QuEChERS-based analytical methods developed for LC-MS/MS multiresidue determination of pesticides in representative crop fatty matrices: Olives and sunflower seeds

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Keywords:
Pesticide residue analysis
Agrochemicals
Liquid chromatography-tandem mass spectrometry
Insecticides
Herbicides
Sample pre-treatment

ABSTRACT
Oilseed crops are greatly extended all over the world. Their high fat content can interfere during pesticide multiresidue analysis through liquid chromatography-tandem mass spectrometry (LC-MS/MS). This work aimed at overcoming this issue by developing and validating two QuEChERS-based methods for LC-MS/MS determination of 42 pesticides in two fatty food matrices: olives and sunflower seeds. Optimization of the extraction method was achieved following a 2^{6-2} fractional factorial design in a highly cost-effective way. Validation of the multi-residue methods demonstrated improved limits of detection, below the established maximum residue levels (MRLs) for almost all compounds, good precision, and trueness, in compliance with SANTE guidelines. Application of these methods to the analysis of real samples from the Iberian Peninsula showed the presence of some pesticides of relevant environmental concern, including four compounds contained in the Pesticide Action Network International list of highly hazardous pesticides, found at levels between 0.03 ng/g and 104 ng/g.

1. Introduction

In the last decades, European policies have aimed to reduce the use of pesticides for the sake of a more sustainable agriculture. Firstly, through the Common Agricultural Policy raised in 1962, and more recently through the European Directive 2009/128/EC (European Commission, 2009), the European Commission (EC) has established an Integrated Pest Management System and recommended reducing pesticide dependence in agriculture. However, in practice, these policies have not achieved their goals. The fact that pesticide sales have not decreased since 2011 indicates that farmers are still strongly relying on these chemicals for growing their crops (Eurostat, 2020). A recent report from the EC explicitly stated that “pesticides are a cause of pollution and have a direct effect especially on the state of biodiversity, water bodies, and soils” (European Commission, 2017). Moreover, many environmentally stable pesticides can also be harmful to humans due to their bio- accumulative and toxic properties (Bernardes, Pazin, Pereira, & Dorta, 2015). Among the EU member states, Germany, Spain, France, and Italy represent over 66% of the total EU pesticide sales volume. Huge amounts of these chemicals are released into the environment and, due to their well-known ubiquity, the monitoring of their levels on the different environmental compartments has become essential. In food, the EC has fixed Maximum Residue Levels (MRLs, the highest level of a pesticide residue that is legally tolerated in or on food), which vary depending on the commodity and the specific pesticide toxicity and environmental occurrence (European Commision, 2005). When a

Table 1 (*continued*)

Target analyte	Abbrev.	HESI mode	SRMs (m/z)	RF lens (V)	CE (eV)	SRM1/SRM2	RT (min) Olives/Sunflower
Fenthion oxon	FENOX	+	263 > 231	62	16	2.3	8.88/8.84
			263 > 216	62	24		
	FENOX-d_3	+	266 > 234	69	17		8.85/8.82
Fenthion sulfone	FENS	+	311 > 125	65	21	3.1	9.39/9.33
			311 > 233	84	17		
	FENS-d_6	+	317 > 131	72	21		9.32
Propanil	PROP	+	218 > 127	53	26	1.1	10.11/10.07
			218 > 162	53	16		
	PROP-d_5	+	223 > 128	59	28		10.05
Methiocarb	METCB	+	226 > 169	35	9	1	10.32/10.29
			226 > 121	35	18		
	METCB-d_3	+	229 > 169	30	9		10.30/10.25
Azinphos methyl	AZM	+	318 > 132	30	15	1.6	10.57/10.54
			318 > 261	30	5		
	AZM-d_6	+	324 > 132	43	15		10.51
Terbuthylazine	TERBZ	+	230 > 174	52	17	8.8	10.57/10.56
			230 > 104	52	31		
	TERBZ-d_5	+	235 > 179	55	17		10.51
Linuron	LINU	+	249 > 160	51	19	1.3	10.78/10.75
			249 > 133	51	34		
	LINU-d_6	+	255 > 185	48	18		10.70
Molinate	MOLI	+	188 > 126	43	12	4.8	12.02/11.99
			188 > 98	43	17		
	METCB-d_3	+	229 > 169	30	9		10.30/10.25
Malathion	MALA	+	353 > 227	70	16	4.9	12.10/12.10
			353 > 307	70	15		
	MALA-d_{10}	+	363 > 237	70	18		12.00
Alachlor	ALA	+	270 > 162	40	21	2.7	12.51/12.50
			270 > 132	40	41		
	ALA-d_{13}	+	283 > 251	45	10		12.35
Metolachlor	METO	+	284 > 252	48	15	2.4	12.39/12.39
			284 > 176	48	26		
	METO-d_{11}	+	295 > 263	48	17		12.25
Terbutryn	TBTN	+	242 > 186	55	19	15.2	12.85/12.19
			242 > 158	55	25		
	TBTN-d_5	+	247 > 191	59	19		12.81/12.16
Irgarol	IRGA	+	254 > 198	57	19	15.2	12.57/12.07
			254 > 108	57	30		
	IRGA-d_9	+	263 > 199	60	19		12.47/12.05
Azinphos ethyl	AZET	+	346 > 137	37	25	1.1	12.70/12.69
			346 > 97	37	31		
	CFVP-d_{10}	+	369 > 170	58	41		12.78
Chlorfenvinphos	CFVP	+	359 > 170	60	41	1.2	12.88/12.90
			359 > 99	60	27		
	CFVP-d_{10}	+	369 > 170	58	41		12.78
Fenthion	FEN	+	279 > 169	63	19	1	14.02/14.05
			279 > 247	63	13		
	FEN-d_6	+	285 > 169	62	19		13.94
Diazinon	DIAZ	+	305 > 169	64	22	1.9	14.62/14.64
			305 > 153	64	21		
	CFVP-d_{10}	+	369 > 170	67	21		12.78
Diflufenican	DIFLU	+	395 > 266	60	24	4.6	15.35/15.38
			395 > 246	60	34		
	DIFLU-d_3	+	398 > 268	84	25		15.31/15.36
Quinoxyfen	QUIN	+	310 > 199	102	33	1.9	17.38/17.31
			310 > 216	102	36		
	QUIN-d_4	+	312 > 162	112	44		17.32/17.27

HESI: Heated Electrospray Ionization; SRM: Selected Reaction Monitoring; RF Lens: Radio Frequency Lens; CE: Collision Energy; DIA: Desisopropylatrazine; DEA: Desethylatrazine; 2,4D: 2,4-Dichlorophenoxyacetic acid; MCPA: 2-methyl-4-chlorophenoxyacetic acid.

