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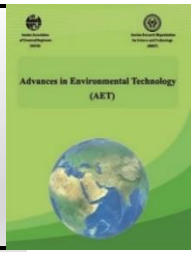

Development and validation of a gas chromatography-tandem mass spectrometry analytical method for the monitoring of ultra-traces of priority substances in surface waters

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ABSTRACT

The 2013/39/EU Directive of the European Parliament provided a list of substances (organic and inorganic compounds); these so-called priority and dangerous priority substances affect the quality status of surface waters. Due to their high level of toxicity, these contaminants have legal limits in the order of µg/L and even some in the order of ng/L and pg/L. To this aim, an effective and sensitive analytical method for monitoring these contaminants was deemed necessary. In this experimental process, a highly effective workflow represented by a pre-analytical and an analytical phase was developed and validated. The pre-analytical phase comprises a liquid-liquid microextraction and a quick, easy, cheap, effective, rugged, and safe purification. The analytical part was performed by a very sensitive and robust multi-residual GC-MS/MS method without the need for derivatization.
This method simultaneously identified and quantified most of these substances (represented by pesticides, chloroalkane hydrocarbons, and polycyclic aromatic hydrocarbons) at very low concentration levels while respecting the analytical concentration limits required by the European directive.

1. Introduction

In recent decades, increased awareness of water's vital importance has led to it being considered a resource worthy of protection. Water, in fact, is a precious asset for every form of life; it is not inexhaustible and must be available to everyone, as recognized in the European Water Charter adopted by the European Council as of May 1968 [1]. Therefore, the study of organic micro-pollutants in water is necessary to assess their environmental impact and any human health risk as an end consumer [2-9]. The release of these contaminants into the environment can occur during production, storage, transportation, or industrial use and by leaching, runoff, and volatilization from landfill sites [10-11]. Some pesticides and the active principles of drugs can enter the food chain, and their metabolites are discharged into the surface waters through wastewater treatment plants. Some of these organic micro-pollutants (e.g., pesticides, polycyclic aromatic hydrocarbon, and chlorinated paraffin) are classified as toxic substances for aquatic invertebrates, persistent organic pollutants (POPs), and bio-accumulative [12-14]; they tend to accumulate in environmental compartments as recalcitrant molecules [15]. For this reason, the use of water for drinking water, agriculture, and livestock exposes humans to the risk of bioaccumulation of plant protection products through the food chain. Furthermore, being liposoluble, they tend to accumulate in animal tissues and fluids, giving a biomagnification effect along the trophic chain [16-18]. In this regard, there is a need to monitor the aquatic environment to establish and eventually recover the quality status defined within the 2000/60/EC Framework Directive. This Directive profoundly modified the legislative framework to reduce the introduction of dangerous substances in water, extending up to the elimination of

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priority hazardous substances [19]. The 2013/39/EU Directive increased the list of priority substances to be monitored in water by adding twelve new pollutants to the already identified thirty-three and also modified the concentration levels for the substances already on the list. Some of these contaminants have legal detection limits in the order of µg/L (group A and B), while others are in the order of ng/L and pg/L (group C). In this regard, many extremely sensitive analytical approaches have been developed to identify and quantify specific and individual classes of contaminants in surface waters (e.g., [20]). Focusing on the pre-analytical phase, a liquid-phase microextraction and a solid-phase extraction have been performed to achieve the following: i) perform the clean-up and/or extraction process in order to limit the presence of interferers; ii) increase the concentration of group A and B ten thousand times and group C 40,000 times; iii) allow the analysis of all the compounds after the entire sample preparation procedure. This effective pre-analytical phase allowed for the development and validation of a multi-residual, high-throughput, and very sensitive GC-MS/MS analytical method that simultaneously identified and quantified numerous priority substances represented by pesticides, chloroalkane hydrocarbons, and polycyclic aromatic hydrocarbons (PAHs).

2. Materials and methods

2.1. Chemicals and standard solutions

All chemicals used in this study were of the highest analytical purity grade. Acetone, 35% hydrochloric acid, anhydrous sodium sulfate, toluene, ethyl acetate, and acetonitrile were obtained from Sigma-Aldrich (Milan, Italy). Standard reference material PCB 101 C13 and PCB 138 C13, heptachlor and heptachlor epoxide C13, PAHs deuterated were obtained from Cambridge Isotope Laboratories (Tewksbury, Massachusetts, US); The Dicofol D8, standard reference material PCB 209, 6 methylchrysene and Chloroparaffin C10-C13 63%, and CAS 85535-84-8 were purchased from Dr Ehrenstorfer (Sesto San Giovanni, Milan, Italy); (supporting information 1). The Perfluorotributylamine (PFTBA) was supplied by Agilent (Santa Clara, California, US). The helium 5.5 and nitrogen 5.5 were supplied by SOL S.p.A. (Monza, Italy). The purified water was obtained from a Milli-Q water system purchased from Millipore (Burlington, Massachusetts, US). In order to avoid any cross-contamination, all the glassware was first washed in hot soapy water and rinsed with deionized (DI) water. Thereafter, the glassware was rinsed with acetone.

