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Single solid phase extraction method for the simultaneous analysis of polar and non-polar pesticides in urine samples by gas chromatography and ultra high pressure liquid chromatography coupled to tandem mass spectrometry

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ABSTRACT

A new multiresidue method has been developed and validated for the simultaneous extraction of more than two hundred pesticides, including non-polar and polar pesticides (carbamates, organochlorine, organophosphorous, pyrethroids, herbicides and insecticides) in urine at trace levels by gas and ultra high pressure liquid chromatography coupled to ion trap and triple quadrupole mass spectrometry, respectively (GC-IT-MS/MS, UHPLC-QqQ-MS/MS). Non-polar and polar pesticides were simultaneously extracted from urine samples by a simple and fast solid phase extraction (SPE) procedure using C_{18} cartridges as sorbent, and dichloromethane as elution solvent. Recovery was in the range of 60–120%. Precision values expressed as relative standard deviation (RSD) were lower than 25%. Identification and confirmation of the compounds were performed by the use of retention time windows, comparison of spectra (GC-amenable compounds) or the estimation of the ion ratio (LC-amenable compounds). For GC-amenable pesticides, limits of detection (LODs) ranged from 0.001 to 0.436 $\mu g\,L^{-1}$ and limits of quantification (LOQs) from 0.003 to 1.452 $\mu g\,L^{-1}$. For LC-amenable pesticides, LODs ranged from 0.003 to 1.048 $\mu g\,L^{-1}$ and LOQs ranged from 0.011 to 3.494 $\mu g\,L^{-1}$. Finally, the optimized method was applied to the analysis of fourteen real samples of infants from agricultural population. Some pesticides such as methoxyfenozide, tebufenozide, piperonyl butoxide and propoxur were found at concentrations ranged from 1.61 to 24.4 $\mu g\,L^{-1}$, whereas methiocarb sulfoxide was detected at trace levels in two samples.

1. Introduction

The release of pesticides into the environment can cause the incorporation into a variety of matrices such as water, soil and crops [1], and this may be a serious hazard to human health [2]. Pesticides exposure can induce a variety of diseases by accumulation of these chemicals in the human body [3]. For this reason, in recent years the scientific interest about the potential adverse health effects that may result from exposure of humans to pesticides has increased [2]. For example, the relationship between exposure to certain pesticides used in agriculture and the risk of suffering cancer has been studied [3]. Thus, it was observed a significant association between prostate cancer risk and exposure to several pesticides such as DDT, simazine and lindane [4]. Furthermore, a relationship between pesticides exposure and diseases such as Parkinson [5] was observed, noting that organochlorine insecticides are more particularly associated with Parkinson disease [6].

The presence of pesticides and their metabolites in several matrices in occupationally exposed people and in non-occupationally exposed people has been studied. They have been detected in several biological matrices such as urine [7], serum [8], meconium [9], hair [10], breast milk [11] and others [12,13], providing information related to the accumulation of these compounds in the body [14]. Furthermore, these compounds can be transformed into derived products that can be excreted by different routes [15], being urine one of the principal routes of elimination of chemicals [14].

The analysis of pesticides is a challenge due to different polarities and different basic-acid properties of these compounds [16]. For this reason and according to the wide variety of contaminants, it is necessary to develop a multiresidue method that allows the simultaneous determination of as many pesticides as possible [3].

Pesticides and their metabolites could be present at low concentrations and therefore sensitive analytical methods are necessary [17]. In literature it has found that limits of detection (LODs) lower than 1 $\mu g\,L^{-1}$ are required for pesticides biomonitoring [18]. Thus, liquid chromatography (LC) and gas chromatography (GC) coupled with selective detectors such as mass spectrometry (MS)

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Table 1
Retention time windows (RTWs) and GC-IT-MS/MS conditions for the monitored pesticides.

Pesticide	RTW (min)	Precursor ion (*m*/*z*)	Quantitation ion (*m*/*z*)[a]	CID RF (*m*/*z*)	CID Amplitude (V)
Caffeine (IS)	16.19–16.42	194	120 + 108	60	52
Dichlorvos	6.81–6.98	221	141 + 145	90	72
Biphenyl	8.29–8.36	154	122:132 + 98:99	70	87
Mevinphos	8.56–8.66	193	127	80	58
Acephate	8.64–9.29	143	157	60	0.2
Clormephos	8.78–8.86	235	171 + 199	90	45
Propoxur	11.32–11.47	168	111	70	49
Propachlor	11.32–11.43	212	170 + 106	85	64
Ethoprophos	11.73–11.84	243	131 + 173	80	52
Cycloate	11.79–11.87	216	154	75	46
Cadusafos	12.62–12.72	271	131 + 159	119.5	59
Dimethoate	13.67–13.92	230	199	100	53
Quintozene	14.17–14.29	237	143	80	78
Lindane	14.44–14.54	219	181:183	100	70
Fonofos	14.60–14.71	246	137 + 109	90	38
Diazinon	14.61–14.70	304	179	110	68
Pyrimethanil	15.01–14.14	198	102 + 155 + 159	75	81
Tefluthrin	15.13–15.21	177	127 + 87 + 107	70	72
Etrimfos	15.31–15.40	292	181	70	45
Pirimicarb	15.70–15.83	166	83	53	46
Dichlofenthion	16.34–16.42	279	223 + 251	110	64
Chlorpyriphos methyl	16.64–16.74	286	208 + 286	85	70
Vinclozoline	16.86–16.95	285	241	100	39
Tolclofos methyl	16.96–17.05	265	220 + 215	100	86
Parathion methyl	16.97–17.13	263	136 + 246	80	48
Transfluthrin	17.05–17.12	163	143	65	69
Metalaxyl	17.25–17.35	206	162 + 132	75	54
S421	17.63–17.72	130	95 + 97	65	80
Pirimiphos methyl	17.80–17.89	290	151	85	64
Fenitrothion	18.03–18.14	260	125	71	55
Terbutryn	18.04–18.12	242	170 + 185	107	71
Ethofumesate	18.09–18.19	207	161 + 179 + 137	80	46
Malathion	18.34–18.43	173	127	75	36
Chlorpyrifos	18.61–18.70	314	258 + 286	170	100
Bromacil	18.53–18.84	205	132 + 162 + 134	80	77
Chlorthal-dimethyl	18.83–18.91	301	273	100	85
Fenthion	18.86–18.96	278	135	112	89
Parathion	19.01–19.14	291	263 + 142	128	0.17
Triadimefon	19.17–19.24	208	144	75	59
Tetraconazole	19.25–19.37	336	218	108	89
Isocarbofos	19.24–19.40	230	155	100	82
Butralin	19.39–19.44	266	236 + 220 + 190	110	74
Dicofol	19.40–19.53	250	215	71	56
Pirimifos	19.53–19.60	318	182 + 166 + 246	120	81
Bromophos methyl	19.60–19.67	331	286 + 316	100	80
Isophenphos methyl	19.83–19.89	199	121 + 167	87	61
Pendimethalin	20.09–20.17	252	208 + 192 + 162	95	59
Fipronil	20.09–20.32	420	351 + 353	150	92
Penconazole	20.42–20.57	248	157	89	77
Isophenphos	20.43–20.50	213	185	93	59
Pyrifenox	20.53–20.63	263	192 + 228	100	90
Chlorfenvinphos	20.54–20.66	267	159	100	82
Phentoate	20.76–20.83	274	121	90	57
Quinalphos	20.83–20.93	146	118	64	54
Furalaxyl	20.89–20.97	242	146 + 95 + 224	90	62
Procymidone	21.04–21.15	283	253:257	80	57
Bromophos	21.39–21.45	359	303 + 331	140	79
Metidathion	21.46–21.61	145	85	64	41
Chlorbenside	21.56–21.65	125	89	60	68
Chinomethionat	21.70–21.86	234	206	83	46
Tetrachlorvinphos	21.74–21.85	331	109	80	56
Chlordane	22.03–22.11	375	266 + 301	130	88
Endosulfan alpha	22.03–22.09	241	170:172 + 204:206 + 136	80	72
Phenamiphos	22.49–22.64	303	195	95	56
Protiophos	22.68–22.73	309	239 + 281	120	70
Chlorfenson	22.68–22.81	175	111	70	47
Hexaconazole	22.68–22.79	231	175	102	70
Fludioxonil	23.15–23.50	248	127 + 182 + 154	89	84
Buprofezin	23.39–23.46	249	191:195	80	50
Oxifluorfen	23.45–23.55	252	169 + 120 + 146	90	87
Myclobutanil	23.41–23.57	179	125	80	65
Bupirimate	23.44–23.52	273	193	120	77
Kresoxim methyl	23.51–23.58	206	116 + 132	91	60
Chlorfenapyr	23.87–23.97	364	363	160	100
Cyproconazole	24.13–24.15	222	125	85	69
Chloropropylate	24.54–24.61	251	139	100	74

Table 1 (*Continued*)

