Kimika Analitikoa Saila

Metodo analitikoen garapena disruptore endokrinoen biokontzentrazioa arrainetan determinatzeko

Development of analytical methods to estimate the bioconcentration of different endocrine disrupting compounds in fish

A thesis submitted for the international degree of
Doctor of Philosophy

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ESKER ONAK!!!!!!!!!!!!!!!!!!

4 urte igaro dira dagoeneko eta sinetsi ezin dudan arren, tesia idatzi dut!!! Nondik hasi ere ez dakit, hainbeste izan zarete tesi osoan zehar laguntzeko prest egon zaretenak…

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Bilbon, ekainak 20

Abbreviations / Laburdurak vii

1-Sarrera

1. Lehentasunezko Konposatuak eta emergenteak 03
    1.1. Konposatu disruptore endokrinoak 07
        1.1.1. Musketa fragantziak 09
        1.1.2. Bisfenola-A 10
        1.1.3. Pestizidak 12
        1.1.4. Estrogenoak 14
        1.1.5. Ftalato esterrak 16
        1.1.6. Alkilfenolak 18
2. Konpostu kimikoak ingurumenean 19
    2.1. Konposatu kimikoak ur-ingurumenean 19
    2.2. Konposatu kimikoak biota laginetan 23
3. Ingurumenean dauden konpostu kimikoen analisia 30
    3.1. Erauzketa-teknikak 30
        3.1.1. Lagin likidoak 30
        3.1.2. Lagin solidoak 33
    3.2. Garbiketa-teknikak 35
    3.3. Hidrolisi-urratsa 37
4. Konpostu kimikoen azterketa 40
    4.1. Gas-kromatografia 40
    4.2. Likido-kromatografia 46
5. Konposatu kimikoen metaketa 49
    5.1. EDCen metaketa arrainetan 50
6. Erreferentziak 53

2-Helburuak / Objectives

1. Helburuak 75
2. Objectives 77

3-Microextraction with polyethersulfone for the analysis of priority and emerging compounds in water samples

1. Introduction 81
2. Materials and methods 83
   2.1. Reagents and materials 83
   2.2. Sampling 84
   2.3. Extraction, desorption and derivatisation procedures 85
   2.4. Gas chromatography-mass spectrometry analysis 86
   2.5. Liquid chromatography-tandem mass spectrometry analysis 87
3. Results and discussions 88
   3.1. Optimisation of LC-MS/MS 88
   3.2. Optimisation of sample preparation conditions 88
      3.2.1. PES desorption solvent nature and time 88
      3.2.2. Extraction parameters evaluation 90
      3.2.3. Optimisation of the stirring speed 91
      3.2.4. Time profile 92
   3.3. Matrix effect evaluation 93
   3.4. Performance of the analytical methods 95
      3.4.1. PES-GC-MS analysis of non-polar compounds 96
      3.4.2. PES-silylation-GC-MS vs PES-LC-MS/MS for polar compounds 99
   3.5. Application of the developed methods to real samples 102
4. Conclusions 103
5. References 105

4-Determination of endocrine disrupting compounds and their metabolites in fish bile

1. Introduction 113
2. Materials and methods 115
   2.1. Reagents and materials 115
   2.2. Bile analysis 117
      2.2.1. Sample collection 117
      2.2.2. Extraction and clean-up of bile samples 118
      2.2.3. Water analysis 119
      2.2.4. GC-MS analysis 119

3. Results and discussions 120
3.1. Optimisation of the derivatisation step 120
3.2. Optimisation of the solid phase extraction step 121
3.3. Study of the matrix effect and optimisation of the clean-up step 123
3.4. Performance of the analytical method 127
3.5. Application to real samples 129
4. Conclusions 135
5. References 136

5-Simultaneous enzymatic hydrolysis and extraction of endocrine disrupting compounds in fish bile using polyethersulfone polymer

1. Introduction 143
2. Materials and methods 149
2.1. Reagents and materials 149
2.2. Synthesis and separation of individual glucuronide compounds 151
2.3. Bile collection 152
2.4. Simultaneous enzymatic hydrolysis and extraction of bile samples 153
2.5. LC-MS/MS analysis 153
3. Results and discussions 154
3.1. Optimisation of sample preparation 154
3.1.1. Evaluation of polymeric material 155
3.1.2. Enzyme amount 156
3.1.3. Time profile 157
3.1.4. Desoprtion solvent nature and time 158
3.2. Study of the matrix effect 159
3.3. Performance of the analytical methods 161
3.4. Application to real samples 164
4. Conclusions 164
5. References 166

6-Determination of endocrine disrupting compounds in fish liver, brain and muscle using focused ultrasounds solid-liquid extraction and dispersive solid phase extraction as clean-up strategy

1. Introduction 169
2. Materials and methods 171
   2.1. Reagents and materials 171
   2.2. Sample collection and pretreatment 172
   2.3. Sample extraction and clean-up 174
   2.4. LC-MS/MS analysis 173
3. Results and discussions 174
   3.1. Optimisation of the clean-up strategy 174
   3.2. Optimisation of the extraction temperature and extractant nature 177
   3.3. Evaluation of the matrix effect 180
   3.4. Performance of the analytical methods 184
   3.5. Application to real samples 186
4. Conclusions 188
5. References 189

7-Galindoko araztegiko ur-efluenteari esposatutako arrainen behazunean neurtutako disruptore endokrinoen biokontzentrazio-mailen determinazioa

1. Sarrera 197
2. Materialak eta metodoak 199
   2.1. Erreaktiboak eta materialak 199
   2.2. Arrainen esposizio esperimentuak 200
   2.3. Ur-laginen tratamendua eta analisia 201
      2.3.1. Ur-laginen azterketa 201
      2.3.2. Arrain-behazunaren azterketa 203
   2.4. Metodoaren kalitate parametroak 204
   2.5. Biokontzentrazio balioen estimazioa 205
3. Emaitzak eta eztabaida 206
   3.1. EDCen metaketa 206
   3.2. Behazunaren metatze/eliminazio gaitasunaren azterketa 213

3.3. Material polimerikoen erabilera arrainaren behazunaren metaketa-ahalmena simulatzeko 214
4. Ondorioak 215
5. Erreferentziak 217

8-Disruptore endokrinoetara esposatutako lupien (*Dicentratchus labrax*) organo desberdinetako metaketa-azterketa, kontrolpeko baldintzetan

1. Sarrera 223
2. Materialak eta metodoak 226
2.1. Erreaktiboak eta materialak 226
2.2. Lagin-bilketa 227
2.3. Laginen azterketa 228
2.3.1. Lagin solidoen azterketa 228
2.3.2. Lagin likidoen azterketa 229
2.3.3. Ur-laginen azterketa 229
2.4. LC-MS/MS-aren bidezko azterketa 230
2.5. Kalitate parametroak 231
3. Emaitzak eta eztabaida 231
3.1. Organoen neurriak eta itxura 231
3.2. Ur-laginak 235
3.3. Konposatuen metaketa arrainen organo desberdinetan 236
3.3.1. Analitoen metaketa behazunean 236
3.3.2. Analitoen metaketa gibelean 238
3.3.3. Analitoen metaketa plasman 239
3.3.4. Analitoen metaketa burmuinean eta muskuluan 240
3.4. Konposatuen metaketa egunetan zehar 241
4. Ondorioak 242
5. Erreferentziak 244

9-Conclusions

Conclusions 251

Supplementary material / Eranskinak III

Abbreviations / Laburdurak

0-9

2,4´-DDD: 2,4-bis(p-chlorophenyl)ethane / 2,4-bis(p-klorofenil)etanoa.

2,4´-DDT: 1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene / 1-kloro-2-[2,2,2-trikloro-1-(4-klorofenil)etil]bentzenoa.

4,4´-DDD: 2,2-bis(p-chlorophenyl)ethane / 2,2-bis(p-klorofenil)etanoa.

4,4´-DDT: 1,1′-(2,2,2-trichloroethylidene)bis[4-chloro] / 1,1′-(2,2,2-trikloroetilideno)bis[4-kloro].

4*n*OP: 4-*n*-octylphenol / 4-*n*-oktilfenola.

4*t*OP: 4-*tert*-octylphenol / 4-*tert*-oktilfenola.

4-*NP-G*: 4-nonylphenol glucuronide / 4-nonilfenol glukuronidoa.

Symbols/ Sinboloak

α-HCH: α-hexachlorocyclohexane / α-hexakloroziklohexanoa.

β-HCH: β-hexachlorocyclohexane / β-hexakloroziklohexanoa.

γ-HCH: γ-hexachlorocyclohexane / γ-hexakloroziklohexanoa.

δ-HCH: δ-hexachlorocyclohexane / δ-hexakloroziklohexanoa.

$[^{2}H_{8}]$-4,4′-DDT: $[^{2}H_{8}]$-4,4′-1,1′-(2,2,2-trichloroethylidene)bis[4-chloro]) / $[^{2}H_{8}]$-4,4′-1,1′-(2,2,2-trikloroetilideno)bis[4-kloro]).

[$^2H_{16}$]-BPA: [$^2H_{16}$]-bisphenol-A / [$^2H_{16}$]-bisfenol-A.

[$^2H_4$]-DEHP: [$^2H_4$]-bis(2-ethylhexyl) phthalate / [$^2H_4$]-bis(2-etilhexil) ftalatoa.

[$^2H_3$]-E2: [$^2H_3$]-17β-estradiol / [$^2H_3$]-17β-estradiola.

[$^2H_{15}$]-MX: [$^2H_{15}$]-musk xylene / [$^2H_{15}$]-musk xilenoa.

[$^2H_4$]-NP: [$^2H_4$]-4*n*-nonylphenol / [$^2H_4$]-4*n*-nonilfenola

A

Ace: acetone / azetona.

ACN: acetronitrile / azetonitriloa.

AHTN: tonalide / tonalidea.

ANOVA: analysis of variance / bariantza-analisia.

APCI: atmospheric pressure chemical ionization / presio atmosferikoko ionizazio-kimikoa.

AP_1EOs: alkyl phenol monoetoxilates / alkilfenol monoetoxilatuak.

AP_nEOs: alkyl phenol polyetoxilates / alkilfenol polietoxilatuak.

APGC: atmospheric pressure gas cromatography / presio atmosferikoko gas-kromatografia.

APPI: atmospheric pressure photo ionisation / presio atmosferikoko fotoionizazioa.

APs: alkylphenols / alkilfenolak.

ASE: accelerated solvent extraction / disolbatzailearen bidezko erauzketa azeleratua.

B

BAF: bioaccumulation factor / bioakumulazio-faktorea.

BBP: butyl benzyl phthalate / butil bentzil ftalatoa.

BCF: bioconcentration factor / biokontzentrazio-faktorea.

Benzocaine: ethyl-4-aminobenzoate salt / etil-4-aminobentzoato gatza.

BPA: bisphenol-A / bisfenol-A.

BSA: N,O-bis (trymethylsilyl) acetamide / N,O-bis (trimetilsilil) azetamida.

BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide / N,O-bis(trimetilsilil)trifluoroazetamida.

C

C_{18}: octadecylsilane / oktadezilsilanoa.

CAS: chemical abstract service / konposatu kimikoen laburdura zerbitzua.

CCD: central composite design / diseinu konposatu zentrala.

CCL: contaminant candidate list / kutsatzaileen hautagai zerrenda.

CE: collision energy / talka-energia.

CF: concentration factor / kontzentrazio-faktorea.

CHL: cholesterol / kolesterola.

CI: chemical ionisation / ionizazio kimikoa.

CIS: cooled injection system / injekzio hotzeko sistema.

C_{TWA}: time-weighted average concentration / denboran zeharreko batazbesteko kontzentrazioa.

CRM: certified reference material / erreferentziazko material egiaztatua.

CV: Cell voltage / gelaxka-boltaia.

D

DAD: diode array detector / diodo ildaxka detektagailua.

DDE: dichloro diphenyldichloroethylene / dikloro difenyldikloroetilenoa.

DDD: dichloro diphenyldichloroethane / dikloro difenildikloroetanoa.

DDT: dichloro diphenyltrichloroehtane / dikloro difeniltrikloroetanoa.

DES: diethylbestrol / dietilbestrola.

DEHP: bis(2-ethylhexyl) phthalate / bis(2-etillhexil) ftalatoa.

DiDecP: diisodecyl phthalate / diisodezil ftalatoa.

DiNP: diisononyl phthalate / diisononil ftalatoa.

DMP: dimethyl phthalate / dimetil ftalatoa.

DOP: dioctyl phthalate / dioktil ftalatoa.

dSPE: dispersive solid phase extraction / fase solidoko erauzketa dispertsiboa.

DTP: ditridecyl phthalate / ditridezil ftalatoa.

E

E1: estrone / estrona.

E2: 17 β-estradiol / 17 β-estradiola.

E2-G: 17 β-estradiol glucuronide / 17 β-estradiol glukuronidoa.

E3: estriol /estriola.

ECs: emerging contaminants / konposatu emergenteak.

EDCs: endocrine disrupting compounds / disrupzio endokrinoko konposatuak.

EE2: 17 α-ethynilestradiol / 17 α-etinilestradiola.

EFSA: European Food Safety Authority/ Europako janariaren segurtasun agentzia.

EH: Basque Country / Euskal Herria.

EI: electron ionisation / elektroi ionizazioa.

ENVI: ENVI-CARB / ENVI-CARB.

EPA: United States environmental protecion agency/ Estatu Batuetako ingurumeneko babes agentzia.

ER: estrogen receptor / estrogeno-hartzailea.

ESI: electrospray ionisation / elektroesprai ionizazioa.

EtOAc: ethyl acetate / etil azetatoa.

EU: European Union / Europar Batasuna.

EQS: environmental quality standards / ingurumeneko kalitate-mugak.

F

FUSLE: focused ultrasound solid liquid extraction / ultrasoinu fokatu bidezko solido-likido erauzketa.

G

GC: gas-chromatography / gas-kromatografia.

GC-MS: gas chromatography–mass spectrometry / gas-kromatografia–masa-espektrometria.

GPC: gel permeation chromatography / gel permeazio kromatografia.

GPC-LC-DAD: gel permeation chromatography-liquid chromatography-diode array detector / gel permeazio kromatografia–likido-kromatografia diodo ildaxka detektagailua.

H

HAc: acetic acid / azido azetikoa.

HCHs: hexachlorocyclohexane / hexakloroziklohexanoak.

Hex: *n*-hexane / *n*-hexanoa.

HHCB: galaxolide / galaxolidea.

HVP: high volume production / bolumen handiko ekoizpena.

I

Isooctane: 2,2,4-trimethylpentane / 2,2,4-trimetilpentanoa.

K

K_{ow}: octanol water partition coefficient / oktanol/ur banaketa-koefizientea.

L

LC: liquid-chromatography / likido-kromatografia.

LD liquid-desorption / desortzio-likidoa.

LDPE low density polyethylene / dentsitate baxuko polietilenoa.

LC-MS/MS: liquid chromatography-tandem mass spectrometry / likido-kromatografia tandem masa-espektrometria.

LLE: liquid-liquid extraction / likido-likido erauzketa.

LODs: limits of detection / detekzio-muga.

LOQs: limits of quantification / kuantifikazio-muga.

LVI: large volume injection / bolumen handiko injekzioa.

LVI-PTV-GC-MS: large volume injection-programmed temperature vaporizing-gas chromatography-mass spectrometry / bolumen handiko injekzioa-tenperatura programatutako baporizatzailea-gas chromatografia-masa espektrometria.

M

MAE: microwave assisted extraction / mikrouhinen bidezko erauzketa.

MALDI: matrix-assisted laser desorption / matrizeak lagundutako laser desortzioa.

MBzP: monobenzyl phthalate / monobentzil ftalatoa.

MDLs: method detection limits / metodoaren detekzio-muga.

MEHP: mono-2-ethylhexyl ester / mono-2-etilhexilesterra.

MeOH: methanol / metanola.

Milli-Q: milli-Q water / milli-Q ura.

MOP: monooctyl phthalate / monooktil ftalatoa.

M-PES: membrane enclosed sorptive coating-polyethersulfone / mintzean gordetako geruza adsorbatzailea-polietersulfona.

MRM: multiple reaction monitoring / erreakzio anitzeko monitorizazioa.

MS: mass spectrometry / masa-espektrometria.

MSTFA: N-methyl-N-(trimethylsilyl) trifluoroacetamide / N-metil-N-(trimetilsilil) trifluoroazetamida.

MS/MS: tandem mass spectrometry / tandem masa-espektrometria.

MV: molecular volume / bolumen molekularra.

MW: molecular weight / pisu molekularra.

N

N_2: Nitrogen / nitrogenoa.

NaCl: sodium chloride / sodio kloruroa.

NP mix: nonylphenol mixture / nonilfenol nahasketa.

NPs: nonylphenols / nonilfenolak.

O

OCP: organochlorine pesticides / pestizida organokloratuak.

OPP: organophosphate pesticides / pestizida organofosfatatuak.

OPs: octylphenols / oktilfenolak.

P

PA: polyamide / poliamida.

PAc: polyacrilate / poliakrilatoa.

PAHs: polycyclic aromatic hydrocarbons / hidrokarburo polizikliko aromatikoak.

PCPs: personal care products / zaintza pertsonaleko produktuak.

PDMS: polydimethylsiloxane / polidimetilsiloxanoa.

PEG: polyethylenglycol / polietilenglikola.

PEM: phthalate ester metabolite / ftalato esterren metabolitoak.

PEs: phthalate esters / ftalato esterrak.

PES: polyethersulfone / polietersulfona.

PG: progesterone / progesterona.

PLE: pressurised liquid extraction / presiopeko likido-erauzketa.

POCIS: polar organic chemical integrative system / konposatu organiko polarren lagin-bilketa integrala.

POM: polyoxymethylene / polioximetilenoa.

POP: persistent organic pollutant / kutsatzaile organiko iraunkorra.

PPCPs: pharmaceutical and personal care products / produktu farmazeutikoak eta zaintza pertsonalekoak.

PSA: primary and secondary amine / amina primario eta sekundarioa.

PTFE: polytetrafluoroethylen / politetrafluoroetilenoa.

PTV: programmed temperature vaporizing / tenperatura programatutako baporizatzailea.

PVC: polyvinylchloride / polibinilkloruroa.

Pyr: pyridine / piridina.

Q

QuEChERs: quick easy cheap effective rugged safe / azkarra, erraza, merkea eraginkorra, sendoa eta segurua.

R

r^2: coefficient of determination / determinazio-koefizientea.

REACH: registration, evaluation and authorization of chemical substances / konposatu kimikoen izen ematea, ebaluazioa, baimena eta murrizketa.

RSD: relative standard deviation / desbiderazio estandar erlatiboa.

S

S/N: signal to noise ratio/ zarata/seinale-erlazioa

SBSE: stir bar sorptive extraction / hagatxo birakariaren bidezko erauzketa.

SIM: select ion monitoring / hautatutako ioi-monitorizazioa

SLE: solid liquid extraction / solido-likido erauzketa.

SPE: solid phase extraction / fase solidoko erauzketa.

SPME: solid phase microextraction / fase solidoko mikroerauzketa.

SPMD: semipermeable membrane device / mintz erdi-iragazkorrezko tresna.

T

TCA: α-2,4'-trichloroacetophenone / α-2,4'-trikloroazetofenona.

TCP: 3,5,6-trichloro-2-pyridinol / 3,5,6-trikloro-2-piridinola.

TD: thermal desorption / desortzio termikoa.

TDU: termal desorption unit / desortzio termikoko unitatea.

TMCS: trimethylchlorosilane / trimetilklorosilanoa.

TMS: trimethylsilyl / trimetilsilanoa.

TOC: total organic carbon / karbono organiko totala.

U

UCMR-3: 3rd unregulated contaminant monitoring rule / arautu gabeko 3. kutsatzaileen monitorizazio araua.

US: ultrasound / ultrasoinua.

USA: United States of America / Ameriketako Estatu Batuak.

USE: ultrasound extraction / ultrasoinuen bidezko erauzketa.

UV: ultraviolet / ultramorea.

W

WFD: water frame directive / uraren zuzentaraua.

WS: water solubility / ur disolbagarritasuna.

WWTP: waste water treatment plant / Hondakin-uren araztegia.

SARRERA

Ezin da ukatu kimika gure eguneroko bizitzaren zati garrantzitsu bat denik. Izan ere, "*Chemical Abstract Service*" (CAS) zerbitzuaren arabera, 110 milioi konposatu kimiko baino gehiago zerrendatu dira gaur egunerarte [1] eta horietatik 100.000 erabiltzen dira gaur egun [2, 3]. Nahiz eta konposatu ezorganiko batzuk ezinbestekoak izan gizakiaren hainbat eginkizunetarako, gaur egun ezagutzen diren konposatu kimiko gehienak konposatu organikoak dira, guztien % 90 inguru. Horrez gain, egunero 15.000 konposatu berri sortzen dira eta horietako gehienak konposatu organikoak dira. Hala ere, konposatu berriak sintetizatzen diren heinean, beste batzuen erabilera debekatu egiten da, gizakiengan zein ingurumenean sortzen dituzten kalteengatik. Aipatzekoa da Europar Batasuneko (*European Union*, EU) biztanleriari ingurumeneko arazoei buruz galdetu zitzaienean, % 47k adierazi zuela beren kezkarik nagusiena uraren kutsadura zela [4]. Kezka hori ikusirik, konposatu kimikoen presentzia inguruan gero eta erakunde gehiagok aztertzen dute, eta hainbat araudi indarrean daude ingurumenaren kalitatea ahalik eta hobekien mantentzeko.

1. Lehentasunezko konposatuak eta emergenteak

Uraren kutsadurak sortzen duen kezka ikusita, arau, legedi eta zuzentarau desberdinak jarri dira indarrean, ingurumeneko uraren kalitatea bermatzeko helburuarekin. Europa mailan, araudirik garrantzitsuena Uraren Zuzentaraua (*Water Frame Directive*, WFD) da. Mundu mailan, WFDaren parekoa den beste zuzentaraua, Ameriketako Estatu Batuek (*United State of America*, USA), Ingurumeneko Agentziak (*Environmental Protection Agency*, EPA) bermatzen duena da. WFDa 2000. urteaz geroztik indarrean dago Europako herrialde guztietan, eta hainbat berrikusketa jasan ditu konposatu berriak, eta horiek ingurumenean egon daitezkeen kontzentrazio maximoak finkatzeko. Helburu nagusia, ingurumeneko ur guztiak (gainazalekoak, lur azpikoak, trantsiziokoak eta kontinentalak) kalitate egokian mantentzea da. Horrenbestez, urek zuzentarauak ezarritako ingurumeneko kalitate-mugak (*Environmetal Quality Standards*, EQS) betetzen dituztenean, kalitate egokia lortu dutela baiezta daiteke. EQS balioak 2008. urteko berrikusketan gehitu ziren [5], uretarako, biotarako eta sedimentuetarako kontzentrazio-mailak finkatuz.

Aipatutako bi zuzentarau horiek konposatu kimiko desberdinek ingurumenean duten presentzia arautzea dute helburu (ikus **1.1 taula**). Konposatuen kontzentrazio-maila EQS gainetik dagoenean eta posiblea den kasuetan, konposatu horien isuria eten egin behar da. Ezinezkoa balitz, gutxienez inolako eraginik sortzen ez duten mailetara jaitsi behar dira (EQS balioen azpitik). Arautuko konposatu horiek lehentasunezko konposatuak dira, ingurumenean duten kalte ezagunagatik. WFDk, 2000. urtean, 33 substantzia sailkatu zituen multzo horretan (2000/60/EC eranskinean) [6], eta azkeneko berrikusketaren ondoren (2013. urtean, 2013/39/EC), beste 12 konposatu gehitu zituen zerrenda horretara. Horrela, gaur egun, 45 konposatu sailkatzen dira lehentasunezko konposatu modura [7] (ikus **1.1 taula**). EPAko zuzentarauak, aldiz, uraren programa erregulatzailearen (*Water Regulatory Program*) barnean, 126 konposatu sailkatu ditu lehentasunezko konposatu bezala [8]. Multzo horretan (ikus **1.1 taula**) zenbait metal, pestizida, ftalato ester (*Phthalate Esters*, PE), hidrokarburo poliziklikoaromatiko (*Polycyclic Aromatic Hydrocarbons*, PAH), alkilfenol (*Alkil Phenols*, AP) eta dioxina aurki daitezke, beste familia batzuen artean.

