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Liquid–liquid extraction followed by solid-phase extraction for the determination of lipophilic pesticides in beeswax by gas chromatography–electron-capture detection and matrix-matched calibration☆

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Abstract

Analytical methods for the simultaneous analysis of lindane, chlorpyriphos, *z*-chlorfenvinphos, endosulfan A and B, 4,4′-DDE, 4,4′-TDE, acrinathrine, bromopropylate, tetradifon, coumaphos and fluvalinate in pure beeswax samples are studied. For the analysis of bleached beeswaxes, a liquid–liquid extraction with acetonitrile followed by a clean-up on polymeric cartridges is the best option in terms of recovery and precision. However, some interferences that hinder the identification and quantification of important varroacides are found when non-bleached beeswaxes are analyzed. The analysis of all compounds in the latter samples require a clean-up by coupling an ODS cartridge before the polymeric cartridge. Considerations about the influence of the matrix in the quantitative analysis by a classical external standard calibration are also made and the use of a matrix-matched calibration is advised. Recoveries resulted to be about 100% with coefficients of variation between 10% and 20% (n = 5) for concentrations of 0.5 and 5 mg/kg.

Keywords: Gas chromatography; Matrix-effects; Sample preparation; Beeswax

1. Introduction

Beeswax secreted by bee glands is a reservoir of lipophilic and non-volatile xenobiotics that can diffuse towards other apiarian products yielded in beehives, such as honey, propolis and royal jelly. Among these products, there are many pesticides that reach the hives as a consequence of the phytosanitary treatments given by apiarists and the picking activity of the bees.

Most of the analytical methods of pesticide and varroacide residues in beeswax deal with the determination of an only compound, although the manuscript tackles the analysis of several analytes. So, methods for the analysis of varroacides such as amitraz [1,2], fluvalinate [3,4], flumethrin [5], bromopropylate [6,7], malathion and coumaphos [8], bromopropylate, cymiazole and chlordimeform [2], thymol and other essential oils [9,10] have been published. As regards the pesticides used in the surrounding crops that can also affect beehives, there are methods for parathion-methyl [11] and bromofenvinphos [12]. Sample preparation procedures for organochlorine compounds such as DDE, polychlorinated biphenyls and chlorobenzenes [13], and for the fungicides benomyl and carbendazime in beeswax have also been described [14]. Those methods are mainly based on acetonitrile/*n*-hexane partitionings and the use of Florisil packed columns, before determining the analytes by a chromatographic technique.

The content of pesticides in pure beeswax is very variable, but relatively high amounts have been found just after beehive treatments, which is symptomatic of the build-up of

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these residues in beeswax. According to studies carried out in Switzerland and Germany, bromopropilate, coumaphos and fluvalinate are compounds often present; their concentrations varied from 0.5 to 5 mg/kg in most cases [15,16].

Then, it is necessary to have reliable analytical methods that allow to establish the possible occurrence of lipophilic compounds, specially pesticides, in beeswax, with the aim of preserving the quality of beehives, and in particular, of the apiarian products intended for human consumption.

In this work, some procedures of sample preparation: a liquid–liquid extraction with acetonitrile or methanol of the beeswax dissolved in *n*-hexane, and a solid-phase extraction (SPE) on different sorbents have been assayed to determine simultaneously often-used varroacides, lipophilic insecticides and two degradation products very widespread in the environment of DDT (4,4′-TDE: 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane and 4-4′-DDE: 2-(4-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene) in beeswax. The cartridges for SPE were based on poly(divinylbenzene–co-*N*-vinylpirrolidone) (Oasis cartridges) and octadecylsilane (ODS) with different carbon loading, endcapping and spatial distribution of the functional groups. The experiments were initially made on bleached beeswax and, afterwards, the selected procedures were applied to non-bleached beeswaxes collected directly from beehives. The determination of the analytes in the extracts was carried out by capillary gas chromatography with electron-capture detection, and the amount of residues really present in the extracts was determined by a standard addition calibration method in order to discuss the influence of the matrix in a conventional quantification, which was performed by a calibration with standards dissolved in organic solvent. The use of a calibration with extracts of spiked beeswaxes was considered for routine analysis.

2. Experimental

2.1. Material and reagents

Residue analysis grade *n*-hexane, acetonitrile, acetone, ethyl acetate, 2-propanol, methanol, chloroform, and dichloromethane were supplied by Scharlau (Barcelona, Spain) and Lab-Scan (Dublin, Ireland). Ultrapure water was obtained from a Milli-Q plus apparatus (Millipore, Milford, MA, USA). Pesticide and degradation product certified standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Mechanical shakers were obtained from Selecta (Barcelona, Spain) and a RE-111 rotary evaporator from Büchi (Plawil, Switzerland). For sample preparation, PTFE disposable syringe filter units, 0.50 μm pore size, were obtained from Microfiltration Systems (Dublin, CA, USA). Industrial gases (99.999% minimum purity) were obtained from Carburos Metálicos (Barcelona, Spain).