Pesticide	RTW (min)	Precursor ion (*m*/*z*)	Quantitation ion (*m*/*z*)[a]	CID RF (*m*/*z*)	CID Amplitude (V)
Endosulfan beta	24.74–24.86	241	170:172 + 206 + 136	80	72
Ethion	24.91–24.98	231	175 + 203	100	63
Oxadixyl	24.99–25.18	163	132	71	46
Aclonifen	25.20–25.33	264	229 + 211	115	78
Benalaxyl	25.96–26.04	148	118	50	46
Carbophenothion	26.13–26.21	342	199 + 157	131	58
Cyanofenphos	26.25–26.36	157	139 + 110	60	53
Norflurazol	26.45–26.66	302	234 + 260	95	82
Propiconazol	26.52–26.54	259	191 + 173	114	77
Nuarimol	27.20–27.31	235	139	75	56
Tebuconazole	27.33–27.46	250	125	75	57
Iprodione	28.63–28.74	314	245 + 271	125	84
Bifenthrin	28.76–28.80	181	165	50	30
Bromopropylate	28.89–28.96	341	181:187	110	78
Fenoxycarb	29.17–29.19	116	88	51	36
Fenpropathrin	29.25–29.32	265	210	95	72
Bifenox	29.68–29.79	341	281 + 311	130	86
Furathiocarb	29.88–29.92	325	194	140	77
Tetradifon	30.13–30.23	356	159:161 + 227:229	120	65
Phosalone	30.34–30.49	182	111 + 138	87	0.24
Azinphos methyl	30.66–30.68	132	104	60	59
Pyriproxyfen	30.79–30.84	136	96	59	57
Cyhalothrin	30.79–30.85	181	152	80	88
Acrinathrin	31.66–31.72	181	152	80	88
Pyrazophos	31.67–31.73	265	210	80	51
Fenarimol	31.68–31.78	139	111	50	48
Azinphos	31.99–32.09	132	104	60	60
Permethrin	33.28–33.30	183	165	70	0.34
Pyridaben	33.49–33.55	309	147	100	55
Prochloraz	33.64–33.75	308	202 + 244	130	97
Cyfluthrin	34.76–34.78	206	150	86	96
Boscalid	35.38–35.46	342	307 + 230	100	60
Cypermethrin	35.22–35.30	163	127	70	52
Flucythrinate	35.50–35.53	157	107	79	69
Silafluofen	35.82–35.86	286	258	115	56
Fenvalerate	36.56–36.58	225	119	70	49
Esfenvalerate	36.56–36.58	225	119	70	51
Tau fluvalinate	36.56–36.62	250	200 + 214	71	56
Difenoconazole	37.16–37.18	323	265	122	87
Deltamethrin	37.51–37.59	253	172 + 174	90	61
Azoxystrobin	37.82–37.90	345	273 + 210	115	92
Dimethomorph	38.30–38.32	301	165	120	88

[a] Numbers separated by + (e.g. 120 + 108) means that the two masses were used individually for quantitation; numbers separated by: (e.g. 122:132) means that the masses ranging from 122 to 132 were used for quantitation.

analyzers have been used for the analysis of pesticides in urine [19,20], because MS allows the sensitive and unambiguous detection, avoiding most of the matrix interferences.

However, biological fluids such as urine are very complex matrices and have a large number of components [3]. Therefore the determination of pesticides in urine implies the extraction of the pesticides from the matrix and generally a clean-up step previous chromatographic analysis is necessary [8,21]. Besides, and despite the low amount of proteins in urine, some methods include a preliminary step to precipitate the proteins [22]. Among the most applied techniques, liquid–liquid extraction (LLE) has been used for many years as routine technique [23,24], but there are several disadvantages such as it is time-consuming and it requires large amount of organic solvents [16,21], which are expensive, increasing the operator exposure to solvent vapours. Furthermore, an evaporation step is sometimes needed, which can lead to degradation of some compounds [25]. In this sense, solid phase extraction (SPE) has been developed as a powerful alternative, due to its simplicity and because it needs less organic solvents than LLE [19,26]. Besides, when SPE is used, a cleaner extract is obtained and a better chromatographic outline is achieved [7]. Moreover, it is a technique that can be easily automated and reproducible results are usually obtained. Furthermore, selecting an adequate cartridge, it is able to extract multiple components, which present different chemicals properties such as polarity [27]. However, this technique has been used for the extraction of a few compounds with similar properties [28], using C_{18} [22,29] or new sorbents [15] for the extraction.

Despite of the advantages of SPE, microextraction techniques such as solid-phase microextraction (SPME) [8,30] and stir bar sorptive extraction (SBSE), has been developed [31], although they have several problems such as high cost, sample carry-over and a decline in performance with time [32] or they are not allowed the extraction of polar pesticides [33]. Therefore SPE is still the accepted technique for the extraction of pesticides from urine.

The aim of this work has been the development of an extraction method that allows the simultaneous extraction of a wide range of polar and non-polar pesticides in urine applying SPE. Extracted polar and non-polar pesticides were determined by ultra high pressure liquid chromatography (UHPLC) and GC coupled to triple quadrupole (QqQ-MS/MS) and ion trap (IT-MS/MS) systems, respectively.

2. Materials and methods

2.1. Chemicals and materials

Certified pesticide standards (including carbamates, organochlorine, organophosphorous, pyrethroids, herbicides and insecticides) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), Riedel-de-Haën (Seelze-Hannover, Germany)

Table 2
Retention time windows (RTWs) and UHPLC-QqQ-MS/MS conditions for the selected pesticides.

Pesticide	RTW (min)	Cone voltage (V)	Quantitation transition[a]	Confirmation transition[a]	Ion ratio
Methomyl	2.19–2.24	10	185.0 > 128.0 (15)	185.0 > 88.0 (15)	0.54
Demeton-S-methyl sulfone	2.27–2.29	30	263.0 > 169.0 (17)	263.0 > 121.0 (17)	0.40
Carbendazim	2.29–2.32	30	192.2 > 160.1 (13)	192.2 > 132.2 (19)	0.02
Thiamethoxam	2.32–2.35	25	292.0 > 132.0 (25)	292.0 > 181.0 (25)	0.59
Dicrotophos	2.58–2.60	30	238.0 > 112.0 (10)	238.0 > 193.0 (10)	0.37
Ethiofencarb sulfone	2.64–2.66	25	258.0 > 107.0 (18)	258.0 > 201.1 (5)	0.61
Thiabendazol	2.66–2.68	20	202.2 > 175.1 (30)	202.2 > 131.1 (30)	0.89
Imidacloprid	2.74–2.77	25	256.2 > 209.3 (15)	256.2 > 175.1 (15)	0.97
Tiofanox sulfone	2.85–2.87	18	251.0 > 57.2 (10)	251.0 > 76.1 (6)	0.48
Vamidothion	2.94–2.96	15	288.2 > 146.0 (13)	288.2 > 118.0 (20)	0.21
3-Hydroxy carbofuran	2.98–3.00	20	238.0 > 181.0 (10)	238.0 > 163.0 (16)	0.94
Acetamiprid	3.01–3.03	25	223.3 > 126.0 (18)	223.3 > 56.1 (18)	0.36
Chloridazon	3.06–3.09	30	222.0 > 92.0 (30)	895.1 > 104.0 (30)	0.55
Cymoxanil	3.18–3.20	20	199.0 > 128.0 (8)	199.0 > 111.0 (18)	0.86
Thiacloprid	3.28–3.30	25	253.3 > 156.0 (16)	253.3 > 186.1 (16)	0.10
Butocarboxim	3.49–3.51	24	213.0 > 75.0 (15)	213.0 > 156.0 (10)	0.10
Aldicarb	3.52–3.54	30	213.0 > 116.0 (11)	213.0 > 89.0 (16)	0.58
Phosphamidon	3.62–3.77	28	300.0 > 127.0 (25)	300.0 > 174.0 (14)	0.90
Simazine	4.01–4.03	25	202.3 > 104.0 (25)	202.3 > 96.0 (25)	0.55
Bendiocarb	4.04–4.06	20	224.1 > 167.1 (10)	224.1 > 109.0 (15)	0.81
Carbofuran	4.04–4.06	20	223.3 > 165.1 (14)	223.3 > 123.0 (20)	0.64
Ofurace	4.06–4.08	20	282.2 > 160.2 (25)	282.2 > 254.3 (15)	0.53
Demeton S methyl	4.10–4.12	10	231.1 > 89.1 (10)	231.1 > 61.2 (30)	0.10
Carbaryl	4.17–4.32	10	202.3 > 145.1 (6)	202.3 > 127.0 (30)	0.15
Desmetrine	4.22–4.24	35	214.1 > 172.1 (20)	214.1 > 82.1 (30)	0.43
Imazalil	4.29–4.31	20	297.2 > 159.0 (25)	297.2 > 201.1 (20)	0.14
Thiodicarb	4.35–4.42	30	377.0 > 88.0 (20)	377.0 > 113.0 (20)	0.34
Azadirachtin	4.36–4.39	35	743.1 > 724.9 (30)	743.1 > 665.2 (35)	0.44
Disulfoton sulfoxide	4.54–4.56	24	291.0 > 185.0 (14)	291.0 > 97.0 (31)	0.75
Disulfoton sulfone	4.63–4.65	24	307.0 > 97.1 (28)	307.0 > 153.1 (12)	0.44
Flutriafol	4.70–4.72	32	302.0 > 70.2 (18)	302.0 > 123.0 (29)	0.28
Fensulfothion	4.79–4.82	30	309.1 > 281.2 (15)	309.1 > 157.0 (26)	0.76
Fenpropimorph	4.87–4.89	10	304.4 > 147.1 (30)	304.4 > 97.9 (35)	0.85
Forchlorfenuron	4.89–4.92	30	248.1 > 128.7 (13)	248.1 > 155.0 (13)	0.25
Azaconazole	4.92–4.96	20	300.1 > 159.0 (23)	300.1 > 231.0 (16)	0.37
Diuron	4.92–4.94	25	233.0 > 72.0 (25)	233.0 > 160.0 (25)	0.05
Diethofencarb	5.24–5.26	20	268.3 > 226.3 (10)	268.1 > 152.1 (20)	0.38
Linuron	5.36–5.39	25	249.1 > 160.1 (17)	249.1 > 182.1 (17)	0.60
Ethiprole	5.43–5.47	30	397.5 > 256.0 (25)	397.5 > 352.0 (20)	0.18
Methiocarb	5.43–5.47	20	226.2 > 121.1 (20)	226.2 > 169.2 (20)	0.12
Terbuthylazine	5.44–5.47	25	230.4 > 174.1 (18)	249.1 > 95.9 (25)	0.16
Flutalonil	5.54–5.59	27	324.4 > 242.3 (25)	324.4 > 262.3 (20)	0.81
Paclobutrazol	5.55–5.57	25	294.2 > 70.0 (20)	294.2 > 125.0 (30)	0.07
Promecarb	5.56–5.59	15	208.3 > 151.1 (10)	208.3 > 109.0 (13)	0.30
Methoxyfenozide	5.62–5.64	30	369.1 > 149.1 (18)	369.1 > 313.2 (8)	0.45
Flurochloridone	5.74–5.79	20	312.0 > 292.0 (20)	312.0 > 145.0 (30)	0.10
Bromuconazole	5.77–4.79	40	376.0 > 70.1 (25)	376.0 > 158.9 (35)	0.37
Triazophos	5.77–5.81	25	314.3 > 162.1 (18)	314.3 > 119.1 (30)	0.26
Iprovalicarb	5.78–5.86	25	321.4 > 119.1 (15)	321.4 > 203.3 (8)	0.65
Triadimenol	5.85–4.87	16	296.3 > 70.0 (8)	296.3 > 99.0 (15)	0.12
Diphenylamine	5.85–5.87	20	170.2 > 93.0 (25)	170.2 > 152.1 (25)	0.14
Fluquinconazole	5.86–5.88	40	376.0 > 307.0 (30)	376.0 > 349.0 (20)	0.86
Mepanipyrim	5.90–5.92	15	224.0 > 106.0 (20)	224.0 > 77.0 (35)	0.71
Fenhexamide	5.92–5.95	25	302.2 > 97.1 (25)	302.2 > 55.2 (30)	0.27
Mecarbam	5.93–5.95	20	329.8 > 226.8 (10)	329.8 > 198.8 (8)	0.12
Epoxiconazole	6.00–6.02	25	330.2 > 121.1 (20)	330.2 > 141.1 (20)	0.09
Fenbuconazole	6.12–6.18	30	337.3 > 70.1 (25)	337.3 > 125.0 (20)	0.64
Cyprodinil	6.15–6.18	20	226.3 > 93.0 (30)	226.3 > 108.0 (30)	0.79
Tebufenozide	6.19–6.21	19	353.0 > 133.0 (20)	353.0 > 297.0 (8)	0.96
Flusilazole	6.19–6.21	25	316.1 > 247.3 (20)	316.1 > 165.1 (20)	0.83
Diflubenzuron	6.19–6.25	20	311.1 > 158.1 (12)	311.1 > 141.1 (23)	0.68
Captafol	6.20–6.36	20	362.0 > 220.0 (20)	362.0 > 188.0 (20)	0.56
Diclobutrazol	6.33–6.35	30	328.2 > 70.2 (20)	328.2 > 159.0 (30)	0.08
Furmecyclox	6.41–6.44	20	252.3 > 170.2 (13)	252.3 > 110.0 (20)	0.47
Coumaphos	6.46–6.48	30	363.0 > 303.0 (18)	363.0 > 289.0 (18)	0.20
Pyraclostrobin	6.59–6.61	25	388.0 > 163.0 (20)	388.0 > 194.0 (20)	0.27
Triflumuron	6.63–6.67	25	359.1 > 156.1 (17)	359.1 > 139.1 (35)	0.90
Bitertanol	6.70–6.72	30	338.3 > 99.1 (15)	338.3 > 296.2 (10)	0.79
Pencycuron	6.77–6.79	30	329.4 > 125.0 (15)	329.4 > 218.3 (15)	0.13
Diniconazole-M	6.85–6.87	30	326.0 > 70.0 (30)	326.0 > 159.0 (30)	0.10
Indoxacarb	6.87–6.89	25	528.1 > 293.3 (15)	528.1 > 249.3 (15)	0.60
Trifloxystrobin	6.90–6.92	25	409.4 > 186.2 (15)	409.4 > 206.2 (15)	0.39
Triflumizole	6.98–7.02	25	346.3 > 278.3 (20)	346.3 > 43.1 (35)	0.90
Tebufenpyrad	7.24–7.27	25	334.2 > 145.0 (25)	334.2 > 117.0 (35)	0.86
Terbufos	7.24–7.26	15	289.3 > 103.0 (8)	289.3 > 57.0 (20)	0.20
Piperonyl butoxide	7.34–7.39	15	356.0 > 177.0 (10)	356.0 > 119.0 (30)	0.23