Lehentasunezko konposatuez gain, badira aipaturiko zuzentarauetan lehentasunezko moduan sailkatzen ez diren beste hainbat konposatu; konposatu emergenteak alegia. Konposatu horiek gizakiarengan zein ingurumenean kaltea eragin dezaketelako susmoa dago. Gainera, konposatu asko eta asko ingurumenean maiz aurkitzen diren konposatuak dira. Informazio nahikorik ez dago egun lehentasunezko kutsatzaile moduan sailkatzeko, baina monitorizazio-programetan sartzea gomendatzen da hurrengo azterketa batean lehentasunezko multzoan sartu behar diren ala ez erabakitzeko. Talde horren barnean, zaintza pertsonaleko produktuak (*Personal Care Products*, PCPs) eta hormonak bezalako produktu farmazeutikoak daude (ikus **1.1 taula**) WFD [9] eta EPA [10] zuzentarauetan. EPAk bi multzotan sailkatzen ditu konposatu emergenteak. Alde batetik, edateko uraren kalitatea bermatzeko, kutsatzaileen hautagaien zerrenda (C*ontaminant Candidate List*, CCL) kaleratzen du 5 urtean behin. Azken eguneratzean, 116 konposatu gehitu ziren CCL-3 zerrendara. Beste alde batetik, EPAk arautu gabeko kutsatzaileen monitorizazioaren 3. araua (*3rd Unregulated Contaminants Monitoring Rule*, UCMR-3) plazaratu zuen. Zerrenda horren bidez,

EPAk etorkizunean lehentasunezko moduan sar daitezkeen 30 konposaturen monitorizazioa egitea du helburu eta 5 urtez behin berritzen da. **1.1 taulan**, EPAk emergente moduan sailkatu dituen CCL-3 eta UCMR-3 arauetako konposatuak ikus daitezke.

Konposatu emergenteen multzoan egon beharreko konposatuak finkatzeko, Europako eta Ipar Ameriketako zientzialariek eta erakundeek lanean dihardute 2005. urteaz geroztik NORMAN izeneko proiektuan. NORMAN proiektuan ikertzen duten lan-taldeen helburua, konposatu emergenteak neurtzeko metodoak bateratzea eta horien monitorizazioari buruzko arauak ezartzea da. WFDko eta EPAko zuzentarauek kontuan hartu ez dituzten hainbat konposatu, kutsatzaile emergenteen taldean sartzea proposatu dute NORMANeko kideek (ikus **1.1 taula**). Horietatik aipagarrienak musketa fragantziak, surfaktanteak, produktu farmazeutikoak, hormonak, pestizida polarrak eta biozidak dira. Guzti horiek gizakien eta animalien sistema endokrinoan eragiten dituzten aldaketak direla eta [11], konposatu disruptore endokrino (*Endocrine Disrupting Compound*, EDC) bezala ezagutzen dira.

1.1 taula: *WFD eta EPA zuzentarauetan eta NORMAN lan-taldean lehentasunezko eta emergente moduan sailkatu diren konposatu batzuk.*

Familia	Analitoa	WFD	EPA	NORMAN	EDC
Musketa fragantziak	Tonalidea			X	X
	Galaxolidea			X	X
BPA	Bisfenol-A	X[*]	X[**]	X	X[1]
Estrogenoak	17 β-estradiola	X[E**]	X[E]	X	X
	17 α-etinilestradiola	X[E**]	X[E]	X	X
	Dietilstrilbestrola			X	X
APak	Nonilfenol-nahasketa	X[P]	X[***]		X[2]
	4-*tert*-oktilfenola	X[P]			X[2]
	4-*n*-oktilfenola	X[P]			X[3]
PEak	Bis-(2-etilhexil) ftalatoa	X[P]	X[P]		X[1]
	Butilbentzil ftalatoa		X[P]	X	X[1]
	Dioktil ftalatoa		X[P]	X	X[3]
Pestizidak	Hexakloroziklohexano isomeroak	X[P]	X[P]		X[1]
	Diklorodifeniltrikloroetano eta diklorodifenildikloroetano isomeroak	X[P]	X[P]		X[1]
	Klorfirifosa	X[P]			X
	Klorfenbinfosa	X[P]			X

[p] lehentasunezko konposatuak; [e] konposatu emergenteak.
* lehentasunezko moduan sailkatua izateko hautatutako substantzia 2008/105/EC zuzentaraueko 3. Eranskinean.
** 2015/495 behaketa zerrendan aipatuak, hurrengo berrikusketan lehentasunezko konposatu moduan sailkatzen diren edo ez erabakitzeko.
*** CCL-3 araudian sartzeko proposatua, baina azkenean ez hautatua.
[1, 2, 3] Europar Batasunak EDCak sailkatu dituen multzoa [12].

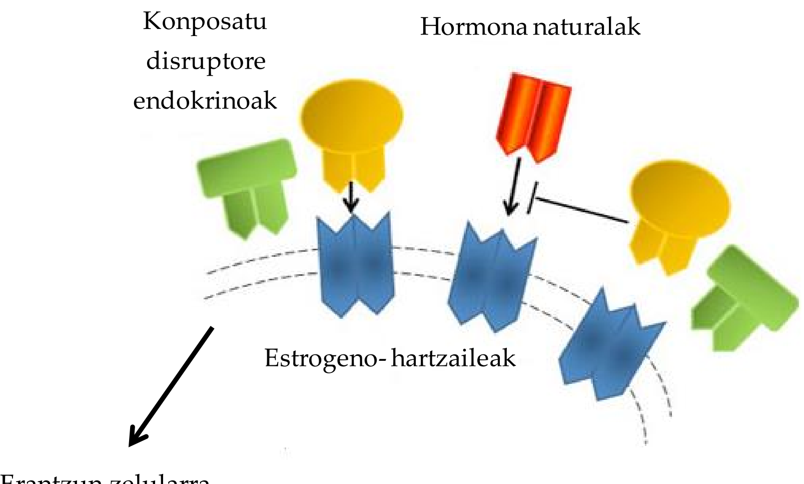
1.1. Konposatu disruptore endokrinoak

EDCak gorputzaren sistema endokrinoan aldaketak sortzen dituzten substantzia exogenoak edo horien nahasteak dira. Konposatu horiek organismo osasuntsuetan edo organismo horien ondorengoetan kalteak sor ditzakete [13]. Disrupzio endokrinoa elikaduran edo ingurumenean dauden kutsatzaileek sor dezakete. EUk 3 multzo nagusitan sailkatu ditu EDCek duten disrupzio-mailaren arabera edo ingurumenean dauden kopuruaren arabera [12] (ikus **1.1 taula**). Konposatuak talde bakoitzean sailkatzeko irizpideak honako hauek dira:

- 1. taldea: disrupzio endokrinoa eragiten dutelako susmo handia duten konposatuak edo ingurumenean maila altuan dauden konposatuak.
- 2. taldea: disrupzio endokrinoa eragiteko aukera handia duten konposatuak edo ingurumenean maila ertainean dauden konposatuak.
- 3. taldea: disrupzio endokrinoa eragiten ote duten garbi ez dagoen konposatuak edo ingurumenean maila baxuan dauden konposatuak.

Araudi horretan, EDCak izateko susmopean dauden 564 konposatu aztertu dituzte eta 66 konposatu 1. multzoan sailkatu dira. 1. multzoko konposatuetatik, gizakiak 60 konposatutara esposatuta egoteko aukera handia du. 2. taldean beste 52 konposatu sailkatu dira eta azkenik, 3. multzoa beste 118 konposatuk osatzen dute. Gainontzeko konposatuek ez dute EDC moduan sailkatuak izateko ebidentzia nahikorik erakutsi.

EDCek gorputzean kalteak eragiteko bi mekanismo nagusi dituzte. Alde batetik, gorputzean era naturalean dauden hormonen jokaera imita dezakete eta, beste aldetik, hormonen produkzioan, metabolismoan edo eliminazioan parte har dezakete [13, 14]. **1.1 irudian** ikus daitekeen moduan, egoera normal batean, hormona hormona-hartzaileari lotzen zaio. Haatik, EDC bat jatorrizko hormonarekin lehian dagoenean, EDCa hormona-hartzaileari lotu dakioke hormonaren erantzun normala aldatuz.



1.1 irudia: *Disruptore endokrinoak eta hormona naturalak estrogeno-hartzailearekin lotzen diren adibidea.*

Erantzun desberdin horien ondorioz, hainbat izan dira uretan bizi diren espezietan aurkitutako arazoak: malformazioak [15], ugalketa-arazoak [16, 17], espezieen tamainaren aldaketa [18] edo intersexualitatea [18-20], adibidez. Arazo horiek, bai ingurumenean jasotako arrainetan bai laborategiko arrainetan aurkitu izan dira, arrainek pairatu duten esposizio-maila oso baxua izanda ere.

Intersexualitatea edo "ovotestis"a, garapen-maila desberdinean egon daitezkeen obozitoak barrabilen ehunaren barruan daudenean gertatzen den fenomenoa da [19, 21, 22]. Ohikoa ez den egoera horren ondorioz, arrainetan ugalketa arazoak ikusi dira eta, kasurik larrienetan, populazio osoa desagertzeko zorian egon da [23, 24]. Ezaguna da intersexualitatea sortzen duen faktorerik garrantzitsuenetako bat EDCekiko esposizioa dela [18, 19, 21, 25], bai larba garaian baita heldutasun sexuala lortu dutenean ere [26].

EDC gehienek jatorri sintetikoa dute. Edonola ere, badaude jatorri naturala duten EDCak, hormona naturalak eta sojatik eratorritako fitoestrogenoak, adibidez [27-30]. Konposatu sintetikoen artean, PEak, bisfenol-A (*Bisphenol-A*, BPA), musketa fragantziak, APak, hormona sintetikoak eta pestizidak aipa daitezke, besteak beste (ikus **1.1 taula**). Konposatu horietatik gehienak, lehenago aipatutako

zuzentarauetan aipatzen dira espezifikoki sortzen dituzten kalteengatik. Hala ere, dietilestrilbestrola (*Diethylestrilbestrol*, DES) eta musketa fragantziak bezalako konposatuak, nahiz eta EDC moduan sailkatu diren, NORMAN lan-taldean baino ez dira aipatzen. Jarraian, aipatutako familien deskribapen sakonagoa egingo da.

1.1.1. Musketa fragantziak

Musketa fragantziak, duten usain bereziagatik antzinatik erabili izan diren konposatuak dira. Jatorrian, orein (*Moschus moschiferus*) arrek ipurdi aldean duten guruin batetik lortzen ziren, lortutako konposatua muskona izanik. XIX. mendera arte, musketa fragantzia naturalak bakarrik erabiltzen ziren, baina arrazoi ekonomikoak eta etikoak direla medio, musketak sintetizatzen hasi ziren. Orein bakoitzaren jariakinen % 0,5-2 baino ez zen erabilgarria lurringintzan eta 40 orein behar ziren lurrin pote bat egiteko. Horrela, muskona produktu natural garestiena da, 30.000-50.000 $/Kg prezioa duela [31]. Gaur egun, musketa sintetikoen erabilera guztiz hedatuta dago munduan zehar. Europa mailan, 1.000-5.000 tona/urteko ekoizten dira [32, 33] eta EPAk bolumen handiko ekoizpen-produktu (*High Volume Production*, HVP) bezala sailkatu ditu, urtean 1 milioi libra edo gehiago ekoizten direlako [34, 35]. Musketa sintetikoen artean, musketa poliziklikoak dira erabilienak, galaxolidea® (HHCB) eta tonalidea® (AHTN) batez ere [35, 36]. Musketa poliziklikoen ezaugarririk nagusienak usaina finkatzeko duten gaitasuna eta usaina bera dira. Ondorioz, garbiketarako xaboietan, xanpuetan edo perfumeetan erabiltzen dira gehigarri gisa [32, 33, 35, 37, 38]. Une honetan, musketa fragantziak ez daude WFD edo EPA erakundeetan araututa, baina etorkizunean monitorizazio-programetan sartzeko aukera asko dituzte erabilera handiagatik, baita sortzen dituzten kalteengatik ere [35, 38]. Musketa fragantziek aktibitate estrogenikoa erakusten dute [32, 35, 39], organismo akuatikoetan metatzeko joera dute [40-42] eta erresistentzia multixenobiotikoen inhibitzaileak dira [31, 42].

1.1.2. Bisfenol-A

Bisfenol konposatuen artean, 7 dira aurki daitezkeen analogoak (ikus **1.2 irudia**). Horietatik garrantzitsuena BPA dela esan daiteke, gehien erabiltzen den analogoa baita [43]. Orokorrean, bibliografian aurkitutako lan gehienetan BPA bakarrik determinatzen dute [2, 29, 44-46]. BPAren gehiengoa fenolen, poliakrilatoen, poliesterren eta epoxi-erretxinen polimerizazioan erabiltzen da eta gutxiengoa gehigarri moduan [43, 47-49]. Asko dira BPA eduki dezaketen produktuak: jostailuak, tresneria elektronikoa, medikuntzan erabilitako materiala eta jakien eta edarien ontziak. Ontzi horiek BPA edukitzeak sortzen du kezkarik handiena gizakion artean; azken finean, jakien eta edarien bidez, BPA gorputzean barneratu eta kalteak sor baititzake. Europako janariaren segurtasunerako agentziak, (*European Food Safety Authority*, EFSA-k) gizakiak egunero barneratu dezakeen BPA-muga berria ezarri zuen 2015. urtean. Muga hori, 50 μg/gp (gorputzaren pisua) izatetik 4 μg/gp izatera pasatu zen. Egindako ikerketen arabera, gizakiak muga berri horretatik behera barneratzen du BPA egunero, osasunari inolako kalterik ekarri gabe [43]. Zenbait ikerlarik adierazi dute BPA gizakiengan minbizia, ugalketa-sisteman aldaketak eta aldaketa neuronalak sortzeko gai dela [50-52]. Uretan bizi diren arrainentzat dosi toxikoa 1-10 mg/L-koa zela aipatu zuten Nikfar eta lankideek [53]. BPAren barneratze-maila hori murrizteko asmoz, zenbait neurri hartu dira herrialde desberdinetan. USAko zenbait estatutan (New Yorken eta Minesotan, adibidez), BPA duten produktuak guztiz debekatu dira eta Kanadan, Taiwanen eta EUan haurren elikadura botiletan zein ontzietan debekatuta dago [47]. Une honetan, BPA da araututa dagoen bisfenolaren analogo bakarra, baina gainontzeko 6 analogoak, antzeko propietate fisiko-kimikoak izanik, BPAren ordezkariak izateko hautagaiak dira. Tamalez, antzekotasun hori dela eta, analogo horiek efektu toxiko altua dutela eta disruptore endokrino moduan joka dezaketela baieztatu da [48, 54-56].

Bisfenol-A

Bisfenol-S

Bisfenol-F

Bisfenol-Z

Bisfenol-AF

Bisfenol-P

Bisfenol-AP

1.2 irudia: *Bisfenolaren 7 analogoen egitura kimikoa.*

1.1.3. Pestizidak

Pestizidak, urtetan zehar, intsektuen kontrolerako erabili izan diren konposatu kimikoak dira. Erabilera nagusiena nekazaritzan izan den arren, neurri txikiago batean etxeetako landareak zaintzeko ere erabili izan dira. Pestizidak bi talde nagusitan bana daitezke beren egitura kimikoaren arabera: pestizida organokloratuak (*Organochlorine Pesticides*, OCPak) eta pestizida organofosfatatuak (*Organophospate Pesticides*, OPPak). OCPak gehien erabili diren pestizidak izan dira, batez ere 1940-1980 urte bitartetan [57, 58]. OCPrik ezagunenak diklorodifeniltrikloroetanoak (*Dichloro Diphenyl Trichloroehtane*, DDT), diklorodifenildikloroetanoak (*Dichloro Diphenyl Dichloroethane*, DDD), diklorodifenildikloroetilenoak (*Dichloro Diphenyl Dichloroethylene,* DDE*)* hexakloroziklohexanoak (*Hexochlorocyclohexane*, HCH) eta beraien isomero guztiak dira. Konposatu horiek hainbat kalte sortu dituztela frogatu dute hainbat ikerlarik [57, 59, 60]. Horietatik aipagarrienak jaiotza goiztiarra, abortuak, bularreko minbizia eta nerbio-sisteman eragindako kalteak dira. Arazo guzti horiek direla eta, egun herrialde gehienetan beraien erabilera debekatuta dago. Debeku horren atzean, oso garrantzitsua izan zen USAn egondako mugimendu ekologista. 1962. urtean, Rachel Carson idazleak eta itsas-biologo estatu batuarrak “udaberri isila” izeneko liburua kaleratu zuen. Liburu horrek mugimendu soziala sortu zuen eta ondorioz, EPA erakundearen sorrera bultzatu zen. Erakunde horrek DDTa debekatu zuen salbuespen gutxi batzuekin. Zenbait herrialdetan, erabilera legezkoa da malaria eta tifusa garraiatzen dituzten eltxoak hiltzeko. Beraien propietate fisiko-kimikoak direla eta, ingurumenean oso iraunkorrak dira degradaziorik jasan gabe [57-59, 61]. Luo eta lankideen arabera [61], 22 egun-30 urteko erdibizitza dute ingurumenean eta gizakiongan 6-10 urtekoa. Degradazio baxu horren ondorioz, ingurumen-matrize desberdinetan antzeman daitezke debekatutako pestizidak. Adibide moduan, populazioaren % 95ak DDEa gorputzaren barnean duela estimatu da [57]. OCPak EDC moduan sailkatu dira [59] sistema endokrinoan sortzen dituzten aldaketengatik. Horretaz gain, toxikoak eta minbiziaren eta diabetesaren eragileak dira, abortuak eragiteko gai dira eta espermaren kalitatearen jaitsierarekin erlazionatu dituzte [57, 59, 60]. Koureas eta lankideek alzheimerrarekin eta dementziarekin ere erlazionatu dituzte OCPak [57].

OPPak, ordea, efizientzia altuko pestizidak dira, eta ingurunean ez dira hain iraunkorrak. Hori dela eta, oraindik zenbait pestizidaren erabilera indarrean dago [62], nahiz eta WFDk klorfirifosa (*Cholpyrifos*) eta klorfenbinfosa (*Chlorfenvinphos*) adibidez, lehentasunezko konposatu bezala definitu dituen.

Euskal Herri (EH) mailan, Bizkaian batez ere, HCH isomeroen gaia oso ezaguna da, bertako bi enpresak HCH isomeroak ekoizten baitzituzten 1947-1987 eta 1950-1982 urteen bitartean. HCH isomeroak 5 diren arren, pestizida moduan aktiboa den bakarra γ-HCH isomeroa da, lindano izenaz ezaguna dena. Sintesian, isomero guztiak ekoizten dira. Erabilgarriak ez direnak baztertu egiten dira egun, baina lehen hondakinetara botatzen ziren. Sintesian aktiboa den lindanoa ~ 9:1 erlazioan dago; beraz, ekoizten diren 10 tonetatik bakarra da erabilgarria, gainontzekoa hondakina izanik. Hasiera batean, aipatutako bi enpresek sintetizaturiko HCH nahaste osoa saltzen zuten, baina urte batzuk geroago, isomeroen bereizketa egiten hasi ziren aktiboa zena salduz, eta ondorioz, hondakinak pilatuz. Bizkaian bakarrik 80.000 tona inguru ekoiztu zirela uste da. Ekoiztutako α-HCH eta β-HCH isomeroak lindanoa bera baino toxikoagoak dira. Garai hartan, hondakinak lurrarekin nahasten ziren eta Bizkaiko leku desberdinetan sakabanatzen ziren, ingurumeneko arazo larriak sortaraziz. Gaur egun ere, oso zaila da hondakinak behar bezala tratatzea arazo ekonomikoak direla eta. Euskal Erkidegoko gobernuak hondakinak bilatu eta segurtasun-zelda desberdinetan (Argalario eta Loiu) pilatzea proposatu zuen. Horrenbestez, 2002. urteaz geroztik topatu diren HCH hondakinak bertan daude pilatuta eta bertan ingurumeneko hainbat kontrol egiten dira [63].

1.1.4. Estrogenoak

Sexu-estrogeno esteroideak pisu molekular baxuko molekula lipofilikoak dira eta hormonalki aktiboak dira [64, 65]. Esteroide guztiak kolesterol (*cholesterol*, CHL) molekularen eratorriak dira. Beraien eginkizun nagusienak sexu-bereizketa egitea, ugaztunen ziklo menstruala kontrolatzea eta emeen ugaltze-aparatua eta hainbat organoren funtzionamendu normala ahalbidetzea dira. Horretaz gain, koipearen, azukrearen eta proteinen metabolismoan paper garrantzitsua dute, baita odolaren koagulazioan ere [64, 66]. Hormonak bi multzo nagusitan banatzen dira. Alde batetik, hormona naturalak daude. Talde horretako estrogenorik garrantzitsuenak estrona (E1), 17 β-estradiola (E2) eta estriola (E3) dira. Beste alde batetik, hormona sintetikoak edo hormona exogenoak daude. Multzo horretan ezagunena 17 α-etinilestradiola (EE2) da [64-67]. Hormona sintetikoak gizakion eta animalien beharretarako erabiltzen dira batik-bat, eta gaur egun sendagairik erabilienak dira. Gizakion kasuan, EE2 emakumeen hilerokoa kontrolatzeko piluletan erabiltzen da. Pilula bakoitzak 30-50 μg EE2 ditu eta gainontzekoa progesterona (PG) da. Horretaz gain, menopausia-sintomak arintzeko eta prostatako minbizia sendatzeko ere erabiltzen da [67]. Halarik ere, hormonen gehiegizko erabilerak bularretako minbizia sor dezakeela adierazi dute hainbat zientzialarik [67-69]. Albaitaritzan, hormona sintetikoen erabilera legala da gaur egun edozein gaixotasun sendatzeko eta kontrolatzeko. Hazkuntza azkartzeko helburuarekin, ordea, ezin da erabili. [64]. Helburu horrekin hormonak erabili nahi izanez gero, merkatu beltzera jo daiteke. DESa da, adibidez, merkatu beltzean lor daitekeen hormona sintetiko ohikoena. DESa esteroidea ez den sexu-hormona sintetikoa da. Hormona esteroideen antza handia daukanez eta gorputzean hormona sintetikoen antzeko eraginak sor ditzakeenez, hormona sintetikoen multzoan sailka daiteke [64, 66]. Akuikulturan, aldiz, sexu bakarreko arrainak, emeak normalean, lortzeko erabiltzen dira hormona sintetikoak [64, 66, 67, 70, 71]. Hormona naturalek zein sintetikoek dituzten erabilera ugarien ondorioz, etengabe isurtzen dira ingurumeneko uretara, normalean araztegien bidez. Hormonak dauden uretan bizi diren arrainek intersexualitatea garatzen dutela adierazi dute hainbat ikerlarik [72, 73]. Azken finean, akuikulturan gertatzen den fenomenoaren antzekoa da baina, kasu honetan, kutsadurak eragindakoa. Steele eta lankideen

[74] esanetan, araztegien irteeran 1-50 ng/L hormona nahikoa dira arrainek intersexualitatea gara dezaten. Hormonek duten egitura kimikoaren arabera, aktibitate estrogeniko desberdina dute. Nahiz eta kimikoki oso antzekoak izan, E2 molekula, 17 α-estradiol (ikus **1.3 irudia**) baino 10 aldiz aktiboagoa da estrogeno-hartzaileen (*Estrogen Receptor*, ER) aldean [64]. Hormona naturalen aktibitate estrogenikoak hurrengo hurrenkera dauka: E2 > E1 >> E3 > 17α-E2 [66, 75].

