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Simultaneous Determination of Multiclass Pesticides and Antibiotics in Honey Samples Based on Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract A simple, fast, and efficient method was developed for simultaneous determination of 79 pesticides and 13 antibiotics compounds of different chemical classes of pesticides and antibiotics in honey samples by ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS). The sample preparation procedure includes homogenization with McIlvaine buffer 0.1 mol L^{-1} (pH 4), followed by extraction with acetonitrile and cleanup with florisil, using dispersive solid phase extraction (d-SPE). The proposed method was validated with good results, such as linearity (r^2>0.9901), normality, and independence of the evaluated data, as well as recoveries between 70 and 120 % with relative standard deviation (RSD) <20 % for most of the compounds spiked from 0.1 to 200 μg kg^{-1}. The experimental method limits of detection and quantification were from 0.03 to 1.51 μg kg^{-1} and from 0.1 to 5 μg kg^{-1}, respectively, for the pesticides. For the antibiotics, the decision limits (0.1 to 2 μg g^{-1}) and the detection capacity (0.12 to 2.81 μg g^{-1}) were below the maximum residue limits (MRLs) established for honey by the Brazilian and European legislation. The method was successfully applied to real samples from different botanical and geographic origins. From them, 44 % presented residues from 0.12 to 10 μg kg^{-1} of one or more analytes. The proposed method combines the advantages of a quick sample preparation step with the selectivity and sensitivity of the UHPLC-MS/MS and proved to be suitable for routine analyses.

Keywords Honey · Pesticides · Antibiotics · UHPLC-MS/MS

Introduction

Honey presents therapeutic and medicinal properties and is considered a healthy food with high nutritional value (Gomes et al. 2010). Concerning food safety, honey must be free of toxic and carcinogenic chemical contaminants, especially pesticides and antibiotics (Zacharis et al. 2012). The exposure of bees to these compounds causes different acute toxic effect, leading to a short-term mortality as well as the contamination of honey and consequent effects on health of consumers (Tapparo et al. 2013).

Neonicotinoid pesticides, as imidacloprid, clothianidin, and thiamethoxam, are harmful to bees (Gbylik-Sikorska et al. 2015). These compounds are associated with colony collapse disorder (CCD), which reports mass disappearance of pollinator insects (Wu et al. 2012). The main consequence of CCD is the low pollination of food crops, including fruits and vegetables, leading to a decrease of 45 % in world production of these major commodities (vanEngelsdorp and Meixner 2010). Therefore, the European Commission has prohibited for 2 years the use of neonicotinoids on crops that are attractive for bees (Jovanov et al. 2015). Macrolide and sulfonamide antibiotics are compounds applied in bee colonies for the treatment of bacterial diseases, such as American foulbrood (AFB) and European foulbrood (EFB) (Juan-Borrás et al. 2015). However, high concentration of antibiotic

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residues is very harmful to human health and might develop disease resistance (Bedendo et al. 2010).

Maximum residue limits (MRLs) for antibiotics, pesticides, and environmental contaminants in honey are established by different food regulatory agencies. The European Union (EU 2015) has set MRLs for some pesticides, with levels higher or equal to 10 μg kg^{-1}, and prohibited the use of antibiotics (Gómez-Pérez et al. 2012). In Brazil, the National Program for Control of Residues and Contaminants (PNCRC) coordinated by the Ministry of Agriculture, Livestock and Supply (MAPA) apply MRLs ranging between 0.3 and 50 μg kg^{-1} for honey (MAPA 2013). In this context, the development of sensitive, efficient, and reliable analytical methods is important to help the monitoring programs and to ensure food safety.

Currently, chromatographic techniques as gas chromatography (GC) and ultra-high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) are the techniques more frequently employed for multiresidue determination, permitting to achieve good selectivity and sensitivity for determination of residues and contaminants in honey samples (Pirard et al. 2007; Frenich et al. 2014).

Sample preparation is a critical step to develop multiresidue methods for the determination of pesticides and/or antibiotics in honey, mainly due to the complexity of the matrix. Honey is essentially composed of a complex mixture of carbohydrates, of which fructose and glucose account for nearly 85 % and other substances, such as, organic acids, amino acids, minerals, proteins, vitamins, and lipids (Gomes et al. 2010). In the last years, different sample preparation procedures were applied for residue and contaminants determination on honey samples (Jovanov et al. 2013). Sheridan et al. (2008) employed solid phase extraction (SPE) and LC-MS/MS for determination of 14 sulfonamides and chloramphenicol, achieving limits of detection (LODs) below 10 μg kg^{-1} for all compounds and 0.2 μg kg^{-1} for chloramphenicol. Campillo et al. (2006) proposed a method for determination of 16 pesticides using solid phase microextraction (SPME) and GC with microwave-induced plasma atomic emission, with LOD from 0.02 to 10 μg kg^{-1}. Bianchin et al. (2014) employed headspace solid phase microextraction (HS-SPME) for screening of volatile components in honey, combining multiple extraction temperatures by GC-MS. Blasco et al. (2003) determined residues of 28 organophosphorus and five carbamates using stir bar sorption extraction (SBSE) by LC-MS, with LOD between 10 and 80 μg kg^{-1}. Jovanov et al. (2015) developed and optimized a method for determination of seven neonicotinoids employing dispersive liquid-liquid microextraction (DLLME) and LC with diode array detection (LC-DAD), achieving LODs from 1.5 to 2.5 μg kg^{-1}. Bezerra et al. (2010) developed a method for determination of four pesticides using matrix solid phase dispersion (MSPD) and GC-MS, with LODs from 20 to 80 μg kg^{-1}.

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method proposed by Anastassiades et al. (2003) brings the advantages of analysis of many compounds with different chemical classes in a short time using small amounts of solvent. This method also provides suitable accuracy and precision and provides an easy and efficient cleanup step (Prestes et al. 2009). Orso et al. (2014) proposed a method for determination of 24 residues of pesticides in honey using QuEChERS method and GC with electron capture detection (GC-ECD). Recovery results were between 71 and 119 % for most of the compounds, with relative standard deviation (RSD) <20 %. The proposed method enables to achieve LODs method between 3 and 6 μg kg^{-1}. Shendy et al. (2016) determined nitrofuran and nitroimidazole residues using a modified QuEChERS sample preparation. Results for decision limit (CCα) and detection capacity (CCβ) were 0.12–0.74 and 0.21–1.27 μg kg^{-1}, respectively, with recovery between 91 and 105 % and RSD from 2.6 to 12.6 %.

Frenich et al. (2010) compared different sample preparation procedures (QuEChERS, SPE, MSPD, and solvent extraction) for simultaneous determination of different types of antibiotics (sulfonamides, tetracyclines, macrolides, quinolones, and anthelmintics). Solvent extraction proved to be the most reliable technique for determination of the selected compounds. Rissato et al. (2007) applied this method for determination of 48 pesticides of different classes (organohalogen, organophosphorous, organonitrogen, and pyrethroids) in honey samples by GC-MS/MS. The recovery results ranged from 76 to 95 %, and the LODs were lower than 10 μg kg^{-1}.

Nowadays, these sample preparation procedures are widely used as an alternative for some drawbacks like extract interference concentration and solvent waste. However, there are some limitations when multiresidue analysis was applied in honey samples, due to the different chemical compound properties and the complexity of this matrix.

Therefore, considering the importance of honey for consumer health and for the economy, this study aims to develop and validate a rapid and effective method for the simultaneous determination of 79 pesticides and 13 antibiotics using a water-acetonitrile extraction step followed by a cleanup with dispersive solid phase (d-SPE) and UHPLC-MS/MS analysis applying the selected reaction monitoring (SRM) mode. For this purpose, several modifications of the extraction method were tested using different sorbents for cleanup. The method was applied for 43 honey samples from different regions of Rio Grande do Sul State, Brazil.

Experimental

Chemicals and Apparatus

Analytical standards and triphenylphosphate (TPP), used as internal standard (IS), were acquired from Dr. Ehrenstorfer (Germany). Atrazine-d5, used as surrogate standard (SS), was purchased from CDN Isotopes (Canada). Acetonitrile HPLC grade was acquired from J.T. Baker (USA), and purified water was provided by a Direct QUV system (resistivity of 18.2 MΩ cm) from Millipore (France).

The analyzed compounds were selected based on compounds with MRL established for honey at European Union and PNCRC (Brazil). Standard stock solutions (1000 mg L^{-1}) of each compound were prepared in acetonitrile and/or methanol (HPLC grade) considering the purity of solid standard. From these individual solutions, one mixture at 10 mg L^{-1} for pesticides and one for antibiotics, both in acetonitrile, were prepared. All these solutions were kept at −20 °C and are stable for 1 year. These solutions were used to prepare one mix at 1.0 mg L^{-1} in acetonitrile for pesticides and other for antibiotics that were renewed every month.