Table 2 (*Continued*)

Pesticide	RTW (min)	Cone voltage (V)	Quantitation transition[a]	Confirmation transition[a]	Ion ratio
Bioallethrin	7.36–7.38	20	303.0 > 93.0 (18)	303.0 > 107.0 (18)	0.62
Lufenuron	7.37–7.39	30	511.5 > 158.2 (20)	511.5 > 141.1 (20)	0.14
Teflubenzuron	7.39–7.49	26	380.9 > 158.0 (20)	380.9 > 140.9 (40)	0.99
Quinoxyfen	7.51–7.55	40	308.0 > 197.0 (30)	308.0 > 162.0 (30)	0.02
Hexythiazox	7.52–7.54	30	353.0 > 228.0 (15)	353.0 > 168.0 (26)	0.77
Tricresyl phosphate	7.56–7.61	30	369.1 > 166.2 (30)	369.1 > 90.9 (30)	0.76
Spiromesifen	7.59–7.61	16	371.0 > 273.0 (10)	371.0 > 255.0 (24)	0.14
Flufenoxuron	7.68–7.70	30	489.1 > 158.0 (15)	489.1 > 141.0 (30)	0.42
Fenpyroximate	7.78–7.80	30	422.2 > 366.1 (15)	422.2 > 138.0 (15)	0.32
Fenazaquin	8.08–8.10	30	307.3 > 161.2 (17)	307.3 > 147.0 (23)	0.22
Abamectin	8.19–8.21	70	895.1 > 1830.1 (50)	895.1 > 327.6 (50)	0.51

[a] Collision energy (eV) is given in brackets.

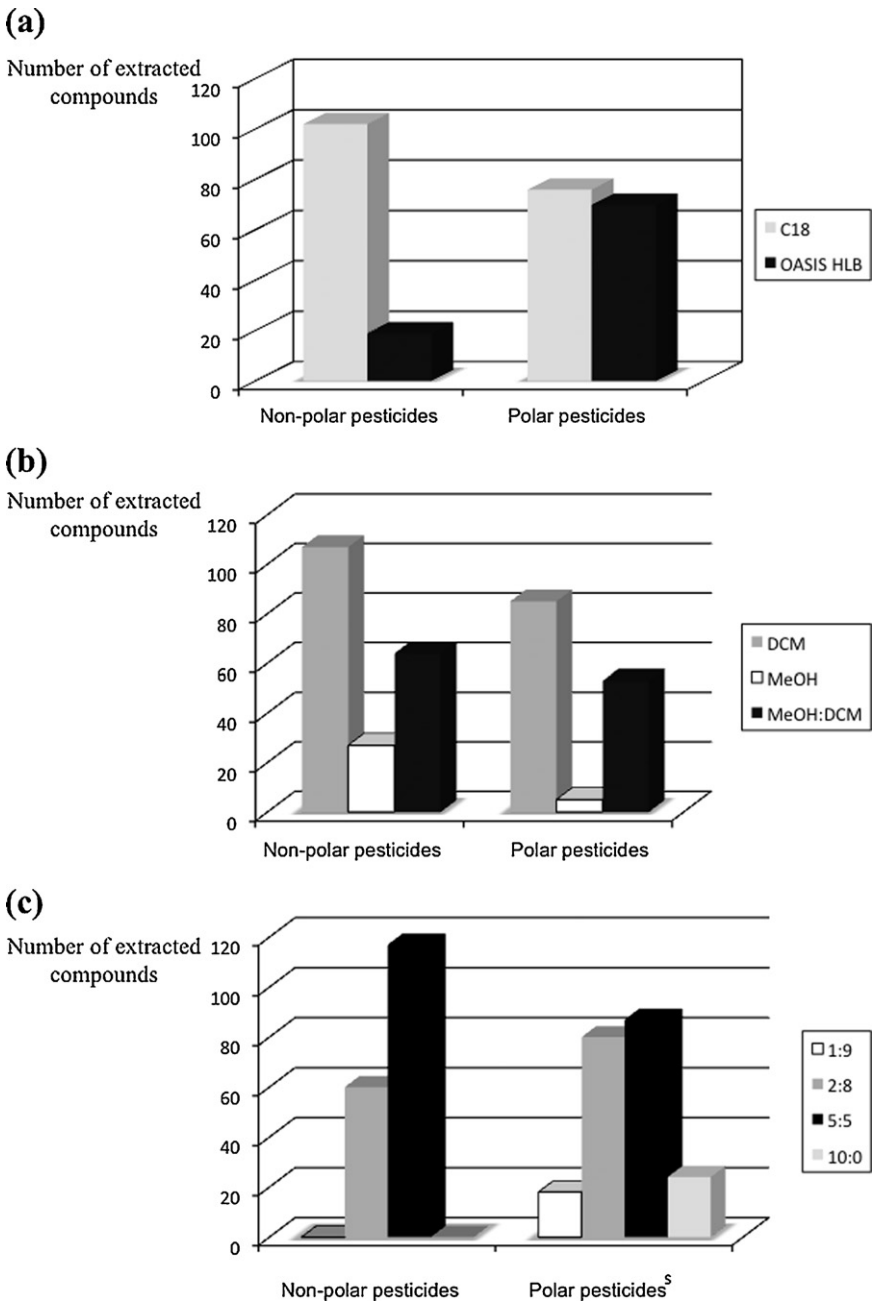

Fig. 1. Effect of (a) type of cartridge, (b) type of solvent and (c) dilution of sample (1:9, 2:8, 5:5 and 10:0, urine:water) on the number of extracted compounds when a blank urine sample was spiked at 10 $\mu g\,L^{-1}$. *Abbreviations*: DCM, dichloromethane; MEOH, methanol.

