Screening Of Pesticides Used In Marine Aquacultures With The Aid Of Lc-High Resolution Orbitrap Ms

Margarita Kapsi *, Christina Nannou, Vasiliki Boti, Triantafyllos Albanis

Laboratory of Analytical Chemistry, Department of Chemistry, University of Ioannina, Ioannina, Greece

Corresponding author: mkapsi@cc.uoi.gr

Abstarct

A wide range of chemicals are used in aquaculture, including antibiotics, pesticides, hormones, anesthetics, various pigments, minerals, and vitamins. The concerns about the use of chemicals center on both their potential effects on human health and on natural ecosystems. These compounds may accumulate in aquacultured fish through contaminated feed ingredients while also; certain biocides (e.g. Irgarol) are applied directly to the water in aquaculture ponds to control weeds and algae. It is, therefore, needed to collect data on these chemicals for a better knowledge of its fate in natural waters and for the risk assessment. The aim of this work was to develop an efficient method on the basis of solid phase extraction (SPE) technique for the determination of antifouling compounds and pesticides such as Irgarol 1051, Azamethiphos, and Deltamethrin in aquaculture sea water samples. Sea water collected from Epirus region (North-Western Greece), was used to validate an analytical method. Analysis was carried out with ultra-high performance liquid chromatography (UHPLC) high-resolution Orbitrap mass spectrometry.

Introduction

Sea lice are a major problem in aquaculture, as they can seriously damage or even kill farmed fish. To control infestations, managers treat the fish with veterinary pesticides such as azamethiphos or deltamethrin. Irgarol is also commonly used as antifouling agent to control algae and weed growth. Such residues in the aquatic environment have been proved to exert toxic and adverse effects on the marine ecosystem. Their detection and reduction is one of the major challenges for the preservation and sustainability of the environment since the rapid expansion leads to the requirement of wider use of drugs, disinfectants and antifouling compounds to eliminate the microorganisms in the aquaculture facilities. There are risks associated with the use of biocides in aquaculture since predators and humans may ingest the fish and the shellfish accumulating these contaminants. Moreover high risk for the aquatic environment posses the development of antibiotic resistance in bacteria (1). Thus, their levels in water must be monitored regularly, especially in sources of water (2,3).

Last years, an effort has been made to shift the existing methods of analysis into multi-methods, thus enabling the simultaneous determination of various classes of compounds in one analysis. Until recently, this was achieved employing LC systems coupled to mass spectrometry. However selective, sensitive and precise that methods are, a few limitations may occur due to the targeted acquisitions needed. This means that only analytes included in MS acquisition method will be detected, so the number of analysed compound is limited (4).

Orbitrap mass spectrometers overcomes this obstacle, rendering feasible untargeted or post target analysis, enabling so the analysis of numerous compounds and offering a retrospective view to investigate unknown compounds in samples. The ion frequencies are measured by acquisition of time-domain image current transients and converted to m/z measurements using Fourier Transform (5). The high resolution power in the Orbitrap is inversely proportional to the measurement time, thus higher resolution data require longer measurement times.

The orbitrap mass analyzer function is based on ion trapping on a central spindle electrode using a quadrologarithmic field (Hoogenboom, Peterman). The detected ions are measured by the frequency of the harmonic ion oscillations along the axis of the electric field.

High resolution mass spectrometry provides ultra-high resolution and mass accuracy as well, supporting in this way a variety of applications, such as multi-methods, semi-targeted and non-targeted analysis as well.

Materials And Methods

Standards and reagents

Analytical standards were purchased by Fisher Scientific (Leicestershire, UK), purity >98.4%. Stock standard solutions were prepared at a concentration of 1–2 μg/L in methanol and were stored at −20°C. The solvents used, including methanol and water LC-MS grade, were supplied by Merck (Darmstadt, Germany). Oasis™ HLB cartridges (divinylbenzene/N-vinylpyrrolidone copolymer, 200 mg, 6 cm^3) were purchased from Waters (Mildford, MA, USA). Formic acid (purity, 98%) was obtained from Fluka (Buchs, Germany). The selected pesticides were: Table 1 lists the main physicochemical properties of the target compounds.

Sample preparation and solid phase extraction

Isolation of target analytes from sea water samples were performed off-line, using a standard 16-port SPE manifold connected to a vacuum pump. HLB cartridges were first activated with 6 mL methanol and 6 mL distilled water (without letting the cartridges to become dry). Afterwards, a volume of 250 mL of water sample was percolated. The analytes were eluted with 3mL of dichloromethane, hexane and acetone, consecutively. Finally they were evaporated to dryness under a gentle stream of N_2 and reconstituted into the desirable volume of methanol. The final sample was filtered directly into an analysis vial using a 0.45 μm PVDF syringe filter (Millex-HV, Millipore, Cork, Ireland) and injected to a liquid chromatography - LTQ Orbitrap MS system.