Pesticide multiresidue analysis in food represents a challenging task due to the frequently low concentrations of these chemicals and the complexity of the different matrices (Abbaspour et al., 2019; Parrilla Vazquez et al., 2016; Romero-Gonzalez et al., 2014; Valverde et al., 2018). To overcome these difficulties, sample pre-treatment remains as one of the most important steps to be considered during method opti- mization. For the extraction of pesticides from food, diverse methods have been reported in the last years, including solid phase extraction (SPE) (Huo et al., 2016; Shamsipur et al., 2016), solid phase micro- extraction (SPME) (Choi et al., 2020; Kasperkiewicz & Pawliszyn, 2021; Liang et al., 2017; Pelit et al., 2015) and dispersive liquid-liquid micro-extraction (DLLME) (Chu et al., 2015; Farajzadeh et al., 2017; Ghoraba et al., 2018). However, over these extractive procedures, the QuEChERS method (acronym of quick, easy, cheap, effective, rugged, and safe), a two-step procedure consisting of a salting-out solid-liquid extraction and a dispersive-SPE (d-SPE) clean-up, has become the pre- treatment of choice for most laboratories worldwide (Barchanska et al., 2018). Two international standard organisations have in fact established two different versions of the original QuEChERS method as official methods for determination of pesticides in food: the European Committee for Standardisation CEN ((CEN) Standard Method EN 15662, n.d.) and the AOAC International (Lehotay & Collaborators, 2007). This responds to the broad applicability of the methodology to a wide range of organic compounds and food matrices. In addition, QuEChERS extraction, when combined with chromatographic techniques coupled to mass spectrometry (MS), allows multiresidue analysis without compromising the

method sensitivity and selectivity.

Oilseed crops are greatly extended all over the world as they play an important role in human nutrition. Their high fat content makes them a suitable energy source but, at the same time, represents a relevant source of interferences in the analysis of pesticide residues in these matrices. Therefore, great efforts have been made in the last years to improve clean-up procedures in such matrices (Madej et al., 2018). For instance, Cunha et al. evaluated different sorbents for the d-SPE of pesticides from olives, including $MgSO_4$, primary secondary amine (PSA), graphitized carbon black (GCB), and octadecylsilane (C_{18}), and concluded that the combination of all sorbents gave the cleanest extracts (Cunha et al., 2007). Another study showed better results with QuEChERS, as compared to matrix solid-phase dispersion, for the extraction of >100 pesticides from olives, using the aforementioned sorbents (Gilbert-Lopez et al., 2010). Also in olives, negligible advantages were observed with the use of advanced sorbents such as Z-Sep or the novel Enhanced Matrix Removal-Lipid (EMR), obtaining a superior removal efficiency of co-extracts with the combination of PSA + C18 (Lopez-Blanco et al., 2016). Freezing is also a common direct clean-up for fatty commodities, due to the different melting points of pesticides and fat. However, poor recoveries and the relatively long time of the procedure make this purification approach unsuitable for the analysis of low melting point pesticides (Kaczynski, 2017). Apart from this, most of the methods described in the literature for the analysis of pesticides in fatty food matrices, with few exceptions (Gilbert-Lopez et al., 2010; Kaczynski, 2017), cover only a limited number of pesticides and they often belong to the same chemical class.

In this context, the objectives of this work were (1) to develop and validate a practical LC-MS/MS method for the multiresidue analysis of up to 42 moderately polar pesticides, including neonicotinoids, triazines, phenylureas, organophosphates or anilines, in two representative fatty commodities: olives and sunflower seeds, and (2) to apply the methodology developed to the analysis of various samples of different origin.

2. Materials and methods

2.1. Chemicals and reagents

High purity (96-99.9%) standards of 42 pesticides and 34 isotopically-labelled analogues used as surrogate standards for quantification were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany) or Dr. Ehrenstorfer (LGC Standards, Teddington, UK). Target analytes are shown in Table 1, whereas relevant physical-chemical properties (molecular mass, solubility, acid dissociation constant (pKa), and octanol water partition coefficient (log K_{ow}), among others) are provided in Table S1 as Supporting information (SI). 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 °C. Working solutions at different concentrations (from 0.01 to 500 ng/mL) containing all analytes were prepared by appropriate dilution of the stock individual solutions in methanol. 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 2000 ng/mL was also prepared and used for method optimization, validation, and real sample quantification. Pesticide-grade solvents MeOH, aceto- nitrile (ACN), and LC-grade water were supplied by Merck (Darmstadt, Germany).

2.2. Sample preparation and extraction procedure

Olives were pitted and the edible part was milled in a coffee grinder. In the case of sunflower seeds, ultra-freezing with liquid nitrogen was performed prior to the grinding to obtain a better specific surface area to work with after milling. Then, samples were stored in the dark frozen at –20 °C. At the time of analysis, 7.5 g of pitted olives and sunflower seeds (containing kernel and husk) was weighed in 50 mL polypropylene centrifuge tubes. Then, 375 gL of the surrogate standard mixture was added to the samples (final concentration of 50 ng/g). In the case of sunflower seeds, 7.5 mL of deionized water was also added and manually shaken for matrix hydration (Kaczynski, 2017). The prepared samples were left under the hood for 60 min to allow the MeOH of the added surrogate standard solution evaporate. For extraction, 15 mL of ACN (with 1% formic acid) was added to the sample and vigorously mixed. Afterwards, the salting out step was performed with 6 g $MgSO_4$ and 1.5 g sodium acetate provided by Bekolut® GmbH & Co. KG (Hauptstuhl, Germany) as SALT-Kit-AC2. The sample was then manually and vortex shaken for 1 min and, after that, tubes were centrifuged for 5 min at 3220 Relative Centrifugal Force (RFC). Then, 7 mL of the supernatant was transferred into a 15-mL centrifuge tube for clean-up. Two different clean-up sorbents were used for each matrix: a) Olives: 1200 mg $MgSO_4$, 400 mg PSA, 400 mg GCB, 400 mg C18e (Bekolut® PSA-Kit-08A), b) Sunflower seeds: 900 mg MgSO4, 150 mg PSA, 150 mg C18e (Bekolut® PSA-Kit-04). After vigorous manual and vortex shaking during 1 min, tubes were centrifuged for 5 min and 3220 RFC. Final extracts of olives and sunflower seeds, without any intermediate evap- oration step with nitrogen, were acidified with 0.7% and 0.5% of formic acid, respectively, and transferred into 2 mL vials for LC-MS/MS analysis.

