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Residue Determination in Honey

Simone Moretti, Giorgio Saluti and Roberta Galarini

Additional information is available at the end of the chapter

Abstract

The use of antibiotics to fight bacterial and fungal honeybee diseases is documented since 1940s. Although at present in some countries certain antibiotics are authorized in apiculture, only few law systems provide maximum residue limits in honey. In addition, residues of worldwide banned antibiotics such as chloramphenicol, nitrofurans and nitroimidazoles have been frequently found. Therefore, the availability of reliable analytical methods able to detect concentrations at few parts per billions is fundamental. After a general overview of the available sample treatment strategies and analytical techniques, the most significantly published methods are discussed. Aminoglycosides and, to a lesser extent, tetracyclines are the more difficult classes to analyse. The current trends are the development of multiclass procedures and of micro-extraction techniques to improve the cost-effectiveness of residues control in the globalization era.

Keywords: honey, antibiotics, honeybee diseases, sample preparation, liquid chromatography-mass spectrometry

1. Introduction

Antimicrobials are used in food-producing animals to prevent and/or treat animal diseases. Although epidemiological data on the real magnitude of their adverse effects are very scarce, they indicate that the presence of antibiotic residues in food could be an important vehicle for the development of antibiotic-resistant bacterial strains. Because of these concerns, many countries have restricted the use of antibiotics in farm. The major honeybee diseases for which antibiotics are applied are American foulbrood, European foulbrood infections and nosemosis. Foulbrood infections are caused by bacteria, whereas *Nosema* disease is caused by a fungus. Currently, in the European Union the maximum residue limits (MRLs) for antibiotics in food are listed in Regulation (EU) No 37/2010 [1]. This regulation stipulates that each antibiotic

must have a MRL before it can be used on a food-producing animal. European Union does not allow the use of antibiotics for treatment of honeybees, and therefore, there are not MRLs in honey for these substances. The lack of harmonized rules with regard to acceptable control methods, limits of detection or sampling methods, results in different interpretations by European Member States. Some Member States and Switzerland have established action limits or tolerance levels [2, 3]. In the CRL Guidance Paper (2007) [4], the European Union References Laboratories (EURLs, ex CRLs) proposed recommended concentrations for analysis of macrolides, streptomycin, sulphonamides and tetracyclines in honey within the national residue control plans carried out in accordance with Council Directive 96/23/EC [5] (**Table 1**). These recommended concentrations, however, have no real legal basis. They are used as reference during method development since detection capability (CCβ) for screening methods or decision limit (CCα) for the confirmatory ones [6] should be lower than recommended concentrations. All the veterinary drugs just mentioned belong to the Group B of Annex I of Council Directive 96/23/EC [5], that is, they are permitted substances with fixed MRLs in several food commodities. On the other hand, in the case of banned substances (Group A, Annex I of Council Directive 96/23/EC) such as chloramphenicol (CAP) and nitrofurans (NFs), the European Union has set minimum required performance limits (MRPLs) of 0.3 and 1.0 µg/kg, respectively. MRPLs are foreseen in Article 4 of Commission Decision 2002/657/EC

Country	Approved substance	MRLs[a] (µg/kg)	RCs[b] (µg/kg)	Source
EU	Streptomycin	–	40	European Regulation 37/2010 [1] CRL Guidance Paper [4]
	Tetracyclines	–	20	
	Sulphonamides	–	50	
	Erythromycin Tylosin	–	20	
USA	Lincomycin	–	–	CFR—Code of Federal Regulations—Title 21 [8]
	Oxytetracycline	–	–	
	Tylosin	–	–	
Canada	Fumagillin	25	–	List of Maximum Residue Limits (MRLs) for Veterinary Drugs in Foods [9]
	Oxytetracycline	300	–	
	Erythromycin	–	30[c]	
	Tylosin	200[d]	–	
Australia/New Zealand	Oxytetracycline	300	–	Food Standards Code (standard 1.4.2—Schedule 20) [10]
Japan	Oxytetracycline	300	–	Positive List System for Agricultural Chemical Residues in Foods [11]
	Amoxicillin	8	–	
	Ampicillin	9	–	

[a]Maximum residue limits (MRLs) or tolerances (legal limits).
[b]Recommended concentrations (RCs) which only represent a reference point for analytical method performances.
[c]Working residue level (WRL) below which there is considered to be undue risk to human health.
[d]Sum of tylosin A and B.

Table 1. Worldwide limits for antibiotics in honey.

[6] and they are reference point for action. They are intended to harmonize the analytical performance of methods ensuring the same level of consumer protection in the European Union. Among banned substances, also the use of nitroimidazoles has been documented in beekeeping practice. However, no MRPLs have been fixed for nitroimidazoles, the European Union has not fixed the relevant MRPLs, and during the development of analytical methods, the recommended concentration of 3 µg/kg (CRL Guidance Paper [4]) is taken into account.

The worldwide standard, the Codex Alimentarius, has not fixed any MRL for antibiotics in honey [7]. The Codex Alimentarius or "Food Code" was established by FAO and the World Health Organization in 1963 to develop harmonized international food standards, which protect consumer health and promote fair practices in food trade. Similarly, in United States, no tolerances for antibiotics in honey have been established, although oxytetracycline is approved for longtime in beekeeping practice to control American foulbrood. At present, lincomycin and tylosin are authorized, too. The MRLs (or tolerances) for residues of antibiotics in food are set by the US Food and Drug Administration (USFDA) and listed in the Code of Federal Regulations, Title 21 [8]. Conversely, Canada, Australia, New Zealand and Japan have established MRLs for oxytetracycline (300 µg/kg) [9–11]. In addition, also MRLs for fumagillin, oxytetracycline and tylosin are provided by Canadian authorities (**Table 1**). For erythromycin, a working residue level is provided, below which no risk to human health is considered.

Sulphathiazole and oxytetracycline are probably the first antibiotics used to fight honeybee diseases. Starting from 1980s, analytical methods have been developed for these two drugs in honey at trace levels mainly based on liquid chromatography coupled to UV-Vis (LC-UV-Vis) and fluorescence detectors (LC-FLD). In the early 2000s, the availability of liquid chromatography systems coupled to mass spectrometric analysers (LC-MS) at bench level involved the progressive development of procedures using this technique which allows a more selective and universal detection than the traditional detectors based on UV absorption (quite universal, but not selective) or fluorescence (selective, but not universal). Therefore, existing methods have been progressively converted using LC-MS improving performances and sample throughput, and new challenging analytical problems have been solved thanks to this technique equipments (e.g. the detection of nitrofurans metabolites in food).

From a toxicological point of view, in the European Union law system the distinction is between permitted drugs (aminoglycosides, lincomycin, macrolides, quinolones, sulphonamides and tetracyclines) and banned drugs (chloramphenicol, nitrofurans and nitroimidazoles) belonging to substances of group B and A, respectively (Annex I of Directive 96/23 [5]). As discussed before, there are not MRLs for antibiotics in honey (**Table 1**). Hence, in this context, "permitted drugs" are drugs with an MRL in food commodities other than honey (meat, milk, liver, etc), whereas the banned ones (chloramphenicol, nitrofurans and nitroimidazoles) cannot be used in any food-producing species generally not only in European Union, but also in several other countries. This distinction is also fundamental to choose the analytical technique to develop confirmatory methods, which are procedures fulfilling Commission Decision 2002/657/EC criteria [6]. For the banned substances, the use of mass spectrometric detectors is mandatory, whereas, for the permitted ones, traditional detectors, UV-Vis or FLD, are suitable, too. In addition, for banned drugs, the required method limits are in the range from 0.1 to 1 µg/kg; for permitted drugs, limits of about one order of magnitude greater can be acceptable (**Table 1**).

The use of liquid chromatography coupled to mass spectrometry equipments and the worldwide improvement of law systems probably explains the decrease in the incidence of veterinary drug residues in honey and honeybee products (royal jelly and propolis). The number of cases per year in 2003 was 40, whereas in 2015 only six notifications have been recorded by the European Rapid Alert System for Food and Feed (RASSF) as shown in **Table 2** [12]. In place since 1979, RASSF enables information to be shared efficiently between its members [national food safety authorities of EU Member States, the EU Commission, the European Food Safety Authority (EFSA), Norway, Liechtenstein, Iceland and Switzerland]. It provides an efficient service to ensure that urgent notifications are sent, received and responded to in the shortest time possible. Thanks to RASSF, many food safety risks had been averted before any harm to European consumers was caused.

Year	No of notifications	No of found substances[a]	Number (substance)
2002	45	57	34 (CAP), 13 (STR/DSTR), 7 (SAs), 3 (TCs)
2003	40	53	20 (SAs), 17 (CAP), 11 (STR), 3 (TCs), 2 (NFs)
2004	25	27	10 (SAs), 7 (CAP), 5 (NFs), 5 (STR)
2005	41	49	25 (CAP), 8 (STR), 6 (SAs), 5 (TCs), 4 (NFs), 1 (MAC)
2006	16	17	7 (CAP), 6 (SAs), 2 (STR), 1 (NFs), 1 (TCs)
2007	20	41	24 (SAs), 6 (QNs), 6 (TCs), 2 (MACs), 1 (CAP), 1 (STR), 1 (NFs)
2008	27	29	9 (MACs), 7 (TCs), 5 (SAs), 3 (CAP), 2 (QNs), 2 (STR), 1 (NFs)
2009	10	10	4 (TCs), 3 (NFs), 2 (STR), 1 (SA)
2010	8	9	3 (lincomycin), 2 (STR), 1 (TC), 1 (NMZ), 1 (MAC), 1 (QN),
2011	6	6	3 (SAs), 2 (NMZs), 1 (lincomycin)
2012	6	7	5 (SAs), 2 (NFs),
2013	4	4	2 (SAs), 1 (NFs), 1 (TCs),
2014	1	1	1 (SA—sulphamethoxazole)
2015	6	10	3 (CAP), 3 (STR/DSTR), 2 (TCs), 1 (NMZ), 1 (SA)

[a]In the same sample more than one residue could be present.

Table 2. RASFF notifications in the period 2002–1015 (hazard category "residues of veterinary medicinal products"; product category "honey and royal jelly").

In Section 4, an extensive overview of the main published analytical methods for the determination of residues of the antibiotics (legally or illegally) used in apiculture is carried out. The analytical steps of each selected method (sample treatment, analytical technique and detection limits) are summarized in **Tables 3–11**.

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
CAP	100 mM NaAc buffer (pH 5.0)/ Oasis HLB-SPE, phosphate buffer (pH 6.5), ACN:DCM (80:20, v/v)	Symmetry Shield RP18 (150 × 2.1 mm, 3.5 μm)	Gradient: water/ ACN	LC-MS/MS (ESI−)	0.021	[17]
CAP	50 mM NaAc buffer (pH 5.2)[b]/ DCM (Extrelut[c])	Acquity UHPLC BEH C18 (50 × 2.1 mm, 5.0 μm)	Gradient: 25% NH$_3$ in 10% CAN/25% NH$_3$ in ACN	LC-MS/MS (ESI−)	0.013	[18]
CAP	Water/ACN, CHCl$_3$	Purospher Star RP-18 (55 × 4.0 mm, 3.0 μm)	Gradient: 0.15% FA/MeOH	LC-MS/MS (ESI−)	0.01	[19]
CAP	Water/MIP-SPE	Ascentis C18 (100 × 2.1 mm, 3.0 μm)	Isocratic: 30% ACN in 10 mM NH$_4$Ac (pH 6.7)	LC-MS/MS (ESI−)	0.03	[20]
CAP	Water/ MWCN-SPE	Halo fused-core C18 (50 × 2.1 mm, 2.7 μm)	Gradient: 0.1% FA/ACN	LC-MS/MS (ESI−)	0.008	[21]
CAP	Water/EtAc	Luna C18 100 Å (50 × 2.0 mm, 5.0 μm)	Gradient: 2 mM NH$_4$Ac/MeOH	LC-MS/MS (ESI−)	0.10	[22]
CAP and FF, FFA, TAP	1% NH$_3$/Oasis HLB-SPE	Ascentis express phenyl-hexyl (100 × 2.1 mm, 2.7 μm)	Gradient: 5 mM NH$_4$Ac buffer (pH 5.0)/MeOH	UHPLC-MS/ MS (ESI−)	0.03	[23]
CAP	Water/EtAC	Luna C18 (150 × 4.6 mm, 5.0 μm)	Gradient: Water/ ACN	LC-MS/MS (ESI−)	0.09	[24]
CAP and FF, TAP	QuEChERS (1% AcOH in ACN)	LMA-MAA-EDMA monolithic[d] (150 mm × 250 μm)	Gradient: Water/ ACN-MeOH (90:10, v/v)	LC-MS/MS (ESI−)	0.045	[25]

[a]FF, florfenicol; FFA, florfenicol amine; TAP, thiamphenicol.

[b] An enzymatic hydrolysis was carried out to deconjugate CAF in the muscle sample included in the method scope.

[c]Extrelut (diatomaceous earth) was used to help the liquid-liquid extraction process.