LC Conditions

The LC LTQ-FT Orbitrap MS system consisted of an Accela AS autosampler, an Accela quaternary gradient U-HPLC-pump and an LTQ Orbitrap XL 2.5.5 SP1 mass spectrometer (Thermo Fisher Scientific, Inc. GmbH, Bremen, Germany). The linear ion trap (LTQ) part of the hybrid MS system was equipped with an Ion Max Electrospray Ionization (ESI) probe. Full-scan accurate mass spectra were obtained at high resolution (60000 FWHM). The analytes were eluted on a 50mm x 2.1mm i.d. Hypersil GOLD column, 1.9 μm, using a gradient elution system consisting of water (A) and methanol (B) both containing 0.1% formic acid and 5mM ammonium formate at 300 μL/min flow rate.

The advantage of this hybrid instrument is that fragmentation can be carried out either in the linear trap or the HCD cell. The same applies to resulting fragments that can be measured either with the linear ion trap or the orbitrap detector. In the case of the orbitrap detector a high resolution mode was applied to obtain MS and MS/MS with high accuracy.

Identification of target compounds was based both on the calculated accurate mass and retention time while for further confirmation the MS fragmentation was used (Table 2).

Chromatographic separations were carried out using an Accela LC system (Thermo Scientific, Hemel Hempstead, UK),. The injected sample volume was 5 μL. The gradient solvent was methanol and water both containing 0.1% formic acid

Confirmation criteria

The guidelines of the EU Document No. SANCO/12495/2011 were followed for the identification of the pesticides and the analytical method validation as well. To be more specific, the positive findings confirmation criteria were: (i) Chromatographic separation: An interval of ±2.5% in the retention time (RT) of each analyte elution in the real sample and the quality control sample of the same analytical sequence is acceptable. (ii): Mass spectrometric detection: The diagnostic ion (relative ion intensity >10% in the full scan mass spectrum of an analyte in the sample) must correspond to that of the same analyte in the quality control sample in the sequence. Moreover, the measured accurate mass of the $[M+H]^+$ should identify with the theoretically calculated one with a mass tolerance of ±5 ppm, while product ions must fit to those of the calibration standard within a discrepancy of ±15 %. Another useful tool for further confirmation can be provided by the characteristic isotopic pattern (chlorine, bromine, etc) by obtaining accurate masses for these isotopic signals.

Method validation

The applied method was validated according to the Guideline of Method Validation and Quality Control Procedures for Pesticide (6).

Method performance was evaluated in the terms of linearity, selectivity, extraction recoveries and matrix effects and precision. For the calculation of the limit of detection (LOD), responses of consecutive injections with decreasing concentrations of standards were measured to find at which concentration the standard could give a peak value of approximately 10^4. It is noteworthy that in high resolution mass spectrometry detectors, the chromatogram may not give us information about the background noise, rendering thus impossible to calculate the signal-to-noise ratio (S/N). Hence, it is necessary to take into account the above-mentioned LOD.

The (%) value of the matrix effect indicates the matrix-induced suppression or enhancement, depending on being less or greater than 100, respectively

The SPE UHPLC-LTQ-Orbitrap MS method recoveries and relative standard deviations (RSDs) were calculated by spiking blank samples at three levels: the LOQ, a medium (100 ng L^{-1}) and a high level (250 ng L^{-1}) and analysed five times (n=5). The precision of the method was estimated by repeatability and lab reproducibility studies, expresses as RSD (%). To measure the intra-day precision, the standard deviation of the recovery percentages of the spiked samples ran during the same day was determined, while the inter-day precision was determined in the same way but analyzing the spiked samples in five distinct days.

The present FT Orbitrap MS method was successfully applied to the analysis of various pesticides in river water samples. According to the calculated LODs (), the sensitivity is really good and could be comparable to this of triple quadropole instruments that are commonly used for such analysis of organic contaminants. To sum up, it seems that FT Orbitrap MS contributes significantly to ion discrimination of 2.

