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RESEARCH ARTICLE

DEVELOPPEMENT AND VALIDATION OF A QUECHERS EXTRACTION BASED GAZ CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY ``GC-MS/MS`` METHOD FOR THE DETERMINATION OF 7 POLYCHLORINATED BIPHENYLS RESIDUES IN BIVALVES "OYTERS".

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Abstract

Polychlorinated biphenyls have been determined in bivalve molluscs collected from OUALIDIA region in Morocco. It is a popular seaside resort of Moroccans, it is located 70 km south of El Jadida in the direction of Safi. Her Celebrity is due to its lagoon and oyster parks in September 2016.

High-performance analytical techniques are required for the identification and quantification of these persistent organic pollutants. The objective of this study is to develop a multi-residual analysis method for polychlorinated biphenyls in bivalves by gas chromatography coupled with quadrupole triple mass spectrometry. A global protocol comprising a QuECHERS version of the extraction step followed by a step of purifying the extract obtained on a mixture of the adsorbents was carried out. The developed method allows the quantification of 7 polychlorinated biphenyls in "Oyster" bivalves. Linearity ($R \geq 0.995$), specificity and selectivity, having an average efficiency greater than 70%, repeatability, reproducibility, accuracy, are checked. As a result, the potential of the developed method has been demonstrated.

Introduction:-

Polychlorinated biphenyls, more commonly known as PCBs, are the synthetic organic substances, considered as priority chemical pollutants in both the United States and Europe, based on the lists established by the United States Environmental Protection Agency (EPA) and the Commission of the European Communities (Official Gazette of 14 n / 82) [1,2]. They are synthetic chemicals and their commercial production began in 1929. These products are non-flammable, inert to acids, bases and other corrosive chemicals and require a temperature above 1000 ° C to allow their degradation complete.

Environmental contamination, both terrestrial and marine, cases of human poisoning and of animals caused by ingestion of foods accidentally contaminated with PCBs have motivated, over the last 30 years, the study of behavior and toxic effects of these compounds in the environment [2,4,8].

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PCBs that are widely distributed in the environment are classified as persistent organic pollutants (POPs), such as dioxins and polycyclic aromatic hydrocarbons (PAHs). There are two types of PCBs according to their mechanism of action: PCBs known as "Dioxin-Like" or PCB-DL. Their mechanism of action on cells is similar to that of dioxins: it binds to the same cell receptor and their toxicity is expressed as toxic equivalent factor in relation to the toxicity of TCDD (2,3,7,8- Tetrachloro-Dibenzo para-Dioxin) more commonly known as Seveso dioxin while PCBs called "Non Dioxin-Like" or PCB-NDL. They are found in larger quantities in river fish than Dioxin-Like PCBs. Of all PCBs, seven molecules are particularly found in contaminated products. They constitute indicator PCBs whose dosage is used to quantify the contamination of the products.[3,6,9]

Depending on the nature of the studied material, PCB analysis is carried out according to a more or less complex protocol comprising several steps: extraction of residues, purification of the extract, possible separation into groups of compounds, instrumental analysis by coupled gas chromatography triple quadrupole mass spectrometry and ultimately chemical confnmation.[4,5,10]

A variety of GC and HPLC methods have been developed for multi-residue determination of organochlorine pesticides employing a variety of sample preparation and cleanup techniques. In recent years, due to its rapidness, easy to use, effectiveness, reliability and safety, the QuEChERS method, as a new extraction method, has become a new sample pretreatment technology that is extensively adopted at home and abroad and is also widely applied in the gas or liquid chromatographic analysis for various pesticides.[1] [7][9]

In this work, the QuEChERS-GC-MS-MS method is used and validated to quickly determine the polychlorinated biphenyls residues : 2,4,4'-trichlorobiphenyl, 2,4',(-tetrachlorobiphenyl, 2,2',4,5,5'-Pentachlorobiphenyl, 2,3',3,4,4',5-Pentachlorobiphenyl, 2,2',3,4,4',5'-Hexachlorobiphenyl, 2,2',4,4',5,5'-Hexachlorobiphenyl, 2,2',3,4,4',5,5'-Hexachlorobiphenyl) in Moroccan bivalves "OYTERS".

Materials and Methods:-

Equipment:-

GC-MS/MS system Agilent Triple Quadrupole Mass Spectrometry 7890Awith Sensor Technologies 7000 MSD were used. Analysis is performed in MRM "Multiple reaction monitoring". The instrument is equipped with injector type Multi mode and an automatic sample changer 7693. The software type is mass hunter allows the acquisition to data processing and reporting. A refrigerated centrifuge SIGMA 4 bucketsand an Ultra-Turrax homogenizer were used for in extraction and purification steps.

The 50ml tubes are used for the deposition of the extract, 2 ml tubes of centrifuge EFF and an analytical balance for weighing the samples.