[d]LMA-MAA-EDMA: poly(lauryl methacrylate-co-methacrylic acid-co-ethylene glycol dimethacrylate).

Table 3. Confirmatory methods for chloramphenicol (CAP).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
LIN, TYL	Na$_2$CO$_3$– NaHCO$_3$ buffer (pH 9.0)/ C18-SPE	Zorbax C8 (150 × 2.1 mm, 5.0 μm)	Gradient: 0.1% TFA/0.1% TFA in ACN/MeOH	LC-MS (APCI+)	7–10	[31]
ERY, OLE, SPI, TILM, TYL	100 mM NaH$_2$PO$_4$ buffer at pH 8.0/Oasis HLB-SPE	YMC ODS-AQ S-3 120 Å 50 × 2.0 mm	Gradient: 1% FA/water/ACN	(a) LC-MS (ESI+) (b) LC-MS/MS (ESI+)	(a) <1 (b) 0.01–0.07	[32]
ERY, LIN, JOS, SPI, TILM, TYL	TRIS[b] buffer (pH 10.5)/Oasis HLB-SPE	Synergi Hydro-RP (150 × 2.0 mm, 4.0 μm)	Gradient: 10 mM NH$_4$Ac (pH 3.5)/ACN	LC-MS/MS (ESI+)	0.24–2.10	[33]
TYL, TYLB, TYLC, TYLD	100 mM Na$_2$CO$_3$– NaHCO$_3$ buffer (pH 9.0)/ Strata-X-SPE	Luna C18(2) 100 Å (150 × 4.6 mm, 5.0 μm)	Gradient: 1% FA/ACN/ MeOH	(a) LC-MS (ESI+) (b) LC-DAD	(a) 2–3 (b) 49–57	[34]
ERY, NEO, OLE, SPI, TILM, TYL, TYLB	100 mM NaH$_2$PO$_4$ buffer (pH 8.0)/ Oasis HLB-SPE	(a): YMC ODS-AQ S-3 120 Å (50 × 2.0 mm) (b): Acquity BEH C18 (100 × 2.1 mm, 1.7 μm)	(a) Gradient:10 mM NH$_4$Ac/ ACN (b) Gradient: Water/1% FA/ ACN	(a) LC-MS/MS (ESI+) (b) UHPLC-HRMS/MS (Q-TOF) (ESI+)	(a) 0.01–0.5 (b) 0.2–1.0	[35]
ERY, TYL	100 mM Na$_3$PO$_4$ (pH 8.0)/C18-SPE	Gemini C18 110 Å (50 × 2.0 mm, 5.0 μm)	Isocratic: water/ACN (30:70, v/v)	LC-MS/MS (ESI+)	5.0–5.2	[36]
AIVT, AZI, CLA, ERY, JOS, SPI, TILM, TYL	Water/Oasis HLB-SPE	C18HCE (100 × 2.1 mm, 5.0 μm) (home-made)	Gradient: 0.2% FA/0.2% FA in ACN	LC-MS/MS (ESI+)	0.01–0.5	[37]

[a]AIVT, acetylisovaleryltylosin (tylvalosin); AZI, azithromycin; CLA, clarithromycin; ERY, erythromycin; JOS, josamycin; LIN, lincomycin; NEO, neospiramycin; OLE, oleandomycin; SPI, spiramycin I; TILM, tilmicosin; TYL, tylosin A; TYLB, tylosin B; TYLC, tylosin C; TYLD, tylosin D.
[b]TRIS, tris(hydroxymethyl)aminomethane.

Table 4. Confirmatory methods for lincomycin and macrolides (MACs).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
AHD, AMOZ, AOZ, SEM	[b]/EtAc, Lichrolut EN-SPE	Symmetry Shield C18 (150 × 2.1 mm, 3.5 μm)	Gradient: 0.025% AcOH/ ACN	LC-MS/MS (ESI+)	0.12–0.56	[39]
AHD, AMOZ, AOZ, SEM, Ft, Fz, Nt, Nz	100 mM HCl/ Oasis HLB-SPE[c], AF buffer to pH 6–7, Oasis HLB-SPE	Inertsil ODS3 (150 × 2.0 mm, 3.0 μm)	Gradient: 20 mM AF buffer (pH 3.8)/ACN	LC-MS/MS (ESI+)	0.15–2.1	[40]

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (µg/kg)	References
		Column	Mobile phase			
AHD, AMOZ, AOZ, SEM	10% NaCl/Oasis HLB-SPE[c], (NaCl), hexane, EtAc	Inertsil ODS3 (150 × 2.1 mm, 3.5 µm)	Gradient: 10 mM NH$_4$Ac/ MeOH	LC-MS/MS (ESI+)	0.2	[41]
AHD, AMOZ, AOZ, SEM	[b]/hexane, EtAc	Acquity UHPLC BEH C18 (100 × 2.1 mm, 1.7 µm)	Gradient: 0.5 mM NH$_4$Ac/ MeOH	UHPLC-MS/MS (ESI+)	0.09–0.14 (CCα)	[42]
AHD, AMG, AMOZ, AOZ, DNSH, NPIR, PSH, SEM and CAP	[b]/EtAc, Oasis HLB-SPE	Acquity UHPLC BEH C18 (50 × 2.1 mm, 1.7 µm)	Gradient: 0.37% NH$_3$ in 10 mM NH$_4$Ac:MeOH (80:20, v/v)/ MeOH	UHPLC-HRMS/ MS (Q Exactive Plus) (ESI±)	0.05–2.3	[43]
AHD, AMOZ, AOZ, SC	[b]/EtAc, hexane	Synergy Hydro-RP (150 × 2.0 mm, 4.0 µm)	Gradient: water:MeOH (80:20, v/v)/0.1% AcOH in MeOH	LC-MS/MS (ESI+)	0.22–0.57	[44]
AHD, AMOZ, AOZ, SEM and DMZ, RNZ	[b]/QuEChERS (ACN) without d-SPE	Zorbax Eclipse XDB-C18 (150 × 4.6 mm, 5.0 µm)	Gradient: 5 mM AF buffer in water:MeOH (90:10, v/v) (pH 3.0)/MeOH	LC-MS/MS (ESI+)	0.21–0.53	[45]

[a]AHD, nitrofurantoin metabolite; AMG, nitrovin metabolite; AMOZ, furaltadone metabolite; AOZ, furazolidone metabolite; DMZ, dimetridazole; DNSH, nifursol metabolite; Ft, furaltadone; Fz, furazolidone; Nt, nitrofurantoin; Nz, nitrofurazone; NPIR, nifurpirinol; PSH, nifuroxazid metabolite; RNZ, ronidazole; SEM, nitrofurazone metabolite.

[b]Derivatization with 2-NBA in HCl solution with subsequent neutralization.
[c]Derivatization with 2-NBA and HCl after the indicated purification step.

Table 5. Confirmatory methods for nitrofurans (NFs).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (µg/kg)	References
		Column	Mobile phase			
DMZ, HMNNI, IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ, TNZ, TRZ[b] and CAP	Water/ACN (NaCl), hexane	Zorbax Eclipse Plus C18 (100 × 2.0 mm, 1.8 µm)	Gradient: 0.1% AcOH/0.1% AcOH in ACN	LC-MS/MS (ESI ±)	0.13–2.0	[47]
DMZ, MNZ, RNZ	Water (NaHCO$_3$)/ EtAc, Silica-SPE	Sunniest C18 (150 × 2.0 mm, 3.0 µm)	Gradient: water/MeOH	LC-MS/MS (ESI+)	0.05–0.2	[48]
DMZ, HMNNI, IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ	Water/QuEChERS (without d-SPE), Alumina-N-SPE	Pentafluorophenyl-propyl-bonded silica (150 × 2.0 mm, 3.0 µm)	Gradient: 0.01% AcOH/ ACN	LC-MS/MS (ESI+)	0.03–0.15	[49]
DMZ, HMNNI, MNZ, MNZ-OH, RNZ	0.1% FA, 10 mM NH$_3$ to pH 7.0/ Strata-SDB[c]-SPE	Kinetex XB C18 (100 × 3.0 mm, 2.6 µm)	Isocratic: 0.1% FA/MeOH (88:12, v/v)	LC-MS/MS (ESI+)	0.05–0.1	[50]

Compounds[a]	Extraction/clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
DMZ, HMMNI, IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ, SCZ, TRZ[b]	10 mM NH₄Ac (pH 6.0)/MIP	Kinetex XB C18 (150 × 2.1 mm, 2.6 μm)	Gradient: 0.1% FA/0.1% FA in ACN	LC-MS/MS (ESI+)	0.18–0.51	[51]

[a]DMZ, dimetridazole; HMMNI, 2-hydroxymethyl-1-methyl-5-nitroimidazole; IPZ, ipronidazole; IPZ-OH, ipronidazole metabolite; MNZ, metronidazole; MNZ-OH, metronidazole metabolite; RNZ, ronidazole; TRZ, ternidazole; CAP, chloramphenicol.

[b]Other less common NMZs are included in the method scope.

[c]Styrene-divynilbenzene copolymer (RP-SPE).

Table 6. Confirmatory methods for nitroimidazoles (NMZs).

Compounds[a]	Extraction/clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
(a) CIPRO, DANO, ENRO, MARBO, NOR, SARA[b] (b) FLUME, NALI, OXO	(a) 2% AcOH in ACN, SCX-SPE (b) ACN, 50 mM Na₂HPO₄ pH 11.0, SAX-SPE[c]	(a) Zorbax RX C8 (250 × 4.6 mm, 5.0 μm) (b) Kromasil C8 (250 × 3.2 mm, 5.0 μm)	(a) Isocratic: 10 mM Phosphate buffer (pH 3.0)/ACN (b) Isocratic: 10 mM OA/ACN/MeOH (60:30:10, v/v/v)	LC-FLD	5–50	[52]
CIPRO, DANO, DIFLO, ENRO, FLUME, MARBO, NALI, NOR, OXO, SARA	NaH₂PO₄/Na₂HPO₄ buffer (pH 6.3)/hexane, Oasis HLB-SPE	XBridge MS C18 (100 × 2.1 mm, 3.5 μm)	Gradient: 1% FA/ACN	LC-MS/MS (ESI+)	0.07–0.66	[53]
CIP, DAN, DIFLO, ENRO, NOR, SARA[b]	MacIlvaine buffer (pH 4.0) (Na₂EDTA)/Oasis HLB-SPE, MCAC-SPE	Inertsil ODS-4 (150 × 4.6 mm, 3.0 μm)	Isocratic: 1 mM SDS, 20 mM citrate buffer (pH 3.1)/ACN (70:30, v/v)	LC-FLD	0.33–4.4	[54]
CIPRO, DANO, DIFLO, ENRO, MARBO, SARA[b]	ACN/hexane	WondaSil C18 (150 × 4.6 mm, 5.0 μm)	Isocratic: 1% FA/MeOH (71:29, v/v)	LC-DAD	0.4–19	[55]
CIPRO, DANO, DIFLO, ENRO, FLUME, MARBO, OXO	30 mM NaH₂PO₄ buffer (pH 7.0)/QuEChERS (5% FA in ACN)	Zorbax Eclipse Plus HHRD (50 × 2.1 mm, 1.8 μm)	Gradient: 0.02% FA/ACN	UHPLC-MS/MS (ESI+)	0.2–1.7	[56]
CIP, ENR, NOR	Water, H₂SO₄ to pH 1.0/PS-MSLM[d]	Zorbax Eclipse XDB-C18 (150 × 4.6 mm, 5.0 μm)	Isocratic: MeOH/ACN/0.34% PA, 0.6% TEA[e] (15:5:80, v/v)	LC-FLD	0.067–0.35	[57]

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (µg/kg)	References
		Column	Mobile phase			
ENRO, FLUME, MARBO, OXO	50 mM SDS (pH 3.0)	Kromasil C18 (150 × 4.6 mm, 5.0 µm)	Isocratic: 50 mM SDS, 10 mM NaH$_2$PO$_4$, HCl to pH 3.0/TEA[e]/1-propanol (87:12.5:0.5, v/v/v)	LC-FLD	10–100	[58]

[a]CIPRO, ciprofloxacin; DANO, danofloxacin; DIFLO, difloxacin; ENRO, enrofloxacin; FLUME, flumequine; MARBO, marbofloxacin; NALI, nalidixic acid; NOR, norfloxacin; OXO, oxolinic acid; SARA, sarafloxacin.

[b]Other less common QNs are included in the method scope.

[c]SCX: strong cation exchange and SAX: strong anion exchange.

[d]PS-MSLM: phase separation-based magnetic-stirring salt-induced liquid-liquid microextraction method (LLE).
[e]Triethylamine.

