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ORIGINAL PAPER

Multi-residue analysis of traces of pesticides and antibiotics in honey by HPLC-MS-MS

Delphine Debayle · Guy Dessalces · Marie Florence Grenier-Loustalot

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Abstract The aim of this work was to develop an analytical method for simultaneous assay of residues of two families of antibiotics, and three pesticides, in honey. The assays involved a mixture of five tetracyclines, four sulfamides, and the pesticides coumaphos, carbendazim, and amitraz (two metabolites). All the compounds were extracted from honey and preconcentrated by optimised solid-phase extraction (SPE). Analysis was by high-performance liquid chromatography-mass spectrometry-mass spectrometry (HPLC-MS-MS) using a triple-quadrupole spectrometer in multiple reaction monitoring (MRM) mode in order to identify and quantify the compounds present (Sheth et al J Agric Food Chem 38:1125–1130, 1990). During development of the analytical method a strong matrix effect was found that depended on the floral origin of the honey. This led to the development of a standard additions method to quantify the contaminants sought.

Keywords Honey · Antibiotics · Pesticides · Multi-residues · HPLC-MS-MS · Solid-phase extraction

Introduction

Honey is a complex product that has always been considered as a natural and healthy food, i.e. free of any traces of impurities [1]. The beekeeper, however, must combat two major threats—diseases and poisoning of bees by insecticides. This explains the need to verify the potential presence of undesirable residues in hive products (honey, wax, propolis (bee glue), etc.) [2].

D. Debayle · G. Dessalces (✉) · M. F. Grenier-Loustalot
Service Central d'Analyse du CNRS,
Echangeur de Solaize, BP 22,
69390 Vernaison, France
e-mail: g.dessalces@sca.cnrs.fr

Substances that could be present in non-negligible quantities in honey may be authorised or prohibited veterinary substances, and contaminants from the environment such as pesticides. The aim of this work was to develop a multi-residue analytical method to simultaneously detect residues of antibiotics and pesticides in honey.

Two families of antibiotics were investigated, tetracyclines and sulfamides, because these antibiotics are frequently used in beekeeping to combat foulbrood [2]. We also included several pesticides, amitraz and coumaphos [3, 4], which are used in beekeeping to combat varroatosis, and carbendazim, which is widely used as an agricultural fungicide [5], especially to treat canola (rapeseed), a frequent honey source of bees.

Tetracyclines are effective antibiotics frequently used in human and veterinary medicine. This family of antibiotics can be found in a variety of foods such as milk [6, 7], eggs [8], and bovine meat [9, 10]. Similarly, sulfamides are used as anti-infection agents and are relatively non-toxic to humans. At the present time the maximal authorised quantity of sulfamides in honey has not been defined by the European Union [11].

Concerning pesticides, amitraz is valuable to beekeepers in the fight against varroatosis that started to develop in France in the early 1980s. There are few publications on the persistence of amitraz in the honey matrix, but the existence of breakdown products was reported by Jimenez [12] who detected five, of which only two remained in most samples after two weeks. Coumaphos is an organophosphate used as an insecticide/acaricide to combat varroatosis and small hive beetles. In 2002, the United States Environmental Protection Agency (EPA) established an upper limit of 0.1 mg kg^{-1} in honey and 100 mg kg^{-1} in beeswax. The European Community tolerates 0.1 mg kg^{-1} in honey [13]. While these two products are used to combat parasites in

Table 1 Published data on the analysis of contaminants in honey

Compounds	Techniques	Matrices	Limits	Ref.
Coumaphos + 35 pesticides	GC-MS	Honey	Spiked honeys, 0.02–1.6 mg kg^{-1}	[15]
Pesticides	LC-MS	Honey	0.01–0.15 mg kg^{-1}	[20]
Sulfamides	Thin-layer chromatography	Honey	20 μg kg^{-1}	[18]
Tetracyclines	LC-UV	Milk	2–4 μg kg^{-1}	[7]
Tetracyclines	LC-MS-MS	Water	0.2–3.8 μg L^{-1}	[19]

the hive environment, carbendazim, on the other hand, is a fungicide [5] used to treat crops, fruit trees, and vegetable cultures. We chose this fungicide for a number of reasons. It is used on crops that may be used by bees to gather honey (canola, sunflower). Furthermore, analysis of carbendazim enables the analysis of another fungicide, benomyl that is detected in the form of carbendazim [14].

