**Analysis of 52 pesticides in fresh fish muscle by QuEChERS extraction
followed by LC-MS/MS determination.**

Maria Vittoria Barbieri¹, Cristina Postigo^{1,2}*, Nuria Guillem-Argiles¹, L. Simon
Monllor-Alcaraz¹, Julliana Izabelle Simionato³, Elisa Stella⁴, Damià Barceló^{1,5}, Miren
López de Alda¹

ABSTRACT

Pesticide pollution in water has been well described; however, little is known on pesticide accumulation by aquatic organisms, and to date, most studies in this line have been focused on persistent organochlorine pesticides. For this reason, a method based on QuEChERS extraction and subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been developed and validated for the determination of 52 medium to highly polar pesticides in fresh fish muscle. Target pesticides were selected on the basis of use and occurrence in surface waters. Quantification is carried out following an isotope dilution approach. The method developed is satisfactory in terms of accuracy (relative recovery between 71-120%), precision (relative standard deviation below 20.6%) and sensitivity (limits of determination in the pg/g or low ng/g f.w. range for most compounds). The application of the validated methodology to fish specimens collected from the Adige River (Italy) revealed the presence of trace levels of diazinon, dichlorvos and diuron, and measurable levels of metolachlor, quinoxifen, irgarol, terbutryn, and acetamiprid, but in all cases at concentrations below the default maximum residue level of 10 ng/g established for pesticides not specifically regulated in fish. Metolachlor and quinoxifen were both the most ubiquitous and abundant pesticides, in agreement with their high potential of bioaccumulation. Both are toxic to aquatic organisms, and therefore, their potential effects on aquatic ecosystems should be further explored.

Keywords: bioconcentration, biota, herbicides, insecticides, exposure, liquid chromatography-mass spectrometry

30 **1. Introduction**

31 Pesticides are among the most used chemical substances worldwide, with an annual
32 production of over 3 million tons (Roser and Ritchie, 2017). Their use in agriculture has
33 allowed increasing the quality and the quantity of food production. However, regardless of
34 their merits, they have been appointed as some of the most toxic substances in the
35 environment and consequently represent a risk for human health (Furio et al., 2015). For this
36 reason, and based on the available information, the protection of water resources and
37 aquatic ecosystems from pesticide pollution has motivated the adoption of several
38 regulatory measures. For instance, residues of selected pesticides that are considered
39 priority substances in the environment must be strictly controlled in European water bodies
40 and biota, so that their levels remain below established environmental quality standards
41 (EQS) (EC, 2013).

42 Research on the environmental occurrence of medium to highly polar pesticides has
43 been very much focused in the water compartment while PBT (persistent, bioaccumulative,
44 and toxic) pesticides have been usually targeted in biota due to their high octanol-water
45 partition coefficients (K_{ow}) and hence capacity to partition into lipids. However, ionizable and
46 ionic pesticides, despite their low K_{ow} values, are also likely to bioaccumulate in aquatic
47 organisms via ion specific sorption mechanisms. The knowledge on the bioaccumulation
48 potential of this type of pesticides is nowadays very limited but essential for proper risk
49 assessment and pesticide regulation. To gain insights in this respect pharmacokinetic models
50 and novel screening tools for prediction of sorption of ionic chemicals in fish according to
51 their physical-chemical properties are being developed (Bittermann et al., 2018). Moreover,
52 analytical methods to study the concentrations of medium to highly polar pesticides in biota
53 need also to be available so that the fate of these chemicals in different aquatic organisms
54 can be evaluated and the aforementioned models validated.

Besides keeping pesticide residues in the environment low, protection of public health also requires controlling pesticide residues in food or feed. This has been achieved through the establishment of maximum residue levels (MRL), i.e., the highest pesticide levels legally tolerated after their correct application in food products (EC, 2005). MRLs set for the different pesticides by the European Food Safety Authority (EFSA) takes into account the toxicity of the compound, the maximum levels expected on food, and the different diets in Europe. Such standards have been set for pesticides currently in use or used in the past for food production in or outside the European territory and for 315 food products in total. However, MRLs of pesticides do not exist for fish products despite the fact that these organisms may be exposed to trace pesticide concentrations continuously released into the aquatic environment. In this case, and in any other case where a pesticide is not specifically mentioned, a general default MRL value of 10 ng/g can be applied. Determination of pesticide residues in biota or in any other high fat content food sample, expected to contain low ng/g levels, requires the development of advanced multi-residue analytical methods with high sensitivity and selectivity instrumental technologies, such as gas chromatography (GC) or liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) (Picó, 2016; Villaverde et al., 2016). Because of the complexity of these matrices, efficient extraction and clean-up protocols are also needed. For analyte extraction, approaches based on Soxhlet extraction, solid-liquid extraction, pressurized solvent extraction (PSE) (Choi et al., 2016; Chung and Chen, 2011), or microwave assisted extraction (MAE) have been used (Chung and Chen, 2011; LeDoux, 2011). Solid phase extraction (SPE) has usually been the method of choice for clean-up of the extracts (Chung and Chen, 2011; LeDoux, 2011; Santhi et al., 2012). Many analytical approaches have also included as a first step a lipid removal approach so that fatty interferents were not co-extracted (Chung and Chen, 2011).

In recent years, extraction with QuEChERS (quick, easy, cheap, effective, rugged and safe) has received increasing use for preparation of complex samples like fish (Baduel et al.,

2015; Belenguer et al., 2014; Farré et al., 2014; Kaczyński et al., 2017; Lazartigues et al., 2011; Morrison et al., 2016; Nácher-Mestre et al., 2014; Portolés et al., 2017). This could be attributed to the advantages that QuEChERS offer over traditional extraction methods, such as high analyte recoveries, accurate results, fast sample treatment, little use of solvent, and small lab-space and equipment requirements. However, as aforementioned, only a few methodologies are currently available for the analysis of medium to highly polar pesticides in fish samples.

In this context, this work presents a multi-residue target analytical method based on a QuEChERS extraction approach and LC-MS/MS analysis for the determination of fifty-two pesticides in fresh fish muscle. Selection of target pesticides was made based on their feasibility for LC-MS analysis, their environmental relevance in terms of being considered as priority substances (EC, 2013) or included in the European Watch List (EC, 2018), their occurrence in surface water, and their extent of use at European level (Eurostat, 2007). The list of investigated compounds contains ten pesticide transformation products. The compounds covered belong to many different chemical classes and present a very wide range of physical-chemical properties (K_{ow} , pK_a , water solubility, etc). The list includes twelve organophosphates, seven triazines, four phenylureas, two chloroacetamides, five neonicotinoids, four acidic pesticides, and nine compounds belonging to other chemical classes. This represents an analytical challenge in terms of developing a single multi-residue method for all of them.

To the authors' knowledge, previous works that investigate highly to medium polar pesticides in fish samples within the last decade (Badel et al., 2015; Belenguer et al., 2014; Ernst et al., 2018; Farré et al., 2014; Franco-Barrios et al., 2014; Kaczyński et al., 2017; Kaonga et al., 2015; Lazartigues et al., 2011; Morrison et al., 2016; Nácher-Mestre et al., 2014; Portolés et al., 2017; Shin, 2006; Vorkamp et al., 2014; Xiao et al., 2013) did not cover 23 % of

the pesticides selected in this study (i.e., 2,4-D, azinphos-methyl oxon, bentazone, bromoxynil, clothianidin, fenitrothion oxon, malaoxon, MCPA, mecoprop, oxadiazon, thifensulfuron-methyl, and triallate). In addition, the analytical methods used in these studies were in a few cases qualitative, developed for wide-scope pesticide screening (Nácher-Mestre et al., 2014; Portolés et al., 2017), and in other cases quantitative approaches that either used matrix-matched calibration curves (Belenguer et al., 2014; Farré et al., 2014; Lazartigues et al., 2011) or a few deuterated compounds as surrogate standards (Kaczyński et al., 2017; Lazartigues et al., 2011; Xiao et al., 2013) for quantification. In contrast, the analytical method proposed in the present study is based on the isotope dilution quantification method, which ensures the reliability of the results. Finally, the application of the method to real fish samples proved its suitability for the analysis of these compounds in this matrix and provided a first picture on the occurrence of some of the investigated pesticides in fish tissues.