17-α-E2 17- β-E2

1.3.irudia: *α-E2 eta β-E2 molekulen egitura kimikoa.*

1.1.5. Ftalato esterrak

PEak edo ftalato esterrak 1920. urtetik aurrera sintetizatzen hasi ziren konposatu kimikoak dira [76, 77]. PEen sintesian bentzeno dikarboxilikoa alkohol desberdinekin esterifikatzen da PE desberdinak emanez. Erabili daitezkeen alkoholak linealak, aromatikoak edo adarkatuak izan daitezkeenez (ikus **1.4 irudia**), sortzen diren PEen kopurua oso handia da. Ftalatorik txikiena (pisu molekularra, *Molecular Weigth*, MW, 195) dimetil ftalatoa (*Dimethyl Phthalate*, DMP) da eta astunena (MW=531), aldiz, ditridezil ftalatoa (*Ditridecyl Phthalate*, DTP) [78]. Konposatu horiek dituzten propietate fisiko-kimiko desberdinen ondorioz, bai industrian bai eguneroko bizitzan ere asko erabiltzen dira. 1975. urtean, mundu mailan 1,8 milioi tona ekoiztu ziren eta 2010. urtean 6 milioi tona inguru. Ekoizpen horren laurdena bis(2-etilhexil) ftalatoak (*bis-2-ethylhexyl phthalate*, DEHP) hartu zuen [76, 79, 80]. PEak plastikoei malgutasuna, iraunkortasuna eta egonkortasuna emateko erabiltzen dira [77, 81, 82]. Erabilerarik aipagarrienak elektronika, polibinilkloruroaren (*Polyvinyl Chloride,* PVC) sintesia, eraikuntzako, jostailuen industriako, medikuntzako, garbiketako produktuetako eta zaintza pertsonaleko produktuak dira [77, 82]. Aipatu beharra dago PEak plastikoen sintesian gehigarri moduan erabiltzen direla eta, ondorioz, ez daudela plastikoei kimikoki lotuta [76, 78, 80, 81]. Arrazoi horregatik, oso erraz askatzen dira plastikoetatik ingurumenera edo janarien ontzietatik jakietara. Gizakiak janariaren bidez barneratzen du PEen kopururik handiena [77, 81]. Hala ere, PEen propietate fisiko-kimikoen ondorioz, ftalatorik arinenak (DMP) arnas-sistemaren bidez barneratzen dira, eta MWa igotzen doan heinean, barneratzea azalaren bidez ematen da. Azkenik, PE astunenen (MW ~390-tik gora) iturririk nagusiena elikadura da [77]. Propietate fisiko-kimiko desberdinek toxikologian ere eragina dute [78]. PEak minbizi eragileak direla ziurtatu dute zenbait ikerlarik [80, 83] eta EPAk [84] berak. Horrez gain, espermaren kopurua murriztu dezakete [82, 85] eta ugalketa arazoak sortu [77]. Ingurumenean eta bizidunengan duten kaltea ikusirik, hainbat erakundek PEen erabilera arautu dute. Garrantzitsuenak, umeen jostailuekin lotura duten arauak dira. DEHPa, dibutil ftalatoa (*Dibutyl Phthalate*, DBP) eta bentzil butil ftalatoa (*Benzyl Butyl Phthalate*, BBP) umeen jostailu guztietan debekaturik daude pisuaren % 0,1 pisutan baino kontzentrazio-maila altuagoetan, eta diisononil

ftalatoa (*Diisononyl Phthalate*, DiNP), dioktil ftalatoa (*dioctyl phthalate*, DOP) eta diisododezil ftalatoa (*Diisodecyl Phthalate*, DiDecP) ezin dira jostailuetan erabili % 0,1 baino maila kontzentratuagoetan, umeek jostailua ahoan sartzeko arriskua baitago [86]. **1.4 irudian**, ftalato desberdinen egiturak ikus daitezke.

a) Dioktil ftalatoa (DOP)

b) Butilbentzil ftalatoa (BBP)

c) Bis-2-etilhexil ftalatoa (DEHP)

1.4 irudia: **a)** *alkohol lineala*, **b)** *alkohol aromatikoa eta* **c)** *alkohol adarkatua duten PE molekulak.*

1.1.6. Alkil fenolak

APak, alkilfenol polietoxilatuen (*Alkylphenol Ethoxylates*, AP_nEOs-en) eta alkilfenol monoetoxilatuen (*Alkylphenol Monoethoxylates*, AP_1EOs-en) degradazio produktuak dira [87-89], araztegietan (*Wastewater Treatment Plant*, WWTP) jasaten dituzten degradazio aerobikoen ondorioz sortuak (ikus **1.5 irudia**). Sortzen diren degradazio produktuak jatorrizkoak baino egonkorragoak eta toxikoagoak dira [90]. APak, surfaktante ez-ionikoak dira eta detergenteetan, emultsionatzaile moduan, garbiketa produktuetan eta plastikoen sintesian erabiltzen dira [90, 91]. AP guztien % 55k industrian du jatorria. Gainontzeko % 30 garbiketa industrialean sortzen da eta, % 15 etxeetan erabiltzen diren garbiketarako produktuetan topa daitezke [90]. 500.000 tona/urteko ekoizten dira mundu-mailan eta guzti horren % 60k edo ingurumenean amaitzen du [90]. AP konposatuak nonilfenol (NP) eta oktilfenol (OP) linealez eta adarkatuez osatuta daude. Komertzialki eskuragarri dagoen NPa, *orto-* eta *para*-isomeroez osatutako nahastea da. Nahiz eta isomero guztien konposizioa guztiz ezezaguna izan ez, zenbait egilek gutxienez 100 isomero daudela uste dute, nahiz eta gehienez 66 identifikatu diren [92-95]. Isomero bakoitzak toxikotasun-maila desberdina du. Beraz, oso garrantzitsua da isomero guztiak identifikatzea [96-98]. *Orto*-isomeroek *para*-isomeroek baino iraunkortasun baxuagoa dute ingurumenean eta aktibitate estrogeniko txikiagoa [99]. Era berean, karbono atomo kopuruak eta adarkadura motak ere eragina dute; tertziarioek sekundarioek baino estrogeneizitate handiagoa dute, esaterako [97]. APek orokorrean, ingurumenean bizi diren organismoengan garapen toxikotasuna erakutsi dute [96] eta gainera eragin estrogenikoa dute; zehatzago esanda, estrogeno-hartzailearekiko antagonistak dira [97, 98]. NPa estrogeno ahul moduan definitu dute hainbat egilek, E2ak baino 10^{-6} estrogenitate gutxiago duelako [97, 98].

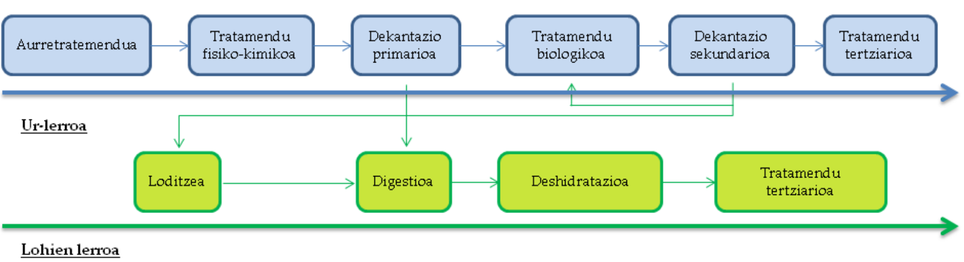
1.5 irudia: *APnEO konposatuek jasaten duten degradazio bidea.*

2. Konposatu kimikoak ingurumenean

Aipatu berri den moduan, milioika dira gaur egun erabiltzen diren konposatu kimikoak, eta ondorioz, milioika dira ingurumenean amaitzen duten konposatuak ere. Konposatu organikoen propietate fisiko-kimikoek, uretan eta biotan duten disolbagarritasun-maila (*Water Solubility*, WS) finkatzen dute, beste faktore batzuen artean. Orokorrean, oktanol-ur banaketa-koefizientea (*octanol/water Partition Coefficient*, K_{ow}) baxuena duten konposatu kimikoek, uretan disolbagarriagoak dira. Haatik, K_{ow} balioa handituz doan den heinean, biotan metatzeko aukera handitu egiten da.

2.1. Konposatu kimikoak ur-ingurumenean

Ura gizakion bizitzarako ezinbestekoa den arren, esfortzu txikia egiten da osasuntsu mantentzeko. Urteetan zehar gizakiaren jardueren ondorioz (industria, nekazaritza, abeltzaintza, etab.) sortutako hondakinek ingurumeneko uretara isurtzen ziren. Produktu kimikoen erabilera handitzen joan ahala, gero eta produktu gehiago isuri dira ingurumeneko uretara. Arazo horri aurre egin nahian, 19. mendearen amaieran araztegiak sortu ziren. Araztegien helburua ur industrialak edota etxeko urak jaso da eta, tratamendu fisikoak, kimikoak edo biologikoak aplikatu ostean, lortutako "ur garbia" ingurumenera bueltatzea da teorian, ingurumenerako kaltegarriak ez diren baldintzetan. Araztegi gehienek 2 tratamendu burutzen dituzte, nahiz eta 3. tratamendu bat ere egon daitekeen. Araztegien tratamenduaren eskema, **1.6 irudian** ikus daiteke.



1.6 irudia: *Araztegi batean urak jasaten dituen tratamenduen eskema ingurumenera bueltatu aurretik.*

Urek, araztegira heltzen direnean, aurretratamendu urrats batzuk jasaten dituzte lehen objektu handiak (enborrak edo plastikozko poltsak), objektu txikiak, uretan disolbatuta dauden areak edo antzeko tamaina duten partikulak eta gainazalean dauden koipeak kentzeko. Aurretratamendu urratsa oso garrantzitsua da hurrengo pausoetan erabiltzen den tresneriaren bizitza luzatzeko.

Aurretratamenduaren ostean, urek lehen mailako tratamendua jasaten dute ahalik eta materia organiko gehien dekantatzeko. Urari tratamendu kimiko desberdinak egiten zaizkio, koagulazio/malutapen prozesuak erabiliz. Tratamendu horien ondorioz, solidoak dekantatzen dira bi azpi-produktu sortuz; ur-laginak eta solidoak edo lohiak. Ondorengo urratsetan, urek eta lohiek tratamendu desberdinak jasango dituzte.

Ondoren, urek bigarren mailako tratamendua jasaten dute. Bertan, mikroorganismo desberdinak erabiliz, uretan disolbatuta dagoen materia organikoaren deskonposizioa eragiten da non, oxigenoaren laguntzaz, mikroorganismoek materia organikoa deskonposatzen duten, eta bestalde ura eta partikula solidoak bereizten diren. Kasu askotan, araztegien arazketa prozesua urrats horretan amaitzen da.

Tratamendu tertziarioaren helburua, ultramore (*Ultraviolet*, UV) izpiak edo klorazioa erabiliz, metal astunen eta hainbat konposatu kimikoren degradazioa egitea da. Gero eta gehiago dira tratamendu hori burutzen duten araztegiak.

Aipatu berri den bezala, araztegietan hainbat prozesu fisiko-kimiko egiten dira urak ingurumenera bueltatu aurretik. Halaz guztiz, hainbat lanetan aipatu da araztegiak ez direla gai konposatu kimiko guztiak ezabatzeko [28, 46, 70, 100-103]. Araztegiak sortu ziren garaian, gaur egun ezagutzen diren hainbat konposatu organiko ez ziren ezagutzen eta ondorioz araztegietan egiten diren tratamenduak ez dira gai konposatu organiko guztiak arazteko, ez baitago tratamendu espezifikorik oraindik ere indarrean. Horrez gain, aipatu beharra dago araztegien irteerako urek konposatu organikoen aurrekontzentrazio gisa joka dezaketela.

Estuarioetako, erreketako edo lakuetako uretan aurki daitezkeen konposatu organikoen kontzentrazioak araztegien irteeren ondoan aurki daitezkeenak baino baxuagoak dira diluzio efektua dela eta. **1.2 taulan** munduan zehar jasotako ingurumeneko uretan aurkitu diren zenbait konposatu organikoren kontzentrazioak laburbildu dira. Oro har, estuarioko eta errekako uretan ng/L-ko mailan daude konposatu gehienak, NPak [101] eta PEak [104, 105] izan ezik, ng/mL mailan aurkitzen baitira. Hala ere, aipatutako kontzentrazio horiek araztegietako irteeretan neurtutakoak baino baxuagoak dira eta kasu askotan μg/L-ko mailakoak dira.

1.2 taula: *Ingurumeneko ur desberdinetan neurturiko EDC desberdinen kontzentrazioak mundu mailan.*

Analitoa	Matrizea	Herrialdea	Urtea	Kontzentrazioa (ng/L)	Ref
BPA	Araztegiko irteera	Espainia	ea	603-3.690	[100]
	Araztegiko irteera	EH	2011	12-2.615	[46]
	Errekako ura	Txina	2012	7-75	[106]
Musketa fragantziak	Araztegiko irteera	EH	2011	82-259	[103]
	Araztegiko irteera	USA	2009	87-3.817	[107]
	Estuarioa	EH	2012	< 32	[35]
E2/EE2	Araztegiko irteera	EH	2011	< 1-232	[46]
	Araztegiko irteera	Espainia	ea	< 0,05-33	[108]
	Errekako ura	Txina	ea	100	[101]
DES	Araztegiko irteera	EH	2011	< 1-3	[46]
	Errekako ura	Txina	ea	20	[101]
OPak	Araztegiko irteera	Espainia	ea	282-4.085	[100]
	Errekako ura	Txina	ea	150	[101]
NPak	Araztegiko irteera	EH	2011	2-18.896	[46]
	Araztegiko irteera	Espainia	ea	658-11.361	[100]
PEak	Araztegiko irteera	Hegoafrika	2010-2011	nd-60	[109]
	Errekako ura	Suedia	2012	170-6.800	[105]
DDTak	Araztegiko irteera	EH	2011	< 9-226	[46]
	Errekako ura	Espainia	ea	1-39	[100]
HCHak	Araztegiko irteera Errekako ura	Espainia	ea	190-212 3-125	[100]
OPPak	Araztegiko irteera	EH	2011	< 7-60	[46]
	Errekako ura	Espainia	2004-2006	10-71	[110]

Musketa fragantziak (HHCB eta AHTN), OPak (4*t*OP eta 4*n*OP), NPak (NP isomero-nahastea), PEak (BBP, DEHP eta DOP), DDTak (2,4'-DDD, 4,4'-DDD, 2,4'-DDT, 4,4'-DDT), HCHak (α-HCH, β-HCH, δ-HCH, γ-HCH), OPPak (klorfirifosa eta klorfenbinfosa). ea: ez aipatua, nd: ez aurkitua.

2.2. Konposatu kimikoak biota laginetan

Ingurumeneko uretan dauden konposatu kimikoen kontzentrazioa ezagutzea ingurumenean bizi diren espezie urtarren osasuna ezagutzeko ezinbestekoa da. Aipatu berri den moduan, konposatu organiko gehienak oso kontzentrazio baxuetan egoten dira ingurumenean, ng/L-ko mailan gehienetan. Kontzentrazioak toxikotasun-mailakoak baino baxuagoak diren bitartean, balio horiek ez lukete arriskurik suposatu beharko. Alabaina, etengabeko esposizioak arazoak ekar ditzake uretan bizi diren espezieetan [19, 111-114]. Arrainek konposatu toxikoak barneratzeko hurrengo 3 bide nagusi dituzte:

Elikadura

Elikaduraren bidez, konposatu kimikoak barneratzeko bi bide dituzte arrainek. Alde batetik, uretan disolbatuta dauden konposatuak bertan dauden elikagaiekin elkar daitezke eta, ondoren, arrainek barneratu. Bestetik, barneraketa arrain handiek kutsatuta dauden arrain txikiak jaten dituztenean gertatzen da. Azken fenomeno horri biomagnifikazioa deritzo eta kate trofikoan goian dauden espezieetan edo gizakiengan du garrantzi handiena. Behin arrainak konposatu kimikoak barneratu dituela, gorputzak liseritzen ditu. Gorputzak absorbatu ez dituen konposatuak gorotzen edo gernuaren bidez kanporatzen dira eta gainontzekoak gibelera garraiatzen dira absortzio prozesuarekin jarraitzeko. Bide horretatik jasotako konposatu kimikoak nahiko azkar heltzen dira organismora; horregatik, kalte larriak sor ditzake arrainetan.

Larruazala

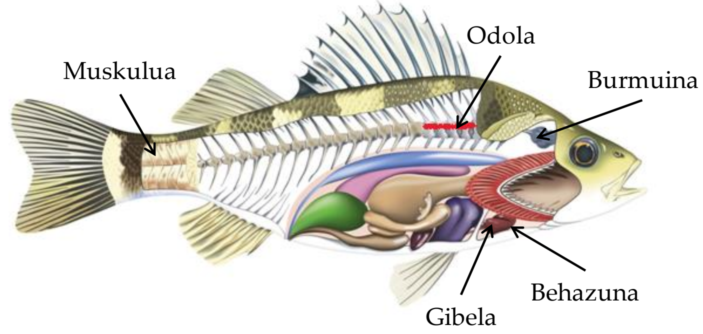
Arraina uretan disolbatuta dauden konposatu kimikoekin kontaktuan dago etengabe. Nahiz eta konposatu kimikoak oso kontzentrazio-maila baxuetan egon (ng/L), denbora luzez egoten dira azalarekin kontaktuan eta konposatuek gorputzean barneratzeko aukera dute. Molekula polarrak eta txikiak (< 600 Dalton) azaleko poroen bidez barneratzen dira. Haatik, molekula handiagoak (> 600 Dalton) eta hidrofobikoak gune lipidikoen bidez garraiatzen dira mintza zeharkatzeko. Behin konposatuek arrainaren azala zeharkatu ondoren, dermisean

dauden odol-hodietara iristen dira eta konposatuen propietate fisiko-kimikoen arabera, gorputz osoan zehar garraiatzen dira edo muskuluan metatzen dira. Uretan disolbagarritasun altuena duten konposatuak, odolean disolbatu eta garraiatuko dira, eta uretan disolbagarritasun baxua dutenak, aldiz, hau da, lipidoetan metatzeko joera dutenak, muskuluan metatuko dira. Arrainak badu gorputz patogenoei eta estres kimiko, biologiko eta fisikoari aurre egiteko defentsa mekanismo bat. Defentsa mekanismo hori, arrainak etengabe sortzen duen muki jariakina da [115-117]. Aipatutako patogenoen artean konposatu kimikoak daude. Hala ere, ezinezkoa da muki jariakinaren eraginkortasuna % 100koa izatea, eta denboraren bortxaz hainbat konposatuk metatzea lortzen dute.

Arnasketa

Arrainak zakatzak erabiltzen ditu uretan dagoen oxigenoa eta karbono dioxidoaren arteko elkartrukea egiteko. Arrainaren odolak bihotzetik zakatzetarako bidea egiten du, bertan oxigenatzen da eta gainontzeko organoetara ponpatzen da oxigenoa garraiatzeko. Odolak, oxigenoaz gain, zakatzetatik sartzen diren konposatu kimikoak ere garraiatzen ditu. Era horretan, konposatuak gorputz osoan zehar garraiatzen dira eta organoetan metatzeko aukera dute.

Arnasketa bidearen bitartez konposatuak metatzeko joera sakonago aztertuko da, kaltetutako organoen funtzioa azalduz. **1.7 irudian** EDCen metaketan garrantzia duten arrainen zenbait atal ikus daitezke.



1.7 irudia: *EDCen metaketa arrainetan aztertzeko garrantzitsuak diren zenbait atal*.

Odola

Arrainek odol-sistema itxia dute. Bihotzak ponpatzen duen odola oxigenatzeko zakatzetara joaten da lehenbizi eta ondoren gorputz osotik zehar garraiatzen da. Zakatzen bidez barneratutako konposatu organikoek odola erabiltzen dute garraiobide moduan. **1.3 taulan** ikus daitekeen moduan, konposatu organikoek ez dute odolean metatzeko joera handirik erakusten eta ng/mL-ko maila oso baxuetan antzeman [118, 119] dira; zenbait kasutan, uretan dauden kontzentrazio-maila berdinean.

Gibela

Konposatu kimikoak odolaren bidez heltzen dira gibelera gorputzetik kanporatuak izan aurretik. Gibelak, konposatu arrotzak egonkorragoak eta polarragoak diren konposatuetara metabolizatzen ditu, behazunaren bidezko kanporatzea laguntzeko. Konposatuek bi erreakzio-mota jasan ditzakete gibelean. Lehenik eta behin, 1. fasea (*Phase I*) deritzon prozesuaren bidez, konposatu organiko batzuk (ez-polarrenak) eraldatu egiten dira polarragoak diren metabolitoetan [120, 121]. Orokorrean, metabolito berri horiek alkohol (-OH) edo azido (-COOH) talde bat daukate. Bigarren erreakzioari 2. fasea (*Phase II*) deritzo. Bertan, metabolito berri horien –OH edo –COOH taldeei glukuronido [44, 122, 123], glukosido [124] edo sulfato [44, 125] talde bat lotzen zaie, konposatu polarragoak lortuz; hartara, behazunaren bidez kanporatzeko errazagoak diren konposatuak bilakatuz. Konposatu polarragoak (eraldatu gabe –OH edo –COOH taldea daukatenek) ez dute 1. fasea pairatzen eta zuzenean 2. fasean transformatzen dira.

PE molekulek jasaten duten 1. fasearen adibidea **1.8 irudian** agertzen da laburbildurik. PE molekulak dituzten 2 ester taldeetatik, R talde bat erreakzioan askatu egiten da eta -COOH taldea aske geratzen da. 1. faseko erreakzioa jasan dezaketen molekulen artean PEak [120, 126, 127], klorfirifos [128-130] eta klorfenbinfos pestizidak eta HHCBa [33] daude. Transformazio horren ostean, konposatu horien metabolitoek 2. fasea pairatzen dute. BPAk, hormonak eta APak, zuzenean 2. fasera pasatzen dira, jadanik glukuronizatu, glukosidatu edo sulfatatu

daitezkeen –OH (edota –COOH) taldeak baitituzte. Legler eta lankideen arabera, adibidez, E2 hormonaren % 90 glukuronido moduan askatzen da [131]. Konposatu glukuronidoak ez dira estrogenikoki aktiboak [132, 133], baina, araztegietan dauden bakterioek konposatu glukuronidoak hidroliza ditzakete jatorrizko konposatuak bihurtuz.

Diester ftalatoa — Monoester ftalatoa (1. fasea) — Monoester glukuronidoa (2. fasea)

1.8 irudia: *PE molekulek jasaten duten 1.eta 2. faseko erreakzioaren eskema.*

Odolarekin gertatzen ez den bezala, konposatu kimikoek gibelean metatzeko joera dute (ikus **1.3 taula**) [29, 134-136]. Aipatu behar da, OPPen kasuan adibidez, sortzen diren metabolitoak jatorrizko pestizidak baino toxikoagoak direla [130]; beraz, oso garrantzitsua da gibelean sor ditzaketen ondorioak aurreikusteko metatzen diren konposatuak aztertzea.