2.2. Sample preparation

One L of water was added to 2 ml of concentrated hydrochloric acid (to stabilize pentachlorophenol) and was spiked with the following surrogate standards: i) 32 µL of PCB 101 C13 and PCB 138 C13 both in acetone, 80 ppb as the final concentration; ii) 10 µL of heptachlor and heptachlor epoxide C13 in acetone, 10 ppb as final concentration; iii) 20 µL of PAHs deuterated in acetone, 50 ppb as the final concentration; iv) 10 µL of Dicofol D8 in acetone, 500 ppb as final concentration. Amendola et al. used the same extraction method for tributyltin analysis in water by GC-MS/MS [21]. The remaining 1 L of water was added to the mixture, and the sample of water was extracted with 7 mL of toluene for 1 hour under magnetic stirring conditions. After the extraction, the sample was left for approximately 15 minutes, allowing for the stratification of the extract. The extract had the appearance of an emulsion, and, to this purpose, it was completely recovered with a Pasteur pipette together with 2-3 ml of water and transferred in a centrifuge tube where three teaspoons of anhydrous sodium sulphate (approximately 3 g) were added to break the emulsion. After centrifugation with a Speed Master 14R centrifuge (Euroclone, Milan, Italy) at 5500 rpm for 10 minutes, the extract was recovered and transferred into a vial; in order to avoid the transfer of water droplets, 2 Pasteur of toluene, for rinsing purposes, were added. A couple of spatulas of anhydrous sodium sulphate were added to eliminate the residues (micro drops) of water contamination (this step is mandatory in case of water contamination of the extract). The extract was recovered and transferred to a second vial where the sodium sulphate was washed with toluene using a Pasteur to guarantee a quantitative recovery. The extract was then concentrated to 100 µL by using a slight nitrogen flow into a TurboVap-XcelVap instrument (Horizon Technology Group, Italy); the temperature was set at 40°C and the pressure from 3 to 10 psi. Sixty µL of toluene, 30 µL of PCB 209 (150 ppb as the final concentration), and 10 µL of 6-Methylchrysene (50 ppb as the final concentration) as internal standards were added before the GC-MS/MS analysis (obtaining a final volume of 200 µL) to monitor group A and B for priority substances (Table 1). To monitor the priority substances of group C in Table 1, the residual extract, after injection, was purified; this procedure is described in the following section.

2.3. Extract purification to analyze the group C substances

The purification of the extract was based on the "QuEChERS" (Quick, Easy, Cheap, Effective, Rugged, and Safe) approach using the Solid Phase Extraction (SPE) technique. 185 µL of the residual extract were loaded on the cartridges (5 µL were injected into GC-MS/MS to analyze the group A substances and 10 µL into the group B substances as described below), which consisted of a double phase of black coal/PSA (6 ml of Supelclean EnviCarb-II/SAX/PSA cartridge with 500 mg of ENVI-Carb and 500 mg of PSA) that were previously washed with 2 mL toluene and a 3 mL of a mixture of ethyl acetate-acetonitrile 1:2. After the capture phase, the analytes were eluted into a vial using 5 ml of ethyl acetate-acetonitrile 1:2. The purified extract was concentrated to 10 µL using once again the TurboVap-XcelVap instrument; the temperature was set at 40°C and

the pressure from 3 to 10 psi. Finally, pure toluene was added to reach a 50 µL as the final volume. This purification procedure allowed us to concentrate the analytes 40,000 times compared to the initial water sample. The syringe standards previously added to analyze the group A substances were used in this stage (considering that they are now four times more concentrated).

Table 1. Priority Substances. *Groups A and B* represents the analytes with the required limit of quantification (LOQ) of µg/L or ng/L; *group C* comprises those contaminants with legal detection limits in the order of ng/L or pg/L.

Group A
4- Nonylphenol, Aclonifen, Alaclor, Aldrin, Bifenox, Cybutryne, Chlorpyrifos, Dicofol, Dieldrin, Endosulfan, Endrin, hexachlorobenzene, hexachlorobutadiene, Hexachlorocyclohexane, Isodrin, octylphenol 4, pentachlorobenzene, Pentachlorophenol, ppDDT, terbutryn, Trifluralin, ppDDE, opDDD, ppDDD+opDDT, PBDE28, PBDE47, PBDE100, PBDE99, PBDE154, PBDE153, Dichlorvos
Group B
Chloroalkanes C10-C13
Group C
Heptachlor epoxide, Heptachlor, Hexabromocyclododecane, Cypermethrin