For SPE, 100 and 500 mg ODS, 500 mg terc-ODS, 500 mg Florisil and polymeric Oasis HLB 200 mg cartridges were supplied by Waters (Milford, MA, USA) and Merck (Darmstadt, Germany). Estrata 500 mg ODS-E cartridges were purchased from Phenomenex (Torrance, CA, USA). Florisil of 60–100 mesh was supplied by Fisher Scientific Company (Springfield, NJ, USA).

Pesticide stock solutions were made in acetone. Mixtures of these pesticide solutions were used to make the assays and the conventional calibration standards in the 0.01–1 mg/L range; for this purpose, dilutions were made with acetone. Assays were made on bleached beeswax from Fluka (Steinheim, Germany) and beeswax samples supplied by beekeepers.

2.2. Sample preparation

Some beeswaxes obtained from beekeepers required a previous rinse step to remove strange substances: honey, bees, propolis, etc. This step started with the boiling of a water–beeswax mixture for 20 min. Then, the mixture was cooled at room temperature, the water was substituted for fresh water and the heating was repeated once again. So, impurities were dissolved in water and placed at the bottom of the solidified beeswax (less dense than water), impurities from beeswax were then removed with a scraper.

A solution of analytes in acetone was added to beeswax to achieve a concentration of 0.5 and 5 mg/kg for each pesticide. The beeswax was melted at 70 °C, then, the pesticide standard solution was added and the mixture was homogenized with a rod before solidifying.

Spiked samples were kept at room temperature and darkness for 5 days at the maximum.

2.3. Liquid–liquid extraction

Beeswax (0.1 g) was dissolved in *n*-hexane and placed in a decantation funnel. A volume of 10 mL of solvent was added and the mixture was mechanically shaken for 15 min. Acetonitrile and methanol were studied as extraction solvents, it was necessary to add 2 mL of methanol after the extraction to improve the separation of the phases when acetonitrile was used. The number of extractions (one or two) with this latter solvent was also studied. The collected extract was evaporated and the residue was dissolved in 1 mL of acetone. Finally, the extract was filtered through PTFE, 0.50 μm pore size.

2.4. Solid-phase extraction and clean-up

2.4.1. Commercial cartridges

A 10 mL extract obtained after the extraction with acetonitrile by the above-described procedure was diluted with 200 mL of water. A study about the extraction of the target-compounds from the water–acetonitrile mixture by 500 mg ODS, 500 mg ODS-E, 500 mg terc ODS and 200 mg Oasis HLB cartridges was done. Firstly, the cartridges were rinsed by successive elution of 10 mL of methanol and 10 mL of water. After that, the sample was percolated through the cartridge at about 5 mL/min using a suction system. Then, the

solvent was removed from the cartridges by pulling nitrogen through it for about 20 min. The extract was eluted with 4 mL of an organic solvent after leaving the solid phase to soak for 5 min. Ethyl acetate, methanol and acetone were tested as eluents. Finally, the eluate was evaporated and the residue dissolved in 1 mL of acetone.

2.4.2. Clean-up procedures coupling two cartridges

Two different clean-up procedures were assayed: the coupling of two cartridges during the extraction step of the water–acetonitrile mixture, and the coupling of two cartridges during the elution of the analytes. To this purpose, 200 mL of water were added to the 10 mL acetonitrile extract, Oasis cartridges were used to retain the analytes, and ethyl acetate to elute them from the cartridges (these working conditions were the best ones, according to previous experiences).

For the extraction of the water–acetonitrile mixture, 100 or 500 mg ODS cartridges, conditioned by elution of 10 mL of methanol and 10 mL of water, were coupled before the previously conditioned Oasis cartridges by using an adaptor (Fig. 1A). The sample was eluted through the cartridges with the help of a suction system and the ODS cartridge was discarded. Then, the Oasis cartridge was dried, the analytes were eluted with ethyl acetate and the evaporated residue was dissolved in 1 mL of acetone, as above-mentioned.

For the Oasis cartridge elution, 500 mg Florisil or 100 mg ODS cartridges, both conditioned by elution of 10 mL of ethyl acetate, were coupled behind the Oasis extraction cartridges (Fig. 1B). Ethyl acetate (4 mL) was percolated through the two cartridges and evaporated. The residue dissolved in acetone (1 mL) was ready for injection in GC.