2.3.Analytical conditions

Chromatographic separation was carried out with an LC system Aria Mx equipped with two Transcend quaternary pumps (max pressure 600 bars) connected in series with a triple quadrupole TSQ Quantiva mass spectrometer, and interfaced with a heated electrospray ionization (HESI) source (Thermo Fisher Scientific Inc.). A 10 gL volume of extract was injected into the chromatographic column (a Purospher STAR RP- 18e column, 150 x 2.1 mm, 2 ^m particle diameter from Merck (Darmstadt, Germany)) using a CTC Pal autosampler. Retained analytes were eluted using a mobile phase at a flow rate of 0.2 mL/min. Starting with 10% of ACN (organic constituent of the mobile phase) in water (aqueous constituent of the mobile phase), a linear organic gradient was established as follows: after 1 min in isocratic organic conditions, the organic phase proportion increased to 50% in 2.5 min. Then, during the following 10 min, the ACN proportion achieved 80% and, finally, 100% in 1 min. Afterwards, isocratic conditions were maintained for 2.5 min and then initial conditions were restored in 2 min and kept for 7.5 min to ensure complete re-equilibration of the column sorbent.

Selected reaction monitoring (SRM) was used for the MS/MS acquisition. Two SRM transitions were chosen for each target compound (one for quantification and another one for confirmation), and one SRM transition for each surrogate standard, based on the intensity and selectivity of the fragments. Positive and negative ionization modes were alternated, allowing the determination of all target pesticides in one single run. Optimum conditions of each transition, regarding collision energy, RF lens voltage, and m/z are summarized in Table 1. With respect to the MS general detection conditions, ion spray voltage was set to 3500 V in the positive ion mode, and –2500 V in the negative ion mode, ion transfer tube temperature was 350 ˚C, and vaporizer temperature was 280 ˚C. Nitrogen was used as sheath, sweep and auxiliary gas for the nebulization stage at the HESI, and argon was used as the collision gas at a pressure of 2.5 mTorr. Instrument setup and control, data acquisition and quantification were performed by Thermo Scientific Xcalibur v.4.1.31.9 software (Thermo Fisher Scientific Inc.).

2.4.Method optimization and validation

The extraction procedure was optimized through a 2^{6-2} fractional factorial design of experiments (DOE). This DOE was used to accommodate two 3-levels continuous factors with two 2-levels categorical factors (Table S2). A total of 16 randomized elemental experiments were performed. The regression model adjusted for each compound included lineal effects of all factors, quadratic effects of continuous factors and also their interaction (Table S2). Statistical evaluation of the optimization results was carried out with the software JMP 12.1.0 (SAS Institute Inc., Cary). Validation of the optimized analytical methods was per- formed in terms of compound recoveries, matrix effects, linearity, precision, and limits of detection and quantification (LOD and LOQ, respectively) following the guidelines described in Document No. SANTE/12682/2019 (European Commission, 2019). A representative (pool of various samples) matrix of olives and sunflower seeds was employed for the multiresidue method validation and, as no blank matrices were available, the background concentration of the target analytes, if present, was taken into consideration in the calculations. Quantification was performed by the internal standard method, in which surrogate standards are added at the beginning of the analytical method to account for all possible sources of errors throughout the method (analyte losses, poor recoveries and matrix effects). Surrogate com- pounds were the deuterated analogues of the target pesticides in most cases (Table 1). Method linearity was determined by the coefficient of determination (r^2) of the weighed (1/x) linear regression model obtained through the 11-point calibration curves built for each compound within the linearity range 0.01 ng/mL – 500 ng/mL (equivalent to 0.02 ng/g-1000 ng/g, respectively).

Analyte recoveries and precision of the method were calculated through the analysis of n = 6 replicate samples fortified at two different concentration levels (10 ng/g, as the default minimum MRL, and 100 ng/g). Absolute recoveries were obtained by comparing analyte peak areas in spiked samples and methanolic solutions at equivalent con- centrations. Relative recoveries were calculated by comparing the ab- solute recovery of the analyte and that of the isotopically-labelled compound used as surrogate standard. The relative standard deviation (RSD %) of the n = 6 replicates analysed at both concentration levels (10 ng/g and 100 ng/g) was used to assess method precision.

Matrix effects were obtained by comparing analyte peak areas of the samples spiked after extraction, prior to LC-MS analysis, and methanolic standards at the same concentration (50 ng/mL, equivalent to 100 ng/ g). Method LODs and LOQs were calculated through the signal to noise (S/N) method. A matrix-matched calibration curve in the low linearity range (0.1-10 ng/g) was generated for each compound and the S/N obtained in these solutions was used for LOD and LOQ calculation. Moreover, each integrated peak was visually checked for an extra confirmation. A S/N ratio of 3 was used for the LOD, and a S/N ratio of 10 was used for the LOQ. Then, the limit of determination (LDet) was established as the LOD of the SRM2 when higher than the LOQ of SRM1, and indicates the minimum concentration that can be quantified (SRM1) and confirmed (SRM2).

2.5.Real samples determination

After validation, the analytical method was applied and evaluated for the determination of the target pesticides in olives and sunflower seeds harvested from different sites of the Iberian Peninsula during 2018, 2019 and 2020. MCMG, SBG and JMG codes correspond to sun- flower seed samples, whereas JMO (Cordovil variety), JMC (Cordovil variety), LBO (Verdeal variety), Farga, Sevillenc, Mor, and Sossis correspond to olive samples.