2.4. Gas chromatography MS/MS Analysis of A and C groups substances

This experimental work was performed on an Agilent 7890A GC coupled to an Agilent G7010 Triple Quadrupole GC/MS with an electron ionization (EI) source. The GC system was equipped with an Electronic Pneumatics Control (EPC), a Multimode Inlet (MMI) and a programmable temperature vaporizing (PTV) inlet. The Zebron ZB-XLB capillary column (60 m × 0.25 mm i.d. ×0.25 µm film thickness, Phenomenex) was used to provide the analyte separation and a highly inert flow path into the detector. Five µL of the extract sample before the purification step and 50 µL of the purified extract sample were injected. Agilent MassHunter software was used for instrument control and qualitative and quantitative data analysis. Helium was used as the carrier gas at a constant flow rate of 1.5 ml/min. The GC-MS/MS analysis of both the extract sample before the purification step and the purified extract sample were performed by using the instrument parameters described in Table 2. The MMI and oven temperature to perform the GC separation were programmed as described in Table 2. Finally, the MS/MS acquisition was performed using the parameters described in Table 3a. The mass spectrometer was calibrated on a monthly basis with PFTBA. The optimized MS/MS acquisition was carried out with the fragments reported in Table 3b. One multiple reaction monitoring (MRM) transition was selected for the quantitation and qualification for each analyte, and the collision energy was optimized.

The qualitative analysis was performed by comparing the retention times of the analytes in the sample with those in the standards and the presence of characteristic fragmentations with a ratio of their abundance that was compared with that present in the standard. The resolution of the peaks was set to > 0.5, and the symmetry index of the latter between 0.66 and 1.50. The quantitative analysis is based on the abundance of the primary characteristic fragmentation of each analyte (peak area) compared to the relative response of the primary characteristic fragmentation of the internal standard (peak area). In the same manner, the percentage of surrogate standards is quantified in order to assess the effectiveness of the extraction process. This percentage must be between 50% and 150%. It is critical to review the data in a matrix before setting up a quantitation method.

2.5. Gas chromatography MS/MS analysis of B group substances

The method was developed using an Agilent triple quadrupole GC/MS-MS system 7890A/G7010. The chromatographic separation was performed by a Zebron ZB-XLB capillary column (60 m X 0.25 mm i.d. X 0.25 µm film thickness, Phenomenex). Helium was used as the carrier gas at a constant flow rate of 2.25 mL/min. The injection volume was 10 µL. The injector was programmed in solvent by vent mode with the following parameters: heat temperature of 70 °C to 250°C; pressure 17.319 psi; total flow 54 mL/min; septum purge flow 3 mL/min; purge flow to split vent 100 mL/min at 9 min; vent flow 300 mL/min; and vent pressure 0 psi until 0.4 min. The GC oven temperature was programmed as follows: initial temperature 55°C held for 4 minutes; 25 °C/min up to 150 °C and held for 0 min.; 6 °C/min up to 240 °C and held for 15 minutes; 2 °C/min up to 340 °C and held for 1 minute. The total GC analysis time was 88 minutes (Table 2). A quicker method to perform an analyte screening was also developed. It has a running time of 73 minutes, and the program for the GC oven provides an initial temperature of 55°C held for 4 minutes; 25°C/min up to 150°C and held for 0 min.; 15°C/min up to 240°C and held for 5 minutes; 10 °C/min up to 340°C and held for 10 minutes (Table 2). The first method, being a longer process, allows for discriminating Chloroalkanes C10-C13 in the presence of interfering compounds, such as medium-chain chlorinated paraffins (MCCPs). The mass spectrometer was operated in electron ionization mode (EI, 70 eV) and MS/MS mode (MRM). For the MS/MS experiments, nitrogen 5.5 was used as the collision gas, and the collision cell flow was set at 1.5 mL/min. The temperature of the transfer line and ion source was set at 310 and 250 °C, respectively. The transition 136-65 (20eV collision energy) was selected because it is more specific in respect to the general transition 102-67, as it allows for the isolating of Chloroalkanes C10-C13 and limits the interferences. All chlorinated paraffins have shown a high sensitivity to the general transition 102-67 [22-23], but the transition 136-65 is more selective for short chained chlorinated paraffins;

therefore, it allows them to be separated from other interfering substances (supporting information 2). The Electro-multiplier was increased by 600V. The optimized MS/MS acquisition was carried out with the transitions reported in Table 2; one MRM transition was selected for the quantitation and qualification, and the collision energy was optimized.

Table 2. Multimode Inlet temperature program for GC analysis (Panel A) and oven temperature program for the GC analysis (Panel B). The injection mode is solvent vent.

Panel A

Group A Injection volume 5 µl	**Rate (°C/min)**	**Value (°C)**	**Hold Time (min)**	**Run Time (min)**
Initial		70	0.6	0.6
Ramp 1	900	300	9	9.8556
Ramp 2	900	90	0	75.434
Group B screening method Injection volume 10 µl	**Rate (°C/min)**	**Value (°C)**	**Hold Time (min)**	**Run Time (min)**
Initial		70	0.5	0.5
Ramp 1	900	250	9	9.7
Ramp 2	900	70	0	38.8
Group B completed method Injection volume 10 µl	**Rate (°C/min)**	**Value (°C)**	**Hold Time (min)**	**Run Time (min)**
Initial		70	0.5	0.5
Ramp 1	900	250	9	9.7
Ramp 2	900	70	0	88.8
Group C Injection volume 50 µl	**Rate (°C/min)**	**Value (°C)**	**Hold Time (min)**	**Run Time (min)**
Initial		75	2.1	2.1
Ramp 1	900	300	30	32.35
Ramp 2	900	75	0	54.864