Analysis by GC-MS/MS:-

The analysis of the molecules is done by MRM "Multiple reaction monitoring". A database of molecules provided by Agilent is composed by:

- Retention time
- Ions Relatives (Precursor ions) (m / z)
- Ions Son (product ions) (m / z)

The conditions for chromatographic analyzes are detailed in Table 1:

Table 1:- GC-MS/MS conditions

Colonne	Agilent 19091S-433HP-5MS 350°C: 30 m*250µm In: front MM inlet He Out: aux EPC 4
Equilibration time	0.5 min
Max temperature	350 °C
Oven programme	70°C for 2min Then 25°C/min to 150°C for 0 min Then 3°C/min to 200°C for 0 min Then 8°C/min to 280°C for 1min
Run time	32.867min 5min post (run time)

He quench gaz N_2collision gaz	2.25 ml/min 1.5 ml/min
Mode	MRM solvent vent
Heater	70°C
Pressure	33.274psi
Temperature programme	70°C for 0.07 min Then 600°C/min to 325°C for 5min
Run time	32.867min

Chemicals:-
Acetonitrile is used as solvent for PCBs residues analysis. Also, the mixture of salts "anhydrous sulphate crystallized magnesium, hydrogen citrate, disodium citrate sesquihydrate and dihydratetrisodium" are used to saturate the aqueous phase and thus to achieve separation with the organic phase. This product is calcined at about 550 ° C in an oven to remove all traces of phthalates, eg. FlukaNo. 63136. Adsorbents bulk of PSA (Primary and Secondary Amine) and adsorbent Suplclean LC-18, which are used for cleanup, have been selected for their physicochemical properties. The Preparation of standard solutions is based on the purity of standard organochlorine pesticides (Table 2).

Table 2:- The purity of PCBs

Organochlorine pesticides	Purity %
PCB28 (2,4,4'-trichlorobiphenyl)	99
PCB52 (2,4',(-tetrachlorobiphenyl)	99
PCB101 (2,2',4,5,5'-Pentachlorobiphenyl)	99
PCB 118 (2,3',3,4,4',5-Pentachlorobiphenyl)	99.5
PCB138 (2,2',3,4,4',5'-Hexachlorobiphenyl)	98.5
PCB 153 (2,2',4,4',5,5'-Hexachlorobiphenyl)	99
PCB180 (2,2',3,4,4',5,5'-Hexachlorobiphenyl)	99.5

Preparation of standard solutions:-
The stock solutions of compounds were prepared individually from compounds of high purity, at 1 mg/mL in acetone. From these solutions, a standard multi-substance mixture was prepared in 10 ppm of each compound in acetone. This mixture was used to determine the characteristics of ions of Q1 and Q3 SIM modes and transitions MRM mode.

In order to correctly assess the quantification limits and establish relevant calibration ranges, the mixture solutions were prepared in acetone depending on the compounds response coefficient. A wide range of concentrations was used. The previously prepared standard solutions are added to extracts of blank samples to compensate for the effect of matrix samples. The amount of extracts of blank samples used is equivalent to the extract of the sample concentration ready for analysis. These analytical standard solutions also contain quantities of internal standard equivalent to that contained in the extract of the sample ready for injection.

Sample preparation Method:-
Sample of oysters harvested from Walidia (Morocco) region, was placed in containers containing seawater and stored at -20 ° C until use. After crushing, ground fibers were stored in a refrigerator (-20 °C).

Extraction Method:-
The procedure for sample preparation is described in Figure 1.Weigh 10g ∓ 0.03g of the ground and homogenized sample, then put it into a centrifuge tube "FEP" 50 ml. Add, with a micropipette in all samples except the "blank samples fortified and White reactive", an amount of internal standard "or surrogate trace the sulfotep". Then, add a volume of 10ml of the acetonitrile extraction solution, on the Farm tubes and shake vigorously for one minute.

Next, add the mixture of previously prepared buffer salts (4g of anhydrous magnesium sulfate, 1 g of NaCl, 0.5g of Disodium hydrogen citrate sesquihytdrate and 1 g of trisodium citrate dehydrate) to the prepared suspension, on Farm tubes, shake vigorously for one minute without delay and ensure that the solvent reacts well with the entire

sample and that agglomerates formed crystals were sufficiently dispersed.Then, centrifuge tubes at 3000rpm for 2 minutes.

Cleanup

An aliquot of the upper organic phase is transferred to a tube containing "150 mg of MgSO4, 50mg PSA and 100mg LC18" per ml of extract. After a mixing and centrifuging step, the purified extract is analyzed by GC-MSMS.