Table 2
Description of 16 elemental experiments of the 2^{6-2} fractional factorial DOE for extraction optimization.

Pattern	Factors			
	CAN (*formic acid*)[a]	Formic acid in final extract[b]	Extractive salts[c]	Clean-up sorbent[d]
− − − − − −	−1	−1	Citrate	4
− − − + + +	−1	0	Acetate	8A
− − + − + +	−1	0	Acetate	8A
− − + + − −	−1	+1	Citrate	4
− + − − + −	0	−1	Acetate	4
− + − + − +	0	0	Citrate	8A
− + + − − +	0	0	Citrate	8A
− + + + + −	0	+1	Acetate	4
+ − − − − +	0	−1	Citrate	8A
+ − − + + −	0	0	Acetate	4
+ − + − + −	0	0	Acetate	4
+ − + + − +	0	+1	Citrate	8A
+ + − − + +	+1	−1	Acetate	8A
+ + − + − −	+1	0	Citrate	4
+ + + − − −	+1	0	Citrate	4
+ + + + + +	+1	+1	Acetate	8A

Pattern: ACN (formic acid) and formic acid extract are continuous factors. "- -" corresponds to level −1, "-+" or "+-" corresponds to level 0, and "++" corresponds to level + 1. The first 2 symbols of the pattern determine the level of ACN (formic acid), the third and fourth determine the level of Formic acid extract and, then, the fifth and sixth determine the extractive salt and clean-up sorbent levels, respectively.

[a] level −1 represents 0% formic acid in ACN; level 0, 0.5% formic acid; level + 1, 1% formic acid.

[b] -1 represents 0% formic acid in the final extract; 0, 0.5% formic acid; +1, 1% formic acid.

[c] Citrate represents Bekolut® SALT-Kit-CIT; Acetate, Bekolut® SALT-Kit-AC2.

[d] 4 represents Bekolut® PSA-Kit-04; 8A, Bekolut® PSA-Kit-04.

3. Results and discussion

3.1.Method optimization

For the extraction optimization, a fractional factorial design of experiments was performed. Two 3-levels continuous factors and two 2- levels categorical factors were studied, conforming a 2^{6-2} fractional factorial design. For each continuous factor, a central point was established to evaluate possible quadratic effects. To accommodate factors with 3 levels, two artificial factors with 2 levels were used for each of them. Having a total of 6 factors with 2 levels each, a fraction of the complete 2^6 design was selected conforming the definitive 2^{6-2} fractional factorial design (Kuehl, 2000; Montgomery, 2001). During the extraction, different factors can affect the process efficiency and, considering the wide range of physical-chemical parameters of the target pesticides, optimum values for each factor should be adopted with an overall vision. Firstly, for the extractive salts, two buffered variants of

Table 3
Absolute and relative recoveries and repeatability of the method validation.

Pesticide	Absolute recoveries (%)				Relative recoveries (%)				Repeatability (% RSD)			
	Olives		Sunflower seeds		Olives		Sunflower seeds		Olives		Sunflower seeds	
	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g
2,4D	N.D.	N.D.	N.D.	57	N.D.	N.D.	N.D.	115	N.D.	N.D.	N.D.	5.6
Acetamiprid	9.2	8.1	63	56	87	87	89	108	4.8	6.3	3.6	4.2
Alachlor	74	39	85	73	94	80	87	105	8.2	6.6	5.7	4.6
Atrazine	42	53	128	87	75	81	85	104	2.0	3.8	6.0	2.4
Azinphos ethyl	N.D.	44	89	85	N.D.	77	67	100	N.D.	4.5	5.3	3.1
Azinphos methyl	N.D.	101	111	88	N.D.	91	86	104	N.D.	8.0	6.8	4.1
Bentazone	48	36	82	92	72	79	79	100	3.6	4	4.3	1.3
Bromoxynil	N.D.	N.D.	55	44	N.D.	N.D.	106	115	N.D.	N.D.	13	11
Chlorfenvinphos	77	66	104	87	88	116	79	102	2.3	5	7.6	4.3
Chlortoluron	26	22	61	66	111	120	82	117	7.7	5.8	5.3	2.5
Chlothianidin	N.D.	17	52	42	N.D.	100	100	108	N.D.	4.6	4.5	1
Cyanazine	64	76	125	94	102	82	83	112	5	2.8	3.7	2.6
DEA	43	53	169	121	129	113	89	111	3.3	6.4	4.1	3.1
DIA	27	32	105	79	116	132	77	101	0.9	5.4	6	2.9
Diazinon	70	80	123	83	118	133	82	104	9.1	13	6.1	2.9
Dichlorvos	N.D.	248	N.D.	N.D.	N.D.	92	N.D.	N.D.	N.D.	6.2	N.D.	N.D.
Diflufenican	57	44	130	84	93	89	78	116	17.8	7.3	5.0	2.9
Dimethoate	17	16	76	74	112	102	91	113	3.6	5.3	5.7	0.5
Diuron	29	26	66	70	96	91	72	103	5.6	6.3	9.2	3.7
Fenthion	N.D.	76	N.D.	89	N.D.	90	N.D.	118	N.D.	4.9	N.D.	10
Fenthion oxon	68	56	95	82	98	97	79	102	1.6	3	4.5	2
Fenthion oxon sulfone	51	32	132	119	101	93	95	109	4.4	3.7	4.7	2.3
Fenthion oxon sulfoxide	33	43	64	63	98	91	98	133	2.3	0.8	8.2	3
Fenthion sulfone	N.D.	46	N.D.	55	N.D.	71	N.D.	91	N.D.	10	N.D.	6.8
Fenthion sulfoxide	42	35	95	80	98	96	80	135	12	14	13	7.6
Imidacloprid	16	15	64	54	103	91	89	110	4.6	2.8	5.9	1.9
Irgarol	74	76	81	71	92	85	81	111	2.3	3.8	5.6	0.8
Isoproturon	49	53	80	75	106	108	87	114	2.1	7.5	4.5	1.9
Linuron	172	147	141	129	89	114	83	97	6.9	4.5	5.7	4.9
Malaoxon	73	52	95	81	104	101	128	145	4.5	3.2	8.3	3.1
Malathion	N.D.	19	39	36	N.D.	96	120	131	N.D.	13	8.1	2.8
MCPA	N.D.	N.D.	N.D.	9.2	N.D.	N.D.	N.D.	93	N.D.	N.D.	N.D.	18
Methiocarb	104	88	113	103	83	88	92	110	3.5	7	2.9	3.5
Metolachlor	73	54	79	72	98	96	78	99	1.5	14	4.3	1.6
Molinate	116	75	105	76	70	76	80	102	19	14	7.6	3.3
Propanil	209	169	175	166	113	113	89	103	10	7.7	7	5.4
Quinoxyfen	29	13	71	51	103	84	72	80	11.6	8.2	6.8	13
Simazine	37	49	138	95	80	97	82	99	1.4	3.8	4.5	2.8
Terbuthylazine	96	92	119	79	89	103	90	107	3	4.7	2.8	2
Terbutryn	80	59	80	77	97	100	79	109	1.3	1.4	2.6	0.9
Thiacloprid	13	12	58	51	75	88	87	106	7.1	8.5	5.2	1.3
Thiamethoxam	24	25	54	48	103	77	81	101	4.6	3.4	5.6	0.8