2. Materials and methods

2.1 Chemicals and reagents

High purity (96-99.9%) standards of 52 pesticides and 45 isotopically-labeled compounds used as surrogate standards for quantification were purchased from Fluka (Sigma–Aldrich, Steinheim, Germany) or Dr. Ehrenstorfer (LGC Standards, Teddington, UK). Table 1 lists the target analytes, whereas relevant physical-chemical properties (average molecular mass, solubility, and K_{ow} among others) are provided in Table 1. Stock standard solutions of the individual analytes were prepared in methanol (MeOH) (dimethyl sulfoxide in the case of simazine) and stored in amber glass bottles in the dark at $-20\text{ }^{\circ}\text{C}$. Working methanolic solutions at different concentrations (between 0.01 and 1000 ng/mL) containing all analytes were prepared by appropriate dilution of the stock individual solutions. They

were used to construct calibration curves and as spiking solutions during method development and in the validation study. A solution containing the surrogate standard mixture at a concentration of 1000 ng/mL was also prepared. Pesticide-grade solvents MeOH, acetonitrile (ACN), and LC-grade water were supplied by Merck (Darmstadt, Germany).

2.2 Extraction and clean-up

Ten g of fresh fish muscle tissue were weighed into a 50 mL polypropylene centrifuge tube for QuEChERS extraction containing the extraction salts used in the EN 15662 CITRATE buffered method (4 g MgSO_4 , 1 g NaCl, 1 g NaCitrate and 0.5 g disodium citrate sesquihydrate (DCS)) provided by Bekolut® GmbH & Co. KG (Hauptstuhl, Germany) as Bekolut® Citrate-Kit- 01. Then the sample was spiked with the surrogate standard mixture at a concentration of 50 ng/g and manually shaken. The dispersive solid phase extraction of the sample was carried out using 10 mL of acetonitrile as extraction solvent (1:1 w/v). After vortex extraction at 2500 revolutions per min (rpm) for 1 min, tubes were centrifuged for 5 min at 4000 rpm. Seven-mL of the supernatant was transferred into a 15 mL centrifuge tube for clean-up with Bekolut® PSA-Kit-04A (400 mg primary secondary amine (PSA), 400 mg C18 and 1200 mg MgSO_4). The use of PSA sorbent aims at removing sugars and fatty acids, whereas C18 is added to remove remaining lipids. The role of MgSO_4 is to remove water (necessary for GC-MS applications) and to increase the process efficiency. After vortex extraction (1 min at 25000 rpm) and centrifugation (5 min, 4000 rpm) the supernatant was transferred into a 2 mL vial for LC-MS/MS analysis.

155 **Table 1.** Main physical-chemical properties of the target pesticides.

Pesticide	Type	MM (g mol ⁻¹) [‡]	Solub (mg L ⁻¹) [‡]	Koc (mL g ⁻¹) [‡]	Kow logP [‡]	Henry's (Pa m ³ mol ⁻¹) [‡]	GUS [‡]	Pka [‡]
2,4-D	Alkylchlorophenoxy	221.04	24300	39	-0.82	4.0 X 10 ⁻⁰⁶	1.69	3.40
Acetamiprid	Neonicotinoid	222.67	2950	200	0.80	5.3 X 10 ⁻⁰⁸	0.40	0.7
Alachlor	Chloroacetamide	269.77	240	335	3.09	3.2 X 10 ⁻⁰³	1.08	0.62
Atrazine	Triazine	215.68	35	100	2.70	1.5 X 10 ⁻⁰⁴	3.2	1.7
Azinphos-ethyl	Organophosphate	345.38	4.5	1500	3.18	3.1 X 10 ⁻⁰⁶	1.4	n/a
Azinphos-methyl	Organophosphate	317.32	28	1112	2.96	5.7 X 10 ⁻⁰⁶	1.42	5
Azinphos-methyl-oxon	Metabolite	301.26	2604*	10 *	0.77*	6.2 x 10 ⁻¹³ *	-	n/a
Bentazone	Benzothiazinone	240.30	7112	55	-0.46	7.2 X 10 ⁻⁰⁵	2.89	3.51
Bromoxinil	Hydroxybenzoxitrile	276.90	38000	302	0.27	8.7 X 10 ⁻⁰⁷	0.03	3.86
Chlorfenvinphos	Organophosphate	359.60	145	680	3.80	-	1.83	n/a
Chlorpyrifos	Organophosphate	350.58	1.05	5509	4.70	0.478	3.63	n/a
Chlortoluron	Phenylurea	212.68	74	196	2.50	1.4 X 10 ⁻⁰⁵	3.02	n/a
Cyanazine	Triazine	240.69	171	190	2.10	6.6 X 10 ⁻⁰⁶	2.07	12.9
Clothianidin	Neonicotinoid	249.68	340	123	0.90	2.9 X 10 ⁻¹¹	4.91	11.1
Desisopropylatrazine	Metabolite	173.60	980	130	1.15	980	-	n/a
Desethylatrazine	Metabolite	187.63	2700	110	1.51	1.6 X 10 ⁻⁰⁴	4.37	n/a
Diazinon	Organophosphate	304.35	60	609	3.69	6.1 X 10 ⁻⁰²	1.14	2.6
Dichlorvos	Organophosphate	220.98	18000	50	1.90	2.6 X 10 ⁻⁰²	0.69	n/a
Diflufenican	Carboxamide	394.29	0.05	3.19°	4.20	1.2 X 10 ⁻⁰²	1.51	n/a
Dimethoate	Organophosphate	229.26	25900	25*	0.75	1.4 X 10 ⁻⁰⁶	1.01	n/a
Diuron	Phenylurea	233.09	35.6	813	2.87	2.0 X 10 ⁻⁰⁶	1.83	n/a
Fenitrothion	Organophosphate	277.23	19	2000	3.32	9.9 X 10 ⁻⁰³	0.48	n/a
Fenitrothion oxon	Organophosphate	261.17*	301 *	21*	1.69*	4.0 X 10 ⁻¹ *	-	n/a
Fenthion	Organophosphate	278.33	4.2	1500	4.84	2.4 X 10 ⁻⁰²	1.26	n/a
Fenthion oxon	Metabolite	262.26*	213.5*	57 *	2.31*	3.0x10 ⁻⁹ *	-	n/a
Fenthion oxon sulfone	Metabolite	294.03*	7602*	13*	0.28*	2.4 x 10 ⁻¹¹ *	-	n/a
Fenthion oxon sulfoxide	Metabolite	278.26*	1222*	11*	0.15*	9.5 x 10 ⁻⁸ *	-	n/a
Fenthion sulfone	Metabolite	310.33*	190.4*	542*	2.05*	1.1x10 ⁻⁸ *	-	n/a
Fenthion sulfoxide	Metabolite	294.33*	3.72*	466*	1.92*	7.0x10 ⁻⁶ *	-	n/a
Fluroxypir	Pyridine compound	255.03	6500	-	0.04	169 X 10 ⁻¹⁰	2.42	2.94
Imidacloprid	Neonicotinoid	255.66	610	6719	0.57	1.7 X 10 ⁻¹⁰	3.74	n/a
Irgarol	Triazine	253.37	7	1569	3.95	1.3 x 10 ⁻⁰⁷ *	-	n/a
Isoproturon	Phenylurea	206.28	70.2	251*	2.5	1.5 X 10 ⁻⁰⁵	2.07	n/a
Linuron	Phenylurea	249.09	63.8	843	3	2.0 X 10 ⁻⁰⁴	2.21	n/a
Malaaxon	Organophosphate	314.29*	7500*	4650*	0.52*	1.2 X 10 ⁻⁰⁸ *	-	n/a
Malathion	Organophosphate	330.36	148	1800	2.75	1.0 X 10 ⁻⁰³	-1.28	n/a
MCPA	Metabolite	200.62	29390	29*	-0.81	5.5 X 10 ⁻⁰⁵	2.94	3.73
Mecoprop	Aryloxyalkanoic acid	214.65	250000	47	-0.19	2.2 X 10 ⁻⁰⁴	2.29	3.11
Methiocarb	Carbamate	225.31	27	182*	3.18	1.2 X 10 ⁻⁰⁴	0.55	n/a
Metolachlor	Chloroacetamide	283.80	530	120	3.40	2.4 X 10 ⁻⁰³	2.10	n/a
Molinate	Thiocarbamate	187.30	1100	190	2.86	6.9 X 10 ⁻⁰¹	2.49	n/a
Oxadiazon	Oxidiazole	345.2	0.57	3200	5.33	3.8 X 10 ⁻⁰²	2.40	n/a
Pendimethalin	Dinitroaniline	281.31	0.33	17491	5.40	2.7 X 10 ⁻⁰³	-0.32	2.8
Propanil	Anilide	218.08	95	149	2.29	4.4 X 10 ⁻⁰⁴	-0.51	19.1
Quinoxifen	Quinoline	308.13	0.05	23°	4.66	3.2 X 10 ⁻⁰²	-0.93	n/a
Simazine	Triazine	201.66	5	130	2.30	5.6 X 10 ⁻⁰⁵	2	1.62
Terbuthylazine	Triazine	229.71	6.6	329*	3.40	3.2 X 10 ⁻⁰³	3.07	1.9
Terbutryn	Triazine	241.36	25	2432	3.66	1.5 X 10 ⁻⁰³	2.4	4.3
Thiacloprid	Neonicotinoid	252.72	184	615°	1.26	5.0 X 10 ⁻¹⁰	0.14	n/a
Thiamethoxam	Neonicotinoid	291.71	4100	56	-0.13	4.7 X 10 ⁻¹⁰	4.69	n/a
Thifensulfuron methyl	Metabolite	387.39	54.1	28	-1.65	3.3 x 10 ⁻⁰⁸	0.44	4
Triallate	Thiocarbamate	304.7	4.1	3034	4.06	0.89	0.69	n/a