Bondesil C18 (40 μm) and primary secondary amine (PSA) (40 μm) were acquired from Agilent (USA); Oasis HLB® from Waters (USA); anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) from J.T. Baker (USA); and sodium hydrogen citrate sesquihydrate ($C_6H_6Na_2O_7$), ammonium formate, and sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7$) from Sigma-Aldrich (Japan). For the preparation of 400 mL of the McIlvaine buffer 0.1 mol L^{-1} in accordance with Solliec et al. (2015), 14.8 g of disodium ethylenediaminetetraacetic acid (Na_2EDTA), 7.7 g of anhydrous citric acid, and 21.4 g of dibasic sodium phosphate heptahydrate p.a. (Na_2HPO_4 $7H_2O$) from Synth (Brazil) were used. Florisil® 60-100 mesh from Mallinckrodt (Ireland) was prepared for use as described by Ramos et al. (1997) heating, in muffle, at 550 °C overnight and transferred to an oven at 130 °C for 5 h and immediately deactivated through the addition of 8 % (*m*/*v*) Milli-Q water.

Vortex shaker (model QL-901) from BioMixer (Brazil), analytical balances (UX-420H) from Shimadzu (Japan) and APX-200 from Denver Instruments Ltda (Brazil), refrigerated centrifuges NT 825 from Novatecnica (Brazil) and SL 703 from Solab (Brazil), thermostatic bath 398 (De Leo, Brazil), and nylon filters of 13 mm (0.2 μm) were used.

UHPLC-MS/MS Conditions

Chromatographic analyses were carried out on a UHPLC-MS/MS from Waters (USA) equipped with Acquity UPLC™ liquid chromatography; Xevo TQ™ MS/MS triple quadrupole detector; an autosampler, a binary pump, and a column temperature controller; nitrogen generator model NM30L-MS (Peak Scientific, Scotland); argon gas 6.0 used as collision gas; and a data acquisition software MassLynx V4.1. For chromatographic separation, an analytical column Acquity UPLC™ BEH C18 (100×2.1 mm, 1.7-μm particle size), maintained at 40 °C, was used. The quadrupole mass spectrometer was operated in selected reaction monitoring (SRM) mode using two transitions, one for quantification and another for confirmation with their collision energy (CE), as shown in Table 1. The used UHPLC-MS/MS conditions were as follows: capillary voltage 2 kV, desolvation temperature 500 °C, desolvation gas flow (nitrogen) 600 L h^{-1}, spray flow 80 L h^{-1}, collision gas flow (argon) 0.15 mL min^{-1}, source temperature 150 °C, and injection volume 10 μL. Mobile phase consisted of (A) water with formic acid 0.1 % (*v*/*v*) and ammonium formate 5 mmol L^{-1} and (B) methanol with formic acid 0.1 % (*v*/*v*) and ammonium formate 5 mmol L^{-1}. The mobile phase gradient started at 5 % of B and remained constant until 7.74 min, increasing to reach 100 % of B in 14 min, returning to 5 % of B from 14.01 to 15 min. The flow rate was constant at 0.2 mL min^{-1}. As described by Kemmerich et al. (2015), the mobile phase composition with the additives formic acid and ammonium formate provided good results for the analysis of pesticide residues by UHPLC-MS/MS.

Sample Preparation Optimization

For sample preparation procedure, some preliminary assays were carried out using citrate QuEChERS method (Jovanov et al. 2014) and solvent extraction, in order to establish the best conditions for sample preparation. First, samples were heated in a water bath at 40 °C for 5 min. After, 2 g of honey sample was weighed in a 50-mL polypropylene tube and 20 μL of the surrogate standard was added resulting a concentration of 20 μg kg^{-1}.

In the first assay, the citrate QuEChERS method was evaluated through the addition of McIlvaine buffer 0.1 mol L^{-1} (pH 4) for sample homogenization before addition of acetonitrile. Extraction was achieved using acetonitrile, and for partition, sodium hydrogen citrate sesquihydrate and sodium citrate tribasic dihydrate salts were added. For the cleanup step, different combinations of sorbent were evaluated using a gravimetric test described by Tejera-Garcia et al. (2012), in which the extracts are evaporated and the mass of coextractives in the final extract is calculated. The following tests were conducted with homogenization and extraction like the first test, but without the partition step. The cleanup in this case was evaluated using 50 mg of each sorbent: C18+PSA; florisil+C18; and florisil+PSA, C18, Oasis HLB, PSA, and florisil. In all the tests, 1 mL of extract and 150 mg of $MgSO_4$ were used.

Table 1 UHPLC-MS/MS parameters for the determination of selected pesticides and antibiotics, class, linear range, and coefficient of determination (r^2)

Compounds	Class	t_R (min)	ESI	SRM transitions, *m/z* (CE, eV)		Linear range (μg kg^{-1})	r^2
				Quantification	Confirmation		
Acephate	I	2.88	+	184>143 (8)	184>125 (8)	5–200	0.9988
Acetamiprid	I	4.91	+	223>126 (20)	223>56 (15)	0.1–100	0.9971
Atrazine	H	6.82	+	216>174 (18)	216>96 (23)	0.1–200	0.9934
Azinphos ethyl	I	7.67	+	346>77 (36)	346>132 (16)	1–150	0.9993
Azinphos methyl	I	7.08	+	318>160 (8)	318>261 (8)	1–200	0.9983
Azoxystrobin	F	7.12	+	404>329 (30)	404>372 (15)	2–200	0.9937
Boscalid	F	7.36	+	343>307 (20)	343>140 (20)	0.1–200	0.9986
Bromophos methyl	I	9.17	+	365>147 (26)	365>309 (12)	0.1–200	0.9986
Carbaryl	I	6.41	+	202>145 (22)	202>117 (28)	1–200	0.9950
Carbendazim	F	4.16	+	192>160 (18)	192>132 (28)	0.1–200	0.9999
Carbofuran	Ac/I/N	6.2	+	222>165 (16)	222>123 (16)	1–200	0.9919
Carbofuran 3-OH	I	4.89	+	238>163 (16)	238>181 (10)	2–150	0.9991
Carboxin	F	6.38	+	236>87 (22)	236>143 (16)	0.1–150	0.9909
Cyproconazole	F	7.50/7.68	+	292>125 (27)	292>70 (24)	1–200	0.9954
Chloramphenicol	An	5.29	+	321>152 (18)	321>257 (12)	0.1–100	0.9981
Chlorpyrifos ethyl	I	8.82	+	345>97 (32)	350>198 (20)	0.1–150	0.9986
Chlorpyrifos methyl	I	8.38	+	322>125 (20)	322>290 (16)	1–200	0.9957
Clothianidin	I	4.63	+	250>132 (18)	250>169 (12)	1–200	0.9984
Dichlorvos	I	6.15	+	221>79 (34)	221>109 (22)	0.1–150	0.9963
Diethofencarb	F	7.2	+	268>226 (10)	268>124 (40)	1–200	0.9953
Difenoconazole	F	8.31	+	406>251 (25)	406>111 (60)	0.1–150	0.9908
Dimethoate	Ac/I	4.93	+	230>125 (20)	230>199 (10)	1–150	0.9959
Dimoxystrobin	F	7.94	+	327>116 (21)	327>205 (10)	1–150	0.9933
Diniconazole	F	8.34	+	326>70 (25)	326>159 (34)	1–200	0.9969
Diuron	H	6.93	+	233>72 (18)	233>46 (14)	0.1–200	0.9925
Emamectin benzoate	An	8.79	+	887>126 (38)	887>158 (37)	0.1–200	0.9993
Epoxiconazole	F	7.75	+	330>101 (50)	330>121 (22)	1–150	0.9909
Erythromycin ABC	An	6.84	+	734>158 (30)	734>576 (20)	1–150	0.9971
Etofenprox	I	9.48	+	394>107 (43)	394>177 (15)	0.1–200	0.9971
Etrimfos	I	8.08	+	293>125 (26)	293>265 (16)	1–150	0.9973
Fenarimol	F	7.33	+	331>81 (34)	331>268 (22)	1–100	0.9979
Fenpropathrin	Ac/I	8.82	+	350>125 (14)	350>97 (34)	1–150	0.9982
Fenpropimorph	F	7.41	+	304>147 (28)	304>57 (30)	0.1–200	0.9926
Fipronil	I	7.8	-	435>250 (26)	435>330 (16)	2–100	0.9923
Flusilazole	F	7.84	+	316>165 (28)	316>247 (18)	1–200	0.9910
Flutolanil	F	7.4	+	324>65 (40)	324>262 (18)	0.1–100	0.9988
Fluvalinate	I	9.21	+	503>181 (30)	503>208 (12)	0.1–100	0.9979
Furazolidone	An	3.88	+	226>95 (14)	226>139 (15)	1–200	0.9988
Hexaconazole	F	8.21	+	314>70 (22)	314>159 (28)	2–200	0.9963
Imazalil	F	6.87	+	297>159 (22)	297>69 (22)	0.1–200	0.9911
Imidacloprid	I	4.55	+	256>175 (20)	256>209 (15)	1–200	0.9983
Iprovalicarb	F	7.62	+	321>119 (16)	321>203 (10)	0.1–200	0.9971
Isoxaflutole	H	6.81	+	360>220 (40)	360>251 (14)	1–200	0.9987
Lincomycin	An	8.31	+	407>252 (24)	407>338 (16)	0.1–100	0.9968
Linuron	H	7.29	+	249>160 (18)	249>181 (17)	0.1–100	0.9977
Malathion	I	7.43	+	331>99 (24)	331>127 (12)	1–200	0.9971
Mepanipyrim	F	7.69	+	224>106 (25)	224>77 (40)	0.1–100	0.9901
Mepronil	F	7.47	+	270>91 (44)	270>119 (28)	1–200	0.9931
Methacrifos	Ac/I	7.03	+	241>125 (20)	241>209 (8)	0.1–200	0.9971
Metalaxyl	F	5.79	+	280>192 (17)	280>220 (13)	0.1–200	0.9962