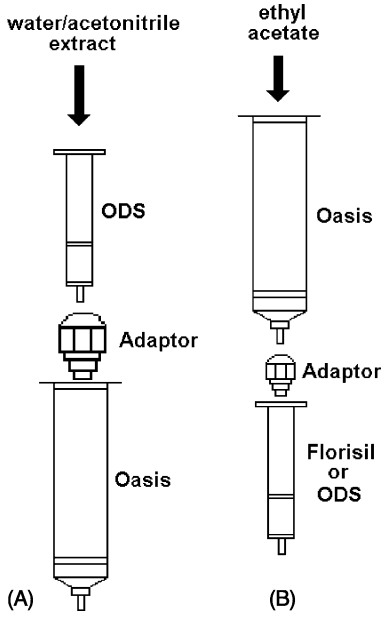

Fig. 1. Scheme of the combination of cartridges assayed. (A) During the extraction of the analytes from beeswax. (B) During the elution of the analytes retained.

2.4.3. Florisil packed columns

Florisil was activated by heating at 120 °C for 4 h. A glass column of 1 cm ID was prepared with a slurry of 4 g of Florisil in *n*-hexane and compacted with a rod. Once ready, the column was loaded with 1 mL of beeswax solution in chloroform (0.1 g/8 mL) and eluted by gravity with 25 mL of solvent. Two solvents, ethyl acetate and 1:1 *n*-hexane/dichloromethane mixture, were used, always preventing the column from drying. Then, the eluate was evaporated in a rotary evaporator at 40 °C, under vacuum, and the residue dissolved in 0.5 mL of acetone.

2.5. Gas chromatography analysis

An Hewlett-Packard (Avondale, PA) 5890 gas chromatograph equipped with an HP7673 auto-sampler, an electron-capture detection (ECD) system and two 60 m × 0.25 mm capillary columns coated with a 0.25 μm thick film of 5% phenylmethylpolysiloxane from Phenomenex and Hewlett-Packard, were used. The oven temperature program was as follows: initial temperature 50 °C, held for 1 min, then a 15 °C/min ramp to 160 °C, and finally a 2.1 °C/min ramp to 300 °C, held for 60 min. The carrier gas (He) flow-rate was 0.8 mL/min, measured at 50 °C. Splitless injection (3 μL) was carried out at 225 °C, and the purge valve was on at 1 min. Argon–methane (90:10) was used as an auxiliary gas for ECD, whose temperature was 300 °C.

3. Results and discussion

3.1. Liquid–liquid extraction of the analytes in bleached beeswax

Table 1 shows the recoveries obtained with acetonitrile and methanol for two spiking levels of bleached beeswax. It can be inferred from the results that the recoveries with methanol were low in general. The recoveries obtained with acetonitrile were abnormally high in all cases, higher than 100%; moreover, they were higher for the lowest concentration, 0.5 mg/kg. This fact was attributed to the influence of the beeswax matrix in the injection port of the chromatograph [17–20].

As regards the number of extractions with acetonitrile, the recoveries were higher than 100% after an only extraction, and in many cases, similar to those obtained with two extractions. For the following experimentation, one extraction with acetonitrile was used to carry out the liquid–liquid extractions of the beeswax dissolved in *n*-hexane, which simplified the sample preparation procedure. The baseline and profile of the chromatograms were similar regardless the solvent or extraction number.

To correct the quantitative errors arisen from the influence of the matrix in the injection port of the chromatograph, some calibration methods are possible: a standard-addition calibration, an external-standard calibration with extracts of

Table 1
Recoveries (in %) obtained after liquid–liquid extraction with methanol and acetonitrile of bleached beeswax dissolved in *n*-hexane ($n = 5$)

	0.5 mg/kg				5 mg/kg			
	Methanol		Acetonitrile		Methanol		Acetonitrile	
	One extraction	Two extractions	One extraction	Two extractions	One extraction	Two extractions	One extraction	Two extractions
Lindane	60	85	150	247	24	37	115	126
Chlorpyrifos	58	77	193	303	34	44	148	145
z-Chlorfenvinphos	154	184	314	390	68	87	168	183
Endosulfan A	9	4	97	223	20	22	102	121
4,4′-DDE	24	18	108	230	19	24	116	116
4,4′-TDE	45	90	222	340	35	37	156	181
Endosulfan B	5	7	149	280	18	17	149	147
Acrinathrine	20	18	143	238	19	25	153	157
Bromopropylate	214	318	820	930	39	84	264	452
Tetradifon	54	77	208	346	25	38	146	206
Coumaphos	–	10	634	412	60	64	110	199
Fluvalinate isomer 1	31	42	179	277	29	35	112	209
Fluvalinate isomer 2	51	56	192	309	27	40	115	216

0.5 mg/kg and 5 mg/kg represent the spiking level; methanol and acetonitrile are the extracting solvents; (–) represents not detected.

a beeswax spiked with increasing amounts of the analytes and a calibration with extracts of a free-analyte beeswax spiked with the compounds after the extraction. In all cases, the same sample procedure must be accomplished for samples and calibration standards.