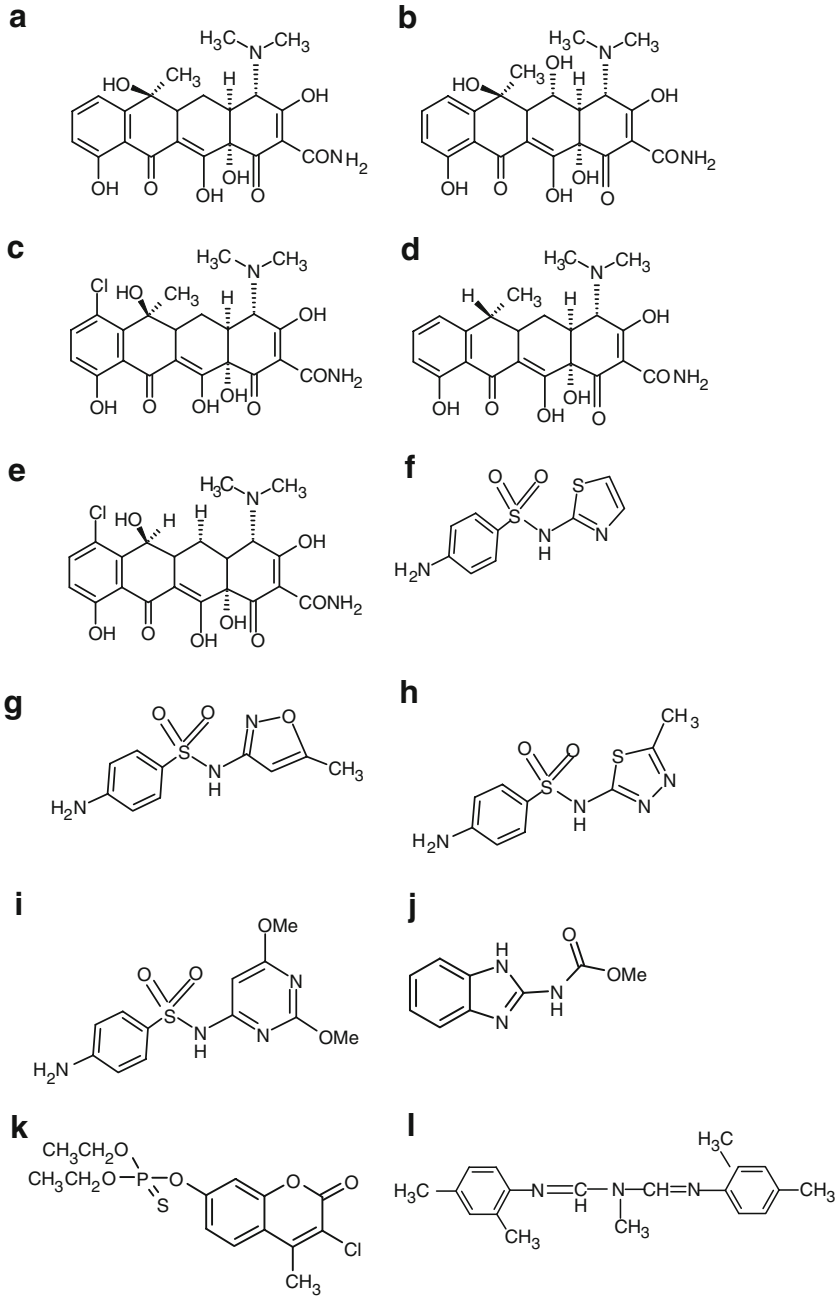

Fig. 1 Chemical structures of the compounds analysed: (**a**) tetracycline (TC), (**b**) oxytetracycline (OTC), (**c**) chlortetracycline (CTC), (**d**) doxycycline (DC), (**e**) demeclocycline (DMC), (**f**) sulfathiazole (STZ), (**g**) sulfamethoxazole (SMX), (**h**) sulfamethizole (SMZ), (**i**) sulfadimethoxin (SDM), (**j**) carbendazim (CDZ), (**k**) coumaphos (CMP), (**l**) amitraz (AMZ)

It is not easy to monitor residues of tetracyclines, sulfamides, or pesticides in some matrices (water, meat, milk, honey), because the low concentrations present impose relatively low limits of detection and quantification. Published data, listed in Table 1, show that the data are neither concordant nor satisfactory from the point of view of concentrations and limits of detection. The present work was thus undertaken in order to develop and validate a multi-residue method.

Experimental

Reagents and products

We optimised our analysis with nine antibiotics and three pesticides (Fig. 1). Antibiotics of the tetracycline family (tetracycline (TC), oxytetracycline (OTC), doxycycline (DC), demeclocycline (DMC), and chlortetracycline (CTC)) and antibiotics of the sulfamide family (sulfathiazole (STZ), sulfadimethoxin (SDM), sulfamethoxazole (SMX), and sulfamethizole (SMZ)) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). The tetracyclines were hydrochlorides.