Panel b

Group A	**Rate (°C/min)**	**Value (°C)**	**Hold Time (min)**	**Run Time (min)**
Initial		70	0.6	0.6
Ramp 1	900	300	9	9.8556
Ramp 2	900	90	0	75.434
Group B screening method	**Rate (°C/min)**	**Value (°C)**	**Hold Time (min)**	**Run Time (min)**
Initial		55	4	4
Ramp 1	25	150	0	7.8
Ramp 2	15	240	5	18.8
Ramp 3	10	340	10	38.8
Group B completed method	**Rate (°C/min)**	**Value (°C)**	**Hold Time (min)**	**Run Time (min)**
Initial		55	4	4
Ramp 1	25	150	0	7.8
Ramp 2	6	240	15	37.8
Ramp 3	2	340	1	88.8
Group C	**Rate (°C/min)**	**Value (°C)**	**Hold Time (min)**	**Run Time (min)**
Initial		75	0.1	0.1
Ramp 1	80	90	4.8	5.08571
Ramp 2	40	155	1	7.7125
Ramp 3	9	240	10.5	27.657
Ramp 4	2.8	255	10	43.014
Ramp 5	100	280	6	49.264
Ramp 6	100	340	5	54.864

Table 3. MS conditions used for all the samples (Panel A) and Retention time, precursor/product ions, and collision energy of investigated analytes and injection standard (Panel B).

Panel A

MS source	Source temp. (°C)	Quadrupole temp. (°C)	Transfer line temp. (°C)	Solv delay (min)	He gas (ml/min)	N_2 collision gas (ml/min)	Acquisition mode	MS1/MS2 resolution	Time segments	Acquisition parameters
EI, 70 eV	270	150	310	8	2.25	1.5	MRM mode	Wide	Refer to Table 6	Refer to Table 6

Panel B

Analyte	Retention Time	Precursor Ion Q1 (m/z)	Fragment Ion Q3 (m/z)	Collision Energy (eV)
4- Nonylphenol	19.4	220.0	107.0	10
Aclonifen	29.4	212.0	182.0	10
Alaclor	19.5	188.0	160.0	10
Aldrin	21.0	263.0	193.0	35
Antracene	18.5	178.0	178.0	10
Benzo(a)pirene	49.4	252.1	252.1	10
Benzo(b)fluorantene	47.2	252.1	252.1	10
Benzo(K)fluorantene	47.3	252.1	252.1	10
Benzo(ghi)perilene	60.5	276.1	276.1	10
Bifenox	36.8	189.0	126.0	20
Cybutryne	22.5	182.0	109.0	10
Chlorpyrifos	20.7	199.0	171.0	15
Dicofol	32.94	251	139	20
Dieldrin	26.3	263	193	30
Endosulfan	α:24.8 β:29.8	α:195.0 β:241.0	159.0 206.0	5 30
Endrin	27.8	263.0	193.0	35
Fluorantene	23.8	202	202	10
hexachlorobenzene	17.52	284.0	249.0	15
hexachlorobutadiene	10.6	227.0	190.0	15
Hexachlorocyclohexane	α17.3; β18.9; γ18.2; ʎ19.85	217.0	181.0	5
Indeno(123, cd)pirene	58.5	276.1	276.1	10
Isodrin	22.3	193.0	123.0	30
octylphenol 4	17.5	206.0	107.0	10
pentachlorobenzene	15.3	250.0	215.0	20
Pentachlorophenol	18.1	266.0	167.0	25
ppDDT	29.2	235.0	165.0	20
terbutryn	20.1	185.0	170.1	5
Trifluralin	16.0	306.0	264.0	5
ppDDE	25.6	264.0	176.0	30
opDDD	26.2	235.0	165.0	30
ppDDD+opDDT	28.2	235.0	165.0	20
PBDE28	28.6	408.0	248.0	20
PBDE47	37.4	326.0	217.0	30
PBDE100	43.6	565.6	405.6	20
PBDE99	44.5	565.6	405.6	20
PBDE154	56.4	643.6	483.6	20
PBDE153	56.9	643.6	483.6	20
PCB 209	49.75	498.0	428.0	30
dichlorvos	11.05	187	93	10
heptachlor epoxide	22.70	353	263	15
heptachlor	19.82	272	237	15
hexabromocyclododecane	α26.45; β26.6; γ26.85	239	131	20
cypermethrin	I=48; II=48.6; III=48.9 IV=49.15	163	127	5
PCB 138	31.35	369.9	299.9	28
PCB 101	23.9	335.9	266	28
Chloroalkanes C10-C13	20-32	136	65	20

2.6. Data analysis

Agilent Mass Hunter Quantitative Analysis software was used to calculate the concentration of the single compound in the water sample. The angular coefficient and the intercept of the straight line were obtained from the linear regression of the calibration standards, by which the unknown analyte concentrations were calculated with respect to the area of the normalized peak for the area of the syringe standard in that sample (relative answer). In the case of the presence of an analyte at a concentration higher than that present in the standard, in the calibration line, the extract was appropriately diluted with the working solution of the internal standard.