The target analytes were detected as $[M+H]^+$ adduct ions for irgarol and azamethiphos while deltamethrin was detected as $[M+NH_4]^+$, all under positive ion mode. Mass accuracy of measured ions was calculated below 3 ppm at 50ppb, in all cases. The variables involved in the chromatographic process were optimized in instrument auto tune sections. The selected conditions proved to be excellent within 10 min elution with the quantification LTQ-Orbitrap LC-MS limit down to approximately 0.25 ppb. Under optimized conditions, the fragmentation process in the LTQ system, taking the above-mentioned adduct ions as precursor ones and using CID energy of 35% yields more than enough structural information for positive identification based on monitoring the basic fragment ion. SPE extraction conditions were validated using spiked seawater samples (previously

ensured to be free of the analytes of interest) fortified with the target analytes at two concentration levels, 20 ng/L and 500 ng/L respectively. The analytical method proved to be linear over a wide range of concentration, exhibited satisfactory repeatability and reached trace level in the order of low ng/L.

Results and discussion

Twenty two pesticides were chromatographically separated within 8 min. The separation was performed using a mobile phase consisted of water and methanol (0.1 % formic acid). The whole system turned out to be considerably effective for the separation.

The target analytes were detected as $[M+H]^+$ adduct ions for irgarol and azamethiphos while deltamethrin was detected as $[M+NH_4]^+$, all under positive ion mode. Mass accuracy of measured ions was calculated below 3 ppm at 50ppb, in all cases. The variables involved in the chromatographic process were optimized in instrument auto tune sections. The selected conditions proved to be excellent within 10 min elution with the quantification LTQ-Orbitrap LC-MS limit down to approximately 0.25 ppb. Under optimized conditions, the fragmentation process in the LTQ system, taking the above-mentioned adduct ions as precursor ones and using CID energy of 35% yields more than enough structural information for positive identification based on monitoring the basic fragment ion. SPE extraction conditions were validated using spiked seawater samples (previously ensured to be free of the analytes of interest) fortified with the target analytes at two concentration levels, 20 ng/L and 500 ng/L respectively. The analytical method proved to be linear over a wide range of concentration, exhibited satisfactory repeatability and reached trace level in the order of low ng/L.

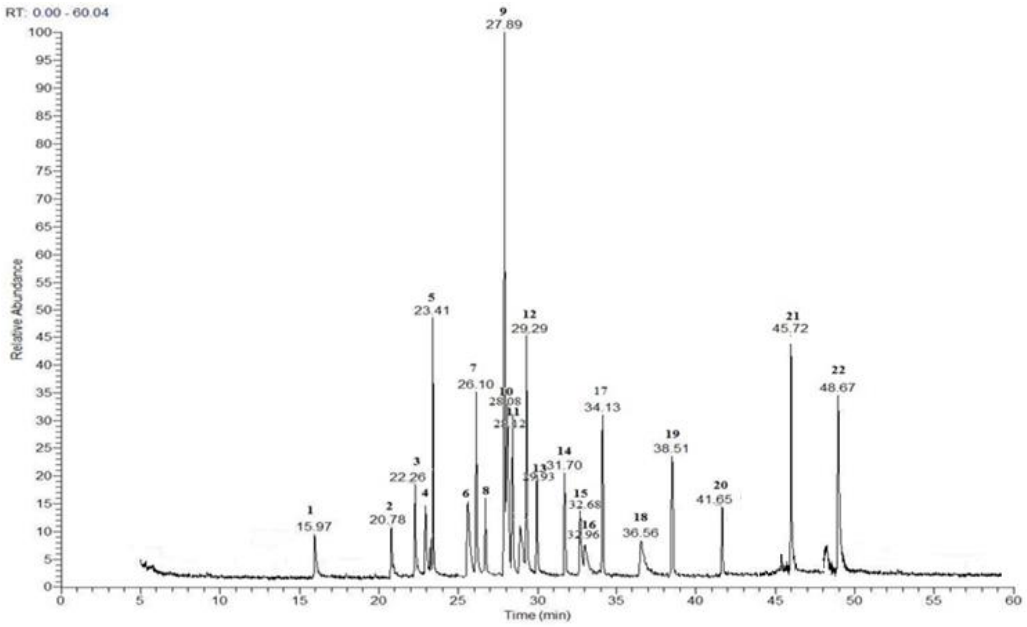

Figure 1. *Typical chromatogram of gc-ms 1.Eptc, 2.Molinate, 3. Propachlor, 4.Ethoprophos, 5.Trifluralin, 6.Atrazine, 7.Terbuthylazin, 8.Disulfoton, 9.Dimethenamid-p, 10.Chloropyrifos-methyl, 11.Acetochlor, 12.Pirimiphos-methyl, 13.Metolachlor, 14.Pendimethaline, 15.Quinalphos, 16.Triadimenol A, 17.Endosulfan-alpha, 18.Myclobutanil, 19.Endosulfan-beta, 20.Endosulfan-sulfate, 21.Azinphos-ethyl, 22.Quizalofop-ethyl*

Table 1. Retention time and characteristic ions selected for each compound.