The three pesticides, amitraz (AMZ), coumaphos (CMP), and carbendazim (CDZ), were purchased from Cil-Cluzeau (Paris, France). For amitraz, the two main breakdown products, 2,4-dimethylphenylamine (Amitraz I) and *N*-(2,4 dimethylphenyl) formamide (Amitraz II) were followed (Fig. 2).

The purity of all products was higher than 95% and they were used as received, with no further purification.

The honey samples were French honeys from untreated hives and of different floral origin (honeys from organic agriculture). They were supplied by different beekeepers and before use it was verified that the compounds monitored in this study could not be detected by HPLC-MS-MS.

Stock solutions were prepared by dissolving each compound in methanol at a concentration of 1 g L^{-1}; these solutions were stored frozen at -16° C. Standard solutions were prepared by diluting the stock solutions with 90:10 (*v*/*v*) water-acetonitrile. They were stored in darkness in a refrigerator at +4° C for a maximum of one week.

Methanol, acetonitrile and dichloromethane, all HPLC grade, were obtained from Fisher Scientific (Illirch, France). Formic acid, purity 98–100%, was purchased from Riedel-de Haën (St Quentin Fallavier, France). Ammonia (20%), hydrochloric acid (37%), and citric acid were from Prolabo, RP Normapur (Pessac, France).

SPE cartridges were obtained from Waters (St Quentin en Yvelines, France)

Equipment

Separation was with an Alliance 2695 chromatograph (Waters, St Quentin en Yvelines, France) equipped with an Inertsil ODS2 column (Hichrom, Reading, UK) and coupled to a Quattro-Micro triple-quadrupole mass spectrometer (Micromass-Waters). The chromatograph was equipped with independent thermostatted chambers for the column and injected samples.

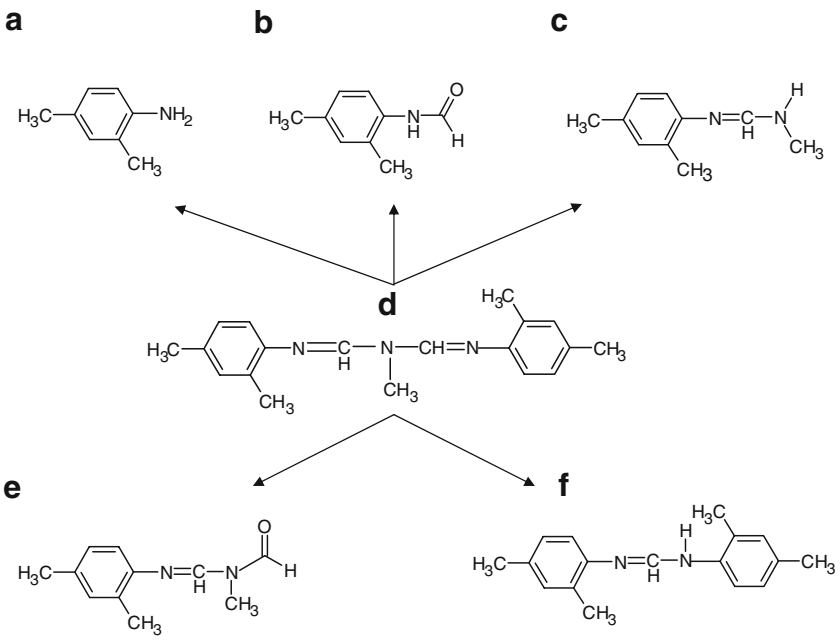

Fig. 2 Compounds arising from the breakdown of amitraz: (**a**) amitraz I, (**b**) amitraz II, (**c**) amitraz III, (**d**) amitraz, (**e**) amitraz IV, (**f**) amitraz V

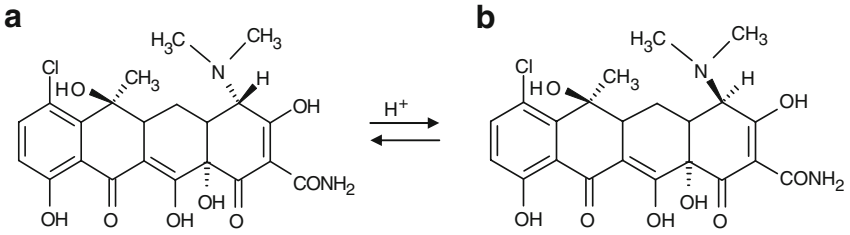

Fig. 3 The principal epimers of chlortetracycline: (**a**) chlortetracycline, (**b**) epichlortetracycline

Method

Solid-phase extractions (SPE) developed manually were subsequently adapted for a Rapidtrace automatic extractor (Zymark, Roissy, France), leading to better reproducibility.