N.D.: not detected; RSD: relative standard deviation

QuEChERS methodology were studied: AOAC 2007 method, which uses sodium acetate as buffer (Lehotay & Collaborators, 2007), and CEN method, which uses citrate buffer instead ((CEN) Standard Method EN 15662, n.d.). Acid dissociation constant (pka) and log P are important parameters that can influence the salting out partitioning of pesticides between aqueous and organic phases, and, thus, pH adjustment could affect their extraction. Moreover, formic acid has been used for the stabilization of some pH-labile compounds during sample extraction (i.e. alachlor) (Kaczynski, 2017; Mastovska & Lehotay, 2004). Therefore, the proportion of formic acid in the extraction solvent (ACN) was investigated (levels: 0% – 1%, with a central point at 0.5%). Due to the complex composition of both matrices, i.e., medium-high proportion of fat (10-25% in the case of olives and 51% in the case of sunflower seeds (Garrido-Fernandez et al., 1997; Madej et al., 2018)), and a high pres- ence of pigments (chlorophyll and carotenoids in the case of olives (Minguez-Mosquera & Garrido-Fernandez, 1989)), the selection of the clean-up sorbents could be determinant in the extraction efficiency. PSA is frequently used for the removal of fatty components, among others; non-polar compounds are effectively cleaned-up by C18e sorbent, while GCB remove planar compounds such as pigments (Madej et al., 2018). Thus, two different combinations of these sorbents were tested to cover both commodity matrices: Bekolut® PSA-Kit-08A, containing 1200 mg MgSO4, 400 mg PSA, 400 mg GCB and 400 mg C18e in each tube and Bekolut® PSA-Kit-04, with 900 mg MgSO4, 150 mg PSA, 150 mg C18e. In the end, the pH of the final extract could affect the ionization efficiency of the target pesticides at the HESI probe in the mass spectrometer. Consequently, the addition or not of 1% of formic acid to the final extract was also evaluated (levels: 0% – 1%, with a central point at 0.5%). A summary of the experimental design is shown in Table 2.

In sunflower seeds, the acetate buffer showed a clearly higher extraction efficiency than the citrate buffer and was thereby selected as optimum. Particularly, the acidic herbicide 4-chloro-2-methylphenoxy- acetic acid (MCPA), which usually represents an analytical challenge, showed a higher response with the acetate buffer than with the citrate buffer. In olives, given the overall similar performance obtained with both buffered salts,

the acetate buffer was also selected. The use of formic acid in ACN during the extraction step favoured the process for almost all compounds, with a few negligible exceptions (bentazone, bromoxynil and malaoxon in olives; and MCPA, thiametoxam, and desethyl-atrazine (DEA) in sunflower seeds) and, so, 1% of formic acid in ACN was established as the optimum in both matrices. Regarding the clean-up, no significant differences were observed between both sorbent mixtures in the case of olives. Thus, Bekolut® PSA-Kit-08A was selected for the method according to the matrix components (high content of fats and pigments). Besides, clean-up optimization in sunflower seeds led to the use of Bekolut® PSA-Kit-04, as it showed comparatively better

Table 4
Linearity and sensitivity of the method and MRLs.

