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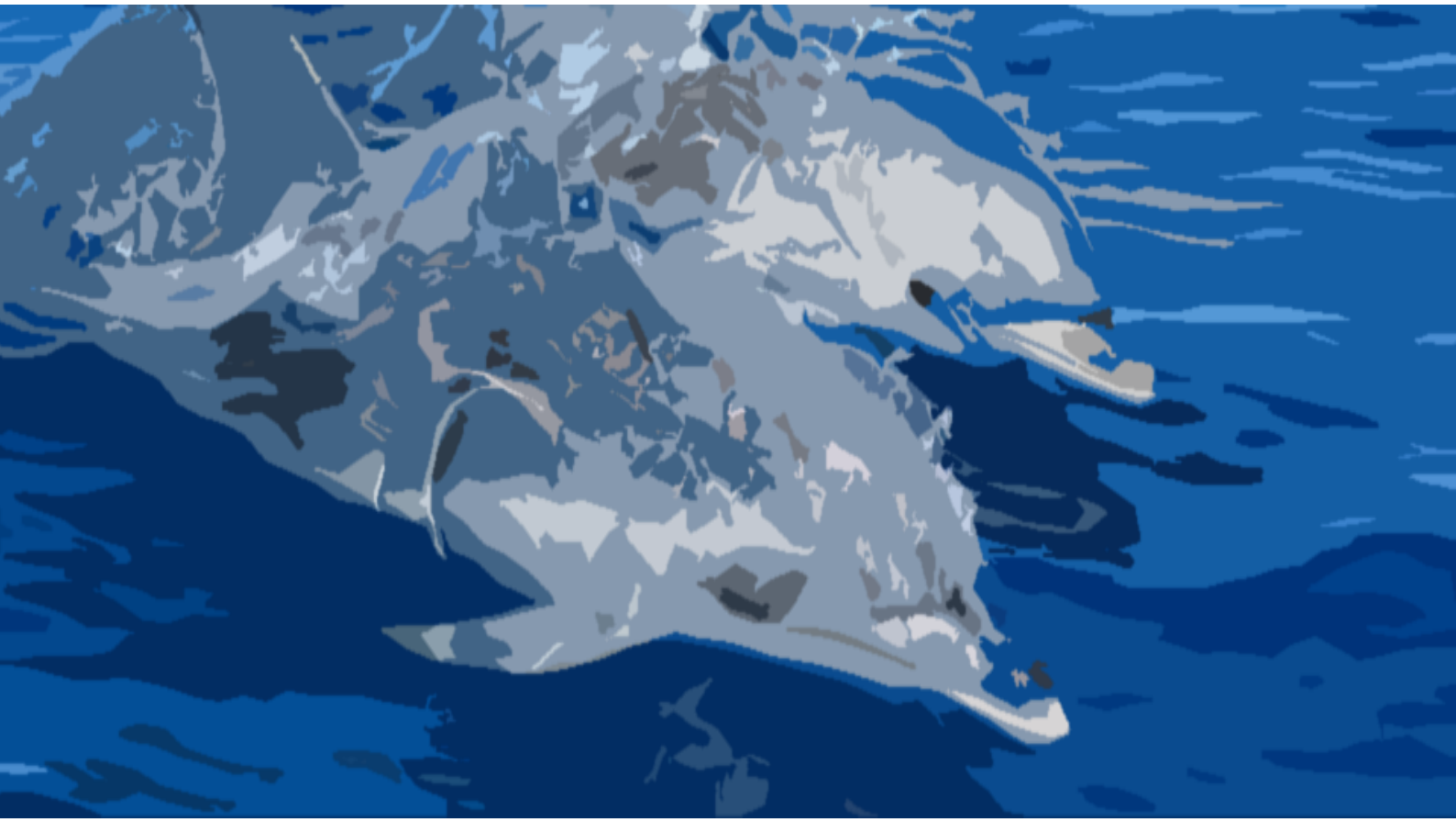
DEVELOPMENT OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF EMERGING POLLUTANTS IN TISSUES OF STRANDED CETACEANS



Emily González Bareiro

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Tutors:

Sarah Montesdeoca Esponda

Jesús De la Fuente Márquez

Final Project to obtain the Degree in Marine Sciences

“Development of an analytical method for the determination of emerging pollutants in tissues of stranded cetaceans”

This work is the result of a collaboration between two research institutes of the University of Las Palmas de Gran Canaria, Instituto Universitario de Estudios Ambientales y Recursos Naturales (IUNAT) and Instituto de Sanidad Animal y Seguridad Alimentaria (IUSA)**.**

Personal details:

Emily González Bareiro

Degree in Marine Sciences

Course 2020/2021

University of Las Palmas de Gran Canaria

Supervisors details:

Sarah Montesdeoca Esponda

IUNAT, University of Las Palmas de Gran Canaria

Jesús De la Fuente Márquez

IUSA, University of Las Palmas de Gran Canaria

_____

Emily González Bareiro

_____ _____

Sarah Montesdeoca Esponda **Jesús De la Fuente Márquez**

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Gran Canaria

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Abstract

Research on marine pollution has been commonly focused on compounds like pesticides or heavy metals. However, emerging pollutants such as ultraviolet filters (UVFs) and stabilizers (UVSs) are of increasing concern because their effect in the environment is not completely know. UVFs and UVSs are substances that are added to personal care products (PCPs) like cosmetics and products related with the sun care.

Once released into the aquatic ecosystem, these compounds can be bioaccumulated by marine organisms. To test this fact in cetaceans, a method based on microwave-assisted extraction combined with ultra-high performance liquid chromatography and mass spectrometry detection has been optimized to determine twelve UVFs and UVSs compounds in dolphin blubber samples.