The analysis of antibiotics and pesticides in honeys requires an initial extraction step to separate the compounds of interest from the matrix, and also to pre-concentrate them, while maximally limiting risks of interference from other compounds during the analysis (matrix effect). It was necessary to find a methodology adapted to the physico-chemical characteristics of each family of residues sought.

Tetracyclines are amphoteric substances slightly soluble in water at pH 7. They are relatively basic and are unstable in acid or alkaline media. An important problem in the quantitative analysis of tetracyclines is the possible formation of epimers in samples as a function of pH [10, 22]. Among the six tetracyclines, only demeclocycline does not form epimers. Chlortetracycline, tetracycline, doxycycline, and oxytetracycline all contain the 4-epimer (Fig. 3). Recent European Union legislation on MRLs (maximum residue levels) [23] stipulates that for each tetracycline, calculated residual values must include the sum of the parent compound and its 4-epimer, the most easily quantifiable.

Sulfamides are derivatives of *para*-aminobenzenesulfonic acid and hydrolysis in required for their quantification in order to break their bond with sugars present in honey [24–26].

All the pesticides except coumaphos contain sites that can ionise as a function of pH. For amitraz, only the two major breakdown products were sought, 2,4-dimethylphenylamine (amitraz I) and *N*-(2,4-dimethylphenyl) formamide (amitraz II), because of its rapid breakdown in honey [12].

Contaminants were recovered by placing 6 g honey in a centrifuge tube and diluting to 12 g with 2 mol L^{-1} hydrochloric acid. The sample was mixed manually for 3 min before sonicating for 10 min at room temperature. It was then left in darkness for 5 min to release the sulfamides from their bonds with sugars by acid hydrolysis, as described in the literature [26]. Samples were then centrifuged for 10 min at 7,000 rpm at 20° C to eliminate proteins. Supernatant (10 g) was then removed and diluted with 10 mL 0.2 mol L^{-1} citric acid. The presence of citric acid enables an easier adjustment of the pH to around 4 with 20% ammonia. An Oasis HLB 60-mg extraction cartridge (Waters) was successively conditioned with 3 mL acetonitrile, 3 mL water, and then 3 mL 0.1 mol L^{-1} HCl before depositing 10 g honey solution at pH 4. The cartridge was then rinsed with 1.5 mL deionised water and dried under nitrogen for 4 min. Compounds of interest were eluted with 3 mL acetonitrile-methanol-dichloromethane (50:25:25, *v*/*v*/*v*). The eluate was evaporated to dryness under a nitrogen stream at 40°C, and the residue was taken up with 500 µL water-acetonitrile (90:10, *v*/*v*) before HPLC-MS-MS injection.

Results and discussion

Liquid chromatography coupled to mass spectrometry is the most widely used technique for all the compounds studied [9, 20, 24]. Development of the analytical method initially involved optimising the chromatographic separation, then finding a suitable technique for recovering residues from honey and finally quantifying them by mass spectrometry [21].

Optimising separation by high-performance liquid chromatography

The first step involved optimising the chromatographic separation of the different compounds by use of aqueous reference solutions.

Table 2 The HPLC columns tested

Column	Length (mm)	Inner diameter (mm)	Particle size (µm)	Bonding	Eluent
Inertsil ODS2	50	2.1	5	C_{18}	Water-acetonitrile
X-Terra	100	3	5		Water-acetonitrile
Nucleodur	50	2.1	3		Water-acetonitrile