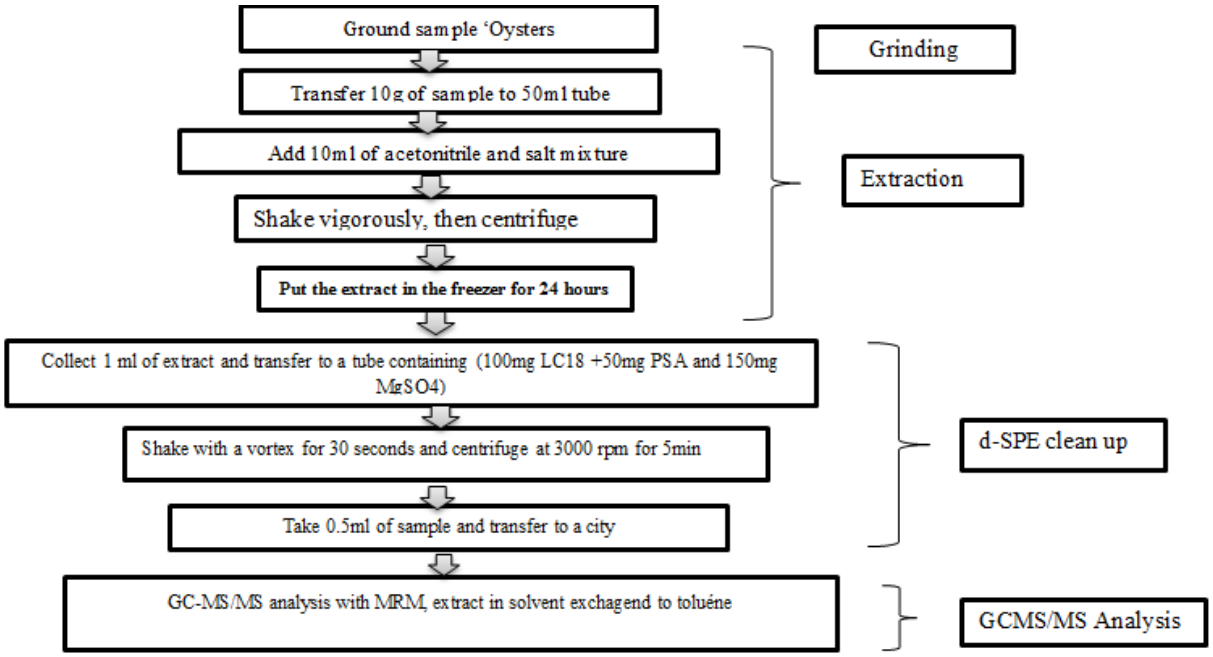


Figure 1:- Organigramme of sample preparation protocol according to QuEChERS method

Chromatographic Analysis:-

The injection of 0.2ppm mixed standard (7 PCBs) gave results presented in Figure 2 and Table 3.

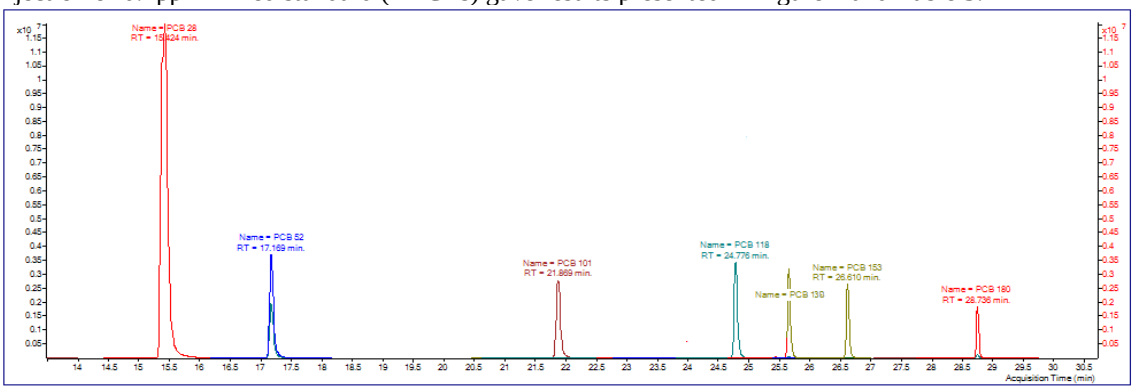


Figure 2:- Chromatogram of standard solution of 7 PCBs (0.2 ppm)

Table 3:- Retention times of standard solution of 7 polychlorinated biphenyls

OrganochlorinePesticides	Retention Time (min)
PCB28 (2,4,4'-trichlorobiphenyl)	15.24
PCB52 (2,4',(-tetrachlorobiphenyl)	17.16
PCB101 (2,2',4,5,5'-Pentachlorobiphenyl)	21.86
PCB 118 (2,3',3,4,4',5-Pentachlorobiphenyl)	24.77
PCB138 (2,2',3,4,4',5'-Hexachlorobiphenyl)	25.68
PCB 153 (2,2',4,4',5,5'-Hexachlorobiphenyl)	26.61
PCB180 (2,2',3,4,4',5,5'-Hexachlorobiphenyl)	28.73

Method Validation:-

Validation study of developed method, in this work, was studied according to NF T90-210 and the guidelines of Analytical Quality Control and Method Validation Procedures for Pesticide Residue Analysis in Food and Feed SANTE/11945/2015.