Behazuna

Gibelean metabolizatutako konposatuak behazunera gorputzetik kanporatuak izateko helburuarekin heltzen dira [114, 122, 137]. Hori dela eta, behazuna da konposatu organikoak maila altuenean dauden ehuna eta beraz, metaketa-maila altuena duena. **1.3 taulan** ikus daitekeen moduan, konposatuak aurreko ehunetan baino kontzentrazio altuagoetan daude behazunean eta zenbait kasutan ng/μL-ko mailara hel daitezke. Adibide moduan, aipagarria da Ingalaterrako araztegi bateko irteeran arrantzatutako arrain baten behazunean neurtutako NP maila 12 ng/μL baino handiagoa zela [123]. Esan beharra dago elikagaiak digeritzeko erabiltzen den bakoitzean, behazun-xixkoa hustu egiten

dela. Hori dela eta, behazunean aurki daitezkeen kutsatzaileak esposaketa laburrekoak dira, Martinez-Gomez eta lankideen arabera ordu gutxi eta zenbait egun bitartekoak [114]. Arrazoi horregatik eta behazunak duen metaketa ahalmenagatik, ikerlari askok behazuna proposatzen dute biomonitorizazioetan neurtu beharreko ehun bezala [44, 114].

Muskulua

Azaletik barneratzen diren konposatuek epe luzean muskuluan metatzeko joera dute. Orokorrean, aztergai diren konposatu organikoak ng/mL maila baxuetan metatzen dira muskuluan (ikus **1.3 taula**). Egia da kontzentrazio horiek ingurumeneko uretan aurki daitezkeenak baino handiagoak direla. Horregatik, oso garrantzitsua da arrainaren muskuluan metatzen diren konposatuen kontzentrazioak ezagutzea, ez arrainean izan dezakeen toxikotasunagatik bakarrik, baita muskulua jatean gizakiengan sor daitezkeengatik arazoengatik ere.

Burmuina

Arrainen burmuinak lipidoen metabolismoaren, sexu-hormonen jariatzearen, hazkuntzaren, osmoregulazioaren eta garapen sexualaren portaera kontrolatzen du, besteak beste. Heldugabeko arrainetan, esteroideek burmuinean dagoen gonada pituitarioaren garapenean ezinbesteko garrantzia dutela uste da. Azken horrek, arrain heldu baten ugalketa-zikloa kontrolatuko du [138]. Beraz, burmuinean dauden EDCen kontzentrazioa jakitea ezinbestekoa da arrainaren garapenean arazorik egon daitekeen ala ez aurresateko.

1.3 taula*: Ingurumeneko ur desberdinetan jasotako arrainen atal desberdinetan neurtutako EDCen kontzentrazioak.*

Analitoa	Matrizea	Espeziea	Herrialdea	Ingurumena	Urtea	Kontzentrazioa	Ref
BPA	Behazuna	*Rutilus rutilus*	Ingalaterra	Araztegiko irteera	2007	763-1.951[a]	[123]
	Behazuna	*Parophrys vetulus*	USA	Estuarioa	2005	< 6-52[a]	[139]
	Gibela	*Cyprinus carpio*	Iran	Zingira	2010	2.150[b]	[134]
	Gibela	*Cyprinus carpio*	Txina	Lakua	2009	107[b]	[29]
	Odola	*Morone saxatilis*	USA	Lakua	2011	1-6[a]	[118]
	Muskulua	*Oreochromis niloticus niloticus*	Taiwan	Erreka	2010-2011	nd-12[c]	[47]
	Muskulua	*Cyprinus carpio*	Iran	Zingira	2010	1.580[b]	[134]
Musketa fragantziak	Gibela	*Salmo salar*	Frantzia	Estuarioa	2011	4-6[b]	[135]
	Muskulua	*Mullus surmuletus*	Mediterraneoa	Itsasoa	2013	1-9[b]	[140]
	Muskulua	*Liza aurata*	Portugal	Estuario	2013	4-12[b]	[141]
E2/EE2	Behazuna	*Rutilus rutilus*	Ingalaterra	Araztegiko irteera Erreka	2007	17-2.503[a] < 0,4-56[a]	[123]
	Behazuna	*Parophrys vetulus*	USA	Estuarioa	2005	< 6-310[a]	[139]
	Gibela	*Cyprinus carpio*	Txina	Lakua	2009	12-19[b]	[29]
	Odola	*Platichthys flesus*	Frantzia	Estuarioa	ea	< 0,2-14[b]	[119]
NPak	Behazuna	*Rutilus rutilus*	Ingalaterra	Araztegiko irteera Erreka	2007	5.531-12.678[a] < 60-2.453[a]	[123]
	Behazuna	*Cyprinus carpio*	Txina	Erreka	2011	950-4.648[c]	[44]
	Gibela	*Cyprinus carpio*	Iran	Zingira	2010	3.210[b]	[134]
	Gibela	*Cyprinus carpio*	Txina	Lakua	2009	68[b]	[29]
	Muskulua	*Mugil cephalus*	Taiwan	Erreka	2010-2011	34-232[c]	[47]
	Muskulua	*Cyprinus carpio*	Iran	Zingira	2010	1920[b]	[134]

1.3 taula: *Jarraipena. Ingurumeneko ur desberdinetan jasotako arrainen atal desberdinetan neurtutako EDCen kontzentrazioak.*

Analitoa	Matrizea	Espeziea	Herrialdea	Ingurumena	Urtea	Kontzentrazioa	Ref
OPak	Behazuna	*Cyprinus carpio*	Txina	Erreka	2011	15-39[c]	[44]
	Gibela	*Cyprinus carpio*	Txina	Lakua	2009	15[b]	[29]
	Muskulua	*Cyprinus carpio*	Iran	Zingira	2010	3240[b]	[134]
PEak	Muskulua	*Liza subviridis*	Taiwan	Erreka	2004-2005	0,05-254[b]	[142]
HCHak	Behazuna	*Hypophthalmichthys molitrix*	Txina	Erreka	ea	1,5-30[a]	[136]
	Gibela					0,07-18[c]	
	Muskulua					0,15-14[b]	
	Muskulua	*Cyprinus carpio*	Pakistan	Erreka	2007-2009	0,1-2,4[c]	[143]
DDTak	Behazuna	*Hypophthalmichthys molitrix*	Txina	Erreka	ea	nd-0,04[a]	[136]
	Gibela					nd-0,37[c]	
	Muskulua					nd-0,33[b]	
	Muskulua	*Cyprinus carpio*	Pakistan	Erreka	2007-2009	0,3-16[c]	[143]

[a] ng/mL; [b] ng/g (pisu lehorra); [c] ng/g (pisu hezea). Musketa fragantziak (HHCB eta AHTN), NPak (NP isomero nahastea), OPak (4*t*OP eta 4*n*OP), PEak (BBP, DEHP eta DOP), DDTak (2,4′-DDD, 4,4′-DDD, 2,4′-DDT, 4,4′-DDT), HCHak (α-HCH, β-HCH, δ-HCH, γ-HCH), OPPak (klorfirifosa eta klorfenbinfosa). ea: ez aipatua, nd: ez aurkitua.

3. Ingurumenean dauden konposatu kimikoen analisia

3.1. Erauzketa-teknikak

Kimika analitikoaren erronka nagusienetako bat, nagusiena ez bada, laginen aurretratamendua ahalik eta era egokienean egitea da. Ahalegin handia egin behar da laginaren aurretratamendu egokia gauzatzeko, baina pausu horren egokitasun ezak analisi osoaren porrota ekar dezake. Beraz, kimika analitikoaren helburua, aurretratamenduaren ikuspuntutik, doiak, zehatzak, sentikorrak, sendoak eta selektiboak diren metodoen garapena da.

3.1.1. Lagin likidoak

Likido-likido erauzketa (*Liquid Liquid Extraction*, LLE) eta fase solidoko erauzketa (*Solid Phase Extraction*, SPE) izan dira lagin likidoen konposatu organikoen erauzketarako orain arte gehien erabili izan diren bi teknika analitikoak [46, 144-147]. Bi teknika horien abantailarik nabarmenena haien erauzketa-eraginkortasun handia da eta, gainera, ez da tresneria sofistikaturik behar. Kontrara, laginak erauzteko denbora luzeak behar dira eta kimika berdearen printzipioekin ez datoz bat, disolbatzaile organikoen bolumen handiak erabili behar baitira. Azken urteotan, ingurumenarekiko sortu den kezka dela eta, disolbatzaile organiko gutxiago, edo disolbatzaile organikorik behar ez duten mikroerauzketa teknikak garatu dira, "sorptive" (adsortzio edo absortzio moduan) erauzketa-metodoetan oinarrituz [148, 149]. Teknika horien aitzindaria fase solidoko mikroerauzketa (*Solid Phase Microextraction*, SPME) da. Teknika hori Pawliszyn eta lankideek erabili zuten lehenbizikoz 1990. urtean [150] eta ordutik ona, oso teknika erabilia izan da konposatu organiko desberdinen azterketarako ur-laginetan [148, 151], arrainen behazunean [152], giza-plasman [153, 154] eta giza-gernuan [151, 155]. Teknika horren oinarria banaketa-orekan datza. Analitoa, lagina eta solidoa edo likidoa izan daitekeen fase hartzailearekin orekan dagoenean sortzen den egoeraren konstantea da [156]. Teknika guztiek bezala, SPMEk desabantaila garrantzitsu bat du. Fase polimerikoaren kopuru txikia bakarrik erabil daitekeenez, lortzen diren detekzio-mugak (*Limits Of Detection*,

LODs) ez dira behar bezain baxuak ingurumeneko uren analisia zuzenean burutzeko [102, 147, 157]. Arazo horri aurre egiteko, oreka-banaketa berdina erabiltzen duen beste teknika batek hartu zuen indarra 1999. urtean [158]. Teknika hori, hagatxo birakarien bidezko erauzketa (*Stir Bar Sorptive Extraction*, SBSE) da. Twister® moduan ere ezaguna da. Twister-hagatxoetan, iman bat fase polimerikoaz estalita dago. Hagatxoa lagin likidoarekin kontaktuan jartzean, analitoak fase polimerikoan absorbatuta/adsorbatuta geratzen dira.

Hagatxorik ohikoenak polidimetilsiloxanoz (*Polydimethylsiloxane,* PDMS) estalita daude. PDMSa, analito ez-polarrak erauzteko polimero egokiena da [145, 157, 159]. Analito polarrak erauzteko, ordea, hagatxo desberdinak garatu dira; Acrylate twister® eta EG Silicone twister®. Lehenbizikoa, poliakrilato (*Polyacrilate*, PAc) eta polietilenglikol (*Polyethylen Glycol*, PEG) nahaste polimerikoez osatuta dago eta bigarrena, ostera, PEG eraldatutako silikonaz [149]. SBSE erauzketan eragina duten parametroen artean, fase polimerikoaren mota eta kantitatea (luzera eta lodiera), hagatxoaren bira-abiadura, pHa, gatz inerte baten adizioa (NaCl normalean), disolbatzaile organiko baten adizioa (MeOH normalean), erauzketa-denbora (orekan lan egin ohi da), erauzketa-tenperatura eta laginaren bolumena aurkitzen dira. Parametro horiek erauzketaren orekan eta eraginkortasunean eragina dute eta erauzi nahi den analitoaren arabera eragin positiboa edo negatiboa eduki dezakete. Adibidez, gatz inerte baten gehipenarekin PDMS zuntzen bidez konposatu polarren erauzketa hobe daiteke (salting-out efektu bezala ezaguten dena). Disoluzioaren indar ionikoa handituz (NaCl gehituz) konposatu polarren kasuan batez ere, uretan duten disolbagarritasuna jaitsi daiteke PDMS polimeroan absorbatzeko joera faboratuz [160-162]. Konposatu organiko ez-polarren kasuan, ordea, NaClaren gehipenak kontrako joera eragin dezake (salting-in efektua).

Erauzketako parametroak optimizatu ostean, analitoak fase polimerikotik askatzeko (desorbatzeko) bi bide nagusi daude: desortzio termikoa (*Termal Desorption*, TD) eta desortzio likidoa (*Liquid Desorption*, LD). TDa erabili ahal izateko, analitoak bereizteko gas-kromatografia (*Gas-Chromatography*, GC) erabili behar da. Kasu horretan, hagatxoak gas-kromatografoaren injektoreari lotuta

dagoen desortzio termikoko unitatean (*Thermal Desorption Unit*, TDU) desorbatzen dira. Modu horretan, erauzitako analito guztiak zuzenean GCan injektatzen direnez, detekzio-muga baxuak lor daitezke. TDU bat eskuragarri ez dagoenean edo analisia likido-kromatografia (*Liquid Chromatography*, LC) teknikaren bidez aurrera eraman nahi denean, LDa izaten da analitoak hagatxotik desorbatzeko aukera bakarra. Azken kasu horretan, hagatxoa aurretik aukeratutako disolbatzaile organiko egoki batez estali ondoren eramaten da aurrera desortzioa eta, horrela, analitoak disolbatzaile oraganikoan geratzen dira disolbatuta [149, 163].

Ingurumeneko laginetan dauden konposatu kimikoek ezaugarri fisiko-kimiko desberdinak dituzte. Kimika analitikoaren erronketako bat ezaugarri fisiko-kimiko desberdinak dituzten familia desberdinetako konposatuen aldibereko determinazioa egitea da. Orain arte aipatutako teknika desberdinek (LLE, SPE, SPME eta SBSE) ez dituzte teknika analitikoen betebehar guztiak asetzen; ingurumeneko arazoengatik, denbora arazoengatik edo analito desberdinak erauzteko gaitasun ezarengatik. Arazo guzti horiei irtenbidea emateko, polimero desberdinak erabili izan dira mikroerauzketan oinarritutako metodoekin konposatu polarrak eta ez-polarrak aldi berean aztertzeko. Emaitza oparoak eskaini dituen polimeroetako bat polietersulfona (*Polyethersulfone*, PES) izan da (ikus **1.9 irudia**).

1.9 irudia: *PES polimeroan egitura kimikoa.*

PES polimeroa konposatu polarrak zein ez-polarrak erauzteko erabili izan da. 0,07-6,88 bitarteko log K_{ow} balioa duten analitoetarako egokia dela frogatu da [145]. Musketa fragantziak, APak, hormonak, konposatu farmazeutikoak, PEak, pestizidak, konposatu perfluorinatuak ur-laginetatik erauztea lortu dute hainbat ikerlarik [102, 145, 164]. PES polimeroak, polartasun desberdineko konposatuak erauzteaz gain, kostu baxua (~ 0,05 €/analisi) du komertzialki saltzen diren hagatxoekin alderatuz. Kostu baxu horri esker, ez dago hagatxoak berrerabili

beharrik. Horrek oroimen-efektua eta hondatze kimikoak edo mekanikoak sor ditzakeen erauzketa-eraginkortasunean aldaketak saihestea suposatzen du [145]. Kontrara, PES polimeroa ezin da momentuz TDan erabili; beraz, LDa erabili behar da beti analitoak polimerotik desorbatzeko. Nolanahi ere, ng/L mailako detekzio-mugak lortzen dira dagoeneko [102, 164].

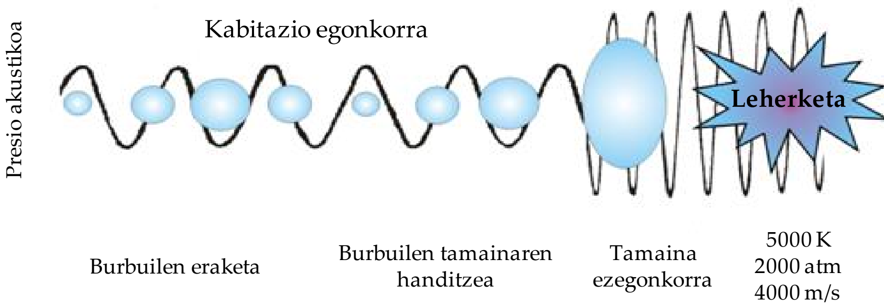
3.1.2. Lagin solidoak

Solido-likido erauzketa (*Solid-Liquid Extraction*, SLE) tekniken artean Soxhlet erauzketa izan da urtetan zehar gehien erabili izan dena [82, 165-167]. Lagin likidoen erauzketan gertatzen den moduan, teknika klasikoek disolbatzaile organikoen bolumen handiak (100-250 mL) behar izaten dituzte eta erauzketa-urratsa burutzeko nahiko denbora luzeak. Teknika analitiko berrien garapenarekin erauzketa-metodo berriak garatu dira. Horien artean aipagarrienak mikrouhinen bidezko erauzketa (M*icrowave Assisted Extraction*, MAE), ultrasoinuen bidezko erauzketa (*Ultrasound Extraction*, USE) eta presiopeko likido erauzketa (P*ressurised Liquid Extraction*, PLE) daude. Azken hori disolbatzailearen bidezko erauzketa azeleratua (*Accelerated Solvent Extraction*, ASE) moduan ere ezagutzen da. PLEaren eta MAEaren kasuan (sistema itxian lanean ari bagara), disolbatzailea presiopean dago eta, presioa igoz, disolbatzaile organikoaren irakite tenperatura ere igotzen denez, tenperatura altuagoetan lan egin daiteke eta ondorioz erauzketa faboratu [168, 169] eta erauzketa denbora txikitu egiten da. Erauzketa-teknika horiekin, erauzketan erabili behar den disolbatzaile organikoaren bolumena ere asko murriztu da (10-25 mL).

Disolbatzaile organiko gutxi erabiltzen duen beste teknika bat, lehenago adierazi bezala, USE da. USE erauzketan, lagina disolbatzaile organiko batekin edo disolbatzaile-nahasketa batekin jartzen da kontaktuan eta ultrasoinu-energiaren bidez erauzten dira konposatuak. USE tekniken artean, ultrasoinu fokatu bidezko solido-likido erauzketa (*Focused Ultrasound Solid Liquid Extraction*, FUSLE) garrantzia hartzen hasi da azken urteotan biota laginetatik APak [170], hormonak [170], PAHak [171, 172] eta konposatu perfluorinatuak [173] aztertzeko, ohiko ultrasoinu-bainuak baino errepikakorragoa baita. FUSLEn, disolbatzaile organikoan murgilduta dagoen titaniozko edo beirazko punta baten bidez egiten

da sonikazioa eta lortzen den indarra ultrasoinu-bainuan lortzen dena baino 100 bider handiagoa da [174]. Energia-fokatze horren ondorioz erauzketa-denborak segundo eta minutu gutxi batzuetara labur daitezke [175].

Ultrasoinuak intentsitate nahikoa lortu duenean, burbuilak edo kabitateak sortzen dira hedapen-zikloan zehar. Burbuilak sortu eta lehertzeko fenomenoari kabitazioa deritzo [176]. Kabitazio-burbuila bat lagin solido baten partikulen gainazaletik gertu lehertzen denean, disolbatzailearen tantak abiadura handian sakabanatzen dira solidoaren gainazaletik zehar eta gainazala zulatzen eta erosio mekanikoa sortarazten du. Horren ondorioz, partikulen apurketa gertatzen da, tamaina txikiko partikulak sortuz [177, 178]. Burbuilen apurketarekin batera, mikroskopikoki tenperatura (< 5.000 K) eta presio (< 2.000 atm) oso handiak lortzen dira eta horrek ere erauzketaren baldintza makroskopikoak aldatu barik, erauzketa faboratzen du [177]. Irradiazio ultrasonikoa disolbatzailearen bidez transmititzen denean, perturbazioa sortzen da (eta horren errepikapenaz), hedapen- eta konpresio- zikloak sortzen dira [179] (ikus **1.10 irudia**).



1.10 irudia: *FUSLE erauzketan sortzen den kabitazio fenomenoaren eskema.*

Aipatu diren teknika guztiak, bai lagin likidoetatik analitoak erauztekoak bai lagin solidoetatik erauztekoak, ez dira oso selektiboak eta intereseko analitoez gain hainbat interferentzia ere erauzten dituzte [175, 180]. Kasu gehienetan interferentziak ezabatzeko garbiketa urrats bat egin behar izaten da analisiaren aurretik.

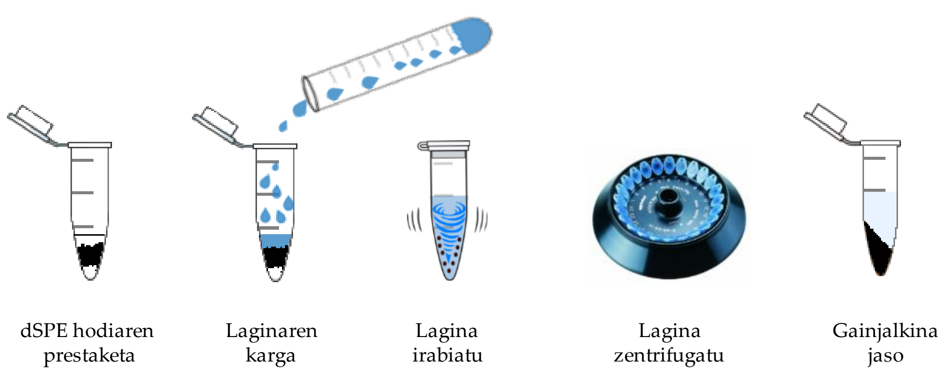
3.2. Garbiketa-teknikak

Lagin solidoen eta likidoen garbiketa-urratsa egiteko, garbiketa-teknika berdinak erabil daitezke. Hala ere, aztertu nahi den matrizearen arabera, erauzitako interferentziak desberdinak dira eta horrek finkatzen du aukeratu beharreko garbiketa-teknika.

Garbiketa-teknika klasikoen artean, SPE delakoa da erabiliena [175]. SPE teknikak lagin likidoetatik analitoak erauzteko erabiltzen diren teknikek duten oinarri berdina du. Florisil®, silizea eta alumina dira ur- [46, 181] eta biota-laginen [182, 183] garbiketarako gehien erabiltzen diren adsorbatzaileak. Garbiketa urratsean erabiltzen diren adsorbatzaileen masak handiak izan ohi dira (150-5.000 mg) eta, ondorioz, kartutxoa egokitzeko, interferentziak ezabatzeko eta analitoak eluitzeko erabili behar den disolbatzaile organikoaren bolumena ere handia da. Gel-permeazio kromatografia (*Gel Permeation Chromatography*, GPC) asko erabili izan den beste garbiketa-teknika bat da, batez ere biota-laginek duten koipea kentzeko [45, 184]. Zutabe kromatografiko bereziak erabiliz, tamainaren araberako banaketa egiten da, koipeak zutabean atxikituta geratzen dira eta intereseko molekulak bakarrik eluitzen dira. GPCan, SPEan bezala, disolbatzaile organikoen bolumen handiak erabili behar izaten dira; hala eta guztiz ere, gaur egun asko erabiltzen dira [185, 186].

Erauzketa-teknikekin gertatzen den moduan, kimika analitikoan egindako aurrerapenei esker, ingurumenarekin egokiagoak diren teknikak garatu dira. Garbiketa-metodo klasikoei irtenbidea emateko, fase solidoko erauzketa dispertsoaren (*Dispersive Solid Phase Extraction*, dSPEaren) erabilera asko hedatu da azken urteotan [15, 144, 175]. dSPE teknikaren erabilera Anastassiadeek eta lankideek [187] proposatu zuten estrainekoz "QuEChERS"(*Quick Easy Cheap Effective Rugged Safe*, azkarra erraza merkea eraginkorra sendoa segurua) metodoarekin batera. dSPE metodoaren abantailarik nagusienak erabiltzen diren disolbatzaile organikoaren bolumen txikia eta prezio merkea dira. Ohiko SPE zutabeetan erabiltzen diren adsorbatzaileak erabili daitezke dSPEan. Gehien erabili diren adsorbatzaileak hauexek dira: amina primarioak eta sekundarioak (*Primary*

and Secondary Amine, PSA) gantz azidoak kentzeko, ENVI-CARBa (ENVI) esterolak eta pigmentuak kentzeko eta oktadezilsilanoa (*Octadecylsilane*, C_{18}) interferentzia ez-polarrak kentzeko [144, 180, 186]. Zenbait kasutan, ur-arrastoak kentzeko $MgSO_4$ ere gehitzen zaio. dSPE urratsean aukeratutako fasea edo faseen nahasketak $MgSO_4$ eta intereseko analitoak dituen erauzia tutu batean nahasten dira eta irabiagailu baten laguntzaz irabiatzen da nahastea fase solidoa dispertsatzeko. Fasea dekantatu ostean, intereseko analitoak disolbatzailean geratzen dira eta interferentziak fasean adsorbaturik (ikus **1.11 irudia**). Interferentziekiko afinitate altua duen adsorbatzailea aukeratu behar da eta analitoekiko afinitate handia duen disolbatzailea, aldiz. dSPE metodoa BPA [180, 188], hormonak [180, 188, 189] eta pestizidak [185] biota-laginen garbiketarako erabili dira maiz, baita konposatu organikoak plasmatik [144, 190], gernutik [190] eta elikagaietatik [191] erauzteko ere.