MM: molecular mass; Solub: solubility in water at 20 °C ; Koc: organic carbon partition coefficient; Kow: octanol–water partition coefficient; Henry's law constant at 25°C; GUS: leaching potential index; Pka: dissociation constant at 25 °C; n/a: data not available. ‡ The PPDB, Pesticide Properties Database. <http://sitem.herts.ac.uk/aeru/footprint/index2.htm> - Lewis KA, Tzilivakis J, Warner D, Green A. (2016). An international database for pesticide risk assessments and management. Human and Ecological Risk Assessment: An International Journal, 22(4), 1050-1064. *Data estimated using the US EPA EPISuite™ <http://www.Chemspider.com>. ° Kegley SE, Hill BR, Orme S, Choi AH, PAN Pesticide Database, Pesticide Action Network, North America (Oakland, CA, 2016), <http://www.pesticideinfo.org>

2.3 LC-MS/MS analysis

The instrumentation used for analysis consisted of an LC system Aria Mx with two Transcend quaternary pumps (max pressure 600 bars) coupled to a TSQ Quantiva triple-quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI) source (Thermo Fisher Scientific Inc.). Sample injection was carried out by means of a CTC pal autosampler. For LC-MS/MS analysis, 20 μ L of the final QuEChERS extract was injected into the chromatographic column, a Purospher STAR RP-18e column (150 $\times$ 2.1 mm, 2 μ m particle diameter) from Merck (Darmstadt, Germany). Chromatographic separation was achieved with an organic linear gradient using ACN and water as the mobile phase at a flow rate of 0.2 mL/min. The starting composition of the mobile phase was 10% ACN in water. The organic phase proportion was increased to 50% in 2 min, to 80% in the following 10 min, and finally to 100% in 1 min. Pure organic conditions were maintained for 3 min to ensure column cleaning, and then initial conditions were achieved in 1 min and maintained for 8 min to ensure column equilibration.

MS/MS detection was carried out in the selected reaction monitoring (SRM) mode acquiring two SRM transitions per target pesticide and one SRM transition per surrogate compound. The analytical system allowed switching between positive and negative ionization modes without affecting method sensitivity, and consequently, all analytes were determined in a single analytical run (45 compounds in the positive ionization mode and 7 compounds in the negative ionization mode). Table 2 shows the optimum conditions for MS/MS determination of target pesticides. Other MS detection conditions were as follows: ion spray voltage, 3500 V (positive ionization mode) and 2500 V (negative ionization mode); ion transfer tube temperature, 350 $^{\circ}$ C; vaporizer temperature, 280 $^{\circ}$ C. Nitrogen was used as sheath gas, auxiliary gas, and sweep gas; whereas argon was used as collision gas, 2.5 mTorr. Instrument control and data acquisition and evaluation were performed by Thermo Xcalibur 3.0.63 software (Thermo Fisher Scientific Inc.).

Table 2. LC-MS/MS conditions for target pesticides and surrogate compounds (in italics).

Target analyte	Abbrev.	RT (min)	HESI mode	SRMs (m/z)	RF Lens (V)	CE (eV)	SRM1/ SRM2
Fluroxypyr	FLUXP	4.3	-	255>197 255>235	33	16 6	2.7
Bentazone	BEN	4.4	-	239>132 239>117	68	28 33	20.3
	<i>BEN-d₆</i>	4.4	-	245>132	79	29	-
2,4-dichloro-phenoxy acetic acid	2,4D	4.9	-	219>162 219>125	35	16 28	20.1
	<i>2,4D-d₃</i>	4.9	-	224>166	32	17	-
4-chloro-2-methylphenoxy acetic acid	MCPA	5.1	-	199>142 199>105	38	17 30	380.6
	<i>MCPA-d₃</i>	5.0	-	204>146	38	18	-
Deisopropylatrazine	DIA	5.2	+	174>104 174>132	58	23 18	2.3
	<i>DIA-d₅</i>	5.1	+	179>137	57	18	-
Thiamethoxam ^a	THIAM	5.3	+	292>132 292>181	47	24 23	1.1
	<i>THIAM-d₃</i>	5.3	+	295>214	43	13	-
Fenthion oxon sulfoxide	FNOSX	5.3	+	279>264 279>262	68	20 22	4.2
Mecoprop	MECO	5.6	-	213>142 213>140	39	17 17	2.9
	<i>MECO-d₃</i>	5.6	-	218>147	38	18	-
Clothianidin ^a	CLOTD	5.7	+	250>169 250>132	43 47	15 18	2.2
	<i>CLOTD-d₃</i>	5.6	+	253>172	45	15	-
Imidacloprid ^a	IMID	5.8	+	256>209 256>175	51	20 20	1.1
	<i>IMID-d₅</i>	5.8	+	261>214	44	19	-
Desethylatrazine	DEA	5.8	+	188>146 188>104	66	18 25	5.2
	<i>DEA-d₆</i>	5.8	+	194>147	59	20	-
Thifensulfuron methyl	THIFEN	6.0	+	388>167 388>205	57 54	18 27	6.2
	<i>THIFEN-d₃</i>	5.9	+	391>167	64	18	-
Dimethoate	DIME	6.0	+	230>125 230>157	35	22 21	13.8
	<i>DIME-d₆</i>	5.9	+	236>131	44	23	-
Acetamiprid ^a	ACET	6.0	+	223>126 223>90	53	22 33	5.5
	<i>ACET-d₃</i>	6.0	+	226>126	55	22	-
Bromoxynil	BROMX	6.0	-	276>81 276>79	82	30 30	1.1
	<i>BROMX ¹³C₆</i>	6.2	-	282>79	90	30	-
Fenthion oxon sulfone	FENOXS	6.1	+	295>217 295>91	74	20 34	9.9
	<i>FENOXS d₃</i>	6.1	+	298>218	77	20	-
Azinphos methyl oxon	AZMO	6.4	+	324>132 324>148	84	22 17	17.1
Thiacloprid ^a	THIAC	6.6	+	253>126 253>90	59	23 35	6.8
	<i>THIAC-d₄</i>	6.6	+	257>126	60	23	-