2.7. Method validation

According to the European legislation UNI CEI EN ISO/IEC 17025:2018, the validation of the analytical method was mandatory because the entire workflow was designed and developed in our laboratory. To validate the method for the analysis of Groups A and C, we first generated the calibration curves by loading all analytes at known concentrations in the GC column. The first point of the curve is represented by the LOQ of each analyte, the second point is two times the LOQ, and the third point is five times the LOQ. Normally, the LOQ of all the analytes has been calculated as 30% of the maximum limit allowed by current legislation, which corresponds to the minimum required performance. The purpose of this phase was to verify the linearity of the instrumental response on all contaminants being analyzed in the application range. Confirmation was given by the values of the correlation coefficient R^2, which must be greater than 0.98 for all the analytes. In order to extract the sample before the purification step, the curve was generated by contaminating the Milli-Q water with the standards of the examined analytes for three equally distributed concentration levels in the range of the analysis range and prepared as described above; as shown in Table 4a, the concentration range varies for each substance. Concerning the group C analytes, due to the required ultra-trace concentration, the calibration curve was generated by also considering a fourth point represented by ten times the LOQ of each tested analyte. The concentration range was performed as described in Table 4b.

For each of the two extraction phases, six blanks (in matrix for the extract sample before the purification step and in solvent for the purified extract sample) and six samples spiked at the level of the LOQ for each analyte were prepared; the blank preparation is identical to the sample with the exception of the analytes. The calculated concentration of the samples was corrected with the blank value injected in the same analytical session. By means of the calibration curves, blanks, and recoveries, it was possible to estimate the limit of quantification and the measurement uncertainty for each analyte using the calculation sheets supplied by our laboratory. These calculation sheets are based on the statistical metrological method (refers to UNI CEI EN ISO/IEC 17025:2018), which provides for assessing all the contributions that give support to the measurement value of the uncertainty. It is an Excel document (validated by our agency's quality system) that includes two datasheets for each analyte. In the first sheet, field 1 shows the relationship between the analyte peak area and the peak area of the internal standard. The relationships between the two areas are shown for the different levels of the standards, so the system creates the calibration line starting from the data that is entered. In field 2, the uncertainty characteristics, due to the nominal concentration of the reference material, were inserted. In field 3, the uncertainties due to the use of flasks and pipettes for the dilution of the initial standard solution have been reported. In field 4, the results of six white matrix replicas were inserted (the ratios between the peak area of the blank and the peak area of the internal standard were evaluated), which have been used for the limit of detection (LOD) and LOQ calculation.

Table 4. Three concentration levels for each analyte of group A (Panel A) and four concentration levels for each analyte of group C (Panel B).

Panel A

Analyte	LOQ (ppb)	2LOQ (ppb)	5LOQ (ppb)
hexachlorobutadiene	60	120	300
pentachlorobenzene	60	120	300
Diclorvos	2	4	10
hexachlorobenzene	10	20	50
PCF	1000	2000	5000
trifluralin	60	120	300
Nonylphenol	150	300	750
Hexachlorocyclohexane	20	40	100
Octylphenol	60	120	300
Alaclor	150	300	750
PAHs (anthracene, fluoranthene, benzo b and benzo k fluoranthene, Benzo[a]pyrene and Benzo[ghi] perylene)	2	4	10
Cyclodiene pesticides	10	20	50
Cybutryne	10	20	50
Summation DDT	10	20	50
endosulfan	20	40	100
Chlorpyrifos	60	120	300
Summation PBDE	200	400	1000
Aclonifen	20	40	100
pp DDT	10	20	50
Bifenox	10	20	50
EDHP	150	300	750
Dicofol	2	4	10
terbutryn	60	120	300

Panel B

Analyte	LOQ (ppb)	2LOQ (ppb)	5LOQ (ppb)	10LOQ (ppb)
heptachlor + heptachlor epoxide	0,04	0,08	0,2	0.4
Cypermethrin	0,25	0,50	1,25	2.5
hexabromocyclododecane	6,66	13,32	33,3	6.6

The recovery, the uncertainty, the LOD, and the LOQ are calculated during the validation phase through the spreadsheet described above; the analyte concentration in the sample is calculated by Agilent software using the following formula:

