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Analytical Methods

Multiclass method for pesticides quantification in honey by means of modified QuEChERS and UHPLC–MS/MS

Patrícia Amaral Souza Tette [a], Fabiano Aurélio da Silva Oliveira [c], Elba Nathália Corrêa Pereira [c], Gilsara Silva [c], Maria Beatriz de Abreu Glória [a], Christian Fernandes [b,*]

[a] LBqA – Laboratório de Bioquímica de Alimentos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, bloco 3, sala 2091, Pampulha, CEP 31210-901 Belo Horizonte, MG, Brazil

[b] Laboratório de Controle de Qualidade de Medicamentos e Cosméticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, bloco 2, sala 4029, Pampulha, CEP 31210-901 Belo Horizonte, MG, Brazil

[c] LANAGRO-MG, Ministério da Agricultura Pecuária e Abastecimento, MAPA, Pedro Leopoldo, MG, Brazil

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ABSTRACT

Bee products can be produced in an environment contaminated by pesticides that can be transported by honey bees to the hive and incorporated into the honey. Therefore, rapid and modern methods to determine pesticide residues in honey samples are essential to guarantee consumers' health. In this study, a simple multiresidue method for the quantification of 116 pesticides in honey is proposed. It involves the use of a modified QuEChERS procedure followed by UHPLC–MS/MS analysis. The method was validated according to the European Union SANCO/12571/2013 guidelines. Acceptable values were obtained for the following parameters: linearity, limit of detection (0.005 mg/kg) and limit of quantification (0.010 and 0.025 mg/kg), trueness (for the four tested levels the recovery assays values were between 70 and 120%), intermediate precision (RSD < 20.0%) and measurement uncertainty tests (<50.0%). The validated method was applied for determination of 100 honey samples from five states of Brazil.

1. Introduction

Honey is one of the most used products of the hive, both naturally and in several industrialized forms (Komatsu, Marchini, & Moreti, 2002). Known since ancient times, honey has always attracted the attention of man, especially because of its sweet taste (Bera & Almeida-Muradian, 2007; Rossi, Martinelli, Lacerda, Camargo, & Victória, 1999). Furthermore, several hive products have been appreciated due to their antimicrobial and antiseptic properties. However, in recent years, the pesticide monitoring in honey has become a public health issue in view of the growth of the levels of these chemicals in bee products (Li et al., 2013; Rial-Otero, Gaspar, Moura, & Capelo, 2007). Therefore, the monitoring of pesticide residues in honey is important to evaluate the potential risk of these products to consumers' health. Also, such monitoring can provide information about the use of pesticides in crop fields around the hives and in their neighborhoods. In this case, honey can be used as a bio-indicator for the evaluation of environmental impact (Rissato, Galhiane, Knoll, Andrade, & Almeida, 2006).

In this context, analytical methods for the determination of pesticides in honey must be available for routine analysis. The determination of pesticide residues in foods requires a prior step of sample preparation due to the low concentrations of the analytes in the sample, the distinct chemical properties of the analytes and the complexity of the matrices (Prestes, Friggi, Adaime, & Zanella, 2009). Although most of these procedures are carried out by conventional techniques, such methods are generally not applicable to all food matrices, do not produce clean extracts and generate low recovery. These disadvantages have led to the development of new approaches with an emphasis on the practicality of implementation, the use of significantly lower amounts of organic solvents, and the ability to detect analytes in very low concentrations. In recent years, efforts in the field of analytical chemistry focused on the miniaturization of sample preparation associated with improvement in selectivity and sensitivity (Melwanki & Fuh, 2008). However, these efforts are far from being considered ideal, due to the limitation of application, quickness, sensitivity and reliability of the results (Martínez-Vidal, Liébanas, Rodríguez, Frenich, & Moreno, 2005). In this context, QuEChERS

* Corresponding author.
E-mail address: cfernandes@farmacia.ufmg.br (C. Fernandes).

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(an acronym for quick, easy, cheap, effective, rugged, and safe), developed by Anastassiades, Mastovska, and Lehotay (2003), is an appropriate alternative. This technique, which has the advantages of being fast, easy, economical, effective, robust and secure, can be applied in any laboratory, due to the simplification of the steps (Prestes et al., 2009). This approach has become popular for sample preparation at international level (Cieslik, Sadowska-Rociek, Ruiz, & Surma-Zadora, 2011).

Besides the extraction and purification procedures, the choice of appropriate separation and detection techniques is a step of fundamental importance. Technological advances in mass spectrometry technique allow meeting the criteria of sensitivity and selectivity (Chiaradia, Collins, & Jardim, 2008). Accordingly, the performance of liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has shown great success in multiresidue pesticide analysis in complex food matrices such as honey (Barganska, Slebioda, & Namiesnik, 2013; Jovanov et al., 2013; Lopez, Pettis, Smith, & Chu, 2008; Orso et al., 2016; Tomasini et al., 2012; Wiest et al., 2011). This technique provides information regarding the characteristic ion of each analyte as well as two or more transitions of these ions, useful to quantify and confirm the analytes at concentrations consistent with maximum residue levels (MRLs) established (Martins Júnior, Bustillos, & Pires, 2006).

Several studies on multiresidue determination of pesticides in honey have been reported in the literature. Kasiotis, Anagnostopoulos, Anastasiadou, and Machera (2014) developed a method to investigate the occurrence of 115 pesticides of different chemical classes such as neonicotinoids, organophosphates, triazoles, carbamates, dicarboximides and dinitroanilines in honey from different areas of Greece using modifications of the QuEChERS technique and LC–MS/MS. The total chromatographic run time was 35 min. Similarly, the method developed by Cotton et al. (2014) evaluated the occurrence of 83 pesticides and antibiotics of different classes in honey from France using QuEChERS and LC–MS/MS in a run time of 30 min. Kujawski et al. (2014) determined pesticides in honey after 14 min run using two extraction techniques, QuEChERS and extraction on a diatomaceous earth support (SLE). However, the developed method was applied to only 30 pesticides including acaricides, insecticides, herbicides and fungicides. Rapid methods for multiresidue analysis of pesticides in honey have not been described in the literature. Gómez-Pérez, Plaza-Bolanosa, Romero-Gonzáleza, Martínez-Vidala, and Garrido-Frenicha (2012) created a method for the simultaneous analysis of more than 350 pesticides and veterinary drugs in honey using ultra-high performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry (UHPLC-Orbitrap-MS) in a run time of 14 min, but the liquid liquid extraction was time consuming, due to the 1 h agitation required for the extraction of the compounds.

Therefore, the aim of this study was to develop and validate a rapid, sensitive and selective method for determination of 116 pesticide residues from 35 different classes (acylamino acid, anilinopyrimidine, aryloxyphenoxypropionate, benzimidazole, benzofuran, carbamate, carbanilate, carboxamide, chloroacetamide, cyanoimidazole, diacylhydrazine, dicarboximide, dinitroaniline, hydroxyanilide, imidazole, morpholine, neonicotinoid, organophosphate, oxadiazine, phenylamide, phenylpyrazole, phenylurea, phosphorothiolate, pyrazole, pyrethroid, pyridazinone, pyridine, pyrimidine, strobilurin, sulphite ester, tetrazine, tetronic acid, triazine, triazole, urea and other pesticides unclassified) in honey using QuEChERS and ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). The developed method was validated according to European Union SANCO/12571/2013 guideline (SANCO, 2013). Also, measurement uncertainty was evaluated as well as method performance by means of participation in a proficiency test. Finally, the method was used to evaluate the quality of the honey produced in five states from Brazil.

2. Experimental

2.1. Material

2.1.1. Honey samples

Honey samples were purchased from consumer stores or provided by honey producers or cooperatives: 66 from the state of Minas Gerais (49 wild flower honey, 4 from eucalyptus, 1 from *Vernonia polyanthes* and 12 without flower type), 9 samples from São Paulo (1 wild flower honey and 8 without flower type), 18 samples from Santa Catarina (all wild flower honey), 2 samples from Espírito Santo (all wild flower honey) and 5 from Pará (all wild flower honey). All collected samples were produced by *Apis mellifera* honey bees except one sample from Pará, which was produced by *Melipona scutellaris*. The blank honey samples were acquired from the consumer market. The samples were stored at ambient temperature (20 °C) until analysis. Honey sample from the provider BIPEA, code 18-3619-0038, analyzed in the proficiency test, was maintained under refrigeration (5 °C) until analysis.