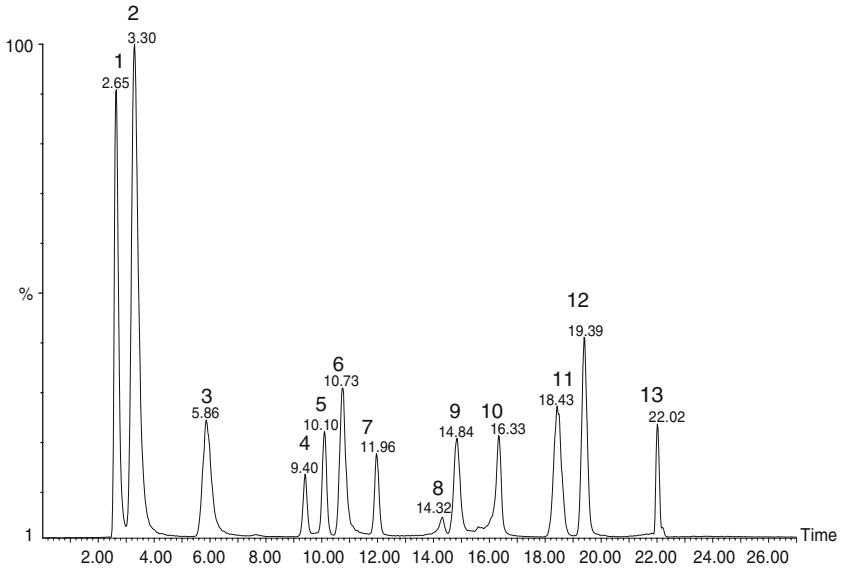

Fig. 4 Chromatogram obtained from all the compounds studied. *1*: amitraz I; *2*, carbendazim; *3*, sulfathiazole; *4*, oxytetracycline; *5*, tetracycline; *6*, sulfamethizole; *7*, demeclocycline; *8*, chlortetracycline; *9*, sulfamethoxazole; *10*, doxycycline; *11*, amitraz II; *12*, sulfadimethoxin; *13*, coumaphos

Taking the nature of the antibiotics into account, specially designed columns for basic products were used, with an elution gradient. Three columns were tested (Table 2).

The compounds were very poorly separated with the Nucleodur column and asymmetric peaks were obtained. The X-terra column gave relatively fine peaks for all the compounds, but it was impossible to separate two co-eluting pairs—carbendazim with amitraz I and amitraz II with sulfadimethoxin.

The Inertsil ODS2 column was selected since it furnished better separation efficiencies (Fig. 4). Separation was with an elution gradient using a water-acetonitrile mixture, each solvent containing 0.2% formic acid (Table 3). Column flow-rate was 200 μL min^{-1} and sample and column temperatures were 5 and 25° C (thermostatted compartments on the chromatograph).

Optimising mass spectrometry parameters

In order to follow trace amounts of residues in a matrix, they must first be unambiguously identified and then quantified. In liquid chromatography it is thus obligatory to work in MS-MS mode, most often with a triple quadrupole analyser [9, 20].

Table 3 HPLC solvent gradient

Time (min)	Water (%)	Acetonitrile (%)
0	98	2
16	76	24
17	2	98
19	2	98
19.1	98	2

Each solvent contained 0.2% formic acid

Contaminants were detected with an electrospray source (ESI) in positive-ion mode. After optimising the parameters, the following settings were used: capillary voltage 3.10 kV, RF lens at 2 V, source and desolvation temperatures 120 and 350° C. The flow-rates of the nebuliser and desolvation gases (N_2) were 80 and 600 L h^{-1}. The pressure of the collision gas (argon) was close to 0.307 Pa. Two fragmentation reactions characteristic of pseudo-molecular ions were selected for each residue in MRM (multiple reaction monitoring) mode. Fragmentation

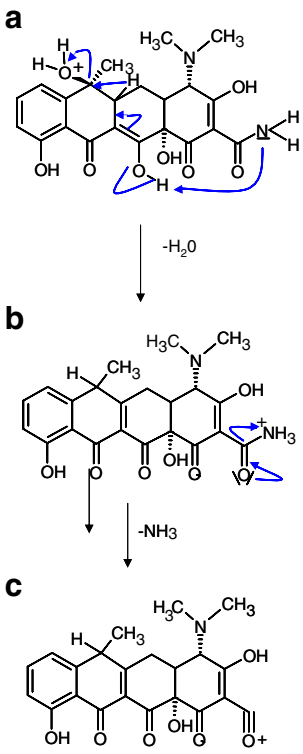

Fig. 5 Fragmentation of tetracycline (**a**) 445 *m/z*, (**b**) 427 *m/z*, (**c**) 410 *m/z*

of the tetracyclines was consistent with published data [27–29], with the loss of water and ammonia, as can be seen in Fig. 5 for tetracycline. The optimised parameters are listed in Table 4.

Extraction of contaminants

Given the complexity of the honey matrix [2], a pre-concentration and purification step was necessary. Several published methods were tested [21].

We first tested a liquid-liquid extraction method, since it was used by Verzegnassi to recover ten sulfamides from honey, using solvents such as acetonitrile and dichloromethane [25]. In parallel, Jimenez [12] extracted compounds arising from the breakdown of amitraz in honey using a mixture of hexane and acetone. Nevertheless, none of these methods, each specific for a single family of compounds, provided acceptable recovery yields for all of the compounds we studied.

Based on work in our laboratory [21] we tested solid-phase extraction (SPE). We first used Oasis MCX cartridges (Waters), working with a mechanism of ion exchange combined with hydrophobic retention. Most of the compounds to extract except coumaphos contain amine groups that can ionise as a function of pH. After depositing the honey in acid solution and rinsing, the compounds were eluted with acetonitrile-ammonia mixtures (95:5 and 70:30 *v*/*v*). The extraction yields were highly dispersed for the family of tetracyclines since they are strongly retained on the Oasis MCX stationary phase.