Table 7. Confirmatory methods for quinolones (QNs).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (µg/kg)	References
		Column	Mobile phase			
STR	10 mM HClO$_4$ (pH 2.0)/SCX-SPE, C18-SPE	Hypersil BDS (100 × 4.0 mm, 3.0 µm)	Isocratic: 10 mM AHS[b], 0.4 mM, NQS in 20% ACN (pH 3.3)/ACN (97:3, v/v)	LC-FLD (post-column derivatization)	5	[59]
STR	Water/ WCX-SPE	Zorbax C18 (150 × 2.1 mm, 5.0 µm)	Isocratic: 20 mM HFBA[c]/ ACN (70:30, v/v)	LC-MS/MS (ESI+)	2	[60]
STR	0.1% PA/SCX-SPE, C18-SPE	Hypersil ODS2 (150 × 4.6 mm, 5.0 µm)	Isocratic: 10 mM AHS[b], 0.4 mM NQS (pH 3.3)/ACN (28:72, v/v)	LC-FLD (post-column derivatization)	5	[61]
STR, DSTR	50 mM AHS[b], [25] mM Na$_3$PO$_4$ buffer to pH 2.0/ C18-SPE	Alltima C18 (150 × 2.1 mm, 5.0 µm)	Isocratic: 1.9 mM PFPA[d], [3].2 mM AF/ ACN (85:15, v/v)	LC-MS/MS (ESI+)	<1–2	[62]
APR, AMI, DSTR, GEN, KAN, NEO, PAR, SPC, STP	Water/ WCX-SPE	ZIC®-HILIC, 150 × 2.1 mm, 3.5 µm, SeQuant AB	Gradient: 175 mM NH$_4$Ac (pH 4.5)/0.2% FA in ACN	LC-MS/MS (ESI+)	17–99	[63]
STR, DSTR	KH$_2$PO$_4$-Na$_2$EDTA-TCA buffer (pH 4.0), NaOH to pH 7.5/Oasis HLB-SPE	HILIC Atlantis (150 × 2.1 mm, 3.0 µm)	Gradient: 0.05% FA/0.05% FA in ACN	LC-MS/MS (ESI+)	19–20	[64]

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (µg/kg)	References
		Column	Mobile phase			
DSTR, GEN SPC, STR	20 mM K_2HPO_4 buffer (pH 7.4)/ MIP-SPE	XAmide HILIC (150 × 4.6 mm, 5.0 µm)	Isocratic: 20 mM AF (pH 3.0)/ACN (40:60, v/v)	LC-MS/MS (ESI+)	1.8–6.0	[65]
AMI, APR, DSTR, GEN, HYG, KAN, NEO, PAR, SIS, SPC, STR, TOB	Water/10 mM NH_4Ac, 0.4 mM Na_2EDTA, 0.5% NaCl, 5% TCA, 1–10 M NaOH, WCX-SPE	Obelisc R 100 Å, (100 × 2.1 mm, 5.0 µm)	Gradient: 1% FA/1% FA in ACN	LC-MS/MS (ESI+)	1–12	[66]
DSTR, GEN, SPC, STR	5 mM K_2HPO_4 buffer (pH 11.0)/PVA-Sil-SPE[f] (home-made sorbent)	TE[g]-Cys HILIC (150 × 3.0 mm, 3.0 µm)	30 mM AF/ FA (99/1, v/v)/ ACN/water/FA (80:19:1, v/v/v)	LC-MS/MS (ESI+)	2.3–6.1	[67]
APR, DSTR, GEN, NEO, PAR, SPC, STR	50 Mm KH_2PO_4 buffer (pH 7.0)/ MIP-SPE	Bare fused-silica capillary (90 cm × 50 µm × 375 µm)	200 mM FA/7 mM NH_3	CZE-IT-MS[e]	6–103	[68]

[a]AMI, amikacin; APR, apamycin; GEN, four isomers of gentamicin; HYG, hygromycin; KAN, kanamycin; NEO, neomycin; PAR, paromomycin; SIS, sisomycin; SPC, spectinomycin; TOB, tobramycin.

[b]AHS, sodium 1-heptanesulphonic acid (ion-pairing reagent).

[c]HFBA, heptafluorobutyric acid (ion-pairing reagent).

[d]PFPA, pentafluoropropionic acid (ion-pairing reagent).

[e]CZE-IT-MS: capillary zone electrophoresis coupled to ion trap mass spectrometry.

[f]PVA-Sil: polyvinyl alcohol-Silica.

[g]TE: thiol-ene.

Table 8. Confirmatory methods for streptomycin/dihydrostreptomycin (STR/DSTR).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (µg/kg)	References
		Column	Mobile phase			
STZ	Acetone/1 M HCl, diethyl ether	µBondapack phenyl (300 × 3.9 mm)	Isocratic: KH2PO4 buffer (pH 3.0) in 10% ACN	LC-UV	60	[69]
SCP, SDT, SDX, SDZ, SMP, SMR SMX, SMZ, SPD, STZ	10% TCA, 1 M Na_2HPO_4 to pH 6.5/ACN, DCM	Nucleosil C18 HD (50 × 2.0 mm, 3.0 µm)	Gradient: 0.3% FA in water:ACN (95:5, v/v)/0.3% FA in ACN	LC-MS/MS (ESI+)	<10	[70]
SCP, SDT, SMP, SMR, SMX, SPD[b]	0.1% PA (pH 2.0)/SCX-SPE, AHS[c], PA to pH 6.0, Oasis HLB-SPE	Symmetry Shield C18 (150 × 3.9 mm)	Isocratic: 10 mM KH_2PO_4 (pH 3.5–4.0)/ACN (73:27, v/v)	LC-FLD (with derivatization)	2–5	[71]

Compounds[a]	Extraction/clean-up	Separation		Equipment	CCβ or LOD (µg/kg)	References
		Column	Mobile phase			
SCP, SDA, SDX, SGN SMP, SMR, SMT, SMZ SNL, SPD, STZ[b]	2 M HCl. 5 M NaOH and 1.2 M NaAc to pH 5.0/ACN, 1% AcOH, SCX-SPE	Purospher Star RP-18 EC (150 × 4.6 mm, 5.0 µm)	Gradient: NaAc buffer:ACN (98:2, v/v)/NaAc buffer:ACN (68:32, *v/v*)	LC-FLD (with derivatization)	1–2	[72]
SCP, SDA, SDT, SDX, SMR, SMZ, SMP, SMM, SMX, SPD, STZ[b]	0.1% PA (pH 2.0)/SCX-SPE, AHS[c], PA to pH 6.0, Oasis HLB-SPE	Atlantis dC18 (150 × 2.1 mm, 3.0 µm)	Gradient: 0.2% FA/0.2% FA in ACN	LC-MS/MS (ESI+)	0.5–6.0	[73]
SDM, SMX, STZ[b] and 4 TCs, 4 pesticides	2 M HCl/200 mM Citric acid, 20% NH$_3$ to pH 4.0, Oasis HLB-SPE	InertSil ODS2	Gradient: 0.2% FA/0.2% FA in ACN	LC-MS/MS (ESI+)	0.2–6.2	[74]
SCP, SDA, SDX, SMM, SMP, SMR, SMT, SMX, SMZ, SNL, SPD, SQX, STZ[b] and CAP	2 M HCl, 5% NaOH to pH 8.5/Oasis HLB-SPE	Xterra C18 (150 × 2.1 mm, 3.5 µm)	Gradient: 0.15% AcOH/0.15% AcOH in MeOH	LC-MS/MS (ESI+)	0.5–5	[75]
SDT, SDX, SMR, SMX, SMZ, SPD, STZ and DAP, TRM	2 M HCl, 300 mM Citric acid, 25% NH$_3$ to pH 4.0/Oasis HLB-SPE	Xterra MS C18 (150 × 2.1 mm, 3.5 µm)	Gradient: 0.1% FA/0.1% FA in ACN	LC-MS/MS (ESI+)	0.3–0.9	[76]
SDT, SDX, SDZ, SGN, SMM, SMP, SMR, SMX, SMZ, SNL, SQX, STZ[b]	300 mM Citric acid (pH 1.8)/SCX-SPE	Symmetry C18 (100 × 2.1 mm, 3.5 µm)	Gradient: 0.2% FA/ACN	LC-MS/MS (ESI+)	2.2–17.4	[77]
SCP, SDX, SDZ, SGN, SMM, SMP, SMR, SMX, SMZ, SQX, SSZ STZ[b] and DAP, TRM	100 mM AcOH (pH 5.0)/acetone:DCM (50/50, v/v)	Zorbax XDB-C18 (75 × 4.6 mm, 3.5 µm)	Gradient: 5 mM AF (pH 3.5)/5 mM AF in MeOH	LC-MS/MS (ESI+)	1.5–5.3	[78]

[a]DAP, dapsone; SCP, sulphachloropyridazine; SDA, sulphadiazine; SDT, sulphadimethoxine; SDX, sulphadoxine; SGN, sulphaguanidine; SMM, sulphamonomethoxine; SMR, sulphamerazine; SMP, sulphamethoxypyridazine; SMX, sulphamethoxazole; SMZ, sulphamethazine; SNL, sulphanilamide; SPD, sulphapyridine; SQX, sulphaquinoxaline; STZ, sulphathiazole; TRM, trimethoprim.

[b]And less common SAs are included in the method scope.

[c]AHS, sodium 1-heptanesulphonic acid (ion-pairing reagent).

Table 9. Confirmatory methods for sulphonamides (SAs).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (µg/kg)	References
		Column	Mobile phase			
CTC, DC, MINO, MTC, OTC, TC	MacIlvaine buffer (Na$_2$EDTA) (pH 4.0)/ phenyl-SPE	Discovery RP-Amide C16 (5.0 µm)	Gradient: 0.09% OA (pH 3.0)/ACN	LC-DAD	15–30	[80]
CTC, DC, OTC, TC	50 mM oxalate buffer (pH 4.0)/ Oasis HLB-SPE	Atlantis dC18 (150 × 2.1 mm, 3 µm)	Gradient: 1% FA/ ACN:MeOH (50:50, v/v)	LC-MS/MS (ESI+)	3.3	[81]
CTC, OTC, TC	MacIlvaine buffer (Na$_2$EDTA) (pH 4.0)/hexane, PLS-2-SPE[b], MCAC-SPE	Hydrospher C18 HS-301–3 (100 × 4.6 mm, 3.0 µm)	Isocratic: 1 M Imidazole buffer/MeOH (82:18, v/v)	LC-FLD	5–9	[82]
CTC, OTC, TC	Citrate buffer, (Na$_2$EDTA)/ PLS-2-SPE[b]	Tsk-gel ODS-80Ts (150 × 4.6 mm)	Isocratic: 1 M Imidazole buffer/MeOH (75:25, v/v)	LC-DAD	10–20	[83]
CTC, DC, OTC, TC	5% HCl/ MIP-SPE	Restek C18 (150 × 2.1 mm, 5.0 µm)	Isocratic: 100 mM OA/ ACN/MeOH (70:20:10, v/v/v)	LC-MS/MS (ESI+)	0.1–0.3	[84]
CTC, OTC, TC	ACN/SPE (home-made sorbent)	ShodexRSpak DE-613 (150 × 6.0 mm)	Isocratic: 0.05% TFA/ACN (60:40, v/v)	LC-MS/MS (ESI+)	3–20	[85]
CTC, OTC, TC	50 mM NH$_4$Ac buffer (pH 5.5)/ MCAC-SPE, Oasis HLB-SPE	Waters Phenyl (100 × 2.1 mm, 3.5 µm)	Gradient: 0.1% FA/0.1% FA in ACN:MeOH (50:50, v/v)	LC-MS/MS (ESI+)	7.2–7.7	[86]
CTC, DC, OTC, TC	MacIlvaine buffer (Na$_2$EDTA) (pH 4.0)/ Strata-X-SPE	Symmetry C18 (150 × 2.1 mm, 3.5 µm)	Gradient: 0.05% AcOH/0.05% AcOH in ACN	LC-MS/MS (ESI+)	5.5–9.2	[87]
CTC, DC, MTC, OTC, TC	Water/chitosan-modified graphitized MWCN	SB-C18 (50 × 4.6 mm, 5 µm)	Gradient: 0.1% FA/MeOH	LC-HRMS (Q-TOF) (ESI+)	0.61–10	[88]

[a]CTC, chlortetracycline; DC, doxycycline; DMC, demeclocycline; OTC, oxytetracycline; MINO, minocycline; MTC, methacycline; TC, tetracycline.
[b]PLS-2, polystyrene-divinylbenzene polymer (RP-SPE).

Table 10. Confirmatory methods for tetracyclines (TCs).