This validation was performed by the accuracy, precision, linearity and limit of quantification (LOQ).
Accuracy and precision data were obtained with recovery studies by spiking samples with polychlorinated biphenyls standards at levels of 0.2ppm, 0.1ppm,0.05ppm,0.02ppm,0.01ppm. The spiked samples were analyzed in 5 replicates. Precision of the method was evaluated through the relative standard deviations (%RSD) associated with PCBs measurements during recovery.

Linearity was determined by plotting calibration curve with standard solutions in acetonitrile containing five different concentrations (0.01, 0.02, 0.05, 0.1 and 0.2 ppm). Five injections were made at each of the five concentration levels.

The limit of quantification (LOQ) was determined according standard guidelines of Analytical Quality Control and Method Validation Procedures for Pesticide Residue Analysis in Food and Feed SANTE/11945/2015. Five independent analysis of oyster samples are prepared at a pre-assumed LQ value of 0.01ppm and then analyzed under intermediate fidelity conditions (VIM 2.24) « AFNOR 2010 NF V 03-110 ».

Statistical Analysis:-

The data were statically analyzed by using one way ANOVA. All statistical calculations have been done using Excel.

Results:-

Table 4:- Regression Equations and Criteria of Linearity.

PCB	$y = a_0 + a_1 x + a_2 x^2$			Coefficient of Determination (r)	Correlation coefficient (r^2)
	a_0	a_1	a_2		
PCB28	-8.81	6323.3	4277	0.9999	0.99996
PCB52	-3.04	1081.3	607.3	0.9996	0.9998
PCB101	-1.52	878.54	643.1	0.9998	0.9999
PCB118	-2.91	1197.05	510.6	0.9997	0.9999
PCB138	-0.48	707.43	648.8	0.9999	1.0000
PCB153	-1.51	643.44	147.3	0.9999	0.9999
PCB180	-1.17	453.16	161	0.9998	0.9999

The linearity is the ability within a definite range to obtain results directly proportional to the concentration of the analyte. The concentrations of the validation standards were back calculated from the calibration curve. A linear regression model was fitted on the back-calculated concentrations as a function of the introduced concentrations. The intercept, the slope and the coefficient of determination of the equations obtained are presented in table (4). The slopes values close to 1 demonstrate the linearity of the method. The linearity was demonstrated for all PCBs in the range (0.01 to 0.2 ppm) because the coefficient determination are more than 0.995 (r^2).

Table 5:- Suitability test to the calibration mode**l**.

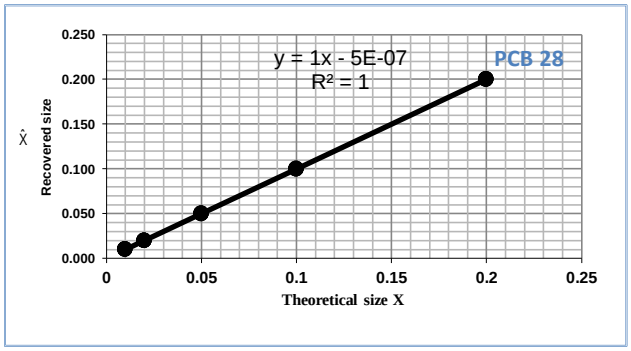
PCB	Interpretation	Observed value	Critical Value * $\alpha = 1\%$	Conclusion
PCB28	Field calibration	2.53	4.10	The calibration domain is validated
PCB53		2.45		
PCB101		1.90		
PCB118		3.89		
PCB138		1.11		
PCB153		3.27		
PCB180		3.45		

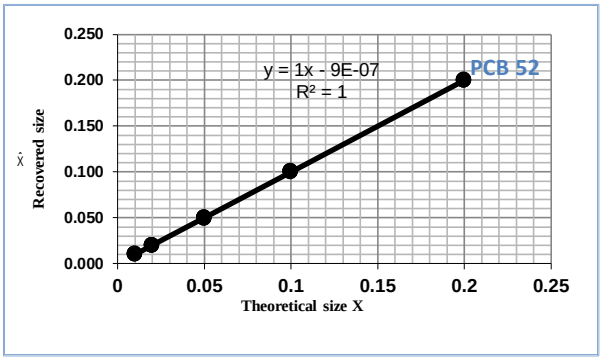
*: The critical value of Fisher with a 1% risk equals F (5, 20, 1%) corresponding to 4.10 (See fractile of Fisher law). « AFNOR 2009, NF T 90-210 »

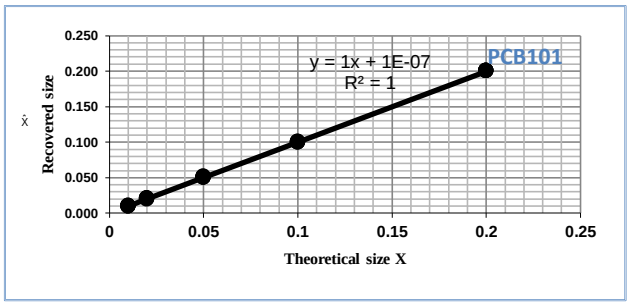
The calibration function was verified by the Fisher test (Table 5). F ratio is less than the critical value of F corresponding to Fisher variable for a risk of 1%. Then, the results show that the linear range is validated and the regression model is acceptable.