2.1.2. Chemicals and reagents

Acetonitrile and glacial acetic acid were supplied by Merck (Darmstadt, Germany), methanol, ethyl acetate and formic acid were obtained from Tedia (Ohio, USA), all HPLC grade. Polymerically bonded ethylenediamine-*N*-propyl phase (PSA) (Varian, Palo Alto, CA, USA), anhydrous magnesium sulfate (purity $\geqslant$ 97%-Sigma-Aldrich, Saint Louis, MO, USA), Florisil (Mallinckrodt, St. Louis, USA), and anhydrous ammonium acetate and sodium acetate (Vetec-Rio de Janeiro, RJ, Brazil) were of analytical grade. The solutions were prepared with ultra pure-water (Milli-Q Plus system; Millipore Corp., Billerica, MA, USA). All reference standards were of high purity grade (>98.0%) and were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Individual stock solutions were prepared at 1000 mg/L in acetonitrile or methanol and stored in a freezer at −18 °C. The working solutions were prepared through appropriate dilutions of the stock solutions.

2.2. Apparatus

2.2.1. Chromatography parameters

The UHPLC system (Shimadzu LC20ADXR) comprised a binary pump (Shimadzu LC20ADXR), an auto sampler (Shimadzu SIL20ACXR) and a column oven (Shimadzu CTO20AC). Chromatography was carried out using a Shim-pack XR-ODSII column (2.0 × 100 mm, 2.2 μm particle size) with a mobile phase consisting of ammonium acetate (10 mmol/L) (phase A) and methanol (phase B) both acidified with 0.1% formic acid at a flow rate of 0.5 mL/min. The gradient elution program was as follows: 0 min, 50% B; 6 min, 80% B; 10 min, 90% B; 10.5 min, 50% B; 10.5–13 min, 50% B. The total chromatographic run time was 13 min. Injection volume was 5 μL and the column temperature was set at 60 °C. The chromatographic method was previously developed by Madureira et al. (2012) and was adapted for the UHPLC system.

2.2.2. Mass spectrometry parameters

Mass spectrometry analysis was performed using a 5500 Triple Quadrupole mass spectrometer (Applied Biosystems, MDS SCIEX, Ontario, Canada). The instrument was operated using electrospray ionization (ESI) in the positive ion mode. Instrument settings, data acquisition and processing were controlled by the software Analyst (Version 1.5.1, Applied Biosystems). Source parameters were optimized as follows: ion spray voltage 4.5 kV for ESI (+), curtain gas 20 psi, collision gas 8 psi, nebulizer gas and auxiliary gas 30 psi and ion source temperature 500 °C. Retention time, precursor

Table 1
Retention time windows (RTWs) and MS/MS conditions for each compound.

Compound	RTWs (min)	Quantification transition (CE[a]; V; CXP[b]; V)	Confirmation transition (CE[a]; V; CXP[b]; V)	DP[c] (V)
3-Hydroxy carbofuran	0.76–0.80	238.1 < 163.1 (21; 4)	238.1 > 181.2 (15; 2)	82
Acetamipride	0.74–0.78	223.1 > 126.0 (29; 12)	223.1 > 73.0 (71; 8)	51
Alachlor	5.55–5.75	270.1/272.1 > 238 (15; 22)	270.1/272.1 > 162.1/240.0 (27; 14/ 15; 22)	76/71
Aldicarb	1.18–1.25	208;1 > 116.0 (11; 3)	208;1 > 88.9 (20; 3)	51
Allethrin	7.99–8.41	303;1 > 135.1 (17; 12)	303;1 > 91.1 (55; 8)	106
Ametryn	4.20–4.40	228.0 > 186.0 (25; 16)	228.0 > 116.0 (35; 10)	71
Azinphos ethyl	5.07–5.33	346.0 > 132.2 (23; 12)	346.0 > 160.2 (15; 12)	76
Azinphos methyl	3.34–3.52	318.1 > 132.1 (23; 12)	318.1 > 261.1/160.0 (9; 24/11/16)	106
Azoxystrobin	3.99–4.20	404.1 > 371.9 (21; 34)	404.1 > 343.9 (29; 34)	101
Benalaxyl	6.21–6.52	326.0 > 148.0 (31; 12)	326.0 > 294.0 (15; 28)	81
Bitertanol	6.53–6.87	338.1 > 269.1 (13; 24)	338.1 > 99.0 (21; 10)	51
Buprofezin	8.15–8.30	306.2 > 201.1 (17; 18)	306.2 > 116.0 (21; 10)	56
Cadusafos	7.17–7.30	271.1 > 159.0 (19; 18)	271.1 > 215.0 (13; 10)	76
Carbaryl	1.95–2.05	202.2 > 145.1 (15; 14)	202.2 > 127.1 (39; 12)	66
Carbendazim	0.95–1.00	192.0 > 160.1 (25; 14)	192.0 > 132.1 (41; 12)	56
Chlorbupham*	3.86–4.06	241.1 > 172.0 (17; 16)	241.1 > 154.0 (29; 14)	51
Chlorfentezine	6.82–6.97	303.0 > 137.9 (21; 12)	303.0 > 102.0 (53; 8)	21
Chlorpyrifos-methyl	6.77–7.12	321.9 > 125.0 (27; 12)	321.9 > 289.8 (23; 26)	106
Chlortiophos	8.80–8.92	361.0 > 304.8 (23; 28)	361.0 > 192.0 (39; 16)	86
Cinidon-ethyl*	7.68–8.10	410.9 > 347.9 (31; 32)	410.9 > 365.9 (25; 34)	51
Cyazofamid	5.25–5.52	324.9 > 108.0 (19; 10)	324.9 > 261.0 (13; 24)	66
Cyhalofop butyl*	7.42–7.52	375.1 > 256.0 (23; 22)	375.1 > 120 (41; 10)	61
Cyproconazole	4.74–5.00	292.1 > 70.1 (23; 8)	292.1 > 125.0 (37; 12)	81
Cyprodinil	5.98–6.28	226.1 > 92.9 (45; 34)	226.1 > 76.9 (63; 34)	71
Desmedipham*	3.35–3.60	318.1 > 182.0 (19; 16)	318.1 > 136.0 (37; 12)	46
Diazinon	6.32–6.65	305.1 > 97.0 (49; 10)	305.1 > 169.1 (31; 16)	71
Difenoconazole	6.63–6.97	406.1 > 250.9 (35; 24)	406.1 > 337.2 (23; 24)	96
Dimethomorph	4.52–4.92	388.1 > 300.9 (29; 26)	388.1 > 165.1 (43; 14)	66
Diniconazole	6.86–7.00	326.1 > 70.0 (59; 12)	326.1 > 70.1 (61; 8)	76
Disulfoton sulfone	2.57–2.71	307.0 > 153.0 (17; 14)	307.0 > 171.0 (17; 14)	91
Diuron	3.00–3.20	233.1 > 72.0 (23; 8)	233.1 > 159.9 (35; 14)	81
Ethion	7.93–8.34	385.0 > 199.1 (15; 18)	385.0 > 171.0 (23; 18)	91
Ethiprole	4.36–4.55	397.0 > 350.9 (29; 30)	397.0 > 254.9 (47; 22)	156
Ethofumesate*	3.93–4.14	304.1 > 121.1 (29; 12)	304.1 > 161.2 (31; 12)	71
Ethoprophos	5.29–5.57	243.1 > 131.0 (27; 12)	243.1 > 96.6 (41; 10)	91
Etrinphos	5.98–6.29	293.1 > 125.0 (33; 12)	293.1 > 265;1 (21; 12)	66
Fenamiphos	5.58–5.87	304.1 > 217.1 (29; 20)	304.1 > 202;0 (45; 20)	11
Fenamiphos sulfone	1.82–1.92	336.0 > 188.0 (39; 16)	336.0 > 266;0 (27; 14)	131
Fenamiphos sulfoxide	1.66–1.75	320.1 > 232.9 (33; 20)	320.1 > 171.1 (31; 16)	131
Fenarimol	5.07–5.34	330.9 > 268.0 (31; 24)	330.9 > 139.0 (47; 12)	101
Fenazaquin	9.60–9.75	307.2 > 57.0 (37; 10)	307.2 > 91.0 (87; 14)	66
Fenhexamid	5.13–5.40	302.1 > 97.2 (31; 10)	302.1 > 55.1 (55; 8)	116
Fenpyroximate	9.15–9.27	422.1 > 366.1 (25; 34)	422.1 > 135.0 (41; 12)	81
Fenpropimorph	10.47–11.00	304.3 > 147.1 (37; 14)	304.3 > 117.1 (73; 10)	66
Fluazifop p-butyl	7.75–8.15	384.1 > 282.0 (29; 26)	384.1 > 328.0 (23; 30)	116
Flumethrin*	10.68–11.2	527.0 > 267.0 (21; 24)	527.0 > 239.0 (31; 22)	46
Fluquinconazole	4.92–5.17	376.0 > 307.0 (33; 28)	376.0 > 349.0 (33; 28)	11
Flusilazole	5.88–6.02	316.1 > 247.0 (25; 22)	316.1 > 165.1 (37; 14)	86
Flutriafol	2.70–2.83	302.1 > 122.9 (35; 12)	302.1 > 109.0 (43; 12)	85
Fosthiazate	2.55–2.80	284.1 > 104.0 (27; 10)	284.1 > 227.9 (11; 20)	91
Furathiocarb	7.64–8.04	383.2 > 195.2 (17; 3)	383.2 > 252.2 (24; 3)	72
Hexaconazole	6.29–6.61	314.2 > 70.0 (53; 12)	314.2 > 159.2 (37; 12)	86
Hexythiazox	8.18–8.60	353.0 > 228.0 (21; 20)	353.0 > 168.1 (35; 16)	61
Imazalil	5.92–6.23	297.0 > 159.0 (29; 14)	297.0 > 200.9 (23; 14)	81
Indoxacarb	7.15–7.52	528.0 > 203.1 (59; 18)	528.0 > 150.1 (31; 14)	136
Iprovalicarb	5.14–5.41	321.2 > 119.0 (23; 3)	321.2 > 203.2 (12; 2)	61
Isoproturon	2.86–3.01	207.3 > 72.1 (23; 8)	207.3 > 165.1 (19;14)	71
Linuron	3.71–3.90	249.1 > 159.9 (25; 4)	249.1 > 182.0 (21; 4)	76
Malathion	4.48–4.72	330.9 > 127.1 (17; 12)	330.9 > 285.1 (11; 26)	111
Metalaxyl	3.05–3.21	280.2 > 220.1 (19; 20)	280.2 > 192.2 (25; 18)	66
Metazachlor	2.89–3.04	278.1 > 134.1 (29; 12)	278.1 > 210.1 (15; 18)	51
Metconazole	6.39–6.72	320.1 > 70.1 (59; 6)	320.1 > 125.1 (57; 12)	96
Methidathion	3.15–3.32	303.0 > 145.0 (13; 14)	303.0 > 85.1 (29; 8)	86
Methiocarb	3.90–4.10	226.1 > 169.1 (13; 14)	226.1 > 121.1 (25; 10)	76
Methiocarb sulfoxide	0.68–0.72	242.1 > 185.1 (19; 16)	242.1 > 122.1 (39; 12)	81
Methoxifenozide	4.90–5.04	369.1 > 149.0 (23; 14)	369.1 > 313.1 (11; 28)	71
Mevinphos	0.83–0.89	225.1 > 127.1 (21; 12)	225.1 > 193.0 (11; 16)	66
Monocrotophos	0.54–0.57	224.1 > 127.0 (23; 12)	224.1 > 98.0 (17; 12)	71
Monolinuron	2.16–2.28	215.1 > 125.9 (27; 12)	215.1 > 148.0 (19; 12)	91
Myclobutanil	4.64–4.88	289.1 > 70.1 (33; 10)	289.1 > 125.1 (39; 10)	91
Nuarimol	3.90–4.20	314.9 > 252.0 (31; 22)	314.9 > 81.1 (51; 8)	81
Omethoate	0.44–0.47	214.1 > 183.0 (15; 16)	214.1 > 125.0 (29; 12)	56
Oxamyl*	0.50–0.53	237.1 > 72.1 (25; 8)	237.1 > 90.0 (11; 10)	51
Paclobutrazol	4.48–4.72	294.0 > 70.1 (55; 6)	294.0 > 125;0 (55; 12)	81/51