1.11 irudia: *dSPE garbiketan jarraitu beharreko urratsak.*

3.3. Hidrolisi-urratsa

Konposatu organikoek, behazunetik zehar kanporatu nahi direnean, 2.2 atalean aipatu den moduan, gibelean 2. faseko (*Phase II*) metabolismoa jasaten dute glukuronido, glukosido edo sulfato bihurtzeko. Molekula konjokatu horiek zuzenean azter daitezke analisi zuzena deitzen den metodo multzoen bidez, edo analisi ez-zuzenen bidez. Azken metodo-multzo horretan, molekula konjokatuak hidrolisatu egiten dira jatorrizko analitoen moduan aztertzeko.

Bibliografian oso lan gutxi aurkitzen dira analisi zuzena erabiltzen dutenak [192-194]. Estrogenoak era konjokatuan (glukuronido, glukosido eta sulfatoak) aztertu zituen lehenbiziko lana 2002. urtean plazaratu zen [195]. Talde berak, urte batzuk geroago, araztegiko sarrerako eta irteerako uretan estrogeno konjokatuak aztertu zituen [194]. Erauzketa teknika moduan SPEa erabili zuten aipatutako bi lanetan, baina ez zuten inolako garbiketa urratsik erabili, eta horrek kromatografiako detekzio-urratsean % 80-90 seinale galera eragin zuen laginak elektroesprai ionizazio (*Electrospray Ionisation*, ESI) sistemaren bidez neurtu zirenean [195]. Antzeko emaitzak lortu zituzten Pedrouzo eta lankideek [196]. Estrogeno konjokatuak oso polarrak dira eta garbiketa-urrats egokia egitea ez da erraza, orokorrean lortzen diren berreskurapenak oso baxuak izanik [133]. Komori eta lankideek [197] erauzketa-urratsaren ostean SPE metodoan oinarritutako bi garbiketa-urrats egin zituzten Florisil® eta NH_2 adsorbatzaileak erabiliz, baina lortu zituzten berreskurapenak oso baxuak izan ziren araztegiko irteerako (% 18-81) eta sarrerako (% 6-15) uretan. Bi garbiketa-urrats nahikoak ez zirela zirudien eta horregatik, ikerketa talde berak 3 urratseko garbiketa proposatu zuen. Ur purifikaturako lortu zituzten emaitzak onargarriak izan ziren (% 63-81), baina ez zuten inolako berreskurapen azterketarik egin araztegiko urekin. Azkenik, Koh eta lankideek [198] SPE bidez erauzitako laginetan, GPC eta SPE garbiketa-urratsen ostean, emaitza oparoak lortu zituzten (% 95-99).

Estrogeno konjokatuak neurtzeko teknikarik ohikoena LCa da, ESI ionizazioa erabiliz, baina aipatu den moduan, garbiketa-urrats egokia lortzea zaila izaten da eta zuzenenean neurtzen denean seinaleen galera handia da. Arazo horri

irtenbidea emateko, elektroforesi kapilarra kromatografia-sistema erabili zen eta emaitza oparoak lortu ziren. Tamalez, teknika hori ez da ingurumeneko laginak neurtzeko guztiz aproposa ESIren bidez lortutako detekzio-mugak baino 20 aldiz altuagoak lortzen baitira [199].

Analisi zuzenak egiten direnean, lagin bakoitzaren analisi-denbora asko laburtzen da, ikerlari askoren aburuz hidrolisi-urratsa baita mugatzailea [133, 200]. Analisi zuzenaren bidez glukuronido, glukosido eta sulfato guztiei buruzko informazioa jaso daiteke, baina aipatu beharra dago molekula konjokatu guztiak ez direla komertzialki eskuragarriak eta horrek erauzketa-metodoa eta metodo kromatografikoaren garapena oztopa dezakeela. Beraz, zenbait kasutan, ezinbestekoa da analisi ez-zuzena egitea.

Hidrolisia, orokorrean, bi modutan eraman daiteke aurrera. Alde batetik, hidrolisi kimikoa eta, beste aldetik, hidrolisi entzimatikoa. Hidrolisi kimikoa hidrolisi basiko (saponifikazio), azido, solbolisi azido eta amonolisiaren bidez egin daiteke, eta 4 bideak beren artean osagarriak dira. Gernuan estrogeno konjokatuen hidrolisien bidez egindako lanek ez dute emaitza egokirik eman gernuan egon daitezkeen interferentzien erruz. Horregatik, zenbait ikerlarik interferentzia horiek ezabatzeko, hidrolisi urratsaren aurretik LLE-a egitea proposatu izan dute [201-203]. Hidrolisiaren etekina handitzeko egindako azken saiakera, hidrolisia disolbatzaile organiko ez-polarretan egitea izan da. Disolbatzaile polarrekin alderatuta, emaitza oparoak lortu dituzte Liu eta lankideek [133]. Behazunaren matrizea urtsua dela kontuan hartuz, azken hidrolisiaren hobespen hori baztertua izan da eta aukerarik egokiena hidrolisi entzimatikoa dela onartu. Nahiz eta hobekuntza nabaria egin izan den, hidrolisi kimikoaren eraginkortasuna % 100 izatetik urrun dago oraindik. Gainera, hidrolisi kimikoan erabili beharreko baldintzak nahiko bortzitzak izan daitezke eta horrek analitoen galerak ekar ditzake [122].

Hidrolisi entzimatikoa hidrolisi kimikoa baino askoz gehiago erabili izan da, honek eskaintzen dituen dekonjokazio-maila altuengatik [133]. Hidrolisi entzimatikoa erabiltzen hasi zenean entzimak, β-glukuronidasa eta sulfatasa, animalia hornodunengandik lortzen ziren, baina berehala erabaki zuten

Eschericia Coli eta barraskilotik (*Helix pomatia*) lortzea [204, 205]. Barraskilotik lortzen den entzima β-glukuronidasaz eta sulfatasaz osatuta dago. Entzima mota horiekin, Bloch eta lankideek [204] hidrolisi azidoarekin alderatuta, % 70-100 kontzentrazio altuagoa lortu zuten. Hidrolisi entzimatikoan aktibitate entzimatikoa, denbora, tenperatura eta pH-a kontrolatzea ezinbestekoa da dekonjokazio-maila maximoa lortzeko. Asko dira bibliografian hidrolisi entzimatikoa egin duten ikerlariak, baldintza zeharo desberdinak erabili dituztenak. β-glukuronidasa eta sulfatasa entzimak erabiltzen dituzte ikerlari gehienek, hidrolisia 37 °C-an 12-24 ordu bitartean burutzen dute [114, 123, 125, 152, 204, 206, 207]. Hidrolisi-denbora laburtzeko, zenbait ikerlarik hidrolisiaren tenperatura 40-55 °C-ra igo zuten eta 2-3 ordura laburtu zuten hidrolisi-urratsa [119, 139, 208, 209]. Nahiz eta hidrolisiaren denbora asko laburtu, analisi osoaren denborak luzea izaten jarraitzen du. Kimika analitikoaren garapenari esker, teknika berriak erabili izan dira hidrolisia azkartzeko. Álvarez-Sánchez [210] eta Vallejo [122] ikerlariek, US-energia erabili zuten hidrolisia azkartzeko. Álvarez-Sánchezek hidrolisia burutzeko 30 minutu behar izan zituen estrogenoen kasuan, eta Vallejo eta lankideek AP eta E2 konposatuen hidrolisirako 20 minutu. Aurrerapausu izugarria izan da denbora murrizketa hori, baina laginak banan-banan hidrolisatu behar direnez, lagin asko aztertu behar badira analisi-denbora ez dela gehiegi murrizten aipatu beharra dago. Analisi osoaren denbora are gehiago laburtu nahian, Kawaguchi eta lankideek [211] aldibereko hidrolisia eta erauzketa metodo bat proposatu zuten gernu-laginetan 4-nonilfenol glukuronidoa (*4-Nonylphenol Glucuronide*, 4-NP-G) SBSE bidez aztertzeko. 90 minutu nahikoa izan zen hidrolisia eta erauzketa berreskurapen egokiarekin burutzeko. SBSE edo antzeko tekniken bidez ur-laginak erauzteko normalean 12h-tik gora behar direla aipatu behar da [102]. Matrize eta analito desberdinekin proba gehiago egin beharko lirateke, baina badirudi, bide hori izan daitekeela analisi-denborak laburtzeko eta, bide batez, ingurumenari begira egokiagoak diren teknikak lortzeko modua.

4. Konposatu kimikoen azterketa

Kromatografia, eskaintzen duen bereizketa ahalmenagatik, konposatu organikoen azterketarako gehien erabiltzen den teknika da [2, 180, 212]. Konposatuen propietate fisiko-kimikoen arabera, GCa edo LCa izan daiteke aukera. Jarraian sakonago deskribatuko da teknika bakoitza.

4.1. Gas-kromatografia

GCa azkarra, sinplea, erlatiboki merkea eta errepikakorra den bereizketa-teknika da [213] eta lurrunkorrak edo erdi-lurrunkorrak eta termikoki egonkorrak diren konposatuak bereizteko erabiltzen da. Bereizketa hori, analitoen irakite-tenperaturaren arabera egiten da gehienbat. Fase organiko batean disolbatuta dauden analitoak tenperatura altuan (260-300 °C) dagoen injektore batean lurrundu ostean, tenperatura kontrolatzen duen labe batean dagoen zutabe kapilar batean sartzen dira gas garraiatzaile baten laguntzaz (He, H_2 edo N_2). Zutabe kapilar horiek, luzera (10 m-60 m) eta barne diametro (100 µm–320 µm) desberdinak edukitzeaz gain, barrualdeko paretak, lodiera desberdineko fase geldikor batez (0,1 µm-1 µm) estalita daude. Zutabearen parametro horiek eta labearen tenperatura kontrolatuz, analitoen bereizketa eraginkorra lor daiteke. Zutaberik erabilienak % 5-(fenil)-% 95-metil polisiloxanoz eginda daude, 30 m-ko luzera dute, 250 µm-ko barne diametroa eta 0,25 µm-ko fase geldikorreko lodiera [46, 145, 161, 185]. GC bidez bereiz daitezkeen konposatuen artean, musketa fragantziak [32, 103, 145, 214], pestizidak [145, 161, 185], PEak [46, 76, 80], APak [92, 102, 167], BPA [45, 46, 146] eta estrogenoak [46, 119, 122] daude (ikus **1.4 taula**).

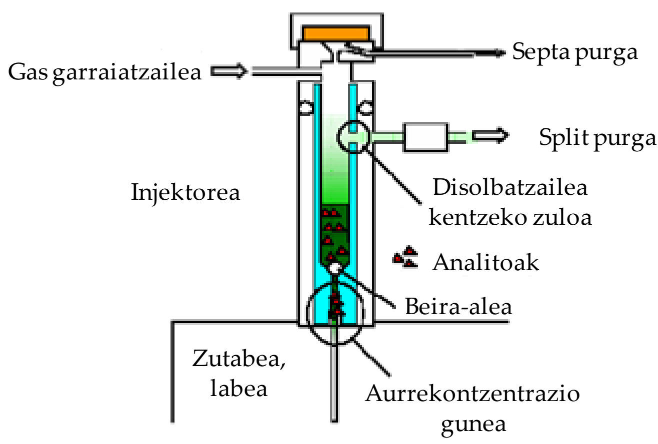
GCaren bidez berizten diren analitoen analisia edo detekzioa egiteko gaur egun erabiltzen den detektagailurik ohikoena masa-espektrometroa (*mass spectrometry*, MS) da (ikus **1.4 taula**). MSa detektagailu unibertsala, selektiboa eta sentikorra da. Gas-kromatografia–masa-espektrometria (*Gas Chromatography-Mass Spectrometry*, GC-MS) teknika ingurumeneko laginen azterketarako oso teknika erabilia da bi arrazoi nagusirengatik. Alde batetik, GC-MS teknika erabiltzen denean, konposatuen identifikazioa egiteko erabil daitekeen espektroen datu-basea

unibertsala eta nahiko handia da [215, 216] eta, beste aldetik, ingurumeneko lagin desberdinetarako lortzen diren detekzio-mugak egokiak dira [46, 145, 161, 185] (ikus **1.4 taula**). Orain arte, MSa izan da detektagailurik erabiliena, baina gaur egun, gero eta gehiago erabiltzen dira tandem masa-espektrometrian (*tandem mass spectrometry*, MS/MS) oinarritutako metodoak [80, 100, 123, 217]. Kuadrupolo hirukoitzari esker, selektibitatea igo daiteke eta horri esker positibo faltsuak saihes daitezke. Horrez gain, matrize-interferentziak murrizten ditu, zenbait kasutan matrize-efektua guztiz saihestuz. Horrek zarata/seinale erlazioa hobe dezake, sentikortasuna handituz [80, 100].

Selektibotasuna hobetzeko beste aukera bat ionizazio-teknika desberdinak erabiltzea da. MS detektagailuetan erabiltzen den ionizazio-mota ohikoena elektroi-ionizazioa (*Electron Ionisation*, EI) da [29, 46, 162]. EI ionizazio-teknika oso indartsua da, eta askotan jatorrizko molekularen MWari buruzko informazio guztia galdu egiten da. Informazio hori ezagutzea garrantzitsua izan daiteke molekula baten identifikaziorako. PEak dira horren adibide. EI bidez neurtu nahi direnean, PE guztietan oso egonkorra den egitura berdina lortzen da eta horrek ia masa-espektro berdina ematea eragiten du edozein dela jatorrizko PEaren adarkadura. Arazo horri irtenbidea emateko, ionizazio-teknika ahulagoak erabil daitezke, ionizazio kimikoa (*Chemical Ionisation*, CI) [218, 219] edo presio atmosferikoko gas-kromatografia (*Atmospheric Pressure Gas Cromatography*, APGC), [146] adibidez.

Beste alde batetik, sentikortasuna igotzeko beste aukera bat, detektagailuz aldatu gabe, injekzio-sistema desberdinak erabiltzea da. Orokorrean, GC sisteman 1-3 μL lagin injekta daitezke split/splitless injektorean (ikus **1.4 taula**). Azken urteotan, bolumen handiko injekzioa (*Large Volume Injection*, LVI) garrantzia hartzen hasi da split/splitless injekzio sistemarekin alderatuz gero [220]. LVI bidez, bolumen handiak (100 μL-raino) injekta daitezkeenez, LOD eta LOQ txikiagoak lor daitezke. Hala ere, aipatu beharra dago lagin gehiago sartzearekin batera matrize gehiago ere sartzen dela sistemara, eta MDLak ez direla zertan hobetu LOD eta LOQ mugekin gertatzen den moduan. LVIa tenperatura programatutako baporizatzaile (*Programmable Temperature Vaporizing*, PTV, (ikus **1.12 irudia**))

sistemari akoplatuta dago. Bertan, injektatzen den erauziaren disolbatzailea lurrunduz doa fluxu eta tenperatura (disolbatzailearen irakite tenperatura baino 10 gradu baxuago) kontrolatu batean eta, horregatik, analitoak injektorearen atorrean (linner) kontzentratzen dira. Lurruntze-urrats horren ostean, injektorearen tenperatura oso azkar igotzen da, analito guztiak lurrun daitezen, eta GCko zutabera garraiatzen dira (splitless moduan) [220].



1.12 irudia: *LVI-PTV injekzio sistemaren eskema.*

Azkenik, GCa erabiltzen denean, analitorik polarrenek (karboxilo, hidroxilo eta amino taldeak dituzten molekulak) lurrunkortasun baxua izaten dute eta beren bidezko bereizketa ez da bideragarria GC zutabe kapilar ez-polarrak erabiltzen direnean. Beste zenbait kasutan, konposatuak lurrunkorrak dira baina zutabean adsorbatuta geratzen dira edo deskonposatzen dira, mamu-gailurrak eta buztandun gailurrak eratuz eta, ondorioz, sentikortasuna galduz [213]. Hori guztia saihesteko, deribatizazio-erreakzio baten bidez, konposatu polarrak, konposatu ez-polarrago bilakatzen dira, beren lurrunkortasuna handituz eta GC bidez neurtzeko aproposagoak bihurtuz (zutabe ez-polarrekin). Estrogenoak GC bidez neurtu nahi badira, deribatizazio-urratsa ia ezinbestekoa da [46, 119, 122, 146], eta APen eta BPAren kasuan, deribatizazioak sentikortasuna hobe dezake [221, 222]. Nolanahi ere, urrats hori ez da beti burutzen analisiari etapa bat gehitzea dakarrelako (ikus **1.4 taula**).

***1.4 taula**: EDCak neurtzeko GC teknika erabiltzen duten metodoen laburpena, GC teknikaren parametrorik esanguratsuenak laburbilduz.*

Konposatuak	Matrizea	Erauzketa	Garbiketa	Deribatizazioa	Injekzioa	Bereizketa eta detekzioa	Detekzio-mugak (ng/L)	Ref
Pestizidak, PEak	Araztegiko irteera Araztegiko sarrera	SPE (Oasis-HLB)	SPE (Florisil)	Ez	LVI-PTV	GC-MS	1-322[a]	[46]
BPA, APak, estrogenoak	Araztegiko irteera Araztegiko sarrera	SPE (Oasis-HLB)	SPE (Florisil)	BSTFA(50 µL) + Piridina (125 µL)	LVI-PTV	GC-MS	1-55[a]	[46]
Pestizidak	Itsasoko ura	SBSE (PDMS)	Ez	Ez	TDU	GC-MS	0,0-7,5	[161]
BPA, APak	Errekako ura Arrain muskulua Arrain gibela	SPE (C_{18}) MAE MAE	Ez GPC GPC	BSTFA(50 µL) + Piridina (30 µL)	Split-Splitless	GC-MS	0,2-12[c] 0,3-8[c]	[45]
Pestizidak	Arrain muskulua	QuEChERS (dispersive SPE)	dSPE (PSA)	Ez	LVI-PTV	GC-MS	1-2[c]	[185]
Estrogenoak, APak	Arrain behazuna	SPE (Oasis-HLB)	Ez	BSTFA(25 µL) + Piridina (125 µL)	Split-Splitless	GC-MS	2-13	[122]
APak, BPA, estrogenoak	Arrain behazuna Errekako ura	SPE (Oasis-HLB)	Ez	Pentafluorobentzilazioa (20 ng BPFBB)	Split-Splitless	NCI-GC-MS	1-10[b]	[44]
Estrogenoak	Arrain odola Arrain behazuna	SPE (C_{18})	SPE (NH_2)	MSTFA/ NH_4I β-merkaptoetanol (30 µL, % 99.1:0,5:0,4)	Pulsed splitless	GC-MS	100[a]	[119]
Musketa fragantziak	Araztegiko sarrera Araztegiko irteera	LLE (toluenoa)	Ez	Ez	Split-Splitless	GC-MS	10-100[b]	[32]
Musketa fragantziak	Araztegiko irteera	HS-SPME (PDMS/DVB)	Ez	Ez	Split-Splitless	GC-MS	40[a]	[214]
Pestizidak, estrogenoak	Araztegiko sarrera Araztegiko irteera	PES tutuak	Ez	Ez	LVI-PTV	GC-MS	2-3[a]	[145]
APak, BPA, estrogenoak, musketa fragantziak, pestizidak	Araztegiko irteera	SBSE (PDMS)	Ez	Azido azetikoa (500 µL)	Split-Splitless	APGC-TOF-MS	0,02-2[c]	[146]

***1.4 taula**: Jarraipena. EDCak neurtzeko GC teknika erabiltzen duten metodoen laburpena, GC teknikaren parametrorik esanguratsuenak laburbilduz.*

Konposatuak	Matrizea	Erauzketa	Garbiketa	Deribatizazioa	Injekzioa	Bereizketa eta detekzioa	Detekzio-mugak (ng/L)	Ref
PEak	Arrain muskulua Arrain gibela	US (Hex:Ace 80:20)	SPE (Florisil)	Ez	Split-Splitless	GC-MS	8-11	[76]
APak, BPA	Errekako ura	SBSE (PDMS)	Ez	Azido azetikoa (200 µL)	TDU	GC-MS	0,5-1[a]	[147]
APak, BPA	Arrain behazuna Arrain odola Arrain muskulua	SPE (Oasis-HLB) SPE (Oasis-HLB) US (MeOH)	Ez Ez SPE (Oasis-HLB)	EtOAc (180 µL) + MTBSTFA (20 µL)	Split-Splitless	GC-MS	0,2-0,6[b] 0,2-0,6[b] 0,2-0,6[b]	[118]
Estrogenoak	Araztegiko irteera	SPE (Oasis-HLB)	Ez	BSTFA(70 µL) + EtOAc (35 µL)	Split-Splitless	GC-MS	0,05-0,06[a]	[108]
Pestizidak, APak, BPA, musketa fragantziak	Arrain gibela	QuEChERS (T10-Ultra torrax)	dSPE (PSA + C_{18})	Ez	Split-Splitless	GC-MS	ea	[227]
Estrogenoak	Arrain odola	SPE (C_{18})	SPE (NH_2)	MSTFA:TMSI:DTE	Split-Splitless	GC-HRMS	0,002[a] (ng/g)	[228]

[a]: LOD (detekzio-muga), [b]: LOQ (kuantifikazio-muga), [c]: MDL (metodoaren detekzio-muga), [d]: MQL (metodoaren kuantifikazio-muga).
PEak (ftalato esterrak); BPA (bisfenol-A); SPE (fase solidoko erauzketa); LVI-PTV (bolumen handiko injekzioa-tenperatura programatuko baporizatzailea); GC-MS (gas kromatografia-masa espektrometria); BSTFA (N,O-Bis(trimetilsilil)trifluoroazetamida); SBSE (hagatxo birakarien bidezko erauzketa); PDMS (polidimetilsiloxanoa); TDU (desortzio termikoko unitatea); MAE (mikrouhinen bidezko erauzketa), GPC (gel permeazio kromatografia); Quechers (azkarra, erreza, merkea, eraginkorra, sendoa eta segurua); dSPE (fase solidoko erauzketa dispertsoa); PSA (amina primarioa eta sekundarioa); BPFBB (1,4-bispentafluorobenzylbenzene); MSTFA (N-metil-N-(trimetilsilil)trifluoroazetamida); LLE (likido-likido erauzketa); HS-SPME (buru-gunea-fase solidoko mikroerauzketa); DVB (dibinilbentzenoa); PES (polietersulfona); US (ultrasoinua); Hex (hexanoa); Ace (azetona); EtOAc (etil azetatoa); MTBSTFA (N-tert-Butildimetilsilil-N-metiltrifluoroazetamida); C_{18} (oktadezil silanoa); TMSI (iodotrimetilsilanoa); DTE (ditioritriola); GC-HRMS (gas kromatografia-erresoluzio altuko masa espektrometria). Musketa fragantziak (HHCB, AHTN), APak (4*t*OP, 4*n*OP, NP mix), pestizidak (2,4′-DDD, 4,4′-DDD, 2,4′-DDT, 4,4′-DDT, α-HCH, β-HCH, δ-HCH, γ-HCH, klorfiribosa, klorfenbinfosa), estrogenoak (E2, EE2), PEak (DEHP, DOP, BBP).
ea: ez aipatua. Lagin likidoen kasuan mugak ng/mL unitateetan daude eta lagin solidoen kasuan ng/g-tan.