Two alternatives have been assayed in this work to reduce the errors derived from the matrix: a calibration with extracts of beeswax spiked (before the extraction) with the pesticides and a standard-addition calibration. For the latter option, the extract was divided into aliquots to which increasing volumes of a standard mixture were added, always keeping the final volume virtually constant.

Table 2 shows the recoveries, precisions and correlation coefficients obtained by both methods. For the external-standard and matrix-matched calibration, a non-bleached beeswax was used to obtain the calibration graph. As it can be seen in Table 2, the calibration with extracts is valid to correct the quantitative errors. The recoveries were close to 100% with variation coefficients ranging from 4 to 12%. The analysis of high concentrations was more precise. The addition standard method allowed to determine the concentrations really present in the extracts, so, it was observed that the recoveries obtained by the liquid–liquid extraction at a concentration of 0.5 mg/kg were about 100% except for compounds such as fluvalinate, acrinathrine, bromopropylate, DDE and TDE, for which the recoveries decreased up to 70–80%. As for the concentration of 5 mg/kg, it was verified that the extraction was slightly effective for the pyrethroids fluvalinate and acrinathrine, besides coumaphos. The values of R.S.D. ranged from 3 to 15% ($n = 2$).

If the recoveries obtained by standard-addition calibration are compared with those obtained by a conventional external-

Table 2
Recoveries, relative standard deviations (R.S.D.s) and coefficients of correlation (r) obtained by an external standard calibration method with extracts in the 0.01–1 mg/L range and by a standard addition method after liquid–liquid extraction

	External standard calibration ($n = 5$)					Standard addition calibration ($n = 2$)				
	0.5 mg/kg			5 mg/kg		0.5 mg/kg			5 mg/kg	
	r	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	r	Recovery (%)	R.S.D. (%)	r	Recovery (%)	R.S.D. (%)
Lindane	0.994	101	8.4	92	7.2	0.992	94	6.9	0.978	114	4.2
Chlorpyrifos	0.999	106	9.2	94	6.3	0.995	102	5.0	0.991	115	5.5
z-Chlorfenvinphos	0.998	96	10.0	99	8.2	0.994	100	8.6	0.995	107	9.5
Endosulfan A	0.994	97	11.3	93	9.0	0.976	92	13.1	0.989	103	10.4
4,4′-DDE	0.994	94	7.2	92	5.0	0.990	70	12.4	0.999	103	6.9
4,4′-TDE	0.996	102	8.3	100	4.3	0.990	72	8.0	0.999	85	7.6
Endosulfan B	0.993	94	10.3	98	6.7	0.994	96	8.7	0.984	94	3.9
Acrinathrine	0.998	97	9.3	109	7.2	0.999	84	3.5	0.998	40	3.4
Bromopropylate	0.992	102	10.3	102	9.5	0.991	82	6.0	0.996	96	4.2
Tetradifon	0.986	104	8.0	105	6.4	0.971	106	7.7	0.981	89	7.8
Coumaphos	0.982	104	11.2	105	9.9	0.980	90	5.0	0.996	68	9.8
Fluvalinate isomer 1	0.989	110	12.3	104	7.7	0.990	72	6.8	0.997	37	11.2
Fluvalinate isomer 2	0.984	108	10.4	97	6.8	0.988	74	8.4	0.995	38	14.5

0.5 mg/kg and 5 mg/kg represent the spiking level.

standard calibration with standards that do not match the beeswax matrix, it can be noted that the mean concentrations calculated by the classical calibration are about 205% and 95% higher than the real ones for the spiking levels of 0.5 and 5 mg/kg, respectively. However, the increase of the recovery varied a lot depending on the compound; for instance, endosulfan A was not practically affected by the matrix, whereas bromopropylate and coumaphos were the most influenced.

3.2. Solid-phase extraction of the analytes in bleached beeswax by Florisil-packed columns

Table 3 shows the recoveries and precisions achieved after eluting the columns with *n*-hexane–dichloromethane (1:1) and ethyl acetate and performing two different calibration methods. The recoveries were higher in the elution with ethyl acetate for the external standard calibration, mainly at 5 mg/kg; chlorfenvinphos and coumaphos were not detected at a concentration of 0.5 mg/kg. As deduced from the standard addition method, the recoveries were close to 100% for the lowest spiking concentration, whereas the recoveries were notably lower for the highest concentration.

