Talanta 112 (2013) 1–10

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development and validation of an easy multiresidue method for the determination of multiclass pesticide residues using GC–MS/MS and LC–MS/MS in olive oil and olives

C. Anagnostopoulos *, G.E. Miliadis

Laboratory of Pesticide Residues, Department of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute, 8 St. Delta str., GR-145 61 Kifissia (Athens), Greece

ARTICLE INFO

Article history:
Received 22 November 2012
Received in revised form
14 March 2013
Accepted 20 March 2013
Available online 27 March 2013

Keywords:
GC–MS/MS
LC–MS/MS
Olive
Olive oil
Validation
Dispersive solid phase extraction

ABSTRACT

Olives and olive oil are two of the most important commodities produced in the Mediterranean region. Due to their significant economical importance, the usage of pesticides in their production is systematic, by using a wide range of plant protection products with a variety of modes of action. As a consequence, monitoring of their residue levels in this products is a necessity. In the present study a gas and liquid chromatography-tandem mass spectrometry multiresidue method, with a short sample preparation step, based on acetonitrile extraction is developed and validated according to the European Union guidelines (SANCO Doc. No. 12495/2011) in olives and olive oil, with a large scope that includes pesticides of different chemical classes. Good sensitivity and selectivity of the method were obtained with limits of quantification at 10 μg/kg. All pesticides had recoveries in the range of 70–120%, with relative standard deviation values less than 20–25%, at both validation levels. Excellent linearity was achieved with $r \geq 0{,}99$ for both matrices. The method is easy, with low consumption of reagents, is characterized by reliability, sensitivity and therefore is suitable for the monitoring the levels of multiclass pesticides residues in olives and olive oil. The method was applied to 262 samples of the Greek market, of which 7% were found positive for the present of pesticides. In some of the samples 2–8 different analytes were detected.

1. Introduction

The most important cultivation in the Mediterranean region since ancient times to today is the olive tree. The European Union is the biggest consumer of olive oil by 1.3 million tons annually. According to the Greek Ministry of Rural Development and Food, in 2009 over 150 million trees were cultivated in Greece to produce table olives and olive oil. The importance of olive tree cultivation in Europe and worldwide makes the intensive use of agrochemicals essential. In Greece alone, about 180 formulations are approved for use in the cultivation of olive trees, containing active substances belonging to various chemical groups.

Monitoring of pesticide residues is crucial, as it assures that applications are made according to the proposed good agricultural practices and the product is safe for the consumer. Due to the importance of olive production, the number and the variety, as to their chemical class, of the active substances used in plant protection products is continuously growing and as a result the determination of their residues in the final product is becoming more and more challenging.

The main ingredients of olives and olive oil that play important role in the determination of residues of pesticides, in the terms of coextracted matrix interferences, are lipids [1] and pigments. Most analytical methods focus on the removal of lipids, since besides separation and sensitivity problems, the continuous analysis of many samples, during routine work, can cause problems in the chromatographs (e.g. block the analytical column or liner contamination). The most common approaches for cleanup are solid phase extraction (SPE) [2–4], dispersive solid phase extraction [5–7] and gel permeation chromatography (GPC) [8,9]. Other approaches like on-line reversed phase LC-GC [10], are also used in a smaller scale. The above extraction procedures are mostly combined with GC or LC and mass spectrometry (MS). The use of tandem mass spectrometry (MS/MS) in combination with GC or LC is gaining ground as it provides better sensitivity and confirmation reliability.

In the current study the use of a tandem mass spectrometry (MS/MS) technique combined with GC and LC was investigated for the determination of pesticide residues in olives and olive oil. The sample preparation procedure, based on acetonitrile, was developed as to be easy, economic and with sufficient clean up for both fatty component and pigments. The method was assessed as for the sub-sampling homogeneity, the matrix effect and was validated according to EU guidelines [1] for a variety of analytes, with

* Corresponding author. Tel.: +30 210 8180359.
E-mail address: c.anagnostopoulos@bpi.gr (C. Anagnostopoulos).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.03.051

different physicochemical properties, as to assure the flexibility of its scope.

2. Experimental

2.1. Reagents and standards

Pesticide reference standards (purity > 98%) of all analytes were purchased from ChemService and Ehrenstorfer GmbH. All solvents, namely acetonitrile, methanol and water were of HPLC grade. Primary secondary amine sorbent (PSA, 40 μm, Bondesil) was purchased from Varian Inc., USA. Magnesium sulfate dried was purchased from Acros Organics and graphitized carbon black (GCB) was purchased from Sigma-Aldrich Chemie GmbH.

2.2. Apparatus

GC–MS/MS analysis was performed by a Varian 3800 gas chromatograph connected to a triple quadrupole mass spectrometer (Varian model 1200 L). Samples were injected with a CP 8400 autosampler, using a 10 μL syringe, into a 1079 programmed temperature injector operated with the large volume injection technique (PTV-LVI). A Factor Four Capillary Column VF-5 ms 30 × 0.25 mm I.D. × 0.25 μm film thickness, with a guard column (fused silica untreated capillary column 5 × 0.53 mm I.D. cyanophenyl-methyl deactivated) from Varian Inc. was used for the chromatographic separation of the compounds. The mass spectrometer was operated at the electron impact mode (EI). For instrument control, data acquisition and processing, Varian MS Workstation software version 6.8 was used.

The LC–MS/MS analysis was performed by an Agilent Series 1200 liquid chromatograph with a degasser (G1379B), autosampler (Hip/ALS G1367A) with thermostat (FC/ALS Therm G1330B), binary pump (G1312A), a thermostated column department (TCC G1316A) equipped with a reverse phase Zorbax Eclipse XDB C_{18} 3.5 μm particle size, 150 mm × 2.1 mm analytical column (Varian, Palo Alto, CA, USA). and a triple quadrupole mass spectrometer (Agilent Triple Quad 6410) equipped with an electrospray ionization interface operating at positive mode (ESI+).