Table 4 Choice of MRM transition for the compounds studied [17]

Compound	Chemical formula	Retention time (min)	Precursor ion $[M + H]^+$	Cone potential (V)	Transition	Collision potential (V)
Tetracycline 5	$C_{22}H_{24}N_2O_8$	10.1	445	18	445 → 427	11
					445 → 410	15*
					445 → 154	24
Doxycycline 10	$C_{22}H_{24}N_2O_8$	16.3	445	20	445 → 428	15*
					445 → 154	29
Oxytetracycline 4	$C_{22}H_{24}N_2O_9$	9.4	461	18	461 → 443	9
					461 → 426	14*
Chlortetracycline 8	$C_{22}H_{23}CIN_2O_8$	14.32	479	18	479 → 462	14*
					479 → 444	15
Demeclocycline 7	$C_{21}H_{21}CIN_2O_8$	11.96	465	20	465 → 447.8	12*
					465 → 429.9	16
					465 → 154	23
Sulfamethoxazole 9	$C_{10}H_{11}N_3O_3S$	14.8	254	20	254 → 156	12*
					254 → 108	22
					254 → 92	25
Sulfathiazole 3	$C_9H_9N_3O_2S_2$	5.9	256	20	256 → 156	12*
					256 → 108	22
					256 → 92	23
Sulfamethizole 6	$C_9H_{10}N_4O_2S_2$	10.7	271	18	271 → 156	12*
					271 → 108	20
					271 → 91	23
Sulfadimethoxin 12	$C_{12}H_{14}N_4O_4S$	19.4	311	24	311 → 156	17*
					311 → 108	25
					311 → 92	25
Amitraz I 1	$C_8H_{11}N$	2.7	122	25	122 → 107	14*
					122 → 79	26
Amitraz II 11	$C_9H_{11}NO$	18.4	150	24	150 → 107	18*
					150 → 123.2	13
Carbendazim 2	$C_9H_9N_3O_2$	3.3	192	18	192 → 160	14*
					192 → 132	28
					192 → 65	35
Coumaphos 13	$C_{14}H_{16}CIO_5PS$	22.1	363	25	363 → 307	15
					363 → 227	27*

*Indicates the most intense daughter ion, selected for quantification

Table 5 Extraction yields for Oasis HLB, 60 mg, and a forest honey

Compound	Test 1	Test 2	Test 3	Test 4	Test 5	Mean yield
TC	63.1	71.6	58.2	58.2	66.4	63.5
DC	111.9	112.3	109.9	111.5	101.6	109.4
OTC	86.2	88.3	88.1	84.9	86.7	86.8
CTC	88.7	93.0	92.6	87.3	88.3	90.0
STZ	40.2	41.2	47.4	42.7	46.4	43.6
SMX	49.9	46.4	54.5	52.6	55.5	51.8
SMZ	50.1	46.3	57.5	53.4	57.1	52.9
SDM	51.7	48.5	54.4	55.8	57.4	53.5
AMZ I	60.0	60.2	76.2	62.1	77.7	67.2
AMZ II	57.1	65.7	52.7	58.3	60.9	58.9
CDZ	94.0	92.5	102.3	92.8	93.4	95.0
CMP	89.7	79.9	89.9	89.7	88.9	87.6

Yields for the sulfamides varied from 60 to 80% depending on the chemical structure of the antibiotic. For the pesticides, yields were 35% for amitraz II, about 70% for carbendazim, 100% for amitraz I, and about 65% for coumaphos.

Many authors have used Oasis HLB cartridges (Waters), working with hydrophilic and hydrophobic retention mechanisms, while others prefer C_{18} bonded silica stationary phases. These two types of phase are also used to extract antibiotics and pesticides from a variety of matrices such as meats [9], water [18], milk [6], and honey [15, 26, 29].

Considerable testing was required to obtain acceptable yields for all the compounds; this resulted in the procedure described in the experimental section. Under the working conditions described, extraction yields varied with the compound sought (Table 5). For the tetracyclines, yields varied from 64% for tetracycline to 87% for oxytetracycline, 90% for chlortetracycline and 109% for doxycycline. For the sulfamides, they were only of the order of 50%, regardless of the sulfamide. Finally, for the pesticides, the values varied with the pesticide family. The yield for amitraz I was 67%, while that of amitraz II was 59%, that of carbendazim was 95%, and that of coumaphos 88%. It can be seen that the lower yields involved compounds containing free NH_2 functions and it will be seen below that they can vary with the type of honey.

Even though recovery yields were not all close to 100%, we considered them acceptable since they were reproducible. As is the case in all multi-residue methods, the final procedure is often a compromise that furnishes reproducible and reliable results provided calibrations are done under the same conditions.