Compounds	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (µg/kg)	References
		Column	Mobile phase			
1 QN, 16 SAs, 3 TCs (20)	2 M HCl, 300 mM Citric acid/ Oasis HLB-SPE	Nucleosil 100–5, C18 HD (50 × 2.0 mm, 5 µm)	Gradient: 0.3% FA in Water:ACN (95:5, v/v)/0.3% FA in can	LC-MS/MS (ESI+)	0.4–11	[89]
2 amphenicols, 3 AGs, 8 β-lactams, 7 MACs, 17 SAs, 5 TCs (42)	(1) ACN; (2) 10% TCA/ ACN, 12.5% NH₃; (3) NFPA/ ACN, 12.5% NH₃; (4) Water, 1 M Na₂HPO₄ (pH 12.0)/ ACNᵃ	Zorbax SB-C18 (50 × 2.1 mm, 1.8 µm)	Gradient: 1 mM NFPAᵇ in 0.5% FA/ ACN:MeOH (50:50, v/v)	LC-MS/MS (ESI+)	29–81	[90]
CAP, lincomycin, MACs, 5 QNs 1 SA, 4 TCs and others (17)	Water/ Strata-X-SPE	Polar-RP Synergi (50 × 2.0 mm, 4 µm)	Gradient: 0.1%FA/0.1% FA in ACN	LC-MS/MS (ESI±)	0.13–6.7	[29]
4 MACs, 5 QNs, 4 SAs, 4 TCs (17)	100 mM Na₂EDTA (pH 4.0), Oasis HLB-SPE	Acquity UHPLC BEH C18 (100 × 2.1 mm, 1.7 µm)	Gradient: 0.05% FA/ MeOH	UHPLC-MS/ MS (ESI+)	0.1–1.0	[91]
12 β-lactams, 23 MACs, 8 NMZs, 16 QNs, 24 SAs, 6 TCs and others (112)	50 mM Succinate buffer (pH 5.0)/ ACN, 12.5% NH₃ to pH 6.5, water, Evolute ABN-SPE	Kinetex C18 (150 × 2.1 mm, 2.6 µm)	Gradient: 0.3% FA in Water:ACN (95:5, v/v)/0.3% FA in water:ACN (5:95, v/v)	LC-HRMS (Exactive) (ESI+)	Not providedᶜ	[92]
3 lincosamides, 10 MACs, 13 QNs, 7 TCs, TRMᵈ (36)	MacIlvaine buffer (Na₂EDTA) (pH 4.0)/Oasis HLB-SPE	Aqua (150 × 2.0 mm, 3.0 µm)	Gradient: 0.2%FA/0.2% FA in ACN	LC-MS/MS (ESI+)	9.4–20	[93]
2 amphenicols, 6 MACs, 4 NMZs, 5 QNs, 12 SAs, 1 TC and others (391)	Water/1% FA in ACN	Hypersil GOLD aQ C18 (100 × 2.1 mm, 1.7 µm)	Gradient: 4 mM AF in 0.1% FA/4 mM AF, 0.1% FA in MeOH	UHPLC-HRMS (Exactive) (ESI±)	10–50	[94]
2 amphenicols, 6 MACs, 3 QNs, 13 SAs and others (31)	Water (Na₂EDTA)/ QuEChERS (1% AcOH in ACN) without d-SPE	Acquity UHPLC BEH C18 (100 × 2.1 mm, 1.7 µm)	Gradient: 0.1% FA, 10 mM AF/ ACN	UHPLC-HRMS/MS (Q-TOF) (ESI+)	1–20	[95]
9 NMZs, 8 QNs, 10 SAs (27)	2 M HCl, water/hexane, SCX-SPE	Poroshell 120 EC-C18 (100 × 3.0 mm, 2.7 µm)	Gradient: 0.1% FA/0.1% FA in ACN	LC-MS/MS (ESI+)	0.19–2.5	[96]
CAP, 3 MACs, 7 SAs, other drugs (2) and 79 pesticides (92)	MacIlvaine buffer (pH 4.0)/ ACN, Florisil (d-SPE)	Acquity UHPLC BEH C18 (100 × 2.1 mm, 1.7 µm)	Gradient: 5 mM AF in 0.1% FA/5 mM AF, 0.1% FA in MeOH	LC-MS/MS (ESI±)	0.12–2.8	[97]

Compounds	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
3 AGs, LIN[e], 5 MACs, 6 SAs, 8 TCs (22)	Water, 2 M, HCl in MeOH, Na₄EDTA to pH 2.0/PSA (d-SPE)	Zorbax SB-C18 (100 × 2.1 mm, 3.5 μm)	Gradient: 100 mM HFBA[f]/ water/ACN	LC-MS/MS (ESI+)	7–33	[98]

[a]Four subsequent LLE steps were carried out.

[b]NFPA, nonafluoropentanoic acid (ion-pairing reagent).

[c]CCβs for permitted antibiotics (lincomycin, MACs, QNs, SAs, TCs) were provided considering a hypothetical MRL of 100 or 200 μg/kg. For banned substances (NMZs), CCβs were in the range 1.2–2.6 μg/kg.

[d]TRM, trimethoprim.

[e]LIN, lincomycin.
[f]HFBA, heptafluorobutyric acid (ion-pairing reagent).

Table 11. Multiclass confirmatory methods.

2. Honeybee diseases

Honeybees are affected by fungal, bacterial, viral (*Thai Sac brood*) and acarine (*Varroa*) diseases. Antibiotics are generally used to fight bacterial and fungal diseases such as American foulbrood, European foulbrood and nosemosis [3].

American foulbrood is by far the most virulent brood disease known in honeybees. The disease is caused by the spore-forming bacterium, *Paenibacillus larvae*. Larvae up to 3 days old become infected by ingesting spores that are present in their food. Spores germinate in the gut of the larva and the vegetative form of the bacteria begins to grow, taking its nourishment from the larva. Infected larvae normally die after their cell is sealed. The vegetative form of the bacterium, before to die, produces many millions of spores which are extremely resistant to desiccation and can remain viable for more than 40 years in honey and beekeeping equipment. Because of this persistence, in most countries official apiary inspectors are required to burn all infected colonies. Other countries (e.g. USA, Canada, and Argentina) allow the use of antibiotics, such as oxytetracycline and tylosin, to keep the disease in control. However, antibiotics are not a cure or a treatment of the infection since they affect only the vegetative stage of American foulbrood, inhibiting its development in the gut of the larvae. This may prevent the rapid diffusion within a colony.

European foulbrood is closely related to American foulbrood in symptomatology, and until 1906, these two diseases were not differentiated. The causative organism of European foulbrood is the bacterium *Melissococcus plutonius*, which does not produce spores, and therefore, this disease is considered less severe than American foulbrood. European foulbrood occurs primarily in spring when numbers of *M. plutonius* reach their peak. The bacterium is ingested by honey bee larvae and it replicates in mid-gut. If the bacteria out-compete the larva, the larva will die before the cell is capped. Alternatively, the bee may survive until adulthood if the larvae has sufficient food resources. Some antimicrobials, for example, oxytetracycline, have been demonstrated to be an effective treatment.

Nosemosis, caused by the fungus *Nosema apis* or *Nosema ceranae*, is historically considered the most serious disease of adult bees. Infection is acquired when spores are swallowed by bees and infect the epithelial cells of the hind gut, giving rise rapidly to large numbers of spores and impairing the digestion of pollen which shortens the life of honeybees. *N. ceranae* was originally a parasite of the Asian honeybee (*Apis cerana*), but now is widespread in some European regions, too. In recent years, the disappearance of adult honeybees, known as colony collapse disorder, has been devastating a great number of beehives worldwide. This problem has caused serious damage to apiculture and also to agricultural activities that use honeybees as pollinators. Among the possible causes of the disappearance of honeybees, nosemosis has been reported as a primary candidate.

3. Methods for the determination of drug residues in honey: sample treatment

3.1. Matrix-analyte

Sample treatment is fundamental in the residue analysis of food, since the achievement of low detection limits (some parts per billions) and suitable selectivity involves extensive purification of generally complex food matrices. The sample preparation process consists of the extraction followed by one or more purification steps. Rarely, the purification step is omitted. To decide the sample treatment strategy, main aspects have to be considered: the characteristics of both sample matrix and he physico-chemical properties of analyte(s) have to be taken into account, together with, in addition, the already developed procedures (literature searching).

Because of the hydrophilic nature of honey, frequently, the extraction coincides with the sample dissolution in pure water or in acidified aqueous solutions or in buffers. After that, besides the traditional liquid-liquid extraction (LLE) and solid-phase extraction (SPE) purifications, more recent clean-up methodologies have been applied such as quick, easy, cheap, effective, rugged and safe (QuEChERS), molecularly imprinted polymers (MIPs) and multi-walled carbon nanotubes (MWCNs). These two latter are particular kinds of SPE, whereas QuEChERS methodology is a variation of LLE, followed by a dispersive solid-phase extraction (d-SPE) step. It is important to keep in mind that, despite the proliferation of dozens of new purification approaches with various acronyms, essentially all these fall into LLE or SPE techniques. Some additional examples are microextraction by packed sorbent (MEPS), stir bar sorption extraction (SBSE), dispersive liquid-liquid microextraction (DLLME) and phase separation-based magnetic-stirring salt-induced liquid-liquid microextraction (PS-MSLM). These recent methodologies give also evidence of the current trend towards "micro", that is, towards a lower consumption of reagents and materials during the sample treatment. Less common and expensive purification systems such as turbo-flow chromatography are not here considered.

Dissociation constants (pK_as) and lipophilicity are key parameters to understand the behaviour of drugs, and therefore, to perform appropriate extraction and purification strategies, physico-chemical properties of a drug molecule are described by its pK_a(s) and its polarity pK_a (dissociation constant) is a measure of the strength of an acid or a base. It determines the

charge on a molecule at any given pH. The lipophilicity polarity is measured by the partition coefficient, P, or better, by the distribution coefficient, D, which are the key parameters to understand the behaviour of molecules, and therefore, to design appropriate purification strategies during the method development, P is the ratio of the concentration of a compound in octanol to its concentration in water P (Eq. (1)):

$$P = \frac{[\text{drug}]_{\text{octanol}}}{[\text{drug}]_{\text{water}}} \qquad (1)$$

P is generally expressed as logarithm of the log P. Log P is a constant for the molecules under its neutral form, and its value is a measure of lipophilicity or hydrophobicity. On the other hand, the distribution coefficient (D, or better, its logarithm, log D) takes into account all neutral and charged forms of the molecule. Therefore, for ionizable solutes, such as drugs, the pH-dependant lipophilicity descriptor, that is, the distribution coefficient (D), is more appropriate. D is the ratio of the sum of the concentrations of all forms of the compound (ionized plus un-ionized) in each of the two phases, octanol and water, (Eq. (2)):

$$D = \frac{[\text{drug molecule}]_{\text{octanol}}}{[\text{drug molecule}]_{\text{water}} + [\text{drug ion}]_{\text{water}}} \qquad (2)$$

Roughly, when log D < 0, the molecule is polar (hydrophilic) and vice versa. Because the charged forms hardly enter the octanol phase, this distribution varies with pH. In the pH region where the molecule is mostly unionized, log D = log P. Acids are neutral when protonated and negatively charged (ionized) when deprotonated. Bases are neutral when deprotonated and positively charged (ionized) when protonated. Therefore, the log D of a compound is strongly influenced by its acid-base dissociation constant(s), pK_a. However, log D values cannot furnish precise information about the ionization status of the compound mainly because frequently more than one acidic or basic centre can be present in its structure. Only the knowledge of the pK_as allows the understanding of the predominant forms at the various pH values. In **Figures 1–3**, the plots of log D versus pH of one representative compound per class are shown. These plots were obtained applying the MoKa® package (Molecular Discovery Ltd.) [13]. This software package is able to predict also the pK_as. Ranges of pHs increasing log D (lipophilicity) can favour RP-SPE and LLE purification strategies, which are based on the analyte transfer from a more polar medium (honey solution) to a less polar one. On the other hand, selective purifications such as ion-exchange SPE are enabled when the analytes are in their ionized form and, therefore, in pH intervals where log D values are lower (higher hydrophilicity).