2.3. Selection of pesticides

A wide variety and number of pesticides are used in olive tree cultivation. For the validation of the method, a representative group of analytes was selected as to ensure that acceptable performance is achieved for all other analytes of the same chemical classes. The selected pesticides were insecticides, fungicides and herbicides of 32 different chemical groups, including: *Acylalanines*, *Anilinopyrimidines*, *Aromatic hydrocarbons*, *Aryloxyphenoxypropionic acids*, *Aryloxyphenoxypropionic esters*, *Benzamides*, *Benzenedicarboxylic acids/esters*, *Benzilates*, *Benzimidazoles*, *Benzofurans*, *Carbamates*, *Chloroacetamides*, *Cinnamic acids*, *Dicarboximides*, *Dinitroanilines*, *Imidazoles*, *Morpholines*, *Neonicotinoids*, *Organochlorines*, *Organophosphorous*, *Oxadiazoles*, *Phenylureas*, *Phosphorothiolates*, *Pyrethroids*, *Pyridinecarboxamides*, *Pyrimidinamines*, *Pyrimidinols*, *Strobilurins*, *Tetrazines*, *Triazines*, *Triazoles* and *Ureas*. Important pesticide metabolites e.g. aldicard sulfone and sulfoxide, metabolites of the carbamate fungicide aldicab, were also selected, since they are of high toxicity and are therefore included in the residue definition of the parent compound.

2.4. Preparation of standard solutions

The stock solutions of the individual pesticide standards were prepared by accurately weighing 10–50 mg of each analyte in volumetric flasks (certified 'A' class) and dissolving in 10 mL acetone, acetonitrile or methanol depending on the analytes solubility. The stock standard solutions were stored at −20 °C. A single composite working standard solution was prepared by combining aliquots of each stock solution and diluting in acetonitrile to obtain a final concentration of 1 mg/L. The working standard solution was also stored at −20 °C and before each use was left to reach room temperature. From this working standard solution, 3 series of calibration standards were prepared within the range of 5–100 μg/L by serial dilution in acetonitrile, olive extract and oil extract respectively.

2.5. Extraction procedure and analysis

Five grams of homogenized sample were weighted in a 50 mL polypropylene centrifuge tube and extracted with 10 mL acetonitrile for 1 min, shaking vigorously by hand. The sample was then centrifuged at 4000 rpm for 5 min and stored in the freezer (−20 °C) for at least 12 hours. Freezing is a critical part of the extraction procedure as it helps to partly remove some additional co-extractives with limited solubility in acetonitrile while the major part of fat and waxes solidify and precipitate. No additional cleanup of fatty components is conducted or required. An aliquot of 6 mL of the still cold acetonitrile phase was transferred into a 15 mL centrifuge tube containing 150 mg of PSA, 12.5 mg GCB and 900 mg of $MgSO_4$, the tube was shaken vigorously for 1 min and centrifuged for 5 min at 4000 rpm. The final extract was transferred into a screw cup storage vial, taking care to avoid sorbent particles of being carried over, and stored in the freezer until analysis. Before injecting in the chromatographic system, the final solution was filtered through a 0.45 μm disposable PTFE syringe filter,. Following this extraction procedure the concentration C in mg/kg of the analytes in the sample correspond to $2 \times C$ in μg/mL of the analytes in the final extract.

2.6. Method Validation

The validation of the method was preformed according to the newest EU guidelines [1]. Analytical parameters evaluated were mean recovery (as a measure of trueness), repeatability (as a measure of precision) linearity, homogeneity during sub-sampling, and sensitivity.

2.7. Confirmation criteria

For initial identification of the analytes the retention time (R.T.) criterion was used. The R.T. of the analyte was matched based on a calibration standard at a tolerance of ± 0.5% for GC and ± 2.5% for LC. The final confirmation of a target compound, initially identified by RT, was done according to the criteria laid down in document SANCO 12495/2011 [1]. The permitted tolerances for the relative ion intensities (% of base peak) in MS/MS techniques according to this document are as follows:

- For relative intensity more than 50% the tolerance is ± 20%.
- For relative intensity between 20–50% the tolerance is ± 25%.
- For relative intensity between 10–20% the tolerance is ± 30%.
- For relative intensity less than 10% the tolerance is ± 50%.

3. Results and discussion

3.1. Optimization of MS/MS parameters

The ionization and fragmentation of the pesticides and metabolites was studied prior to the validation of the method. A large volume injection was used in GC in order to increase sensitivity.

Table 1
LC–MS/MS and GC–MS/MS quantification and qualifier transitions, capillary voltages (CV), collision energy (CE) and retention times (RT) used for the studied analytes.

Analytes	Quantitation transition		CV (V)	CE (eV)	Qualifier		CV (V)	CE (eV)	RT (min)
System	**GC–MS/MS**								
Acrinathrin	181	152	–	15	208	181	–	15	35,1
Aldrin	263	192	–	25	293	257	–	10	15,7
Boscalid	140	76	–	20	140	112	–	10	20,6
Chlorfenvinfos	318	248	–	15	318	248	–	15	14,74
Chlorthal dimethyl	291	81	–	15	305	180	–	15	13,6
DDD op	235	165	–	15	235	200	–	15	15,5
Fenthion	272	237	–	15	272	109	–	15	13,5
HCH a	219	183	–	15	219	145	–	15	10,29
HCH b	219	183	–	15	219	145	–	15	11,13
Isophenphos methyl	198,1	183	–	15	199	93	–	25	13,9
Permethrin	183	153	–	15	183	168	–	15	19,3+19,6
System	**LC–MS/MS**								
Acetamiprid	223	126	36	27	223	90	51	21	3.8
Alachlor	270	238	31	25	270	162	31	25	23,09
Aldicarb	208	89	10	20	208	116	10	20	5.3
Aldicarb sulfone	240	148	40	15	240	86	25	25	1.4
Aldicarb sulfoxide	207	89	42	18	207	132	40	15	1.2
Azinphos ethyl	346	160,2	135	15	346	132,2	135	15	23,13
Azoxystrobin	404.1	371.9	36	19	404.1	343.9	36	29	9.8
Benalaxyl	326.2	148.2	26	27	326.2	208.2	26	21	12.0
Benfuracarb	411	252	135	25	411	195	35	25	27,3
Benzoxymate	364	199	135	15	364	105	135	15	25,8
Bitertanol	338	99	125	25	338	70	125	25	25,8
Bromuconazole	378	159	46	30	378	70	46	15	10.5
Bupirimate	317,1	166,1	95	35	317,1	108,1	95	35	22,7
Buprofezin	306	116	40	21	306	201	40	17	14.2
Caduzafos	271	215	35	15	271	159	45	25	10.0
Carbaryl	202	145	40	15	202	127	40	50	7.6
Chlorobromuron	292,9	204	36	45	292,9	182,1	36	23	21,4
Chlorpyriphos ethyl	350	198	21	20	350	125	21	20	28,4
Chlortoluron	213.1	72	36	33	213.1	140	36	33	8.1
Clofentezine	303	138	56	15.5	303	102	56	47	13.2
Cyanazine	241.1	214.1	41	23	241.1	104.1	41	41	6.5
Cymoxanil	199	128	46	13	199	111	41	25	4.4
Cyprodinil	226	93	170	30	226	77	170	70	24,1
Demeton S methyl	230,8	89	30	10,5	248	61	6	47	5.84
Demeton S methyl sulfone	263	121	75	25	263	169	65	25	1.4
Diazinon[a]	305	169.1	21	29	205	96.6	21	41	24.3
Difenoconazole	406.1	250.9	41	37	406.1	337	41	23	13.2
Dimethoate	230	125	11	29	230	199	11	13	2.9
Dimethomorph	388	301	60	17	388	165	60	28	9.9
Disulfoton	275	89	9	5					10.1
Disulfoton sulfone	307	153	65	15	307	261	65	15	7
Disulfoton sulfoxide	291	185	45	25	291	213	45	15	6.7
Dodemorph	282,3	116,1	72	16	282,3	98,2	72	22	19,15
EPN	324	296	135	25	324	157	135	25	26,05
Epoxiconazole	330.1	121	36	27	330.1	101.2	36	63	11.3
Ethion	385	199,1	135	15	385	171	135	15	28,9
Ethofumesate	304	121.1	36	27	304.1	161.2	36	31	9.8
Ethoprophos	243	173	45	25	243	131	65	35	10.9
Etoxazole	360	141	66	37	360	113	66	50	15.1
Famoxadone	392.2	238	16	23	392.2	330.9	16	15	12.9
Fenamidone	312	92	41	33	312	236	41	19	9.9
Fenamiphos sulfone	336	266	135	25	336	188	135	25	16,9