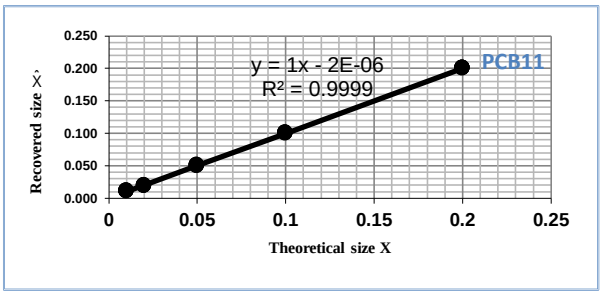
In conclusion, the calibration function is validated on the studied domain with a 1% risk because the error of the model is significantly negligible compared to the experimental error observed and the criterion observed is less than the critical value. Then the calibration function is linear over the analyzed range [0,01; 0.2 ppm]. (Figure 3 and 4)

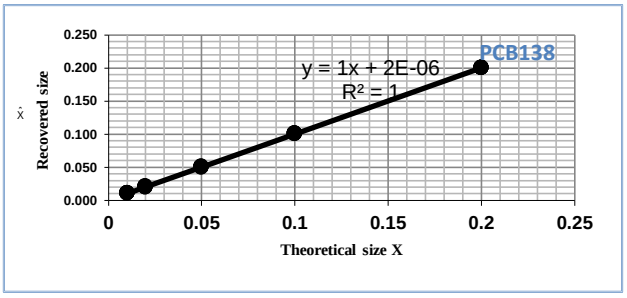
Figure 3:- Linearity graphs for a quadratic form

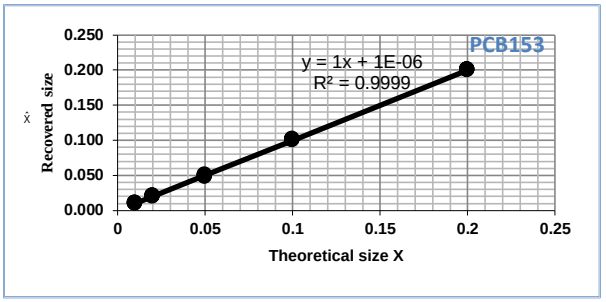












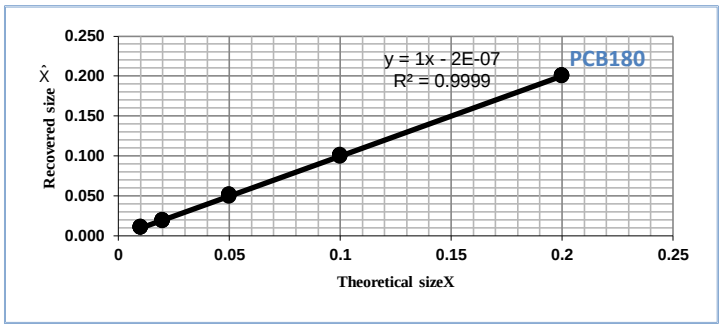
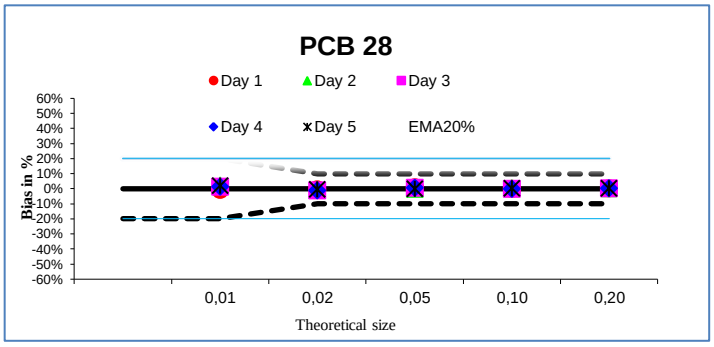
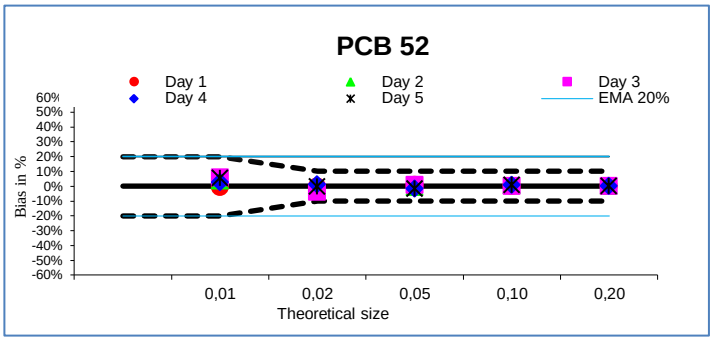
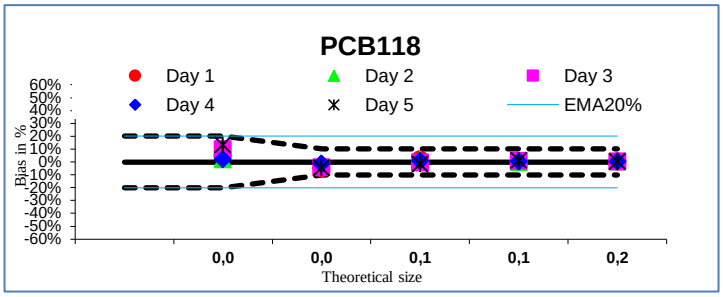
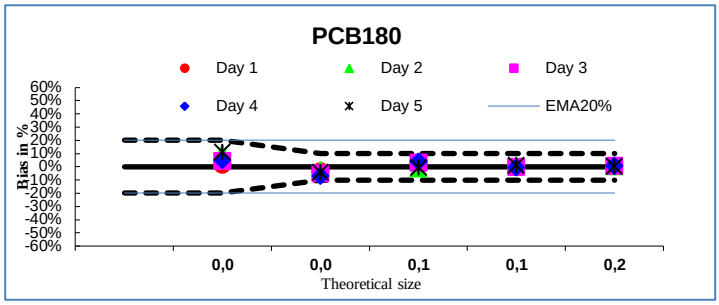