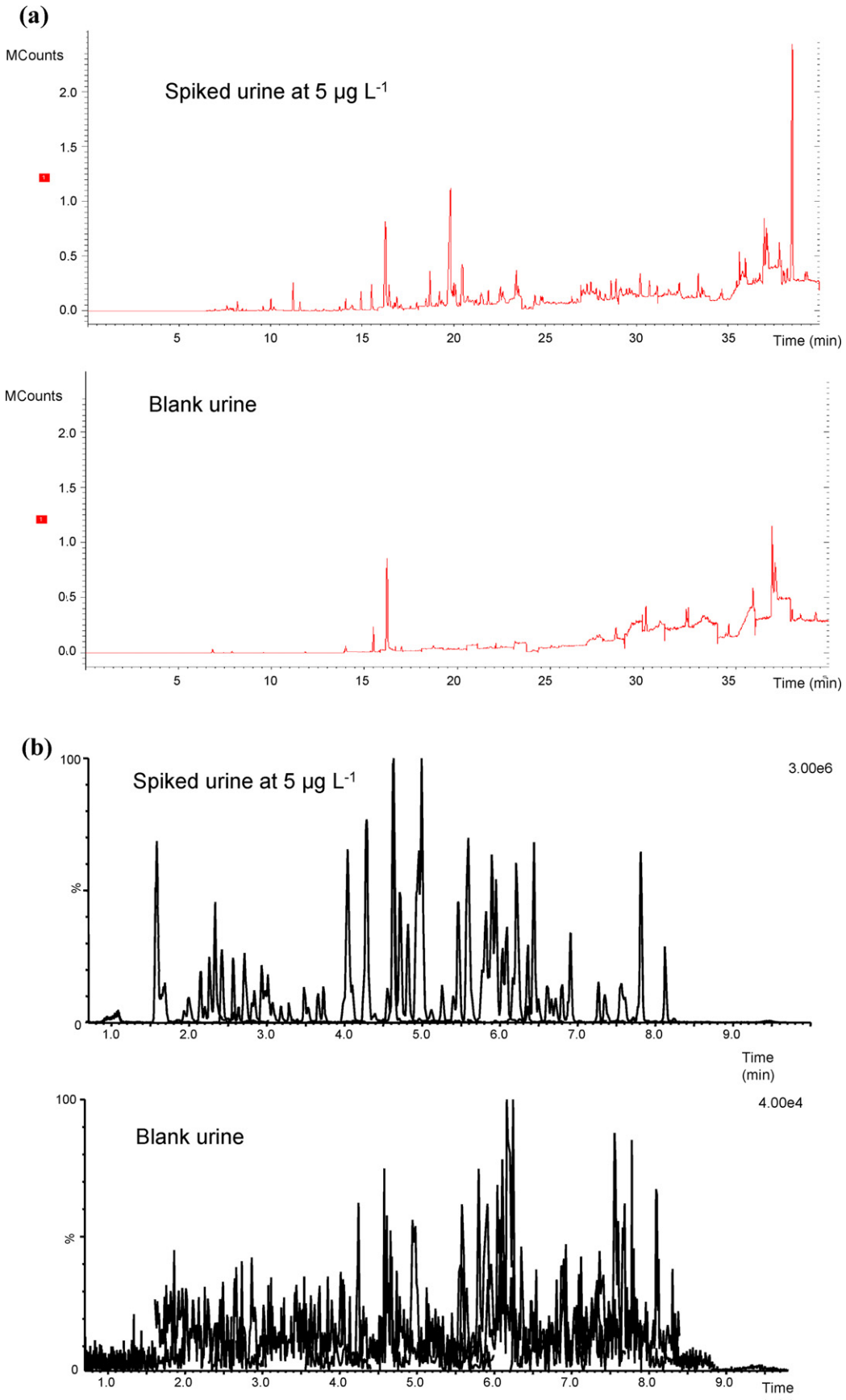

Fig. 2. Total ion chromatograms (TIC) of a urine sample spiked with the target pesticides at 5 µg L^{-1} and blank urine by (a) GC-IT-MS/MS and (b) combined UHPLC-QqQ-MS/MS, based on quantitation MS/MS transitions.

Table 3
Summary of the performance parameters evaluated in the validation protocol for GC-amenable pesticides.

Pesticide	Recovery (%)[a]			Interday precision[b]	LOD ($\mu g\,L^{-1}$)	LOQ ($\mu g\,L^{-1}$)
	5 $\mu g\,L^{-1}$	10 $\mu g\,L^{-1}$	50 $\mu g\,L^{-1}$			
Dichlorvos	89 (13)	74 (8)	82 (3)	15	0.040	0.181
Biphenyl	64 (17)	78 (14)	65 (12)	18	0.053	0.176
Mevinphos	84 (10)	109 (8)	93 (6)	13	0.023	0.091
Acephate	63 (20)	72 (13)	61 (7)	22	0.019	0.089
Chlormephos	77 (16)	78 (10)	62 (5)	17	0.124	0.388
Propoxur	73 (6)	91 (6)	97 (5)	8	0.012	0.022
Propachlor	68 (8)	106 (4)	97 (1)	12	0.024	0.079
Ethoprophos	70 (7)	103 (7)	97 (1)	10	0.080	0.265
Cycloate	73 (4)	111 (5)	76 (4)	13	0.004	0.015
Cadusafos	72 (17)	81 (13)	81 (6)	19	0.047	0.156
Dimethoate	69 (6)	88 (3)	108 (0)	12	0.067	0.145
Quintozene	77 (18)	81 (14)	69 (7)	21	0.144	0.479
Lindane	70 (20)	112 (18)	86 (13)	22	0.136	0.453
Fonofos	79 (11)	99 (9)	81 (2)	13	0.023	0.078
Diazinon	76 (9)	119 (6)	89 (4)	11	0.022	0.073
Pyrimethanil	118 (18)	106 (10)	82 (7)	21	0.022	0.073
Tefluthrin	70 (17)	98 (15)	75 (11)	19	0.007	0.024
Etrimfos	72 (11)	118 (10)	108 (4)	14	0.073	0.243
Pirimicarb	87 (15)	90 (13)	86 (7)	17	0.047	0.158
Dichlofenthion	87 (7)	98 (7)	77 (3)	11	0.021	0.071
Chlorpyriphos methyl	70 (13)	109 (12)	117 (7)	18	0.127	0.423
Vinclozoline	88 (17)	82 (12)	77 (8)	21	0.011	0.036
Tolclofos methyl	72 (10)	113 (6)	94 (3)	11	0.021	0.068
Parathion methyl	77 (2)	91 (2)	81 (1)	7	0.012	0.088
Transfluthrin	72 (5)	105 (5)	84 (1)	11	0.014	0.046
Metalaxyl	79 (11)	119 (5)	99 (3)	13	0.070	0.233
S421	71 (15)	69 (8)	70 (6)	17	0.066	0.134
Pirimiphos methyl	76 (7)	114 (5)	95 (1)	12	0.008	0.025
Fenitrothion	78 (12)	93 (10)	86 (5)	16	0.010	0.039
Terbutryn	78 (6)	118 (6)	95 (2)	11	0.110	0.367
Ethofumesate	82 (12)	88 (5)	89 (4)	18	0.030	0.101
Malathion	93 (15)	117 (9)	74 (4)	17	0.028	0.068
Chlorpyrifos	89 (13)	100 (8)	83 (5)	19	0.010	0.034
Bromacil	83 (13)	86 (7)	80 (6)	17	0.086	0.286
Chlorthal-dimethyl	85 (5)	111 (2)	61 (2)	11	0.001	0.003
Fenthion	70 (5)	87 (3)	91 (1)	9	0.161	0.535
Parathion	85 (10)	84 (9)	111 (4)	10	0.023	0.099
Triadimefon	76 (21)	107 (6)	83 (9)	24	0.024	0.080
Tetraconazole	76 (6)	102 (5)	71 (2)	10	0.100	0.334
Isocarbofos	89 (17)	71 (19)	113 (10)	23	0.112	0.456
Butralin	62 (16)	90 (15)	77 (16)	19	0.030	0.102
Dicofol	77 (11)	110 (6)	116 (3)	14	0.019	0.062
Pirimifos	70 (18)	97 (17)	65 (11)	21	0.022	0.073
Bromophos methyl	84 (13)	101 (11)	104 (9)	17	0.033	0.096
Isophenphos methyl	77 (9)	113 (6)	75 (3)	12	0.005	0.016
Pendimethalin	65 (13)	96 (10)	77 (3)	17	0.010	0.035
Fipronil	83 (10)	77 (5)	72 (2)	13	0.055	0.182
Penconazole	75 (8)	106 (5)	81 (6)	11	0.095	0.316
Isophenphos	73 (9)	111 (6)	88 (3)	12	0.031	0.104
Pyrifenox	74 (6)	110 (5)	86 (3)	11	0.044	0.146
Chlorfenviphos	75 (9)	106 (8)	78 (6)	14	0.033	0.108
Phentoate	86 (21)	99 (15)	120 (7)	24	0.115	0.384
Quinalphos	78 (20)	105 (17)	107 (5)	22	0.104	0.346
Furalaxyl	75 (16)	109 (10)	102 (6)	19	0.077	0.258
Procymidone	70 (4)	110 (4)	96 (2)	9	0.410	1.367
Bromophos	70 (15)	90 (10)	84 (4)	18	0.016	0.053
Metidathion	82 (18)	107 (16)	107 (8)	22	0.034	0.089
Chlorbenside	66 (7)	91 (4)	67 (3)	11	0.007	0.024
Chinomethionat	79 (11)	78 (7)	72 (7)	16	0.026	0.087
Tetrachlorvinphos	71 (15)	83 (10)	87 (8)	22	0.058	0.108
Chlordane	73 (6)	101 (2)	82 (3)	9	0.090	0.299
Endosulfan alpha	79 (9)	106 (2)	79 (1)	9	0.047	0.156
Phenamiphos	75 (15)	79 (9)	86 (3)	21	0.034	0.114
Protiophos	79 (21)	99 (13)	91 (7)	24	0.042	0.140
Chlorfenson	82 (17)	107 (16)	83 (11)	23	0.003	0.010
Hexaconazole	78 (9)	90 (3)	72 (2)	13	0.100	0.335
Fludioxonil	90 (13)	109 (7)	78 (5)	18	0.021	0.071
Buprofezin	84 (20)	103 (19)	87 (11)	25	0.101	0.336
Oxifluorfen	67 (14)	91 (9)	75 (11)	15	0.045	0.149
Myclobutanil	89 (13)	100 (10)	95 (7)	17	0.004	0.015
Bupirimate	74 (12)	114 (10)	76 (5)	18	0.023	0.078
Kresoxim methyl	70 (9)	117 (7)	102 (2)	12	0.005	0.016
Chlorfenapyr	61 (18)	92 (13)	86 (9)	25	0.182	0.605
Cyproconazole	83 (14)	104 (7)	84 (7)	18	0.003	0.011