It was verified from the data achieved by eluting the analytes with ethyl acetate that the beeswax matrix increased the mean recoveries obtained by the classical calibration in 112% and 174% for the levels of 0.5 and 5 mg/kg, respectively, which differed from the liquid–liquid extraction results, in which the quantifications errors were higher for the lowest concentration. The error by excess was higher for bromopropylate, coumaphos and chlorfenvinphos.

3.3. Solid-phase extraction on ODS and polymeric cartridges after liquid–liquid extraction of the bleached beeswax

Table 4 lists the recoveries and precisions obtained by using a conventional calibration and ethyl acetate as an eluent in the bleached beeswax analysis. To this end, 200 mL of water were added to the 10 mL extract obtained with acetonitrile, and the mixture was percolated on four types of cartridges. The recoveries always resulted to be higher for the concentration of 5 mg/kg. The ODS and terc-ODS cartridges provided the worst recoveries and precisions. The ODS-E cartridges supplied, in general terms, a better precision in comparison with the other ODS-based cartridges, and a similar precision in relation to the Oasis cartridges. The latter were considered as the most suitable as a consequence of the highest recoveries and precisions obtained.

As regards the influence of the elution solvent on the recoveries and precisions obtained for Oasis cartridges, the recoveries were similar or higher in the elution with methanol and acetone than with ethyl acetate for 5 mg/kg, although the precision was better with ethyl acetate. At the concentration of 0.5 mg/kg, the recoveries were generally higher if methanol was used, although the precision was also poorer

Table 3
Recoveries R.S.D. values and coefficients of correlation (*r*) obtained by solid-phase extraction of the analytes in bleached beeswax with 4 g Florisil columns after eluting them with different solvents and using two calibration methods

	Conventional external standard calibration						Standard addition calibration					
	0.5 mg/kg (n = 5)			5 mg/kg (n = 5)			0.5 mg/kg (n = 5)			5 mg/kg (n = 5)		
	Hexane–dichloromethane		Ethyl acetate	Hexane–dichloromethane		Ethyl acetate	Ethyl acetate			Ethyl acetate		
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	r	R.S.D. (%)	Recovery (%)	r	R.S.D. (%)
Lindane	153	9.4	135	17.2	123	18.6	116	9.7	94	0.997	15.6	62	0.945	9.1
Chlorpyrifos	220	22.7	267	13.9	153	12.4	154	19.9	94	0.993	15.8	72	0.998	14.6
z-Chlorfenvinphos	22	78.6	–	–	52	67.4	235	21.9	–	–	–	49	0.991	13.0
Endosulfan A	83	12.4	135	15.2	120	14.5	117	9.5	106	0.993	12.5	66	0.948	7.3
4,4′-DDE	154	10.1	143	11.0	113	22.8	115	10.7	85	0.997	10.0	54	0.952	9.4
4,4′-TDE	173	10.6	197	14.5	157	10.1	149	13.4	81	0.999	18.9	60	0.956	14.6
Endosulfan B	175	20.4	153	20.0	94	45.0	133	21.2	103	0.997	14.7	71	0.994	17.5
Acrinathrine	184	20.7	166	27.9	101	47.3	153	11.4	102	0.997	24.5	78	0.996	12.0
Bromopropylate	221	54.9	878	25.6	53	39.3	298	20.3	137	0.996	19.9	74	0.965	20.1
Tetradifon	142	63.6	157	23.3	83	50.2	140	10.7	107	0.999	11.5	67	0.948	9.4
Coumaphos	–	–	–	–	–	–	192	38.3	–	–	–	34	0.947	35.0
Fluvalinate isomer 1	124	15.4	119	15.0	77	38.7	146	15.6	92	0.999	12.5	61	0.998	16.9
Fluvalinate isomer 2	159	35.3	116	13.0	77	32.3	146	16.0	88	0.999	13.3	59	0.999	8.4

0.5 mg/kg and 5 mg/kg represent the spiking level; hexane–dichloromethane and ethyl acetate are eluents; (–) represents not detected.