3.2. Purification

Liquid-liquid extraction (LLE) is one of the first sample preparation approaches and continues to be widely used. LLE is based on the transfer of an analyte from the aqueous sample to a water-immiscible solvent based on its distribution coefficient, D. The water-immiscible solvents can be ethyl acetate, dichloromethane and chloroform. Nevertheless, some shortcomings, such as emulsion formation, the use of relatively large sample volumes and toxic organic solvents, make the traditional LLE (relatively) expensive and environmentally harmful. To avoid emulsion formation, supported liquid extraction (SLE) can be applied.
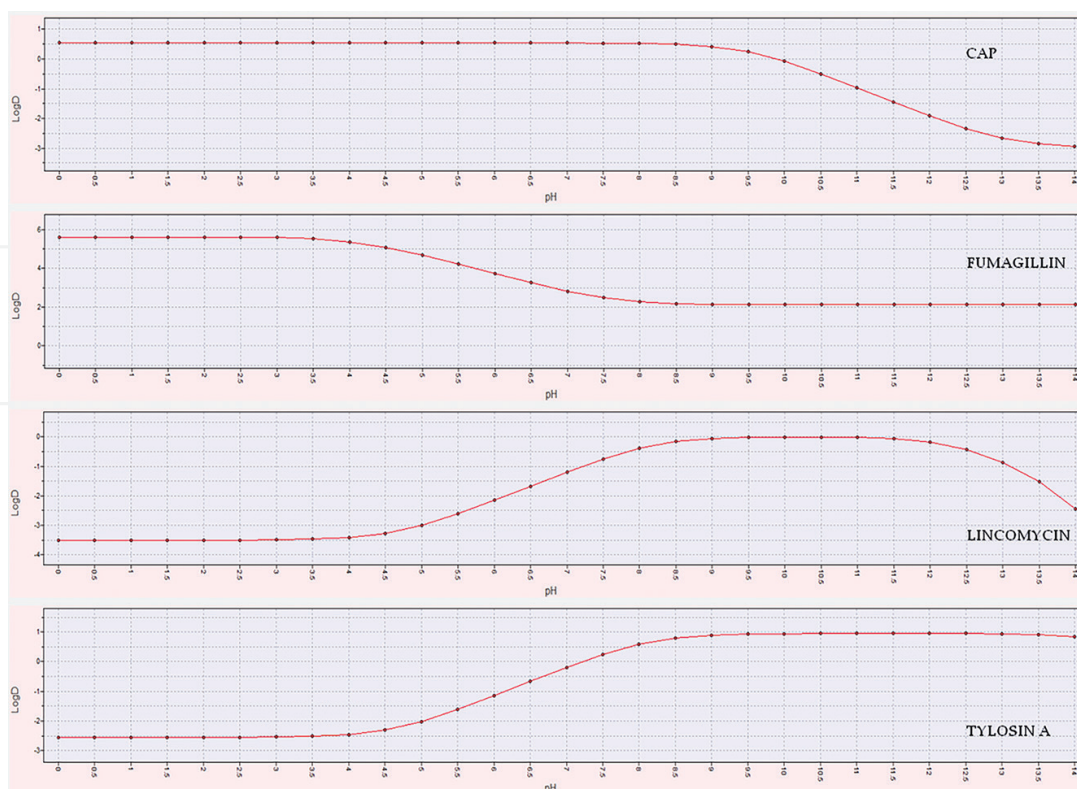
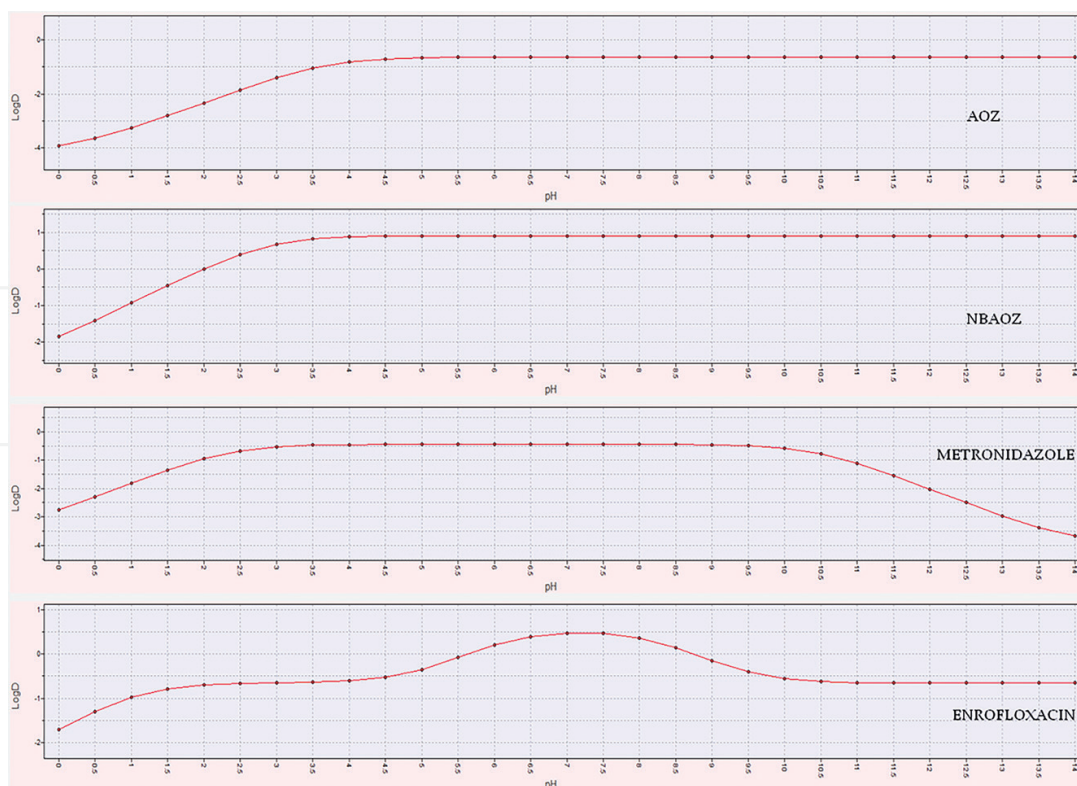**Figure 1.** Log D versus pH for chloramphenicol (CAP), fumagillin, lincomycin and tylosin A (MAC).

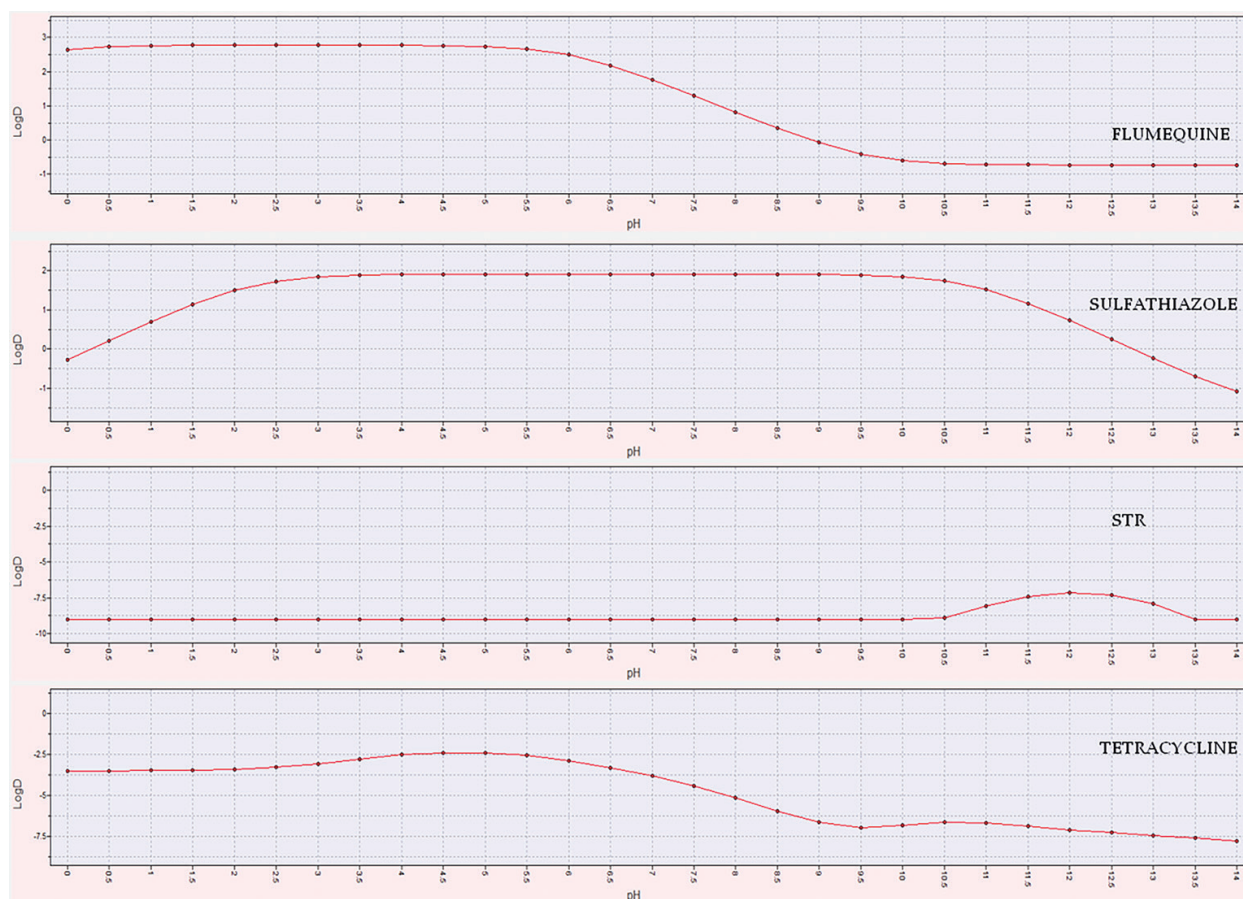**Figure 2.** Log D versus pH for AOZ, derivatized AOZ (NBAOZ), metronidazole (NMZ) and enrofloxacin (QN).

Figure 3. Log D versus pH for flumequine (QN), sulfathiazole (SA), streptomycin (STR) and tetracycline (TC).

Its principle is simple: a chemically inert, high surface area support, highly purified, graded diatomaceous earth (Extrelut®, Hydromatrix®, Celite®, etc.) serves as a stationary vehicle for the aqueous phase of the liquid-liquid extraction experiment. The aqueous-based sample (e.g. diluted honey) is added to the dry sorbent and allowed to wet the diatomaceous earth. A small volume of immiscible organic extraction solvent is then added and allowed to percolate by gravity through the supported aqueous phase. Because the aqueous sample has been widely dispersed throughout the solid support, the organic solvent has intimate contact with the thin film of aqueous phase and rapid extraction (equilibration) occurs.

Even today, probably, solid-phase extraction (SPE) is the most used sample purification tool in trace analysis. This technique was developed in the mid-1970s as an alternative to LLE. The degree of selectivity of SPE technique can be very different, depending on the attractive forces between the analytes and the functional groups on the sorbent surface. SPE sorbents are most commonly categorized by the nature of their primary interaction or retention mechanism with the analyte(s) of interest. The sorbent can interact with analytes by hydrophobic (non-polar/non-polar), hydrophilic (polar-polar, hydrogen bonding, dipole-dipole, π-π interactions) and cationic-anionic interactions. The most common SPE sorbents packing can be classified into non-polar phases (reversed phases—RP), polar phases (normal phases—NP), ion-exchange and immunoaffinity adsorbents.

Non-polar sorbents are used under RP chromatography conditions and are suitable for the extraction of hydrophobic or polar organic analytes from aqueous matrices. Accordingly, reversed phase is the most used SPE sorbent type to purify honey, which is a water-soluble matrix. These sorbents comprise alkyl silica and polymer-based materials. Alkyl silica sorbents are manufactured by bonding alkyl or aryl functional groups, such as octyl (C8), octadecyl (C18) and phenyl (Ph) to the silica surface. It should be noted that in SPE, the interactions described above are not found in pure form, but in combination. For example, C18 silica-based sorbents are non-polar sorbent, but it still possess free silanol groups, which can produce hydrophilic secondary interactions. The retention of analytes under RP conditions is due primarily to the van der Waals attractive forces between the carbon-hydrogen bonds in the analytes and the functional groups on the silica surface. The elution of adsorbed compounds is generally made by using a non-polar solvent (compared to water) to disrupt the forces that bind the compound to the sorbent. However, silica-based bond phases contain non-uncapped silanols, which can cause the strongly binding of some group of compounds (i.e. tetracyclines), and in addition, they can be used only in a limited pH range (2–8). Currently, silica materials have been more and more replaced by polymeric sorbents. The macroporous wettable hydrophilic-lipophilic balance (HLB) polymeric sorbent (divinylbenzene-N-vinylpyrrolidone) was at first introduced by Waters Company (Oasis HLB). Later, other manufacturers commercialized similar reversed-phase proprietary polymeric sorbents such as Strata-X (surface-modified styrene-divinylbenzene; Phenomenex), LiChrolut EN (highly cross-linked polystyrene-divinylbenzene; Merck, Darmstadt, Germany) and Evolute ABN (cross-linked polystyrene-divinylbenzene functionalized with oligomeric hydroxyl groups; Biotage). These cartridges have been widely applied in honey purification of almost all antibiotic classes.

The intrinsic honey characteristics undoubtedly favour the wide application of RP-SPE purification approaches since NP-SPE is more suitable to isolate a polar analyte in a mid- to non-polar matrix (acetone, chlorinated solvents, hexane, etc). The most common polar stationary phases are silica, alumina and florisil. Retention of an analyte under NP conditions is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface (hydrogen bonding and π-π interactions, among others). The passing of a solvent that disrupts the binding mechanism, usually a solvent that is more polar than the sample matrix, allows the elution of the adsorbed compounds. To the best of our knowledge, examples of NP-SPE purification applied to determination of veterinary drug residues in honey are limited to nitroimidazole family (**Table 6**). This is probably why nitroimidazoles are very polar compounds. The application of this kind of sorbents generally involves a preliminary liquid-liquid extraction step to transfer the analytes from the aqueous phase (solubilized honey) to an organic phase (non-polar matrix) which is then loaded onto the cartridge.