Table 1 (*continued*)

Compound	RTWs (min)	Quantification transition (CE[a]; V; CXP[b]; V)	Confirmation transition (CE[a]; V; CXP[b]; V)	DP[c] (V)
Paraoxon-ethyl	2.75–3.00	276.0 > 220.0 (21; 20)	276.0 > 174.0 (33; 16)	81
Penconazole	5.90–6.21	284.2 > 70.1 (21; 8)	284;2 > 159.0 (41; 14)	46
Pencycuron	6.72–7;07	329.0 > 125.0 (31; 12)	329;0 > 218.0 (23; 20)	91
Pendimethalin	8.15–8.57	282.2 > 212.1 (15; 20)	282.2 > 91.0 (33; 8)	36
Phenthoate	5.80–6.10	321.0 > 79.1 (51; 16)	321.0 > 163.1 (17; 16)	96
Phorate sulfoxide	2.46–2.60	276.9 > 199.0 (13; 18)	276.9 > 142.9 (27; 12)	111
Phosphamidon	1.25–1.55	300.0 > 127.0 (27; 12)	300.0 > 226.9 (19; 20)	91
Phosmet	3.42–3.59	318.0 > 133.0 (51; 12)	318.0 > 160.0 (19; 12)	96
Picolinafen	7.71–8.10	377.2 > 238.3 (35; 14)	377.2 > 145.0 (69; 14)	91
Pirazophos	6.51–6.85	374.1 > 222.1 (29; 20)	374.1 > 194.1 (43; 20)	86/91
Pirimiphos-ethyl	7.85–8.26	334.2 > 198.0 (32; 18)	334.2 > 182.1 (31; 18)	61
Pirimiphos-methyl	6.63–6.97	306.1 > 164.1 (29; 14)	306.1 > 108.1 (39; 10)	51
Profenofos	7.42–7.81	372.9 > 302.9 (25; 28)	372.9 > 97.0 (35; 28)	126
Propaquizafop	8.07–8.20	444.1/446.2 > 370.9 (21; 34)	444.1/446.2 > 100.0 (23; 10)	111/86
Propargite*	8.56–9.00	368.1 > 231.1 (15; 20)	368.1 > 175.1 (23; 16)	41
Propham	2.61–2.74	180.1 > 138.1 (13; 14)	180.1 > 120.1 (25; 14)	61
Propoxur	1.68–1.77	210.1 > 111.0 (19; 3)	210.1 > 168.1 (11; 3)	61
Pyraclofos	6.84–6.94	361.0 > 256.9 (31; 24)	361.0 > 111.0/138.0 (81; 10/ 55; 12)	111
Pyraclostrobin	6.46–6.80	388.0 > 194.1 (17; 18)	388.0 > 163.1 (33; 14)	51
Pyridaben	9.43–9.95	365.1 > 309.1 (17; 30)	365.1 > 147.2 (31; 30)	41/21
Pyrifenox	7.99–8.40	294.2 > 93.1 (27; 8)	294.2 > 92.1 (83; 8)	86/66
Pyriftalid	3.81–3.97	319.0 > 139.0 (37; 12)	319.0 > 220.1 (33; 20)	96
Pyrimethanil	4.00–4.21	200.2 > 107.1 (33; 10)	200.2 > 80.0 (39; 8)	41
Pyriproxyfen	7.99–8.40	322.0 > 96.0 (21; 10)	322.0 > 78.1 (75; 6)	71
Pyroquilone	1.60–1.85	174.1 > 132.0 (33; 12)	174.1 > 117.0 (41; 12)	91
Quinalphos	5.73–6.03	299.1 > 163.1 (33; 14)	299.1 > 147.1 (31; 14)	61
Quinoclamine	1.40–1.65	208.1 > 105.0 (33; 10)	208.1 > 89.0 (51; 8)	106
Quizalofop-P-ethyl	7.77–7.88	373.0 > 299.0 (27; 26)	373.0 > 271.0 (35; 22)	151
Spiromesifen	8.80–8.92	371.1 > 273.0 (21; 22)	371.1 > 255.1 (31; 20)	141
Tebuconazole	5.98–6.29	308.1 > 70.1 (57; 8)	308.1 > 125.1 (53; 12)	71
Tebufempirade	7.80–8.20	334.1 > 145.1 (39; 4)	334.1 > 117.1 (67; 6)	111
Temephos	8.10–8.20	466.9 > 418.9 (25; 34)	466.9 > 125.0 (41; 12)	86
Tetraconazole	5.45–5.60	372.0/374.0 > 159.0 (39; 14)	372.0/374.0 > 161.0 (39; 14)	101/81
Thiacloprid	0.80–0.85	253.3 > 126.0 (29; 12)	253.3 > 186.0 (21; 12)	101
Thiobencarb	6.96–7.08	258.0/260.1 > 125.0 (23; 12)	258.0/260.1 > 127.0 (25; 14)	56
Thiodicarb	2.05–2.16	355.1 > 88.1 (27; 3)	355.1 > 108.0 (21; 3)	60
Triadimefon	4.67–4.91	2940 > 197.0 (21; 18)	2940 > 225.0 (17; 20)	66
Triadimenol	4.84–5.09	296.1/298.0 > 70.1 (31; 8)	296.1/298.0 > 70.0 (33; 8)	46
Trichlorfon	0.79–0.84	257.0 > 109.0 (23; 10)	257.0 > 221.0 (15; 20)	101
Tricyclazole	1.00–1.25	190.1 > 163.0 (31; 14)	190.1 > 136.0 (39; 12)	61
Trifloxystrobin	7.20–7.57	409.1 > 186.1 (23; 16)	409.1 > 145.1 (63; 14)	66
Triflumizole	7.12–7.48	346.0 > 278.0 (15; 26)	346.0 > 73.1 (21; 8)	51
Triforin	3.51–3.69	434.9 > 389.8 (17; 36)	434.9 > 215.1 (37; 20)	56

The precursor ion for most of the pesticides was $[M+H]^+$, except for * which were $[M+NH_4]^+$

[a] CE-collision energy potentials.