Table 4
Recoveries and precisions (R.S.D.s) achieved in the clean-up carried out by solid-phase extraction of the acetonitrile extract-water mixture on different commercial cartridges

	500 mg ODS[a]				200 mg Oasis HLB[a]				500 mg ODS-E[a]				500 mg terc-ODS[a]			
	0.5 mg/kg		5 mg/kg		0.5 mg/kg		5 mg/kg		0.5 mg/kg		5 mg/kg		0.5 mg/kg		5 mg/kg	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Lindane	101	31.7	74	7.1	142	3.8	188	11.7	135	25.1	114	21.5	87	70.3	98	21.8
Chlorpyrifos	143	28.4	127	14.7	142	16.5	228	5.8	76	11.9	169	28.3	85	17.6	121	9.3
z-Chlorfenvinphos	18	34.2	24	6.4	66	15.3	153	6.3	63	12.0	76	12.3	10	40.2	35	20.3
Endosulfan A	81	21.9	75	12.5	62	15.7	102	7.4	15	6.9	75	30.7	32	36.8	57	20.7
4,4′-DDE	60	21.1	55	1.8	43	9.1	88	0.2	20	14.3	57	36.0	32	24.5	45	32.5
4,4′-TDE	89	20.8	61	5.5	85	9.6	161	7.0	63	7.3	89	16.1	43	7.6	83	37.7
Endosulfan B	91	28.2	71	1.4	92	15.0	168	6.6	38	17.6	132	6.9	60	60.4	85	33.1
Acrinathrine	40	35.7	69	0.9	99	12.7	122	18.8	39	3.1	136	16.1	68	72.3	61	19.9
Bromopropylate	44	36.5	45	8.3	71	13.3	162	5.2	58	7.4	88	11.8	26	53.6	42	21.1
Tetradifon	28	76.3	56	2.0	61	18.5	146	10.4	61	22.1	76	11.7	59	76.5	74	31.2
Coumaphos	10	40.6	25	7.2	53	10.3	143	17.3	48	6.9	94	17.4	13	25.4	33	26.7
Fluvalinate isomer 1	59	38.5	65	11.9	124	2.0	165	9.4	88	4.4	130	8.8	81	53.2	88	0.7
Fluvalinate isomer 2	62	50.7	66	13.3	126	3.5	162	10.6	90	2.6	123	6.6	88	57.7	88	12.0

0.5 mg/kg and 5 mg/kg represent the spiking level; cartridges eluted with ethyl acetate ($n = 5$).
[a] Cartridge

than in the elution with ethyl acetate. The chromatograms were also similar.

The possibility of making a correct quantification by a calibration with extracts of spiked beeswax was also considered after selecting the use of Oasis cartridges and ethyl acetate as an eluent. As it can be observed in Table 5, the calibration with extracts is a valid alternative to overcome the quantitative errors derived from the matrix; the recoveries are about 100% with coefficients of variation ranging from 6.4 to 11.9%. The amounts really present in the extracts were higher for the concentration of 5 mg/kg, except for chlorpyrifos, endosulfan A and DDE, as checked by the addition standard method. The mean error by excess was now of 39% and 89% for 0.5 and 5 mg/kg, respectively.

3.4. Selection of the extraction procedure and application to non-bleached beeswaxes

The liquid–liquid extraction, followed or not by a solid-phase extraction on polymeric cartridges as a clean-up mode, was the most advisable procedure for the analysis of bleached beeswax on account of the bad precision achieved in the use of Florisil-packed columns. The liquid–liquid extraction without a clean-up step or the procedure that also involved the solid-phase extraction were similar: both required a matrix-matched calibration, the chromatograms were acceptable, and the precisions and detection limits were of the same order. These detection limits were calculated as three times the signal-to-noise ratio on diverse chromatograms, and varied between about 0.005 and 0.04 mg/kg, according to the analyte.

When these extraction procedures were applied to samples of non-bleached beeswax collected from beehives after rinsing them with boiling water, the chromatograms had a higher number of chromatographic peaks, some of them hindered the identification and determination of target compounds such as coumaphos and fluvalinate, two widely used varroacides. The extraction with Florisil-packed columns did not improve substantially the chromatograms and the gas chromatography with columns of different manufacturers was not useful either to separate the interferences. It was ascertained by mass spectrometry that the interfering compounds eluted at the end of the chromatogram were mainly flavones, which color the beeswaxes. As a consequence, the study of new clean-up procedures of the extracts was necessary.