Cyanazine	CIANZ	7.3	+	241>214 241>104	59	18 30	6.2
	<i>CIANZ-d₅</i>	7.3	+	246>219	59	18	-
Simazine ^b	SIMAZ	7.4	+	202>132 202>104	61	20 25	1.2
	<i>SIMAZ-d₁₀</i>	7.3	+	212>137	63	21	-
Dichlorvos ^b	DICV	7.4	+	221>109 221>145	57	14 18	11.4
	<i>DICV-d₆</i>	7.3	+	227>115	69	19	-
Fenthion sulfoxide	FENSOX	7.5	+	295>280 295>109	68	19 33	2.0
	<i>FENSOX-d₆</i>	7.5	+	301>286	53	18	-
Malaoxon	MALOX	7.5	+	315>99 315>125	48	23 33	16.4
Fenitrothion oxon	FENTOX	7.8	+	262>216 262>104	66	19 22	1.2
	<i>FENTOX-d₆</i>	7.8	+	268>222	66	19	-
Chlortoluron	CHLOR	8.1	+	213>140 213>104	51	25 33	5.0
	<i>CHLOR-d₆</i>	8.0	+	219>78	58	19	-
Isoproturon ^b	ISOPR	8.4	+	207>134 207>91	51	23 37	1.9
	<i>ISOPR-d₆</i>	8.4	+	213>134	53	23	-
Atrazine	ATRZ	8.7	+	216>174 216>104	58	18 28	4.6
	<i>ATRZ-d₅</i>	8.6	+	221>179	59	18	-
Diuron ^b	DIUR	8.7	+	233>160 235>133	51	27 41	1.4
	<i>DIUR-d₆</i>	8.7	+	239>160	59	28	-
Fenthion oxon	FENOX	8.9	+	263>231 263>216	62	16 25	2.3
	<i>FENOX-d₃</i>	8.8	+	266>234	69	17	-
Fenthion sulfone	FENS	9.4	+	311>125 311>233	64	21 40	3.0
	<i>FENS-d₆</i>	9.3	+	317>131	72	17	-
Propanil	PROP	10.1	+	218>127 218>162	53	27 16	1.0
	<i>PROP-d₅</i>	10.1	+	223>128	59	28	-
Methiocarb ^a	METCB	10.4	+	226>169 226>121	35	9 19	1.4
	<i>METCB-d₃</i>	10.3	+	229>169	30	10	-
Terbutylazine	TERBZ	10.6	+	230>174 230>104	52	18 32	9.1
	<i>TERBZ-d₅</i>	10.5	+	235>179	55	18	-
Azinphos methyl	AZM	10.6	+	318>132	30	16	1.3
		10.6	+	318>261	30	7	
	<i>AZM-d₆</i>	10.4	+	324>132	43	16	-
Linuron	LINU	10.8	+	249>160 249>133	51	19 34	1.6
	<i>LINU-d₆</i>	10.7	+	255>185	48	18	-
Molinate	MOLI	12.0	+	188>126 188>98	43	13 17	6.6

192 **Table 2.** (continued)

Irgarol ^b (Cybutryne)	IRGA	12.1	+	254>198 254>108	57	19 30	15.9
	<i>IRGA-d₉</i>	12.0	+	263>199	60	19	-
Terbutryn ^b	TBTN	12.2	+	242>186 242>158	55	19 26	16.1
	<i>TBTN-d₅</i>	12.2	+	247>191	59	19	-
Malathion	MALA	12.2	+	353>227 353>306	70	17 15	7.4
	<i>MALA-d₁₀</i>	12.1	+	363>237	70	18	-
Metolachlor	METO	12.5	+	284>252 284>176	48	16 26	2.8
	<i>METO-d₁₁</i>	12.3	+	295>263	48	17	-
Alachlor ^b	ALA	12.6	+	270>162 270>132	40	21 42	3.7
	<i>ALA-d₁₃</i>	12.4	+	283>251	45	11	-
Fenitrothion	FEN	12.5	-	262>152 262>122	52	21 34	1
	<i>FEN-d₆</i>	12.4	-	265>153	51	22	-
Azinphos-ethyl	AZET	12.8	+	346>137 346>97	37	25 32	1.6
	<i>AZET-d₁₀</i>	12.7	+	356>138	39	25	-
Chlorfenvinphos ^b	CFVP	13.0	+	359>170 359>99	60	42 27	1.2
	<i>CFVP-d₁₀</i>	12.9	+	369>170	58	41	-
Fenthion	FENT	14.1	+	279>169 279>247	63	20 13	1.3
	<i>FENT-d₆</i>	14.0	+	285>169	62	20	-
Diazinon	DIAZ	14.8	+	305>169 305>153	64	22 22	1.9
	<i>DIAZ-d₁₀</i>	14.6	+	315>170	67	23	-
Diflufenican	DIFLU	15.5	+	395>266 395>246	60	24 34	6.4
	<i>DIFLU-d₃</i>	15.4	+	398>268	84	25	-
Oxadiazon	OXDZ	16.8	+	345>303 345>220	60	13 20	1.5
	<i>OXDZ-d₇</i>	16.8	+	352>304	60	10	-
Quinoxifen ^b	QUIN	17.4	+	310>199 310>216	102	34 36	1.9
Chlorpyrifos ^b	CPF	17.2	+	350>198 350>97	54	22 40	2.3
Pendimethalin	PENDI	17.3	+	282>212 282>194	33	12 19	7.5
	<i>PENDI-d₅</i>	17.3	+	287>213	43	12	-
Triallate	TLLT	18.0	+	304>142 304>83	55	40 47	1.7
	<i>TLLT-¹³C₆</i>	17.9	+	310>143	55	29	-

^aCompound included in the EU watch list (2018/840/EC)

^bCompound included in the EU list of priority substances (2013/39/EC)

CE: collision energy; HESI: electrospray ionization; RT: retention time; SRM: selected reaction monitoring, SRM1/SRM2: peak area ratio

2.4 Method validation performance

The performance of the analytical methodology was evaluated in terms of linearity, sensitivity, accuracy, matrix effects, and precision according to the guidelines set for pesticide residues analysis in food and feed (EC, 2017). The validation study was carried out using fresh muscle from a trout individual purchased at a nearby supermarket. For method validation, twenty pesticides were added to the initial pesticide list (32 pesticides, see Table 3) investigated during the optimization of the extraction approach.

Table 3. Recovery rates of pesticides in fish muscle tissue.

Absolute recovery (%)	1	2	3	4	5	6	7	8	9
Alachlor	38	28	31	31	23	30	28	32	46
Atrazine	71	90	120	115	100	94	100	73	133
Azynphos methyl	26	22	16	23	10	20	25	28	35
Azynphos ethyl	52	50	56	77	34	56	45	47	59
Chlorfenvinphos	26	27	24	41	15	35	31	46	61
Chlorpyrifos	10	8	11	10	6	10	10	9	20
Chlortorluron	55	51	73	72	64	57	61	51	76
Cyanazine	89	62	76	66	69	60	72	67	81
Deisopropylatrazine	52	52	54	38	68	62	43	28	51
Desethyl atrazine	40	45	64	51	47	45	57	47	51
Diazinon	31	30	38	41	33	39	38	41	52
Dichlorvos	84	70	70	83	57	84	79	92	64
Dimethoate	34	33	52	43	38	31	43	29	36
Diuron	31	28	40	33	33	33	42	28	42
Fenitrothion	5	1	5	2	1	0	2	3	2
Fenthion oxon	37	32	54	42	44	40	52	40	52
Fenthion oxon sulfone	10	30	166	13	15	109	22	32	8
Fenthion oxon sulfoxide	179	128	140	95	155	130	150	85	144
Fenthion sulfone	25	22	26	27	18	20	18	11	31
Fenthion sulfoxide	94	77	97	100	85	86	86	86	98
Irgarol	45	48	56	73	47	59	48	56	72
Isoproturon	77	84	91	96	89	92	102	89	120
Linuron	37	50	46	52	44	55	48	47	59
Malaoxon	100	66	76	80	68	70	82	71	82
Malathion	4	-7	0	11	-6	1	3	7	14
Metholachlor	47	40	46	51	33	44	40	46	62
Molinate	23	25	31	42	32	44	25	29	40
Propanil	2	2	2	2	2	2	3	2	3
Quinoxifen	9	8	13	14	7	10	9	12	17
Simazine	56	35	49	45	42	41	39	41	43
Terbutylazine	35	46	47	61	39	50	45	46	61
Terbutryn	40	47	56	70	45	53	45	52	64

Quantification was performed by the internal standard method, based on the peak areas obtained for each analyte and its corresponding surrogate compound, which was a deuterated analog in most cases. Up to eleven-point calibration curves were constructed using least squares linear regression models within the concentration range 0.01 ng/mL - 1000 ng/mL (equivalent to 0.01 and 1000 ng/g f.w., respectively). Method linearity was expressed by the coefficient of determination (r^2) of the linear model obtained for each analyte.