Quantification of species

We initially used the external standard method with aqueous reference solutions, but it was found that this technique was unreliable for honey. Calibration curves in the range of 20–200 μg kg^{-1} using spiked honeys showed a strong matrix

Table 6 Slopes of the calibration curves: peak area = *f* (residue content), for spiked water and different honey types

Residue	Water	Forest	Mixed flowers	Chestnut	Acacia	Sunflower	Canola	Lavender	Mountain	Heather
TC	165	153	182	57	176	88	153	150	79	77
OTC	133	130	163	57	152	67	149	152	97	76
DC	264	220	270	124	252	145	298	234	162	165
CTC	54	54	63	29	37	32	43	44	27	33
STZ	572	131	211	57	166	129	166	163	133	122
SMX	284	106	151	147	114	124	115	108	96	91
SMZ	424	131	147	57	171	125	158	168	122	103
SDM	603	215	297	275	197	225	202	203	187	186
A I	245	312	199	492	98	258	235	118	297	236
A II	167	174	161	150	165	160	122	143	128	109
CDZ	2472	1803	1880	225	1188	748	166	1546	1173	609
CMP	32	26	13	35	34	25	18	28	34	18

Table 7 Limits of detection and quantification of the residues (ng g^{-1}) [19]

Compound	LD acacia	LQ acacia	LD forest	LQ forest	LD lavender	LQ lavender
Tetracycline TC	0.4	1.4	2.2	3.7	0.6	2.0
Oxytetracycline OTC	0.5	1.7	1.3	2.2	1.0	3.6
Doxycycline DC	1.6	5.4	6.2	7.3	1.1	3.8
Chlortetracycline CTC	0.2	0.7	2.1	1.3	1	3.5
Sulfamethoxazole STZ	0.7	2.3	1.7	2.1	0.4	1.3
Sulfathiazole SMY	0.7	2.4	1.9	2.1	0.4	1.5
Sulfamethizole SMZ	0.6	1.9	1.8	1.9	0.5	1.6
Sulfadimethoxin SDM	0.2	0.6	1.2	2.2	0.4	1.3
Carbendazim CDZ	0.6	1.9	1.4	2.0	0.4	1.3
Amitraz I AI	0.7	2.4	1.6	2.5	0.5	1.7
Amitraz II AII	0.6	2.0	2.1	4.9	0.8	2.8
Coumaphos CMP	1.8	6.1	3.5	3.3	1.6	5.6

effect whose intensity differed depending on the floral origin of the honey. This can be seen in Table 6 that lists the calibration line slopes for different types of honey.

For the tetracyclines (TC, OTC, DC, CTC), there was a considerable disparity in the calibration lines, depending on the type of honey used. The spiked aqueous solution did

Table 8 Results of interlaboratory trial IHC 2005

Laboratory	Honey 3 (contaminated by TC)		Honey 4 (spiked with TC 20 ppb)		Honey 5 (spiked with STZ 50 ppb)	
	Results (μg kg^{-1})	Z-score	Results (μg kg^{-1})	Z-score	Results (μg kg^{-1})	Z-score
01 [a]	25.8	-0.66	12.5	-0.65	36	-0.06
02 [a]	22	-1.23	9	-1.74	42	0.68
03 [a]	27	-0.48	16	0.44	49	1.56
08 [a]	32	0.28	13	-0.49	19	-2.18
09 [a]	26	-0.63	11	-1.11	37	0.06
12 [a]	46.7	2.49	27.4	4.00	13.2	-2.90
14 [a]	38	1.18	19	1.38	34	-0.31
17 [b]	8.2	-3.31	ND		20.9	-1.94
18 [b]	18	-1.83	10	-1.43	46	1.18
21 [a]	33	0.43	14.3	-0.09	29.9	-0.82
22 [b]	38	1.18	14	-0.18	24	-1.56
23 [b]	14	-2.44	5	-2.99		
27 [a]	44	2.09	21	2.00	36	-0.06
28 [a]	36.6	0.61	15.2	0.04	49.5	1.25
29 [a]	27.9	-0.34	19.5	1.54	42.3	0.72
30 [a]	43.1	1.95	ND		51.5	1.87
31 [a]	33.3	0.47	15.2	0.20		
35 [a]	23.6	-0.99	ND		21.6	-1.86
36 [b]	37	0.80	ND		23/27	-1.43
37 [a]	23.14	-1.06	8.79	-1.80	30.41	-0.76
38 [a]	30.4	0.04	17.9	1.04	31	-0.68
39 [a]	32.1	0.29	15.2	0.20	<10	
40 [a]	31	0.13	15	0.13	45	1.06
41 [a]	26.2	-0.93	11.7	-1.49	27.5/26.6	-1.18
42 [a]	25.9	-0.64	9.3	-1.64		
45 [a]	27.3	-0.43	18.6	1.26	8.9	-3.44
46 [a]	101.2	10.71	37	6.99		
Mean value	30.2		14.6		35.4	

[a] HPLC-MS-MS
[b] HPLC-DAD
ND: non detected

not automatically give values higher than those obtained with honey, meaning that there may be a positive or negative matrix effect depending on the floral origin of the matrix. The lowest responses for the four tetracyclines studied were obtained with sunflower, heather, mountain, and chestnut honeys. All flowers, canola, and acacia honeys gave the highest responses.

