Development and validation of a multianalyte immunoassay for the quantification of environmental pollutants in seawater samples from the Catalonia coastal area.

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ABSTRACT

Five different enzyme-linked immunosorbent assays (ELISAs) have been developed and applied for the detection of five representatives of important families of chemical pollutants in seawater: Irgarol 1051® (triazine biocide), sulfapyridine and chloramphenicol (antibiotics), 17β-estradiol (hormone) and domoic acid (algae toxin). The assays were validated by high-performance liquid chromatography (HPLC) coupled to high-resolution mass spectrometry (HRMS) showing good correlation between both immunochemical and chemical techniques. A process of extraction and clean-up was added prior to the analysis based in solid phase extraction (SPE). The multianalyte platform presented good specificity for each compound and adequate sensitivity, with limits of detection (LOD) after the SPE treatment of 0.124 ± 0.006 , 0.969 ± 0.09 , 0.20 ± 0.05 , 1.11 ± 0.012 and 1.39 ± 0.09 ng L⁻¹ for Irgarol 1051®, sulfapyridine, chloramphenicol, 17β-estradiol and domoic acid, respectively. No matrix effects were noticed in working with the seawater

extracts. Afterward, seawater samples from the Mediterranean Sea (coastal area of Catalonia) were analysed by both techniques and only one sample presented one contaminant, 17 β -estradiol, in the concentration of $0.011 \pm 0.04 \mu\text{g L}^{-1}$.

KEYWORDS

ELISA, multianalyte immunoassay, HPLC, environmental analysis, validation

1. INTRODUCTION

During last decades there has been many efforts in understanding the origin and levels of pollutants in aquatic environments, since they are considered both reservoirs of biodiversity and areas with great economic potential [1]. Considering, in particular, coastal areas as regions that are exposed to contaminants coming from many different sources, the need of controlling the occurrence of contaminants becomes essential. Some groups of contaminants such as endocrine disruptor chemicals (EDC) can cause severe negative effects due to its accumulation in water and soil samples, or aquatic organisms; also, EDC may affect the hormonal balance of fishes and other organisms [2,3]. Furthermore, some examples of antibiotics can be highlighted as interesting contaminants to be monitored considering its massive use for combating infections and infestations in aquaculture facilities [4]. Most of the pollutants found in the environment are directly generated by humans, however, there is an increasing concern about the marine biotoxins which are being produced by algal blooms which can produce harmful effects, affecting both the environment and causing economical losses [5].

As a result of this scenario and during the last years, several global and regional monitoring programs have been proposed [6,7] aiming for the establishment of early warning systems and screening methods that could provide information about multiple contaminants with high sensitivity and selectivity. Several environmental quality standards (EQS) have been set by the European Commission in order to control the allowed concentration of certain chemical species that could be related to environmental or human health risks (established in the Environmental Quality Standards Directive 2008/105/EC as amended by the Priority Substances Directive 2013/39/EU).

Is within these initiatives that the development of multianalyte and/or multiplexed platforms able to measure simultaneously several analytes from a single sample became a hot topic. Several multiresidue or multiplexed analytical procedures for the analysis of a huge number of chemicals in environmental samples have been reported in the last years based on chromatographic techniques [8-10], however, due to its features of being rapid, simple and low cost methods altogether with their high specificity, potential portability and high throughput capabilities, immunochemical techniques have been considered great candidates for environmental monitoring at low concentration levels. From microbial based assays[11,12] to enzymatic assays [13], it is described different detection systems based on fluorescence [14,15], microarrays [16,17] or biosensors [18,19], several examples that have been presented in the literature regarding multianalyte or multiplexed systems aiming to detect pollutants in environmental samples.

For the development of this work, some representative compounds pertaining to different family groups of contaminants and with different chemical natures, commonly present in seawater, have been considered. These compounds are; the biocide Irgarol 1051® (Irg), two antibiotics sulfapyridine (Spy) and chloramphenicol (CAP), the hormone 17 β -estradiol (E2) and the marine biotoxin domoic acid (DA). The analysis of the selected compounds has been carried out through a multianalyte ELISA platform and then, and the results have been compared and validated by a reference analytical technique such as high-performance liquid chromatography (HPLC) coupled to high resolution mass spectrometry (HRMS).