Analyte recovery and repeatability were calculated from the analysis of n=6 fortified fish muscle samples at two different concentration levels (10 ng/g f.w. and 500 ng/g f.w.). Absolute recovery values were calculated by comparing analyte peak areas in fortified fish samples and methanolic solutions with the standards mixture at equivalent concentrations (10 ng/mL and 500 ng/mL). Background concentrations in the fish tissue used for the recovery study were taken into account for the calculations. Relative recovery values were calculated by comparing the absolute recovery of the analyte and that of the corresponding isotopically-labeled compound used as surrogate standard.

Method repeatability was calculated as the relative standard deviation (RSD) of the analyte peak areas obtained after the replicate (n = 6) analysis of fish muscle samples fortified with the standard mixture at concentrations of 10 ng/g f.w. and 500 ng/g f.w.

The method limits of detection (LODs) and quantification (LOQs) were estimated from the analyte signals observed after LC-MS/MS analysis of fortified (10 ng/g f.w.) fish muscle samples as the concentration that provided a signal to noise ratio of 3 in the case of the LOD and 10 in the case of the LOQ. The LOQ of the analyte SRM1 and the LOD of the analyte SRM2 were considered in the provision of the analyte limit of determination (LODet), which is the minimum concentration of analyte that can be quantified with the SRM1 and confirmed with the SRM2.

Matrix effects were evaluated comparing analyte peak areas obtained after analysis of samples fortified after the extraction process and standard solutions at equivalent concentrations (500 ng/g f.w.), according to the following equation: $((A_{matrix} - A_{std}) / A_{std}) * 100$, where A_{matrix} is the analyte area obtained in a fish sample extract fortified with the standard mixture after QuEChERS extraction and A_{std} is the analyte area obtained in a methanolic standard solution. Negative values indicate ionization suppression matrix effects whereas positive values indicate ionization enhancement matrix effects.

2.5 Sample collection

The validated method was applied to the analysis of the target pesticides in fish muscle tissue of individuals collected from the Adige River basin (Italy). This river basin is highly impacted by agricultural activities, such as apple tree cultivation, and also by hydroelectric energy production. Thirteen fishes were caught during a sampling campaign carried out in July 2015. Sampling locations were distributed along the basin as shown in Figure 1.

Riverine brown trout (*Salmo trutta fario*) or marble trout (*Salmo trutta marmoratus*), bullhead (*Cottus gobio*) or grayling (*Thymallus thymallus*), and chub (*Leuciscus cephalus*) were collected as representative species of predator, benthivorous and omnivorous ecological groups, respectively (Kračun-Kolarević et al., 2016). Table 4 shows the details of the species sampled in the different sampling locations. Fish were caught by net and/or electrofishing after obtaining the corresponding permit from local authorities. Samples were kept at 4°C and transported to the laboratory. Fish muscle (350 g tissue of one individual or 350 g of tissue blend of small individuals) was pulverized in a mixer, separated in different portions in polyethylene vessels and stored in the dark at -20 °C. Samples were shipped on dry ice to the analytical laboratory and upon reception, they were also stored at -20 °C until analysis.

Figure 1. The Adige River basin and a detailed map of fish sampling sites.

Table 4. Fish species sampled in the different sampling points, with details of diet habits (predator, benthivorous or omnivorous) and the code assigned to classify them.

CODE	SITE	SPECIE	CLASS
1_Brown trout	1	<i>Salmo trutta fario</i>	Predator
2_Brown trout	2	<i>Salmo trutta fario</i>	Predator
3_Brown trout	3	<i>Salmo trutta fario</i>	Predator
4_Brown trout	4	<i>Salmo trutta fario</i>	Predator
4_Bullhead	4	<i>Cottus gobio</i>	Benthivorous
5_Marble trout	5	<i>Salmo trutta marmoratus</i>	Predator
5_Bullhead	5	<i>Cottus gobio</i>	Benthivorous
6_Marble trout	6	<i>Salmo trutta marmoratus</i>	Predator
6_Chub	6	<i>Leuciscus cephalus</i>	Omnivorous
6_Grayling	6	<i>Thymallus thymallus</i>	Benthivorous
7_Marble trout	7	<i>Salmo trutta marmoratus</i>	Predator
7_Chub	7	<i>Leuciscus cephalus</i>	Omnivorous
7_Grayling	7	<i>Thymallus thymallus</i>	Benthivorous

3. Results and discussion

3.1 Optimization of the extraction procedure

In the present work, a fractional factorial planning (4-1) was used to determine the best approach to extract pesticides from fish muscle tissue samples. To this end, different extraction salts, extraction solutions, and agitation times were evaluated (see Table 5). These experiments were carried out only with 32 of the target compounds.

Table 5. Experimental planning to optimize the QuEChERS extraction procedure.

EXP.	EXTRACTING SALTS	EXTRACTING SOLVENT	AGITATION TIME (sec)	
			EXTRACTION	CLEAN-UP
1	AOAC – ACETATE	ACN/1%HOAc	30	30
2	EN – CITRATE	ACN/1%HOAc	30	90
3	AOAC – ACETATE	ACN	30	90
4	EN – CITRATE	ACN	30	30
5	AOAC – ACETATE	ACN/1%HOAc	90	90
6	EN – CITRATE	ACN/1%HOAc	90	30
7	AOAC – ACETATE	ACN	90	30
8	EN – CITRATE	ACN	90	90
9	ORIGINAL - CHLORIDE	ACN/0.5%HOAc	60	60

AOAC – ACETATE: 6 mg MgSO₄, 1.5 g NaAc

EN – CITRATE: 4 mg MgSO₄, 1 g NaCl, 1 g NaCitate, 0.5 g DCS

ORIGINAL-CHLORIDE: 6 mg MgSO₄, 1.5 g NaCl

As for the extraction salts, the original QuEChERS unbuffered methodology that uses MgSO₄ (6 mg) and NaCl (1.5 g) (Anastassiades et al., 2003), and two variations of this methodology, i.e., the method AOAC 2007.01 that uses NaAc (1.5 g) instead of NaCl (AOAC, 2007), and the European method EN15662 that requires the use of MgSO₄(4 mg), NaCl (1 g), NaCitate (1 g) and DCS (0.5 g) (EURL-SRM, 2015), were tested. A clean-up process based on PSA (PSA-kit-04A, BEKOLut®) was applied in all cases because it was the clean-up recommended for cleaning high lipid content extracts. As extraction solvent, acetonitrile with and without acetic acid (1%) were tested.

Pareto charts were constructed for each pesticide with the absolute recoveries obtained in the extraction optimization study so that the significance of each of the tested parameters and the effects of the interactions among them on analyte recovery could be evaluated. Pareto charts for average recovery for 20 selected pesticides are provided as supplementary information (Figures 2-22).