The developed method was successfully applied to the determination of the target compounds in blubber tissue samples of five common bottlenose dolphin (*Tursiops truncatus*). Three of the twelve studied compounds, namely 2-Hydroxy-4-methoxybenzophenone (BP3), 2-ethylhexyl 2-cyano-3,3-diphenylprop-2-enoate (OC) and 3-methylbutyl (E)-3-(4methoxyphenyl) prop-2-enoate (IMC), were found. Among the identified compounds, OC was present in all samples and at the highest concentration, in the range from to 52,61± 18,59 to 107.99 ± 11,32 ng g^{-1}.

Keywords: UV filters, UV stabilizers, Dolphin, Blubber, MAE, UHPLC-MS/MS.

1. Introduction

Despite the high dilution power of seawater, it's a fact that the marine environment is currently suffering an increasing pollution by the discharge of massive quantities of chemicals. Some of this contamination is caused by emerging pollutants (EPs) that are substances that may be harmful to human health or environment, such as endocrine disruptors, pharmaceutical, personal care products (PCPs), nanoparticles and chemicals used in packaging products (Bo et al., 2015).

Research on water pollution has been commonly focused on compounds like pesticides or heavy metal (Alonso et al., 2014; Bachman et al., 2014; Borrell, 1993; Jarman et al., 1996). Around the 20th century, contamination by EPs has become a matter of interest to the scientific community because of their potential impact on the environment. Some of them are included in European regulations but the majority are not regulated by any legislation. 17β-oestradiol and 17α-ethynylestradiol hormones are examples of compounds recently regulated (Guedes-Alonso et al., 2020).

The entry of pollutants such as PCPs into the marine environment can be indirect through wastewater discharges or direct through recreational activities such as swimming or water sports. Given their diverse behavior and multiple sources of production, the detection and quantification of these contaminants is a challenge for researchers. Although many studies have demonstrated their bioaccumulation, the available data are still very limited, therefore, more research is needed.

UV Filters (UVFs) and UV stabilizers (UVSs) are compounds that are added to PCPs to protect skin and hair from the sunlight, and also in other industrial goods such as paint, wax, plastic, or textile to prevent photodegradation of polymers and pigments (Gago-Ferrero et al., 2013).

UVFs group include several compounds with different chemical characteristics and behavior, most of them used in sunscreens for decades. Among UVSs, benzotriazole UV stabilizers (BUVSs) are a family of substances very used in a variety of cosmetics products (Montesdeoca-Esponda et al., 2020). Indeed, both UVFs and BUVSs are considered EPs and their use is only regulated in some countries. For example, in Hawaii and Palau, the use of some of these compounds has been recently prohibited, while others are not regulated because there are not enough studies to confirm their negative effects (Mendoza, 2018).

UVSs and BUVSs have been studied in different matrices such as sediments (Tsui et al., 2015; Zhang et al., 2011), water (Jeon et al., 2006), seaweeds (Pacheco-Juárez et al., 2019) and marine organisms (Gimeno-Monforte et al., 2020).

Among organisms, most studies have focused on fishes (Mottaleb et al., 2009, Balmer et al., 2005, Buser et al., 2006, Meinerling et al., 2006, Zenker et al., 2008, Fent et al., 2008). and it has been demonstrated their adverse effects, particularly affecting fertility and reproduction.

Although the concentrations found in organisms are generally small, they can be accumulated and biomagnified through the trophic chain (Gago-Ferrero et al., 2012).

Since cetaceans are top predators situated at high trophic level with a worldwide distribution, these animals could present high concentrations of organic pollutants in their tissues. The impact that pollutants can have on them depends on feeding strategy, diet, nutritional status, sex, age, etc. Besides long lifespan, they possess a large absorption capacity and slow elimination, which increases the accumulation of contaminants (Álvarez, 2017). Therefore, marine mammals are considered significant bioindicators of ecosystem and public health (Bossart, 2011; Schaefer et al., 2015).

In order to evaluate the contamination to which cetaceans are exposed, the presence of a variety of environmental pollutants have been studied, such as anthropogenic and natural organohalogen compounds (Vetter et al., 2001), different persistent organic pollutant (POPs) (Bachman et al., 2014), polychlorinated biphenyl (PCB) and dichlorodiphenyltrichloroethane (DDT) (Borrell, 1993), polybrominated diphenyl ethers (PBDEs) (Leonel et al., 2014), isovaleric acid (Koopman et al., 2003), organochlorine compounds, including dibenzo-p-dioxins (PCDDS), and dibenzofurans (PCDFS) (Jarman et al., 1996). However, studies of the presence of EPs in cetaceans are very scarce.

Considering that human health depends to a large extent on the ocean environment and that cetaceans are considered as bioindicators of the ecosystem, this work has the aim of develop and validate a procedure for determining 12 UVFs and UVSs in dolphins. The selected compounds present log K_{ow} in the range 6-12 (*Table 1*), which indicates that they are highly non-polar compounds with a tendency to bioaccumulate. To the best of our knowledge, only three works have been conducted to determine UVFs and UVSs in dolphins (*Table 2).* Nakata et al. (2010) determined BUVSs in blubber of dolphin, while UVFs were studied in blood (Gago-Ferrero et al., 2012) and liver (Gago-Ferrero et al., 2013).