3 deribatizazio-metodo desberdin daude: banaketa aurreko deribatizazioa, zutabeko deribatizazioa (*on column*) eta banaketa ondoko deribatizazioa. Lehenengo biak GCan erabiltzen dira analitoen lurrunkortasuna handitzeko, eta hirugarrena LCan gehienbat, molekulen detekzioa hobetzeko. Zutabeko deribatizazioan analitoak eta deribatizatzaileak injekzio-portuan nahasten dira [223]. Deribatizazio-mota horrek analisi denbora laburtzen du baina deribatizatzailea soberan gehitzeak zutabearen fase geldikorraren degradazioa ekar dezake eta, ondorioz, ez da guztiz gomendagarria zutabearen bizitza asko murrizten baita. Hori dela eta, banaketa aurreko deribatizazioa izaten da ohikoena. Deribatizazio-erreakzio desberdinak daude. Ezagunenak azetilazioa (azido azetiko anhidroa erabiliz) [146, 147] eta sililazioa [45, 46, 119, 122] dira, nahiz eta azken hori izan erabiliena. Sililazioa ordezkapen nukleofilikoan oinarritzen da, non alkilsilil talde elektrofilikoa, orokorrean trimetilsilil (*Trymethylsilyl*, TMS) taldea, gune nukleofilikora elkartzen den bertako H bat kanporatuz [160]. Orokorrean, TMS eratorrien analogoak dira egonkorrenak. Silil-emaile talderik ezagunenak N,O-bis (trimetilsilil) trifluoroazetamida (*N,O-bis (trimethylsilyl) Trifluoroacetamide*, BSTFA), N,O-bis (trimetilsilil) azetamida (*N,O-bis (trimethylsilyl) Acetamide*, BSA) eta N-metil-N-(trimetilsilil) trifluoroazetamida (*N-methyl-N-(trimethylsilyl) Trifluoroacetamide* (MSTFA) dira. Sililazio-erreakzioaren desabantailarik nagusiena hurrengoa da: TMS taldea urarekin edo hezetasunarekin hidrolizatzen da eta ondorioz erreakzioa gelditu egiten da. EE2aren deribatizazioan, BSTFA eta MSTFA erabiltzen direnean piridina edo formaldehidoa erabili behar dira disolbatzaile gisa EE2tik E1erako oxidazioa saihesteko eta bi alkohol taldeen deribatizazioa lortzeko (ikus **1.13 irudia**) [224-226].

1.13 irudia: *E2 molekularen 2 alkohol taldeen deribatizazioa TMS taldea erabiliz.*

Deribatizazio-urrats hori saihestu nahian, ikerlari askok LC bidezko bereizketa eta analisia nahiago dute.

4.2. Likido-kromatografia

GC bidez neurtu ezin diren molekulak (konposatu ez-lurrunkorrak) edo deribatizazio-urratsa pairatu behar dutenak izaten dira LC bidez bereiztu eta neurtzen direnak. Gutxi gorabehera, molekula guztien % 80 ez da GC bidez neurtzeko aproposa. Azken urteotan, LCaren garapenarekin batera, gero eta gehiago dira APak, BPA eta estrogenoak neurtzeko LCa erabiltzen duten ikerlariak [2, 15, 47, 212] (ikus **1.5 taula**). Aipatutako konposatuez gain, LC bidez, 1. faseko eta 2. faseko metabolismo prozesuetan sortutako metabolitoak eta glukuronidoak, glukosidoak eta sulfatoak neur daitezke [126, 180, 193, 194, 229]. Halaz guztiz, LCak badu desabantaila nagusi bat, hots: GCarekin alderatuz gero, matrize-efektua neurketan nabarmenagoa izan daitekeela. Matrize-efektuak seinalearen desagerpena edo handitzea ekar dezake eta, horrekin batera, galerak zehaztasunean edota doitasunean [230, 231]. Ondorioz, garbiketa-urrats egokia egitea gomendagarria da lagina LC bidez aztertu aurretik.

GCan bezala, LCan detektagailurik erabiliena MSa da, eta azken urteotan MS/MS detektagailua indarra hartzen hasi da [15, 180, 229] (ikus **1.5 taula**). GCan ez bezala, MS edo MS/MS detektagailuez gain, beste detektagailu mota batzuk

erabiltzen dira. Gehien erabiltzen diren detektoreak ultramore-ikuskor (*Ultraviolet-Visible*, UV-Vis) detektagailuak eta fluoreszentzia-detektagailuak dira, biak ez-suntsikorrak. UV-Vis detektagailuen artean, diodo ildaxka detektagailua (*Diode Array Detector*, DAD) oso erabilia da. Detektagailu horiek ez dira MS edo MS/MS bezain sentikorrak, baina eraztun aromatikoak dituzten molekulak neurtzeko oso aproposak dira [232, 233].

LCan, analitoen bereizketa egokia egiteko ezinbestekoa da fase mugikor egokia aukeratzea. Fase mugikorrak, bereizketa egokia egiteaz gain, MS ionizazio ahalmena handitu dezake. Orokorrean, bi fase mugikor egoten dira, A eta B, eta analisiak iraun bitartean, A eta B proportzio desberdinetan nahasten dira ponpa kuaternario baten laguntzaz bereizketa egokia ahalik eta denbora laburrenean egiteko. Fase mugikorrik ohikoenak ur eta azetonitrilo (*Acetonitrile*, ACN) edo ur eta metanol (*Methanol*, MeOH) nahasteak dira, eraldatzaile desberdinekin. Eraldatzaile ohikoenak azido formikoa, azido azetikoa, amonio azetatoa eta amonioa dira [2, 15, 47, 180] (ikus **1.5 taula**). Eraldatzaile horiekin bi helburu bete nahi dira. Alde batetik, fase mugikorraren gainazal-tentsioa aldatzeko gatzak gehitzea eta, beste aldetik, fase mugikorraren pHa aldatzea. Aldaketa horiekin, bereizketa eta ionizazioa fabora daitezke. APen, estrogenoen eta BPAren neurketetan fase mugikor azidoak erabiltzen ziren hasiera batean. Hala ere, hainbat ikerlarik ikusi dute fase mugikor basikoek (amonioa erabiliz) sentikortasuna igotzen dutela [234, 238].

1.5 taula: *EDCak neurtzeko LC teknika erabiltzen duten metodoen laburpena, LC teknikaren parametrorik esanguratsuenak laburbilduz.*

Konposatuak	Matrizea	Erauzketa	Garbiketa	Fase mugikorra A/B	Zutabea	Ionizazioa	Detekzioa	Mugak (ng/L)	Ref
BPA, estrogenoak	Arrain muskulua	QuEChERS (Quechers Kit)	dSPE (PSA + C_{18})	MeOH/Ura pH=9, amonioa	C_{18} 1,7 µm, 50 mm x 2.1 mm	ESI (-)	HPLC-MS/MS	0,003-3[c]	[15]
BPA, estrogenoak, estrogeno konjokatuak	Arratoi barrabilak	QuEChERS (Sampli Q)	dSPE (PSA)	Ura (amonio azetatoa 0,01 mM)/ACN	Zorbax eclipse plus C_{18} 1,7 µm, 50 mm x 2,1 mm	ESI (-)	LC-MS/MS	0,55-7[a]	[180]
BPA, APak	Arrain muskulua	US DCM:Ace (7:3)	SPE (NH_2)	Ura (2 mM amonio azetatoa)/ACN	Polar RP 4 µm, 2 mm x 150 mm	ESI (-)	LC-MS/MS	0,3[c] (pisu hezea)	[47]
Estrogenoak	Ingurumeneko urak	SBSE (PDMS)	Ez	Ura (% 10 ACN)/ACN	Tracer Excel 120 C_{18} 5 µm, 150 mm x 4 mm	Ez	HPLC-DAD	25-100[a]	[212]
BPA, PEak, estrogenoak, pestizidak	Araztegiko irteera	SPE (Oasis-HLB)	Ez	Ura/ACN (% 0,1 azido formikoa)	Zorbax eclipse plus C_{18} 1,8 µm, 50 mm x 4,6 mm	ESI (-) ESI (+) ESI (+) ESI (+)	LC-TOF-MS	23-592[b]	[2]
BPA, APak	Itsasoko ura	DLLME (1-oktanola)	Ez	Ura/MeOH % 0,05 amonioa	Hypersil Gold C_{18} 3 µm, 50 mm x 2,1 mm	ESI (-)	LC-MS/MS	5-30[d]	[234]
BPA	Loihak	PLE (EtOAc)	Ez	Ura/MeOH % 0,0025 amonioa	Gemini C18 3 µm, 100 x 2 mm	APCI (-)	LC-MS/MS	5[a] ng/g	[235]
Estrogenoak	Araztegiko sarrera Araztegiko irteera	SPE (DVB)	SEC	Ura/MeOH	ea	APCI (-)	LC-MS/MS	6-8[b]	[236]
APak	Loihak	PLE (Hex:Ace, 1:1)	SPE (C_{18})	Ura/MeoH (5 mM NH_4OAc)	Luna C_{18} 5 µm, 150 x 4,6 mm	APCI (-)	LC-MS	30[a]	[237]
PEak	Arrain muskulua	US (DCM:hex, 1:1)	SPE (Florisil)	MeOH/Ura (5 mM NH_4OAc)	C_8 5 µm, 250 x 2 mm	ESI (+)	LC-MS/MS	4[c]	[78]
BPA, estrogenoak	Arrain behazuna	SPE (Strata-X)	Ez	Ura/ACN:Ura (98:2) (1 mM NH_4OAc)	Guard RP C_{18} 1,7 µm, 150 x 2,1 mm	ESI (-)	UPLC-MS/MS	6-13[b]	[139]
BPA, APak, estrogenoak	Araztegiko irteera	MASE (kloroformoa)	SPE (Florisil)	MeOH/Ura (% 0,05 NH_4OH)	Zorbax extend C_{18} 1.8 µm, 50 x 2,1 mm	ESI (-) APCI (+)	HPLC-MS/MS	1-30[a] (ESI) 10-61[a] (APCI)	[238]

[a]: LOD (detekzio-muga), [b]: LOQ (kuantifikazio-muga), [c]: MDL (metodoaren detekzio-muga), [d]: MQL (metodoaren kuantifikazio-muga).

BPA (bisfenol-A); PEak (ftalato esterrak); APS (Alkil fenolak); Quechers (azkarra, erreza, merkea, eraginkorra, sendoa eta segurua); dSPE (fase solidoko erauzketa dispertsoa; PSA (amina primarioa eta sekundarioa); C_{18} (oktadezil silanoa); MeOH (metanola); ESI (elektroesprai ionizazioa); HPLC-MS/MS (bereizmen altuko likido kromatografia-tandem masa espektrometria); ACN (azetronitriloa); LC-MS/MS (likido kromatografia-tandem masa espektrometria); US (ultrasoinua); DCM (diklorometanoa); Ace (azetona); SPE (fase solidoko erauzketa); SBSE (hagatxo birakarien bidezko erauzketa); PDMS (polidimetilsiloxanoa); HPLC-DAD (bereizmen altuko likido kromatografia-diodo ildaxka detektagailua); LC-TOF-MS (likido kromatografia-hegaldi denbora-mas espektrometria); DLLME (likido-likido mikroerauzketa dispertsoa); PLE (presiopeko likido erauzketa); EtOAc (etil azetatoa); APCI (presio atmosferikoko ionizazio kimikoa); DVB (dibinilbentzenoa); SEC (tamainaren bidezko bazterketa kromatografia); Hex (hexanoa); MASE (mintzen bidezko erauzketa). APak (4*t*OP, 4*n*OP, NP mix), pestizidak (2,4′-DDD, 4,4′-DDD, 2,4′-DDT, 4,4′-DDT, α-HCH, β-HCH, δ-HCH, γ-HCH, klorfirifosa, klorfenbinfosa), estrogenoak (E2, EE2), PEak (DEHP, DOP, BBP). ea: ez aipatua. Lagin likidoen kasuan mugak ng/mL unitateetan daude eta lagin solidoen kasuan ng/g-tan.

LCan erabiltzen diren ionizazio-teknikei dagokienez, ESI eta presio atmosferikoko ionizazio-kimikoa (*Atmospheric Pressure Chemical Ionization*, APCI) dira gehien erabiltzen direnak, nahiz eta ionizazio-teknika gehiago ere badauden, presio atmosferikoko fotoionizazioa (*Atmospheric-Pressure Photo Ponisation*, APPI) eta matrizeak lagundutako laser desortzioa (*Matrix-Assisted Laser Desorption*, MALDI), besteak beste. ESIa izaten da ionizazio-teknikarik erabiliena [2, 15, 47, 180] (ikus **1.5 taula**) sentikortasun handiagoa [238] eta erabilgarritasun gehiago dituelako. Nolanahi ere, ESI ionizazio teknikan matrize-efektuak eragin handiagoa daukala aipatu beharra dago, APCIarekin alderatuz gero [239]. ESI molekula polar eta ionikoetarako erabiltzen da nagusiki eta APCIa, ostera, txikiak eta termikoki egonkorrak diren molekula ez-polarrak eta polarrak ionizatzeko. **1.5 taulan** ikus daitekeen moduan, BPAk, APak eta estrogenoak, ESI eta APCI bidez neurtu dira nahiz eta lehenengo ionizazio-metodoa erabiliena izan den sentikorragoa delako [238].

5. Konposatu kimikoen metaketa

Lan honetan zehar aipatu bezala, konposatu organiko gehienak oso kontzentrazio-maila baxuetan (ikus **1.2 taula**) daude ingurumeneko uretan, askotan detekzio-mugaren azpitik. Arrainek, ordea, konposatu horiek metatzeko gaitasuna dute (ikus **1.3 taula**) eta hori dela bide, kontzentrazio-maila altuagoetan aurki daitezke. Beraz, ikerlari askok arrainak eta muskuiluak erabili dituzte biomonitorizazio-tresna moduan [240, 241]. Espezie bizidunekin lan egiteak emaitzen heterogenotasuna ekar dezake, arrainen sexua, tamainua, adina eta organismoaren egoera fisiologikoa oso desberdinak izan daitezkeelako [241]. Berezko aldakortasunaren efektua murrizteko lagin asko aztertu behar dira. Horrez gain, oso organismo gutxi bizi daitezke kutsadura handiko lekuetan. Beraz, biomonitorizazio-azterketak era erraz, merke eta ingurumenari begirunea erakutsiz egiteko ikerlariek mekanismo kimiko desberdinak erabili dituzte arrainen eta muskuiluen metaketa-mekanismoa simulatzeko orduan. Muskuiluen kasuan, mintz erdi-iragazkorreko tresna (*Semipermeable Membrane Devide*, SPMD) [241] eta konposatu organiko polarren lagin-bilketa integrala (*Polar Organic Chemical Integrative Sampler*, POCIS) [240] erabili dira, eta arrinen

biomonitorizazioa simulatzeko, berriz, POCISak [242].

POCISak eta SPMDak konposatu organikoen biomonitorizaziorako sarri erabili izan dira lagin-bilketa pasibo teknika moduan [243-245]. Lagin-bilketa pasiboak denboran zeharreko batezbesteko kontzentrazioa (*Time-Weighted Average Concentration*, C_{TWA}) lortzea ahalbidetzen du [246]. Aipatu berri den moduan, POCISak eta PDMSak dira erabili izan diren mekanismoetako batzuk, baina azken urteotan material polimeriko desberdinak ere baliatu izan dira emaitza oparoak lortuz [244, 246, 247]. Konposatu organikoen erauzketa egiteko material polimerikoak hasi dira erabiltzen lagin-bilketa pasiboan ere. Horrenbestez, ingurumeneko biomonitorizazioan baliatzea pentsa liteke. Material horien bidezko erauzketak adsortzioan eta absortzioan oinarritzen diren teknikak dira eta denborarekiko menpekotasuna erakusten dute. Material sorta handiak eskaintzen dituzten abantailaz baliatuz, arrainen atal desberdinen metaketa-gaitasuna aztertzeko erabilgarriak izan daitezkeela pentsa liteke. Hala ere, gai horren inguruan lan handia dago egiteko.

5.1. EDCen metaketa arrainetan

Arrainek duten metaketa-gaitasuna neurtzeko biokontzentrazio-faktoreak (*bioconcentration factor*, BCF) erabiltzen dira. Faktore horrek, modu errazean erlazionatzen ditu konposatu batek ehun jakin batean daukan kontzentrazioa ($K_{analito\ ehunean}$) konposatu berak uretan daukan kontzentrazioarekin ($K_{analito\ uretan}$) (ikusi **1.1 ekuazioa**). BCFak kalkulatzeko konposatuak orekara iritsi behar dira. Laborategietan kontrolpean egiten diren esperimentuetan kutsatzailearen metaketa orekara heldu dela ziurtatu daitekeen bitartean, lagin errealetan hori ziurtatzea ezinezkoa da. Ondorioz, laborategiko baldintzetan eta lagin errealetan ikusitako BCF balioak desberdinak izan daitezke.

$$BCF = \frac{K_{analito\ ehunean}}{K_{analito\ uretan}}$$

1.1 ekuazioa

BCFez gain, bioakumulazio- edo biometatze-faktoreak (*Bioaccumulation Factor*, BAF) ere kalkula daitezke. BAFak kalkulatzeko, uretako kontzentrazioa neutzeaz gain, arrainaren kutsatzaile organikoekiko esposizio osoa hartu behar da kontuan, elikadura barne [248]. Kontrolpeko esperimentuetan janarian eta airean dauden konposatuen kontzentrazioak neur daitezke, baina ingurumeneko azterketan nahiko zaila izaten da; beraz, ikerlari gehienek BCF parametroa erabiltzen dute. Konposatu bat maila handian metatu den ala ez aurresaten duen balio finkorik ez dago. Kanadako gobernuak plazaratu zuen gida baten arabera [249], BCF balioa 5.000 baino handiagoa denean konposatu horiek biokontzentratu direla esan daiteke. Lee eta lankideen arabera, ordea [47], BCFa 1.000 baino handiagoa denean esan daiteke konposatuak biokontzentratu direla. Inoue eta lankideen arabera [250], aldiz, BCF balioa 2.000-5.000 artean badago, konposatuek metatzeko joera dutela esan daiteke, eta BCFa 5.000 baino handiagoa denean, berriz, metatzeko oso joera handia dagoela diote.

BCF teorikoak kalkulatzeko ikerlari askok log k_{OW} balioak erabili dituzte [248, 251-253]. BAF eta BCF faktoreak esperimentalki kalkulatzea ezinezkoa denean. Kanadako gobernuak, kasurako, log K_{ow} 5 edo handiagoa duten konposatuek zuzenean metatzeko joera dutela adierazten du. Eredu horren arabera BCFa eta log K_{ow} aren arteko erlazioa zuzena dela onartzen da. Zenbait ikerlarik, ordea, erlazio hori parabolikoa dela baieztatu dute [252, 253]. Arlo horretan ere, BCF eta BAF faktoreak teorikoki kalkulatzeko lan asko dago egiteko, parametro bakarrean guztia oinarritzeak ez baitauka zentzurik.

BCF teorikoak kalkulatzeko metodo desberdinak daudela ikusi da. BCF esperimentalak hainbat faktoreren menpekoak dira: arrain-espeziea, sexua, tamaina, elikadura-ohiturak eta ingurumeneko baldintzak, besteak beste [45, 47]. Oso zaila da ikerketa lan batean lortu diren BCF balioak beste ikerketa lanetakoekin erkatzea; hala eta guztiz ere, **1.6 taulan** bibliografian topatutako BCF desberdinak ikus daitezke laburbildurik.

Esperimentalki kalkulatu diren BCF gehienak muskulu-laginei dagozkie; azken finean, hau baita gizakiok jaten dugun atala. **1.6 taulan** ikus daitekeen moduan, BCF faktoreak nahiko baxuak dira, beti ere Lee eta lankideek [47]

proposatzen duten 1.000 baliotik beherakoak, konposatu eta arrain espezie desberdinetarako. Kontrako muturrean behazunean kalkulatutako BCFak daude. Horiek ingurumenean jasotako arrain-espezie desberdinetarako 1.000-20.000 izatera iritsi dira. Muskuluan ez bezala, behazunean kalkulatu diren BCFak ikusita, behazuna biomonitorizazio-ehun bezala erabil daitekeela esan daiteke. Gainera, behazunak berrikitan barneratutako konposatuei buruz ematen du informazioa.

1.6 taula: A*rrain espezie desberdinetako atal desberdinetan neurtutako BCF balioak.*

Konposatua	Ingurunea	Espeziea	Atala	BCFa	Ref
APak BPA	Errekako ura	*Cyprinus carpio*	Behazuna	1.648-12.960 3.583-14.178	[106]
APak BPA	Lakuko ura	*Cyprinus carpio*	Muskulua	74 29	[29]
APak BPA	Lakuko ura	*Carassius auratus*	Muskulua	53 18	[29]
APak BPA	Lakuko ura	*Anabarilius alburnops*	Muskulua	77 49	[29]
APak BPA	Errekako ura	11 espezie	Muskulua	80-1.000 1-274	[47]
Musketa fragantziak	Errekako ura	*Carassius auratus* *Cyprinus carpio* *Hypophthalmichthys molitrix*	Muskulua	52.370-54.070* 66.030-72.220* 34.940-39.400*	[254]
APak	Laborategia	*Oncorhynchus mykiss*	Behazuna Odola Gibela Burmuina Muskulua	68.794 91 1.020 116 101	[111]
APak BPA	Errekako ura	5 espezie	Behazuna	1.050-20.200 998	[255]
BPA	Laborategia	*Cyprinus carpio*	Behazuna	3-1.340	[255]
Pestizidak	Laborategia	*Dania rerio*	Arraina	2.754-6.918	[256]
PEak	Lakuko ura	*Tilapia guineensis*, *Chrysichthys nigrodigitatus*	Muskulua Gibela	0,4-2 0,5-5	[82]
APak BPA	Errekako ura	*Cyprinus carpio*	Behazuna	3.583-14.178 1.500-12.960	[44]

* BAF balioak, nahiz eta gure BCF moduan kalkulatu diren. APak (4*t*OP, 4*n*OP, NP mix), pestizidak (2,4′-DDD, 4,4′-DDD, 2,4′-DDT, 4,4′-DDT, α-HCH, β-HCH, δ-HCH, γ-HCH, klorfirifosa, klorfenbinfosa), PEak (DEHP, DOP, BBP).

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Helburuak/Objectives

Disruptore endokrinoak (EDC), 21. mendeko arazo larrienetako bat bilakatu dira organismo urtarretan sor ditzaketen kalteak direla eta. EDCak etengabe isurtzen dira ingurumeneko uretara, araztegietatik batez ere, hauek ez baitira gai konposatu guztiak kentzeko. Zentzu horretan, hainbat ikerlarik ingurumeneko uretan, araztegien irteera korronteetan batez ere, EDCen presentzia salatu dute. Nahiz eta aipatutako konposatuak oso kontzentrazio-maila baxuan egon (ng/L edo baxuago), askotan dosi hilgarria baino baxuagoa, esposizioa kronikoa bada kontzentrazio hori arrainetan kalteak sortzeko nahikoa izan daiteke .