Table 1 (*continued*)

Analytes	Quantitation transition		CV (V)	CE (eV)	Qualifier		CV (V)	CE (eV)	RT (min)
Fenbuconazole	337	125	41	37	337	70	41	33	11.7
Fensulfothion oxon	293	265	65	35	293	237	75	35	4.8
Fenthion oxon	263	231	135	25	263	216	135	25	19,7
Fenthion oxon sullfoxide	279	149	135	25	279	104	135	25	12,4
Fenthion sulfone[a]	311	125	135	25	311	109	135	25	16.7
Fenthion sulfoxide	295	280	135	25	295	109	135	25	16,88
Fluazifop	384	328	60	30	384	282	60	30	27,8
Flusilazole	316,1	247,1	85	25	316,1	165	95	45	23,7
Fosthiazate	284	228	61	15	284	104	61	27	7.8
Furathiocarb	383	195	51	23.5	383	252	51	19	13.9
Haloxyfop Methoxyethyl ester	376	316	65	35	376	288	75	45	11.8
Hexaconazole	314	70	30	20	314	159	26	30	12.7
Imazalil	297	159	135	30	297	109	135	30	18,03
Imidacloprid	256	209	45	20	256	175	45	25	3.3
Iprodione	330	245	101	23	330	101	101	33	23,24
Iprovalicarb	321	119	51	23	321	203	51	20	10.7
Isoproturon	207	135	135	35	207	72	135	35	18,6
Kresoxyl methyl	314.1	115.9	16	21	314.1	206.1	16	13	11.8
Malaoxon	315	127	31	17	315	99,2	31	31	16,4
Metalaxyl M	280.1	220	46	19	280.1	159.9	46	31	8.4
Methidathion	303	145	130	5	303	85	130	5	19,7
Methiocarb sulfoxide	275	122	25	25	258	122	25	45	4.7
Metoxuron	229	156.1	26	31	229	72.1	26	35	5.6
Monolinuron	215.1	125.9	61	25	215.1	148	61	19	7.7
Myclobutanil	289	125	36	41	289	70	36	33	10.7
Omethoate[a]	214	125	50	30	214	109	20	35	2.5
Oxadiazon	362	220	35	25	362	177	35	25	28,5
Oxamyl	237	72	36	15	237	90	36	15	1.4
Penconazole	284	158,9	41	39	284	70	41	29	24,35
Pendimethalin	282	212	80	5	282	194	80	10	28,8
Permethrin	408,1	183,1	31	25	–	–	–	–	32,2
Pirimicarb	239,1	181,9	16	21	239,1	72,1	16	31	13,8
Pirimiphos methyl	306,1	108,1	26	39	306,1	67	26	29	25,7
Procloraz	373	303	40	20	373	97	30	30	13.8
Profenofos	373	303	40	20	373	97	30	30	13.8
Propagite	368	231	30	10	368	175	1	21	15.2
Pyrazophos	374	222	35	15	374	194	35	15	26,02
Pyriproxyfen	322	96	15	21	322	185	11	29	14.6
Spiroxamine	298	144	41	27	298	100	41	35	10.7
Terbufos sulfone	321	171	65	15	321	265	65	15	7.7
Tetraconazole[a]	372	159	40	10	372	70	36	45	23.5
Thiabentazole	202	175	40	30	202	131	40	30	9,7
Thiacloprid	253	126	81	29	253	186	76	19	5.2
Thiamethoxam	292	211	51	17	292	181	56	31	2.2
Thiodicarb	355	108	30	25	355	88	30	25	8.1
Tolclofos methyl	301	268,9	135	15	301	175	135	15	26,3
Triticonazole	318	125	135	25	318	70	135	25	22,8
Vamidothion	288	146	10	30	288	118	10	40	3.9
Zoxamide	336	187	135	35	336	159	135	35	25,3

[a] Not included in the initial validation.

Table 1 presents the transitions and MS conditions used for data acquisition in both LC–MS/MS and GC–MS/MS systems. The ionization technique used for GC–MS/MS determination was electron impact (EI) and for LC–MS/MS was positive electron spray (ESI+) ionization. Due to the stronger ionization occurring with electron impact the pseuro-molecular ion is hardly used as a precurson ion in GC–MS/MS determination, in contrast to LC–MS/MS in which, the proton adduct [H^+] of the molecular ion was the precursor ion in all cases.

The MS/MS optimization was conducted by performing multiple injections of each analyte at a concentration of 100 μg/L at different collision energy and capillary voltage values. MS/MS spectra were acquired to obtain information about the abundance of each compound transitions. The optimum capillary voltage values varied from 1 to170 eV (applies to LC–MS/MS only) and respective collision energy values from 5 to 70 eV for LC–MS/MS and 10 to 25 eV for GC–MS/MS, depending on the analyte.

In both systems a time—scheduled data acquisition sequence was applied, that involved overlapping segments, each of 1 min. In each segment 2 to 45 transitions were monitored. By this technique an artificial window of a maximum space ± 2 min from the retention time of each analyte was created. Therefore the instrument consumes the ideal amount of time as to have a successful acquisition with less time shifts that can easily cause loss of a peak and sufficient dwell and scan time, without stacking a lot of transitions in one time segment. This technique is useful in the development of multiresidue methods with a large number of transitions in one run.