Table 1 (continued)

Compounds	Class	t_R (min)	ESI	SRM transitions, *m/z* (CE, eV)		Linear range (μg kg^{-1})	r^2
				Quantification	Confirmation		
Methidathion	I	7,0	+	303>85 (20)	303>145 (10)	1–100	0.9972
Metolachlor	H	7.87	+	284>176 (25)	284>252 (15)	0.1–200	0.9915
Metsulfuron methyl	H	6.06	+	382>167 (16)	382>199 (22)	1–200	0.9957
Mevinphos	Ac/I	4.89/5.36	+	225>127 (15)	225>193 (8)	0.1–200	0.9975
Monolinuron	H	6.52	+	215>99 (34)	215>126 (22)	1–200	0.9986
Omethoate	Ac/I	3.13	+	214>125 (22)	214>183 (11)	2–200	0.9995
Oxadixyl	F	5.79	+	279>132 (34)	279>219 (10)	1–100	0.9993
Paraoxon ethyl	I	6.68	+	276>220 (17)	276>248 (16)	1–200	0.9968
Penconazole	F	8.06	+	284>159 (34)	284>70 (16)	0.1–100	0.9904
Phosmet	Ac/I	7.08	+	318>77 (46)	318>160 (22)	0.1–100	0.9977
Picoxystrobin	F	7.85	+	368>145 (22)	368>201 (10)	0.1–200	0.9918
Pirimicarb	F	5.92	+	239>182 (16)	239>72 (21)	1–200	0.9949
Pirimiphos ethyl	I	8.66	+	334>198 (23)	334>182 (25)	0.1–200	0.9944
Pirimiphos methyl	Ac/I	8.23	+	306>108 (32)	306>164 (22)	0.1–150	0.9966
Profenofos	Ac	8.57	+	373>128 (40)	373>303 (20)	1–150	0.9992
Profoxydim	H	9.07	+	466>280 (16)	466>238 (19)	1–150	0.9969
Propargite	H	8.88	+	368>175 (15)	368>231 (15)	1–150	0.9980
Propoxur	F	6.16	+	210>111 (16)	210>168 (10)	0.1–200	0.9987
Pyraclostrobin	F	8.13	+	388>163 (25)	388>194 (12)	1–150	0.9990
Pyrazophos	I	8.22	+	374>194 (32)	374>222 (22)	1–200	0.9975
Pyridate	I	9.35	+	379>207 (18)	379>351 (10)	1–100	0.9914
Pyrimethanil	I	7.18	+	200>107 (24)	200>82 (24)	0.1–200	0.9946
Salinomycin	Ac	9.57	+	774>403 (61)	774>431 (50)	2–150	0.9945
Simazine	H	6.2	+	202>96 (22)	202>124 (16)	1–200	0.9987
Sulfachloropyridazine	An	4.34	+	285>92 (28)	285>156 (15)	2–150	0.9980
Sulfadiazine	An	3.16	+	251>108 (30)	251>156 (15)	0.1–150	0.9948
Sulfadimethoxine	An	5.21	+	311>92 (32)	311>156 (20)	0.1–150	0.9973
Sulfamethazine	An	4.1	+	279>92 (28)	279>186 (16)	0.1–150	0.9987
Sulfamethoxazole	An	4.4	+	254>108 (27)	254>156 (16)	0.1–150	0.9920
Sulfaquinoxaline	An	5.38	+	301>92 (30)	301>156 (16)	0.1–150	0.9928
Sulfathiazole	An	3.34	+	256>92 (25)	256>156 (15)	0.1–200	0.9991
Tebuconazole	F	8.05	+	308>70 (22)	308>125 (40)	1–200	0.9984
Terbuthylazine	H	7.39	+	230>174 (16)	230>96 (28)	1–200	0.9919
Thiacloprid	I	5.24	+	253>126 (20)	253>90 (40)	0.1–200	0.9989
Thiamethoxam	I	4.02	+	292>132 (22)	292>211 (12)	2–200	0.9962
Tolclofos methyl	F	8.25	+	301>125 (17)	301>175 (29)	1–200	0.9982
Triadimefon	F	7.5	+	294>69 (20)	294>197 (15)	1–200	0.9961
Triazophos	F	7.57	+	314>162 (35)	314>119 (18)	1–100	0.9955
Trichlorfon	F	8.69	+	257>127 (15)	257>257 (7)	0.1–150	0.9965
Trifloxystrobin	F	8.32	+	409>145 (40)	409>186 (16)	1–150	0.9958
Triflumizole	F	8.44	+	346>278 (10)	346>60 (10)	1–200	0.9975
Tylosin	An	6.65	+	916>101 (45)	916>174 (40)	0.1–200	0.9971

I insecticide, *F* fungicide, *H* herbicide, *Ac* acaricide, *An* antimicrobial, *B* bactericidal, *N* nematicide. Source: PPDB (2015)

The optimized sample preparation procedure presented in Fig. 1 consisted in weight 2 g of honey sample, spike at 20 μg kg^{-1} with the surrogate standard (atrazine-d5), and extract as follows: 2 mL McIlvaine buffer 0.1 mol L^{-1} (pH 4) was added for sample homogenization and extraction was performed with 2 mL of acetonitrile and 2 min of agitation in vortex. Samples were centrifuged for 6 min at 2137×*g*. For cleanup step, 1 mL of supernatant was transferred to an Eppendorf tube of 2 mL containing 150 mg of anhydrous $MgSO_4$ and 50 mg of activated florisil, followed by shaking

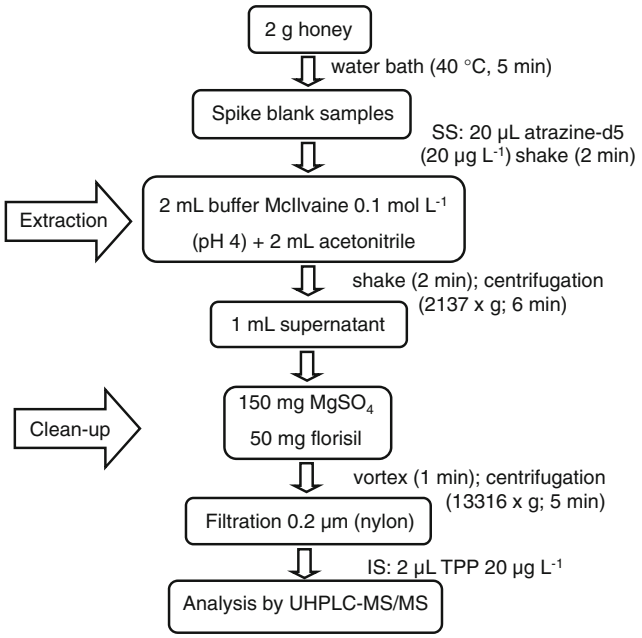

Fig. 1 Representation of the proposed method for analysis of pesticides and antibiotic residues in honey samples

in vortex for 1 min. Tubes were centrifuged for 5 min at 13, 316×*g*, the extract was filtered (0.2 μm), and 2 μL of the internal standard triphenylphosphate (TPP) was added at the concentration 20 μg L^{-1} for subsequent UHPLC-MS/MS analysis.