C extract = (a x Rr - b) x C int standard

where C extract= analyte concentration (µg/L) in the sample; a= angular coefficient of calibration line; Rr= extracted relative response: ratio between the area of the analyte peak and the internal standard peak; b= intercept of the calibration line; and C int standard= internal standard concentration (µg/L); C sample = C extract/10000. Moreover, the results of six recoveries at the lowest point of the curve have been entered (the ratios between the sample area and the internal standard peak area have been calculated) in order to evaluate the repeatability. Concerning the second datasheet, the worksheet generated the values of LOD, LOQ, and uncertainty measurement for each analyte. The latter was calculated by summing the various contributions due to the calibration line, the purity of the standard, the dilutions of the working solutions, the uncertainty on the blanks, and the replicates of the control standard. For the validation of the method for the analysis of Chloroalkanes C10-C13, two linear calibration curves were calculated by three calibration points in the extract: 5 ppm, 10 ppm, and 20 ppm. PCB 209 was employed as an injection standard to eliminate instrumental imprecision as described above. Six blanks and six spiked samples were

analyzed to evaluate the recovery of the developed methods. The measured signal was corrected by subtracting the blank signal to eliminate all factors that independently influence the analyte concentration. The linear range, recovery, and both LOD and LOQ limits were evaluated. The collected data were statistically processed. The LOD was estimated as three times the standard deviations at the lowest (injected) spiked concentrations of Chloroalkanes C10-C13; the LOQ was estimated as ten times the standard deviations at its lowest (injected) spiked concentrations of the Chloroalkanes C10-C13.

3. Results and discussion

The aim of this study was to develop and validate a method based on capillary gas chromatography-triple quadrupole mass spectrometry to monitor priority substances in inland surface waters and applicable in the analysis of the marine samples. The challenge was to develop a method that did not require a derivatization step, able to identify and quantify these priority substances at ultra-trace levels. The analyzed substances have chemical-physical characteristics that make them suitable for gas chromatography analysis since they are semi-volatile and analogous to an organic phase that allows the method to decrease to very low concentrations. Using the Solvent Vent technique, the entire extracted sample from group C was injected into the GC-MS/MS system, increasing by 40,000 times the concentration of dichlorvos, heptachlor epoxide, heptachlor, hexabromocyclododecane, and cypermethrin simultaneously.

3.1. Optimization of MS/MS parameters

In order to detect the priority substances by using MRM mode, full-scan and product spectra were acquired to define the retention time, precursor/product ions (m/z), and collision energy of the injected standard of those investigated analytes not present in the Agilent G9250AA-Database-V1.1-Mp2 (Table 5). As shown in Table 3a, the dissociation conditions have been optimized for each compound (not present in the database) with regard to the MRM transitions and fragmentation energy; the precursor ions of each analyte were selected as a compromise between selectivity (m/z) and sensitivity (intensity). In regard to the Chloroalkanes C10-C13, they were found as a mixture of 63% chlorination (the percentage of chlorination required by the 2000/60/CE) [24, 25], but the problem was overcome with the optimal chromatographic conditions as described above. Various tests were carried out to improve the instrumental sensitivity. The temperatures of the source and the injector were changed until the optimal condition was identified. Therefore, a temperature of 250°C was applied to the source and the injector. In this manner, it was possible to discriminate the chlorinated paraffin based on the length of the carbon chain and the amount of chlorine. The van Deemter law was sacrificed to shorten the chromatography times; the peaks maintained good resolution throughout the analysis.

Table 5. Compounds for which the most abundant fragmentations were taken from the Agilent G9250AA-Database-V1.1-Mp2 and compounds whose most abundant fragmentations were determined by analyzing the compound in Product Ion Scan modes.

Analyte	From agilent G9250AA-database	By product ion scan
Hexachlorobutadiene	✓	
Pentachlorobenzene	✓	
Diclorvos	✓	
Hexachlorobenzene	✓	
PCF	✓	
trifluralin	✓	
Nonylphenol		✓
Hexachlorocyclohexane		✓
Octylphenol		✓
Alaclor	✓	
PAHs (anthracene, fluoranthene, benzo b and benzo k fluoranthene, Benzo[a]pyrene and Benzo[ghi]perylene)		✓
Cyclodiene pesticides	✓	
Cybutryne	✓	
Summation DDT	✓	
Endosulfan	✓	
Chlorpyrifos	✓	
Summation PBDE	✓	
Aclonifen	✓	
pp DDT	✓	
Bifenox	✓	
EDHP	✓	
Dicofol	✓	
Terbutryn	✓	
Heptachlor + Heptachlor		✓
Cypermethrin	✓	
Hexabromocyclododecane		✓
Chloroalkanes C10-C13		✓

3.2. Validation

As described in section 2, the validation process of the analytical method allowed us to estimate the limit of quantification with the relative measurement uncertainty associated with each analyte (pesticides, PAHs, and Chloroalkanes C10-C13); these results are in line with the analytical concentration limits required by the European directive and are described in Table 6.

Table 6. Results obtained from the validation process of the analytical method for the analysis of the groups A and C; the limit of quantification with the relative measurement uncertainty associated to each analyte are reported (Panel A). Summary of the validation data for the screening method (Panel B). Summary of the validation data for the longest method (Panel C).