Due to their selectivity, ion-exchange SPE sorbents can be generally used only in single-residue or single-class procedures. These sorbents are very efficient for extraction of charged analytes, such as acidic and basic compounds, from aqueous or non-polar organic samples. Ion-exchange phases are comprised of positively (aliphatic quaternary amine, aminopropyl) or negatively (aliphatic sulphonic acid, aliphatic carboxylic acid) charged groups. Porous polymer, ion-exchange resins have a higher exchange capacity and a wider pH operating range than silica-based materials. Ion-exchange sorbents are usually classified as weak or

strong, depending on the identity of the ionic group and whether its charge is independent of the sample pH (strong ion exchanger) or can be manipulated by changing pH (weak ion exchanger). Antibiotic substances have frequently basic functional groups, and therefore, the application of both strong cation exchange and weak cation exchange has been reported also in honey, mainly for the determination of streptomycin/dihydrostreptomycin (**Table 8**) and sulphonamides (**Table 9**). Finally, the immunoaffinity chromatography is a SPE technique, based on very selective antigen-antibody interactions (immunosorbents); examples of its application to purify honey have been reported, too.

In some cases, both liquid-liquid extraction and solid-phase extraction can be used in an "opposite manner", that is, solubilizing or retaining the interfering substances rather than the analytes. An important example in antibiotic analysis is the so-called defatting to purify food extracts in water-miscible solvents: the added water-immiscible solvent (generally hexane) does not solubilize the analytes of interest, but the highly lipophilic interfering substances (fats), and therefore, it is discarded. Analogously, in the "non-retentive" SPE the sorbent has no affinity for the analytes, but for the sample contaminants. The solid phase is simply used to "filter" the sample: analyte passes through the column without being retained, while (part of) the contaminants are retained. This kind of extraction is generally applied when the analyte is highly soluble in the sample matrix (or in the dilution solvent), and therefore, it cannot be partitioned out onto a solid sorbent (retentive SPE) or an immiscible solvent (LLE).

Among the relatively modern purification approaches, it may be worthwhile to describe the QuEChERS, molecularly imprinted polymers (MIPs) and multi-walled carbon nanotubes (MWCNs) methods. The QuEChERS approach has become particularly popular for the multiresidue analysis of pesticides in various food matrices, and it generally consists of two steps: first, the homogenized sample is extracted and partitioned using an acetonitrile and salt solution ($MgSO_4$ and NaCl), and then, an aliquot of the supernatant is cleaned using a dispersive solid-phase extraction (d-SPE) technique. Dispersive SPE is a "non-retentive" SPE, because the matrix co-extractives are adsorbed onto the sorbent, while leaving analytes of interest in the solvent. In some applications of QuEChERS, the second step (d-SPE purification) can be omitted. MIP sorbents are highly cross-linked polymers with a predetermined selectivity towards a single analyte or group of structurally related analytes. This selectivity is obtained during the synthesis of the polymer by using a template molecule to form cavities with specific shape. The process usually involves initiating the polymerization of monomers in the presence of the template molecule that is extracted afterwards, thus leaving complementary cavities behind. Due to the high selectivity of these sorbents, they generally allow for lower detection limits. In recent years, multi-walled carbon nanotubes, a new kind of carbon material, have attracted much interest that is directed towards the development of solid-phase extraction adsorbents. The MWCNs were promising sorbents because of the larger specific area and the dramatic hydrophobic characteristic of the surface. The adsorption mechanisms involve weak interactions (mainly π-π stacking, van der Waals and electrostatic forces), facilitating the adsorption of analytes in a selective and reproducible manner.

To conclude, the current trends in food sample preparation involve the following issues: the miniaturization of the equipment for sample preparation (micro techniques); the decrease in the amount of sample to be analysed; the reduction in the use of organic solvents; the

development of multiclass procedures; and the development of automated methods for the preconcentration. All these strategies aim at the reduction in the employed reagents/materials and at the increase in the analysis throughput.

4. Methods for the determination of drug residues in honey: analytical techniques

Until early 2000s, LC-UV-Vis and LC-FLD were the most used equipments to detect residues in food. UV-Vis detectors measure solute analytes by their absorbance in the ultraviolet or visible region. A UV detector employs a deuterium discharge lamp (D_2 lamp) as a light source, with the wavelength of its light ranging from 190 to 380 nm. If substances are to be detected at longer wavelengths, that is, in the visible region (380–700 nm), a UV-VIS detector is used with an additional tungsten lamp (W lamp). Nowadays, photodiode arrays and DAD (semiconductor devices) have replaced UV-Vis detectors, and its use is mandatory to definitively confirm the presence of residues of permitted veterinary drugs in food [6]. A DAD detects the absorption in UV to VIS region. While a UV-VIS detector has only one sample-side light-receiving section, a DAD allows the acquisition of full wavelength spectrum at one time thanks to multiple photodiode arrays. Spectra are measured at regular intervals (one second or less) during the LC separation with continuous eluate delivery. Therefore, to identify a compound, in addition to the retention time, DAD enables the comparison between the spectrum of the authentic standard and of the analyte. It is important to underline that according to Commission Decision 2002/657/EC, only the coupling between LC and DAD (not between LC and UV-Vis) allows the definitive confirmation of residues of permitted substances in food.

Fluorescence detectors have greater sensitivity and selectivity over the UV-Vis ones. This is an advantage for the measurement of specific fluorescent species in samples; however, only about 15% of all compounds have a natural fluorescence. Compounds having specific functional groups are excited by shorter wavelength energy and emit higher wavelength radiation. This phenomenon is called fluorescence. Generally, the presence of aromatic conjugated pi-electrons produces the most intense fluorescent signal. Most unsubstituted aromatic hydrocarbons fluoresce with quantum yield increasing with the number of rings, their degree of condensation and their structural rigidity. In addition, aliphatic and alicyclic compounds with carbonyl groups and substances with highly conjugated double bonds fluoresce, but usually to a lesser extent. Among veterinary drugs, quinolones possess native fluorescence; some other antibiotic classes can be efficiently derivatized to give fluorescent compounds (e.g. sulphonamides and aminoglycosides).

For the analysis of residues in food, nowadays, LC-MS is the standard internationally accepted technology already available in most laboratories that is capable of providing structural information about the analytes. Different mass spectrometer platforms have been successfully employed for the analysis of veterinary drugs in honey [14]. Since early 2000s, triple quadrupole mass spectrometer (LC-MS/MS) platform has been introduced in routine worldwide laboratories, and at present, this MS technology is the gold standard for routine analysis of complex sample extracts. The LC-MS/MS, also known as LC-QqQ, is a tandem MS technique in which the first and third quadrupoles act as mass filters and the second, a radio-

frequency-only quadrupole, fragments the analyte through interaction with a collision gas. The most used acquisition mode is multiple reaction monitoring (MRM). Increased selectivity, improved signal-to-noise ratio (S/N), lower limits of quantitation, wider linear range and improved accuracy are some of the benefits of this technique. LC-MS/MS instrumentation tends to give better quantitative precision and improved sensitivity than alternative configurations, making it a superior choice for routine analysis of specific targeted contaminants.

An alternative to LC-MS/MS system is the coupling of liquid chromatography with high-resolution mass spectrometry (LC-HRMS). At the beginning, these analysers were mainly used for research purposes, but after 2007 they started to be applied in routine analysis, too. With HRMS analysers, full-scan spectra are continuously obtained throughout the analytical run allowing for exhaustive qualitative post-acquisition analysis. There are two technologies of high-resolution mass spectrometry: time-of-flight (TOF) and orbitrap. However, single-stage high-resolution mass spectrometry demonstrated to not be suitable for the confirmation of residues at very low concentrations in highly complex matrices such as honey. More recently, hybrid platforms have been available at the bench level such as Q-TOF and Q-Orbitrap combining a quadrupole with an accurate mass analyser. These configurations provide exceptional selectivity and sensitivity over single-stage equipment, and they are increasingly applied in residues analysis of food.

With regard to the chromatographic separation, although the coupling between gas chromatography and mass spectrometry (GC-MS) has been realized before LC-MS, gas chromatography is rarely used for the determination of antibiotics, due to their polar nature, low volatility and thermal instability. Therefore, high-performance liquid chromatography (HPLC) is the technique of choice for antibiotic analysis. Since its introduction in 1970s, HPLC progressively improved mainly thanks to the evolution of packing materials used to carry out the separation. Columns packed with 10 and 5 μm fully porous particles dominated the field for nearly thirty years (1975–2000). In 2004, a great advance in instrumentation and column technology was made achieving very significant increases in resolution, speed and sensitivity. Columns with smaller particles (sub 2-μm) and instrumentation able to deliver mobile phase at 15,000 psi (1000 bar) allowed the achievement of a new level of performance. This new step of HPLC is known as UHPLC technology. In 2007, LC columns with core-shell (superficially porous) particles were introduced. This new generation of microspheres provides the same high efficiency of sub 2-μm UHPLC totally porous particles, but with lower backpressures. The first commercially available core-shell sorbent was the Halo® from Advanced Material Technologies. Currently, the most applied core-shell columns are Kinetex® (Phenomenex), Poroshell® (Agilent), Accucore® (Thermo Fisher Scientific), Ascentis Express® (Supelco), Cortecs® (Waters) and Nucleoshell® (Macherey Nagel). Many of these have been used to determine residues in honey (see **Tables 3–11**).

5. Overview of methods for the determination of drug residues in honey

In the following paragraphs for each compound or class of compounds, an overview of the published confirmatory methods for the determination of residues in honey is given in **Tables 3–11**. Although widely applied in routine laboratories as screening methods, procedures based on bioanalytical techniques such as immunoenzymatic or receptor tests are not considered.

In **Tables 3–11**, for each reviewed procedure, the method limits (CCβs or LODs) are reported. Method limits are generally estimated by the LOD parameter, but, unfortunately, Commission Decision 2002/657/EC [6] introduced a different terminology, that is, decision limit (CCα) and detection capability (CCβ). Although the estimation of method limits is one of the most problematic topics of analytical chemistry [15], from a theoretical point of view, LOD and CCβ (for banned substances) are essentially the same parameter taking into account of both alpha-error (false-positive rate) and beta-error (false-negative rate) [16]. In the relevant column of the tables, CCβs are reported, if available, or, alternately, LODs. They are always given with a maximum of two significant figures.

The most used technique is LC-MS, in particular LC-QqQ platform (**Tables 3–1**). The need of reaching low concentrations involves a progressive decline of LC-DAD- and LC-FLD-based procedures. Methods based on LC-MS (single quadrupole) platform are sporadically described. Finally, in the last few years, high-resolution mass analysers are more and more applied. The ionisation source is almost always electrospray in positive mode (ESI+), except for chloramphenicol for which negative ionization is largely favoured (ESI−). The chromatographic separation is generally achieved in reversed-phase mode, except for aminoglycosides (streptomycin and dihydrostreptomycin) where HILIC columns are frequently applied.

5.1. Chloramphenicol (CAP)

Chloramphenicol is a potent, broad-spectrum antibiotic and a potential carcinogen and has been banned in the European Union since 1994 for use in food-producing animals. The United States and Canada, as well as many other countries, have completely banned its usage in the production of food, too. In January 2002, concerns regarding serious deficiencies of the Chinese residue control system and problems related to the use of banned substances in food-producing animals led the European Union to issue a suspension of imports of all products of animal origin from this country. Meanwhile, a growing number of rapid alert notifications related to the presence of CAP in imported honey have been issued. In beekeeping practice, this antibiotic is mainly used to fight the American foulbrood disease. In 2002, 31 cases out of 34 positive CAP honey detected by the RASFF system (**Table 2**) were from China. These findings were confirmed by Verzegnassi et al. [17] who in the same period analysed 176 raw honeys of various geographical origins, showing very extensive contamination in those of Chinese origin (29 positive samples out of 32). One year later (2003), the percentage of positive chloramphenicol honey from China fell down with only one notification. The import ban was lifted in July 2004.