Overall, the parameters tested did not significantly affect the recovery of most of the target analytes. Only linuron extraction was affected by the salt used in the process, azinphos-methyl extraction was affected by the extraction time, and chlortoluron extraction was affected by the pair salt-agitation time during the clean-up process. The use of longer times (e.g., 5 min) may have resulted in improved extraction of some analytes, but since samples were manually processed, this option was discarded. Although the original unbuffered extraction approach using MgSO_4 and NaCl followed by PSA-based cleanup is the one usually applied for pesticide analysis in fish in the peer-reviewed literature (Belenguer et al., 2014; Ernst et al., 2018; Náchter-Mestre et al., 2014), the method recommended by European Union (EU) regulations, i.e., the citrated buffered extraction, was selected because linuron, a pesticide with relatively poor MS sensitivity, was best recovered under those conditions.

Figure 2. Pareto Chart for the standardize effect of each parameter tested on the average recovery of all pesticides investigated (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

306

307

308 **Figure 3.** Pareto Chart for the standardize effect of each parameter tested on the average
309 recovery of atrazine (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation
310 time for extraction, T_AG2: agitation time for clean-up).

311

312

313 **Figure 4.** Pareto Chart for the standardize effect of each parameter tested on the average
314 recovery of azinphos (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation
315 time for extraction, T_AG2: agitation time for clean-up).

316

317 **Figure 5.** Pareto Chart for the standardize effect of each parameter tested on the average
318 recovery of chlorfenvinphos (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1:
319 agitation time for extraction, T_AG2: agitation time for clean-up).

320

321

322 **Figure 6.** Pareto Chart for the standardize effect of each parameter tested on the average
323 recovery of chlortoluron (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1:
324 agitation time for extraction, T_AG2: agitation time for clean-up).

325

326

327 **Figure 7.** Pareto Chart for the standardize effect of each parameter tested on the average
328 recovery of cyanazine (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation
329 time for extraction, T_AG2: agitation time for clean-up).

330

331 **Figure 8.** Pareto Chart for the standardize effect of each parameter tested on the average
332 recovery of DEA (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation
333 time for extraction, T_AG2: agitation time for clean-up).

334

335

336 **Figure 9.** Pareto Chart for the standardize effect of each parameter tested on the average
337 recovery of DIA (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time
338 for extraction, T_AG2: agitation time for clean-up).

339

340

341 **Figure 10.** Pareto Chart for the standardize effect of each parameter tested on the average
342 recovery of diazinon (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation
343 time for extraction, T_AG2: agitation time for clean-up).

344

345 **Figure 11.** Pareto Chart for the standardize effect of each parameter tested on the average
346 recovery of dichlorvos (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1:
347 agitation time for extraction, T_AG2: agitation time for clean-up).

348

349

350 **Figure 12.** Pareto Chart for the standardize effect of each parameter tested on the average
 351 recovery of dimethoate (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1:
 352 agitation time for extraction, T_AG2: agitation time for clean-up).

353

354

355 **Figure 13.** Pareto Chart for the standardize effect of each parameter tested on the average
 356 recovery of fenthion (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation
 357 time for extraction, T_AG2: agitation time for clean-up).

358

359 **Figure 14.** Pareto Chart for the standardize effect of each parameter tested on the average
 360 recovery of fenthion sulfoxide (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1:
 361 agitation time for extraction, T_AG2: agitation time for clean-up).

362

363

364 **Figure 15.** Pareto Chart for the standardize effect of each parameter tested on the average
365 recovery of irgarol (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation
366 time for extraction, T_AG2: agitation time for clean-up).

367

368

369 **Figure 16.** Pareto Chart for the standardize effect of each parameter tested on the average
370 recovery of isoproturon (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1:
371 agitation time for extraction, T_AG2: agitation time for clean-up).

372

373 **Figure 17.** Pareto Chart for the standardize effect of each parameter tested on the average
374 recovery of linuron (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation
375 time for extraction, T_AG2: agitation time for clean-up).

376

377

378 **Figure 18.** Pareto Chart for the standardize effect of each parameter tested on the average
379 recovery of metolachlor (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1:
380 agitation time for extraction, T_AG2: agitation time for clean-up).

381

382

383 **Figure 19.** Pareto Chart for the standardize effect of each parameter tested on the average
384 recovery of simazine (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation
385 time for extraction, T_AG2: agitation time for clean-up).

386

387 **Figure 20.** Pareto Chart for the standardize effect of each parameter tested on the average
388 recovery of terbuthylazine (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1:
389 agitation time for extraction, T_AG2: agitation time for clean-up).

390

391

392 **Figure 21.** Pareto Chart for the standardize effect of each parameter tested on the average
 393 recovery of malaoxon (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation
 394 time for extraction, T_AG2: agitation time for clean-up).

395

396 **Figure 22.** Pareto Chart for the standardize effect of each parameter tested on the average
 397 recovery of terbutryn (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation
 398 time for extraction, T_AG2: agitation time for clean-up).

399

400 **3.2. Optimization of LC-MS/MS conditions**

401 Optimum conditions for MS/MS determination of the target pesticides were
 402 obtained after flow injection of individual standard mixtures in positive and negative HESI
 403 modes. The identification of the molecular (parent) ions and the optimum RF lens voltage for
 404 their detection was carried out in full scan mode over an m/z range of 50-500, while the
 405 identification of the most abundant product ions and the optimum collision energies was
 406 achieved by means of product-ion scan. The MS/MS method was created in compliance with

European Commission Decision 2002/657/EC (EC, 2002) that establishes the need of acquiring 2 SRM transitions per analyte (one for surrogate standard) to obtain enough identification points. All transitions, regardless of their ionization mode, were recorded within one single acquisition window.

Analyte peak shape and chromatographic separation with different organic linear gradients were evaluated by injecting (20 μ L) a pesticide standard mixture (200 ng/mL). The composition of the mobile phase (ACN and water with no modifiers) was selected on the basis of previous optimization tests performed in the lab for LC-MS/MS analysis of 26 pesticides in sediment samples (Köck-Schulmeyer et al., 2013). This composition allows the simultaneous analysis of all target pesticides during one unique chromatographic run, regardless of their different physical-chemical properties. Extracted ion chromatograms of the target analytes obtained after LC-MS/MS analysis of a fortified fish muscle tissue sample at 10 ng/g f.w. are shown in Figure 23.

3.3. Method performance

Method performance for the different analytes in terms of linearity, trueness (recovery), precision, and sensitivity is summarized in Table 5. Calibration curves constructed within the concentration range 0.01 ng/mL-1000 ng/mL presented coefficients of determination (r^2) above 0.99 for all analytes, except fenthion (0.9886), fluroxypyr (0.9877) and pendimethalin (0.9746). The linearity range, including only calibration solutions not deviating $\pm 20\%$ from theoretical concentrations (as suggested by SANTE/11813/2017 guidelines), expanded from the analyte LOQ to 1000 ng/mL in all cases, with the exception of 2,4-D, azinphos-methyl, diuron, fenthion, fenthion sulfoxide, fluroxypyr, MCPA, mecoprop, oxadiazon, pendimethalin, and thiamethoxam (only up to 500 ng/mL).

Figure 23. Extracted ion chromatograms of the target pesticides after QuEChERS and LC-MS/MS analysis of a fish muscle tissue sample fortified at a concentration of 10 ng/g f.w. (*50 ng/g for those compounds with LOD above 10 ng/g f.w.)

441 **Table 5.** Parameters of method performance: linearity (r^2), absolute and relative recoveries and
 442 repeatability at 10 ng/g and 500 ng/g, and method sensitivity for the different compounds in fish
 443 samples.