Table 1. *Procedures used to determine UVFs and UVSs in different dolphin tissues.*

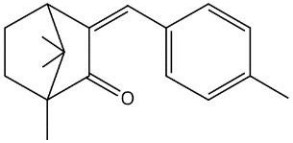
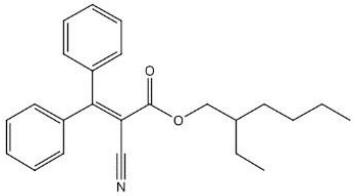
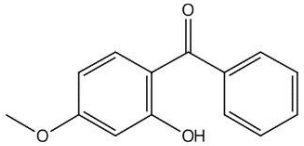
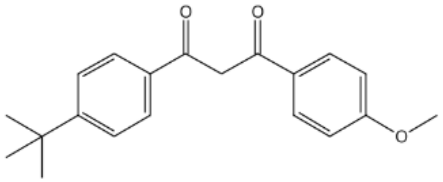
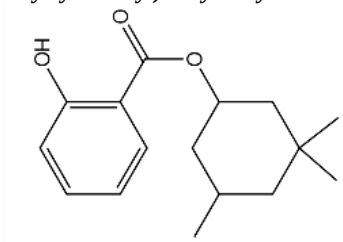
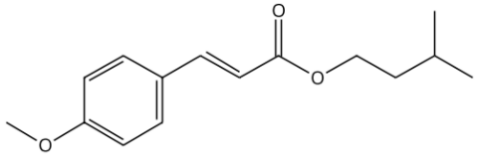
Tissue	Target compounds	Extraction procedure	Determination technique	Reference
Blubber	UV-320 UV-327 UV-328	Soxhlet	GC-HRMS	(Nakata et al., 2010)
Liver	OC	PLE (pressurized liquid extraction)	UPLC-ESI-MS/MS	(Gago-Ferrero et al., 2013)
Blood	UV-328 UV-329 UV-324	LLE (Liquid-Liquid extraction)	UPLC-ESI-MS/MS	(Gago-Ferrero et al., 2012)

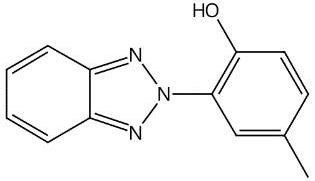
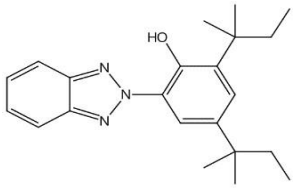
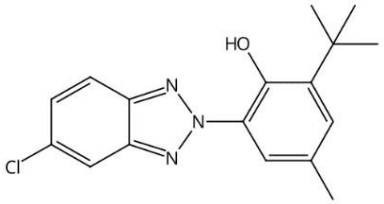
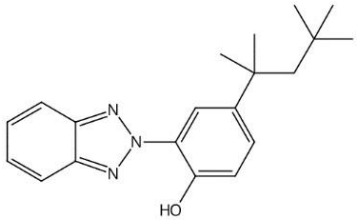
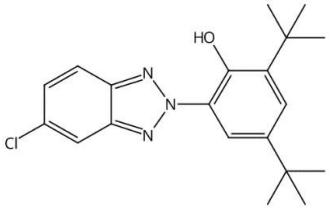
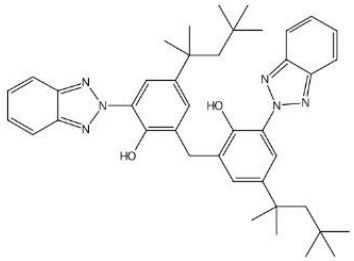
Our study is focused on adipose tissue samples from common bottlenose dolphin (*Tursiops truncatus*). This specie has been studied in Canary Islands regarding the presence of POPs (García-Alvarez et al., 2014; García-Álvarez et al., 2014) and metals (García-Alvarez et al., 2015).

Skin comprises the animal's epidermis, dermis, and hypodermis (Martín Díaz, 2019). This last and deepest layer of the skin, in cetaceans is also known as blubber, which is a dense vascularized layer of fat held by a structure of elastic and collagen fibers. Blubber has long been recognized as the primary and most important site of fat, and thus also energy, storage in cetaceans. Moreover, it has several other important functions such as to serve as an efficient and adjustable thermal insulator, provide buoyancy, help the hydrodynamic locomotion of the animal, etc. (Struntz et al., 2004; Iverson, 2009). Due to its high fat content, this layer can concentrate greatest levels of toxic lipophilic compounds that get into the body through feeding. Thus, dolphins have been proposed as sentinels to evaluate aquatic ecosystem health and identify damaging environmental trends (Bossart, 2011).

That complex matrix requires to implement an efficient extraction procedure to isolate the target compounds from the sample. Microwave assistant extraction (MAE) represents an important alternative for the extraction of certain analytes from solid samples, as it reduces sample processing time and allows the preparation of multiple samples in a single step. In addition to efficiency, MAE allows the use of smaller volumes of organic solvents than other methodologies and facilitates the control of parameters such as time, temperature and power (Esteve-Turillas et al., 2004). After extraction procedure, the separation and detection of target compounds were carried out with ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS).

Table 2. *Characteristics of the compounds of concern.*

UVFs			
Structure	***Compound***	***Structure***	***Compound***
4-Methylbenzylidene camphor ***1,7,7-trimethyl-3 - [(4-methylphenyl) metilen] biciclo [2.2.1] heptan-2-ona*** 	***4MBC*** MF: $C_{18}H_{22}O$ MW: 254.37g/mol CAS: 36861-47-9 $Log\ K_{ow}$ =4.95	***2-ethylhexyl 2-cyano-3,3-diphenylprop-2-enoate*** 	***OC*** MF: $C_{24}H_{27}NO_2$ MW: 361,48g/mol CAS: 6197-30-4 $Log\ K_{ow}$ =6.88
2-Hydroxy-4-methoxybenzophenone 	***BP-3*** MF: $C_{14}H_{12}O_3$ MW: 228,24 g/mol CAS: 131-57-7 $Log\ K_{ow}$ =3.79	***1-(4-tert-butylphenyl)-3-(methoxyphenyl)propane-1,3-dione*** 	***BMDBM*** MF: $C_{20}H_{22}O_3$ MW: 310,39g/mol CAS: 70356-09-1 $Log\ K_{ow}$ =4.51
(3,3,5-trimethylcyclohexyl) 2-hydroxybenzoate 	***HMS*** MF: $C_{16}H_{22}O_3$ MW: 262,34 g/mol CAS: 118-56-9 $Log\ K_{ow}$ =6.16	***3-methylbutyl (E)-3-(4-methoxyphenyl) prop-2enoate*** 	***IMC*** MF: $C_{15}H_{20}O_3$ MW: 248,32 g/mol CAS: 71617-10-2 $Log\ K_{ow}$ =4.33