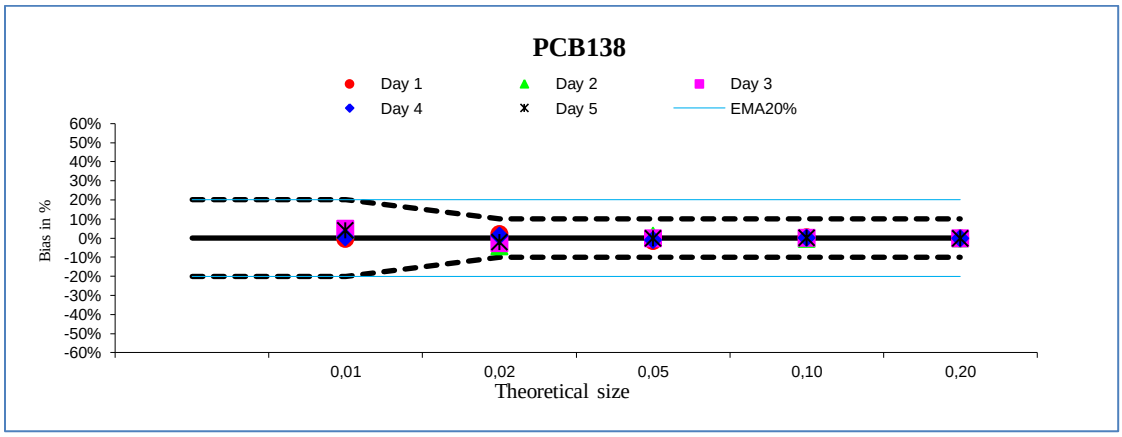
Figure 4:- Breakdown of bias in% according to the levels compared to the calibration EMA defined by the laboratory.

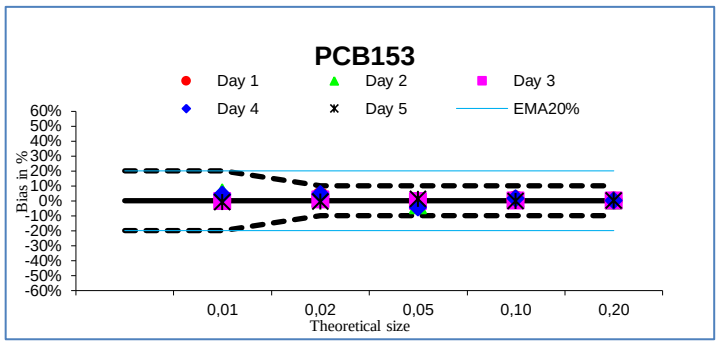


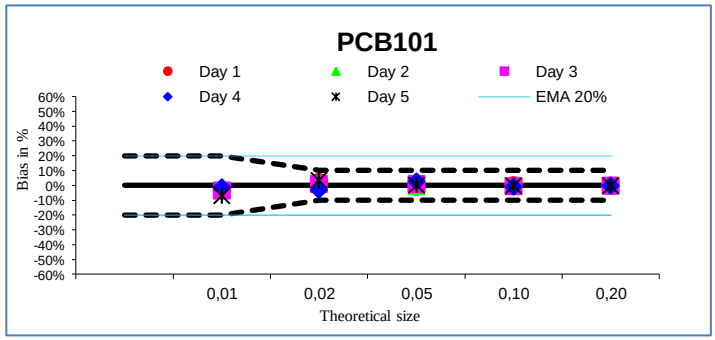












It is necessary to check that all the biases observed on each analyzed standard are acceptable from an acceptable maximum deviation fixed by the exporter.
All biases are lower than the fixed EMA for each standard then the calibration function is considered acceptable in the studied field.
In conclusion $R^2 > 0.995$ for all molecules PCBs and C_{model} is less than $V_{critical\ model}$ Thus the biases observed are lower than the EMA set by the experimenter.
The sensitivity of the used apparatus was estimated by determining limit of quantification (LOQ=0.01ppm) (Table 6). These low LOQ indicate good sensitivity.