Table 3 (*Continued*)

Pesticide	Recovery (%)[a]			Interday precision[b]	LOD ($\mu g\,L^{-1}$)	LOQ ($\mu g\,L^{-1}$)
	5 $\mu g\,L^{-1}$	10 $\mu g\,L^{-1}$	50 $\mu g\,L^{-1}$			
Chloropropylate	67 (16)	100 (8)	72 (1)	21	0.002	0.008
Endosulfan beta	77 (11)	103 (6)	88 (4)	17	0.043	0.142
Ethion	86 (19)	87 (21)	75 (15)	24	0.015	0.050
Oxadixyl	90 (17)	110 (14)	88 (9)	22	0.013	0.043
Aclonifen	77 (13)	89 (11)	80 (3)	15	0.178	0.593
Benalaxyl	96 (11)	114 (8)	72 (5)	19	0.069	0.231
Carbophenothion	79 (22)	99 (15)	108 (6)	24	0.102	0.200
Cyanofenphos	77 (14)	101 (13)	82 (9)	18	0.058	0.192
Norflurazol	83 (8)	112 (2)	97 (1)	12	0.027	0.090
Propiconazol	82 (10)	115 (5)	85 (3)	11	0.028	0.092
Nuarimol	70 (9)	116 (6)	86 (3)	12	0.012	0.041
Tebuconazole	75 (9)	93 (4)	72 (1)	15	0.006	0.018
Iprodione	65 (11)	74 (5)	74 (8)	13	0.436	1.452
Bifenthrin	77 (10)	99 (5)	79 (6)	12	0.026	0.087
Bromopropylate	73 (13)	107 (8)	76 (3)	15	0.007	0.024
Fenoxycarb	85 (14)	71 (16)	83 (10)	17	0.033	0.109
Fenpropathrin	84 (11)	89 (5)	78 (1)	13	0.023	0.075
Bifenox	70 (20)	92 (19)	102 (10)	25	0.013	0.078
Furathiocarb	67 (14)	75 (13)	77 (5)	17	0.015	0.098
Tetradifon	88 (15)	105 (9)	77 (3)	19	0.101	0.338
Phosalone	92 (13)	71 (6)	116 (9)	12	0.046	0.058
Azinphos methyl	99 (10)	82 (8)	75 (5)	20	0.014	0.069
Pyriproxyfen	70 (19)	86 (15)	76 (6)	24	0.015	0.050
Cyhalothrin	68 (10)	115 (16)	86 (7)	15	0.253	0.844
Acrinathrin	69 (12)	73 (12)	75 (6)	22	0.083	0.275
Pyrazophos	70 (19)	76 (14)	112 (8)	23	0.356	1.187
Fenarimol	83 (22)	117 (16)	78 (9)	26	0.020	0.066
Azinphos	109 (18)	74 (18)	75 (12)	22	0.035	0.118
Permethrin	95 (5)	107 (4)	71 (2)	11	0.028	0.093
Pyridaben	78 (11)	90 (5)	70 (2)	13	0.012	0.041
Prochloraz	75 (5)	91 (5)	89 (3)	8	0.112	0.258
Cyfluthrin	77 (18)	76 (14)	85 (8)	24	0.182	0.606
Boscalid	78 (15)	113 (16)	93 (11)	19	0.025	0.085
Cypermethrin	75 (13)	85 (5)	81 (4)	18	0.054	0.180
Flucythrinate	73 (18)	80 (9)	82 (5)	23	0.008	0.027
Silafluofen	64 (10)	103 (5)	81 (1)	17	0.008	0.026
Fenvalerate	88 (8)	90 (3)	74 (2)	15	0.019	0.030
Esfenvalerate	88 (18)	90 (13)	87 (5)	24	0.022	0.046
Tau fluvalinate	71 (15)	85 (12)	85 (8)	20	0.019	0.189
Difenoconazole	77 (16)	97 (18)	82 (12)	21	0.076	0.252
Deltamethrin	62 (12)	65 (13)	79 (6)	17	0.026	0.301
Azoxystrobin	81 (11)	107 (10)	88 (9)	15	0.140	0.466
Dimethomorph	88 (6)	115 (5)	90 (1)	9	0.019	0.063

[a] Repeatability values expressed as RSD are given in brackets ($n = 5$).
[b] RSD values obtained at 5 $\mu g\,L^{-1}$ ($n = 5$).

and Chemservice (Milan, Italy). Isotopically labelled caffeine (caffeine ^{13}C), which was used as internal standard (IS), was provided by Dr. Ehrenstorfer GmbH. Stock standard solutions of individual compounds (with concentrations between 173.1 and 1903.2 mg L^{-1}), were prepared by exact weighing of powder or liquid and dissolution in 50 mL of methanol for LC-amenable pesticides and in 50 mL of acetone for GC-amenable pesticides. These solutions were then stored under refrigeration ($T < 5\,^{\circ}C$). Two multi-compound working standard solutions (LC and GC-amenable pesticides), containing 2 mg L^{-1} of each component, were prepared by further dilution of the individual stock standard solutions with methanol (LC-amenable pesticides) or acetone (GC-amenable pesticides) and stored under refrigeration at 4 °C. A working standard solution of caffeine ^{13}C (20 mg L^{-1}) was prepared by appropriate dilution of the stock solution with acetone and stored under the aforementioned conditions. Analytical grade acetone, dichloromethane and ethyl acetate were obtained from Panreac (Barcelona, Spain), Riedel-de Haën (Seelze-Hannover, Germany) and J.T. Baker (Deventer, Holland), respectively. HPLC-grade methanol was supplied by Sigma (Madrid, Spain). Formic acid was purchased from Fluka (Seelze, Germany). Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA). Cartridges C_{18} Sep-Pak (500 mg) and Oasis HLB (500 mg) cartridges were obtained from Waters (Milford, MA, USA), and they were used for concentration and clean up during optimisation of the extraction procedure.

2.2. Apparatus

SPE was performed using an extraction manifold from Waters connected to a Büchi Vac V-500 (Flawil, Switzerland) vacuum system. An analytical balance AB204-S from Mettler Toledo (Greifensee, Switzerland) and a rotary evaporator R-114 (Büchi, Flawil, Switzerland) were used during extraction.

For GC-amenable pesticides, chromatographic analyses were performed by GC-IT-MS/MS system Varian 3800, equipped with electronic flow control (EFC) and fitted with a Saturn 2000 ion-trap mass spectrometer from Varian (Walnut Creek, CA, USA). Samples were injected with a Varian 8200 autosampler with a 100 μL syringe into an SPI/1079 split/splitless programmed-temperature injector operated in the large-volume injection mode. A fused-silica untreated capillary column (2 m × 0.25 mm i.d.) from Supelco (Bellefonte, PA, USA), was used as guard column connected to a Factor Four Capillary Column VF-5ms (30 m × 0.25 mm i.d. × 0.25 μm film thickness) purchased from Varian (Sunnyvale, CA, USA). MS/MS detection was used and an MS/MS library was created for

Table 4
Summary of the performance parameters evaluated in the validation protocol for LC-amenable pesticides.