2. EXPERIMENTAL SECTION

2.1 Reagents, immunoreagents and equipment.

The chemical reagents and immunoreagents used were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA) and from Sigma Chemical Co. (St. Louis, MO, USA). Domoic acid was purchased from Sigma Chemical Co. as well as the estradiol hapten; β -estradiol 6-(O-carboxymethyl)oxime (6E2). The preparation of the described bioconjugates and antibodies has been performed with the support of the ICTS "NANBIOSIS", more specifically by the Custom Antibody Service (CAbS, CIBER-BBN, IQAC-CSIC). The immunoreagents for Irgarol (Irg), Sulfapyridine (SPy) and Chloramphenicol (CAP) detection used for the development of the

competitive assays in the microarray platform have been described before [20,21]. The preparation of the bioconjugate competitors for 17 β -estradiol and domoic acid have been reported [22]. The monoclonal antibody used on the estradiol assay was purchased from Fitzgerald Industries International. the monoclonal antibody for the detection of domoic acid has been kindly provided by Prof. Chris Elliot from Queen's University [23]. The analytes used in this platform were Irgarol (Irg), Sulfapyridine (SPy), Chloramphenicol (CAP), 17 β -estradiol (E2), and Domoic acid (DA), all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of each analyte were prepared at 10 mM concentration in DMSO and stored at 4°C until its use.

The pH and the conductivity of all buffers and solutions were measured with a pH-meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Dilution plates were purchased from Nirco (Barberà del Vallés, Spain). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Washing steps were performed on a Biotek ELx405HT (Biotek Inc.). Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, CA, USA). The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v4.7 (Molecular Devices) and GraphPad Prism 5.03 (GraphPad Software Inc., San Diego, CA, USA). For the matrix studies, sea salts used to prepare the artificial seawater (aSW) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and prepared at 40 mg mL⁻¹ in Ultrapure water.

2.2 Buffers.

Unless otherwise indicated, phosphate buffer saline (PBS) is 0.01 M phosphate buffer in a 0.8% saline solution at pH 7.5. Coating buffer is a 0.05 M carbonate-bicarbonate buffer, pH 9.6. PBST is PBS with 0.05% of Tween 20. PBT2x is PB (0.02 M phosphate buffer, no saline solution) with 0.1% Tween 20 at pH 7.5. Citrate buffer is 0.04 M sodium citrate, pH 5.5. The substrate solution contains 0.01% 3,3',5,5'-tetramethylbenzidine (TMB) and 0.004% H₂O₂ in citrate buffer. Borate buffer is 0.2 M boric acid/sodium borate, pH 8.7.

2.3. Sampling

Surface seawater samples were collected from 6 different locations along the Catalan coast (NE of Spain, Mediterranean Sea) during the 18th-25th of May 2017; Llafranc (41°53'35.5"N

3°11'35.7"E), Palamos (41°50'53.2"N 3°06'47.2"E), Platja d'Aro (41°48'59.7"N 3°04'10.5"E),
Sant Feliu de Guixols (41°47'26.9"N 3°03'09.5"E), Badalona (41°26'17.0"N 2°14'40.9"E),
Forum-Sant Adria del Besos (41°25'14.6"N 2°14'01.0"E). Samples were stored in amber glass
bottles and preserved at -20 °C until the analysis.

2.4. Sample pre-treatment

Solid phase extraction (SPE) was employed for the general clean-up of water samples. With
minor modifications, the extractions were proceeded following the previously developed and
optimized methods [24-28] with recoveries ranging from 76 to 98 %. Briefly, cartridges were
conditioned with 6 mL of methanol and equilibrated with 6 mL of pure water. For Irgarol1051,
ISOLUTE® ENV+ (220 mg, 6 mL) cartridges were employed, whereas cartridges of OASIS
HLB (200 mg, 6 mL) were used for the rest of compounds. 500 mL of different seawater
samples were loaded into the cartridges at approximately 1 mL min⁻¹. Cartridges were then
washed with 6 mL of pure water to remove the interferences and salts and dried under vacuum
for 20 min. Finally, 6 mL of methanol was used for the elution. All extracts were concentrated
through a flow of N₂ steam of an evaporator Reacti-Therm III of Pierce (Rockford, IL, USA)
and then reconstituted to 0.8 mL of solvent. The extracts analysed by ELISA were reconstituted
with DMSO and diluted 5 times in PBST (20% of final DMSO). Under these conditions it would
allow achieving a preconcentration factor of $625/5 = 125$ times, however, this value should be
corrected with the real recovery obtained after the SPE column treatment (see **Table 1**). The
extracts analysed by HPLC-HRMS were reconstituted with the initial chromatographic
conditions of mobile phase, (9:1) methanol/water.