Panel A

Analyte	Limit of quantification of the extract (µg/l)	Measurement uncertainty (%)
Hexachlorobutadiene	60	44
Pentachlorobenzene	2	44
Diclorvos	2	44
Hexachlorobenzene	6	44
PCF	1200	41
Trifluralin	90	30
Nonylphenol	900	44
Hexachlorocyclohexane	6	38
Octylphenol	30	43
Alaclor	900	41
Cyclodiene pesticides	15	44
Cybutryne	7	44
Summation DDT	75	44
Endosulfan	2	44
Chlorpyrifos	90	44
Summation PBDE	42	44
Aclonifen	36	34
pp DDT	30	38
Bifenox	4	32
EDHP	3900	44
Dicofol	4	44
Terbutryn	19	44
Anthracene	300	44
Fluoranthene	20	44
Benzo b Fluorantene	50	44
Benzo k Fluorantene	50	44
Benzo [a] Pyrene	0.5	44
Benzo ghi Perilene	2	44
Heptachlor + Heptachlor Epoxide	0.01	44
Cypermethrin	0.24	44
Hexabromocyclododecane	2	19

Panel B

Name	Recovery (%)	LOD (ppb)	LOQ (ppb)	Uncertainty (%)	R^2	SQA- MA (ppb)
Chloroalkanes C10-C13	50	0.06	0.12	29	0.9983	0.4

Panel C

Name	Recovery (%)	LOD (ppb)	LOQ (ppb)	Uncertainty (%)	R^2	SQA- MA (ppb)
Chloroalkanes C10-C13	56	0.05	0.06	29	0.9958	0.4

As reported in Tables 6a, 6b, and 6c, the method performance specifications required by the 2013/39/EU Directive have been reached for each analyte (30% of the EQS) except for Heptachlor and Heptachlor Epoxide. For the extract sample before the purification step (µg/L as the concentration level of the analytes), the objective was achieved with positive results in terms of recovery (between 79% and 144%) and uncertainty (between 23.5% and 44%). In the order of the purified extract sample (ng/L and pg/L as concentration levels of the analytes), the following considerations can be made instead: i) a legal limit of 0.08 ng/L is imposed for cypermethrin, and the official EPA method 1699 allows a LOQ of 1 ng/L while our method is able to reach 0.024 ng/L; ii) Dichlorvos has a legal limit of 0.6 ng/L; the official EN 12918 method allows a LOQ of 10 ng/L, and the method described above has a LOQ of 0.18 ng/L; iii) and Heptachlor and heptachlor epoxide are expressed as a sum and has a legal limit of 0.2 pg/L; the LOQ achieved with the proposed method is of 2 pg/L. The lowest LOQ achieved with the official EN ISO 6468 2001 method is 1000 pg/L. The expected concentration of 0.4 ppb (of the Chloroalkanes C10-C13) is measured in accordance to the requirement of the quality detection on the C10-C13 chloroparaffins in water samples [26]. The analysis of the priority substances with the legal limits in the order of ng/L will be improved by making changes in the pre-analytical or analytical phase to increase the robustness of the proposed method. Moreover, regarding Chloroalkanes C10-C13, the coefficients of determination (R2) were 0.9983 for screening the method (Table 6b) and 0.9958 for the longest method (Table 6c).

3.3. Surface water analysis

The validated quantitative method was applied to real samples of surface water (river, lake, sea) from the five provinces of Lazio (Italian region) represented by Rome, Frosinone, Rieti, Latina, and Viterbo (Tables 7 and 8); the monitoring activity was extended over a one year period.
Table 7 (Panels a and b) is very representative of agricultural activity in the Lazio region. Rieti was the province with the least agricultural impact; the lowest number of positive results was found. Chlorpyrifos was only found in the province of Latina, where the most agricultural activity of the Lazio region is concentrated; its high insecticidal capacity makes it particularly suitable for safeguarding the cultivation of the Agro Pontino area. Finally, the established pollution from persistent chlorinated pesticides was verified at Frosinone, where in the past a large industrial center produced them. Out of curiosity, we identified cypermethrin in each province due to its extremely low EQS. Table 8 shows that C10-C13 chloroparaffins were not found in concentrations above the limit of quantification. These results are consistent with the fact that no industries are producing Chloroalkanes C10-C13 in the Lazio region. A comparison between the transitions used for the quantitative analysis of hexachlorocyclohexane in a sample from the Sacco River (province of Frosinone) and a reference standard at the third level of concentration are shown in the supporting information 3.

Table 7a. One year of monitoring of ultra-traces of priority substances of groups A, C in internal surface waters from the three Lazio's provincies represented by Rieti, Frosinone and Rome.

Rieti Analyte	Analyzed Samples	Positive Results obtained	% Positive Result	EQS-AA (µg/l)[a)]	Results out of limit
Cypermethrin	4	1	25	$8x10^{-5}$	1
EDHP	17	1	6	1.3	0
Frosinone Analyte	**Analyzed Samples**	**Positive Results obtained**	**% Positive Results**	**EQS-AA (µg/l)[a)]**	**Results out of limit**
Benzo (a) pyrene	55	5	9	0.00017	2
cypermethrin	17	3	18	$8x10^{-5}$	0
dicofol	55	1	2	$1,3x10^{-3}$	1
EDHP	55	1	2	1,3	0
Heptachlor+heptachlor epoxy	17	3	18	$2x10^{-7}$	3
Hexachlorocyclohexane	55	25	45	0.02	5
Rome Analyte	**Analyzed Samples**	**Positive Results obtained**	**% Positive Results**	**EQS-AA (µg/l)[a)]**	**Results out of limit**
Benzo (a) pyrene	68	8	12	0.00017	0
cypermethrin	21	9	43	$8x10^{-5}$	9
dicofol	68	1	1.5	$1,3x10^{-3}$	1
EDHP	68	1	1.5	1,3	0
Heptachlor+heptachlor epoxy	21	1	5	$2x10^{-7}$	1
Hexachlorocyclohexane	68	10	15	0.02	2

Table 7b. One year of monitoring of ultra-traces of priority substances of groups A, C in internal surface waters from the two Lazio's provincies represented by Viterbo and Latina.