3.5. Clean-up combining two extraction cartridges to analyze non-bleached beeswax

Acetonitrile/*n*-hexane, methanol/*n*-hexane and sodium hydroxide/*n*-hexane partitionings (the two latter previous dissolution of the extract in *n*-hexane after evaporating the acetonitrile) were assayed for the liquid–liquid extraction procedure; they did not supply good results. In the solid-phase extraction, the cartridges were rinsed with

Table 5
Recoveries R.S.D. values and coefficients of correlation (*r*) obtained by an external standard calibration method with extracts in the 0.01–1 mg/L range and by a standard addition method after the clean-up carried out by solid-phase extraction on Oasis cartridges

	External standard calibration (n = 5)					Standard addition calibration (n = 2)			
		0.5 mg/kg		5 mg/kg		0.5 mg/kg		5 mg/kg	
	r	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	*r*	Recovery (%)	*r*	Recovery (%)
Lindane	0.997	106	8.4	104	6.4	0.977	90	0.996	104
Chlorpyrifos	0.997	101	7.2	106	7.3	0.996	99	0.998	60
z-Chlorfenvinphos	0.999	83	11.5	107	11.6	0.997	43	0.999	98
Endosulfan A	0.999	117	9.6	103	8.4	0.987	51	0.996	43
4,4′-DDE	0.943	94	10.3	97	6.5	0.989	42	0.998	37
4,4′-TDE	0.976	92	10.7	93	6.8	0.983	73	0.982	85
Endosulfan B	0.999	86	9.2	101	8.0	0.997	78	0.992	94
Acrinathrine	0.978	108	8.3	106	7.4	0.997	75	0.998	94
Bromopropylate	0.988	97	9.5	108	11.9	0.965	81	0.939	106
Tetradifon	0.952	87	11.3	99	10.3	0.979	46	0.961	103
Coumaphos	0.923	92	12.0	90	10.9	0.933	23	0.908	101
Fluvalinate isomer 1	0.941	93	8.7	103	7.3	0.975	78	0.924	98
Fluvalinate isomer 2	0.937	89	10.0	103	7.9	0.974	84	0.910	101

0.5 mg/kg and 5 mg/kg represent the spiking level.

dichloromethane, methanol, *n*-hexane and water–2-propanol mixtures, but the interferences were not removed either. The cooling of the extracts to −10 °C, and a subsequent filtration, removed beeswax particles, but it was not useful to diminish the interference chromatographic peaks.

Better results were achieved by coupling two cartridges in series, either during the extraction of the acetonitrile/water mixture or during the elution with ethyl acetate of the analytes retained. Table 6 shows the recoveries obtained for a beeswax spiked with 0.5 mg/kg of each pesticide, according to the procedures described in Section 2.4.2 and a conventional calibration. As it can been seen in the Table, the coupling of the cartridges in the analyte elution step (see Fig. 1B) did not resolve the interferences that affected fluvalinate and coumaphos. In the coupling during the extraction step (see Fig. 1A), the 500 mg ODS cartridges retained greatly the target compounds, whereas the coupling of ODS cartridges with lower capacity (100 mg) to the Oasis cartridges was the best option to detect and quantify all the analytes, particularly fluvalinate and coumaphos. The interfering peaks had been removed as it is shown in Fig. 2; in this case, the recoveries decreased in relation to the simple solid-phase extraction, except for coumaphos, and the precision seemed to be similar, between 7.9% and 18.7% (n = 5).

Table 6
Recoveries and precisions (CV, in %) obtained by solid-phase extraction after coupling two cartridges

	Extraction[a]		Extraction[a]		Elution[b]		Elution[b]	
	100 mg ODS[c]		500 mg ODS[c]		200 mg Oasis[c]		200 mg Oasis[c]	
	200 mg Oasis[d]		200 mg Oasis[d]		100 mg ODS[d]		500 mg Florisil[d]	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Lindane	44	14.2	<1	XX	63	15.3	6	31.4
Chlorpyrifos	35	15.6	<1	XX	67	15.5	–	–
z-Chlorfenvinphos	89	12.5	<1	XX	134	11.8	69	12.5
Endosulfan A	36	18.7	<1	XX	18	21.7	–	–
4,4′-DDE	33	12.1	<1	XX	49	17.8	–	–
4,4′-TDE	55	9.6	7	37.4	67	16.6	32	10.2
Endosulfan B	51	14.1	<1	XX	65	14.3	37	9.3
Acrinathrine	37	9.7	<1	XX	67	12.8	34	12.5
Bromopropylate	60	8.5	<1	XX	84	12.7	153	16.8
Tetradifon	84	12.6	<1	XX	22	13.3	130	19.4
Coumaphos	131	8.8	–	–	INTERF	XX	INTERF	XX
Fluvalinate isomer 1	91	8.4	24	15.7	INTERF	XX	94	13.2
Fluvalinate isomer 2	91	7.9	27	17.9	INTERF	XX	91	10.8

Concentration of each pesticide in non-bleached beeswax: 0.5 mg/kg (n = 5); –: not detected; XX: no data; INTERF: matrix compound co-eluted with the analyte.

[a] See Fig. 1A.
[b] See Fig. 1B.
[c] First cartridge.
[d] Second cartridge.