[b] CXP-collision exit potentials.

[c] DP-declustering potential; V-voltage.

ion, transitions, collision energy potentials (CE) and collision exit potentials (CXP) and optimal declustering potential (DP) of all studied analytes are shown in Table 1. Two SRM transitions were used for each analyte, one for quantification and other for qualification to avoid false negatives at trace pesticide levels.

2.3. Sample preparation

The National and Agriculture Laboratory (LANAGRO-MG), from Ministry of Agriculture, Livestock and Food Supply (MAPA), where this study was developed, is accredited by INMETRO (National Institute of Metrology, Quality and Technology) according to ISO 17025:2005 (International Organization for Standardization, 2005) for the analysis of pesticides in several foodstuffs. The methods developed at LANAGRO by means of QuEChERS were used as a starting point in this study. Pesticide free samples were used as blanks for validation experiments. Some parameters that affect QuEChERS extraction were optimized (univariate analysis), such as the amount of sample (2.5, 5 and 10 g), the amount of water for sample dilution (8.5 and 10 mL), the type of extraction solvent (acetonitrile and acetonitrile:ethyl acetate, 70:30 v/v) and the type of clean-up sorbents (50 mg of PSA; 50 mg of Florisil; or 50 mg of PSA together with 50 mg of Florisil) with 150 mg of $MgSO_4$ for 500 µL of extract. The extraction salts were maintained as follows: 4 g of $MgSO_4$ and 1 g of sodium acetate. Fig. 1 shows the flow chart of the QuEChERS method adapted for the analysis of pesticides in honey.

2.4. Method validation

2.4.1. Selectivity and calibration curves

Validation was performed following the European Union SANCO/12571/2013 guideline (SANCO, 2013). The selectivity of the method was evaluated by injecting extracted blank samples. The absence of signal above a signal-to-noise ratio of 3 at the retention times of the target compounds was the parameter used to show that the method was free of interferences. For the preparation of analytical matrix-matched calibration curves (MMC), blank honey extracts were spiked with proper amounts of standard solutions at the final concentrations of 0.005, 0.0075, 0.010, 0.025, 0.050, 0.075, 0.100 mg/kg and injected in random order (n = 6). All solutions were prepared independently. The best type of fit for the regression curve was decided for each compound by applying the homoscedasticity test. Since the data for all analytes were heteroscedastic the weighted least squares method (WLS) was

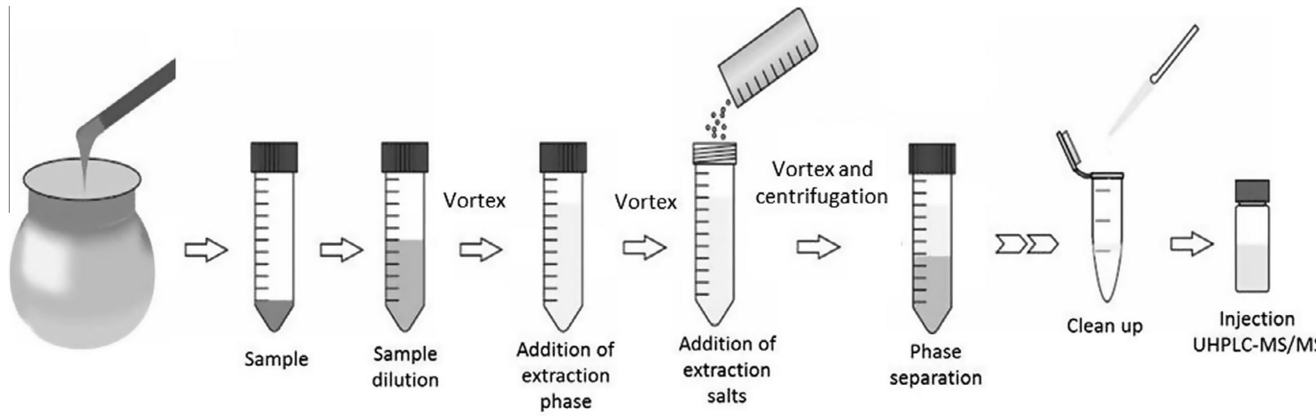

Fig. 1. QuEChERS method adapted for the determination of pesticides in honey.

used. The fit quality and significance of the regression model employed were evaluated using the lack of fit test. The significance level used in all tests was 95%.

2.4.2. Trueness and precision

Trueness was determined on three days and three different analysts. Blank honey extracts were spiked with the analytes at four distinct levels: 0.010, 0.025, 0.050 and 0.100 mg/kg (n = 6 replicates per level). Recoveries were calculated by comparing the concentrations of the extracted compounds with those from the MMC calibration curves. These data were also used to determine the intermediate precision of the method and quantifying the measurement uncertainty (MU). Repeatability, expressed as relative standard deviation (RSD), was evaluated from replicate samples (n = 6) analyzed at the same day for each level. The intermediate precision, expressed as relative standard deviation (RSD), was evaluated through replicates data (n = 18) of the three different days for each level.

2.4.3. Limit of detection, limit of quantification and measurement of uncertainty

The limit of detection (LOD) was experimentally determined using spiked blank honey extracts with all pesticides. The LOD was defined as the lowest concentration of analyte that could be differentiated of the matrix signal with a signal-to-noise ratio (S/N) higher than 6. The LOQ was based on the trueness and precision data, obtained by recovery determination and was defined as the lowest validated spiked level meeting the requirements of a recovery within the range 70–120% and an RSD $\leqslant$ 20%. Measurement uncertainty (MU) was established according to ISO (International Organization for Standardization)/TS 21748:2004 (International Organization for Standardization, 2004) and EURACHEM guide (Eurachem, 2000).

3. Results and discussion

3.1. Extraction method

QuEChERS was chosen for the analysis of pesticides in honey based on the description of several studies in the literature demonstrating its efficiency and good performance for extraction of pesticides in this matrix (Barganska et al., 2013; Kujawski et al., 2014; Tomasini et al., 2012; Wiest et al., 2011). Another criterion used to choose the sample preparation technique was acceptable recoveries for all analytes. After investigating different conditions regarding sample weight, amount of water for sample dilution, type of extraction solvent and type of clean-up phase, the final method was established as: honey (5 g) was weighed in 50 mL tubes and spiked with proper amounts of working standard solutions of pesticides, 10 mL of water was added, and the mixture was vortexed for 30 s. The extraction phase, acetonitrile:ethyl acetate 70:30 with 1% acetic acid (v/v), was added and the mixture vortexed for 1 min. The extraction salts (4 g of magnesium sulfate and 1 g of sodium acetate) were added, vortexed and centrifuged at 1900*g* for 9 min at 20 °C. The supernatant (500 µL) was transferred to a 2 mL tube containing 150 mg of magnesium sulfate, 50 mg of Florisil and 50 mg of PSA for clean-up, and submitted to vortex and centrifugation as already described. Finally, an aliquot of supernatant was transferred to a vial followed by injection at the UHPLC–MS/MS system. The choice of the amount of honey sample and water for dilution as well as the type of extraction solvent and clean-up salts was based on data from recovery and sample cleaning.

The original QuEChERS method consists of two steps, a salting out extraction and a dispersive SPE (dSPE) clean-up (Anastassiades et al., 2003). Since in the QuEChERS approach the sample should have more than 75% of water, an initial dilution of the honey sample was required. The use of ethyl acetate associated with acetonitrile provided less colorful (yellow) extracts, making the clean-up step easier. The use of sodium acetate together with acetic acid buffered the extracts (pKa of acetic acid = 4.75) improving

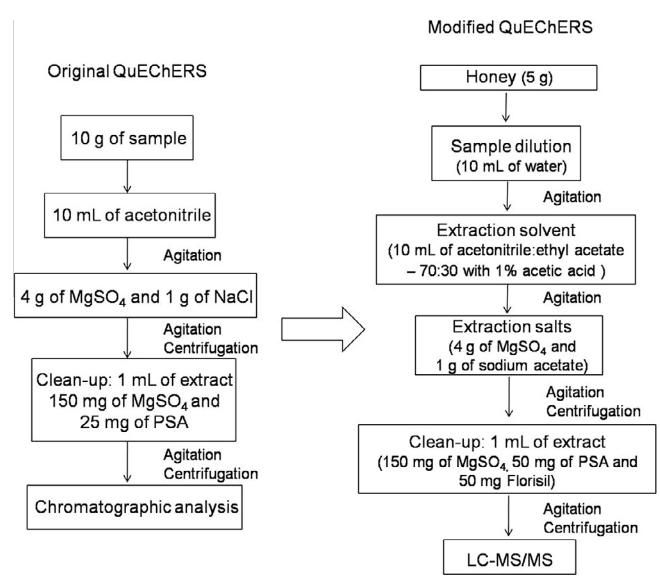

Fig. 2. Steps, reagents and amounts used in the original QuEChERS method and in the QuEChERS modified for the extraction of pesticides in honey.