Table 6:- Results of limite of quantification (LQ=0.01ppm)

PCBs	LQ + 60% * LQ	$\bar{\bar{z}}LQ + 2\ S_{LQ}$	$\bar{\bar{z}}LQ - 2\ S_{LQ}$	LQ - 60% * LQ
PCB28	0.016	0.012572815	0.007987185	0.004
PCB52	0.016	0.011628757	0.008491243	0.004
PCB101	0.016	0.011167194	0.008532806	0.004
PCB118	0.016	0.012348205	0.007111795	0.004
PCB138	0.016	0.011964594	0.006975406	0.004
PCB153	0.016	0.011649222	0.007150778	0.004
PCB180	0.016	0.011221538	0.007578462	0.004

Table 6 shows that the two inequalities ($\bar{\bar{z}}LQ - 2\ S_{LQ} > LQ - 60\% * LQ$ and $\bar{\bar{z}}LQ + 2\ S_{LQ} < LQ + 60\% * LQ$] for all PCBs are verified.

Table 7:- Results of yields study [LQ, 20% and 80%]

PCBs	Average Yield %			Recovery rate [sanco, 2015]
	0.01ppm	0.05ppm	0.16ppm	
PCB28	102.80	86.34	87.94	[70% ; 120%]
PCB52	100.60	79.94	85.73	
PCB101	98.50	80.42	85.54	
PCB118	97.30	79.38	86.09	
PCB138	94.70	80.90	87.49	
PCB153	94.00	79.32	85.88	
PCB180	94.00	78.02	84.11	

From the recovery study (Table 7), it is clear that the method is accurate for quantitative estimation of PCBs in bivalves as the statistical parameters are within the acceptance range (from 70% to 120%) according to the most recent EU guidelines (SANCO, 2015).
For the study of accuracy an example of PCB 28 which is representative for all the other molecules will be shown. The same results were obtained for all PCBs.
The accuracy of the method is investigated on samples of reference value at 0.01ppm, 0.05ppm and 0.16ppm. The used experimental values for the study of accuracy at these three levels of concentrations are derived from the LQ study and the study of yields.
The results are interpreted to verify the accuracy of the method against an acceptable deviation around each reference value set by the laboratory (Tables 8 and 9).

Table 8:- Estimation of the accuracy parameters of the method for three levels "PCB28"

	Level 1	Level 2	Level 3
Reference value	0,01	0,05	0,16
Uref	0,2	0,2	0,2
EMA in %	64	50	45
EMA	0,006400	0,0251	0,072
Number of series	5	5	5
Average	0,01028	0,04317	0,14071
Intermediate fidelity standard deviation	0,001146	0,001663	0,012798
Intermediate fidelity CV	11,2	3,9	9,1

Table 9:- Interpretation of the accuracy parameters of the method for three levels and conclusions « PCB28 »

Standard deviation EN	0,00140	0,03	0,10
Criterion	2,00	2,00	2,00
Accuracy of the method	Verified	Verified	Verified
Réf + EMA	0,02	0,08	0,23
Z + 2 SLQ	0,01	0,05	0,17
Z - 2 SLQ	0,01	0,04	0,12
Réf - EMA	0,00360	0,02	0,09
Z + 2 SLQ <Réf + EMA	Verified	Verified	Verified
Z - 2 SLQ >Réf - EMA	Verified	Verified	Verified
Accuracy of the method	ACCEPTABLE	ACCEPTABLE	ACCEPTABLE

Discussion:-

Figure 2 showing the representative chromatogram for standard of polychlorinated biphenyls mixture. Adequate separation of the 7 PCBs was achieved. No interference peaks were obtained in the chromatogram at the same retention time of the target compounds. The linearity is the ability within a definite range to obtain results directly proportional to the concentration of the analyte. The concentrations of the validation standards were back calculated from the calibration curve. A linear regression model was fitted on the back-calculated concentrations as a function of the introduced concentrations. The intercept, the slope and the coefficient of determination of the equations obtained are presented in (Table 4). The slopes values close to 1 .Therefore, linearity has also been demonstrated for all PCBs in the range (0.01 to 0.2 ppm) because the coefficient determination are more than 0.995 (r^2). The calibration function was verified by the Fisher test (Table 5). F ratio is less than the critical value of F corresponding to Fisher variable for a risk of 1%. And also by a comparison of all observed biases on each standard analyzed are acceptable from an acceptable maximum deviation set by the exporter.