2.5. Competitive ELISA assay

Microtiter plates were coated with the proper concentration of each antigen (in coating buffer,
100µL/well), overnight at 4 °C and covered with adhesive plate sealers. The next day, the plates
were washed four times with PBST (300 µL well⁻¹), using a Biotek ELx405HT microplate
washer form Biotek Inc. (Winooski, Vermont, USA). Then, each analyte was added at different
concentrations for each analyte (50 µL well⁻¹, in PBST, aSW or PBST 20% DMSO, 50 µL well⁻¹)
followed by the specific antibody at their corresponding concentration (50 µL well⁻¹, in PBST
or PBT2x for the individual assays, or the cocktail of antisera for the multiplexed analysis. After
30 min at r.t., the plates were washed as mentioned before, and a solution of anti-IgG-HRP

(single or a mixture of anti-mouse and anti-rabbit IgGs for the multiplexed assays, 1/6000 in PBST), was added to the wells (100 $\mu\text{L well}^{-1}$) and incubated for 30 min at r.t. The plates were washed again, and the substrate solution was added (100 $\mu\text{L well}^{-1}$). Colour development was stopped after 30 min at r.t. with 4 N H_2SO_4 (50 $\mu\text{L well}^{-1}$), and the absorbance was read at 450 nm, with a SpectramaxPlus microplate spectrophotometer from Molecular Devices (Sunnyvale, CA, USA). The standard curves were fitted to a four-parameter equation according to the following formula: $y = (A - B/[1 - (x/C)^D]) + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. For this, the software SoftmaxPro v4.7 of Molecular Devices (Sunnyvale, CA, USA) and GraphPad Prism 5.03 of GraphPad Software Inc. (San Diego, CA, USA) were employed. Unless otherwise indicated, data presented correspond to the average of at least two wells replicates.

2.6. Matrix effects

Matrix studies were performed in single analyte measurements with aSW prepared at 40 mg/mL in ultrapure water. Non-specific interferences produced by the parameters associated with the matrix of interest were studied by preparing a standard curve directly in seawater, or in buffer with an appropriate % of solvent for the multianalyte assays. Measurement of blank sample extracts after the SPE process made possible the study of the hypothetical matrix effect.

2.7. Quality parameters

In order to assess the reproducibility of the assays, these were carried out three times within three different days, with three replicates for each one. The main features of the final assay were described as the mean of all the replicates. The accuracy of each ELISA assay for its specific analyte was assessed by analysing 5 blind SW extracts, including 4 extracts spiked at different concentrations within the working range of each curve's analyte and one blank. The results were fitted to a linear regression curve between the spiked concentrations and the measured ones.

The possible cross-reactivity between the pairs of antibody-competitors used during this work was previously studied, showing in any case significative non-specific interactions [22].

2.8 Immunoassay validation

The multianalyte ELISA format was validated with HPLC-HRMS. The accuracy of the multiplexed ELISA assay for all analyte concentrations was assessed by analysing 6 different real SW samples altogether with 3 more extracts which contained all the analytes spiked at three different levels of concentration (high, medium and low) within the working range of each curve's analyte. Samples analysed by ELISA were preserved in pure DMSO and for its analysis were diluted five times in PBST, obtaining final samples of only a 20% of solvent. All the samples were analysed in parallel by high performance liquid chromatography (HPLC) coupled to high resolution mass spectrometry (HRMS) as a reference technique.

2.9 HPLC-HRMS

An Acquity™ Ultra High-Performance Liquid Chromatograph (UHPLC) from Waters (Massachusetts, USA) was employed for the chromatographic separation. A reversed phase column C18 Synergy (50 mm x 2 mm and 5 µm, 80 Å) from Phenomenex (Torrance, USA) was used as stationary phase. Methanol (A) and water (B) were merged into the mobile phase with an elution gradient program of 10 min in total: 0 min (10 % A) – 5 min (95 % A) – 7 min (95 % A) – 10 min (10% A). The flow corresponded to 0.3 mL min⁻¹, and the volume of injection was 20 µl for each sample.