Pesticide	Olives					Sunflower seeds				
	Linearity	Sensitivity (ng/g)			MRLs* (ng/g)	Linearity	Sensitivity (ng/g)			MRLs* (ng/g)
	r^2	LOD	LOQ	LDet		r^2	LOD	LOQ	LDet	
2,4D	N.D.	N.D.	N.D.	N.D.	50	0.997	2.0	6.6	91	50
Acetamiprid	0.995	0.22	0.75	5.8	3000	0.999	0.06	0.21	0.32	10
Alachlor	0.995	0.24	0.79	3.8	20	0.999	0.91	3.1	3.05	20
Atrazine	0.995	0.05	0.18	0.18	50	0.998	0.03	0.11	0.11	50
Azinphos ethyl	0.997	6.6	22	22	20	0.995	1.8	6.0	6.0	20
Azinphos methyl	0.997	3.9	13	13	50	0.990	2.2	7.3	7.3	50
Bentazone	0.999	0.07	0.24	0.24	30	0.998	0.21	0.70	0.70	30
Bromoxynil	N.D.	N.D.	N.D.	N.D.	10	0.997	2.9	9.6	9.6	10
Chlorfenvinphos	0.999	0.23	0.78	0.78	20	0.998	0.08	0.26	0.26	20
Chlortoluron	0.998	0.47	1.6	2.9	20	0.997	0.45	1.49	1.49	20
Chlothianidin	0.997	14	45	45	90	0.997	1.1	3.8	7.5	20
Cyanazine	0.998	0.09	0.31	0.64	10	0.995	0.03	0.11	0.11	10
DEA	0.983	0.83	2.8	3.4	10	0.976	0.42	1.4	1.4	10
DIA	0.996	1.1	3.8	13	10	0.993	0.60	2.0	2.0	10
Diazinon	0.998	0.23	0.75	0.75	20	0.998	0.03	0.10	0.10	20
Dichlorvos	0.998	13	42	42	10	N.D.	N.D.	N.D.	N.D.	10
Diflufenican	0.998	0.65	2.2	2.1	600	1.000	0.51	1.7	1.7	10
Dimethoate	0.999	2.2	7.3	7.3	3000	0.998	0.12	0.49	1.3	10
Diuron	0.998	2.5	8.4	8.4	20	0.997	1.9	6.2	6.2	20
Fenthion	0.992	12	39	39	10	0.998	12	40	40	20
Fenthion oxon	0.998	0.06	0.20	0.20	10	0.996	0.06	0.21	0.21	20
Fenthion oxon sulfone	1.000	1.9	6.4	29	10	0.998	0.50	1.6	6.6	20
Fenthion oxon sulfoxide	0.996	0.21	0.71	0.94	10	0.997	0.19	0.64	0.64	20
Fenthion sulfone	0.992	18	59	59	10	0.996	12	41	41	20
Fenthion sulfoxide	0.997	0.40	1.3	1.3	10	0.996	0.23	0.77	0.77	20
Imidacloprid	0.999	1.5	5.1	5.1	1000	0.990	0.3	0.98	0.98	100
Irgarol	0.998	0.19	0.62	0.62	10	0.993	0.11	0.38	0.38	10
Isoproturon	0.998	0.49	1.6	2.5	10	0.995	5.9	19	19	10
Linuron	0.998	1.0	3.4	3.4	50	1.000	0.6	2.0	2.0	100
Malaoxon	0.999	0.18	0.61	0.61	20	0.999	0.08	0.27	0.27	20
Malathion	0.997	1.6	5.3	5.2	20	0.997	1.6	5.5	18	20
MCPA	N.D.	N.D.	N.D.	N.D.	50	0.998	5.1	17	17	100
Methiocarb	0.991	0.80	2.7	4.4	200	0.992	0.22	0.73	0.73	100
Metolachlor	0.998	0.08	0.27	0.27	50	0.998	0.05	0.18	0.18	50
Molinate	0.997	0.26	0.85	0.85	20	0.999	0.25	0.82	1.3	20
Propanil	0.996	0.60	2.0	2.0	50	0.994	0.19	0.62	0.62	50
Quinoxyfen	0.999	1.5	4.9	4.9	20	0.996	0.08	0.28	0.28	50
Simazine	0.994	0.16	0.5	0.53	10	0.996	0.11	0.37	0.37	20
Terbuthylazine	0.996	0.01	0.03	0.03	50	0.995	0.03	0.08	0.08	100
Terbutryn	0.999	0.51	1.7	1.7	10	0.997	0.09	0.30	0.30	10
Thiacloprid	0.999	0.66	2.2	3.9	4000	0.998	0.06	0.19	0.19	20
Thiamethoxam	0.995	1.9	6.2	6.2	400	0.995	0.91	3.0	3.0	20

r^2: coefficient of determination, LOD: Limit of Detection, LOQ: Limit of Quantification, LDet: Limit of Determination, N.D.: *not detected*. MRL: *Maximum Residue Level*.
*Extracted from http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=product.selection&language=EN

performance for pesticides such as 2,4-dichlorophenoxyacetic acid (2,4D), bromoxynil, diflufenican and quinoxyfen, among others. Finally, the acidification of extracts presented quadratic effects and, considering the overall desirability results, maximum effects were set at 0.7% and 0.5% of formic acid for olives and sunflower seeds, respectively. These conditions only affected negatively to bromoxynil, for which the presence of protons in the medium could probably hinder the ionization of its hydroxyl group. Prediction profiles for each compound and desirability plots are shown in Figs. S1-S2.

3.2.Method validation

Method performance for the different target compounds under the optimum conditions is shown in Table 3 and Table 4 in terms of trueness (recoveries), sensitivity, repeatability and linearity. As described in the guideline SANTE/12682/2019 (European Commission, 2019), a line- arity range was established from the LOQ to 500 ng/mL (1000 ng/g) for each analyte, through the methanolic solutions used to construct calibration curves that did not deviate ± 20% from the theoretical values. The coefficient of determination (r^2) was used for linearity evaluation, showing values above 0.99 for every pesticide analysed, except for DEA (0.983 in

olives and 0.976 in sunflower seeds).

As shown in Table 3, absolute recoveries were calculated for both matrices, showing good agreement at the two concentrations tested in the validation study. Extraction recoveries were considerably better in sunflower seeds than in olives: 88% and 57% of the target pesticides presented absolute recoveries above 50% after sunflower seed and olives extraction, respectively. Many matrix components from olives co-elute with the target pesticides (Cunha et al., 2007; Lopez-Blanco et al., 2016) diminishing the extraction efficiency rates and subsequent MS response. In sunflower seeds, MCPA is the only pesticide with a highly affected recovery (9%), while in olives low recoveries were obtained for all neonicotinoids (from 9 to 25%), diuron (24%), and dimethoate (17%). Nevertheless, as shown in Table 4, LOQs for these compounds were below the MRLs established by the EC. Moreover, the use of isotopically labelled compounds as surrogates compensates for the los- ses occurred during extraction and analysis, and thus average relative recoveries (n = 6) obtained for both commodities are in compliance with the SANTE guidance (European Commission, 2019). As shown in Table 3, relative recoveries were between 70 and 120% for all com- pounds with the exception of azinphos ethyl, fenthion oxon sulfoxide, fenthion sulfoxide, malathion and malaoxon in sunflower seeds (67, 133, 134, 131 and 145%, respectively), and desisopropylatrazine (DIA) and diazinon in olives (132 and 133%, respectively). However, precision