UVSs			
Structure	***Compound***	***Structure***	***Compound***
2-(benzotriazol-2-yl)-4-methylphenol 	***UV-P*** MF: $C_{13}H_{11}N_3O$ MW: 22525g/mol CAS: 2440-22-4 $Log\ K_{ow}$ =2.99	***2-(benzotriazol-2-yl)-4,6-bis(2-methylbutant-2-yl) phenol*** 	***UV-328*** MF: $C_{22}H_{29}N_3O$ MW: 351,49g/mol CAS: 25973-55-1 $Log\ K_{ow}$ =7.25
2-tert-butyl-6-(5-chlorobenzotriazol-2-yl)-4methylphenol 	***UV-326*** MF:$C_{17}H_{18}ClN3O$ MW: 315,80g/mol CAS: 3896-11-5 $Log\ K_{ow}$ =5.55	***2-(benzotriazol-2-yl)-4,6-bis(2-methylpentan-2-yl) phenol*** 	***UV-329*** MF: $C_{20}H_{25}N_3O$ MW: 323,43g/mol CAS: 3147-75-9 $Log\ K_{ow}{}^{b}$ =6.21
2,4-ditert-butyl-6-(5-chlorobenzotriazol-2-yl) phenol 	***UV-327*** MF: $C_{20}H_{24}ClN_3O$ MW: 357,88g/mol CAS: 3864-99-1 $Log\ K_{ow}$ =6.91	***2-(benzotriazol-2-yl)-6-[[3-(benzotriazol-2-yl)-2hydroxy-5-(2,4,4-trimethylpentan-2-yl) phenyl] methyl]-4-(2,4,4-trimethylpentan-2-yl) phenyl*** 	***UV-360*** MF: $C_{41}H_50N_6O_2$ MW: 658,87g/mol CAS: 103597-45-1 $Log\ K_{ow}$ =12.5

MF: Molecular formula; MW: Molecular Weight; CAS: Chemical Abstracts Service number; Log $K_{ow}{}^{b}$: Octanol-water partition coefficient.

All the information was obtained from SciFinder.

2. Materials and methods

2.1. Reagents and consumables

The studied UVFS were 4-Methylbenzylidene camphor **(4-MBC)**; 2-Hydroxy-4methoxybenzophenone **(BP3)**; 2-ethylhexyl 2-cyano-3,3-diphenylprop-2-enoate **(OC)**; 1-(4-tert-butylphenyl)-3-(methoxyphenyl)propane-1,3-dione **(BMDBM)**; 3-methylbutyl (E)-3-(4-methoxyphenyl)prop-2-enoate **(IMC)** and 3-methylbutyl (E)-3-(4methoxyphenyl)prop-2-enoate **(HMS)**, while the selected BUVSs compounds were 2(benzotriazol-2-yl)-4-methylphenol **(UV-P)**; 2-tert-butyl-6-(5-chlorobenzotriazol-2-yl)4-methylphenol **(UV-326)**; 2,4-ditert-butyl-6-(5-chlorobenzotriazol-2-yl) phenol **(UV327)**; 2-(benzotriazol-2-yl)-4,6-bis(2-methylbutant-2-yl) phenol **(UV-328)**; 2(benzotriazol-2-yl)-4,6-bis(2-methylpentan-2-yl) phenol **(UV-329)**; 2-(benzotriazol-2yl)-6-[[3-(benzotriazol-2-yl)-2-hydroxy-5-(2,4,4-trimethylpentan-2-yl) phenyl] methyl]4-(2,4,4-trimethylpentan-2-yl) phenyl **(UV-360).** Their characteristics are shown in *Table 1*.

Stock solution of 250 ng L^{-1} was prepared in acetone and stored in a glass container at 25 ºC under dark conditions, while the daily standards were prepared in methanol. Target compounds were obtained from Sigma-Aldrich (Madrid, Spain) and Diatomaceous earth from Macherey-Nagel (Duren, Germany). Solvents used as extractants and mobile phases were obtained from Panreac Quimica (Barcelona, Spain). Phree Phospholipid Removal Solid Phase Extraction (SPE) cartridges were bought from Phenomenex España (Madrid, Spain) and the 0.2 µm syringe polyethylene terephthalate (PET) filters from Macherey-Nagel (Dueren, Germany).

2.2. Sample collection and preparation

All the specimens of bottlenose dolphin (*Tursiops truncatus*) analyzed in this work had stranded on the coasts of the Canary Islands. They were necropsied at the "Instituto de Sanidad Animal y Seguridad Alimentaria" (IUSA) and the blubber tissues were stored at -80 ºC until the time of analysis.

2.3. Instrumentation

The microwave oven used for the extraction was a TITAN MPS with 16 vessels were purchased from PerkinElmer (Madrid, Spain). The determination of the compounds was carried out by an ACQUITY-UHPLC (Waters Chromatography, Barcelona, Spain).

It was equipped with a Binary Solvent Manager (BSM), a 2777 autosampler, a column manager and a triple quadrupole mass spectrometry detector (TQD) with an electrospray interface (ESI). All components were monitored with MassLynx Mass Spectrometry software.

2.4. Chromatographic and detection conditions

For the separation of the compounds, an ACQUITY UHPLC Waters BEH C18 column (50 × 2.1 mm and 1.7 µm particle size) was used with a flow rate of 0.4 mL min^{-1} at 35 ºC.