A Thermo Scientific QExactive mass spectrometer was coupled to the chromatography employing a heated electrospray ionization probe (HESI), both acquired from Thermo Fisher Scientific (San Jose, CA, USA). The HESI was working with the following parameters: sheath flow gas, auxiliary gas and sweep gas were 60, 20 and 2 of arbitrary units, respectively. The S-lens RF level was 60 %, the spray voltage 3 kV and the temperatures for the heater and the capillary were 320 and 300°C.

The analysis by HRMS was performed with the acquisition working in the mode of data dependant-MS², obtaining at the same time the full scan from 100 to 700 m/z and the MS/MS spectrum for each compound at the resolution of 70,000 and 35,000 full width at half maximum (FWHM), respectively. In **Table 2** are summarized the corresponding precursor and fragment ions at the optimized collision energy for each compound. Quantification was carried out considering the most intense signal of the fragment ion and confirmation with the second most intense.

3. RESULTS AND DISCUSSION

Recently, it has been reported two examples of platforms designed as proposals for screening and monitoring different pollutants in the environment, one based in a fluorescent multiplexed microarray [22] and another based on an amperometric sensor [29], and each with their own advantages. In the case of the microarray format, the assay protocol is faster, because the secondary antibodies are labelled with a fluorophore, no needing a substrate for obtaining the final signal like in the case of an ELISA protocol [22]. Furthermore, not only the volume of sample is reduced but also the analysis of all the pollutants can be done simultaneously in a single well. In the case of an electrochemical sensor, not only they are considered cheap options for monitoring organic contaminants such the ones selected for this work but are usually more fitted to be used for on-site measures, however, no multiplexation nor multianalyte format was addressed [29].

In order to exploit most of the advantages related to immunoassays the platform chosen for developing this work was an ELISA configuration. Although the assay time is a bit longer than in microarray options the equipment needed for all the protocol is more accessible, relying in absorbance readings instead of fluorescence. The high-throughput capabilities along with the easy protocols related to ELISA, and finally the possibility to develop it in a multianalyte format allowing the detection of several pollutants altogether make the multianalyte-ELISA a great candidate for screening contamination of natural environmental waters.

3.1. Evaluation of immunoreagents in an ELISA format with different matrix conditions and reproducibility of the assays

One of the challenges of detecting and monitoring marine contaminants is related to the low concentration in which they are found in the environment. For that reason, great sensible techniques are required, and therefore, the possibility to include pre-treatment or pre-concentration stages during the quantification of the samples could improve the sensitivity of already established platforms.

Aiming to achieve enough sensitivity to the analysis of seawater samples a SPE clean-up step was performed prior de immunoassay, according to the procedure described before. Therefore, the target samples of this study are seawater samples that have been pre-treated by SPE before its

analysis, in order to, on one hand, pre-concentrate the possible pollutants present in it and, on the other hand, to eliminate possible interferences like other pollutants and salts. That means, that a certain percentage of the elution solvent used in the SPE process will be present in the sample, and accordingly, the evaluation of this solvent in the final multianalyte platform and its comparison with the initial buffer conditions was needed. Both the addition of the solvent and the use of an antibody cocktail was studied and compared to the buffer conditions in terms of detectability, sensitivity and limit of detection (LOD).

The effect of the addition of up to 20% DMSO in the final assay was tested previously for all the analytes (data not shown). In all the cases most of the analytical parameters were maintained, although in some cases the maximum signal slightly decreased, and therefore the antibody concentration were adjusted. The possible cross-reactivity between the pairs of antibody-competitors used during this work was previously studied, showing in any case significative non-specific interactions [22]. In parallel, the reproducibility of all the assays, both in single-analyte and multianalyte format, was approached. In **Figure 3** can be observed that when multiplexed (and even in the presence of a 20% of DMSO), all the assays maintained the levels of detectability and sensitivity (see **Table 3**), achieving LOD of 0.106 ± 0.02 , 0.115 ± 0.06 , 0.034 ± 0.01 , 0.149 ± 0.002 and 0.40 ± 0.03 ppb for Irgarol 1051®, sulfapyridine, chloramphenicol, 17 β -estradiol and domoic acid, respectively. Even though the concentration of the pollutants selected are low in the environment, thanks to the pre-concentration step of the SPE, the detectability could be improved with a factor of 500. All the results correspond to the average of 3 assays performed on different days using three-well replicates. The slightly differences between the multianalyte-ELISA and the individual assays can be explained, on one hand, by the presence of a solvent in the assay buffer in the case of the multianalyte-ELISA, and, on the other hand, by the use of a cocktail of antibodies that can modify lightly the parameters of each assay.