Validation Conditions

The method validation parameters evaluated were the following: selectivity, considering absence of the matrix interferences; analytical curves, in terms of normality and independence; linear range; matrix effect; accuracy; precision (repeatability and intermediate precision); limits of detection (LOD) and quantification (LOQ); decision limit (CCα); and detection capability (CCβ) (Dubreil- Chéneau et al. 2014). For method validation, blank honey samples were collected from a non-agricultural area. The physicochemical analysis of blank samples performed how described by de Almeida-Muradian et al. (2013) presented 36 mEq kg^{-1} of acidity, 20 % of moisture, and 64.8 % of reducing sugars. These results are in accordance with Brazilian legislation for honey quality (MAPA 2000). Calibration curves were prepared in solvent (acetonitrile) and in blank matrix extract at ten different concentration levels (0.1, 1, 2, 5, 10, 20, 50, 100, 150, and 200 μg L^{-1}) with n=6. The normality of residuals of analytical curve was evaluated by Anderson-Darling test in order to check whether data are normally distributed. The evaluation of data independence was performed using Durbin-Watson test, where it was possible to check if the autocorrelation affects the variance of obtained data. Ten levels from linear range were used to these applications. The exclusion of less than 22.2 % of the levels that have not met the acceptance criteria could be used to get a better calibration model (Horwitz 1995). The matrix effect evaluation was performed by comparison between the slopes of analytical solution prepared in solvent and in matrix extracts (Ferrer et al. 2011). The accuracy was evaluated through recovery experiments at 0.1, 1, 2, 5, 10, 20, 50, 100, 150, and 200 μg kg^{-1} levels, while the surrogate (atrazine-d5) was spiked always at 20 μg kg^{-1}. Six replicates were performed for each level, and the accuracy was expressed as the percentage of recovery. The precision (repeatability) was evaluated by the RSD of the recovery studies. The intermediate precision was obtained performing the analytical procedure in different days with blank samples spiked at the intermediate levels of 10, 20, and 50 μg kg^{-1}.

Method LOD and LOQ values were determined experimentally, considering the signal/noise ratio (S/N) of three and ten times, respectively. The decision limit (CCα) was determined considering the lowest level of the spiked analytical curve that presented S/N ratio greater than 10, with acceptable accuracy (between 70 and 120 %) and precision (≤20 %). The detection capability (CCβ) was calculated adding the value obtained for the CCα, which was multiplied by 1.64 times the standard deviation referring to the intermediate precision corresponding to the concentration of CCα.

Results and Discussion

UHPLC-MS/MS Analysis

UHPLC-MS/MS analysis allowed the multiclass determination of 92 compounds, with good selectivity and sensibility. The parameters used in this work, as mobile phase, gradient program, and other parameters, were optimized based on previous studies (Kemmerich et al. 2015; Rizzetti et al. 2016). Figure 2 shows a chromatogram with all analytes prepared in matrix extract at 20 μg L^{-1}, obtained in SRM mode.

Sample Preparation Optimization

The performed tests proved good recovery results (70–120 %) and precision (RSD<20 %) for majority compounds. The first step used in this method was the homogenization of the honey sample, followed by extraction with organic solvent, and finally the cleanup step. The citrate QuEChERS method was ineffective for extraction of antibiotics, which presented low percentages of recovery (40–60 %). The use of the McIlvaine buffer solution (pH 4) provided a better sample homogeneity. Li et al. (2008) verified the importance of this buffer as extraction solvent of antibiotics in honey, besides acting positively in the reproducibility results of their compounds. In contrast, according to the authors, the employment of water resulted in lower recovery values, once the compounds were

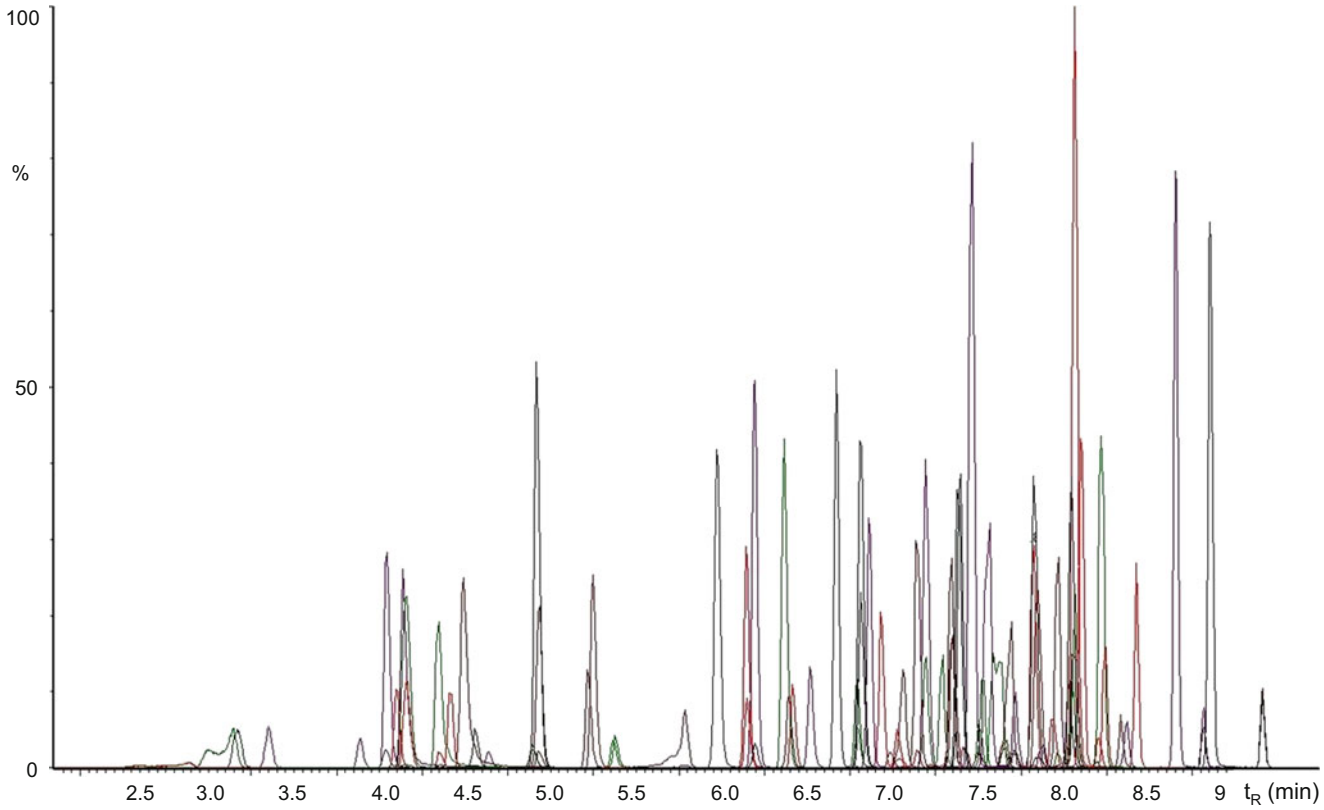

Fig. 2 SRM UHPLC-MS/MS chromatogram of a 20 μg L^{-1} solution of all analyzed compounds prepared in blank matrix extract

retained and complexed with metal residues in the sample. McIlvaine buffer is applied when there are differences of polarity and solubility between the analytes. The presence of Na_2EDTA is necessary to avoid the complexation of macrolide compounds with metals present in honey samples (Frenich et al. 2010).

Acetonitrile was used as an extraction solvent because it is a medium polar solvent which can extract a high variety of compounds with different physicochemical properties (Ho et al. 2012) and according to Prestes et al. (2009) allows the extraction of fewer amounts of lipophilic compounds from the matrix. In addition, acetonitrile is an environmentally friendly solvent and has no persistence in the environment (Anastassiades et al. 2003).

Figure 3 presents the results of the tests performed to establish the optimized conditions for the cleanup step using different sorbents. Considering only the gravimetric assays, the best results were obtained with 50 mg of florisil. Evaluating the recovery results of these preliminary tests, the combination of two sorbents provided lower recoveries than Oasis HLB, PSA, and florisil used separately. The sorbent C18 was not satisfactory, presenting adequate recovery for only 39 compounds, since 33 compounds showed recovery values below 70 %. With the activated florisil, 86 compounds showed satisfactory recoveries (70–120 %) and RSD (<20 %) results, whereas when PSA was applied, only 72 compounds achieve acceptable values. The gravimetric test had indicated that florisil was more effective in removing coextractives compared with PSA. Pinho et al. (2009) analyzed pesticide residues in honey samples and showed better efficiency of florisil for the cleanup step compared to freezing.

Method Validation

The parameters of the proposed method were validated in accordance to CD 2002/657/EC (Commission Decision 2002) for confirmation that the specific requirements are catered. The evaluation of linearity through analysis of the premises of normality and independence of residues has been established for the ten spike levels of the analytical curves. Results of normality and independence test are shown in Table S1.