Pesticide	Recovery (%)[a]			Intermediate precision[b]	LOD ($\mu g\,L^{-1}$)	LOQ ($\mu g\,L^{-1}$)
	5 $\mu g\,L^{-1}$	10 $\mu g\,L^{-1}$	50 $\mu g\,L^{-1}$			
Methomyl	70 (11)	107 (7)	103 (7)	18	0.074	0.246
Demeton-S-methyl sulfone	70 (9)	84 (7)	82 (7)	13	0.032	0.106
Carbendazim	76 (18)	105 (15)	87 (7)	17	0.040	0.135
Thiamethoxam	71 (8)	81 (4)	75 (2)	11	0.217	0.723
Dicrotophos	61 (10)	60 (10)	74 (9)	16	0.060	0.182
Ethiofencarb sulfone	77 (11)	106 (9)	104 (6)	19	0.037	0.123
Thiabendazol	77 (13)	107 (9)	103 (5)	16	0.127	0.424
Imidacloprid	81 (15)	82 (10)	101 (4)	19	0.087	0.289
Tiofanox sulfone	87 (21)	104 (13)	102 (4)	26	0.091	0.301
Vamidothion	70 (10)	77 (8)	77 (2)	17	0.011	0.036
3-hydroxy carbofuran	67 (17)	73 (12)	77 (3)	19	0.247	0.822
Acetamiprid	72 (6)	67 (2)	76 (1)	11	0.012	0.041
Chloridazon	84 (22)	92 (16)	97 (6)	25	0.050	0.167
Cymoxanil	72 (18)	76 (14)	112 (13)	21	0.056	0.187
Thiacloprid	92 (10)	100 (9)	93 (7)	14	0.011	0.036
Butocarboxim	67 (18)	114 (13)	101 (6)	21	0.048	0.160
Aldicarb	72 (19)	120 (15)	119 (4)	20	0.405	1.350
Phosphamidon	80 (9)	66 (7)	73 (4)	14	0.036	0.121
Simazine	83 (8)	67 (8)	65 (5)	11	0.026	0.086
Bendiocarb	70 (19)	107 (7)	125 (6)	23	0.012	0.040
Carbofuran	72 (10)	115 (4)	119 (3)	12	0.020	0.068
Ofurace	87 (9)	90 (5)	85 (3)	18	0.035	0.115
Demeton S methyl	74 (17)	73 (13)	99 (5)	21	0.005	0.016
Carbaryl	73 (5)	107 (4)	90 (2)	12	0.162	0.541
Desmetrine	79 (6)	108 (5)	107 (2)	15	0.006	0.022
Imazalil	67 (13)	62 (10)	104 (2)	14	0.138	0.461
Thiodicarb	65 (20)	111 (17)	101 (11)	23	0.040	0.172
Azadirachtin	77 (16)	104 (9)	105 (6)	18	0.057	0.191
Disulfoton sulfoxide	83 (6)	108 (5)	106 (6)	10	0.038	0.128
Disulfoton sulfone	76 (9)	103 (5)	107 (4)	14	0.031	0.105
Flutriafol	61 (10)	100 (4)	102 30)	12	0.013	0.044
Fensulfothion	79 (3)	104 (2)	80 (1)	10	0.027	0.090
Fenpropimorph	73 (4)	119 (4)	108 (1)	13	0.010	0.033
Forchlorfenuron	81 (15)	106 (8)	83 (2)	21	0.055	0.184
Azaconazole	84 (8)	109 (5)	107 (2)	15	0.014	0.047
Diuron	74 (16)	119 (9)	118 (4)	18	0.071	0.236
Diethofencarb	89 (9)	105 (5)	76 (2)	11	0.041	0.138
Linuron	83 (11)	108 (11)	101 (8)	14	0.032	0.107
Ethiprole	84 (16)	103 (9)	102 (3)	19	0.030	0.124
Methiocarb	75 (8)	108 (6)	100 (2)	15	0.025	0.083
Terbuthylazine	87 (12)	107 (5)	82 (4)	13	0.010	0.032
Flutalonil	89 (7)	74 (4)	79 (2)	11	0.026	0.085
Paclobutrazol	64 (11)	107 (6)	106 (4)	15	0.011	0.037
Promecarb	77 (16)	108 (13)	108 (4)	21	0.036	0.119
Methoxyfenozide	72 (7)	105 (5)	88 (4)	12	0.022	0.056
Flurochloridone	75 (11)	100 (9)	101 (4)	14	0.016	0.054
Bromuconazole	74 (15)	69 (13)	100 (14)	22	0.077	0.257
Triazophos	81 (7)	77 (6)	105 (4)	11	0.017	0.057
Iprovalicarb	77 (7)	101 (2)	100 (1)	12	0.049	0.163
Triadimenol	73 (10)	103 (9)	103 (3)	14	0.025	0.084
Diphenylamine	77 (17)	105 (16)	105 (9)	23	0.016	0.095
Fluquinconazole	101 (7)	108 (4)	109 (1)	14	0.013	0.044
Mepanipyrim	73 (8)	83 (8)	81 (5)	12	0.014	0.046
Fenhexamide	76 (9)	105 (6)	106 (1)	11	0.042	0.139
Mecarbam	93 (6)	112 (7)	104 (4)	12	0.012	0.041
Epoxiconazole	73 (7)	100 (6)	77 (6)	15	0.013	0.043
Fenbuconazole	84 (8)	100 (7)	107 (6)	13	0.029	0.098
Cyprodinil	75 (15)	68 (12)	73 (5)	22	0.032	0.101
Tebufenozide	73 (8)	103 (4)	106 (2)	14	0.061	0.202
Flusilazole	83 (6)	104 (2)	104 (4)	12	0.009	0.031
Diflubenzuron	91 (6)	105 (5)	101 (4)	15	0.031	0.103
Captafol	93 (13)	103 (4)	89 (0)	23	0.607	2.023
Diclobutrazol	75 (18)	103 (15)	103 (8)	22	0.077	0.160
Furmecyclox	75 (5)	96 (9)	112 (2)	12	0.003	0.011
Coumaphos	77 (20)	119 (16)	105 (8)	24	0.054	0.078
Pyraclostrobin	71 (17)	120 (8)	101 (2)	22	0.060	0.199
Triflumuron	73 (20)	103 (6)	109 (6)	24	0.035	0.118
Bitertanol	85 (16)	101 (11)	106 (7)	19	0.078	0.261
Pencycuron	71 (7)	108 (2)	105 (1)	13	0.033	0.111
Diniconazole-M	60 (24)	104 (13)	107 (9)	27	0.147	0.491
Indoxacarb	73 (24)	103 (11)	103 (8)	26	0.112	0.374
Trifloxystrobin	73 (14)	107 (12)	108 (5)	15	0.005	0.017
Triflumizole	70 (20)	101 (19)	106 (18)	22	0.244	0.813
Tebufenpyrad	71 (15)	105 (9)	109 (6)	17	0.029	0.096

Table 4 (*Continued*)

Pesticide	Recovery (%)[a]			Intermediate precision[b]	LOD ($\mu g\,L^{-1}$)	LOQ ($\mu g\,L^{-1}$)
	5 $\mu g\,L^{-1}$	10 $\mu g\,L^{-1}$	50 $\mu g\,L^{-1}$			
Terbufos	74 (6)	117 (4)	105 (2)	15	0.059	0.197
Piperonyl butoxide	73 (14)	109 (5)	106 (3)	17	0.047	0.155
Bioallethrin	79 (12)	108 (11)	105 (5)	14	0.098	0.140
Lufenuron	72 (15)	105 (11)	110 (8)	19	0.032	0.069
Teflubenzuron	73 (13)	111 (16)	118 (11)	20	1.048	3.494
Quinoxyfen	78 (4)	120 (3)	119 (2)	11	0.017	0.055
Hexythiazox	60 (9)	111 (7)	105 (3)	14	0.010	0.033
Tricresyl phosphate	77 (15)	104 (14)	111 (8)	17	0.038	0.125
Spiromesifen	67 (21)	96 (16)	120 (10)	24	0.038	0.125
Flufenoxuron	76 (9)	111 (8)	124 (4)	14	0.069	0.230
Fenpyroximate	81 (14)	108 (12)	101 (8)	18	0.008	0.026
Fenazaquin	70 (7)	100 (5)	107 (3)	15	0.026	0.086
Abamectin	89 (19)	111 (9)	120 (4)	24	0.153	0.510

[a] Repeatability values expressed as RSD are given in brackets ($n = 5$).
[b] RSD values obtained at 5 $\mu g\,L^{-1}$ ($n = 5$).

the target analytes under our experimental conditions, and stored in a computer. The carrier and collision gas was helium (99.9999%), which was used at a constant flow rate of 1 mL min^{-1}. The mass spectrometer was calibrated weekly with perfluorotributylamine. Data were collected by use of Saturn GC/MS Workstation 5.51 software (Varian).

On the other hand, chromatographic analyses of LC-amenable pesticides were carried out using an Acquity UPLC™ system (Waters, Milford, MS, USA) and separations were achieved using an Acquity UPLC™ BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm particle size) from Waters. MS/MS detection was performed using a Quattro Premier XE (Waters, Manchester, UK). Data acquisition was performed using MassLynx 4.1 software with QuanLynx program (Waters).

2.3. GC-IT-MS/MS analysis

Aliquots of 10 μL of sample extract were injected into the GC operating at a syringe injection flow rate of 10 μL s^{-1}. The initial injector temperature of 70 °C, was held for 3.5 min and then increased at 100 °C min^{-1} to 310 °C, which was supported for 10 min. After the injection, the column temperature, initially at 70 °C, was held for 3.5 min and then increased at 50 °C min^{-1} to 150 °C. Then it was increased at 4 °C min^{-1} to 270 °C, and finally increased at 50 °C min^{-1} to 300 °C, which was held for 5 min. Under these conditions, all pesticides were eluted in a reasonably time (<32 min). The ion-trap mass spectrometer was operated in the electron impact (EI) mode. The transfer line, manifold, and trap temperatures were 280, 50, and 200 °C, respectively. The analysis was performed with a filament-multiplier delay of 4.50 min to prevent instrument damage. The automatic gain control (AGC) was activated with an AGC target of 5000 counts. The emission current for the ionization filament was set at 80 μA, generating electrons with energy of 70 eV. The axial modulation amplitude voltage was 4.0 V. The MS/MS process was performed by collision-induced dissociation (CID) with a non resonant excitation for all the compounds studied. The electron multiplier voltage was 1700 V (+200 V offset above the autotuning process). Scan rate and mass range scanned depended on the number of pesticides determined simultaneously. The particular MS/MS experimental conditions used are described in Table 1 for all the target pesticides.