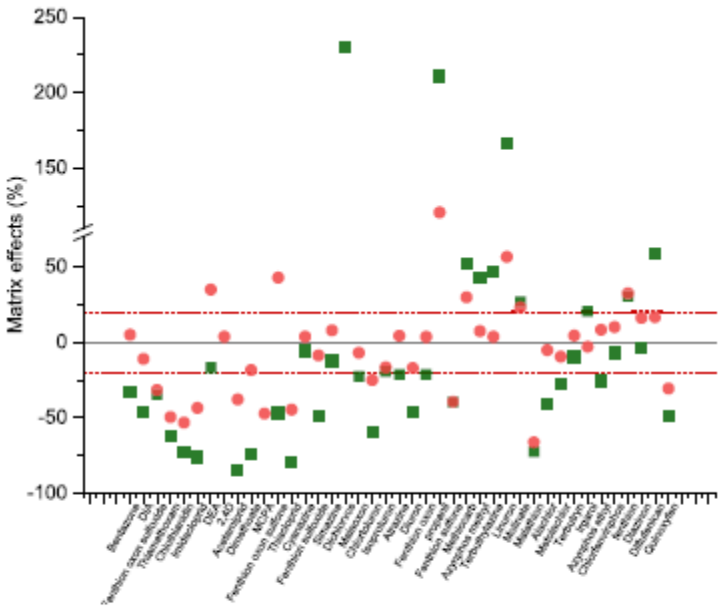

Fig. 1. Matrix effects (%) calculated for olives (green squares) and sunflower (red dots) seeds during method validation. Red dash reference lines represent ± 20%. Compounds ordered according to retention time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was consistent for these compounds with RSD values below 8% (except diazinon, RSD 13.5%). In general, RSD values were below 20% for all analytes, with a 90% of the target pesticides below 10% RSD.

In terms of sensitivity, LDets in olives ranged from 0.03 to 59 ng/g, whereas in sunflower seeds LDets oscillated from 0.08 to 42 ng/g. LDets below the European MRLs established for each commodity were obtained for 37 (olives) and 38 (sunflower seeds) out of the 42 target pesticides (Table 4). Higher LDets were obtained for fenthion and fenthion sulfone (in both matrices), azinphos ethyl, DIA, and dichlorvos in the case of olives, and 2,4D and is oproturon in sunflower seeds. On the other hand, the lowest LODs were achieved for bentazone, cyanazine, metolachlor, and terbuthylazine (in olives), and acetamiprid, atrazine, chlorfenvinphos, cyanazine, diazinon, fenthion oxon, malaoxon, metolachlor, quinoxyfen, terbuthylazine, terbutryn, and thiacloprid (in sun- flower seeds), reaching values below 0.1 ng/g. In general, this responds to an appropriate extraction procedure, which led to good recoveries, and lower matrix interferences enhancing the ionization efficiency in the ESI probe. Moreover, improved LODs were obtained for most of the compounds studied in previously developed methods (Table S3) (Ana-gnostopoulos & Miliadis, 2013; Gilbert-Lopez et al., 2010; Gomez- Almenar & García-Mesa, 2015; Kaczynski, 2017; Lopez-Blanco et al., 2016; Sanchez-Hernández et al., 2016).

As expected, comparatively lower matrix effects were obtained for sunflower seeds due to the matrix complexity of olives. Fig. 1 shows the distribution of matrix effects in both commodities, where, in olives, suppression effects affected to 74% of the compounds and only 18% had a soft matrix effect (±20%). In the case of sunflower seeds, 53% of the pesticides had no significant matrix effects. In this matrix, half of the target compounds presented a signal ionization enhancement, being propanil the most affected (120%). In contrast,

malathion was the pesticide that suffered from highest suppression (-66%). In olives, the maximum ionization suppression was determined for acetamiprid (-85%), imidacloprid (-76%), thiacloprid (-80%) or dimethoate (-74%), which is in accordance with the results of absolute recoveries obtained previously, while dichlorvos, propanil and linuron presented ionization enhancement effects above 100%. As shown in Fig. 1, the use of clean-up sorbents such as C18e, focused on the removal of fatty components, resulted on a higher suppression effect for the most polar compounds (lower RT).