The mobile phase consists of water with 0,1% of formic acid and methanol. The gradient started with A: 25% of water and B: 75% of methanol, both with 0.1 % formic acid, and then reaches 100% B in 3 min, which remains until 5 min. At 6 min the composition come back to initial conditions and the system is allowed to equilibrate up to 7 min before the next injection.

The ESI parameters for the mass spectrometry detection were capillary voltage at 4 kV, extractor voltage at 2 V, cone voltage at 30 V, radio frequency (RF) lens voltage at 1 V, desolvation and source temperatures at 150 and 450 ºC respectively, desolvation gas flow at 500 L hr^{-1} and finally cone gas flow at 50 L hr^{-1}. As desolvation gas Nitrogen was used and Argon as the collision gas.

3. Results and discussion

3.1. Optimization of MAE

The variables that affect the MAE procedure must be optimized in order to achieve the best extraction efficiencies for each compound. The involved parameters are temperature, time and extractant agent. To study the influence of each one, experimental designs were carried out with spiked samples to know their contribution to analytical response and the relation among them.

Given the impossibility to spike dolphin adipose tissues at laboratory, some authors often decide to develop the methodology in a similar matrix such as butter or olive oil (Álvarez, 2017; Nakata et al., 2010). In this work we used butter to optimize the extraction methodology by means of two experimental designs.

First, a 2^3 design (two levels for three variables) was used: 5 and 10 min of extraction time, 50 and 55 ºC of temperature and hexane and acetone as extractant solvent. Taking 100 mg of butter sample spiked with 420 μL of the stock solution of 250 ng L^{-1} , 7 ml of extractant were added and proceeded to extract under the conditions detailed in *Table 3*. Three replicates were used for each experiment.

Table 3. *Summary of the conditions of the first experimental design.*

RUN	Time (min)	Temperature (ºC)	Extractant
1	5	50	Hexane
2	5	55	Hexane
3	10	50	Hexane
4	10	55	Hexane
5	5	50	Acetone
6	5	55	Acetone
7	10	50	Acetone
8	10	55	Acetone

Once the extraction was finished, the sample was transferred to glass vial and the extractant was evaporated. The residue sample was then reconstituted with 1 mL of methanol and sonicated. Since it was observed that the sample became turbid after sonication, a centrifugation step at 3000 rpm for 10 min was implemented. Finally, the extract was filtered through a 0,20 µm syringe filter prior to being introduced into the UHPLC-MS/MS equipment.

The measured responses (area below obtained chromatographic peaks) for each experiment and for each compound were processed in the software Minitab. The mean, standard deviation and relative standard deviation were calculated and Pareto graphs were built.

Figure 1 shows the results obtained for UV-P compound. The interpretation of the graph provides information of the influence of each variable, as well as their combined effect. The significance level represented by a red discontinuous line is the value at which the factor has a significant effect. The blue bars correspond to the factors that exceed the significance level. Finally, the gray bars denote those that do not exceed the significance level. As can be seen, for the UV-P compound, the significance level is 2,35. The factors whose effect exceeds this significance level are time, temperature, extractant and the combined influence of temperature-extractant. The factors individually influence in the following order: time, temperature and extractant.

After the analysis of the Pareto diagrams, the calculation of Pearson correlation allows to know the trend of the effect, with 0 being no influence, -1 the maximum negative effect and 1 the maximum positive effect. Thus, it could be concluded that times present a positive correlation for all compounds except for UV-360. On the contrary, temperature has a negative correlation for all compounds.

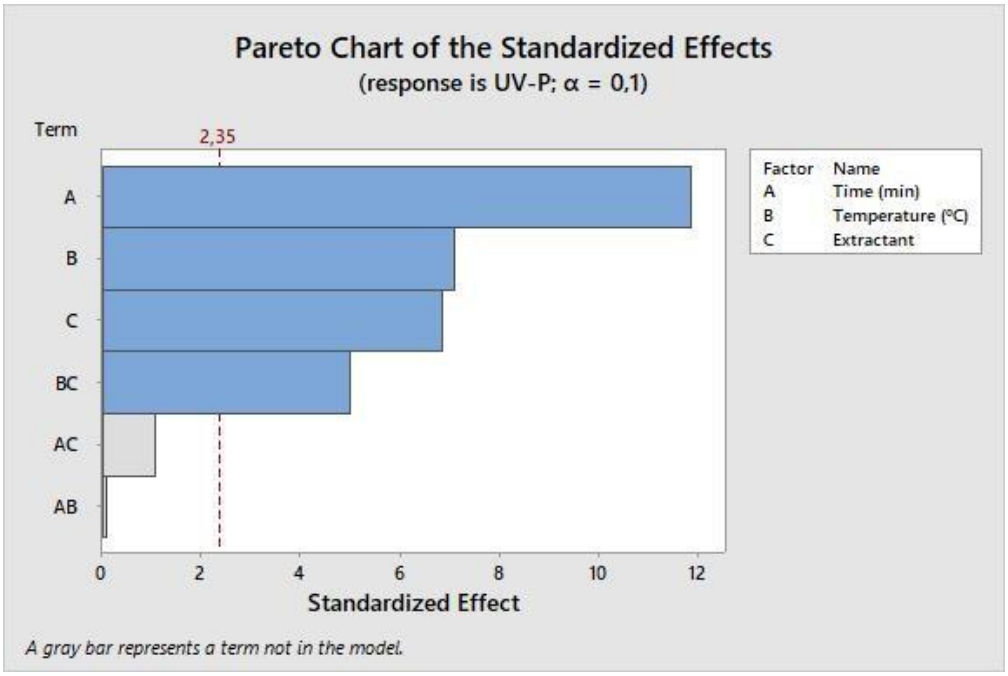


Figure 1. *UV-P Pareto chart of standardized effects for 2^3 experimental design.*

Based on the correlations results, a 3^2 experimental design was built with two variables and three factors: 10, 12 and 14 min and 50, 52 and 54 ºC (*Table 4*). Hexane was chosen as the extractant since no significant influence was found for this variable.