Furthermore, the parameters of IC₅₀ and LOD obtained with the multianalyte-ELISA (without the SPE preconcentration) are maintained in the same range as the ones reported for the multiplexed microarray already mentioned in section 3 (except in the case of domoic acid in which the multiplexed ELISA is slightly better). However, the advantage of the platform presented is the improvement of up to 125 times the detectability levels once the SPE treatment is performed, for instance, the final LOD found for Irgarol 1051, sulfapyridine, chloramphenicol,

17 β -estradiol and domoic acid were 0.124 ± 0.006 , 0.969 ± 0.09 , 0.20 ± 0.05 , 1.11 ± 0.012 and 1.39 ± 0.09 ng·L⁻¹, respectively, compared with the LOD obtained with its corresponding individual assay in seawater that were found in the $\mu\text{g}\cdot\text{L}^{-1}$ range. According to these results, the detectability achieved will be sufficient to reach the environmental levels usually found for most of the analytes selected

3.2. Accuracy studies in the individual ELISA assays

Once the different ELISA assays were established in the desired matrix, the accuracy assays were performed in order to determine the reliability of each assay to determine the specific pollutant selected. Different extracts of seawater previously treated were spiked at 4 different concentrations for each pollutant and were analysed aiming to perform an accuracy assay for each contaminant separately. The matrix effect was also studied analysing a blank sample for each assay, in order to explore if the composition of the seawater treated could have the potential to affect the correct quantification of the target analytes. It was not observed a matrix effect for all the blank samples tested. As represented in **Figure 2**, the linear regression analysis showed slopes near 1 in all cases (being $m=1.00$ the perfect correlation) with good regression coefficients ($R^2 > 0.95$), indicating the great accuracy of the different assays. In parallel, 5 blind samples were analysed for each specific ELISA assay aiming to demonstrate the feasibility of applying the different tools developed for the monitoring of seawater samples. The quantification results are summarized in **Table 4**, showing good levels of accuracy and precision in comparison with the nominal values of those samples. These results demonstrate that the different single-analyte ELISA assay developed can be presented as good candidates for analyte detection in seawater extracts.

3.3. HPLC validation of multiplexed ELISA results: Accuracy assays and real sample evaluation

The development of the multianalyte ELISA platform was performed using a cocktail of all the specific antibodies required for the individual assays, for analysing the different study samples proposed. The features of all the assays are summarized in **Table 3**, whereas the comparison between the multianalyte assays and the individual ones can be visualized in **Figure 3**. Validation of the ELISA platform was assessed with accuracy studies of seawater extracts

fortified at 3 different concentration levels of all the target analytes (within the working range of each curve's analyte) and with the analysis of 6 real samples. The quantification with the multianalyte platform and the HPLC technique had the goal to explore the potential of the ELISA platform developed in analysing complex samples with a combination of the target analytes. In **Table 5** and **Figure 4** the three spiked levels of concentration (High, medium and low) are compared between the two quantification techniques selected. The HPLC-MS technique presents worse accuracy than the ELISA, that could be explained as the ion suppression during the ionization process because of the presence of salts in the extracts even after the SPE. Contrary, it does not affect the response of the immunoassay that does not present matrix effect in using directly seawater within the buffers, as it has been seen in the section 3.1. Finally, 6 seawater samples were obtained from different locations over the Catalan coast. These samples were, as in the previous accuracy assay, pre-treated as commented in section 2.3. to obtain concentrated extracts, which were analysed by ELISA and HPLC simultaneously. As shown in **Table 6**, only one of the samples gave a positive response of 17 β -estradiol in the location of Llafranc and this was good correlated by ELISA and HPLC-HRMS.