Compounds	**Rt**	**Ions (m/z)**		
Eptc	16.20	128	132	189
Molinate	20.82	126	187	127
Propachlor	22.05	120	176	175

Ethoprophos	22.93	158	127	139
Trifluralin	23.47	264	306	248
Atrazine	25.74	200	215	201
Terbuthylazin	26.13	214	173	216
Disulfoton	26.80	129	186	153
Dimethenamid-p	27.97	154	203	230
Acetochlor	28.12	146	174	223
Chloropyrifos-methyl	28.08	286	288	125
Pirimiphos-methyl	29.29	276	290	305
Metolachlor	29.93	162	238	240
Pendimethaline	31.61	251	191	161
Quinalphos	32.68	157	156	146
Triadimenol A	32.96	168	111	128
Endosulfan-alpha	34.13	195	241	170
Myclobutanil	36.49	179	150	181
Endosulfan-beta	38.47	197	231	195
Endosulfan-sulfate	41.62	229	271	241
Azinphos-ethyl	45.69	160	104	105
Quizalofop-ethyl	48.67	299	372	243

Table 2. Analytical parameters and recoveries of compounds anal

Compound	**R^{2} [a]**	**RSD_r% [b]**	**RSD_R% [c]**	**LOD (µg L^{-1})**	**LOQ (µg L^{-1})**	**Linear range (µg L^{-1})**	**Recovery % (0.05 µg L^{-1})**	**Recovery % (0.2 µg L^{-1})**	**Recovery % (0.5 µg L^{-1})**
Eptc	0.999	3.4	4.4	0.012	0.040	0.05-0.75	95.71	100.53	87.06
Molinate	0.995	4.7	2.6	0.014	0.046	0.05-0.75	98.92	92.60	84.30
Propachlor	0.998	3.1	4.6	0.009	0.030	0.05-0.75	77.53	63.26	75.13
Ethoprophos	0.996	2.8	4.8	0.010	0.033	0.05-0.75	75.33	100.37	84.67
Trifluralin	0.998	4.1	2.3	0.006	0.020	0.025-0.5	67.32	89.80	94.70

Atrazine	0.999	3.2	2.5	0.010	0.033	0.05-0.75	100.70	97.45	94.99
Terbuthylazin	0.998	1.6	3.8	0.008	0.026	0.05-0.75	98.60	75.17	80.70
Disulfoton	0.998	4.5	2.4	0.010	0.033	0.05-0.75	91.03	97.27	89.55
Dimethenamid-P	0.999	3.1	4.9	0.005	0.017	0.025-0.5	104.99	88.95	92.27
Chloropyrifos-methyl	0.996	2.8	3.2	0.008	0.026	0.05-0.75	98.32	99.84	96.86
Acetochlor	0.998	4.7	3.9	0.008	0.026	0.05-0.75	96.18	102.02	93.63
Pirimiphos-methyl	0.999	1.7	3.5	0.006	0.020	0.025-0.5	97.62	89.02	90.50
Metolachlor	0.998	3.1	4.9	0.010	0.033	0.05-0.75	97.43	95.73	93.56
Pendimethaline	0.997	4.8	3.1	0.008	0.026	0.05-0.75	96.33	108.46	95.89
Quinalphos	0.994	4.9	3.8	0.012	0.040	0.05-0.75	99.27	76.16	71.55
Triadimenol	0.995	4.1	3.3	0.015	0.050	0.05-0.75	91.14	81.23	90.96
Endosulfan-alpha	0.999	3.8	4.7	0.008	0.026	0.05-0.75	95.20	70.91	80.62
Endosulfan-beta	0.991	2.8	4.1	0.006	0.020	0.025-0.5	97.60	83.58	95.08
Endosulfan-sulfate	0.999	3.5	3.7	0.010	0.033	0.05-0.75	98.16	90.76	100.02
Myclobytanil	0.993	4.6	3.4	0.013	0.043	0.05-0.75	83.49	92.50	78.23
Azinphos-ethyl	0.997	3.9	4.6	0.005	0.017	0.025-0.5	83.74	80.00	88.00
Quizalofop-ethyl	0.993	4.6	3.7	0.006	0.020	0.025-0.5	99.00	76.83	72.43