Table 4. *Summary of the conditions of the second experimental design*

RUN	Time (min)	Temperature (ºC)
1	12	52
2	14	54
3	10	52
4	14	50
5	12	50
6	10	54
7	10	50
8	12	54
9	14	52

From the results of these experiments, it is possible to conclude that lowest tested temperatures and times provides better results, as can be seen in the contour graph corresponding to the OC compound (*Figure 2)*. Same behavior was observed for the majority of studied compounds. Therefore, MAE procedure was established with a time of 10 min and a temperature of 50 ºC.

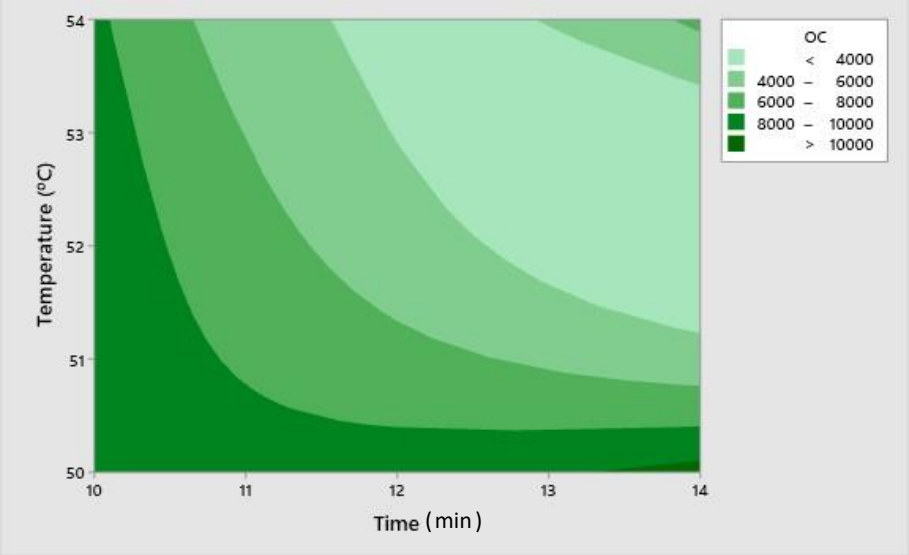


Figure 2. *Response surface for the effect of temperature (ºC) and extraction time (min) on the OC compound extraction*

In summary, the extraction conditions were the following: 100 mg of sample spiked with target analytes was extracted with 7 mL of hexane for 10 min at 50 ºC. The whole procedure is shown in *Figure 3*, where it can be seen that after MAE the extracts were evaporated, reconstituted with 1 mL of methanol, sonicated, centrifugated and filtered prior to being introduced into the UHPLC-MS/MS equipment.

3.2. Validation of the MAE-UHPLC-MS/MS method

Once the method was optimized regarding all the involved parameters, the analytical parameters (detection/quantification limits, relative recovery, precision and matrix effect) was calculated to validate the optimized method for the determination of targets UVFs and BUVs in blubber samples.

Calibration curves were made using eight concentration levels in the range 1-500 ng mL^{-1} of each target compound. Linear correlation coefficients higher than 0,99 were obtained for all of them.

For each compound, MS/MS detection parameters were established, and this information is presented with *table 5*.

Tabla 5. *Determined parameters for BUVSs and UV filter detection*

Compound	Precursor ion (m/z)	Cone voltage (V)	Quantification ion (m/z)	Collision potential (V)	Confirmation ion (m/z)	Collision potential (V)
4-MBC	255.4	25	105	27	171	19
BP-3	229.0	32	151	20	105	25
HMS	263.1	12	139	10	121	30
OC	362.4	28	250	12	232	20
BMDBM	311.2	30	161	23	135	23
IMC	249.1	17	161	15	179	29
UV-P	226.2	40	107	20	120	20
UV-326	316.3	40	57	25	260	20
UV-327	358.3	60	57	30	302	20
UV-328	352.3	50	71	30	282	20
UV-329	324.3	50	57	25	212	25
UV-360	658.6	40	336	25	224	35