The evaluation of data normality was assessed by Anderson-Darling test, at the 5 % significance level. The test rejects normality hypothesis when p values <0.05 are obtained. The following levels have not presented normality of data, i.e., showed heteroscedasticity ($p<0.05$) for some compounds: levels 0.1 and 1 μg kg^{-1}: azoxystrobin, carbofuran, dicofol, mepronil, mevinphos, and triadimefon; levels 150 and 200 μg kg^{-1}: acetamiprid, chloramphenicol, fenarimol,

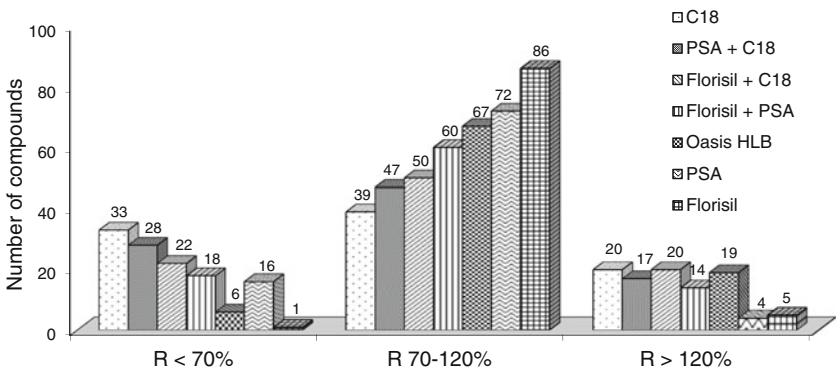

Fig. 3 Number of compounds with recovery below 70 %, between 70 and 120 %, and above 120 % using different sorbents in the cleanup step

fipronil, flusilazole, fluvalinate, phosmet, lincomycin, linuron, mepanipyrim, methidathion, oxadixyl, penconazole, pyridate, and triazophos; levels 0.1 and 200 μg kg^{-1}: azinphos ethyl, carbofuran 3-OH, chlorpyrifos ethyl, dichlorvos, dimethoate, dimoxystrobin, epoxiconazole, erythromycin ABC, etrimfos, all sulfonamides (less sulfathiazole), pyraclostrobin, pirimiphos methyl, profenofos, profoxydim, propargite, salinomycin, trichlorfon, and trifloxystrobin; level 200 μg kg^{-1}: carboxin and difenoconazole. Thus, these levels were excluded for calculation purposes, respecting the limit of 22.2 % of points subjected to exclusion. Results also demonstrated heteroscedasticity for the extreme levels of the analytical curve. The Durbin-Watson test verified which residues were independent, according to the criteria ($p>0.05$). In this way, the data obtained show normal and independent distribution, ensuring the linearity of the analytical curves obtained.

According to Pinho et al. (2010), the matrix effect becomes higher as matrix complexity increases. This effect can suppress or enhance the ionization of analytes in the mass spectrometer source and consequently affect the accuracy and precision results. According to Table 2, most compounds presented positive matrix effect, wherein 73 % showed matrix effect higher than 20 % and 8 % of the compounds presented negative matrix effect, characterized by the suppression of analytical signal. Otherwise, 19 % of the compounds presented matrix effect lower than 20 %. In order to compensate the matrix effect, analytical curves were prepared in blank matrix extract. Galarini et al. (2015) evaluated the matrix effect of honey samples in the determination of 27 antibiotics belonging to sulfonamide, nitroimidazole, and quinolone families by LC-MS/MS. Authors reported high matrix effects.

Accuracy results, in terms of repeatability, were considered efficient because majority compounds achieved recoveries from 70 to 120 %. Good results were obtained for most compounds, except for 21 compounds at the level 0.1 μg kg^{-1}, two compounds at 1 μg kg^{-1}, three compounds at 2 μg kg^{-1}, ten compounds at 5 μg kg^{-1}, six compounds at 10 μg kg^{-1}, two compounds at 20 μg kg^{-1}, 1 compound at 50 μg kg^{-1}, and one compound at 100 μg kg^{-1} presented recovery values above 120 %. The compounds metsulfuron methyl, pyridate, and salinomycin presented recovery below 70 % at 0.1 μg kg^{-1}; clothianidin and simazine at 2 μg kg^{-1}; isoxaflutole and trifloxystrobin at 5 μg kg^{-1}; iprovalicarb at 10 μg kg^{-1}; and picoxystrobin at 20 μg kg^{-1}. The method showed good precision, in terms of repeatability; however, some compounds presented RSD>20 %: chlorpyrifos methyl, clothianidin, diniconazole, furazolidone, simazine, tebuconazole, and tolclofos methyl at 0.1 μg kg^{-1}; mevinphos, picoxystrobin, propoxur, and tebuconazole at 1 μg kg^{-1}; dichlorvos, mevinphos, and profenofos at 2 μg kg^{-1}; carboxin at 5 μg kg^{-1}; dichlorvos and pyraclostrobin at 10 μg kg^{-1}; malathion at 20 μg kg^{-1}; chlorimuron ethyl, dimoxystrobin, linuron, and malathion at 50 μg kg^{-1}; diethofencarb and fenpropimorph at 100 μg kg^{-1}; fenarimol, fipronil, flusilazole, linuron, myclobutanil, pyraclostrobin, salinomycin, and triadimefon at 150 μg kg^{-1}; epoxiconazole, fenpropimorph, flusilazole, metolachlor, and triazophos at 200 μg kg^{-1}.

In relation to the inter-day assay, the compounds carbofuran 3-OH, diethofencarb, etrimfos, fenpropimorph, fluvalinate, linuron, malathion, mepronil, methacrifos, metsulfuron methyl, phosmet, salinomycin, thiamethoxam, triadimefon, triazophos, and triflumizole presented recovery values above 120 % for the 10 μg kg^{-1} level and the compounds azinphos ethyl and chlorpyrifos ethyl for the 50 μg kg^{-1} level. Besides, dimoxystrobin and flusilazole presented recovery values below 70 % for 50 and 20 μg kg^{-1} levels, respectively. The values of RSD were above 20 % for fenarimol, hexaconazole, and triazophos at 10 μg kg^{-1}; boscalid, dimoxystrobin, fenpropimorph, fipronil, methidathion, pirimiphos ethyl, terbuthylazine, and trifloxystrobin at 20 μg kg^{-1}; and boscalid, chlorpyrifos ethyl, salinomycin, tebuconazole, and terbuthylazine at 50 μg kg^{-1}. These results are similar to those obtained by Gómez-Pérez et al. (2012) in the analysis of pesticides and veterinary drugs in honey, in which some compounds showed inadequate recovery and RSD at the lowest level investigated (10 μg kg^{-1}).

The recoveries for antibiotics were performed according to Commission Decision 2002/657/EC, in the concentrations of 0.5, 1, and 1.5 times the MRL of each compound. Table 3 presents MRL data and results for recovery and RSD and for

Table 2 Recovery (R) and relative standard deviation (RSD), for repeatability and intermediate precision, method limit of quantification, maximum residue levels and matrix effect (ME) for pesticides