A Wilcoxon matched-pairs signed-ranks test was employed to determine the correlation between the results obtained by both techniques. The nonparametric Spearman coefficient (r) was equal to 0.812 resulting in a significant correlation between the values of concentration determined by ELISA and HPLC-HRMS, at the 0.01 value of confidence. In **Figure 4** can be observed the correlation of all samples measured by ELISA and HPLC-HRMS. Then, the quantification by the immunoassay option showed good levels of correlation with both the spiked value and the HPLC measurements, demonstrating that the presence of other analytes is no impediment for the correct quantification of each pollutant.

4. CONCLUSIONS

The 5 ELISA platforms developed for the analysis of Irgarol 1051®, sulfapyridine, chloramphenicol, 17 β estradiol and domoic acid in complex matrices as seawater presented good response in terms of accuracy, sensitivity and reproducibility. No matrix effect and non-specific responses by other structurally similar compounds were interfering the functioning of the immunoassays. The validation of the immunoassays through HPLC-HRMS indicated a proper

correlation between both immunochemical and analytical techniques. This fact demonstrates the capability of the platform developed for its application in monitoring the levels of several pollutants present in real environmental samples, even at low concentrations. Due to this fact and the urge to obtain multi-residual analytical methods for the detection of contaminants in environmental matrixes and the demonstration of its possible implementation by reference techniques, this article reports a successful multianalyte ELISA platform for the detection of the five pollutants in seawater.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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TABLES

Table 1. SPE recoveries and LOD obtained for the different pollutants				
Pollutant	SPE cartridge	Recovery (%)	Preconcentration factor^a	mLOD^b ($\mu\text{g L}^{-1}$)
Irgarol 1051®	ISOLUTE® ENV+	96.7	120.8	0.004
Sulfapyridine	OASIS HLB	99	123.8	1.09
Chloramphenicol	OASIS HLB	76	95	0.10
Estradiol	ISOLUTE® ENV+	72	90	0.02
Domoic acid	OASIS HLB	98	122.5	5
^a The preconcentration factor was calculated as the theoretical preconcentration factor 625, divided by 5 (due to the 1/5 dilution in PBST) and corrected by the recovery value of each analyte.				
^b mLOD means limit of detection of the HPLC-HRMS method for each pollutant.				

Table 2. Precursor and fragments ions of the 5 compounds at the optima normalised collision energy (NCE).

	Chemical formula	Molecular ion	m/z precursor ion	m/z fragmentation ions		NCE ^a (%)	Retention time (min)
Irgarol 1051®	C ₁₁ H ₁₉ N ₅ S	[M+H] ⁺	254.1425	198.0805	108.0554	65	6.2
Sulfapyridine	C ₁₁ H ₁₁ N ₃ O ₂ S	[M+H] ⁺	250.0635	156.0109	184.0865	35	3.6
Chloramphenicol	C ₁₁ H ₁₂ Cl ₂ N ₂ O ₅	[M-H] ⁻	321.0042	152.0337	194.0446	20	4.6
17β estradiol	C ₁₈ H ₂₄ O ₂	[M-H] ⁻	351.1261	271.1695	79.9555	35	5.9
Domoic acid	C ₁₅ H ₂₁ NO ₆	[M+H] ⁺	312.1430	266.1377	248.1271	30	0.6
^a NCE was established at 10 and stepped at different percentages for each compound. + retention time.							

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Table 3. Analytical parameters for all the target assays in an ELISA format, performed individually in PBST and in a multiplexed ELISA format (PBST 10% DMSO)^a.