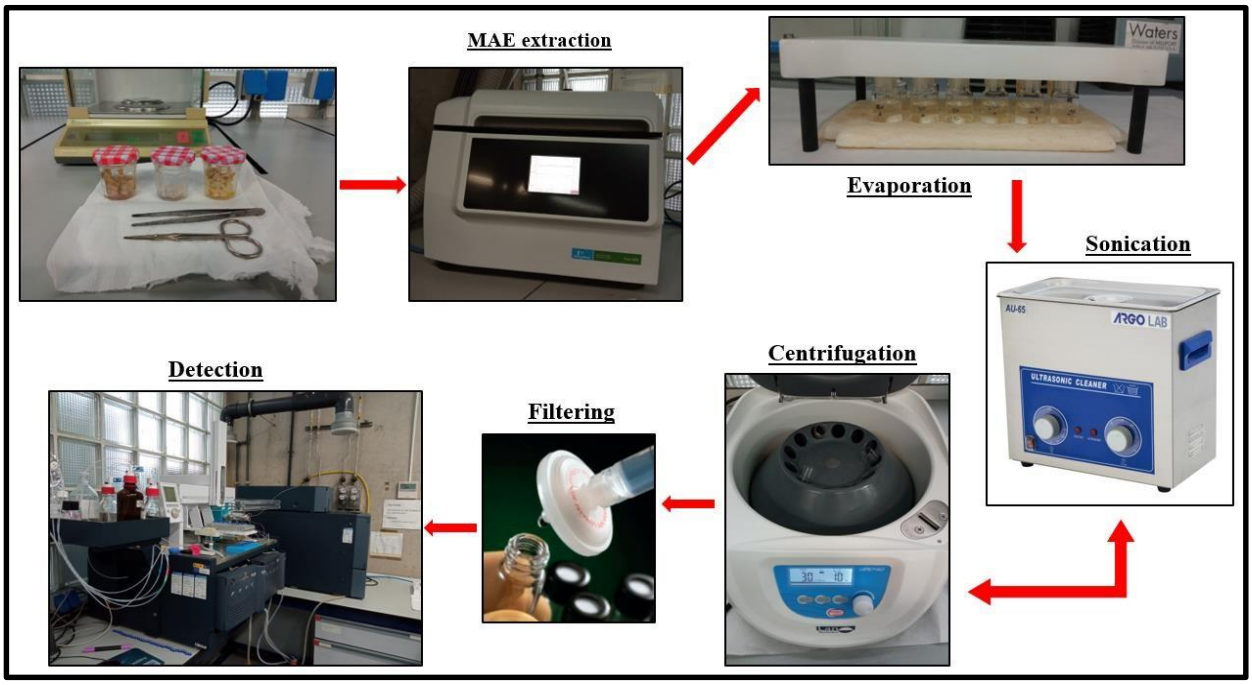


Figure 3. *Schematic summary procedure of the optimized method for UVFs and UVSs.*

The limits of detection (LODs) and the limits of quantification (LOQs) showed in *Table 6* were obtained from the signal/noise (S/N) response of the individual compounds from lowest point of the calibration curve, assuming minimum detectable S/N levels of 3 and 10, respectively. LODs varied between 0,001 and 46,73 ng mL^{-1} while the LOQs were in the range of 0.003to 15.58 ng mL^{-1}.

Table 6. *Instrumental LODs and LOQs of target analytes in UHPLC-MS/MS.*

Compounds	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
4BMC	0,011	0,037
BP3	0,034	0,112
HMS	46,729	155,607
OC	0,088	0,293
BMBDM	28,736	95,690
IMC	0,057	0,189
P	7,772	25,881
326	0,322	1,073
327	19,841	66,071
328	2,447	8,148
329	2,451	8,162
360	0,001	0,003

It must be considered the matrix effect that such complex samples cause in the detection system. High ionic suppression values (in the range 73,43 to 100%, *Table 7*) were calculated by comparison of the signal provided by standards prepared in methanol at three levels (theoretical concentrations of 50, 250 and 500 ng mL^{-1} in the final extract) with others prepared in extracts of blank dolphin sample after MAE procedure. Matrix effect was also calculated in a MAE extract of butter, in order to know if the interferences of this substitutive matrix are comparable with dolphin samples. Results were quite similar, but ionic suppression slightly higher were obtained for butter.

Precision and relative recovery were also tested at the same concentration levels (50, 250 and 500 ng mL^{-1} in the final extract) as shown in *Table 7*. The precision of the method was obtained from the relative standard deviation of three replicates, and it was lower than 20% in most of cases. Some higher values were recorded for some compounds, especially at lowest concentrations. Results of relative recoveries, calculated by comparison of dolphin sample spiked after and before of MAE procedure, were in the range of 55,20 to 97,90 %, except for UV-360, which showed poor extraction with developed method.

Table 7. *Analytical parameters for the developed MAE-UHPLC-MS/MS method*

	Matrix Effect (%)	Precision (%)			Relative recovery (%)		
		50 (ng mL^{-1})	250 (ng mL^{-1})	500 (ng mL^{-1})	50 (ng mL^{-1})	250 (ng mL^{-1})	5000 (ng mL^{-1})
4BMC	86,12-89,94	1,41	11,01	6,36	56,15	55,20	70,67
BP3	84,46-91,18	2,63	3,64	4,22	77,77	55,48	80,71
HMS	97,42-100		-	18,61	-	-	95,00
OC	83,39-87,44	24,66	0,54	13,33	68,25	60,08	62,20
BMBDM	80,58-86,12	22,01	4,03	27,89	70,95	46,91	68,21
IMC	86,20-90,42	24,20	0,50	13,47	60,03	61,52	76,42
P	91,03-94,37	22,63	8,84	17,63	82,30	58,18	71,53
326	83,85-100	-	-	19,52	-	-	90,17
327	91,48-100	-	-	-	-	-	-
328	86,71-95,66	-	-	8,46	-	-	83,42
329	73,43-74,56	7,23	6,66	15,67	97,90	83,96	94,12
360	94,97-97,29	24,15	9,45	16,05	28,47	14,47	24,83

3.3. Presence of UVFs and UVSs in blubber samples of dolphin

Once validated, the developed method was applied to the determination of target compound in five real samples of common bottlenose dolphin (*Tursiops truncatus*). Blubber of individuals stranded on the coasts of the Canary Islands, which information is presented in *Table 8*, was analyzed.