Viterbo Analyte	Analyzed Samples	Positive Results obtained	% Positive Results	EQS-AA (µg/l)[a)]	Results out of limit
Benzo (a) pyrene	73	6	8	0.00017	0
cypermethrin	26	3	12	$8x10^{-5}$	3
Heptachlor+heptachlor epoxy	26	1	4	$2x10^{-7}$	1
Latina Analyte	**Analyzed Samples**	**Positive Results obtaine**	**% Positive Results**	**EQS-AA (µg/l)[a)]**	**Results out of limit**
Benzo (a) pyrene	91	7	8	0.00017	3
cypermethrin	25	11	44	$8x10^{-5}$	6
Chlorpyrifos	91	13	14.3	0.03	3
dicofol	91	13	14.3	$1,3x10^{-3}$	13
EDHP	91	2	2.2	1,3	0
Heptachlor+heptachlor epoxy	25	1	4	$2x10^{-7}$	1
Hexachlorocyclohexane	91	12	13.2	0.02	2

a) Environmental Quality Standards expressed as an Annual Average value (EQS-AA).

Table 8. One year of monitoring of ultra-traces of priority substances of group B in internal surface waters coming from the four provinces of the Lazio.

Rieti Analyte	Analyzed Samples	Positive Results obtained	EQS-AA (µg/l)[b)]	Results out of limit
Chloroalkanes C10-C13	3	0	0.4	0
Frosinone Analyte	**Analyzed Samples**	**Positive Results obtained**	**EQS-AA (µg/l)[b)]**	**Results out of limit**
Chloroalkanes C10-C13	3	0	0.4	0
Rome Analyte	**Analyzed Samples**	**Positive Results obtained**	**EQS-AA (µg/l)[b)]**	**Results out of limit**
Chloroalkanes C10-C13	3	0	0.4	0
Viterbo Analyte	**Analyzed Samples**	**Positive Results obtained**	**EQS-AA (µg/l)[b)]**	**Results out of limit**
Chloroalkanes C10-C13	3	0	0.4	0
Latina Analyte	**Analyzed Samples**	**Positive Results obtained**	**EQS-AA (µg/l)[b)]**	**Results out of limit**
Chloroalkanes C10-C13	3	0	0.4	0
Rieti Analyte	**Analyzed Samples**	**Positive Results obtained**	**EQS-AA (µg/l)[b)]**	**Results out of limit**
Chloroalkanes C10-C13	3	0	0.4	0
Frosinone Analyte	**Analyzed Samples**	**Positive Results obtained**	**EQS-AA (µg/l)[b)]**	**Results out of limit**
Chloroalkanes C10-C13	3	0	0.4	0
Rome Analyte	**Analyzed Samples**	**Positive Results obtained**	**EQS-AA (µg/l)[b)]**	**Results out of limit**
Chloroalkanes C10-C13	3	0	0.4	0
Viterbo Analyte	**Analyzed Samples**	**Positive Results obtained**	**EQS-AA (µg/l)[b)]**	**Results out of limit**
Chloroalkanes C10-C13	3	0	0.4	0
Latina Analyte	**Analyzed Samples**	**Positive Results obtained**	**EQS-AA (µg/l)[b)]**	**Results out of limit**
Chloroalkanes C10-C13	3	0	0.4	0

b) Environmental Quality Standards expressed as an Annual Average value (EQS-AA).

4. Conclusions

To monitor different contaminants, analytical laboratories must apply multiple methods without even reaching the required LOQ, consuming a lot of time and solvents. In this regard, this experimental work aimed to develop an analytical method to simultaneously monitor different priority substances represented by pesticides, chloroalkane hydrocarbons, and PAHs in inland surface waters present in concentrations of the order of µg/L, ng/L, and pg/L, in accordance with the legal limits reported in the European Directive 2013/39/EU. It was made possible thanks to two factors: the development of a sample preparation (*in the pre-analytical phase*) based on a liquid phase microextraction followed by a purification process with carbon cartridges/PSA and the use of a very effective, robust, and sensitive gas chromatography coupled to the tandem mass spectrometry technique in the analytical phase. The method is extremely sensitive since it does not use large quantities of solvents and is suitable for routine analysis for monitoring real samples. Among future perspectives, there will be the possibility of including other analytes whose toxicity evaluation is in progress in this method. These will probably be inserted in the future in the tables of the law.

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