	Irgarol 1051®			Sulfapyridine			Chloramphenicol		
ELISA	4eBSA/ As87			SA2BSA/ As155			CA6BSA/ As226		
Condition	PBST	aSW	Multianalyte ELISA	PBST	aSW	Multianalyte ELISA	PBST	aSW	Multianalyte ELISA
[CA], µg mL ⁻¹	0.25	0.25	0.078	0.25	0.25	0.156	0.0625	0.0625	0.0625
[As] dilution	1/16000	1/16000	1/32000	1/16000	1/16000	1/8000	1/64000	1/32000	1/32000
Abs _{min}	0.03 ± 0.02	0.04 ± 0,004	0.108 ± 0.02	0.03 ± 0.02	0.019 ± 0,015	0.076 ± 0.05	0.030 ± 0.01	0.03 ± 0,02	0.102 ± 0.03
Abs _{max}	1.47 ± 0.09	1.46 ± 0,17	1.65 ± 0.22	1.34 ± 0.08	0.839 ± 0,097	1.64 ± 0.16	0.809 ± 0.02	0.984 ± 0,1	1.39 ± 0.22
Slope	-0.98 ± 0.22	-1.06 ± 0,06	-1.21 ± 0.16	-0.784 ± 0.09	-0,95 ± 0,15	-0.729 ± 0.11	-0.60 ± 0.05	-0,82 ± 0,1	-0.736 ± 0.04
IC ₅₀ (nM)	0.58 ± 0.19	0,43 ± 0,11	3.47 ± 0.11	6.58 ± 1.73	7,41 ± 1,6	10.7 ± 2.64	0.59 ± 0.05	0,94 ± 0,07	2.16 ± 0.16
IC ₅₀ (µg/L ⁻¹)	0.145 ± 0.05	0,111 ± 0,03	0.879 ± 0.16	1.43 ± 0.23	1,84 ± 0,39	2.64 ± 0.66	0.192 ± 0.09	0,324 ± 0,09	0.699 ± 0.05
LOD (µg/L ⁻¹)	0.012 ± 0.007	0.015 ± 0.007	0.106 ± 0.02	0.08 ± 0.02	0.12 ± 0.07	0.115 ± 0.06	0.004 ± 0.01	0.019 ± 0.01	0.034 ± 0.01
LOD* (ng L ⁻¹)	-	-	0.124 ± 0.006	-	-	0.969 ± 0.09	-	-	0.20 ± 0.05
	17β-Estradiol			Domoic acid					
ELISA	6E2BSA/MAb_E2			DABSA/MAb_DA					
Condition	PBST	aSW	Multianalyte ELISA	PBST	aSW	Multianalyte ELISA			
[CA], µg mL ⁻¹	0.3	0.3	0.3	0.6	0.6	0.6			
[As] dilution	1/64000	1/64000	1/64000	1/16000	1/16000	1/16000			
Abs _{min}	0.013 ± 0.003	0.012 ± 0,006	0.125 ± 0.09	0.038 ± 0,01	0.07 ± 0.001	0.128 ± 0.05			
Abs _{max}	1.36 ± 0.20	0.902 ± 0,16	1.17 ± 0.19	0.935 ± 0,12	1.08 ± 0.16	0.826 ± 0.01			
Slope	-1.01 ± 0,07	-0,958 ± 0,04	-1.07 ± 0.02	-0,866 ± 0,13	-0.788 ± 0.15	-1.24 ± 0.13			
IC ₅₀ (nM)	4.01 ± 0,74	2,84 ± 0,05	4.20 ± 0.30	9.69 ± 1.05	9.77 ± 0.87	6.48 ± 0.36			
IC ₅₀ (µg L ⁻¹)	1.09 ± 0.20	1,02 ± 0,02	1.06 ± 0.08	3.02 ± 0.33	2.82 ± 0.14	2.02 ± 0.11			
LOD (µg L ⁻¹)	0.11 ± 0.03	0.10 ± 0.007	0.149 ± 0.002	0.24 ± 0.02	0.17 ± 0.07	0.40 ± 0.03			
LOD* (ng L ⁻¹)	-	-	1.11 ± 0.012	-	-	1.39 ± 0.09			

^aThe results shown are the average a standard deviation of assays performed in three different days. On each day the assays were run using three-weell replicates. The values of LOD* were obtained correcting with the real preconcentration factor (i.e. 120.8 for Irgarol 1051®; 123.8 for sulfapyridine; 95 for chloramphenicol; 90 for 17β-estradiol; and 122.5 for domoic acid) the values of each individual assay (N=3).

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Table 4. Results of the blind's samples quantification by each single-analyte assay ^a .		
	Measured concentration (µg L ⁻¹)	
Blind sample	Nominal concentration	ELISA measurement
Irgarol 1051®	3	4.2 ± 0.7
Sulfapyridine	3	4.1 ± 0.4
Chloramphenicol	6	2.2 ± 0.2
17β-estradiol	14	17.1 ± 2.5
Domoic acid	70	50.6 ± 1.5
^a The ELISA results were obtained as a result of three different assays in different days.		