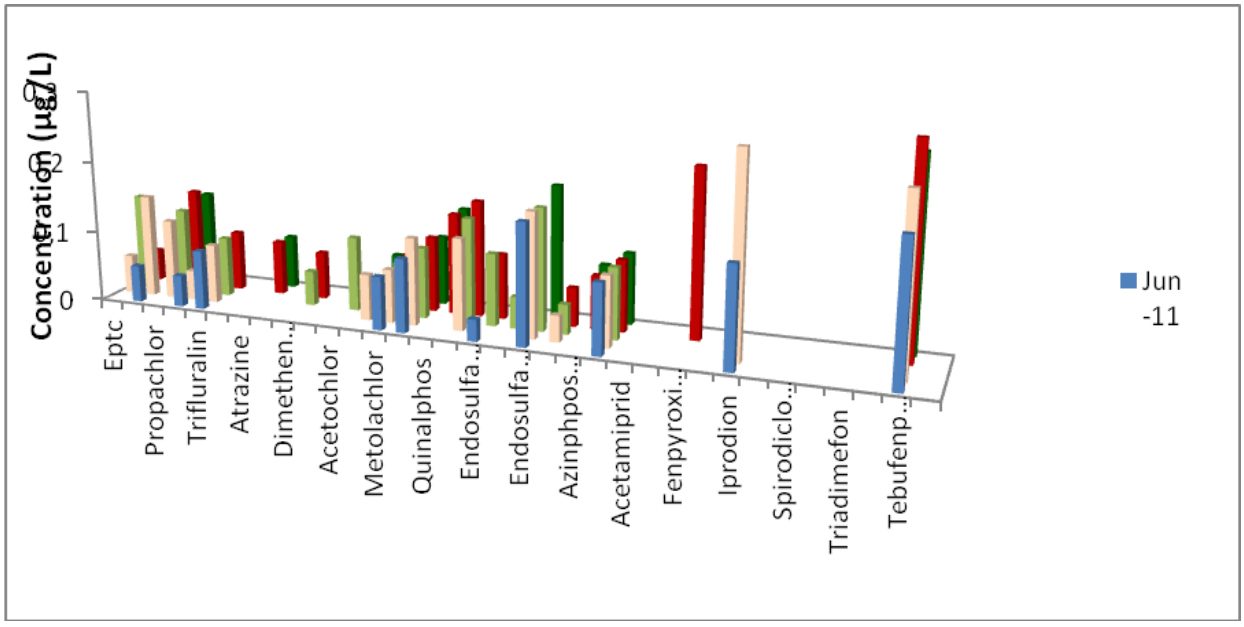

Figure 2. Twenty two (22) pesticides detected in Kalamas river estuaries the area of aquaculture installations start to the North during, June 2015. At highest concentration were detected, Tebufenpyrad 0.29 μg/L, and Fenpyroximate 0.23 μg/L **.**

The present multiresidue methodology was successfully employed for the determination of pesticides residues in aquaculture water environment. It possesses the advantages of SPE (fast, simple, highly sensitive) and could be potentially extended to other classes of pesticides, as a useful tool for monitoring purposes.

Acknowledgments

This project has received funding from European Union's Seventh Framework Programme (FP7-OCEAN-2013) under grant agreement No.614168. The authors would also like to thank the Unit of Environmental, Organic and Biochemical high resolution analysis-ORBITRAP-LC-MS of the University of Ioannina for providing access to the facilities.

References

1. F.A Guardiola, A. Cuesta, J. Meseguer and M. A. Esteban, Int J Mol Sci.; 13(2): 1541–1560 (2012).

2. M.J. Bueno, M.D. Hernando, A. Agüera, A.R. Fernández-Alba, Talanta 77: 1518–1527 (2009).

3.I.K.Konstantinou, T.A. Albanis, Environment International 30: 235 – 248 (2004).

4. Ch. Nannou, Ch. Kosma, T. Albanis, International Journal of Environmental Analytical Chemistry, Vol. 95, 2015 (13), 2015, pp 1242-1262

5. Ch. Nannou, V. Boti, T. Albanis, "Accurate mass screening of pesticide residues in fresh water and sediments using LC-hybrid LTQ/Orbitrap-MS", *SETAC Europe 27th Annual Meeting, May 7-11 2017, Brussels, Belgium*

6. Ch. Nannou, V. Boti, T. Albanis, "Accurate mass screening of pesticide residues in fresh water and sediments using LC-hybrid LTQ/Orbitrap-MS", *SETAC Europe 27th Annual Meeting, May 7-11 2017, Brussels, Belgium*

7. V. Boti, Ch. Nannou, M. Kapsi, T. Albanis, "Determination of pesticides used in marine aquaculture by LC-High Resolution Orbitrap MS", *2nd Progress Workshop, Sea-on-a-chip-project, April 13-15, Ferrara, Italy*