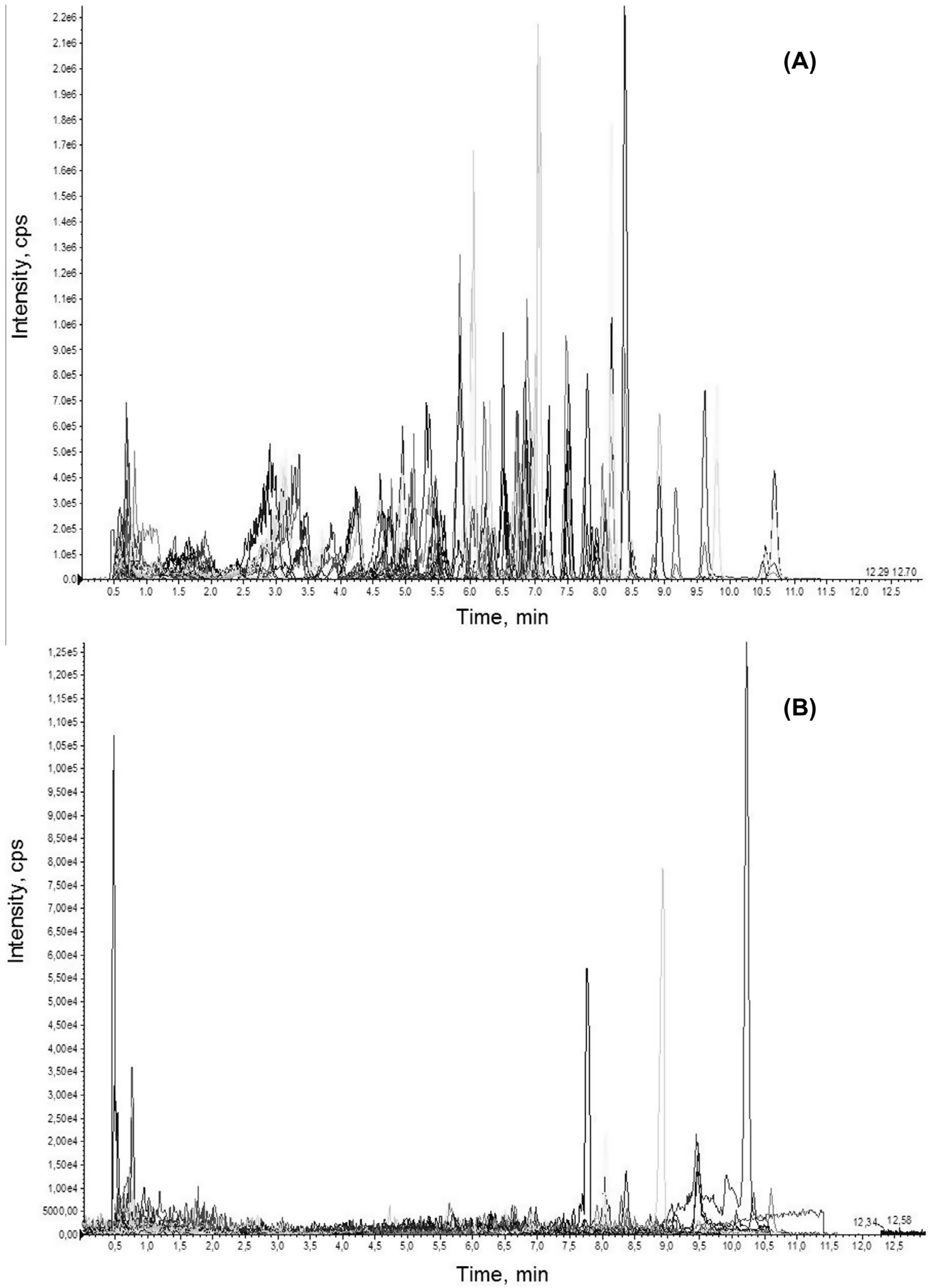

Fig. 3. Total ion chromatograms (TIC) obtained by UHPLC–MS/MS (ESI positive mode) for blank honey extracts spiked with 116 pesticides at 0.1 mg/kg (A) and for a blank sample (B). The y-axis scale is different in the two chromatograms.

Table 2
Validation parameters obtained for the 116 pesticides in the developed method for honey.