In **Figure 4**, the sample preparation protocols proposed by the authors of the nine selected analytical methods listed in **Table 3** are summarized [17–25]. Using the CAF as "case study", the figure generalizes the sample purification concept, which is a modular process composed of one or more LLE and SPE steps. Generally, honey is dissolved in water or in acidic solutions due to better solubility of CAF in organic solvents at these pHs (**Figure 1**), thus enabling subsequent RP-SPE or LLE purification. Only Alechaga et al. [23] solubilize honey in an aqueous basic solution (1% NH$_3$), to favour the adsorption on the stationary phase (Oasis HLB) of florfenicol amine which was included in the same procedure. As explained by its name, florfenicol amine (the main metabolite of florfenicol) is a basic drug non-ionized at pHs exceeding 9. The solubilized honey is then purified with one or two clean-up steps: (a) SPE [20, 21, 23];
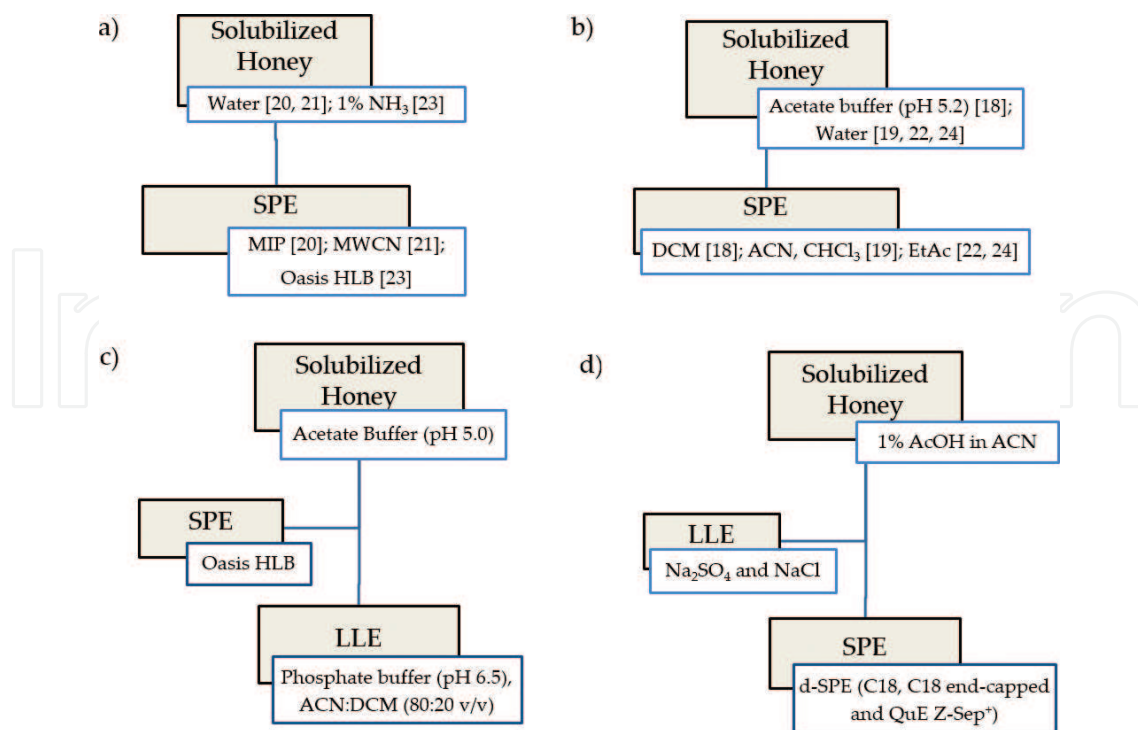
Figure 4. Sample treatment strategies for the determination of **chloramphenicol** residues in honey (**Table 3**): (a) [20, 21, 23]; (b) [18, 19, 22, 24]; (c) [17]; (d) [25].

(b) LLE [18, 19, 22, 24]; (c) SPE and LLE [17]; (d) LLE and SPE [25]. The same scheme could be realized for all the other antibiotic methods summarized in **Tables 4–11**. A complete overview of the sample preparation issues is available in "Analysis of Antibiotic Residues in Food" [26].

5.2. Fumagillin

Fumagillin is a potent amoebicidal agent with properties known since 1950s. This compound is used by apiarists to protect bees from *Nosema apis*. A few articles have reported methods for its determination. The first procedure using LC-MS technique (single quadrupole) has been developed by Nozal et al. in 2008 [27]. In 2011 and in 2015, respectively, Kanda et al. [28] and van den Heever et al. [29] published methods based on LC-MS/MS (triple quadrupole). Nozal et al. [27] and van den Heever et al. [29] applied a quite similar purification approach, solubilizing honey in water and purifying it with polymeric RP-SPE cartridge. They also reached similar LODs ranging from 1 to 4 µg/kg, depending on the honey type (botanical origin). Surprisingly, Kanda et al. [28] reported LODs of two orders of magnitude lower (0.02–0.03 µg/kg), applying QuEChERS extraction with 0.1% FA in acetonitrile followed by non-retentive WAX-SPE. These authors estimate LOD by means of the standard deviation (SD) observed in replicate experiments carried out at a low spiking level, that is, 1 µg/kg (LOD = 3 × SD). However, following the analytical chemistry detection theory, to obtain a reliable estimation of LOD, the spiking level should be close to the found LOD. Clearly, the spiking level reported by Kanda et al. [16] is not suitable, being two orders of magnitude higher than the estimated LOD. This example demonstrates the well-known issues in the estimation of method limits, which can prevent correct compari-

son among method performances. On the other hand, most of the authors do not report how the LODs are obtained, simply declaring that they are calculated according to signal-to-noise (S/N) ratio approach (LOD = 3 × S/N). Finally, it is worthy of note that among the multiclass procedures, only Lopez et al. [30] have included fumagillin within the determined analytes (**Table 11**).

5.3. Macrolides (MACs) and lincomycin

As a result of the development of resistance to oxytetracycline, in the last 15 years two macrolide antibiotics, erythromycin and tylosin, have been widely used for the prevention and treatment of apiculture diseases. Since 1970s, some studies report that tylosin was superior to sulphathiazole in the control of American foulbrood in field colonies of honeybees. In 2005 and in 2013, the US Food and Drug Administration (FDA) and Canada authorities, respectively, approved the use of tylosin in honeybees. In addition, Canada authorities fixed an MRL in honey equal to 200 µg/kg as sum of tylosin A and B (**Table 1**). The most significantly published procedures are summarized in **Table 4** [31–37]. Lincomycin belongs to the group of lincosamides, and its activity against *Paenibacillus larvae* strains has been reported. In 2012, lincomycin was approved by FDA to control tetracycline-resistant American foulbrood disease. Its structure is similar to that of macrolides, and some analytical methods determine simultaneously these substances [31, 33]. Because macrolides are unstable in acidic solution, that is, pH <4, sample extraction is generally carried out in water or in basic buffers (pH 8.0–10.5). Due to their basic nature, at these pHs the reversed-phase solid-phase extraction approach is favoured (**Figure 1**), and all procedures listed in **Table 4** purify the honey extract using silica C18 or polymeric cartridge (Oasis HLB and Strata-X).

5.4. Nitrofurans (NFs)

Nitrofurans have been used for long time in veterinary practice as antibacterial agents for treating infections caused by bacteria and protozoa. At present in Europe and other several countries, these substances are explicitly prohibited or not authorized for all food-producing animals because of their potentially carcinogenic and mutagenic effects on human health. Several studies have showed that animals rapidly metabolize nitrofurans and the in vivo stability of parent drugs is no longer than a few hours. Consequently, the detection of parent drugs in animal tissues is impractical [38]. The covalent binding of NFs with protein tissues has been proven applying the ¹⁴C technique to furazolidone drug. After this observation, analytical methods able to liberate the covalently bound drugs were developed. An acidic hydrolysis followed by a derivatization step with 2-nitro-benzaldehyde (NBA) and subsequent neutralization demonstrated to be the more suitable procedure for NF residue determination. The acid hydrolysis does not release the intact drug, but a structural unit of the parent molecule. 3-Amino-2-oxazolidinone (AOZ), 5-methyl-morpholino-3-amino-2-oxazolidinone (AMOZ), semicarbazide (SEM) and 1-aminohydantoin (AHD) are the released metabolites of furazolidone, furaltadone, nitrofurazone and nitrofurantoin, respectively. It must be underlined that the derivatization with NBA of the cleaved drug metabolites is essential, since AOZ, AMOZ, SEM and AHD are very polar compounds scarcely retained on RP columns and with poor ionization properties in the electrospray interface of MS analysers. It was

thanks to the application of the hydrolysis and derivatization procedure together with the use of LC-MS/MS technique that, in the early 2000s, a large number of contaminated food samples were discovered (**Table 2**). Currently, all the methods are based on this treatment. The analysis of commercialized honey samples demonstrated that furazolidone (AOZ) is the main nitrofuran antibiotic used in apiculture [12]. Inevitably, all methods in **Table 5** apply the LC-MS techniques [39–45]. The first procedure for the determination of metabolites in honey was published by Khong et al. in 2004, using isotopic dilution [39]. Most of the procedures perform the honey solubilization directly in the derivatization mixture (usually an HCl aqueous solution with NBA) [39, 42–44], then purifying the less polar derivatized metabolites (NBAOZ, NBAMOZ, NBSEM and NBAHD). Since after derivatization the solution is neutralized (pH about 7), the LLE and RP-SPE approaches work well (log D about 1 for NBAOZ: **Figure 2**). On the other hand, a limited number of methods perform the derivatization after the first purification step [40, 41]. Tribalat et al. [40] solubilize honey in a 100 mM HCl solution and then carry out a non-retentive RP-SPE (Oasis HLB) since the non-derivatized metabolites are very polar with scarce affinity for non-polar sorbents. As shown in **Figure 2**, at pH < 2 the log D of AOZ is lower than −2. After derivatization, a second (retentive) RP-SPE to isolate NBAOZ, NBAMOZ, NBSEM and NBAHD is carried out. Analogously, Lopez et al. [41] solubilize honey in a 10% NaCl solution, and after a non-retentive RP-SPE (Oasis HLB), they derivatize the metabolites and carry out a LLE using ethyl acetate. For the first time, in 2015, Kaufmann et al. [43] applied an LC-HRMS/MS platform (LC-Q-Exactive) to identify and quantify NFs and CAP, demonstrating acceptable performances for all the four metabolites, except for SEM with CCα and CCβ higher than the fixed MRPL (1 µg/kg).

5.5. Nitroimidazoles (NMZs)

Metronidazole (MNZ), dimetridazole (DMZ), ronidazole (RNZ) and ipronidazole (IPZ) are all nitroimidazole drugs with antibiotic and antiprotozoal activity. NMZs have been traditionally used for treatment and prevention of histomoniasis and coccidiosis in poultry, trichomoniasis in cattle and dysentery in swine. Due to their mutagenicity, genotoxicity and carcinogenicity, in 1990s NMZs have been classified in Europe as prohibited substances for all food-producing species (Group A6 of Annex I of Directive 96/23 [5]). NMZs can prevent and control *Nosema apis*, and in China, these drugs have been used as a cheap alternative to fumagillin. The presence of NMZ residues in honey has been reported only in the last few years [46]. CRL Guidance Paper (2007) [4] requires methods to reach 3 µg/kg. The main published methods based on LC-MS/MS technique are listed in **Table 6** [47–51]. The 5-nitroimidazoles are known to be rapidly metabolized in animals forming the relevant hydroxy metabolites which are generally determined together with the parent drugs because they may have similar mutagenic potential. The first confirmatory procedure in honey has been published by Cronly et al. [47] in 2010, following the detection of metronidazole residues in imported honey from China and from other non-EU countries [12]. Since at pH lower than 2.5 the NMZs are ionized, the solubilization of honey in water or in buffered solution at pH 6–7 favours RP-SPE or LLE purifications (**Figure 2**). On the other hand, some authors have taken advantage of NMZ ionization in strong acidic solutions performing effective cationic-exchange purifications (SCX).

5.6. Quinolones (QNs)

QNs are widely used in veterinary practice because of their rapid effect and broad-spectrum antibacterial activity. Despite the lack of scientific data demonstrating efficacy, the application of these antibiotics in apiculture, especially in Asia, as a prophylaxis for bee diseases increased during the last few years. The first RASFF notifications for the presence of QNs in honey were reported in 2007 in Chinese products. Their use was confirmed by the frequent detection of QN residues in honey also by other control authorities, such as the US Department of Agriculture (USDA) and the Canadian Food Inspection Agency (CCFIA) [14]. To date, the only compounds found in bee products are enrofloxacin, ciprofloxacin and norfloxacin. The native fluorescence of quinolone ring has been extensively exploited to determine these antibiotics in biological fluids and food. Thanks to the high sensitivity of fluorescence detection and the lower cost of equipment compared to LC-MS, this technique is still used to detect and confirm quinolone residues in food. In **Table 7**, the most significant methods are listed [52–58]. Generally, the solubilized honey is purified by reversed-phase SPE [53, 54] or by LLE [55–57]. SPE sorbents, other than reversed-phase types, are reported in the papers published in 1998 by Rose et al. [52] and in 2011 by Yatsukawa et al. [54]. Rose et al. describe two parallel sample treatment protocols using ion-exchange solid-phase extraction: one for nine amphoteric QNs (ciprofloxacin, danofloxacin, enoxacin, enrofloxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin and sarafloxacin) and another for three acidic ones (flumequine, nalidixic acid and oxolinic acid). Amphoteric QNs bear both an acidic group (carboxylic acid) and a basic group (piperazinyl group), and therefore, they are positively ionized at acidic pH, enabling isolation with strong cation-exchange mechanism (SCX-SPE). On the other hand, acidic quinolones can only be neutral, or at basic pHs, they are negatively charged enabling anion-exchange purification. Yatsukawa et al. apply the classical RP-SPE (Oasis HLB) followed by metal chelate affinity chromatography (MCAC). This particular type of SPE acts via the specific chelation of quinolones with ferric ions previously bound to the stationary phase (sepharose fast flow resin). The elution is performed with a buffer (pH 4) containing Na_2EDTA. This is probably the only published application of MCAC to quinolone purification, exploiting their chelating properties. The achievable selectivity allows an efficient removal of interferences also in dark-coloured honey samples such as manuka and buckwheat [54]. On the other hand, MCAC is a well-known stationary phase to purify tetracycline antibiotics using copper (Cu^{2+}) as metal ion (see Section 5.9). Finally, in 2014, Tayeb-Cherif et al. [58] proposed a cheap and simple procedure without any sample purification (**Table 7**): the solubilized honey was just injected in the LC-FLD system. As a result, high detection capabilities (CCβ) are observed (10–100 µg/kg)