Compounds	Repeatability level (μg kg^{-1})[a]										Interm.precision (μg kg^{-1})[a]					
	0.1	1	2	5	10	20	50	100	150	200	10	20	50	LOQm (μg kg^{-1})	MRL[b] (μg kg^{-1})	ME (%)
	Recovery±RSD (%)										Recovery±RSD (%)					
Acephate	121±6	108±9	96±1	96±5	97±6	91±5	89±9	88±3	97±6	101±2	118±16	72±5	71±7	5.0	20	−11.1
Acetamiprid	115±5	98±8	95±13	113±13	115±5	124±7	116±2	104±1	93±1	91±9	119±4	114±8	109±9	0.1	50	9.4
Atrazine	109±10	107±15	101±18	115±14	96±3	98±6	104±3	104±3	98±5	92±2	114±2	92±8	97±11	0.1		58.5
Azinphos ethyl	115±14	115±14	109±10	135±13	115±6	117±6	118±8	122±18	103±10	74±14	117±8	115±15	156±2	1.0		27.5
Azinphos methyl	101±11	102±11	81±14	85±12	96±14	97±8	105±20	105±3	101±9	91±8	117±13	91±16	83±5	1.0		65.2
Azoxystrobin	118±10	88±5	87±12	105±1	93±4	93±14	97±19	103±11	94±6	97±6	100±15	87±6	93±19	0.1	10	32.5
Boscalid	110±10	88±7	115±5	105±9	97±16	92±11	97±17	95±19	94±10	99±5	119±2	82±30	86±25	0.1	500	19.6
Bromophos methyl	105±5	81±10	105±10	106±8	99±5	108±9	96±7	97±6	99±4	86±4	115±14	115±11	120±9	0.1		221.2
Carbaryl	114±14	93±17	100±5	117±20	99±7	106±5	105±3	100±3	101±14	93±8	118±14	94±12	101±13	1.0		98.1
Carbendazim	77±14	112±7	105±10	109±19	109±5	101±9	98±3	98±1	99±8	100±8	110±11	83±9	87±5	0.1	1000	16.0
Carbofuran	124±18	90±12	90±6	105±3	100±2	96±6	98±5	98±3	103±11	99±10	118±9	87±9	87±8	1.0	10	87.4
Carbofuran 3-OH	112±10	145±13	90±18	107±13	115±4	102±3	104±7	99±4	100±12	92±10	125±9	96±13	106±10	2.0		162.0
Carboxin	75±10	91±17	118±12	129±22	113±11	118±3	114±6	105±3	97±6	88±5	112±5	115±10	111±7	0.1		43.2
Chlorpyrifos ethyl	102±7	98±18	86±11	98±11	103±6	104±6	104±5	107±4	101±13	96±11	113±6	104±10	75±2	0.1		9.7
Chlorpyrifos methyl	128±26	98±7	115±17	106±4	107±17	93±11	99±13	105±11	119±8	106±7	117±3	93±6	133±21	1.0		22.6
Chlotianidin	122±36	103±14	67±11	86±13	118±17	110±18	97±6	107±2	101±4	98±6	117±9	97±10	103±18	1.0	10	12.6
Cyproconazole	125±14	70±11	123±7	106±17	84±20	100±10	99±19	105±7	95±20	103±10	115±3	83±18	90±19	1.0	50	15.0
Dichlorvos	112±10	120±18	120±24	122±13	130±21	113±7	113±8	103±4	99±5	91±4	117±9	113±1	110±12	0.1		8.7
Diethofencarb	128±20	95±8	73±19	91±1	82±13	94±14	90±17	105±21	103±19	100±16	127±14	76±18	73±6	1.0		74.8
Difenoconazole	103±10	97±6	95±6	128±5	139±2	119±10	117±4	110±4	99±5	86±6	131±5	120±8	118±2	0.1	50	10.7
Dimethoate	112±12	110±19	125±18	123±8	113±20	115±4	108±3	99±2	98±8	91±8	117±6	119±4	117±12	0.1		82.3
Dimoxystrobin	107±18	91±10	75±13	77±4	80±6	75±18	79±21	80±19	93±9	118±9	92±7	84±25	64±11	0.1	50	413.2
Diniconazole	117±26	101±18	76±19	89±11	92±8	103±7	98±8	104±3	103±5	96±5	119±17	98±9	90±5	1.0	10	32.6
Diuron	118±16	95±8	70±16	84±5	87±6	89±4	100±6	103±3	103±8	93±8	107±4	88±19	100±11	0.1	50	70.1
Epoxiconazole	120±8	112±14	118±15	112±6	128±13	113±13	112±13	118±1	104±4	85±22	113±6	99±15	113±12	1.0	50	30.5
Etofenprox	76±14	82±15	113±16	80±11	79±12	81±9	71±10	85±8	104±13	86±5	114±6	101±20	98±10	0.1	50	442.9
Etrimfos	126±17	95±13	105±16	122±11	120±16	117±7	107±14	114±3	102±4	87±4	128±17	120±2	103±16	1.0		24.4
Fenarimol	125±14	75±12	110±12	73±16	126±18	100±15	95±13	116±5	118±23	89±7	117±25	77±21	75±19	1.0		
Fenpropathrin	123±19	82±16	80±21	106±18	107±5	106±6	103±4	108±2	101±15	94±12	109±7	69±3	96±6	1.0		8.9
Fenpropimorf	115±14	83±20	77±15	73±13	78±5	79±17	87±18	108±21	96±8	110±27	123±4	117±24	107±18	0.1		46.4
Fipronil	91±10	75±19	90±8	115±9	125±18	118±5	100±20	116±16	98±22	77±15	119±8	110±34	107±17	2.0	10	76.8
Flusilazole	122±17	77±3	77±18	78±15	76±17	80±15	70±18	86±18	87±26	102±21	113±5	66±12	80±20	1.0	50	19.6
Flutolanil	77±15	85±12	83±20	84±10	75±13	77±11	79±19	90±18	95±12	109±8	110±20	72±19	51±11	0.1	20	67.0

Table 2 (continued)

Compounds	Repeatability level (μg kg^{-1})[a]										Interm.precision (μg kg^{-1})[a]					
	0.1	1	2	5	10	20	50	100	150	200	10	20	50	LOQm (μg kg^{-1})	MRL[b] (μg kg^{-1})	ME (%)
	Recovery±RSD (%)										Recovery±RSD (%)					
Fluvalinate	103±8	74±19	108±8	105±5	98±11	107±9	99±7	101±3	99±2	95±1	122±16	106±4	104±10	0.1		351.3
Hexaconazole	121±15	77±11	83±10	89±17	83±20	80±17	70±10	111±1	95±3	103±11	101±22	75±17	75±11	2.0		48.3
Imazalil	108±10	105±5	83±18	109±1	108±14	116±6	113±9	92±20	109±6	91±7	117±9	120±10	117±11	0.1	50	49.2
Imidacloprid	117±14	110±17	130±8	104±16	116±9	106±5	105±2	109±3	101±7	96±6	106±5	105±16	94±15	1.0	50	11.3
Iprovalicarb	77±19	75±5	71±18	76±16	68±10	81±5	86±8	99±3	99±12	101±6	112±8	71±15	81±19	0.1	50	92.3
Isoxaflutole	114±2	120±5	75±15	67±14	84±15	80±14	86±7	100±7	107±15	105±8	112±1	70±3	71±14	1.0		71.7
Linuron	119±12	75±9	88±14	101±3	95±13	101±13	81±23	93±1	101±11	89±12	121±16	81±18	83±19	0.1		−94.4
Malathion	90±14	110±6	80±17	78±11	99±17	103±23	99±29	105±14	110±25	99±12	125±3	92±3	109±13	1.0	20	121.6
Mepanipyrim	76±7	70±13	78±4	88±12	80±15	102±16	90±20	107±18	97±18	93±18	125±5	77±8	113±19	0.1	50	80.4
Mepronil	123±20	110±9	88±9	94±14	95±15	93±15	96±18	101±16	110±19	103±16	122±5	76±16	87±19	1.0		37.3
Metalaxyl	116±5	85±12	78±4	97±3	83±1	93±4	99±8	106±12	91±18	96±6	118±13	85±16	98±13	0.1	50	74.7
Methacrifos	87±19	90±20	73±11	85±12	76±7	75±11	87±5	97±5	103±4	103±7	122±13	73±11	77±12	0.1		54.1
Methidathion	118±11	78±19	98±11	127±4	119±14	106±17	103±7	102±3	102±9	91±9	119±13	113±37	99±23	1.0	20	65.9
Metolachlor	96±20	70±18	100±5	90±2	90±16	91±13	87±12	97±10	99±12	93±21	118±6	98±17	89±11	0.1		19.5
Metsulfuron methyl	67±18	100±16	110±5	116±2	103±10	102±4	106±5	96±11	105±12	94±3	121±12	96±7	102±13	1.0		59.2
Mevinphos	118±7	135±22	111±32	118±7	109±4	104±10	104±3	100±2	100±5	96±10	107±4	93±14	94±11	0.1		56.5
Monolinuron	126±15	116±20	102±9	114±14	96±12	89±3	95±4	102±2	98±14	97±11	115±17	91±3	86±6	1.0		56.3
Omethoate	116±8	102±12	79±18	96±4	92±10	89±5	95±9	99±5	103±11	101±9	79±5	89±12	81±4	2.0		80.3
Oxadixyl	121±18	95±14	118±7	108±10	118±7	105±8	112±5	90±3	103±9	93±3	116±17	104±7	105±13	1.0		−23.2
Paraoxon ethyl	96±18	95±6	74±13	84±10	78±3	83±5	85±7	101±6	97±5	98±5	118±6	71±8	79±10	1.0		73.6
Penconazole	72±15	103±6	114±12	124±18	121±11	115±11	122±12	94±10	106±9	82±1	118±11	128±6	114±16	0.1		85.6
Phosmet	117±10	100±7	115±10	118±4	114±15	117±7	109±9	99±14	85±12	87±12	123±16	106±17	106±17	0.1		36.4
Picoxystrobin	118±14	89±22	91±11	89±16	76±5	68±12	82±19	90±17	96±6	105±9	79±3	73±17	84±20	0.1		52.9
Pirimicarb	98±14	105±7	115±5	128±15	115±2	111±8	106±5	117±16	110±28	93±8	120±12	105±3	72±6	1.0	50	123.0
Pirimiphos ethyl	71±16	85±5	84±19	81±15	83±3	93±7	89±5	114±10	105±7	95±13	109±13	102±21	103±1	0.1		25.4
Pirimiphos methyl	105±9	76±15	73±4	93±8	89±5	101±17	98±8	86±12	102±12	91±9	118±5	98±15	85±15	0.1		26.0
Profenofos	122±13	70±7	85±26	89±9	95±8	98±5	91±5	91±11	106±8	98±12	113±7	99±11	93±6	1.0	10	19.2
Profoxydim	128±15	75±16	95±12	76±13	97±14	92±18	89±16	99±3	101±10	88±14	113±27	109±16	80±13	1.0		27.2
Propargite	111±14	80±16	100±18	78±11	85±4	96±9	93±4	108±8	104±17	94±8	114±12	117±12	86±9	1.0		63.1
Propoxur	75±11	105±27	110±15	106±18	113±8	101±7	99±4	112±4	104±9	96±14	120±8	96±13	120±10	0.1		121.1
Pyraclostrobin	105±17	85±5	88±7	73±15	89±21	105±18	99±16	102±3	105±12	90±13	116±7	87±17	93±8	1.0	50	13.7
Pyrazophos	125±19	103±15	89±8	73±15	74±12	93±8	87±10	100±11	107±16	99±20	118±5	82±16	100±19	1.0		43.9
Pyridate	62±16	84±8	91±3	86±5	83±10	90±18	85±13	102±8	102±13	83±9	125±4	115±16	83±16	1.0		