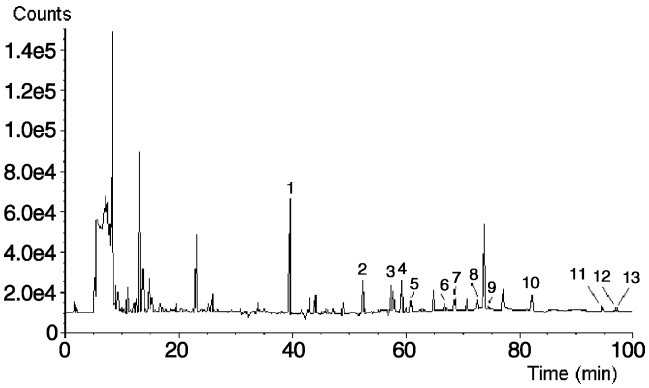

Fig. 2. Chromatogram obtained by solid-phase extraction after coupling the cartridges 100 mg ODS and Oasis during the extraction. Concentration of each pesticide in the non-bleached beeswax: 0.5 mg/kg. Peak identification: 1, lindane; 2, chlorpyriphos; 3, chlorfenvinphos; 4, endosulfan A; 5, DDE; 6, TDE; 7, endosulfan B; 8, acrinathrine; 9, bromopropylate; 10, tetradifon; 11, coumaphos; 12 and 13, fluvalinate.

3.6. Analytical method proposed for non-bleached beeswaxes

Briefly, the liquid–liquid extraction consisted of the dissolution of beeswax in *n*-hexane (0.1 g/10 mL), the extraction of the analytes with 10 mL of acetonitrile by shaking for 15 min, the addition of 2 mL of methanol to favor the phase separation, and the collection of the acetonitrile phase. As regards the clean-up procedure, 200 mL of water were added to the acetonitrile extract and the mixture was eluted on 100 mg ODS and Oasis cartridges, at the same time, under vacuum. The ODS cartridges were placed before the polymeric cartridges (Fig. 1A). After that, the ODS cartridges were discarded, the polymeric cartridges were dried with nitrogen and the analytes were eluted with 4 mL of ethyl acetate after a soaking time of 5 min. Finally, the solvent was evaporated and the residue was dissolved in 1 mL of acetone.

Table 7
Recoveries and R.S.D. values obtained by an external-standard calibration method with extracts of spiked beeswaxes after applying the sample preparation proposed for non-bleached beeswax (n = 5)

	0.5 mg/kg		5 mg/kg	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Lindane	101	11.4	97	14.4
Chlorpyrifos	95	12.5	96	13.5
z-Chlorfenvinphos	99	13.8	104	10.7
Endosulfan A	103	9.9	100	11.8
4,4′-DDE	94	15.8	95	17.0
4,4′-TDE	104	18.7	97	16.3
Endosulfan B	99	19.8	102	12.4
Acrinathrine	97	13.6	101	11.5
Bromopropylate	95	12.8	95	12.8
Tetradifon	107	10.9	99	11.4
Coumaphos	104	11.7	97	13.2
Fluvalinate isomer 1	100	12.0	102	14.5
Fluvalinate isomer 2	94	17.5	103	10.8

0.5 mg/kg and 5 mg/kg represent the spiking level.

The carrying out of a calibration with extracts of bleached beeswaxes spiked and subjected to the extraction/clean-up procedure proposed for the non-bleached beeswaxes resulted in recoveries close to 100% with coefficients of variation ranging between 10% and 20% for pesticide concentrations of 0.5 and 5 mg/kg (see Table 7). The standard addition method revealed that the influence of the matrix still existed, although it had been considerably reduced. The mean increase of the concentration owing to the matrix was near 10% and 40% for the concentrations of 0.5 and 5 mg/kg, respectively. The detection limits were now about three times higher, varying from 0.014 to 0.12 mg/kg.

4. Conclusions

The extraction of a beeswax solution in *n*-hexane with acetonitrile followed by a clean-up that consists of a solid-phase extraction of the acetonitrile extract mixed with water is the best option to analyze pesticides in bleached beeswax. Interferences from the non-bleached pure beeswaxes hinder the determination of fluvalinate and coumaphos by the above-mentioned procedure. The combination, during the solid-phase extraction step, of a low-capacity ODS cartridge before the polymeric cartridge removes these interferences and makes possible the analysis of all the acaricides in non-blanched beeswax.

There are errors by excess in the quantitative analysis when a conventional calibration with standards which do not match the matrix is used. The significance of the error depends on the compound, the analytical method and the concentration level. The error is not always evident if the concentrations in the extracts are relatively low. A matrix-matched calibration is necessary.

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