5.7. Streptomycin and dihydrostreptomycin (STR/DSTR)

Streptomycin and its derivative, dihydrostreptomycin, are aminoglycoside (AGs) antibiotics used in apiculture to protect bees against a variety of brood diseases. They are polybasic cations consisting of two or more sugars, attached to an aminocyclitol ring with glycoside linkage. Despite the fact that streptomycin is not authorized in most countries in beekeeping

practice, its use is often suggested in bee forums and in beekeeping handbooks. Residues of streptomycin and dihydrostreptomycin have been frequently detected in honey and honey-bee products by the EU RASSF system (**Table 2**). Due to the lack of chromophore or fluoro-phore groups, the traditional absorbance or fluorescence detectors cannot be directly applied to AG determination, as shown in **Table 8** [59–68]. Fortunately, the primary amine groups in the aminoglycoside structure react with a number of derivatizing agents. Therefore, espe-cially in the past when mass spectrometry detectors were not commonly available, methods for this antibiotic family were mainly based on liquid chromatography coupled to FLD after post-column derivatization with *o*-phthalaldehyde (OPA) or β-naphthoquinone-sulphonate (NQS). Since aminoglycosides are in polyionic form in aqueous solutions, both their extrac-tion and preconcentration are difficult, and like the sugars of the honey, silica-based C18 sorbents are unable to retain them. The coating of silica C18 sorbents with an ion-pairing reagent such as 1-heptanesulphonic acid (AHS) was experienced to produce a temporary cat-ion exchanger [59, 61, 62], favouring the analyte retention. In contrast, Bohm et al. [64] purify honey extracts with RP-SPE without any addition of ion-pairing reagents, probably thanks to the use of a polymeric sorbent (Oasis HLB), instead of the silica-based C18 stationary phases. Three procedures [60, 63, 66] applied weak cation-exchange extraction (WCX) to clean-up honey. In 2013, Ji et al. [65] synthesized a molecular imprinted polymer (MIP) by polymeriza-tion of methacrylic acid and ethylene glycol dimethacrylate in the presence of streptomycin as template molecule. The observed recoveries for four model compounds in honey (streptomy-cin, gentamicin, spectinomycin and dihydrostreptomycin) ranged from 90 to 110%. Currently, this developed MIP sorbent is commercially available and Moreno-Gonzales et al. applied it to determine aminoglycosides in honey using capillary zone electrophoresis coupled to an ion trap mass analyser [68]. Finally Wang et al. developed a home-made hydrophilic stationary phase (polyvinyl alcohol onto silica gel, PVA-Sil), which demonstrated satisfactory perfor-mances to pre-concentrate aminoglycosides in honey extracts [67].

With regard to chromatographic issues, because of their high polarity, the underivatized aminoglycosides are not sufficiently retained on standard reversed-phase HPLC columns. Therefore, there are two possible choices: (i) the addition of ion-pairing reagents such as alkyl sulphonates (e.g. sodium 1-heptansulphonic acid, AHS) or fluoropropionic acids (e.g. hepta-fluorobutyric acid, HFBA; pentafluoropropionic acid, PFPA) in the mobile phase and (ii) the application of HILIC (hydrophilic interaction chromatography) analytical columns, which are more compatible with MS detection since ion-pairing reagents cause strong ion suppression. HILIC is a variant of normal-phase chromatography that uses water as a strong eluent and water-miscible organic solvents like acetonitrile as organic components of the mobile phase. In **Table 8**, examples applying derivatization [59, 61], ion-pairing reagents [60, 62] and HILIC chromatography [63–67] are reported.

5.8. Sulphonamides (SAs)

As early as 1940s, sodium sulphathiazole was registered for the control of American foulbrood in United States, but its use was later banned because residues of the drug continued to be found many months after its administration. Residues of sulphadiazine, sulphadimethoxine, sulphamerazine, sulphamethazine and sulphamethoxazole have been also detected in honey

[12, 14]. Sulphonamides have good UV absorption with maxima in the range of 260–275 nm, and since the 1980s, confirmatory methods have been developed using HPLC coupled to UV detection. Moreover, after derivatization with fluorescamine, sulphonamides give fluorescence and some procedures apply LC-FLD (with pre- or post-column derivatization), reaching limits of detections (LOD/CCβ) comparable to those of LC-MS methods. In **Table 9**, some example of these applications are listed [69–78]. Since considerable amounts of SAs are bound to honey sugars, in 2000 Schwaiger and Schuch [79] demonstrated the need of an acidic hydrolysis prior to the residue analysis. This step avoids the underestimation of the actual sample contamination.

The solubility of sulphonamides in acids and alkali is conditioned by their amphoteric properties, due to the presence of an anilino amino group (pK_{a1}: 2–2.5) and of an amidic group, which contains a labile hydrogen atom with acidic properties (pK_{a2}: 6–9). Thus, sulphonamides are positively charged in acidic medium at pH <2, neutral at pH 3–6 and negatively charged at pH >6. Therefore, at one hand, exploiting their basic moiety, some procedures use strong cation exchange (SCX-SPE) to isolate sulphonamides from the acidic honey extracts [71–73, 77]. On the other hand, to successfully apply RP-SPE or LLE clean-up, some researchers buffered honey extract in the pH range about 4–6 in which the neutral form of sulphonamides prevails [70, 74–76, 78]. In this interval, the distribution coefficients (D) reach their maximum and the compound lipophilicity is enhanced, as shown in **Figure 3** for sulphathiazole.

5.9. Tetracyclines (TCs)

The efficacy of the oxytetracycline for control of European foulbrood has been widely demonstrated as early as 1950s. In honey, beyond oxytetracycline (brand name: Terramycin®), tetracycline and chlortetracycline residues have been detected, too [12, 14]. Because of their polar nature, tetracyclines have the ability to strongly bind to proteins as well as to chelate with divalent metal ions. Therefore, most extractions incorporate acidic solvents with the addition of metal chelating agents. Frequently, the extraction approaches use Na_2EDTA-McIlvaine buffer (pH = 4). Known as the "universal tetracycline extractant", McIlvaine buffer consists of citric acid and disodium hydrogen phosphate. Other common buffers used for tetracyclines extraction are oxalic acid, succinic acid and citric acid. Another challenge in tetracycline determination is their epimerization. In mildly acidic conditions (pH 2–6), epimerization occurs at position C-4. Accordingly, European Union MRLs in food are established as sum of tetracycline and its epimer, that is, tetracycline and epi-tetracycline, oxytetracycline and epi-oxytetracycline, chlortetracycline and epi-chlortetracycline [1].

As shown in **Table 10** [80–88], besides the classical reversed-phase solid-phase extraction cartridges (phenyl, Oasis HLB, Strata-X and C18), tetracyclines can be selectively purified applying a particular type of solid-phase extraction, that is, metal chelate affinity chromatography (MCAC) [82, 86]. As mentioned before for quinolones (Section 5.6), MCAC exploits tetracycline metal complexing properties to allow for additional clean-up. The sorbent (sepharose resin) is treated with aqueous copper (II) sulphate. The sample extract is then loaded onto the column and TCs are retained. The copper ions give visualization of the clean-up process: the analytes are found where the blue copper ions appear. Initially, the tetracyclines are bound to the blue copper ions on the column until disruption by an EDTA containing buffer and elution of the copper ions, EDTA and tetracyclines.

5.10. Multiclass methods

In efforts to increase the cost-effectiveness of antimicrobial residue enforcement programmes, the development of analytical methods able to detect as many contaminant compounds as possible is highly preferred. However, it is well known that one of the difficulties in the development of these procedures is the incompatibility of selective sample treatments with acceptable accuracies for a wide range of analytes. Therefore, only a generic purification protocol such as liquid-liquid extraction or reversed-phase solid-phase extraction is achievable (**Table 11**). Since generally reversed-phase sorbents provide the least selective retention mechanism when compared to normal phase or ion exchange ones, they allow the most universal solid-phase extraction approach retaining most molecules with any hydrophobic character.

There are some considerations to do before to take on multiclass methods for antibiotics: (i) the extraction of nitrofuran metabolites requires acid hydrolysis and derivatization steps that would be destructive to other analytes of interest. Therefore, this class should be extracted apart from a multiclass method to obtain satisfactory recovery and avoid degradation of acid-labile compounds; (ii) as mentioned before, highly polar compounds, such as aminoglycosides, do not perform well in multiclass methods as they are relatively insoluble in organic solvents and exhibit little or no affinity for non-polar stationary phases used in RP-LC.LC. For this reason, in **Table 11** only two papers include aminoglycosides among the determined classes adding an ion-pairing reagent in the mobile phases; (iii) in addition, in honey, the determination of sulphonamides in honey requires a preliminary hydrolysis step to measure residues bound to sugars, and therefore, also in this case, acid-sensitive antibiotics can be destroyed.

In this context, "multiclass" are procedures involving the determination of more than two drug classes. Probably, the first multiclass method in honey has been published in 2004 by Kaufmann et al. [89], reporting the determination of three antibiotic families, including sixteen sulphonamides together with three tetracyclines and flumequine, a quinolone antibiotic for which until now there is no evidence of use in apiculture. In 2008, Hammel et al. [90] developed an LC-MS/MS protocol for 42 substances including five tetracyclines, seven macrolides, three aminoglycosides, eight beta-lactams, two amphenicols and seventeen sulphonamides. Four subsequent liquid-liquid extraction steps were necessary to adequately extract all the analytes. After this paper, many confirmatory multiclass methods have been published mainly applying triple quadrupole platforms [29, 91–98]. This is in accordance with the general trend in analysis of residues in food started in the late 2000s. Although triple quadrupoles have been introduced in the mid-to late-1990s, only in recent years these equipments have improved their electronics enabling the possibility of acquiring dozens of compounds in the same chromatographic run.

6. Conclusions

The performances of an analytical method are mainly determined by the applied sample preparation and instrumental technique. The coupling honey-antibiotic (matrix-analyte) can be a "case study" to discuss the general strategies of developing methods for trace

analysis in food. It must be kept in mind that the sample preparation protocol has to start from the knowledge of the matrix composition and analyte properties (MW, pK_a, log D, etc). Moreover, the choice of the more suitable clean-up also involves the knowledge of the available methodologies, but in most of the cases the selection is limited to the SPE stationary phases. In the last years, new sorbent materials are more and more produced, enabling new possibilities for more efficient, rapid and cheap protocols. Undoubtedly, aminoglycosides and, to a lesser extent, tetracyclines are the more difficult classes to analyse. Obviously, when multiresidue or multiclass procedures are optimized, the challenge is the achievement of the best compromise among the different properties of each single-class challenging. The current trends in honey sample preparation and, more generally in food, involve the following issues: the miniaturization of the equipment for sample preparation (micro techniques); the decrease in the amount of sample to be analysed; the reduction in the use of organic solvents; and the development of multiclass procedures. All these strategies aim at the reduction in the employed reagents/materials and at the increase in the analysis throughput. The choice of the analytical equipment is less free. Today, LC-QqQ systems (triple quadrupoles) are able to solve almost each analytical problem. With regard to the analyte separation, except for aminoglycosides, reversed-phase stationary phases are generally used. Various column types (traditional, sub 2-μm and core-shell) and manufacturers have been reported in literature to determine the same analyte or class of analytes (**Tables 3–11**), but frequently the applied selection criteria are not explained or compared.

Nomenclatures

Abbreviations

2-NBA	2-Nitrobenzaldehyde
ACN	Acetonitrile
AcOH	Acetic acid
AF	Ammonium formate
AGs	Aminoglycosides
CAP	Chloramphenicol
DCM	Dichloromethane
ELISA	Enzyme-linked immunosorbent assay
EtAc	Ethylacetate
FA	Formic acid
HRMS	High-resolution mass spectrometry
LC-DAD	Liquid chromatography with diode array detector
LC-FLD	Liquid chromatography with fluorescence detection

MACs	Macrolides
MCAC	Metal chelate affinity chromatography
MeOH	Methanol
MIP	Molecular imprinted polymer
MWCN	Multi-walled carbon nanotubes
NaAc	Sodium acetate
NH_4Ac	Ammonium acetate
NFs	Nitrofurans
NMZs	Nitroimidazoles
NQS	Sodium 1,2-naphthoquinone-4-sulphonic acid
OA	Oxalic acid
PA	Orthophosphoric acid
QNs	Quinolones
QuEChERS	Quick, easy, cheap, effective, rugged and safe
SAs	Sulphonamides
SCX	Strong cation exchange
SDS	Sodium dodecyl sulphate
STR/DSTR	Streptomycin/dihydrostreptomycin
TCs	Tetracyclines
TFA	Trifluoroacetic acid
TOF	Time-of-flight
UHPLC	Ultra-high-pressure liquid chromatography

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