In the GC–MS/MS system the dwell times varied between 0.016–0.05 s; therefore the average scan cycle time for the segments varied between 0.1–0.5 s. The number of data points collected is directly related to the MRM cycle time, which is defined by the summation of the dwell times of all the MRM channels. In the LC–MS/MS system, due to the wider peak shape, the number of minimum required data points (approximately 10) can be achieved easier; therefore the dwell times are slightly higher, between 0.05–0.2 s generating scan time cycle up to 2 s.

3.2. GC–(EI)–MS/MS analysis

Aliquots of 5 μL of sample extract were injected into the gas chromatograph. The initial injector temperature 90 °C was held for 0.75 min and then increased at 200 °C $min^{-1}$to 280 °C, and held for 5 min. The injector split ratio was first 30:1. After 0.75 min, splitless mode was set until minute 3. At 3 min, the split ratio was 60:1 and at 6 min, the split ratio was set to 30:1. The column oven temperature program started from 70 °C for 2 min, increased to 180 °C at a rate 30 °C min^{-1}, held for 1 min, then increased to 230 °C at 1.8 °C min^{-1}, held for 1 min, then increased to 280 °C at a rate 30 °C min^{-1}, held for 30 min The helium carrier gas flow rate was 1 mL min^{-1}.

The mass spectrometer was operated at EI-MS/MS mode and selection reaction monitoring (SRM) data acquisition mode. The transfer line, manifold and ionization source temperatures were 280, 40 and 250 °C respectively. The EI energy used was 70 eV as

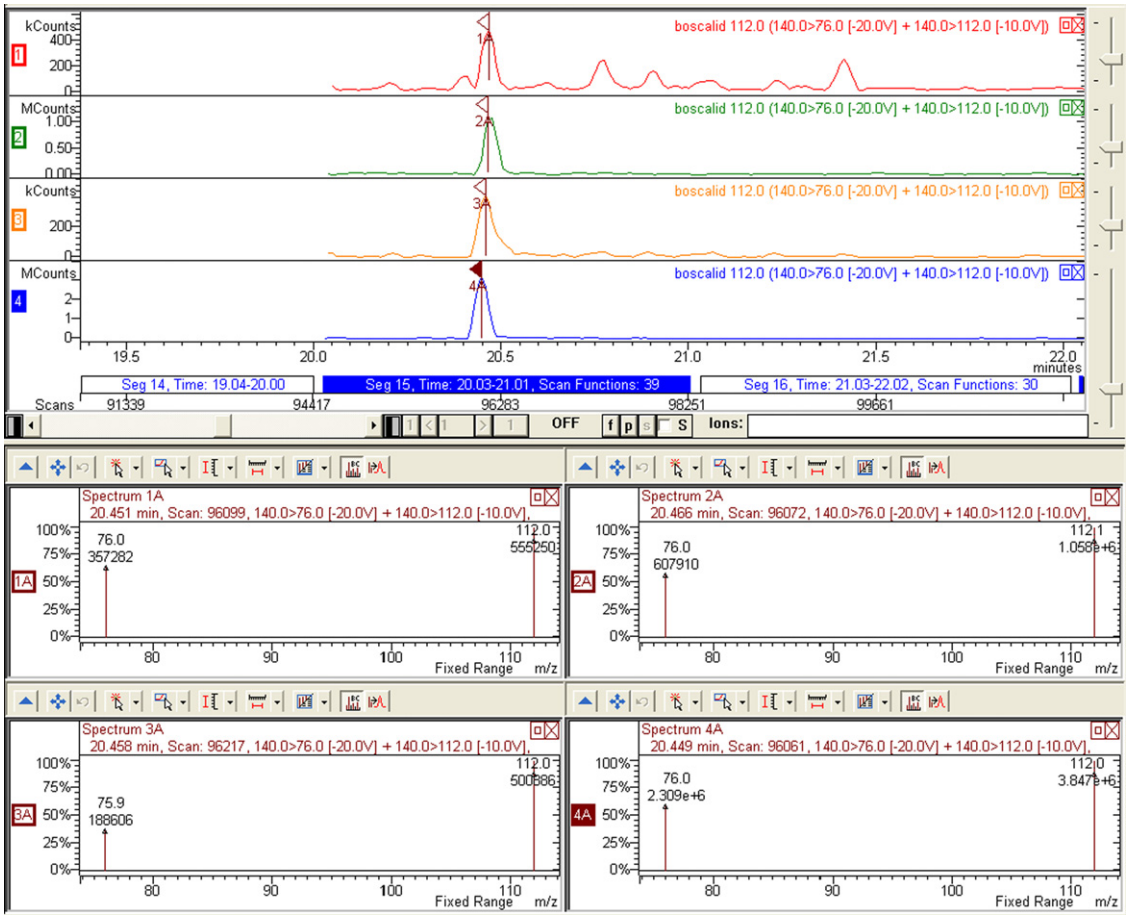

Fig. 1. GC–MS/MS chromatograms of analyte boscalid in: 1A. Chromatogram plot and spectra plot of spiked olive oil sample at 0.01 mg/kg 1B. Chromatogram plot and spectra plot of spiked olive sample at 0.01 mg/kg, 1C. Chromatogram plot and spectra plot of reference standard solution in olive oil extract, at 0.01 μ/ mL, 1D. Chromatogram plot and spectra plot of reference standard solution in olive extract, at 0.01 μ/mL.

in that region the maximum abundance was observed. The collision energy varied from 5–25 eV as mentioned, depending on the precursor and product ions. The analysis was performed with a filament-multiplier delay of 5.50 min. For the operation in MS/MS mode, Argon 99.999% was used as the collision gas at a pressure of 1.3 mTorr. The electron multiplier voltage was set at