Lan honetan lagin likidoen azterketarako erabili diren teknikak, oso ezaguna den fase solidoko erauzketa (SPE) eta modernoagoak diren polimeroen bidezko mikroerauzketa teknikak izan dira. Garbiketa-urratserako, aldiz, adsorbatzaileak era dispertsoan erabiliz (dSPE). Lagin solidoen azterketarako, aldiz, ultrasoinu fokatu bidezko erauzketa (FUSLE) erabili da eta aipatu berri den dSPE garbiketarekin batera. Hortaz, tesi honek bi helburu nagusi ditu:

1. Metodo analitiko desberdinen garapena EDCak ur-laginetan (estuarioko eta araztegiko irteera korronteetan) eta biota-laginetan (arrainen behazuna, plasma, muskulua, gibela eta burmuina) aztertzeko garatutako metodoak sendoak eta ingurumenarekiko errespetagarriak izan behar dira. Aukeratutako analitoak alkilfenolak (APak), bisfenol-A (BPA), zenbait hormona, musketa fragantziak, pestizidak eta ftalato esterrak (PEak) izan ziren. Horrez gain, ftalato eta zenbait pestizidaren kasuan, beraien metabolitoak ere aztertu dira.
2. Aipatutako EDC desberdinen metaketa-azterketa, arrain espezie desberdinetan (*Chelon labrosus* and *Dicentrarchus labrax)* egin da naturalki kutsatutako urak (araztegiko efluenteko urak) eta laborategian kutsatutako itsasoko urak erabiliz.

Endocrine disrupting compounds (EDCs) have become one of the most important issues of the 21st century due to their possible adverse effects on aquatic organisms. EDCs are continuously discharged to the water bodies mainly from the wastewater treatment plants (WWTPs), due to their incomplete removal. In this sense, numerous studies have reported the EDCs presence in the WWTP effluents and in different environmental aqueous samples. Although the mentioned compounds are found at very low concentration levels (ng/L or lower), this sublethal but chronic exposure, seems to be enough to cause alterations in fish populations.

The techniques applied in this work for liquid samples are based on the well-established solid-phase extraction (SPE) and some modern non-exhaustive techniques such as the extraction with disposable polymers or the clean-up based on dispersive solid sorbents. Focused ultrasound solid-liquid extraction (FUSLE) was also applied in the case of solid samples. Therefore, the present PhD thesis has two main objectives:

1. The development of different analytical methodologies for the determination of EDCs in water (estuary and WWTP effluents) and biota (fish bile, plasma, muscle, liver and brain) samples in order to develop robust and environmental friendly analytical methods. The analytes selected were alkylphenols (APs), bisphenol-A (BPA), some hormones, musk fragrances, pesticides and phthalates esters (PEs). In addition, the PEs and some pesticides metabolites were also studied in the present work.
2. The uptake evaluation of several EDCs by different fish species (*Chelon labrosus* and *Dicentrarchus labrax*) under real and laboratory conditions, using naturally contaminated WWTP effluents and fortified seawater.

Microextraction with polyethersulfone for the analysis of priority and emerging compounds in water samples

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1. INTRODUCTION

For about more than 20 years, an increasing input of new organic pollutants has been detected in the aquatic environment. Pharmaceutical residues, estrogens, pesticides and their metabolites generate, together with the priority pollutants, a complex cocktail of chemicals with mostly unknown risks and consequences for the human and environmental health [1].

Currently, according to the European Union Water Framework Directive (WFD 2013/39/EU), the list of priority substances contains only 45 contaminants and/or contaminant groups, which is only a small fraction of a much larger number of possible hazardous pollutants that eventually reach aquatic systems [2]. From this list of 45 priority substances, a group of 21 priority hazardous compounds have been identified as being toxic, persistent and bioaccumulative substances or giving and equivalent level of concern. Alkylphenols (APs), essentially nonyl and octylphenols, and other endocrine disrupting compounds (EDCs) such as phthalates esters (PEs) (bis-(2-ethylhexyl) phthalate, DEHP) are included in the list. Other contaminants, such as natural and synthetic estrogens, are still out of the priority list. On the other hand, bisphenol-A (BPA) is one of the substances to watch, in order to be included as priority contaminant.

The presence of EDCs in aquatic systems is often attributed to the discharges of wastewater treatment plants (WWTPs) [3-5], but this type of scenario is deemed highly complex due to the mixture of contaminants and the high variability of their concentrations. One of the key issues is to establish safe levels of chemical exposure, i.e. with no appreciable health risk to humans, but little is known about the fate and effects of emerging contaminants (EC) mainly due to the large diversity of the chemical structures and the chemical analysis complexity. In fact, the literature shows that long-term exposure of trace pollutants to ECs can cause adverse health effects in most organisms at very low concentrations (low ng/L) [6-8].

Among the different operational units of an analytical procedure, sample preparation, including analyte enrichment and matrix component removal, is one

of the most important steps to achieve fit-for-purpose analytical results [9]. Despite recent advances in this field, conventional sample preparation procedures such as liquid-liquid extraction (LLE) or even solid-phase extraction (SPE), which consume large amounts of organic solvents and require high labour-effort and time, are continuously being used. Hence, the analytical efforts are directed to the development of fast and environmentally friendly methods for the analysis of organic pollutants in different environmental compartments, which at the same time, provide accurate results and low detection limits [9, 10]. One of the most used strategies for the determination of trace organic compounds in liquid matrices is centred on the miniaturisation and automation of sample preparation techniques [11-14]. In addition to the well-established microextraction techniques, mainly solid phase microextraction (SPME) [14-17] and stir bar sorptive extraction (SBSE) [18-25], polyethersulfone (PES) polymer has emerged recently [13, 26, 27] as a very suitable choice for the extraction of more polar species, improving significantly the extraction efficiencies provided by other polymeric materials such as polydimethylsiloxane (PDMS).

The combined use of these outstanding microextraction techniques with sensitive and reliable chromatographic techniques, is always necessary in order to develop analytical methods able to meet legislation requirements. Currently, the suitability of gas chromatography-mass spectrometry (GC-MS) for the identification of a wide variety of organic contaminants in several environmental matrices is widely proved in many research works [28, 29]. However, the analysis of polar compounds such as hormones requires a previous derivatisation step. On the contrary, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) does not require the derivatisation step and offers the possibility to identify and quantify simultaneously a wide variety of organic contaminants of different polarities [30, 31].

In the framework of developing properly validated analytical procedures to support the monitorisation of natural waters and to assess the impact of chronic exposure to EDCs, the main aim of this work was to study the effectiveness of PES tubes for the simultaneous extraction of trace amounts of 2 synthetic musk

fragrances (AHTN (tonalide), HHCB (galaxolide)), 3 APs (4*t*OP (4-*tert*-Octylphenol), 4*n*OP (*n*-octylphenol), NP mix (nonylphenol technical mixture)), 2 hormones (E2 (17 β-estradiol), EE2 (17 α-ethynilestradiol)), BPA, 10 pesticides (2,4′-DDD (2,4-bis(p-chlorophenyl)ethane), 4,4′-DDD (2,2-bis(p-chlorophenyl)ethane), 2,4′-DDT (1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]), 4,4′-DDT (1,1′-(2,2,2-trichloroethylidene)bis[4-chloro]), α-HCH (α-hexachlorocyclohexane), β-HCH (β-hexachlorocyclohexane), γ-HCH (γ-hexachlorocyclohexane), δ-HCH (δ-hexachlorocyclohexane)), chlorpyriphos, chlorfenvinphos and 3 PEs (BBP (butyl benzyl phthalate), DEHP, DOP (dioctyl phthalate)) in effluents and estuary waters. Therefore, parameters affecting the performance of the desorption and the extraction processes were evaluated. In addition, APs, BPA and hormones were quantified by means of GC-MS, after a previous derivatisation of the analytes, and LC-MS/MS to evaluate and compare the suitability of both analytical techniques. Finally, the matrix effect required a further evaluation in order to overcome the presence of organic matter in the aquatic samples and the final methods were applied to the analysis of estuary waters and WWTPs effluents.

2. MATERIALS AND METHODS

2.1. Reagents and materials

The target analytes, including names, abbreviations, CAS number and chemical structures are listed in **Appendix I**. All chemicals were of the highest purity (> 94 %) available. All the analytes were individually prepared at concentrations between ~ 1000-4000 ng/μL in methanol (MeOH, HPLC grade, Lab Scan, Dublin, Ireland) except for DEHP, which was directly obtained at 2000 ng/μL in MeOH, and [$^{2}H_{15}$]-MX, which was obtained at 100 ng/μL in cyclohexane. 100 mg/L dilutions were monthly prepared in anhydrous MeOH. Dilutions at lower concentrations were daily prepared, according to the experimentation.

Anhydrous MeOH (HPLC grade, 99.9 %, Labscan) and sodium chloride (NaCl, Merck, Darmstadt, Germany) were used for matrix modification experiments. Humic acids (technical grade) used for matrix effect assays were obtained from Fluka (Sigma-Aldrich, Germany).

N,O-Bis (trimethylsilyl)trifloroacetamide with 1 % of trimethylchlorosilane (BSTFA + 1 % TMCS, Sylon BFT, 99:1) was used as the derivatisation reagent and was purchased from Supelco (Bellefonte, USA). Pyridine (Pyr, 99.5 %) was purchased from Alfa Aesar (Karlsruhe, Germany).

Ultra-pure water was obtained using a Milli-Q water purification system, (< 0.057 S/cm, Milli-Q model 185, Millipore, Bedford, MA, USA). Ethyl acetate (EtOAc) (HPLC grade, 99.9 %) was obtained from Lab Scan.

MeOH (Optima®, LC-MS quality) used as mobile phase eluent in LC-MS/MS was obtained from Fisher Scientific (Geel, Belgium). Ammonia (25 % as NH_3, Panreac, Reixac, Barcelona, Spain) was used for mobile phase modification.

Extracts were filtered before analysis with Acrodisc syringe (13 mm diameter, 0.2 μm pore size) filters (GHP membrane or PTFE) obtained from Pall Life Sciences (Port Washington, NY, USA).

PES tubes were acquired from Membrane (Wuppertal, Germany) in a tubular format (0.7-mm external diameter, 1.43 g/mL density). Pieces of this polymer (1.5 cm length, ~2 mg) were cut using a sharp blade and soaked overnight in EtOAc previous to their use as sorbent material. Afterwards, the polymer was dried with air and stored until used. Given their reduced cost (c.a. 0.05 €/unit), sorbents were discarded after each use.

2.2. Sampling

Estuary water samples were collected from the estuary of Bilbao (+43° 15' 26.23", -2° 55' 37.82", Bay of Biscay, Spain) whereas wastewater samples were collected at the effluent of the WWTP of Galindo (+43° 18' 19.32", -2° 59' 50.88", Bay of Biscay, Spain), in April 2013 for the analysis of non-polar compounds (i.e. pesticides, musk fragrances and PEs) and in October 2013 for the analysis of more polar compounds (compounds that at least have one -OH group, i.e. hormones, APs and BPA). Samples were collected in pre-washed amber bottles and carried to the laboratory in cooled boxes (4 °C). Samples were filtered through 0.45 μm cellulose filters (Whatman, Kent, UK) and kept in the fridge at 4 °C before

treatment, which was performed within 24 hours.

2.3. Extraction, desorption and derivatisation procedures

Water samples (150 mL) were directly poured into 150 mL extraction vessels with 10 % NaCl (w/v), 5 pre-cleaned pieces of PES in tube format (see section 2.1) and a PTFE covered stirrer was introduced in the vessel. The samples were fortified at 1 ng/mL with the deuterated analogues ([$^2H_4$]-4*n*-nonylphenol ([$^2H_4$]-NP), [$^2H_3$]-17β-estradiol ([$^2H_3$]-E2) [$^2H_{16}$]-bisphenol-A ([$^2H_{16}$]-BPA), [$^2H_4$]-bis(2-ethylhexyl) phthalate ([$^2H_4$]-DEHP), [$^2H_{15}$]-musk xylene ([$^2H_{15}$]-MX) and [$^2H_8$]-4,4′-1,1′-(2,2,2-trichloroethylidene)bis[4-chloro]) ([$^2H_8$]-4,4′-DDT)). Thereafter, vessels were closed and extraction was performed at room temperature with a stirring rate of 1200 rpm overnight, using a 15 position magnetic stirring plate from Gerstel (Mülheim an der Ruhr, Germany). Once the sorption step was over, the polymers were removed and rinsed with Milli-Q water in order to eliminate salt residues, and finally, dried with a clean tissue. Subsequently, the sorbents were chemically desorbed using 300 μL of the appropriate organic solvent and soaked for 16 minutes in an ultrasound bath (USB Axtor by Lovango).

In the case of GC-MS analysis, the polymers were desorbed using EtOAc. On one hand, the 300 μL were placed in a vial and directly analysed by means of large volume injection-programmable temperature vaporiser-gas chromatography-mass spectrometry (LVI-PTV-GC-MS) for the quantification of non-polar compounds (i.e. musk, pesticides and PEs). On the other hand, in the case of more polar compounds (i.e. hormones, APs and BPA), the extract was derivatised prior to GC-MS analysis. The 300 μL EtOAc extract was transferred to a 2 mL vial and evaporated to dryness under a gentle stream of nitrogen in a Turbovap LV Evaporator (Zymark, Hopkinton, USA). Afterwards, Pyr (125 μL) and BSTFA + 1% TMCS (50 μL) were added to the evaporated extract and the mixture was shaken in a vortex and kept in an oven at 65 °C for 45 min. 2 μL of the extracts containing derivatised compounds were subsequently analysed by means of GC-MS.

In the case of the assays performed by means of LC-MS/MS, the polymers were desorbed with 300 μL of MeOH, filtered with PTFE filters and directly

analysed for APs, BPA and hormones determination.

2.4. Gas chromatography-mass spectrometry analysis

Separation and detection were performed in a 6890N gas chromatograph (Agilent Technologies, Avondale, PA, USA) equipped with a large volume injection (LVI) system and an Agilent 5975N electron impact ionisation mass spectrometer. A 40 μL aliquot of sample extract was injected using a 100 μL syringe in a cooled injection system (CIS) which consisted of a septum-less head and an empty baffled deactivated gas liner cooled with liquid nitrogen. LVI-PTV injection parameters were thoroughly optimised and described in a previous work [32]. Briefly, the sample extract was injected at 50 °C while the vent valve was opened for 3 min at a flow rate of 75 mL/min and a vent pressure of 2.9 psi. Subsequently, the analytes were focused on the column in splitless mode for 1.5 min while the temperature of the PTV injection port was increased at 12 °C/s to 300 °C and held for 5 min. Finally, the inlet was further cleaned at a purge flow of 50 mL/min before further injections. Target compounds were separated on a HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm) from Agilent Technologies (Agilent Technologies). The oven temperature was programmed from 60 °C (hold 1 min) to 170 °C at 10 °C/min, then until 250 °C at 15 °C/min (hold 2 min), and finally until 300 °C at 15 °C/min which was held for 3 min.

In the case of derivatised compounds, 2 μL of the extracts were injected into a 6890N gas chromatographer (Agilent Technologies, Avondale, PA, USA) coupled to a 5973N electron impact ionisation mass spectrometer and a 7683 Agilent autosampler. The analysis was performed in the splitless mode for 1.5 min at 300 °C into a HP5-MS capillary column (30 m x 0.25mm, 0.25 μm, Agilent Technologies). The oven program temperature used was as follows: 60 °C (1.5 min), a temperature increase of 10 °C/min to 170 °C to continue rising at 15 °C/min to 300 °C, maintaining it for 5 min.

Hydrogen was used as carrier gas at constant flow of 1.3 mL/min. The MS transfer line temperature was maintained at 310 °C and the ion source and quadrupole at 230 °C and 150 °C, respectively. Measurements were performed in both scan (50-525 m/z) and SIM modes and m/z values are listed in **Appendix II**.

2.5. Liquid chromatography-tandem mass spectrometry

Underivatised extracts were directly analysed in an Agilent 1260 series HPLC equipped with a degasser, a binary pump, an autosampler and a column oven and coupled to an Agilent 6430 triple quadrupole mass spectrometer equipped with ESI source (Agilent Technologies). Before analysis, all samples were filtered through 0.2 μm syringe PTFE microfilters.

The quantitative analysis of the target compounds was performed in multiple reaction monitoring (MRM) mode (see **Appendix III** for LC-MS/MS transitions and optimal conditions of all the analytes). High purity nitrogen gas (99.999 %, Air Liquide, Madrid, Spain) was used as nebuliser, drying and collision gas. MS/MS ionisation parameters were set as follows: N_2 flow rate of 11 L/min, a capillary voltage of 4000 V, nebuliser pressure of 52 psi (358.5 KPa) and source temperature of 325 °C.

MeOH:Milli-Q water with 0.05 % NH_4OH mobile phase was used since recent results in the literature [33] have shown that more basic mobile phases (pH=10.5) provided better sensitivity for APs determination.

Separation of analytes was carried out using an Agilent Zorbax Extend-C_{18} (2.1 mm, 50 mm, 1.8 μm) column (pH range 2.0-11.5) and Zorbax Eclipse XDB-C_{18} pre-column (2.1 mm, 5 mm, 1.8 μm) was used. The column temperature was set to 35 °C. The injection volume was set at 5 μL and the flow rate at 0.2 mL/ min.

Under optimised conditions a binary mixture consisting of Milli-Q water containing 0.05 % NH_4OH (eluent A) and MeOH containing 0.05 % NH_4OH (eluent B) was used for gradient separation of target analytes. Linear gradient was as follows: 30 % B maintained for 4 min, increased to 60 % B in 3 min and to 80 % B in

10 min and maintained constant for 18 min. Initial gradient conditions (30 % B) were then achieved in 5 min where it was finally held for another 5 min.

Agilent 6430 Quantitative analysis software (Mass Hunter, version 05.02) was used for data treatment.

3. RESULTS AND DISCUSSIONS

3.1. Optimisation of LC-MS/MS

MS/MS operating conditions for ESI in the negative ionisation mode were optimised. For this purpose, fragmentor electric voltage and CE were studied in the 70-175 V and 5-45 eV ranges, respectively by injection of individual compounds. Fragmentor electric voltage was chosen in order to maximise the signal of the quasi-molecular ion, while trying to minimise the formation of adducts. Cell accelerator voltage was also evaluated in the 1-7 V range. Optimisation was performed in the full scan MS mode. The fragmentor voltage and the CE were simultaneously optimised by means of the automatic "Optimizer" software (Mass Hunter software) option and the most sensitive two transitions were selected (see **Appendix III**) being comparable to those found in the literature [34, 35].

3.2. Optimisation of sample preparation conditions

For optimisation of PES extraction conditions, all the extracts were analysed by means of GC-MS according to the procedures described in section 2.4.

3.2.1. PES desorption solvent nature and time

The use of organic solvents should show high affinity with the target compounds and must be compatible with the sorptive material. In the specific case of PES, since it is already known that PES may decompose with chlorinated solvents [26], the effectiveness of non-chlorinated polar solvents such as EtOAc and MeOH were evaluated. The extraction was accomplished in a standardised

way, i.e. aliquots of 150 mL of Milli-Q water (without NaCl or MeOH) spiked at 1 ng/mL were extracted for 4 hours. Chemical desorption of the sorbents was performed four times with 300 µL of EtOAc or MeOH each time for 8 min in an ultrasonic bath. 300 µL were chosen as the minimum volume which assures that all PES tubes were completely covered by any of the solvents. The percentage of analytes recovered in the first and second EtOAc fractions were between 85-100 % of the total (sum of 1st and 2nd fractions) for all the target compounds (see **Figure 3.1**), except in the case of DEHP and BPA which showed a constant signal in the rest of the fractions, attributed to blank problems. Similar results were obtained when MeOH was used. Therefore, the desorption conditions were fixed as follows: a single desorption step with 300 µL of EtOAc (in the case of GC-MS analysis) or 300 µL of MeOH (in the case of LC-MS/MS analysis) for 16 min in an ultrasound bath.

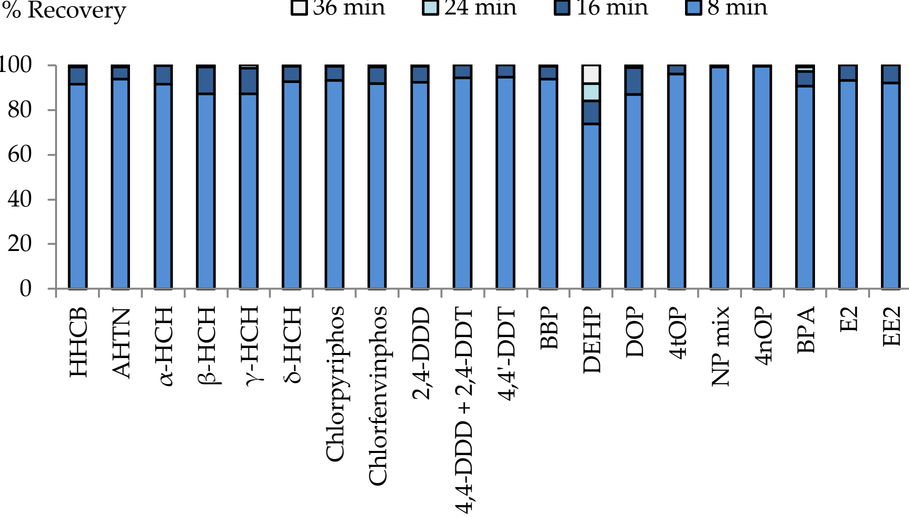

Figure 3.1: *Desorption percentages obtained after 4 desorption cycles (300 µL of EtOAc) of 8 minutes each, using an ultrasound bath.*

3.2.2. Extraction parameters evaluation

Thereafter, factors affecting analyte extraction were evaluated in order to achieve the optimum extraction conditions. The effect of the addition of an inert salt (NaCl) and an organic modifier (MeOH) in the extraction efficiency was simultaneously studied by means of an experimental design approach. To this aim, a Central Composite Design (CCD) was performed using the Statgraphics Centurion XV program and covering a factor space of 0-25 % for NaCl (w/v) and 0-20 % for MeOH (v/v) (with three central points, i.e., 11 experiments). These assays were carried out using fortified Milli-Q water samples at 2 ng/mL concentration level and extracted overnight (ca. 12 h). The precision of the measurements was estimated from the three replicates of the central point, getting relative standard deviation (RSD %) values between 1-19 % for the all studied analytes.

The responses obtained for the CCD were analysed by means of multiple linear regression and response surface analysis including the significant variables (p-values < 0.05). **Figures 3.2a** and **Figures 3.2b** show the response surfaces for α-HCH and 4*t*OP, respectively. Overall, the addition of MeOH was statistically significant (p-value < 0.05) with a negative effect, for all the studied compounds with the exception of ϒ-HCH, chlorfenvinphos and DEHP, which showed not significant effect. This is in agreement with the literature for other sorptive extraction approaches [26, 36, 37]. The presence of MeOH may enhance the extraction efficiency of low polar compounds since the adsorption in the walls of glassware is reduced. On the other hand, lower extraction yields are usually expected for more polar compounds since the solubility increases in presence of an organic modifier. Therefore, in the present work, no MeOH was added in further experiments.