Table 2 (continued)

Compounds	Repeatability level (μg kg^{-1})[a]										Interm.precision (μg kg^{-1})[a]					
	0.1	1	2	5	10	20	50	100	150	200	10	20	50	LOQm (μg kg^{-1})	MRL[b] (μg kg^{-1})	ME (%)
	Recovery±RSD (%)										Recovery±RSD (%)					
Pyrimethanil	94±13	75±7	70±8	81±9	89±15	91±16	83±10	95±3	102±16	97±8	119±7	80±19	98±20	0.1	50	76.4
Salinomycin	124±12	65±14	78±4	90±7	84±2	125±17	87±2	108±15	96±22	78±11	129±18	119±6	119±23	2.0		−12.0
Simazine	80±29	74±8	69±14	95±20	90±8	82±10	90±4	94±2	99±11	104±10	119±14	78±7	78±13	1.0		101.8
Tebuconazole	130±22	70±26	75±17	92±5	80±17	94±15	94±4	91±12	96±3	101±7	118±11	76±17	81±26	1.0	50	46.0
Terbuthylazine	88±10	98±15	103±13	97±10	108±6	103±13	109±13	104±12	104±10	89±7	119±16	102±26	99±24	1.0		36.7
Thiacloprid	106±19	90±13	100±5	102±9	105±9	102±4	102±9	99±1	101±8	97±9	112±10	91±6	91±11	0.1	200	62.2
Thiamethoxam	117±16	92±12	86±4	124±11	119±14	100±11	101±9	103±3	97±6	95±5	126±9	84±15	92±8	2.0	10	26.1
Tolclofos methyl	76±21	75±19	103±8	91±20	109±2	100±6	107±11	98±16	99±2	104±11	118±10	90±11	89±10	1.0	50	9.0
Triadimefon	75±20	75±7	80±17	71±11	79±20	98±11	97±20	100±9	82±23	109±13	122±13	86±20	83±32	1.0	100	34.3
Triazophos	130±19	110±7	75±19	97±20	80±14	104±19	105±16	103±8	113±18	96±27	125±27	85±10	117±18	1.0	10	67.0
Trichlorfon	74±17	120±20	117±10	120±8	117±11	108±15	106±9	99±5	97±16	91±8	110±7	101±18	102±14	0.1	10	158.9
Trifloxystrobin	125±14	80±8	70±19	69±8	99±12	117±10	108±3	113±4	100±12	94±12	115±7	106±23	104±9	1.0	40	8.5
Triflumizole	107±12	70±14	73±14	79±12	83±4	90±11	83±5	102±5	104±17	98±14	126±10	112±19	104±16	1.0		60.6

[a] $n=6$

[b] MRL established by EU

Table 3 Maximum residue levels (MRL), recovery (R), relative standard deviation (RSD), for repeatability and intermediate precision, CCα, CCβ, and matrix effect (ME) for antibiotics

Spike level (μg kg^{-1})[a]										
Compounds	MRL[b] (μg kg^{-1})	Repeatability			Intermediate precision			CCα (μg kg^{-1})	CCβ (μg kg^{-1})	Matrix effect (%)
		0.5 MRL R±RSD (%)	1 MRL R±RSD (%)	1.5 MRL R±RSD (%)	0.5 MRL R±RSD (%)	1 MRL R±RSD (%)	1.5 MRL R±RSD (%)			
Chloramphenicol	0.3	108±10	116±16	114±13	85±8	92±10	107±7	0.10	0.12	202.2
Emamectin benzoate	50	121±19	80±17	85±15	102±16	99±6	84±12	0.10	0.21	−73.1
Erythromycin ABC	10	73±19	87±16	93±3	81±11	73±6	79±19	1.00	1.44	−92.5
Furazolidone	1	113±32	90±21	88±12	79±16	85±17	71±4	1.00	1.72	34.3
Lincomycin	–	120±10	74±18	73±14	64±15	76±13	71±9	1.00	1.17	56.6
Sulfachloropyridazine	–	65±4	85±17	138±18	67±7	78±14	80±4	2.00	2.81	68.7
Sulfadiazine	–	76±14	73±13	103±3	86±12	81±7	75±4	0.10	0.13	8.2
Sulfadimethoxine	50	123±9	105±25	75±16	90±10	84±4	92±3	0.10	0.12	83.8
Sulfamethazine	50	65±14	95±5	98±11	85±10	87±16	96±5	0.10	0.12	29.2
Sulfamethoxazole	–	110±14	90±17	88±3	73±5	78±2	85±7	0.10	0.13	81.0
Sulfaquinoxaline	–	95±17	85±23	83±13	85±7	106±9	89±12	0.10	0.27	81.0
Sulfathiazole	50	127±10	82±6	90±6	76±9	94±16	75±4	0.10	0.12	0.4
Tylosin	10	92±12	76±32	76±16	121±1	89±2	69±2	2.00	2.66	−76.6

[a] n=6

[b] MRL established by EU

repeatability and intermediate assay, CCα, CCβ, and matrix effect for the 13 antibiotics evaluated. The recovery for the antibiotics presented results above 120 % for emamectin benzoate, sulfadimethoxine and sulfathiazole (0.5 MRL), and sulfachloropyridazine (1.5 MRL) in the intra-day assay and for tylosin (0.5 MRL) in the inter-day assay. On the other hand, the compounds sulfachloropyridazine and sulfamethazine (0.5 MRL) in the intra-day assay and lincomycin and sulfachloropyridazine (0.5 MRL) and tylosin in 1.5 MRL concentration in the inter-day assay showed recoveries below 70 %. For the majority of antibiotics, the RSD results showed good method precision in the intra-day assay, except for furazolidone in 0.5 MRL and 1 MRL and sulfaquinoxaline and tylosin in 1 MRL level that presented RSD>20 %. The RSD values for inter-day assay were <20 % for all antibiotics.

The study related by Galarini et al. (2015) showed the determination of 27 antibiotic residues in honey samples following detection by LC-MS/MS. Authors reported results of recovery below 70 % for some antibiotics, such as sulfadiazine and sulfaquinoxaline, with RSD above 20 % for some compounds. This indicates that McIlvaine buffer, containing Na_2EDTA, followed by addition of acetonitrile provides good homogenization, protection for analytes that undergo complexation and extraction.

Intermediate precision results were evaluated using the t test, in four replicates of intermediate levels 10, 20, and 50 μg kg^{-1}. The t test enables to check if the data can be classified as significantly similar or different. The critical value to accept null hypothesis (that the results are significantly different) is of 2.353 for 5 % significance. Results showed good intermediate precision, considering that only hexaconazole, linuron, salinomycin, tebuconazole, and thiamethoxam at 10 μg kg^{-1} and triflumizole at 20 μg kg^{-1} were significantly different. Among these compounds, hexaconazole, linuron, salinomycin, and triflumizole are not monitored in honey and, therefore, have no MRLs established. Thiamethoxam has not presented intermediate precision in this level, affecting the analysis, since their MRL (EU) is 10 μg kg^{-1}. Tebuconazole has not shown proper intermediate precision at 10 μg kg^{-1}, but its MRL is 50 μg kg^{-1} (EU); so, the analysis was not compromised. Negative values of t test have not influenced in the significance of the difference between groups. They indicate that the intermediate precision values are greater than the repeatability.