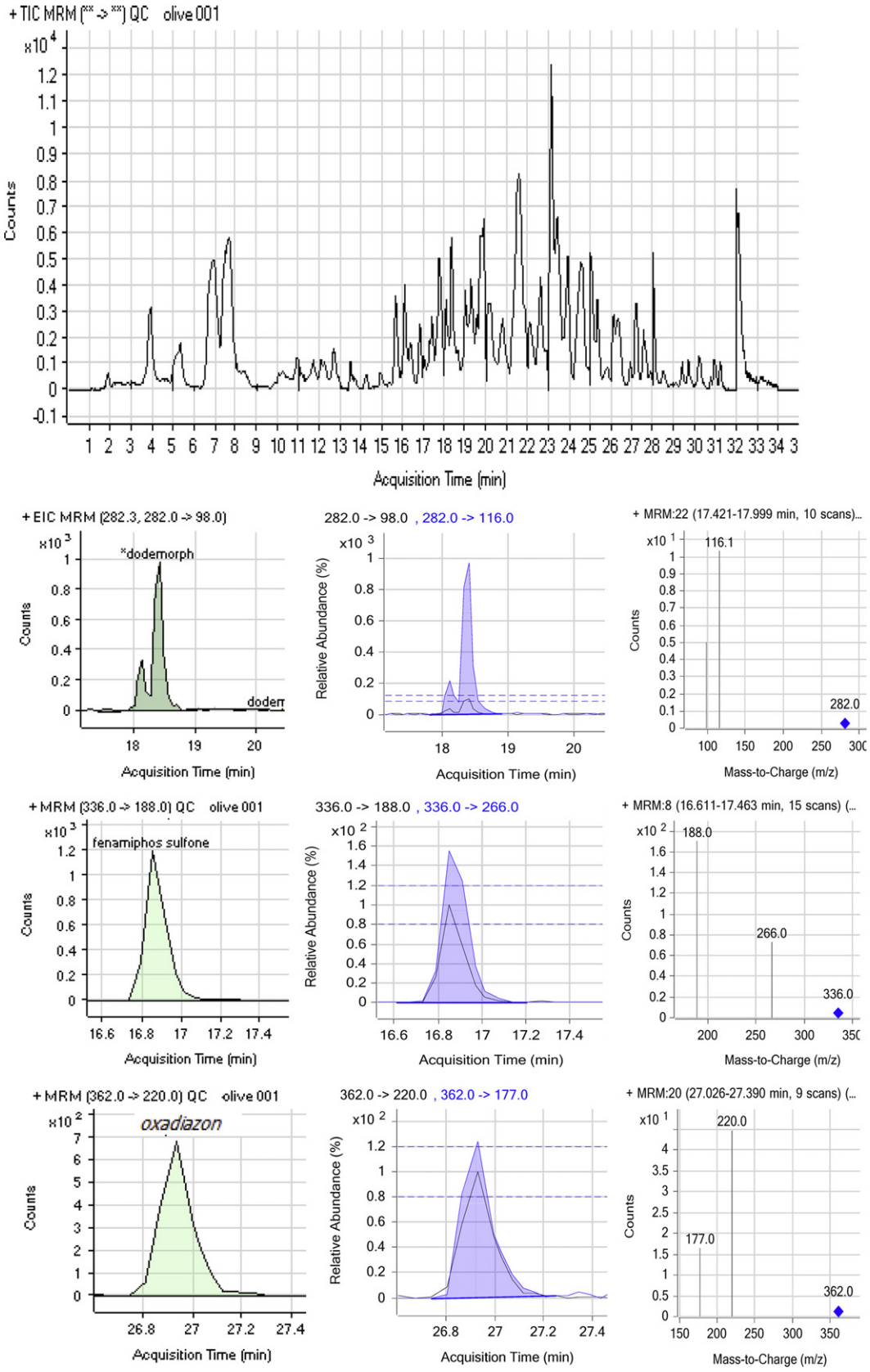

Fig. 2. Total Ion Chromatogram of a QC olive sample spiked at 10 μg/kg and chromatograms of the quantitaion, qualification transitions and spectra plots of the analytes dodemorph, fenamiphos sulfone and oxadiazon.

1500 V. The dwell time was set at 0.05 s for all SRMs. The total GC analysis time was 65 min. In Fig. 1 example GC–MS/MS chromatograms of analyte boscalid in a spiked olive and olive oil samples and in respective matrix matched standard solution are presented.

3.3. LC–(ESI)–MS/MS analysis

Aliquots of 10 μL of sample extract were injected into the liquid chromatograph. The separation was achieved with the C_{18} column at a flow rate of 250 μL/min. The mobile phase consisted of water with 5 mM ammonium formate, 0.1% formic acid and 0.02% acetonitrile (Solvent A) and methanol with 5 mM ammonium formate and 0.1% formic acid (Solvent B). The gradient program started from 20% of solvent B for 2 min., then increased to 60% solvent B over the next 10 min. increased to 100% solvent B over the next 18min.and held at this gradient for 5 minThe column was re-equilibrated for 5 min at the initial mobile phase composition. The total run-time was 40 min. In order to avoid carry-over the needle was washed in the flush port with solvent B for 10 s after each injection.

Typical source parameters were as follows: capillary voltage and collision energy varied as shown in Table 1, drying gas temperature was set at 300 °C. Drying and nebulizing gas was nitrogen generated from a high purity nitrogen generator (Nitroflow Basic Mobile, Parker Filtration & Separation B.V.) and they were set at 7 L/min and 30 psi respectively. For the operation in MS/MS mode, nitrogen was used as collision gas with a pressure of 1.5 mTorr. The multiple reaction monitoring experiments were conducted with a dwell time of 50 m s. For instrument control, Agilent Mass Hunter data acquisition Triple Quad B.01.04 and for data processing Agilent MassHunter Workstation Qualitative Analysis B.01.04 were used. In Fig. 2 example LC chromatograms of the analytes dodemorph fenamiphos sulfone and oxadiazon in QC olive sample are presented.

3.4. Homogenization study during sub-sampling

One of the most important sources of uncertainty is sub-sampling in the laboratory. This parameter is crucial mostly in solid samples, since liquids tend to by homogeneous. Therefore, for the estimation of the homogeneity of the sub-sampling procedure and as to verify if the amount of the analytical sample (5 g) is representative of the whole laboratory sample, a homogenization study was conducted in olives (since olive oil is assumed to be homogeneous).

A laboratory sample of 1 kg of whole olives was purchased from a local market in Athens [12]. The sample was homogenized in a food processor (Thermomix TM31, Vorwerk Group) for 3 min until pure. The whole homogenized sample was spiked with 2.5 mL of a 10 μg/mL working standard solution, containing 72 of the understudy analytes, as to reach a final concentration of 25 μg/kg. The sample was mixed again for 3 min and the sample was left to stand at room temperature for 30 min; and put in the freezer (4 °C) for 24 h, as to ensure that the analytes were homogenously distributed throughout the sample. The next day the sample was left to reach room temperature and stirred with a spatula. Ten sub-samples, each of 10 g (1st *stage sub-samples*) were collected from the laboratory sample and each of them was divided in 2 equal portions of 5 g (2nd *stage sub-samples*). In order to detect if a significant difference was observed between the 10 groups, *Analysis of Variance* (*ANOVA*) was performed. The variance ratio was calculated based on the formula [13]:

$$F_{\text{start}} = \frac{\text{Mean square between classes}}{\text{Mean square within classes}}$$

The critical values of F [p, (df_1−1), df_1 ∗(df_2−1)], at probability 95% (p=0.05), with df_1=2 (number of 2nd stage subsamples) and df_2=10 (number of 1st stage sub-samples) was compared to the F_{stat}. All statistical calculations were performed with SPSS 16.0.