Analyte	Linearity (r^2)	Recovery ^a				Repeatability ^b		Sensitivity ^c		
		Absolute (%)		Relative (%)		RSD (%)		ng/g f.w.		
		10 ng/g	500 ng/g	10 ng/g	500 ng/g	10 ng/g	500 ng/g	LOD	LOQ	LODet
2,4-D	0.9930	24	22	107	103	10.4	11.6	1.28	4.29	9.41
Acetamiprid	0.9993	45	61	86	111	6.1	3.9	0.02	0.07	0.13
Alachlor	0.9985	51	41	94	107	16.3	5.9	0.48	1.59	1.59
Atrazine	0.9959	50	61	88	118	5.5	4.4	0.02	0.05	0.06
Azinphos ethyl	0.9915	77	80	87	114	15.2	11.2	1.07	2.49	2.49
Azinphos methyl	0.9921	39	41	107	108	9.3	19.8	1.70	5.68	5.68
Azinphos methyl oxon ^y	0.9979	33	35	93	88	9.3	8.4	0.05	0.17	0.44
Bentazone	0.9982	79	106	115	116	5.0	12.4	0.36	1.20	1.49
Bromoxynil	0.9912	38	39	95	83	8.0	3.3	0.81	2.73	2.73
Chlorfenvinphos	0.9981	81	71	87	98	9.2	4.2	0.08	0.27	0.27
Chlorotoluron	0.9976	59	60	106	110	7.6	7.2	0.16	0.53	0.53
Chlorpyrifos ⁸	0.9989	BLOD	48	BLOD	81	-	14.9	14.15	28.51	28.51
Clothianidin	0.9963	38	49	105	119	9.4	5.3	0.32	1.05	1.05
Cyanazine	0.9987	49	60	86	115	8.9	3.6	0.03	0.11	0.20
DEA	0.9963	30	38	89	106	4.0	8.0	0.08	0.28	0.42
DIA	0.9960	21	40	71	103	16.6	16.3	0.22	0.73	0.73
Diazinon	0.9960	59	58	91	105	2.4	6.3	0.01	0.04	0.04
Dichlorvos	0.9979	28	81	80	107	18.9	12.6	0.32	3.73	3.73
Diflufenican	0.9939	38	37	81	83	16.3	7.8	0.34	1.13	1.13
Dimethoate	0.9977	46	58	94	117	3.6	3.78	0.03	0.11	0.29
Diuron	0.9943	60	59	102	103	16.7	9.7	0.20	1.23	1.23
Fenitrothion	0.9952	BLOD	76	BLOD	92	-	10.6	22.39	87.07	87.07
Fenitrothion oxon	0.9933	BLOD	86	BLOD	113	-	10.9	12.50	18.21	18.21
Fenthion	0.9886	BLOD	57	BLOD	110	-	13.4	21.46	49.80	49.80
Fenthion oxon	0.9933	65	66	100	116	6.3	5.3	0.01	0.04	0.04
Fenthion oxon sulfone	0.9973	55	62	75	108	15.4	18.6	0.05	0.18	0.52
Fenthion oxon sulfoxide	0.9952	62	69	93	114	3.6	6.3	0.01	0.04	0.06
Fenthion sulfone	0.9924	BLOD	69	BLOD	113	-	9.8	18.00	35.06	60.00
Fenthion sulfoxide	0.9977	62	66	96	117	4.0	6.3	0.02	0.08	0.08
Fluroxypyr ²	0.9877	BLOD	27	BLOD	100	-	6.8	23.25	77.51	77.51
Imidacloprid	0.9941	46	50	100	118	10.9	9.3	0.15	0.49	0.49
Irgarol	0.9938	74	71	109	110	7.9	3.6	0.01	0.05	0.06
Isoproturon	0.9988	73	69	110	112	11.9	5.5	0.12	0.39	0.39
Linuron	0.9974	51	57	95	101	18.2	7.4	1.05	1.48	1.48
Malaoxon [*]	0.9935	47	62	83	115	7.0	9.2	0.32	1.07	1.07
Malathion	0.9953	59	54	84	88	15.8	7.4	0.50	0.92	0.92
MCPA	0.9916	29	25	110	118	16.8	16.3	0.72	2.43	9.89
Mecoprop	0.9924	33	31	109	115	19.5	12.0	0.35	1.18	1.22
Methiocarb	0.9937	43	48	85	93	11.9	4.0	0.34	1.15	1.15
Metolachlor	0.9989	65	53	89	90	5.0	6.6	0.01	0.04	0.04
Molinate ⁶	0.9980	58	52	107	92	15.4	7.0	0.30	1.02	1.02
Oxadiazon	0.9903	BLOD	61	BLOD	120	-	6.6	50	100	100
Pendimethalin	0.9746	BLOD	54	BLOD	103	-	20.6	50	150	150
Propanil	0.9930	44	48	78	83	13.0	10.1	0.60	2.00	2.00
Quinoxifen ^{uu}	0.9921	31	36	80	82	10.6	17.2	0.79	2.65	2.65
Simazine	0.9957	36	51	91	119	6.6	5.4	0.20	0.50	0.50
Terbutylazine	0.9964	41	48	89	115	3.4	4.3	0.02	0.06	0.13
Terbutryn	0.9984	71	73	89	108	5.5	4.7	0.01	0.05	0.06
Thiacloprid	0.9956	47	55	89	117	3.4	4.5	0.02	0.06	0.15
Thiamethoxam	0.9939	55	51	117	103	10.5	3.5	0.33	1.10	1.10
Thifensulfuron methyl	0.9960	27	31	108	120	11.5	1.1	0.23	0.78	0.78
Triallate	0.9906	64	48	116	96	4.3	12.0	2.93	9.79	9.79

444 ^y Compound quantified using *fenthion sulfoxide-d₆* as surrogate standard
 445 ^z Compound quantified using *triallate-¹³C₆* as surrogate standard
 446 ^x Compound quantified using *thiamethoxam-d₃* as surrogate standard² Compound quantified using *mecoprop-d₃* as surrogate
 447 standard
 448 ^a Compound quantified using *chlortoluron-d₆* as surrogate standard
 449 ^b Compound quantified using *linuron-d₆* as surrogate standard
 450 ^w Compound quantified using *methiocarb-d₃* as surrogate standard
 451 ^a Average absolute recovery: comparison of peak areas obtained in n=6 fortified samples and a methanol standard
 452 solution at equivalent concentrations. Average relative recovery: comparison of absolute recoveries obtained for
 453 the analyte and its corresponding surrogate standard (n=5).
 454 ^b Repeatability: relative standard deviation of the relative recoveries observed in n=5 fortified samples.
 455 ^c Sensibility: limit of detection (LOD) and limit of quantification (LOQ) – estimated concentration that would result
 456 in a S/N of 3 and 10, respectively. Calculated from fish fortified samples at 10 ng/g. LODet: limit of determination,
 457 minimum concentration at which the analyte can be quantified (LOQ of SRM1) and confirmed (LOD of SRM2).
 458 BLOD: below limit of detection.

459

460 Analyte absolute and relative recoveries were in general in good agreement at both
 461 investigated concentration levels (10 ng/g f.w. and 500 ng/g f.w.). Average absolute recovery
 462 values ranged from 21 % (deisopropylatrazine (DIA)) to 106% (bentazone), being below 50% for
 463 38% of the analytes. Although poor absolute recoveries affect negatively method sensitivity,
 464 the use of isotopically labeled compounds serves to compensate the two possible causes
 465 behind them, i.e., poor extraction efficiency and/or matrix ionization suppression effects,
 466 which would eventually lead to inaccurate results. The six compounds for which isotopically
 467 labeled analogs were not available were quantified using isotopically labeled compounds
 468 presenting as far as possible similar absolute recoveries, structure, and retention times.
 469 Average relative recoveries were found to range between 71 and 120% for all compounds.
 470 Recovery rates in replicate (n=6) samples were found to be consistent, with RSD values <15%
 471 in most cases, and below 20% in all cases with the exception of pendimethalin at 500 ng/g
 472 f.w. Accuracy and precision values thus overall complied with SANTE/11813/2017 guidelines
 473 for performance acceptability criteria of quantitative methods (average recovery between 70
 474 and 120% and repeatability at each spiking level with RSD below 20%) (EC, 2017).