All biases are lower than the fixed EMA for each standard, the calibration function is considered acceptable in the field studied.Then, the results show that the linear range is validated and the regression model is acceptable. The sensitivity of the apparatus used was estimated by determining limit of quantification (LOQ=0.01ppm) (Table 6). These low LOQ indicate good sensitivity. Table 7 shows that the two inequalities ($\bar{z}$LQ- 2 S_{LQ}> LQ - 60% * LQ and$\bar{z}$LQ + 2 S_{LQ} < LQ + 60% * LQ) are verified. Thus the accuracy of LOQ at 0.01ppm is verified. From the recovery study (Table 8), it is clear that the method is accurate for quantitative estimation of 7 PCBs in bivalves as the statistical parameters are within the acceptance range (from 70% to 120%) according to the most recent EU guidelines (SANCO, 2015). The calculated accuracy error (Table 9) is less than 2. Then, it is considered insignificant [17]. Therefore, the uncertainty associated to accuracy of the method is equal to the uncertainty of the reference material used for testing accuracy study. Table 9 shows the value of Standard deviation EN ≤ Criterion=2 is verified. Therefore the method is just and shows that the two inequalities (Z + 2 SLQ <Réf + EMA and Z - 2 SLQ >Réf– EMA) are verified. Thus the accuracy at 0.05 and 0.16ppm are acceptable. The table below shows the example of PCB 28, which is a summary table of all the results of validation criteria of the same PCBs.

Table 10:- Summary table of all PCB validation criteria 28.

Methode caractirisation										
			Expected performance				Observed value			Conclusions
Calibration										**LINEAR**
Fonction										
Range of standard solutions			0,01	;	0,2	µg/g				
Bias in % For	0,01	µg/g	20%				MAX =	2,0%		Verified
Bias in % For	0,02	µg/g	10%				MAX =	-0,5%		Verified
Bias in % For	0,05	µg/g	10%				MAX =	0,9%		Verified
Bias in % For	0,10	µg/g	10%				MAX =	0,1%		Verified
Bias in % For	0,20	µg/g	10%				MAX =	0,0%		Verified
Fisher Test			4,10				2,53			ACCEPTABLE
Limit of quantification presupposed			0,01			µg/g				**ACCEPTABLE**
Acceptable maximum deviation			0,006			µg/g				
LQ + 40% * LQ			0,016			µg/g	0,01		µg/g	Verified

LQ - 40% * LQ	0,004	µg/g	0,01	µg/g		Verified
Recovery						**ACCEPTABLE**
Fortified sample 1	0	µg/g				
Add 1	0,05	µg/g				
Fortified sample 2	0	µg/g				
Add 2	0,16	µg/g				
Recovery sample 1	70% ; 120%		86,340		%	Verified
Recovery sample 2	70% ; 120%		87,944		%	Verified
Sample 1 Accuracy						**ACCEPTABLE**
Reference value	0,01	µg/g				
Uref	0,2	µg/g				
EMA	0,01	µg/g				
EN	2,0		0,00			Verified
Z + 2 SLQ < Réf + EMA	0,0164	µg/g	0,01		µg/g	Verified
Z - 2 SLQ > Réf - EMA	0,0036	µg/g	0,01		µg/g	Verified
Sample 2 Accuracy						**ACCEPTABLE**
Reference value	0,05	µg/g				
Uref	0,2	µg/g				
EMA	0,03	µg/g				
EN	2,0		0,03			Verified
Z + 2 SLQ < Réf + EMA	0,07512	µg/g	0,05		µg/g	Verified
Z - 2 SLQ > Réf - EMA	0,02488	µg/g	0,04		µg/g	Verified
Sample 3 Accurancy						**ACCEPTABLE**
Reference value	0,16	µg/g				
Uref	0,2	µg/g				
EMA	0,07	µg/g				
EN	2,00		0,10			Verified
Z + 2 SLQ < Ref + EMA	0,23	µg/g	0,17		µg/g	Verified
Z - 2 SLQ > Ref - EMA	0,09	µg/g	0,12		µg/g	Verified

Conclusion:-

In this work, a recently developed method QuECHERS based on accuracy was applied to demonstrate the ability of the GC/MS-MS method to quantify the organochlorine pesticides concentrations in the range of 0.01ppm to 0.2ppm. Figures of merit (Linearity, LOQ, Repeatability intermediate, Precision, Reproducibility and trueness) of the proposed GC/MS-MS procedure were satisfactory for the determination of polychlorinated biphenyls in bivalves ``Oyters``.

The data processing provided precise results and was corroded effectively for the validation of polychlorinated biphenyls. The procedure was successfully applied to the reel bivalve sample.

In conclusion, the QuEChERS method remains a method: fast, easy to implement, inexpensive, specific, exact, sensitive, multi-residual, repeatable and reproducible. According to the results of validation obtained the method proves effective for the detection and the dosage of the 7 polychlorinated biphenyls studied in the oysters of Walidia in "MOROCCO".

The QuEChERS method (Quick, Easy, Cheap, Effective, Rugged and Safe) has been readily accepted by many pesticide residue analysts and many quality control laboratories of marine products in particular and also that are intended for export.

This analytical method should be extended and used for the validation of other bivalves and other organic pollutants.

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