As to ensure that the physicochemical properties of the analytes do not cause any interaction with the matrix, the ANOVA was performed for each analyte separately and the parameter evaluated was the recovery (%). Formulating as null hypothesis that all population means are equal (Ho: $\mu_1=\mu_2=\ldots\ldots\mu_k$), the alternative hypothesis is that at least one mean is different.

From the results no significant differences were observed between the sub-samples for all analytes (the basic statistical parameters of the F analysis are presented in Table S1 of the Supporting Information). Therefore the sub-sampling procedure is sufficient and the amount of 5 g is considered to be an adequate portion representative of the whole laboratory sample.

3.5. Linearity

Calibration curves were constructed from injections of calibration standards in acetonitrile, in olive extract and in olive oil extract. Linearity was evaluated at 8 concentrations levels: 10, 25, 50, 75, 100, 150, 200, and 300 μg/L. Good linearity was achieved in all cases with correlation coefficients better than 0.990 (0.989–1). The majority of the analytes achieved excellent linearity with r values higher than 0.99 (92.8%, 87.5 and 79.2 for standards prepared in olives, olive oil and solvent respectively). For r values between 0.99–0.95 these percentages were 5.2, 8.4 and 17.8% respectively. Only a small minority ($<5\%$) which corresponds to 2–4 analytes (depending on the matrix) had r values lower than 0.95.

The analytes acrinathrin and HCH a in olives, acrinathrin, aldrin, boscalid and propagate in olive oil, and ethoprophos, methiocarb sulfoxide and permethrin in solvent gave r values between 0.95 and 0.98. The analytes ethoprophos and isofenphos methyl in olives, chlorfevinphos, DDD op, fenthion and isofenphos methyl in olive oil and buprofezin, demeton S methyl sulfone and famoxadone in solvent gave r values below 0.95. For these compounds a rough estimation of the analyte concentration is obtained using the calibration line; however for a more accurate quantitation the use of another technique like single point calibration or standard addition is preferable.

3.6. Assessment of matrix effect

Matrix effect is generally the combined effect of all components of the sample, other than the analyte, on the measurement [15]. The matrix effect can be attributed to many sources, as the separation process or the ionization process of the analyte. Cleanup techniques [16] like solid phase extraction [17], dispersive solid-phase extraction [18–20], gel permeation chromatography [21] and freezing out (the last two apply mostly for cleaning fatty matrices) are used in order to reduce the co-extractives in the final extract and as a consequence the matrix effect. Due to matrix co-extractives in a MS/MS system, the peak signal might be enhanced or suppressed.

In order to estimate if the matrix influences in a significant degree the peak area and therefore the sensitivity of the analytes, the slopes of the calibration lines obtained for the 2 matrices (olives and olive oil) and the solvent were compared using a Student t-test. The t_{cal} is defined in the following equation:

$$t_{\text{cal}} = \frac{\left|b_1 - b_2\right|}{\sqrt{S_{b1}^2 - S_{b2}^2}}$$

with b the slope of the calibration line and S_b the standard deviation of the slope.

If the theoretical value (t_{theo}) of 2.306 (d_f=6+6–4=8, for *two-sided critical region*, *at probability 95%*) exceeds the calculated t_{cal} value, the null hypothesis, that there is no significant difference between the two calibration lines, is accepted and therefore the matrix effect is not significant.

Significant difference were observed in some cases, between the slopes of the calibration lines meaning that matrix effect is present and that quantitation should be conducted with matrix matched calibration standards in order to have reliable and accurate quantitation. In both cases the percentage of the analytes that the matrix effect is not significant is 53 and 69% in olive and olive oil matrix respectively. However, these percentages varied from one chemical group to another, as seen in Fig. 3. For olives, in most of the analytes belonging to the chemical classes of carbamates, triazoles, urea and neonicotinooids the influence of the matrix effect was found significant; while in organochlororines and organophosphorus the minority of the analytes presented significant matrix effect. In the case of olive oil, in most chemical groups, (carbamates, organochlororine, organophosphorus, triazoles and urea) the analytes presented significant matrix effect. In the case of neonicotinooids no significant matrix effect was observed.

3.7. Accuracy and lower limit values

Recoveries and repeatability experiments were established in order to evaluate the methods' trueness and precision respectively. The recovery and repeatability experiments were conducted at two levels, 10 μg/kg and 100 μg/kg, with six replicates at each level. The blank matrices used for the experiment were olives and olive oil samples from the market, previously analyzed as to ensure the absence of the analytes under investigation.

Mean recoveries of 70–120% and relative standard deviations (SDr) values below 20% are considered acceptable [1]. As demonstrated in Figs. 4 and 5, the recoveries of the majority of the analytes at both levels ranged between 70.4–120.2% for olives and 69.3–125.7% for olive oil with SDr values below 21.2% for olives and 26.5% for olive oil. The results however varied depending on the chemical class:

- For carbamates, 100% of the analytes in olives and 95% in oil gave acceptable recoveries between 70–120%. All of the analytes gave SDr values below 20% with the majority ranging between 5–10% for both commodities.
- For organochlorines, 100% of the analytes in olives and 87.5% in olive oil gave acceptable recoveries between 70–120%. All of the

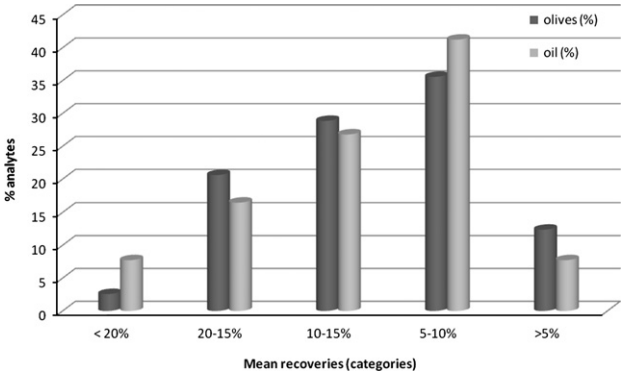

Fig. 4. Graphical distribution of the % mean recoveries of all analytes studied, for both matrices used (olives, olive oil).