2.4. UHPLC-QqQ-MS/MS analysis

Chromatographic analyses were carried out using gradient elution with eluent A being methanol and eluent B consisting of an aqueous solution of formic acid (0.01%, v/v). The analysis started with 10% of eluent A, which was linearly increased up to 50% A in 2.5 min, and then to 90% in 4.5 min. This composition was held for further 1.5 min before being returned to the initial conditions in 1 min, followed by a re-equilibration time of 1.5 min, giving a total time of 11 min. The flow rate was 0.35 mL min^{-1} and the column temperature was set at 30 °C. Aliquots of 5 μL of sample extract were injected.

Pesticides were detected using ESI in positive mode. The ESI source was set as follows: capillary voltage 3 kV, extractor voltage 5 V, source temperature 110 °C, desolvation temperature 350 °C, cone gas flow 80 L h^{-1} and desolvation gas flow 600 L h^{-1}, both gases were nitrogen (>95%). Collision-induced dissociation was performed using argon (99.9999%) as collision gas at a pressure of 4×10^{-3} mbar in the collision cell. The multiple reaction monitoring (MRM) transitions as well as the cone and collision energy voltages applied are summarized in Table 2.

2.5. Sample extraction

The optimised procedure for the simultaneous extraction of polar and non-polar pesticides in urine samples was as follows: C_{18} Sep-Pak cartridges (500 mg) were conditioned with 4 mL of dichloromethane, followed by 4 mL of MilliQ water, without allowing the cartridge to dry out. Then, 5 mL of MilliQ water was added to 5 mL urine and the samples were passed across the conditioned cartridges without vacuum. The cartridges were dried for two hours under vacuum. The retained analytes were eluted with 5 mL of dichloromethane. The extracts were evaporated to dryness with a vacuum rotary evaporator at 40 °C and the residue was dissolved in 2 mL of ethyl acetate solution of the IS (0.5 mg L^{-1}). One mL was taken directly for chromatographic analysis by GC-IT-MS/MS, whereas another millilitre was taken and evaporated under a gentle nitrogen stream. Then, the concentrated extract was re-dissolved with 1 mL of a mixture 1:1 (v/v) of methanol and aqueous solution of formic acid (0.01%, v/v) prior UHPLC-QqQ-MS/MS analysis.

2.6. Validation study

Validation experiments were performed using an uncontaminated urine sample. Linearity was evaluated using matrix-matched calibration, spiking blank extracts at different concentration levels (from 0.1 to 100 $\mu g\,L^{-1}$). Trueness (through recovery studies) was studied spiking blank urine samples at 5, 10 and 50 $\mu g\,L^{-1}$, and evaluating five replicates for each concentration level. Repeatability was evaluated at three concentration levels (5, 10 and 50 $\mu g\,L^{-1}$) and five replicates were carried out. Interday precision was evaluated at 5 $\mu g\,L^{-1}$ and spiked samples were analyzed daily for a period of 5 days. LODs and limits of quantification (LOQs) were estimated as the lowest concentration giving a

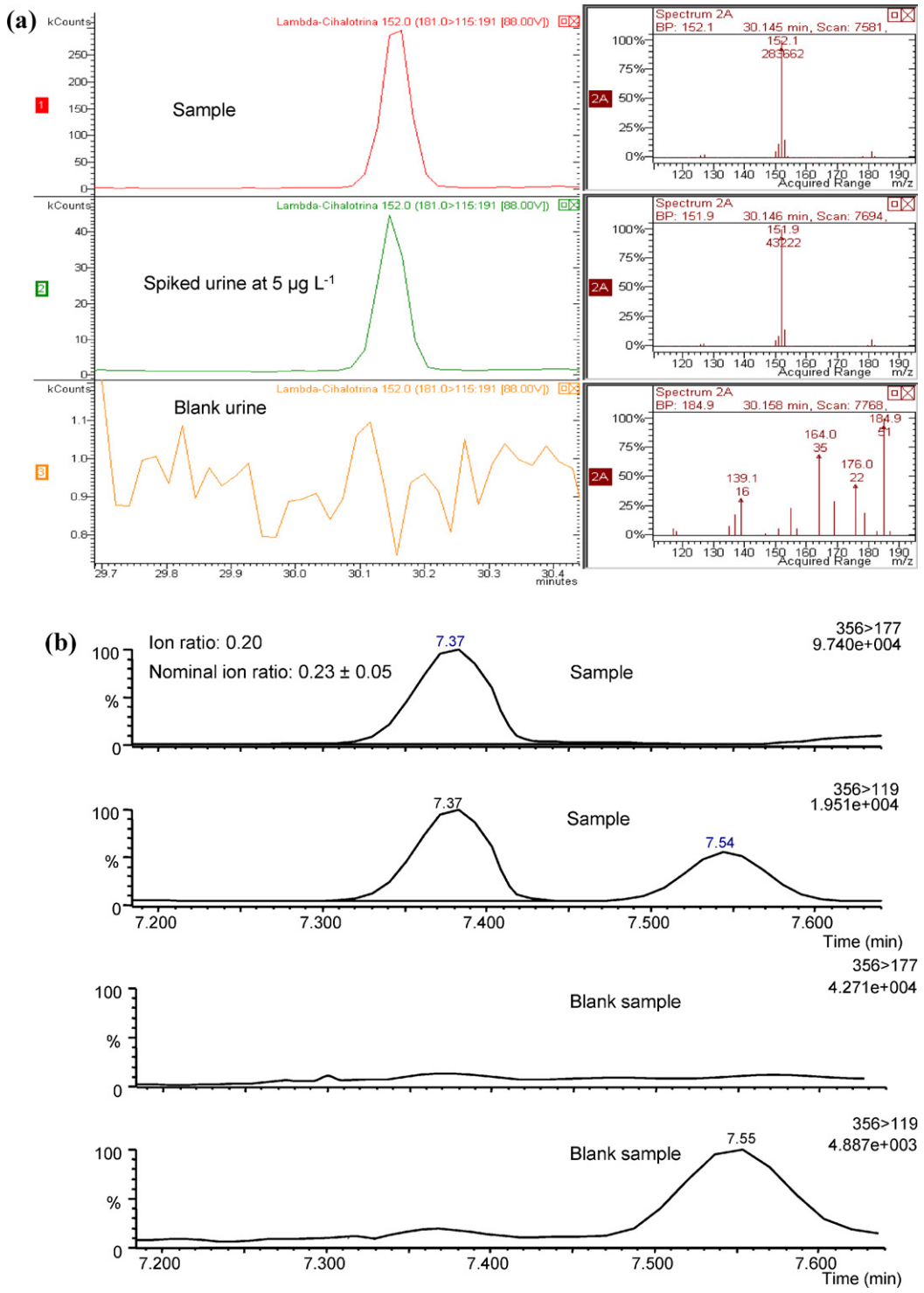

Fig. 3. (a) GC-IT-MS/MS (SRM) chromatogram with the corresponding spectrum of a positive sample containing 24.4 µg L^{-1} of cyhalothrin (above) and a spiked urine sample at 5 µg L^{-1} (middle) and a blank urine (below); (b) UHPLC-QqQ-MS/MS chromatograms obtained from a urine sample containing 3.80 µg L^{-1} of piperonyl butoxide (above) and a blank urine (below).

response of three and ten times the average of the baseline noise, respectively.

2.7. Sample collection

To check the applicability of the method, urine samples of infant from agricultural population from Almeria (Spain) were taken. Urine samples were collected in 15 mL sterile containers and stored at −30 °C immediately until they were analyzed. Before use, the samples were thawed at room temperature.

3. Results and discussion

The aim of this work has been the development and validation of a single extraction procedure for the simultaneous extraction of polar and non-polar pesticides in urine followed by a chro-

matographic determination based on UHPLC-QqQ-MS/MS [34] and GC-IT-MS/MS [1].

3.1. Optimisation of the SPE procedure

Considering the complexity of the matrix urine and the different polarity of the selected compounds, an extraction procedure based on SPE was optimized. Several variables were optimized and for the optimization process, spiked blank urine was used. First, the type of cartridge was studied, and Oasis HLB and C_{18} were evaluated. For this experiment, 2 mL of urine were spiked with 10 $\mu g\,L^{-1}$ of pesticides and were diluted with 8 mL of MilliQ water. Besides, 5 mL of dichloromethane were used as elution solvent. Fig. 1a shows the number of extracted compounds with recoveries ranged from 60 to 120%. It can be observed that the number of extracted GC-amenable pesticides was higher when C_{18} cartridges were used, whereas the type of cartridge has less influence on the extraction of LC-amenable pesticides. Taking into account the obtained results, C_{18} cartridges were selected for further experiments.

Second, the organic solvent used for the elution of the pesticide from the cartridge was evaluated. 2 mL of urine were spiked with 10 $\mu g\,L^{-1}$ of pesticides and were diluted with 8 mL of MilliQ water. Thus, methanol, dichloromethane and a mixture of methanol:dichloromethane (1:1, v/v) were evaluated, showing the number of extracted compounds (recovery ranged from 60 to 120%) in Fig. 1b. Methanol provides the worst results for both types of compounds. For instance, less than 10 and 30 LC and GC-amenable pesticides were extracted respectively, whereas dichloromethane allows the extraction of a higher number of pesticides (more than 70 LC-amenable pesticides and more than 90 GC-amenable pesticides). It can be observed that tested mixture provides an intermediate situation. Therefore, dichlorometane was selected as the more adequate elution solvent for the simultaneous extraction of the selected pesticides.