Compound	Average recovery (%) (Intermediate precision, % RSD)				Uncertainty measurement (%)				LOD (mg/kg)	LOQ (mg/kg)	LMR* (mg/kg)
	Concentration level (mg/kg)				Concentration level (mg/kg)						
	0.010	0.025	0.050	0.100	0.010	0.025	0.050	0.100			
3-Hydroxy carbofuran	–	95.1 (14.9)	94.1 (18.2)	97.7 (15.3)	–	18.4	13.7	13.1	0.005	0.025	–
Acetamipride	–	103.5 (17.5)	92.9 (13.5)	92.0 (17.0)	–	16.4	12.7	13.0	0.005	0.025	0.05
Alachlor	98.4 (13.4)	97.0 (13.0)	95.6 (13.5)	98.3 (10.8)	26.7	14.1	12.4	12.0	0.005	0.010	0.01
Aldicarb	94.0 (14.6)	90.5 (16.4)	99.7 (15.0)	99.3 (19.1)	24.1	14.7	12.5	13.1	0.005	0.010	0.01
Allethrin	94.5 (8.2)	99.8 (13.4)	99.2 (18.9)	95.9 (13.4)	46.4	18.5	13.9	12.9	0.005	0.010	–
Ametryn	99.8 (8.7)	94.9 (12.7)	92.7 (15.1)	100.9 (13.6)	32.5	15.2	12.8	12.5	0.005	0.010	–
Azinphos ethyl	94.2 (17.0)	103.1 (15.6)	97.7 (16.3)	86.6 (16.7)	39.4	17.5	13.7	13.8	0.005	0.010	–
Azinphos methyl	96.8 (11.4)	96.8 (13.1)	98.6 (11.2)	96.9 (12.4)	21.1	13.1	11.9	12.0	0.005	0.010	–
Azoxystrobin	94.3 (15.6)	98.2 (13.2)	98.4 (15.9)	99.7 (11.5)	34.4	15.6	12.9	12.3	0.005	0.010	0.05
Benalaxyl	102.1 (15.9)	102.3 (12.7)	98.0 (9.8)	102.9 (11.8)	24.0	13.5	11.9	12.0	0.005	0.010	–
Bitertanol	97.9 (16.0)	97.2 (16.2)	98.8 (17.0)	97.0 (13.0)	31.4	15.3	13.0	12.3	0.005	0.010	0.05
Buprofezin	102.4 (8.4)	97.5 (11.0)	94.8 (12.8)	93.1 (11.2)	29.7	14.5	12.4	12.1	0.005	0.010	0.05
Cadusafos	104.5 (19.1)	97.3 (19.5)	93.3 (19.0)	90.9 (14.9)	37.8	17.0	13.5	12.8	0.005	0.010	0.01
Carbaryl	99.5 (6.9)	97.2 (6.8)	95.5 (7.4)	93.4 (5.6)	31.7	14.6	11.9	11.7	0.005	0.010	–
Carbendazim	90.6 (10.9)	91.2 (8.7)	91.4 (11.6)	96.2 (12.8)	44.3	17.6	12.8	12.8	0.005	0.010	–
Chlorbupham	–	97.3 (17.3)	101.2 (15.3)	97.0 (13.8)	–	16.5	12.9	12.6	0.005	0.025	–
Chlorfentezine	93.0 (15.6)	93.9 (15.8)	93.8 (15.3)	82.4 (13.4)	41.3	17.4	13.1	12.7	0.005	0.010	–
Chlorpyrifos-methyl	94.1 (17.6)	88.1 (17.4)	89.2 (15.6)	90.7 (12.5)	32.9	15.8	12.8	12.3	0.005	0.010	–
Chlortiophos	94.1 (10.9)	99.1 (9.0)	97.8 (15.3)	94.5 (11.7)	34.3	15.3	12.9	12.3	0.005	0.010	–
Cinidon-ethyl	90.7 (15.2)	86.5 (15.5)	89.6 (15.4)	87.6 (16.6)	43.7	18.0	13.3	13.2	0.005	0.010	–
Cyazofamid	96.4 (13.9)	95.1 (13.7)	92.2 (13.9)	99.1 (12.9)	26.4	14.1	12.4	12.2	0.005	0.010	0.05
Cyhalofop butyl	92.9 (17.3)	93.6 (12.8)	97.4 (15.6)	91.0 (13.9)	48.2	20.0	13.0	16.1	0.005	0.010	0.05
Cyproconazole	–	90.5 (14.3)	87.7 (13.6)	97.6 (9.7)	–	18.7	13.2	12.6	0.005	0.025	0.05
Cyprodinil	100.6 (8.3)	91.7 (14.8)	102.3 (7.0)	98.1 (13.4)	25.6	14.1	11.7	12.2	0.005	0.010	0.05
Desmedipham	99.1 (11.3)	94.5 (13.4)	93.0 (13.5)	88.9 (14.7)	35.1	15.8	12.7	12.7	0.005	0.010	–
Diazinon	102.9 (10.2)	95.0 (12.8)	96.8 (12.3)	96.0 (12.6)	31.8	15.1	12.4	12.3	0.005	0.010	0.01
Difenoconazole	97.2 (13.6)	88.7 (13.8)	96.4 (13.1)	94.1 (9.5)	35.2	15.9	12.6	12.1	0.005	0.010	0.05
Dimethomorph	99.2 (11.5)	96.8 (15.7)	97.7 (12.6)	99.5 (10.7)	37.5	16.4	12.6	12.3	0.005	0.010	0.05
Diniconazole	98.3 (10.7)	102.2 (10.1)	97.2 (12.4)	95.7 (12.4)	24.4	13.4	12.2	12.1	0.005	0.010	0.05
Disulfoton sulfone	99.2 (9.1)	99.1 (9.7)	99.1 (10.5)	94.9 (9.0)	18.2	12.4	11.8	11.6	0.005	0.010	0.01
Diuron	96.4 (7.2)	97.8 (6.4)	94.9 (11.9)	98.7 (9.9)	23.6	13.0	12.1	11.8	0.005	0.010	0.05
Ethion	100.5 (10.6)	94.1 (14.1)	95.2 (16.9)	92.2 (16.4)	38.3	16.6	13.2	13.0	0.005	0.010	0.01
Ethiprole	98.7 (16.5)	91.6 (17.3)	92.2 (19.2)	85.3 (18.7)	47.1	19.0	14.0	13.7	0.005	0.010	–
Ethofumesate	93.9 (9.7)	94.7 (14.3)	96.5 (13.2)	92.5 (11.7)	35.8	16.1	12.6	12.3	0.005	0.010	0.1
Ethoprophos	–	99.2 (13.4)	103.6 (14.2)	108.9 (14.9)	–	22.5	14.2	13.9	0.005	0.025	–
Etrinphos	99.9 (14.9)	100.3 (11.9)	99.5 (13.1)	91.4 (13.4)	23.3	13.4	12.2	12.2	0.005	0.010	–
Fenamiphos	87.2 (11.0)	92.9 (15.7)	82.5 (17.0)	82.0 (15.3)	44.9	18.6	13.8	13.6	0.005	0.010	0.01
Fenamiphos sulfone	97.9 (10.0)	91.9 (12.1)	90.4 (12.5)	88.2 (13.4)	35.8	15.9	12.6	12.5	0.005	0.010	0.01
Fenamiphos sulfoxide	88.8 (7.7)	87.9 (10.4)	90.6 (10.1)	85.5 (6.9)	40.2	17.3	12.8	12.2	0.005	0.010	0.01
Fenarimol	102.7 (16.9)	91.8 (13.5)	86.6 (19.6)	87.4 (18.6)	46.6	18.8	14.3	14.0	0.005	0.010	0.05
Fenazaquin	95.5 (14.2)	99.2 (14.5)	97.6 (14.0)	94.7 (11.9)	44.1	18.5	13.8	13.9	0.005	0.010	0.01
Fenhexamid	–	89.4 (16.1)	85.7 (16.1)	88.0 (15.2)	–	18.5	13.9	13.4	0.005	0.025	0.05
Fenpyroximate	96.6 (8.1)	98.7 (12.7)	98.4 (13.8)	98.1 (12.3)	22.2	13.3	12.3	12.0	0.005	0.010	0.05
Fenpropimorph	91.1 (8.4)	87.1 (12.3)	85.7 (13.1)	86.3 (8.3)	25.5	13.8	12.3	11.7	0.005	0.010	–
Fluazifop p-butyl	98.5 (9.0)	97.6 (13.0)	98.3 (8.7)	97.9 (7.5)	25.8	13.9	11.8	11.7	0.005	0.010	0.05
Flumethrin	101.1 (12.8)	97.0 (10.5)	97.7 (11.9)	92.3 (12.4)	26.3	13.8	12.2	12.1	0.005	0.010	–
Fluquinconazole	–	94.8 (17.6)	93.4 (16.5)	94.9 (15.0)	–	19.6	13.7	13.2	0.005	0.025	0.02
Flusilazole	101.8 (12.3)	98.2 (15.2)	100.3 (13.2)	95.5 (12.0)	27.9	14.6	12.4	12.1	0.005	0.010	0.05
Flutriafol	94.5 (11.0)	90.9 (7.9)	97.3 (6.0)	97.3 (8.5)	17.9	12.2	11.4	11.6	0.005	0.010	0.05
Fosthiazate	99.8 (12.5)	95.8 (12.7)	97.0 (14.0)	89.6 (8.4)	28.5	14.4	12.5	11.8	0.005	0.010	–
Furathiocarb	97.9 (11.4)	98.9 (14.9)	101.0 (14.7)	100.3 (14.2)	29.2	14.8	12.6	12.4	0.005	0.010	0.01
Hexaconazole	98.6 (15.3)	95.0 (14.5)	92.3 (14.1)	89.8 (8.4)	48.5	19.1	13.3	13.2	0.005	0.010	–
Hexythiazox	98.2 (9.4)	102.0 (14.3)	100.0 (14.6)	96.1 (8.5)	29.7	14.8	12.6	11.8	0.005	0.010	0.02
Imazalil	90.8 (15.0)	93.0 (15.1)	91.0 (14.2)	92.4 (15.6)	39.9	17.1	12.9	13.0	0.005	0.010	0.05
Indoxacarb	103.6 (13.4)	101.9 (13.7)	95.7 (11.4)	101.1 (11.0)	41.8	17.4	12.7	12.5	0.005	0.010	0.05
Iprovalicarb	102.7 (12.6)	105.4 (13.6)	96.1 (14.7)	95.9 (12.3)	40.0	16.9	13.0	12.5	0.005	0.010	0.05
Isoproturon	96.7 (8.9)	99.5 (14.5)	95.1 (13.9)	95.6 (13.0)	34.1	15.7	12.7	12.4	0.005	0.010	0.05
Linuron	98.0 (11.2)	96.9 (11.4)	99.2 (13.2)	100.4 (13.0)	36.8	16.0	12.7	12.5	0.005	0.010	–
Malathion	100.5 (16.2)	103.3 (12.3)	100.7 (14.2)	103.2 (9.4)	37.0	16.1	12.8	12.1	0.005	0.010	0.02
Metalaxyl	97.5 (11.2)	101.8 (10.9)	96.5 (10.5)	98.0 (9.6)	29.7	14.5	12.1	11.9	0.005	0.010	0.05
Metazachlor	100.4 (10.3)	102.1 (12.9)	100.0 (13.8)	95.1 (11.5)	29.2	14.6	12.5	12.1	0.005	0.010	0.05
Metconazole	101.9 (11.7)	103.4 (13.3)	92.7 (17.3)	93.3 (15.3)	37.0	16.3	13.2	12.8	0.005	0.010	0.05
Methidathion	95.6 (14.0)	93.4 (13.4)	93.2 (14.0)	94.0 (17.1)	27.6	14.3	12.5	12.8	0.005	0.010	0.02
Methiocarb	103.6 (13.8)	95.5 (13.4)	97.5 (14.9)	92.7 (14.4)	40.1	17.0	13.0	12.8	0.005	0.010	0.05
Methiocarb sulfoxide	87.9 (15.8)	89.0 (10.6)	89.1 (14.5)	95.4 (10.5)	44.3	17.7	13.2	12.5	0.005	0.010	0.05
Methoxifenozide	94.1 (19.5)	101.2 (15.4)	101.4 (17.9)	93.6 (16.2)	46.8	19.3	14.1	13.2	0.005	0.010	0.05
Mevinphos	94.7 (13.9)	99.3 (11.3)	95.9 (14.3)	94.1 (14.8)	36.6	16.0	12.8	12.7	0.005	0.010	–
Monocrotophos	88.6 (18.3)	94.7 (15.8)	88.5 (14.7)	87.0 (14.6)	49.9	19.5	13.5	13.2	0.005	0.010	–
Monolinuron	100.6 (9.9)	99.9 (12.4)	95.9 (13.9)	95.3 (10.4)	33.7	15.5	12.7	12.1	0.005	0.010	–
Myclobutanil	100.9 (11.7)	102.1 (12.9)	100.4 (12.8)	104.2 (10.6)	39.3	16.7	12.7	12.3	0.005	0.010	–

Table 2 (*continued*)