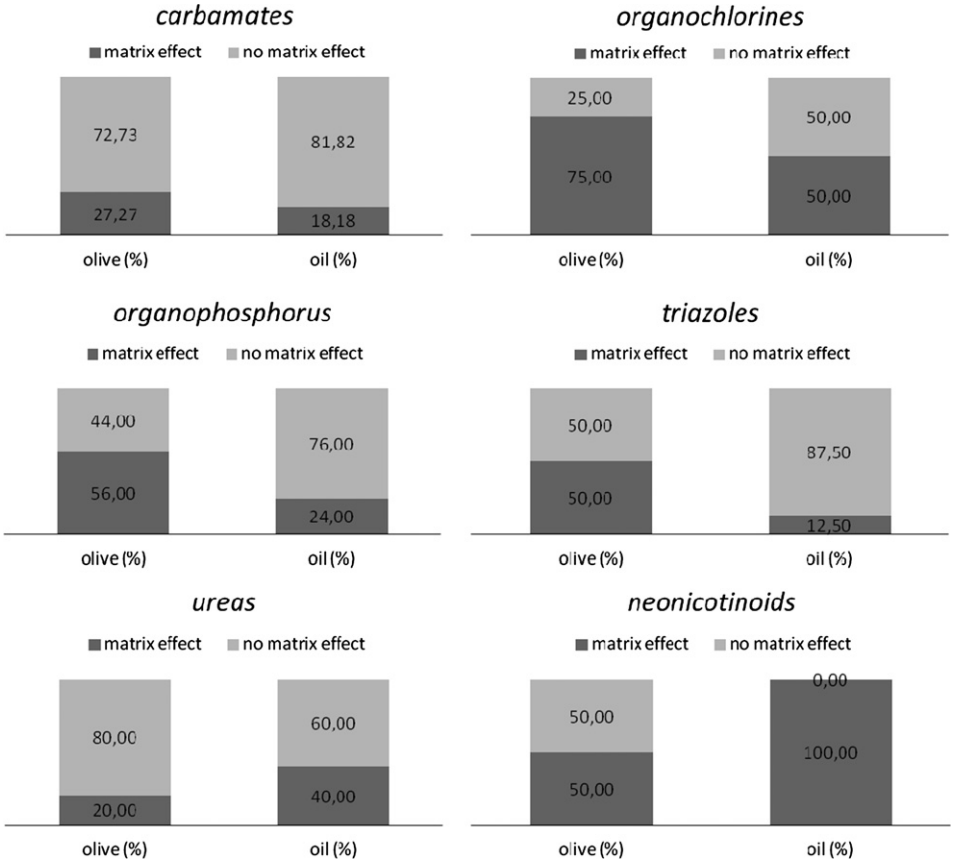

Fig. 3. Distribution of the maxtix effect for the various chemical groups of analytes, according to the significance of the maxtix effect, based on the *t*-test.

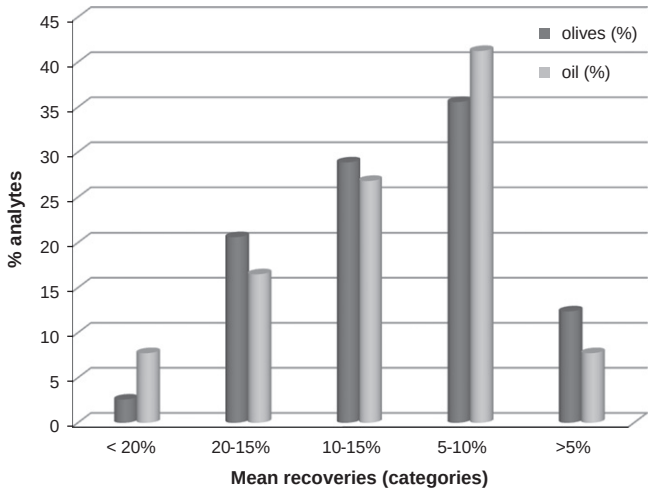

Fig. 5. Graphical distribution of the SDr values of all analytes studied into 5 classes for both matrices used (olives, olive oil).

analytes gave SDr values below 20% except of a 12.5% of the analytes above 20% in olive oil. The majority SDr values ranged between 15–20% for olives and 10–15% for olive oil.

- For organophosphorus, 100% of the analytes in olives and 96% in olive oil gave acceptable recoveries between 70–120%. All of the analytes gave SDr values below 20% with the majority ranging between 5–10% in olive oil and 10–15% in olives.
- For triazoles, 94% of the analytes in olives and 87.5% in olive oil gave acceptable recoveries between 70–120%. Most of the analytes gave SDr values below 20% with the majority ranging between 5–10% for both commodities.
- For ureas, 100% of the analytes in olives and 90% in olive oil gave acceptable recoveries between 70–120%. Most of the analytes gave SDr values below 20% with the majority ranging between 10–15% for both commodities.
- For neonicotinoids, 100% of the analytes in olives and olive oil gave acceptable recoveries between 70–120%. Most of the analytes gave SDr values below 20% with the majority ranging between 5–10% for both commodities.

3.8. Limit of quantification/detection (LOQ/LOD)

The limit of quantification (LOQ) was established as the lowest concentration tested for which recovery and SDr values were satisfactory, in accordance with the criteria established for analysis of pesticide residues in food and with a Signal to Noise (S/N) ratio higher than 10. Therefore as LOQ, the level of 10 μg/kg was set, as it was the lowest validated level with acceptable trueness and precision results. As LOD is related to LOQ with the equation $10 \times \mathrm{LOD} = 3 \times \mathrm{LOQ}$, therefore the LOD of the method was set at the level 3 μg/kg. However, as concentration lower than 10 μg/kg were not studied, concentrations of analytes lower than 3 μg/kg could be detected in many cases.

3.9. Proficiency test

The laboratory participated with the described method in the Proficiency Test COIPT-11 for olive oil organized by the Italian National Reference Laboratory for pesticide residues in products of animal origin and commodities with high fat content (NRL-AO), in cooperation with the IOC (International Olive Council). The matrix of the proficiency test was olive oil. From a target list of 23 pesticides the laboratory accurately determined all analytes present in the distributed sample. All z score values were acceptable between $-1.2 < z < 1$, with an overall z-score value of 0.8, verifying acceptable accuracy for the method.

Table 2
Number of positive findings in olives and olive oil and the width of the calculated concentrations range.