475 As for sensitivity, the method allows detecting and quantifying most of the target
 476 pesticides at concentrations in the pg/g f.w. or the low ng/g f.w. range, thus covering the
 477 values expected for this type of compounds in fish. LODs below 3 ng/g f.w. and LODets below

the general MRL of 10 ng/g applied to pesticides in food, were obtained for 44 out of the 52 pesticides investigated. Compounds showing higher LODets and therefore not sufficing the necessary sensitivity required for their determination in this matrix according to legislation were: chlorpyrifos, fenitrothion, fenitrothion oxon, fenthion, fenthion sulfone, fluroxypyr, oxadiazon, and pendimethalin. In contrast, many other relevant pesticides, such as those included in the list of priority substances in water alachlor (LODet 1.59 ng/g f.w.), atrazine (0.06), chlorfenvinphos (0.27), diuron (1.23), isoproturon (0.39), simazine (0.50), quinoxifen (2.65), irgarol (0.06), dichlorvos (3.73) and terbutryn (0.06) (EC 2013), and those of the Watch List methiocarb (1.15), imidacloprid (0.49), thiacloprid (0.15), thiamethoxam (1.10), clothianidin (1.05) and acetamiprid (0.13) (EC 2018), could be determined at levels down to 0.06-3.73 ng/g f.w.

Figure 24 summarizes the extent of matrix effects affecting pesticide analysis in trout tissue with the described methodology. Matrix effects were not significant for 11 of the 52 target analytes ($\pm 20\%$ variation of the signal). Except in the case of six pesticides, that showed some signal enhancement, matrix effects occurred in the form of signal ionization suppression caused by co-eluting matrix components. The compounds affected the most by signal suppression effects were quinoxifen (-98%), DIA (-72%), and dichlorvos and oxadiazon (-64%). On the contrary, signal enhancement was significant for azinphos-ethyl (+23%) and fenitrothion oxon (+32%). As previously mentioned, the use of isotopically labeled standards compensates for these effects that may also differ among fish species due to their different fat and protein content. Low matrix effects (<20%) for LC-MS analysis of pesticides in hake tissues have been reported by Belenguer et al. (Belenguer et al., 2014), whereas strong ionization suppression effects were observed in carp and perch muscle (up to -94%) by Lazartigues et al. (2011).

Figure 24. Matrix effects found in the analysis of the target pesticides in fish (trout) tissue samples.

506

507 **3.4. Occurrence of target pesticides in real samples**

508 As part of the validation process, the applicability of the method was evaluated
509 through the analysis of the target pesticides in 13 fish muscle tissue samples from the Adige
510 River basin (Italy). Positive identification followed the criteria established by the SANTE
511 guidelines (EC, 2017) regarding pesticide analysis in food: SRM transitions for one analyte
512 fully overlapped, the analyte SRM ratio in the sample extract was within $\pm 30\%$ of the average
513 SRM ratio observed in the calibration standards for the same analytical batch, and the
514 analyte retention time in the extract corresponded to that of the calibration standard within
515 ± 0.1 min. Larger retention time deviations were accepted when the corresponding
516 deuterated analogue retention time was also shifted due to matrix components.

517 Results obtained (concentrations above LOQ in ng/g f.w.) are shown in Figure 25.
518 Twelve out of the 13 analysed samples were found to contain measurable quantities of the
519 target pesticides. However, only 5 compounds were quantified: quinoxifen, terbutryn,
520 metolachlor, acetamiprid and irgarol. Trace levels below the method limit of determination
521 of diazinon (< 0.04 ng/g f.w.), dichlorvos (< 3.73 ng/g f.w.), and diuron (< 1.23 ng/g f.w.) were
522 detected in a few samples.

Figure 25. Concentrations of pesticides (ng g⁻¹ f.w.) quantified in fish muscle tissue samples.

Metolachlor followed by quinoxifen were the most ubiquitous compounds, being present in 11 and 10 samples, respectively. Quinoxifen was below its LOQ in 7 out of the 10 samples that contained it. Irgarol, terbutryn and acetamiprid were found in only one sample.

The compound showing the maximum concentration was quinoxifen (2.97 ng/g f.w. in a grayling (*Thymallus thymallus spp.*) sample collected in sampling location 7), a quinoline fungicide very used in agriculture for disease control in grapes and hops. This compound presented similar concentrations in the other two fish tissue samples where it was found. The second most abundant compound was metolachlor, a chloroacetanilide herbicide used for the control of many kinds of grass and broadleaf weeds in crops. Its average value was 0.59 ng/g f.w. Quinoxifen levels in river fish muscle and liver samples have been also investigated by Kaczynski et al. (Kaczyński et al., 2017), and were found to be below the method limit of detection. As for metolachlor, the levels found in this study were slightly

lower than those measured in fish muscle tissue by Kaczynski et al. (2017) (up to 11 ng/g f.w) and Belenguer et al. (2014) (4 ng/g d.w).

Irgarol and terbutryn, measured at trace levels in trout individuals, are booster biocides used in antifouling paints of boats, and thus fish exposure to these compounds may be of concern. Irgarol has been previously detected in liver samples from sea mullets at concentrations up to 6.9 ng/g (Franco-Barrios et al., 2014). Terbutryn is included in the EU list of potential endocrine disruptors within category 1: substances for which endocrine activity has been documented in at least one study of a living organism (Petersen et al., 2007). These substances are given the highest priority for further studies.

All pesticides detected except acetamiprid present a high bioaccumulation potential (Log Kow from 3.5 to 4.7) (see Table 1). However, the high solubility of acetamiprid (2950 mg/L) may result in a constant exposure of fish to this compound and hence its bioaccumulation, which could occur through sorption mechanisms different from those expected for less polar neutral pesticides. Acetamiprid and other nicotinoids were investigated in eels by Xiao et al. (2013), but were not found at levels above the method LOD in any of the analyzed samples.

Some of the investigated pesticides were found in the benthic species grayling (*Thymallus thymallus spp.*) and bullhead (*Cottus gobio spp.*). This indicates that species living and feeding at the river bottom may also find there a source of pesticides (Belenguer et al., 2014).

Finally, it may be worth noting that five out of the eight detected pesticides are included in either the list of priority substances in water (diuron, dichlorvos, irgarol, quinoxifen and terbutryn) (EC 2013) or in the Watch List of substances for Union-wide monitoring in the field of water policy (acetamiprid) (EC 2018).

4. Conclusions

An analytical methodology, based on QuEChERS extraction and LC-HESI-MS/MS detection has been developed for analysis of 52 pesticides belonging to different chemical classes in fish muscle tissue. The method described shows overall satisfactory performance in terms of repeatability (with RSD values below 20.6 %) and trueness (average relative recoveries between 71 and 120%). The method, with LODs in the low ng/g f.w. range for most compounds, allows determining 44 of the 52 target pesticides at levels below the general MRL of 10 ng/g that applies to pesticides in food when no specific MRLs are regulated. Additional advantages of the methodology developed that make it attractive for regular monitoring and quality control are simplicity and high throughput, if compared to methodologies based on pressurized liquid extraction or ultrasound assisted extraction and solid-phase extraction clean-up, and relatively low cost. Surrogate standards are costly and increase the initial economical investment needed to apply the proposed method. However, their use makes unnecessary the performance of other time-consuming methods of calibration and quantification like matrix-matched calibration or standard addition and it is essential to generate accurate and reliable results in the analysis of complex samples like fish where matrix variability and effects can be very large. Another advantage of using surrogate standards is that their presence in the sample extracts at the expected MS signal response provides evidence that the analytical process has taken place correctly. This obviates the need also for performing replicate analyses of the samples. Application of the method to fish individuals collected in the Adige basin revealed the presence of five of the target pesticides (e.g., irgarol, metolachlor, quinoxifen, terbutryn, acetamiprid) at quantifiable levels. Trace levels of diazinon, diuron, and dichlorvos were also detected. These findings indicate that these compounds do not need to be as persistent as organochlorine compounds to accumulate in aquatic organisms. However, additional and more extensive monitoring

studies are needed to understand the potential of these compounds to bioaccumulate and the potential effects of this bioaccumulation, so that aquatic ecosystems can be preserved.

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