg ranging from 87 to 116% and RSDs being below 18%, whereas chlorpyrifos could not be quantified at this low level. Coumaphos and τ-fluvalinate were studied at higher levels (0.2 and 0.1 mg/kg) because they are used directly inside the hive to protect bees from mites. Good results were obtained for τ-fluvalinate at the level of 0.2 mg/kg (102% recovery and 5% RSD) and for coumaphos at both levels (103 and 109%

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recoveries; 4 and 10% RSDs, respectively). With beeswax being a natural product, differences in composition between samples were expected. Table 5 shows validation data for a group of

Table 5. Percentage Recoveries and RSDs (n = 5) of a selected Group of Pesticides at 0.1 mg/kg in Different Samples Assayed

	commercial beeswax from Germany		commercial beeswax from Uruguay		beeswax from pharmacognosy collection	
	recovery (%)	RSD (%)	recovery (%)	RSD (%)	recovery (%)	RSD (%)
azoxystrobin	111	4	88	4	99	7
boscalid	103	8	76	6	107	7
carbaryl	111	5	84	3	93	4
chlorpyrifos-ethyl	105	6	77	6	94	11
dimethoate	106	6	86	3	94	10
imidacloprid	106	8	86	3	93	3
tebuconazole	99	5	77	1	78	10
thiacloprid	120	5	82	2	99	12
thiamethoxam	103	7	86	3	98	20

selected pesticides spiked onto three different beeswax samples (samples purchased in Germany and Uruguay and the wax from the Pharmacognosy museum). These data further demonstrate the suitability and ruggedness of the method for this complex matrix. The use of different beeswax samples with different incurred residues for analytical validation purposes is a useful tool not only to cross check the obtained results but also to broaden the scope of compounds that can be successfully validated. In summary, a simple variation of the QuEChERS method, involving extraction of wax at high temperatures (~80 °C) to enable liquid–liquid partitioning between the liquefied wax phase and acetonitrile followed by a simple freeze-out plus a dSPE step, proved to be effective for the multiresidue analysis of 51 pesticides in beeswax. Several critical aspects (sample weight, particle size, and different cleanup options) were studied in the course of method development. A comprehensive cleanup involving freeze-out and dSPE using PSA and C18 was shown to remove ca. 75% of the coextractives from the raw extract and ca. 99.6% of the initial wax. Quantitative analysis was accomplished by LC-MS/MS and GC×GC-TOF. Fifty-one pesticides proved to be accurately determined with the developed methodology. The method can be easily expanded to other pesticides that, although not included in the present study, could be found in beeswax samples produced in other environments or agricultural systems. Beeswax is a long-term "pollution reservoir" due to its particular physicochemical properties; it stores the pesticide residues to which the hive is exposed. Therefore, the analysis of this complex matrix will give relevant data on the sustainability of specific agroecosystems.

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Notes

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ABBREVIATIONS USED

QuEChERS, quick, easy, cheap, effective, rugged, and safe; dSPE, dispersive solid phase extraction; LC-MS/MS, liquid chromatography–tandem mass spectrometry; GCxGC-TOF, two-dimensional gas chromatography–time of flight detection; RSD, relative standard deviation; PCB, polychlorinated biphenyl; ISTD, internal standard; MRM, multiple-reaction monitoring; ESI, electrospray ionization; PSA, primary and secondary amine

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