Compound	Average recovery (%) (Intermediate precision, % RSD)				Uncertainty measurement (%)				LOD (mg/kg)	LOQ (mg/kg)	LMR* (mg/kg)
	Concentration level (mg/kg)				Concentration level (mg/kg)						
	0.010	0.025	0.050	0.100	0.010	0.025	0.050	0.100			
Nuarimol	96.8 (12.5)	102.4 (12.5)	100.5 (13.5)	99.1 (11.9)	33.2	15.3	12.6	12.3	0.005	0.010	–
Omethoate	86.6 (15.3)	82.9 (14.2)	89.3 (17.7)	81.6 (14.4)	45.2	18.4	14.0	14.3	0.005	0.010	–
Oxamyl	100.8 (10.8)	98.0 (12.8)	98.4 (15.4)	89.7 (14.8)	49.1	19.6	14.3	15.7	0.005	0.010	0.05
Paclobutrazol	93.5 (9.8)	99.8 (12.6)	99.8 (11.0)	97.8 (12.8)	42.9	17.6	12.7	12.7	0.005	0.010	–
Paraoxon-ethyl	101.0 (8.8)	100.4 (12.0)	98.7 (11.2)	97.5 (11.7)	29.1	14.5	12.2	12.1	0.005	0.010	–
Penconazole	–	100.4 (14.4)	98.5 (12.7)	94.6 (11.6)	–	18.0	12.9	12.6	0.005	0.025	–
Pencycuron	101.2 (16.2)	98.2 (15.9)	96.3 (11.9)	96.9 (11.2)	44.4	18.2	12.9	12.6	0.005	0.010	–
Pendimethalin	100.0 (13.2)	96.1 (13.8)	91.5 (14.8)	91.7 (10.2)	29.4	14.7	12.6	12.0	0.005	0.010	0.05
Phenthoate	–	103.6 (16.3)	96.1 (16.8)	96.2 (13.3)	–	15.0	12.9	12.3	0.005	0.025	–
Phorate sulfoxide	95.3 (12.3)	98.9 (11.0)	100.1 (12.4)	95.0 (9.3)	20.1	12.8	12.0	11.7	0.005	0.010	0.01
Phosphamidon	88.6 (13.1)	95.6 (13.6)	95.8 (14.3)	101.7 (9.5)	32.0	15.2	12.6	12.0	0.005	0.010	–
Phosmet	102.8 (11.6)	89.1 (11.6)	91.4 (14.1)	87.1 (14.4)	34.9	15.6	12.7	12.6	0.005	0.010	0.05
Picolinafen	102.6 (10.7)	95.2 (11.9)	90.7 (12.5)	86.7 (11.2)	39.6	16.7	12.7	12.4	0.005	0.010	–
Pirazophos	102.1 (16.8)	104.4 (15.9)	95.7 (11.4)	94.0 (15.3)	47.7	19.5	13.6	14.3	0.005	0.010	–
Pirimiphos-ethyl	96.4 (9.8)	101.6 (12.4)	98.2 (11.3)	97.5 (7.7)	30.3	14.8	12.2	11.8	0.005	0.010	–
Pirimiphos-methyl	100.8 (15.4)	101.6 (11.1)	98.6 (15.5)	96.7 (14.1)	33.0	15.2	12.8	12.5	0.005	0.010	–
Profenofos	99.4 (10.7)	99.1 (11.7)	98.7 (15.0)	100.1 (10.8)	38.8	16.5	13.0	12.3	0.005	0.010	0.01
Propaquizafop	94.7 (12.1)	98.6 (13.4)	97.0 (11.4)	96.1 (12.0)	39.3	16.8	12.6	12.5	0.005	0.010	0.05
Propargite	98.9 (10.0)	100.0 (13.6)	99.5 (13.6)	97.7 (12.4)	30.4	14.7	12.5	12.3	0.005	0.010	–
Propham	–	93.6 (16.4)	96.9 (13.5)	105.3 (14.5)	–	16.8	12.9	12.7	0.005	0.025	0.05
Propoxur	99.0 (9.2)	96.2 (12.9)	98.1 (14.7)	94.5 (15.1)	39.0	16.7	13.0	12.8	0.005	0.010	–
Pyraclofos	–	100.6 (15.8)	98.7 (14.7)	91.2 (13.7)	–	22.0	14.0	13.5	0.005	0.025	–
Pyraclostrobin	103.2 (15.8)	94.4 (16.0)	89.8 (17.2)	82.9 (18.8)	39.0	17.3	13.8	14.8	0.005	0.010	0.05
Pyridaben	98.6 (9.4)	99.3 (12.0)	98.0 (13.4)	98.3 (11.9)	27.2	14.1	12.4	12.1	0.005	0.010	0.02
Pyrifenox	96.8 (11.9)	103.7 (10.1)	98.6 (12.4)	101.0 (11.2)	34.9	15.5	12.5	12.3	0.005	0.010	–
Pyriftalid	97.2 (13.4)	99.0 (13.1)	99.6 (13.6)	101.4 (11.2)	24.1	13.6	12.3	12.0	0.005	0.010	–
Pyrimethanil	97.1 (7.7)	99.6 (11.7)	96.4 (13.5)	101.0 (8.6)	25.0	13.7	12.3	11.7	0.005	0.010	0.05
Pyriproxyfen	100.0 (9.2)	99.2 (14.2)	91.6 (17.4)	91.3 (15.8)	32.3	15.3	13.1	12.7	0.005	0.010	0.05
Pyroquilone	103.5 (9.1)	95.7 (14.0)	89.6 (13.8)	89.1 (14.4)	34.5	15.8	12.7	12.6	0.005	0.010	–
Quinalphos	101.6 (18.4)	103.0 (14.5)	95.1 (13.5)	91.9 (16.1)	31.6	15.2	12.5	12.8	0.005	0.010	–
Quinoclamine	99.9 (17.4)	101.8 (16.1)	99.6 (14.2)	90.5 (12.8)	30.2	15.0	12.5	12.3	0.005	0.010	0.05
Quizalofop-P-ethyl	103.4 (10.0)	96.4 (11.8)	91.3 (11.8)	85.5 (9.5)	37.9	16.3	12.6	12.2	0.005	0.010	0.05
Spiromesifen	96.7 (8.2)	94.5 (15.3)	90.2 (17.8)	86.9 (16.5)	36.0	16.2	13.3	13.0	0.005	0.010	0.01
Tebuconazole	96.6 (17.9)	103.4 (14.3)	94.7 (14.3)	97.7 (12.6)	44.1	14.2	13.1	12.7	0.005	0.010	0.05
Tebufempirade	99.8 (12.8)	100.4 (12.0)	97.3 (13.7)	98.7 (8.8)	35.5	15.8	12.7	12.0	0.005	0.010	0.05
Temephos	102.8 (12.1)	100.1 12.4	96.1 (13.7)	92.9 (11.8)	32.2	15.1	12.6	12.2	0.005	0.010	–
Tetraconazole	94.4 (16.2)	97.0 (14.8)	97.8 (13.5)	99.5 (13.9)	37.9	16.5	12.8	12.6	0.005	0.010	0.02
Thiacloprid	98.6 (12.1)	102.0 (11.2)	96.2 (10.6)	96.1 (9.6)	31.3	11.2	12.2	12.0	0.005	0.010	0.2
Thiobencarb	94.8 (11.4)	101.8 (11.4)	97.5 (13.7)	97.2 (10.6)	45.8	18.2	13.2	12.6	0.005	0.010	0.05
Thiodicarb	95.7 (13.0)	99.6 (9.3)	95.2 (10.4)	93.7 (9.4)	24.4	13.3	11.9	11.8	0.005	0.010	–
Triadimefon	107.2 (13.9)	101.2 (13.2)	97.6 (18.3)	95.5 (15.9)	44.5	18.0	13.7	13.2	0.005	0.010	0.1
Triadimenol	100.6 (8.7)	100.7 (11.9)	96.9 (10.5)	99.0 (10.4)	36.7	16.1	12.4	12.2	0.005	0.010	0.1
Trichlorfon	94.8 (15.7)	94.7 (14.8)	89.4 (14.9)	85.1 (15.0)	37.9	16.9	13.5	15.1	0.005	0.010	0.01
Tricyclazole	96.9 (8.7)	96.5 (10.5)	94.6 (12.1)	88.0 (9.8)	20.4	12.8	12.0	11.7	0.005	0.010	–
Trifloxystrobin	105.0 (12.7)	99.6 (12.5)	97.2 (14.8)	92.7 (14.1)	27.7	14.2	12.6	12.4	0.005	0.010	0.05
Triflumizole	101.0 (17.6)	99.3 (17.5)	89.9 (17.0)	89.2 (18.1)	42.3	18.0	13.5	13.4	0.005	0.010	–
Triforin	100.1 (12.4)	99.3 (13.5)	99.2 (13.1)	94.9 (10.3)	21.3	13.2	12.2	11.8	0.005	0.010	0.01

Weighted least squares method was the fit regression type for all analytes.
* European Community legislation (European Union, 2015).

pesticides stability and increasing the extraction efficiency. Magnesium sulfate was used in order to ensure dryness of the sample by means of an exothermic reaction, leading to phase separation and extraction of the compounds by the acetonitrile:ethyl acetate solution. To remove the matrix interference, a clean-up step was also performed. A dispersive solid phase extraction employing PSA together with Florisil was performed. PSA had the ability to retain matrix components, such as polar organic acids, sugars and fatty acids. Florisil improved sample clean-up, due to the sugars interaction with the polar surface of this sorbent (Koesukwiwat, Sanguankaew, & Leepipatpiboon, 2008; Kujawski et al., 2014; Prestes et al., 2009).