Table 8. *Information of the dolphins studied*

SAMPLE CODE	SEX	AGE RANGE	SEXUAL MATURITY	BODY CONDITION	STRANDING DATE	ISLAND
CET1020	Female	Juvenile	Immature	Moderate	09/08/2019	Tenerife
CET1042	Male	Adult	Mature	Poor	23/11/2019	Tenerife
CET1103	Male	Juvenile	Immature	Moderate	13/06/2020	Gran Canaria
CET1133	Male	Adult	Mature	Poor	31/10/2020	Fuerteventura
CET1151	Male	Juvenile	Immature	Poor	21/02/2021	Tenerife

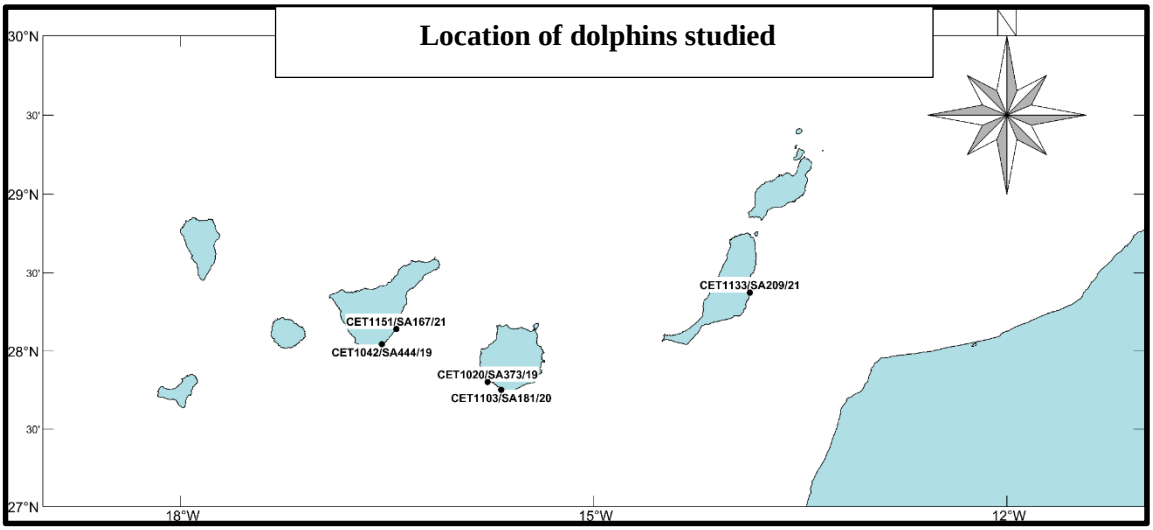


Figure 4. *Stranding area of the dolphins studied.*

Three replicates of 100 mg of blubber of each sample were weighed and 50 mg of diatomaceous earth were used to remove moisture from the adipose tissue sample. Then, optimized MAE-UHPLC-MS/MS procedure was applied and the results of are shown in *Table 9.*

Table 9. *Concentration of target compounds analytes (ng g^{-1}) in blubber samples*

Dolphin Code	BP-3	OC	IMC
CET1020	nd	52,61± 18,59	8,55 ± 1,19
CET1042	5,92 ± 0,04	107,99 ± 11,32	nd
CET1103	nd	54,01 ± 6,01	nd
CET1133	nd	54,93 ± 2,18	nd
CET1151	nd	77,85 ± 4,60	nd

nd: not detected

OC was detected in all the samples, also presenting the highest concentrations among the analyzed compounds (in the range from 52,61± 18,59 to 107,99 ± 11,32 ng g^{-1}). BP3 and IMC were found only in one sample, at 5,92 ± 0,04 ng g^{-1} and 8,55 ± 1,19 ng g^{-1}, respectively.

The presence of OC in all analyzed samples is of great concern as it is a highly lipophilic compound with a log K_{ow} 6.88, stable and resistant to degradation by sunlight, for which some studies show that it can trigger the production of potentially harmful free radicals (reactive oxygen species) when it releases the absorbed energy (Gago-Ferrero et al., 2013). The results seem to demonstrate that dolphins can accumulate relatively high levels

of organic pollutants in their bodies since they occupy a high trophic level in the marine food chain and their metabolic activity is relatively low (Tanabe, 2002).

It is also important to remark the presence of two different compounds in the same specimen, namely BP-3 (5,92 ± 0,04 ng g-1) and OC (107,99 ± 11,32 ng g1) in sample CET1042/SA444/19, and OC (33,59 ± 18,59 ng g-1) and IMC (8,55 ± 1,19 ng g^{-1}) in sample CET1020/SA373/19. This result is worrying as it reflects that the same specimen can accumulate a variety of contaminants that could have different behavior and produce different adverse effects. Moreover, it is possible that interactions between them could produce synergistic effects and greater toxicity.

4. Conclusions and future trends

The study of UVFs and UVSs in cetaceans has a really important purpose, as it is essential to know the possible impact of human discharge of these compounds into the environment. As they are used as indicators of the state of the environment where they live, the presence of these contaminants is alarming because of the possible effects on the organism and the lack of knowledge of the quantity of pollutants present on them.

A MAE-UHPLC-MS/MS method that allows the determination of 12 compounds in blubber of dolphins in a simple and fast way, has been developed. It can perform the simultaneous extraction of sixteen samples at once.

However, the limitations of this methodology must be considered. The blubber, being a fatty tissue, is composed of cells called adipocytes, and although initially the extraction optimization was attempted with the real matrix, this option had to be discarded as it was impossible to spike the sample in a reliable way. Therefore, an alternative matrix with similar properties as the blubber, in this case butter, was employed. Although the behavior of both matrices was quite similar; the interferences of butter were slightly higher. Then, both blubber and butter present an important matrix effect, which cause ionic suppression in MS/MS detection and also problems during the extraction using MAE procedure. Therefore, a more similar matrix is required in order to carry out the validation of the method in a more reliable way, and procedures to avoid interferences must be found. Purification materials such as solid phase extraction could be implemented in the future to remove interferences, especially lipids. Moreover, calibration methods such as matrix match calibration could be useful to overcome the matrix effects.
Regardless of the accuracy of the quantitative determination, the developed method allowed to demonstrate the presence of three target compounds. OC was the compound

most commonly detected and presented the highest concentrations. The no detection of the other target compounds can be caused by their absence in the organisms studied or can be related with the sensitivity of the method caused for the matrix effects. Future studies will search solutions to determine the target compounds in cetaceans samples with better sensitivity and extraction efficiencies.

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