Analyte	Number of findings			C (μg/kg)[a]
	olive oil	olive	Total	
Benalaxyl	1		1	11
Chlorpyriphos ethyl	6	2	8	23–60
Diazinon	1		1	21
Dimethoate	2	6	8	11–184
Endosulfan sulfate	1		1	97
Ethion	1		1	7
Fenthion	1		1	23
Fenthion oxon	2		2	27
Fenthion sulfone	1		1	8
Fenthion sulfoxide	1		1	23
Omethoate	1	8	9	2–458
Pirimiphos methyl	1		1	8
Pyripoxyfen		1	1	50
Tetraconazole	1		1	15
Thiacloprid	1		1	38

[a] Concentrations lower than 10 μg/kg are considered as semi-quantitated

3.10. Application to real samples

The proposed methodology was applied for the analysis of real samples. In the years 2010, 2011 and 2012 a total of 262 olive and olive oil samples (89 olive oil and 72 olives) were obtained from local markets in Greece and analyzed with the current method for the determination of pesticides residue. The sampling methodology was conducted according to Dir. 2002/63/EC [12]. The scope of the sampling was mainly the monitoring of pesticide residues in Greek olive products from the local market. Residues of 15 pesticides were detected in 19 of the samples (7.25%), 7 of them were olive and 12 olive oil samples. In 12 of the samples (8 olives and 4 olive oil) the presence of 1 analyte was detected in 4 samples (3 olives and 1 olive oil) 2 analytes were detected, in 1 olive sample 3 analytes were detected, in 1 olive oil sample 6 analytes were detected and in 1 olive oil sample 8 analytes were detected. The analytes found, the frequency of their presence and their calculated concentrations are presented in Table 2.

The analytes diazinon and tetraconazole were not included in the initial validation. These analytes were added later in the method. The method was validated successfully for other analytes of the same chemical classes (organophosphorus and triazoles) therefore, it is considered that the behavior of the additional analytes would be the same. However as to ensure the validy of the results, QC samples, at a concentration range of 10–50 μg/kg, were conducted for all 4 analytes and were found accepted, with mean recovery and SDr results as follows:

- Diazinon ($n=15$): 86.8% and 16.7% respectively.
- Fenthion sulfone ($n=9$): 91.4% and 14.7% respectively.
- Tetraconazole ($n=9$): 94.2% and 19.7% respectively.
- Omethoate ($n=7$): 98% and 16% respectively.

4. Conclusions

Olives and olive oil are special category of analytical matrices that in most cases require complex, time–money consuming analytical method for the cleanup and the determination of pesticide residues. On the other hand, in the field of pesticide residue analysis in food there is a trend toward simple, cheap and

multiresidue analytical methods. In this study an easy, economic and reliable analytical method for the determination of multiclass pesticides was developed and validated. The advantages of this method are the combination of an easy and cheap extraction procedure based on acetonitrile extraction followed by cleaning of the fat through freezing and of the pigment with GCB. The determination of the analytes in a MS/MS system working in MRM mode and combined with either a GC or LC, gave reliable qualification and quantification of the analytes. The method presented acceptable trueness, precision and linearity with an LOQ set at 0.01 mg/kg. In most cases the matrix effect was found significant and therefore matrix matched calibration standards should be used, as to have more reliable results. The method was applied for the analysis of 262 olive and oil samples. 7.25% of them (19 samples) were found positive for the presence of 15 different pesticides and their metabolites;while in 7 of the samples 2–8 analytes were detected.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.03.051.

References

[1] European Commission, Document N° SANCO/12495/2011, 2011.
[2] C. Yagiie, S. Bayarri, P. Conchello, R. Lazaro, C. Perez-Arquillue, A. Herrera, A. Arifio, J. Agric. Food Chem. 53 (2005) 5105–5109.
[3] E.G. Amvrazi, T.A. Albanis, J. Agric. Food Chem. 54 (2006) 9642–9651.
[4] S. López-Feria, S. Cárdenas, M. Valcárcel, J. Chromatogr. A 1216 (43) (2009) 7346–7350.
[5] M. Anastassiades, S.J Lehotay, D. Stajnbaher, F.J. Schenck, J. AOAC Int. 86 (2003) 412–431.
[6] S.C. Cunha, J.O. Fernandes, M.B.P.P. Oliveira, Talanta 73 (2007) 514–522.
[7] S.C. Cunha, S.J. Lehotay, K. Mastovska, J.O. Fernandes, M.B.P.P. Oliveira, J. Sep. Sci. 30 (2007) 620–632.
[8] J.J. Vreuls, R.J. Swen, V.P. Goudriaan, M.A. Kerkhoff, G.A. Jongenotter, U.A. Brinkman, J. Chromatogr. A. 750 (1–2) (1996) 275–286.
[9] A. García Sánchez, N. Ramos Martos, E. Ballesteros, Anal. Chim. Acta 558 (1-2) (2006) 53–61.
[10] E.M. Diaz-Plaza, J.M. Cortes, A. Vazquez, J. Villen, J. Chromatogr. A 1174 (2007) 145–150.
[12] Commission Directive 2002/63/EC. Off. J. Eur. Commun. 2002, L187, 30–43.
[13] G.W. Snedecor, W.G. Cochran, Statistical Methods, 6th ed., Iowa State University Press, 1967.
[15] A.D. McNaught, A. Wilkinson IUPAC Compendium of Chemical Terminology, The Gold Book, second ed. Blackwell Science Ltd, 1997 available from: ⟨http://old.iupac.org/goldbook/M03759.pdf⟩ (accessed in: 11.07.2010).
[16] S.M. Walters, Anal. Chim. Acta 236 (1990) 77–82.
[17] F.J. Schenck, D.J. Donoghue, J. Agric. Food Chem. 48 (2000) 6412–6415.
[18] S.J. Lehotay, Quick, easy, cheap, effective, rugged and safe approach for determining pesticide residues, in: J.L. Martinez Vidal, A. Garrido Frenich (Eds.), Methods in Biotechnology, Pesticide Protocols, vol 19, Humana Press, Totowa, NJ, 2005, p. 2005.
[19] C.J. Anagnostopoulos, P. Aplada, P Sarli, G.E. Miliadis, S. Haroutounian, Hell. Plant Prot. J. 3 (2) (2010) 71–80.
[20] K.S. Liapis, S.N. Kolokouri, M.A. Koupparis, G.E. Miliadis, European Pesticide Residue Workshop 2010, Strasbourg.
[21] A.B. Gelsomino, B. Petrovicova, S. Tiburtini, E. Magnani, M. Felici, J. Chromatogr. A 782 (1) (1997) 105–122.