Finally, the influence of the dilution of urine samples was investigated. For that purpose a final volume of 10 mL was extracted, using a blank urine sample spiked at 10 $\mu g\,L^{-1}$. Several volumes of spiked urine were used (1, 2, 5 and 10 mL), and they were diluted with water up to 10 mL, showing the obtained results in Fig. 1c. It can be observed that when urine was not diluted, the number of extracted pesticides was lower than 20 (both LC and GC-amenable pesticides). This can be explained taking into account the high number of coextracted interferences that could produce a strong signal suppression, which makes difficult the ionization of the selected compounds. On the other hand, when 1 mL of urine was used (diluted with 9 mL of water), the number of extracted compounds was also low, because the high dilution carried out, and the sensitivity is too low to detect the target compounds at trace levels. However when 2 or 5 mL of urine were used, the number of extracted pesticides increased, observing that 117 GC-amenable pesticides and 87 LC-amenable pesticides were extracted when 5 mL of urine was diluted with 5 mL of water. Therefore, 5 mL of urine, diluted 1:1 (v/v) with MilliQ water, were used for further experiments. It should be indicated that urine dilution presents several advantages such as lower amount of matrix that reduces the matrix effect as well as the compensation of possible variability between urine from different people.

Finally, Fig. 2 shows the corresponding chromatograms of an example of urine sample spiked at 5 $\mu g\,L^{-1}$ of target pesticides and blank urine. It can be observed that GC-amenable pesticides can be separated and detected in less than 40 min, and LC-amenable pesticides in less than 11 min, obtaining a clean chromatogram after the application of the SPE procedure (blank urine chromatogram).

3.2. Validation of the method

Performance characteristics of the optimized method were established by a validation procedure, studying selectivity, matrix effect, linearity, trueness, precision, LODs and LOQs.

First, identification of the selected compounds was performed by the use of retention time windows (RTWs), which were defined as the retention time (RT) average plus or minus 3 times the standard deviation (SD) of the RT (RT ± 3 SD) when 5 spiked samples at 5 $\mu g\,L^{-1}$ were injected. Confirmation in GC-IT-MS/MS was carried out by comparing the sample spectrum with a reference spectrum obtained with a spiked urine blank sample (mid-level calibration standard). Then, a forward search compared both spectra, obtaining a value named FIT ranging from 1 to 1000 (arbitrary units, a.u.), so that a FIT ≥ 700 (a.u.) confirmed the identity of the compound. In the case of UHPLC-QqQ-MS/MS, the confirmation was performed by comparing the intensity ratios of the two MS/MS transitions. Confirmation was considered reliable if the ratio obtained was within the criteria laid down in the European Commission Decision 2002/657 [35], showing in Table 2 the obtained ion ratios.

Bearing in mind that urine is a complex matrix, which has a large amount of compounds that can interfere with the analytes, matrix effect was evaluated in order to ensure bias-free analytical results. Although the best way to compensate matrix effect is the use of isotope internal standard, matrix-matched calibration was used in this study. For that purpose, five concentrations of the pesticides (from 5 to 100 $\mu g\,L^{-1}$) were analyzed in solvent (ethyl acetate for GC-amenable pesticides and mobile phase for LC-amenable pesticides) and in extracted blank urine. Then, slope ratios matrix/solvent for each compound were obtained (data not shown), considering a tolerable signal suppression or enhancement effect if the slope ratio ranged from 0.8 to 1.2, whereas lower values than 0.8 or higher than 1.2 implies a strong matrix effect. It can be noted that the calibration curves obtained from spiked blank extracted urine were significantly different for that obtained by the use of standard solutions for GC-amenable pesticides (strong matrix effect), whereas for LC-amenable pesticides, strong matrix effect was only detected for cyromazine, formetanate, aldicarb sulfone, oxidementon methyl, ethiofencarb sulfone, methiocarb sulfoxide, tiofanox sulfone, cyprodinil, captafol, amitraz, fenbutatin oxide, chloridazon, clofentezin. Therefore, in order to compensate this effect, matrix matched calibration was used for quantification purposes.

Then, linearity was evaluated by spiking extracted blank urine samples with seven different concentrations of pesticides ranging from 0.1 to 100 $\mu g\,L^{-1}$. For GC-amenable pesticides, linear calibration graphs were plotted by least-squares regression of relative peak area (analyte/IS) versus concentration of the calibration standards. Caffeine ^{13}C was used as IS for the determination of GC-amenable compounds, because it has similar physico-chemical properties than some pesticides, and its chromatographic properties are also well known. Determination coefficient (R^2) values were higher than 0.996. For LC-amenable pesticides, linear calibration graphs were plotted by least-squares regression of peak area versus concentration of the calibration standards (no IS was used) and R^2 values were higher than 0.995.

In order to evaluate the trueness of the proposed method, recovery studies were carried out at three concentration levels (5, 10 and 50 $\mu g\,L^{-1}$), performing 5 replicates at each level (Tables 3 and 4). It can be observed that for GC-amenable pesticides (Table 3), recovery values ranged from 61 to 118% (5 $\mu g\,L^{-1}$), 65 to 119% (10 $\mu g\,L^{-1}$) and 61 to 120% (50 $\mu g\,L^{-1}$). For LC-amenable pesticides (Table 4), recoveries ranged from 60 to 101% (5 $\mu g\,L^{-1}$), 60 to 120% (10 $\mu g\,L^{-1}$) and 65 to 125% (50 $\mu g\,L^{-1}$). Although for some compounds, these recoveries are not close to 100%, they can be considered acceptable since they were reproducible (see below).

Precision of the overall method was estimated by performing both repeatability and reproducibility (interday precision) studies. Repeatability was evaluated at the three concentration levels of the recovery studies, performing five replicates at each level (Tables 3 and 4), whereas interday precision was studied at 5 $\mu g L^{-1}$, analyzing daily spiked samples for a period of 5 days. Repeatability values (expressed as relative standard deviation, RSD) were always lower than 25%, as well as interday precision, except for fenarimol, tiofanox sulfone, diniconazol-M and indoxacarb, which have values slightly higher than 25% (see Tables 3 and 4).

LODs and LOQs were calculated analyzing blank samples spiked at 0.001, 0.01, 0.1, 0.5, 1, 2 and 5 $\mu g L^{-1}$, and they were determined as the lowest concentration of analyte for which signal-to-noise ratios were 3 and 10, respectively (Tables 3 and 4). The ranges of LOD and LOQ in urine for GC-amenable pesticides were 0.001 (chlorthal-dimethyl)-0.436 (iprodione) $\mu g L^{-1}$, and 0.003 (chlorthal-dimethyl)-1.452 (iprodione) $\mu g L^{-1}$, respectively. For LC-amenable pesticides, the LOD and LOQ ranged from 0.003 (furmecyclox)-1.048 (teflubenzuron) $\mu g L^{-1}$, and 0.011 (furmecyclox)-3.494 (teflubenzuron) $\mu g L^{-1}$, respectively. It must be indicated that these values allows the determination of these type of compounds at trace levels and they are equal or below other published limits for the determination of pesticides in urine or related matrices [6,36–38].

3.3. Application of the method to real samples

To evaluate the applicability of the proposed method in real samples, 14 samples were analyzed. All samples were obtained from infants who live at an intensive agricultural area in Almeria (Spain). To ensure quality results, an internal quality control was performed in every batch of samples, which implies a matrix-matched calibration, a reagent blank and a spiked blank sample at 5 $\mu g L^{-1}$. Some pesticides were detected in four urine samples, whereas no pesticides were detected in the rest of samples. In one sample, methoxyfenozide, tebufenozide, piperonyl butoxide and propoxur were detected at 4.83, 1.61, 3.80 and 4.00 $\mu g L^{-1}$, respectively. In another sample, cyhalotrin was found at a concentration of 24.4 $\mu g L^{-1}$. Finally, methiocarb sulfoxide was detected at trace levels in two samples. Fig. 3a shows the chromatogram of the positive sample in which cyhalotrin was detected as well as the MS/MS spectrum and a blank urine sample, whereas Fig. 3b shows the UHPLC-QqQ-MS/MS chromatogram for the positive sample of piperonyl butoxide and the corresponding transitions from a blank urine sample. It can be noted that another signal was observed when the confirmation transition was monitored but it appeared outside the RTW for this compound. Therefore, the quantification of piperonyl butoxide was not affected by this signal, allowing a selective determination of this compound. Furthermore, for confirmation purposes, the ion ratio was also evaluated, and it can be noted that the ion ratio of the sample (0.20) was within the acceptance criteria indicated by the UE (0.23 ± 0.05) [35].

4. Conclusions

This work presents a new multiresidue method for the simultaneous extraction of different classes of pesticides such as carbamates, organochlorine, organophosphorous pyrethroids, herbicides and insecticides based on SPE. For that purpose, C_{18} cartridges provide good results for the assayed compounds, and dichloromethane is a suitable eluting solvent. Reliable validation parameters such as trueness, precision, linearity, selectivity and lower limits detection and quantification were obtained. The proposed method can be applied in routine analysis due to sample throughput and a large number of samples can be extracted simultaneously. Furthermore, it can be used in monitoring programs to control the presence of pesticides in urine samples, bearing in mind that the use of a simultaneous extraction step for the different families allows the reduction of sample-handling and sample pre-treatment.

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