Table 5. Results of the accuracy quantification of the different seawater samples spiked with all the target analytes simultaneously^a.

		Measured concentration ($\mu\text{g L}^{-1}$)		
		Nominal concentration	ELISA quantification	HPLC quantification
High level	Irgarol 1051®	1.25	1.25 ± 0.01	1.82 ± 0.04
	Sulfapyridine	2.5	2.48 ± 0.08	2.94 ± 0.02
	Chloramphenicol	2.5	2.58 ± 0.17	2.44 ± 0.21
	17 β -estradiol	2.5	2.49 ± 0.11	2.24 ± 0.14
	Domoic acid	12.5	13.16 ± 0.82	17.2 ± 0.84
Medium level	Irgarol 1051®	1	1.01 ± 0.01	1.46 ± 0.01
	Sulfapyridine	1.25	1.34 ± 0.20	1.00 ± 0.09
	Chloramphenicol	1	1.10 ± 0.06	1.61 ± 0.75
	17 β -estradiol	1.25	1.23 ± 0.12	1.61 ± 0.52
	Domoic acid	10	10.72 ± 0.58	15.8 ± 1.19
Low level	Irgarol 1051®	0.5	0.56 ± 0.05	2.00 ± 0.04
	Sulfapyridine	1	1.12 ± 0.07	0.81 ± 0.19
	Chloramphenicol	0.5	0.58 ± 0.20	0.39
	17 β -estradiol	1	0.95 ± 0.03	1.22 ± 0.59
	Domoic acid	5	5.89 ± 0.82	5.72 ± 0.57

^aThe ELISA results shown are the average of three-well replicates and the experiments were repeated 3 days. For HPLC-HRMS measurements, the results shown corresponds to three replicates within the same day.

Table 6. Comparison of quantification results of the real seawater samples by both platforms, multianalyte ELISA and chromatographic reference technique (HPLC-HRMS)^a.

	Measured concentration ($\mu\text{g L}^{-1}$)					
	Palamós		Sant Feliu		Llafranc	
	ELISA quantification	HPLC quantification	ELISA quantification	HPLC quantification	ELISA quantification	HPLC quantification
Irgarol 1051® Sulfapyridine Chloramphenicol 17 β estradiol Domoic acid	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	0.011 $\pm$ 0.04	0.012
	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Fòrum		Badalona		Platja d' Aro	
	ELISA quantification	HPLC quantification	ELISA quantification	HPLC quantification	ELISA quantification	HPLC quantification
Irgarol 1051® Sulfapyridine Chloramphenicol 17 β estradiol Domoic acid	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	nd	nd	nd	nd	nd	nd
	<LOQ	nd	nd	nd	<LOQ	<LOQ
	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

^aSeawater samples collected from different locations in the Catalanian Coast were passed through the SPE module, eluted with DMSO and the eluate split in two parts, one for analysis with the multianalyte ELISA and the other for the analysis by HPLC-HRMS. For the case of the ELISA, the results shown are the average of three-well replicates and the experiments were repeated 3 days. For HPLC-HRMS measurements, the results shown corresponds to only one replicate.

FIGURES

Figure 1. Chemical structure of the 5 different analytes of this work.

Irgarol 1051

Sulfapyridine

Chloramphenicol

17β-estradiol

Domoic acid

Figure 1

468 **Figure 2.** Results from the accuracy study of the single-analyte assays performed in SPE extracts of real seawater samples spiked at
 469 different concentrations. The graph shows the correlation between the spiked and measured concentration values. The dotted line
 470 corresponds to a perfect correlation ($m = 1$). The data correspond to the average of at least three-well replicates from 2 different days.

Figure 2

474 **Figure 3.** Multianalyte ELISA assay results obtained for each analyte (corresponding to the average of 3 assays performed on
475 different days using 3-well replicates) and the comparison with the calibration curves in buffer conditions (PBST) using each specific
476 antibody against its coating antigen associated (corresponding to the average of 3 assays performed on different days using 2-well
477 replicates).

478
479 **Figure 3**
480

481 **Figure 4.** Correlation of all samples analysed by ELISA and HPLC-HRMS.

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Figure 4