In the case of sulfamides (STZ, SMX, SMZ, SDM), there was a considerable difference in the standards prepared in water and in the spiked matrix. The matrix effect considerably reduced the mass detector response for these compounds. On the other hand, response differences with honeys of different origin were lower than for tetracyclines.

As for the sulfamides, there was a negative matrix effect with carbendazim (CDZ), with a disparity of responses depending on the nature of the honey. All flowers, forest, and lavender honeys had the closest responses to the aqueous solutions.

For the two metabolites arising from amitraz breakdown (A I and A II), certain types of honey had a positive effect on detection, e.g. forest honey. The floral origin of the honey plays an important role in the response and the slopes were shallower for Amitraz II than for Amitraz I. This may be explained by the fact that the former is a formamide and the latter is a primary amine that is more easily ionisable.

In the case of coumaphos (CMP), mountain, acacia, and chestnut honeys gave the best responses, close to those of the aqueous solutions. All flowers honey gave the lowest response while it gave the best responses for the antibiotics, tetracyclines, or sulfamides.

As a result of these comparisons, it can be seen for both the antibiotics and pesticides that the matrix and its floral origin play a major role in the mass detector response and are a very important aspect of quantitative analysis. The only possibility would be to prepare calibration curves using honeys of the same floral origin as that studied, but totally free of traces of the compounds studied. It can easily be seen that this solution is not possible in practice.

Finally, we adopted the standard additions method to overcome the drawbacks detected in the preceding tests. This technique is very widely used when media are complex and have important matrix effects. Thus, use of standard additions avoids having to know the floral origin of the honeys and also allows the importance of the matrix effect and extraction yields to be neglected, since all the tests are done on the same honey. The only disadvantage of this technique is that it requires a larger quantity of sample in order to carry out the different additions and above all that it is costlier in time and material for preparation of spiked solutions.

Validation of the method

Because there is no reference method to which our method can be compared, we decided to validate it using published standards [30, 31] and the SANCO directive [32].

We evaluated the different characteristics selected for validation, i.e. robustness, linearity, limits of detection and quantification, repeatability, accuracy, specificity, and repeatability of extraction yields. Robustness was verified using two parameters, the pH value just before extraction and the time between pH adjustment and extraction. Linearity was monitored in the concentration range 10 to 100 $\mu g\ kg^{-1}$ (ppb). Repeatability and accuracy were verified in six samples of different concentration, each injected six times.

The results obtained show that our method is valid according to the statistical criteria of the methods of published standards. Limits of detection and quantification for different spiked honeys analysed with our method are listed in Table 7. It is seen that the limits of detection vary with the type of honey but are systematically lower than 2.2 ng g^{-1}; limits of quantification are lower than 7.3 ng g^{-1}.

Inter-laboratory test

In order to test our method, we participated in an inter-laboratory study [33]. This collaborative trial involved seven honeys in which tetracycline and sulfathiazole were assayed and that involved 46 laboratories, 23 in Europe, each using the technique of its choice. Several honeys contained no residue, which we confirmed, and the results obtained with the others using the methodology we developed are to be compared with those obtained using other liquid chromatography techniques (Table 8). For the spiked honeys (honeys 4 and 5), it is first seen that the mean of the results obtained by the different laboratories are about 70% of the spike value. This could be the sign of breakdown of the residue or the existence of a fraction not extractible with the techniques used. The overwhelming majority of the laboratories used the HPLC-MS-MS technique and the results are good since the statistical *Z*-score test considers that results are good for values ≤ 2 [34]. The multi-residue method developed and used in our laboratory (laboratory 01; Table 8) is good and can thus be considered as operational.

Conclusion

A multi-residue method for monitoring residues of pesticides and antibiotics used in beekeeping has been developed. It required development of a method for separation of compounds of different polarity and a method of extraction

applicable to honeys of different floral origins. A considerable matrix effect was shown that depended on the floral origin of the honey; that required quantification based on the standard additions method. The analytical method was validated according to the protocols of standard methods and was tested in a collaborative trial involving 46 laboratories.

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