Fig. 2 shows the flow chart of the original QuEChERS method and QuEChERS modified for the extraction of pesticides in honey.

3.2. Method validation

According to the European Union SANCO/12571/2013 guidelines (SANCO, 2013), the precursor (parent) ion and the two transitions (quantification and identification ions) should be present with a signal-to-noise (S/N) ratio greater than 3 (in the lowest calibration level this ratio should be higher than 6); and the ratio of the quantification/confirmation transitions in the sample and the previously injected standard should not differ by more than ±30%. Therefore, two transitions were selected for each compound (Table 1) and these criteria were evaluated. Fig. 3 shows the total ion chromatograms (TIC) obtained from a blank sample and from a sample spiked with all pesticides at 0.01 mg/kg. The absence of signal above a signal-to-noise ratio of 3 at the retention times of the target compounds showed that the method was free of interferences.

The criteria adopted for the selection of the analytical curve levels were the signal-to-noise ratio and the recovery results. From this evaluation five concentrations were selected: 0.010, 0.025, 0.050, 0.075, and 0.100 mg/kg. The 0.005 mg/kg concentration level was injected to confirm the LOD of the method. Over the calibration ranges selected, all calibration curves presented significant linearity according to the lack of fit test and *t*-test on determination coefficients (r^2). The LOD and LOQ of the pesticides are indicated in Table 2. It can be seen that the LODs and LOQs were 0.005 mg/kg and 0.010 mg/kg, respectively, except for 3-hydroxy carbofuran, acetamipride, cyproconazole, chlorbufam, ethoprophos, fenhexamid, fentoate, fluquinconazole, penconazole, pyraclofos and propham, which had a LOQ of 0.025 mg/kg.

Trueness was evaluated by means of recoveries percentage of honey blank samples spiked with 0.010, 0.025, 0.050 and 0.100 mg/kg of the pesticides (n = 6 replicates per level), since reference material was not available. Trueness and precision (repeatability), measured as % RSD, can be seen in Table 2. Almost all results showed recoveries in the range considered acceptable (70–120% – SANCO, 2013) as indicated in Fig. 4. Some analytes (11 from 116) had recoveries out the acceptable range at the level of 0.010 mg/kg and, therefore, the LOQ was higher for these pesticides. Most of the analytes showed recoveries between 91 and 100% and the variation coefficient was, in general, within the range of 10–15% (Fig. 4).

The measurement uncertainty was based on a combination of "top-down" and "bottom-up" methodologies described in the Eurachem guide (Eurachem, 2000). The mass measurements of the standards for the preparation of solutions, the dilution of the standard solutions, the measurements of volume of the extraction solution, the MMC curves and the intermediate precision were the main uncertainty sources associated with the method. It is known that the primary source of total uncertainty for all pesticides validated comes from the MMC curves that encompass all steps from the weighing of standards for preparation of solutions until the final quantification step, including the whole extraction process, the instrumental analysis and data statistical processing (Carneiro et al., 2013; Madureira et al., 2012). The expanded uncertainty, expressed as percentage (MU%, Table 2), for each pesticide was determined in each fortification level for which the assessment of repeatability and reproducibility have been performed. As can be seen in Table 2, the MU calculated for each pesticide showed values below 50%. The uncertainty values at all levels studied were in the range of 11.2%–48.5%. These results were in accordance with the acceptable criteria established in SANCO/12571/2013 document (SANCO, 2013).

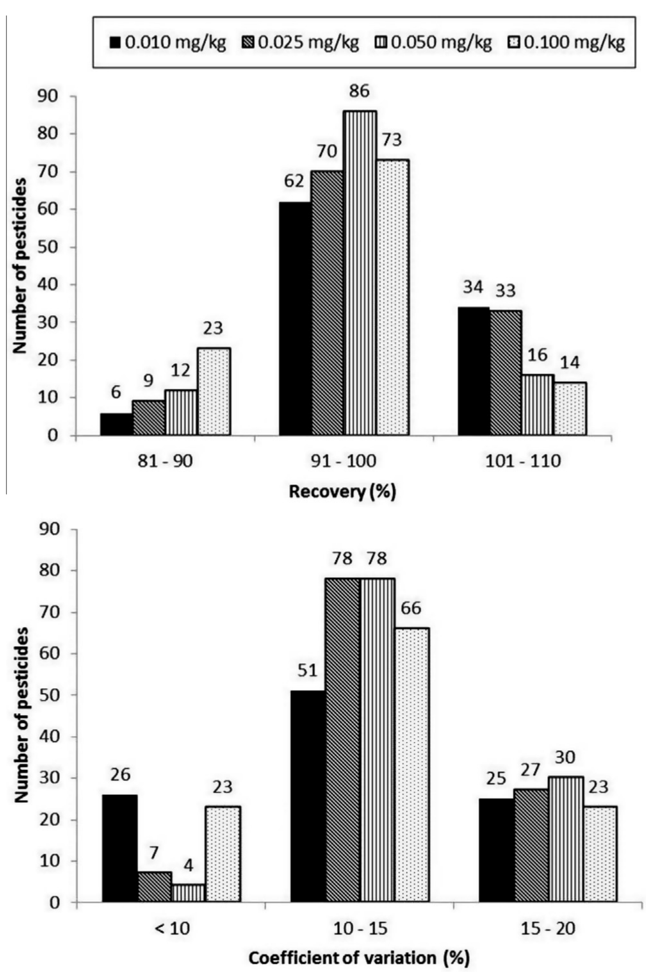

Fig. 4. Recovery and coefficients of variation of the 116 pesticides in honey at each spiked concentration evaluated.

3.3. Sample analysis

The optimized and validated method was applied in the analysis of 100 samples of honey of different brands. The retention time of each analyte and the relative intensities of the quantification and confirmation product ions (obtained by means of single reaction monitoring) in the real samples were compared to those of spiked blank samples at 0.010 and 0.100 mg/kg. Among the samples analyzed one of the 66 samples of Minas Gerais presented trichlorfon at 0.029 mg/kg. This result is above the maximum residue level (MRL) established by the European Union (0.01 mg/kg). Trichlorfon (dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate) is an organophosphate (OP) insecticide used to control a variety of pests and domestic animal ectoparasites and endoparasites (Eraslan, Kanbur, Silici, & Karabacak, 2010). Probably, this contamination has occurred due to the manipulation of this pesticide near the hives with the aim to control parasites in domestic animals or livestock. The presence of this insecticide in honey is worrisome for susceptible populations, including pregnant women and children (Whyatt et al., 2004). According to epidemiological investigations the fetal exposure to OP pesticides can cause inhibition in fetal growth and shortening the period of gestation (Eskenazi et al., 2004).

3.4. Participation in proficiency tests

The validated method was applied in the analysis of honey in a proficiency test. The received sample was submitted to analysis to identify and quantify all possible pesticides within the scope of the laboratory. To analyze the sample, a matrix-matched calibration curve was prepared with a blank extract. No false negative and no false positive results were reported and the z-scores for the identified analytes (from −1.54 to 0.89) demonstrated the method suitability fitness for the purpose, concerning the acceptable limit of ±2.0. The identified analytes were carbendazim, chlorpyrifos methyl, flumethrin, malathion, mevinphos, thiacloprid, cypermethrin, deltamethrin and boscalid. This method will be used in routine analysis of official samples of honey from the Brazilian pesticide residues monitoring program.

4. Conclusions

The validated method using a modified QuEChERS technique as sample preparation and UHPLC–MS/MS was suitable for multiresidue detection and quantitation of 116 pesticides in honey samples. Recoveries between 81.6 and 108.9%, coefficient of variation lower or equal to 20% and expanded uncertainty of up to 48.5% were obtained. The limits of detection (LOD) were 0.005 mg/kg and limits of quantification (LOQ) were 0.01 and 0.025 mg/kg. Accuracy and precision (in intermediate precision conditions) satisfied the European Community recommendations for pesticide residues in SANCO N° 12571/2013 document. In a general way the samples of honey showed appropriate quality in terms of pesticide residues.

The validated method showed to be fast, efficient and reliable and can be used in the monitoring of pesticides in honey and attend the Brazilian National Plan for Residues and Contaminants (PNCRC).

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