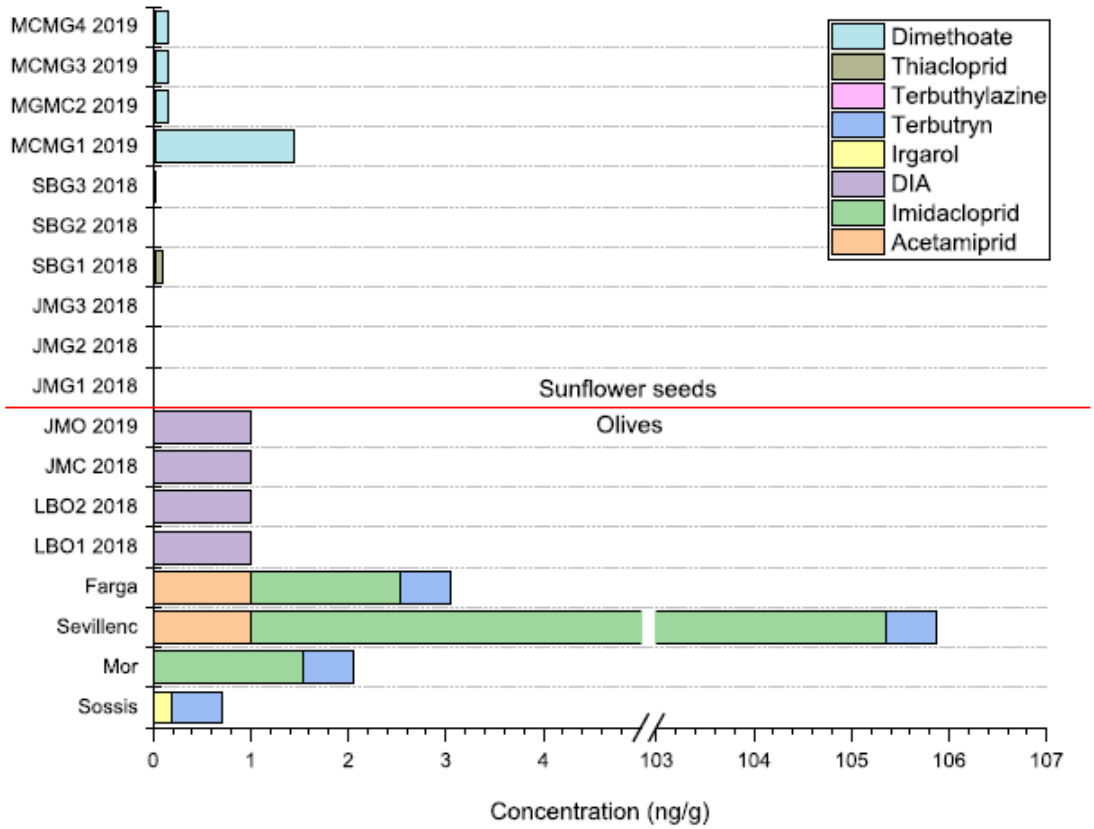

Fig. 2. Pesticide concentrations detected in real samples. LODs are shown for those analytes detected at a level below LDet. MCMG, SBG and JMG samples correspond to sunflower seeds. JMO, JMC, LBO, Farga, Sevillenc, Mor and Sossis samples correspond to olives.

3.3.Method application to real samples

Olive and sunflower crops from Serpa (South of Portugal) harvested during 2018 and 2019, together with samples taken from different fields of Tarragona (NE Spain) during 2020 were analysed as a part of the method evaluation. For the positive identification of the target pesticides in a sample, both SRM transitions should completely overlap regarding retention time (RT) and peak shape, and RT of the extract should correspond to that of the calibration standard in the same batch within ± 0.1 min. If longer RT shift occurred among samples in the same sequence, the corresponding isotopically-labelled compound should have been affected with the same shift to be accepted. Furthermore, the ratio between both SRM transitions in the sample should be within ± 30% of that observed in calibration standards from the same sequence (SANTE/12682/2019).

With these premises, a few pesticides were detected (Fig. 2). For these kinds of oilseed crops, the use of pesticides is predominantly covered by herbicides, insecticides and, to a lesser extent, fungicides (Amvrazi & Albanis, 2009; Debaeke et al., 2017). The neonicotinoid imidacloprid was the pesticide found at the highest concentration in the investigated samples, surpassing 100 ng/g in one olive sample, the “sevillenc” variety. This compound, together with DIA and terbutryn, were the most frequently detected pesticides in olives. Besides, acet amiprid and irgarol were also found at concentrations over the LOQ of their SRM1 but below the LOD of their SRM2, thus preventing quantification. In the case of sunflower seeds, the organophosphorus insecticide dimethoate was present in 4 out of 10 samples, showing the highest concentration at 1.42 ng/g in one of the Portuguese samples from the 2019 campaign. Moreover, terbuthylazine was also detected at very low concentrations in 6 out of 10 sunflower seed samples and the insecticide thiacloprid in one sample.

Thiacloprid, imidacloprid, dimethoate, and terbutryn are included in the Pesticide Action Network (PAN) International list of Highly Hazardous Pesticides (Pesticide Action Network International, 2019). High honeybee toxicity is attributed to dimethoate and imidacloprid, together with a moderate acute human toxicity. Moreover, ecotoxicity of dimethoate includes moderate poisoning of mammals and moderate to very high toxicity to birds. Regarding the neonicotinoid thiacloprid, it represents a well-known moderate hazard to humans and it has been classified as a carcinogen (Pesticide Action Network International, 2019).

4. Conclusions

Two QuEChERS-based analytical methods have been developed and validated for the determination of 42 pesticides, including triazines, organophosphates, phenylureas, anilines, neonicotinoids and others, in two representative food fatty matrices: olives and sunflower seeds. Method extraction was optimized through a fractional factorial design of experiments, which allowed optimizing the method conditions in a cost-effective way. Four different factors were evaluated, including the acidification of the extraction solvent, the type of extractive salts, the type of clean-up salts, and the acidification of the final extract. The optimized methods were successfully validated in terms of linearity, repeatability (with RSD values below 20%) and trueness (with average relative recoveries between 70 and 120% for almost all analytes), in compliance with SANTE guidelines. The sensitivity achieved with the methods, with LOQs in the range of pg/g or low ng/g, allowed deter- mining concentrations below the established MRLs for>90% of the target compounds. Although the use of isotopically-labelled surrogates for the quantification of the target pesticides represents a high initial economical cost, numerous advantages, such as control of the well performance of the analytical method throughout the sequence, compensation of matrix effect regardless of its variability between samples, accuracy of results, and easier quantification (without the need to use recovery factors) are obtained. Thus, the use of this kind of surrogate compounds in the analysis of complex matrices, such as olives and sunflower seeds, are extremely useful to obtain highly reliable results. Furthermore, the advantages of using QuEChERS over other conventional extraction methods are well-known: fast, cheap and safe method performance, beside the wide applicability for multiresidue analysis.

These methods were applied to the analysis of a few real samples from different locations across the Iberian Peninsula. Their application showed the presence of some herbicides and insecticides of relevant environmental concern, including four pesticides from the Highly Hazardous Pesticides list from the PAN International.

CRediT authorship contribution statement

Manuel García-Vara: Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. Cristina Postigo: Methodology, Validation, Writing - review & editing. Patricia Palma: Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. María José Bleda: Methodology, Formal analysis, Resources, Writing - review & editing. Miren Lopez de Alda: Conceptualization, Methodology, Validation, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work has been funded by the European Union through the European Agricultural Fund for Rural Development (FitoFarmGest Operational Group PDR2020-101-030926), the Spanish Ministry of Science and Innovation (Project CEX2018-00794-S) and the Government of Catalonia (2017 SGR 01404). BEKOlut is acknowledged for the gift of QuEChERS kits.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.foodchem.2022.132558.

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This is a post-peer-review version of an article published in Food Chemistry, 2022 following peer review. The version of record García-Vara, M., Postigo, C., Palma, P., José Bleda, M. & López de Alda, M. (2022). QuEChERS-based analytical methods developed for LC-MS/MS multiresidue determination of pesticides in representative crop fatty matrices: Olives and sunflower seeds. Food Chemistry, 386, 1-10. https://doi.org/10.1016/j.foodchem.2022.132558