It was observed that values obtained for CCα ranged from 0.1 to 2 μg kg^{-1}, corresponding to method LOQ and CCβ values ranged from 0.12 to 2.81 μg kg^{-1}. So, the method offers low levels of detection capability, identification, and quantification. Also, these values help to decide if samples do not comply in relation to the permitted limits. The limits achieved are lower, i.e., the method is suitable for determination of all compounds monitored in honey in Europe (EC/2377/90) and in Brazil (MAPA 2014). For those compounds that no MRL was established, the limits should be as low as possible. Kasiotis et al. (2014) obtained limits in the range of

Table 4 Results for 25 positive samples obtained from application of the validated method to real samples

Analytes	Positive samples (μg kg^{-1})																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Acephate	–	–	–	–	0.12	0.52	0.23	0.49	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Azinphos ethyl	–	–	–	–	–	–	–	–	–	–	–	0.33	–	–	0.32	–	0.7	–	–	–	–	–	–	–	–
Azoxystrobin	–	–	–	–	–	–	–	–	–	–	–	–	–	<LOQ	–	–	–	–	–	–	–	–	–	–	–
Boscalid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.86	–	–	–
Carbofuran	0.12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Chlorpyrifos ethyl	1.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.37	–	–	–
Dichlorvos	–	–	–	–	–	–	–	–	–	–	–	2.1	–	4.1	–	–	–	–	–	–	–	–	–	–	–
Difenoconazole	–	–	–	–	–	–	–	–	1.0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Dimethoate	–	–	–	–	–	–	–	–	10	<LOQ	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Dimoxystrobin	0.28	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Diuron	–	–	–	–	–	–	–	–	–	–	–	<LOQ	<LOQ	–	–	–	–	–	–	–	–	–	–	–	–
Emamectin benzoate	–	–	–	–	–	–	–	–	–	–	–	<LOQ	–	–	–	–	–	–	–	–	–	–	–	–	–
Etrimfos	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<LOQ	–	–	–
Erythromycin ABC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5.9	–	–	–	–	–	–	–
Fenthion	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7.3	–	–	–
Flusilazole	0.12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Flutolanil	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.17	–	–	–
Imazalil	–	0.16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Imidacloprid	<LOQ	–	<LOQ	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Linuron	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.0	–	–	–
Paraoxon ethyl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.8	–	–	–
Metalaxyl	–	–	–	–	–	–	–	–	–	–	–	–	–	<LOQ	–	–	–	–	–	–	–	1.8	–	–	–
Oxadixyl	–	–	–	–	–	–	–	–	–	–	–	–	–	<LOQ	–	–	–	–	–	–	–	–	–	–	–
Picoxystrobin	–	–	–	–	–	–	–	–	–	–	0.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pyrazophos	–	–	–	–	–	–	–	–	1.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pirimiphos ethyl	–	–	–	–	–	–	–	–	0.6	0.6	–	–	–	–	–	–	–	–	–	–	–	0.13	–	–	–
Pirimiphos methyl	0.13	–	–	–	–	–	–	–	0.8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Profenofos	0.18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Propoxur	–	–	–	–	–	–	–	–	–	–	–	<LOQ	–	–	–	–	–	–	–	–	–	–	1.2	2.1	–
Salinomycin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.5	–	–	–
Tebuconazole	–	–	–	–	–	–	–	–	–	–	0.15	–	–	–	–	–	–	–	3.8	–	–	–	–	–	–
Tolclofos methyl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<LOQ	–	–	–	–	–
Terbuthylazine	–	–	–	–	–	–	–	–	–	–	–	–	–	<LOQ	–	–	–	–	–	–	–	–	–	–	–
Trichlorfon	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<LOQ
Trifloxystrobin	–	–	–	<LOQ	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Triflumizole	–	–	–	<LOQ	–	–	–	–	0.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

0.1 to 77 μg kg^{-1} for all compounds available, using modified QuEChERS and LC-MS/MS. Dubreil- Chéneau et al. (2014) showed limits (CCα and CCβ) for sulfonamides in honey, in the range of 1.8 to 17.4 μg kg^{-1}. Juan-Borrás et al. (2015) presented limits for antibiotics between 0.7 and 4.5 μg kg^{-1}. The limits achieved for compounds, which MRL has not been established, are sufficiently low to determine these residues. According to the guide SANCO/2006/3228, the limits for compounds, which the MRL does not exist, vary in the range of 0.1 to 50 μg kg^{-1} or L^{-1}, coinciding with the values obtained in the method.

Application to Real Samples

To evaluate the proposed method, 43 honey samples were analyzed, among them are monofloral honey and multifloral honey derived from fruit plants, mainly orange trees, apple trees, peach trees, grape vines, eucalyptus, sunflower blossom, and canola. These samples were acquired directly from beekeepers of different regions of the state of Rio Grande do Sul, Brazil, near to soybeans, corn, and wheat crops.

Of the total analyzed samples, 44 % presented residues of one or more analytes, from 0.12 to 10 μg kg^{-1} in each sample and six samples presented residues below the LOQ (Table 4). The maximum residue limit was not exceeded in any sample. Residues of insecticides and acaricides (acephate, azinphos ethyl, carbofuran, chlorpyrifos ethyl, dichlorvos, dimethoate, paraoxon ethyl, pyrazophos ethyl, pirimiphos ethyl, pirimiphos methyl, profenofos, salinomycin, and tebuconazole), fungicides (boscalid, difenoconazole, dimoxystrobin, flusilazole, imazalil, metalaxyl, picoxystrobin, and propoxur), antimicrobials (erythromycin ABC), and herbicide (linuron) were found at concentrations below the MRLs, according to the limits established by EU and PNCRC (Brazil) for honey. Azoxystrobin, diuron, emamectin benzoate, etrimfos, imidacloprid, oxadixyl, propoxur, tolclofos methyl, terbuthylazine, trichlorfon, and trifloxystrobin showed residues below the LOQ for six samples evaluated.

The residues found in honey samples are due to the proximity of the beehives with soybean, corn, or wheat crops, considering that bee realizes the pollination process, reaching large distances to collect nectar, water, and pollen of flowers (Rissato et al. 2006). For example, acephate and tebuconazole used in citrus, soybeans, and wheat corn were found in four and two samples, respectively (AGROFIT, 2015). The insecticides pirimiphos ethyl, azinphos ethyl, and chlorpyrifos ethyl were found in 23, 16, and 10 % of the samples, respectively. The presence of fungicides occurred in lower extension for the evaluated samples. Therefore, the contamination of the bees occurred indirectly, except for the sample 18, which presented direct contamination by antibiotic erythromycin ABC used in the treatment of bacterial diseases. However, the concentration was below the limit established by PNCRC (Brazil).

Some works reported the presence of pesticide and antibiotic residues in honey samples. Barganska et al. (2013) analyzed 45 samples obtained directly from beekeepers, wherein 29 % presented pesticide residues. Among the analytes, dimoxystrobin, indoxacarb, and pirimicarb were present in 53 % of samples, being below the LOQ of their method, as well as the analyte azinphos ethyl was present in 11 % of the samples. Galarini et al. (2015) analyzed 74 honey samples from different botanical origins and 12 % presented sulfonamide residues below the MRLs. López et al. (2014) analyzed 61 honey samples from four regions of Colombia, and only five samples presented residues of one or more analytes, among them are chlorpyrifos ethyl, profenofos, and fenitrothion. Besides, 28 samples presented residues on the same level or below the MRL established by EU and three samples presented residues above this MRL.

Conclusions

The developed method was effective for determination of 79 pesticides and 13 antibiotics in honey samples. The main feature is the simplicity in the execution of sample preparation, combined with low solvent consumption and cost. Detection of the compounds using UHPLC-MS/MS equipment provided good detectability, selectivity, and accuracy, so it was considered apt for routine laboratory analysis.

The accuracy, evaluated through ten fortification levels, presented recoveries in the range of 62 to 138 %; however, for most compounds, the recoveries ranged from 70 to 120 %. The precision, in terms of repeatability and intermediate precision, was considered suitable after application of the *t* test. Values of LOD and LOQ ranged from 0.03 to 0.6 μg kg^{-1} and 0.1 to 2 μg kg^{-1}, respectively, except for acephate with LOD of 1.5 and LOQ of 5.0 μg kg^{-1}. Values of CCα and CCβ were from 0.1 to 2 and 0.12 to 2.81 μg kg^{-1}, respectively. The proposed method is suitable for monitoring programs since the method LOQ is below the MRL values.

The validated method was applied to 43 samples of honey, and no interference was observed. The results of the analysis demonstrated that 50 % of the samples presented residues of one or more analytes in the samples.

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Compliance with Ethical Standards

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M. G. Bandeira declares that he has no conflict of interest. Osmar D. Prestes declares that he has no conflict of interest. Renato Zanella declare that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study

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