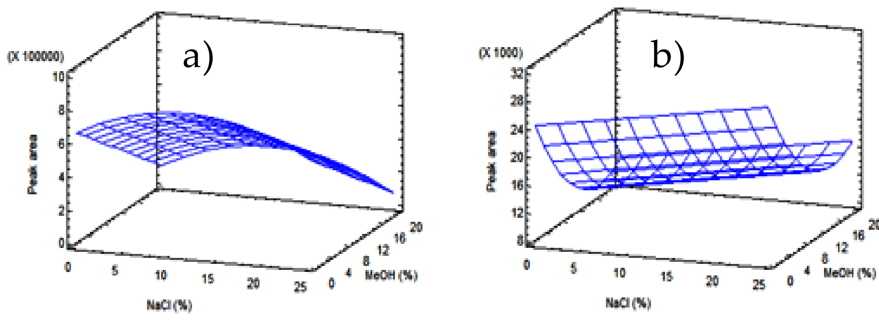

Figure 3.2: *Response surfaces obtained for (a) α-HCH and (b) 4tOP compounds after the study of NaCl and MeOH addition using a CCD design.*

In general, during sorptive extraction, it has been observed that for hydrophobic analytes (octanol/water partition coefficient, log K_{ow} > 3.5) the addition of NaCl does not improve, but even reduces, the extraction efficiency. However, the extraction of polar analytes is favoured when the ionic strength is higher and the response increases with the addition of inert salts [21]. Both trends can be explained on the basis of the salting out effect, which is particularly significant for polar species, and the negative impact of increasing the ionic strength on the kinetics of microextraction processes. According to the results, the addition of NaCl had a negative effect for α-HCH, 2,4'-DDD and DEHP and a positive effect only for E2. On the other hand, chlorpyriphos, 4*t*OP and NP mix showed the best responses when NaCl was added close to mid values (see **Figure 3.2b**) and the rest of the analytes did not show any significant difference. As shown in **Figure 3.2a** for α-HCH, the addition of NaCl up to mid levels did not show a severe loosing in signal and thus, NaCl was fixed at 10 % as consensus.

3.2.3. Optimisation of the stirring speed

The effect of stirring speed on the extraction efficiency was also studied at four levels: 600 rpm, 800 rpm, 1000 rpm and 1200 rpm. For this assessment, extractions were performed in triplicate with 150 mL of fortified Milli-Q water samples (1 ng/mL) under previously fixed extraction conditions (i.e., 10 % of NaCl and no addition of MeOH overnight). In all the cases, high stirring speed affected

positively the extraction process (see **Figure 3.3**). Although no significant differences were observed for most of the analytes between 1000 and 1200 rpm, highest chromatographic responses were obtained at 1200 rpm in the case of β-HCH, chlorfenvinphos, 2,4′-DDD, 2,4′-DDT and NP mix, thus, this extraction speed was selected as optimum.

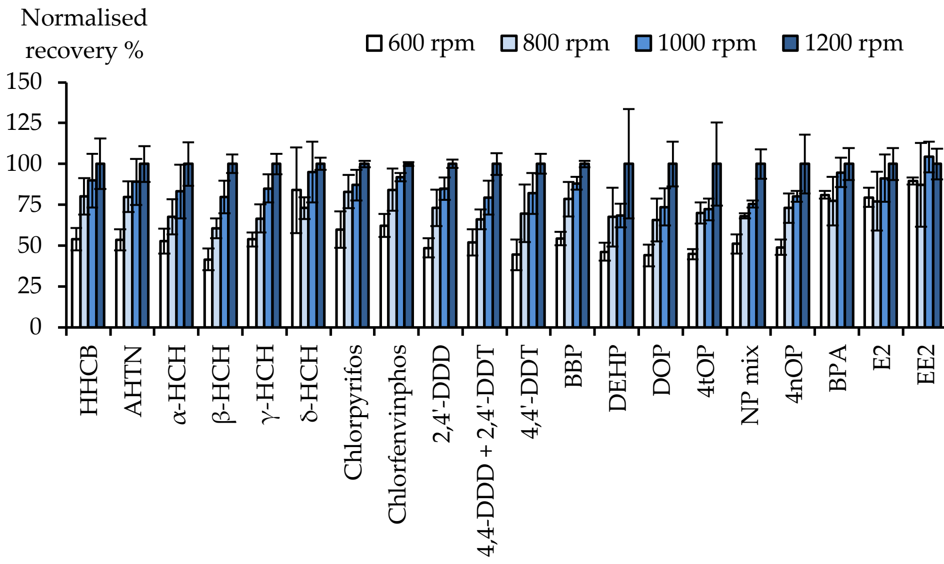

Figure 3.3: *Normalised recoveries (n=3, %) obtained for the analysis of the stirring speed for the target analytes under optimum conditions (10 % NaCl) and no addition of MeOH.*

3.2.4. Time profile

The influence of the extraction time was also investigated for the extraction of 150 mL Milli-Q water samples spiked at 1 ng/mL per compound under optimal conditions during extraction periods ranging between 15 and 720 min. As shown in **Figure 3.4** (for one analyte per family), the equilibrium was reached for all the studied compounds after 540 min, thus it was decided to extract the samples overnight. Even if the extraction time is quite long, it is still comparable to those used with other common microextraction protocols [25, 32].

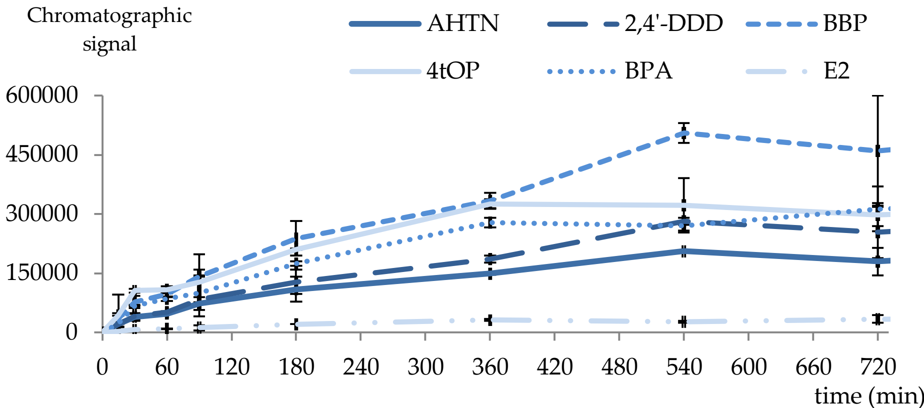

Figure 3.4: *Extraction time (15-720 min) profile (n=3) for one target compound per family under the optimised conditions (10 % NaCl and 1200 rpm).*

3.3. Matrix effect evaluation

Organic matter can reduce the amount of extractable organic compounds and/or interfere in the analysis of target analytes. The influence of organic matter on the extraction efficiency was simulated with Milli-Q water samples in the presence of humic substances. The recoveries of the analytes (500 ng/L each) in Milli-Q water spiked at different concentrations of humic acids (25, 50, 75 and 100 mg/L) were compared to the ones obtained for Milli-Q water spiked at the same concentration, but without the addition of any humic acid.

As it can be observed in **Figure 3.5** for one analyte per family (all of them follow the same tendency), a decrease on the recovery (up to 20 %) was observed for all the analytes as the concentration of humic acid increased, except for BPA.

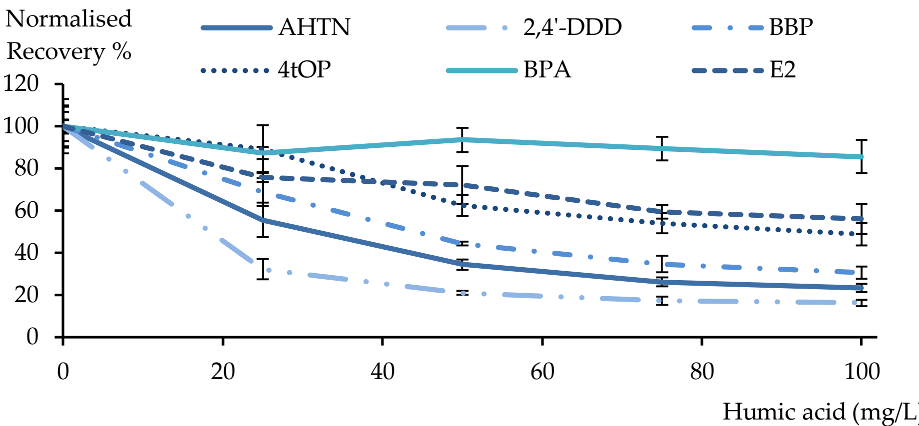

Figure 3.5: *Normalised recovery (%, n=3) for one target compound per family at different humic acid concentrations without surrogate corrections.*

However, the use of surrogates allowed the matrix effect correction created by the presence of humic substances in the samples (see **Figure 3.6**) for all the compounds except for 2,4′-DDD (146 %) at high concentration level (100 mg/L) of humic substances. Thus, total organic carbon (TOC) should be measured to check the applicability of the method in the case of 2,4′-DDD.

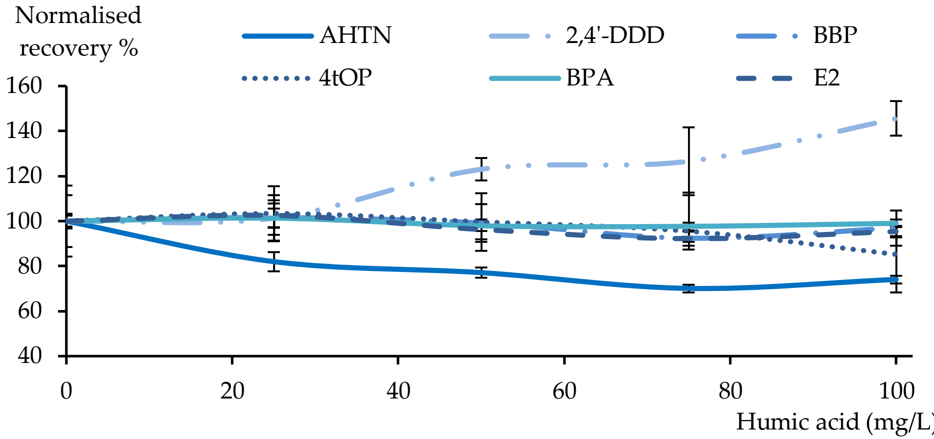

Figure 3.6: *Normalised recovery (%, n=3) for one target compound per family at different humic acid concentrations after the correction with the surrogates.*

3.4. Performance of the analytical methods

The compounds evaluated in the present work were divided in two main groups; the non-polar compounds (i.e. pesticides, musk and PEs), and the more polar compounds (i.e. BPA, hormones and APs).

Although, APs and BPA can also be measured in the GC system without the need of any derivatisation step, according to Kawaguchi et al. [38] and Cavalheiro et al. [39], the chromatographic peak shape and the sensitivity improve after the derivatisation procedure. Thus, it was decided to measure both APs and BPA after a derivatisation step in the case of GC-MS analysis, since the derivatisation of hormones was necessary. Since those compounds can also be measured without any derivatisation step by means of LC-MS/MS, in the present work a comparison between both techniques was also evaluated.

As mentioned before, polar and non-polar compounds were submitted to a derivatisation step before GC-MS analysis. Even though, no transformations of the compounds occurred during the process, the loss of the more volatile compounds was observed, and as a consequence, two different injections were performed. Although, the direct analysis (in Pyr solvent) of the derivatised extract avoids loses of target analytes, the presence of excess of derivatisation reagent can degrade the stationary phase of the column and only few μL can be injected in the GC system. In this sense, the non polar compounds were measured here by means of LVI-PTV-GC-MS and the derivatised compounds using a split/splitless injection system.

The method performance was carried out in terms of calibration linear range (external and procedural calibration), recovery and apparent recovery (%), repeatability in terms of RSD, limits of detection (LODs) and method detection limits (MDLs) and the results obtained for both non polar analytes (see **Table 3.1**) and polar analytes (see **Table 3.2**) were summarised.

3.4.1. PES-GC-MS analysis of non-polar compounds

External standard calibration curves i.e., a set of standards containing target compounds at concentrations ranging from LOD to 250 ng/mL for all the studied compounds were build. Good linearity was obtained for all the target analytes obtaining coefficients of determination (r^2) higher than 0.99 for all the compounds, except for 4,4'-DDT (r^2=0.988). On the other hand, linearity of procedural calibration curves was performed with 150 mL of ultrapure water aliquots fortified with the target analytes between 15 and 450 ng/L (n=7 levels). Good linearity was found out over the wide range of tested concentrations as showed by the r^2 obtained between 0.978-0.999 for all the compounds, except for DEHP ($r^2 < 0.500$).

In order to estimate the recovery and apparent recovery of the method, 150 mL of Milli-Q water samples (n=3) were spiked at 100 ng/L concentration level and followed the whole extraction process under the optimal conditions. Recovery was calculated by comparing the spiked concentration with the concentration obtained from external standard calibration curve without using surrogates. The recovery values ranged between 20-48 % (see **Table 3.1**) for all the compounds except for DOP and DEHP which showed a 4 % of recovery. Similar recovery values were also reported by Betlej et al. [40] using SPME microextraction and PDMS fibers. In general, similar recovery values were also reported in the literature for other microextraction techniques for the rest of the compounds studied in the present work [32, 41].

Table 3.1: *Data obtained for method validation in terms of recovery (%, n=3), apparent recovery (%, n=3), RSD (%, n=3), LODs and MDLs (ng/L, n=6, 99%) for WWTP and estuary water samples using PES-LVI-PTV-GC-MS.*

Analyte	Recovery % (n=3)	Apparent recovery % (n=3)	RSD % (n=3)	LOD (ng/L) (n=3)	MDL (ng/L) (n=6, 99%) Effluent WWTP	MDL (ng/L) (n=6, 99%) Estuary
α-HCH	45	117	14	7	36	25
β-HCH	20	80	19	5	39	25
ϒ-HCH	22	82	11	9	60	17
δ-HCH	25	97	13	9	115	12
AHTN	28	96	4	4	38	26
HHCB	26	84	7	3	132	71
Clorpyrifos	48	78	10	5	16	7
Clorfenvinphos	32	106	9	4	18	15
2,4′-DDD	26	91	8	4	15	10
4,4′-DDD + 2,4′-DDT	29	88	11	6	16	17
4,4′-DDT	43	111	16	21	18	29
BBP	46	80	15	2	13	38
DOP	4	80	4	2	25	15

The apparent recovery (see **Table 3.1**) was calculated by comparing the concentration obtained from the procedural calibration curve with the spiked amount of the target compounds. In the case of DEHP, due to its ubiquitous presence everywhere [42], including in Milli-Q water, it was not possible to build an adequate calibration curve ($r^2 < 0.500$) and as a consequence, the apparent recovery of DEHP could not be measured and it was discarded for further analysis. In general terms, satisfactory apparent recoveries ranging from 66-129 % were obtained for all the target compounds, even not being necessary the use of deuterated analogues. Similar apparent recovery values were also obtained in the literature using different microextraction techniques [32, 43-45].

The repeatability of the methods was assessed using spiked Milli-Q water samples at 100 ng/L for three replicates analysed in the same day. As it can be observed in **Table 3.1**, RSD % values varied from 3 % to 19 %. The obtained repeatability values were comparable to other works dealing with sorptive microextractions of trace organic pollutants determination in environmental samples [32, 46, 47].

Finally, LODs were experimentally determined from three samples (150 mL Milli-Q) and calculated with the average signal of blank (n=3) plus three times the standard deviation. In the case that no signal was detected at the corresponding retention time, LODs were referred to a signal-to-noise ratio of 3. The obtained LODs were below 10 ng/L for all the target compounds, except in the case of 4,4'-DDT which rendered higher LOD values (21 ng/L). These values were comparable to the LODs obtained by SBSE [32, 46, 48, 49].

Afterwards, estuary water samples and WWTP effluents were spiked at the corresponding LOD of each target compound in order to calculate the MDLs as indicated in the guidance for MDL calculation proposed by US Environmental Protection Agency [50]. The signals obtained for non spiked samples were subtracted to spiked samples. Finally, MDLs were calculated at 95 % of confident level for 6 samples ($MDL_{95\,\%} = t_{95,6} * s$, where s is the standard deviation). As detailed in **Table 3.1**, the MDLs were in a range of 7-71 ng/L in the case of estuary and 13-132 ng/L in the case of WWTP effluent water samples. Those values are in

accordance with the values found in the literature for estuary and WWTP effluent samples [32, 51].

3.4.2. PES-silylation-GC-MS vs. PES-LC-MS/MS for polar compounds

External standard calibration curves i.e., a set of standards containing target compounds at concentrations ranging from LOD to 215 ng/mL for 4*t*OP, 4*n*OP, E2 and EE2 and from LOD to 430 for NP mix and BPA were evaluated. Isotopically labelled compounds were also added to use them as surrogates. Good linearity was attained for all the target compounds obtaining r^2 values higher than 0.99 except for NP mix (r^2= 0.61). Procedural calibration curves were tested by spiking Milli-Q water with different amounts of standards (from LOD to 250 ng/L for 4*t*OP, 4*n*OP, E2 and EE2 and from LOD to 500 ng/L for BPA and NP mix). Once again, good linearity ($r^2 > 0.99$) was obtained for all the target compounds, except for NP mix ($r^2 = 0.91$).

Recovery was calculated using 150 mL of Milli-Q water samples spiked at 100 ng/L (4*t*OP, 4*n*OP, E2 and EE2) and at 200 ng/L (BPA and NP mix). The obtained results were statistically comparable ($F_{Experimental}$=1.9-6.5 < $F_{Critical}$=7.7) at 95 % of confidence level according to the analysis of variance, ANOVA, for the two tested detection methods, thus only the values obtained for LC-MS/MS are shown in **Table 3.2**. The obtained recovery values were in the range of 31-54 %, which were in good agreement with other works found in the literature in which these analytes were extracted using other microextraction approaches [25, 52, 53].

Concerning to the apparent recovery calculations, two different approaches were evaluated. On the one hand, a procedural calibration was built (i.e., calibration curves were built with fortified water samples submitted to the whole analytical procedure) and on the other hand, the quantification was performed using external standard calibration method (i.e., calibration curve built with standards and concentrations corrected with the corresponding surrogate).

Table 3.2: *Data obtained for method validation in terms of recovery (%, n=3), apparent recovery (%, n=3), RSD (%, n=3), LODs and MDLs (ng/L, n=6, 99%) for WWTP and estuary water samples using both methodologies, i.e., PES-LC-MS/MS and PES-silylation-GC-MS.*

	Recovery % (n=3)	Apparent recovery % (n=3)		RSD % (n=3)		LOD (ng/L) (n=3)		MDL (ng/L) (n=6, 99%) Effluent WWTP		MDL (ng/L) (n=6, 99%) Estuary	
Analyte	LC-MS/MS	LC-MS/MS	GC-MS	LC-MS/MS	GC-MS	LC-MS/MS	GC-MS	LC-MS/MS	GC-MS	LC-MS/MS	GC-MS
4tOP	55	118	102	9	1	5	22	67	7	40	15
NP mix	52	109	94	45	12	42	76	241	37	93	70
4nOP	54	113	99	8	11	1	8	31	11	79	15
BPA	50	94	101	8	13	11	20	70	25	94	20
E2	38	104	101	16	6	1	2	100	27	72	54
EE2	31	81	103	10	7	14	25	169	72	131	136

In the case of derivatised compounds analysed by GC-MS, the use of external calibration approach did not provide satisfactory recoveries (90-241 %) and it was automatically discarded. On the contrary, good accurate values were obtained using the procedural calibration approach, being the apparent recoveries obtained between 94-103 % for all the studied compounds (see **Table 3.2**).

Regarding to the underivatised compounds detected by LC-MS/MS, adequate apparent recoveries; 93-115 % for all the analytes, except for NP mix (37 %) were obtained after the use of a procedural calibration and the quantification performed using external standard calibration method also provided satisfactory results in terms of apparent recoveries (81-118 %) for all the target compounds (see **Table 3.2**).

The repeatability of the methods was assessed in terms of RSD (%) using Milli-Q spiked at 100 ng/L for 4*t*OP, 4*n*OP, E2 and EE2 and 200 ng/L for BPA and NP mix, for three replicates analysed within a day. As it is summarised in **Table 3.2**, good precision values were obtained for all the target compounds using both methods, i.e., PES-silylation-GC-MS method (RSD < 12 %) and PES-LC-MS/MS method (RSD < 16 %), except for NP mix analysed using LC-MS/MS (RSD ≤ 45 %), which restricts the applicability of this method.

Finally, LODs were calculated using blank samples (150 mL Milli-Q water samples), being the values between 2 and 76 ng/L and between 1 and 42 ng/L for PES-silylation-GC-MS and PES-LC-MS/MS methods, respectively. These values were similar to those found in the literature for APs [54], BPA [38, 54, 55] and hormones [56] using different extraction approaches and MS detection. Better LOD values compared with some studies using other detection techniques like ultraviolet (UV), were obtained in the present work for APs [57], BPA [57, 58] and hormones [57].

Afterwards, MDLs were also determined by spiking effluent and estuary waters at the corresponding LOD of each target compound as indicated in the guidance for MDL calculation proposed by USEPA [50]. For the extracts analysed by GC-MS, the samples were spiked at 25 ng/L (4*t*OP and 4*n*OP), 50 ng/L (BPA)

and 200 ng/L (NP mix, E2 and EE2) and for the extracts analysed by LC-MS/MS, the water samples were fortified at 50 ng/L (4*t*OP, 4*n*OP and E2) and 200 ng/L (NP mix and EE2). The values obtained were in the range of 6-89 ng/L and 29-221 ng/L for PES-silylation-GC-MS and PES-LC-MS/MS, respectively (see **Table 3.2**).

3.5. Application of the developed methods to real samples

The optimised extraction and detection methods were applied to estuary water and WWTP effluent water samples. The average concentration (n = 3) obtained for all the analytes with their corresponding uncertainties at 95 % of confidence level are shown in **Table 3.3**. The rest of the compounds were below the MDLs (see **Tables 3.1** and **3.2** for non-polar and more polar compounds, respectively) for both water samples. In the case of more polar compounds the results obtained by means of GC-MS after the derivatisation and by means of LC-MS/MS were statistically comparable ($F_{Experimental}$=1.7-4.0 < $F_{Critical}$=7.7 and $F_{Experimental}$=1.3-3.6 < $F_{Critical}$=7.7, according to the analysis of variance, ANOVA, for WWTP effluent and estuary, respectively) at 95 % of confidence level, thus, only the values obtained by GC-MS are shown in **Table 3.3**.

Table 3.3: *Concentrations found (n=3, 95 %) at ng/L for the target analytes in WWTP effluent and estuary water samples using PES-LVI-PTV-GC-MS and PES-silylation-GC-MS methodologies.*

	WWTP effluent	Estuary
AHTN	127 ± 12	34 ± 12
HHCB	537 ± 43	155 ± 12
δ-HCH	124 ± 11	26 ± 5
4,4-DDT	124 ± 10	< MDL
NP mix	99 ± 19	83 ± 2
BPA	88 ± 16	347 ± 15

Regarding to the concentrations found at environmental waters (wastewater and estuary), slightly higher concentrations were found in general at the WWTP effluent with the exception of BPA which reported the highest concentration values in the case of estuary sample (see **Table 3.3**).

Similar concentration values were reported in the literature for musk fragrances by Posada-Ureta and co-workers [41] and Cavalheiro et al. [11] for the same WWTP and estuary and Lee at al. [59], but higher concentrations were reported in the literature in the case of WWTP effluents for both musk fragrances in Spain [60] and USA [61].

Concerning to the pesticides detected in the present work, δ-HCH and 4,4′-DDT were detected at the same concentrations in WWTP effluent while the later was not found in estuary waters. It has to be mentioned that the use of 4,4′-DDT was banned in Spain at the late 1970s [62], even its concentration has also being reported in previous works from our research group with similar concentrations [63]. Comparable concentrations of HCH isomers were also reported by Martí et al. [64] and Bizkarguenaga et al. [63] in effluent waters from the Mediterranean and Gernika, respectively.

NP mix was the only APs detected with similar concentration at 99 ng/L and 83 ng/L for WWTP and estuary waters, respectively. Lower concentrations were reported for the same WWTP [63], but higher concentrations were reported in Denmark [65] and Catalonia [66].

Finally, BPA was detected at 88 ng/L in effluent whereas a higher concentration (347 ng/L) was detected at the estuary. Similar concentrations were reported for the WWTP effluents of Gernika [63] by our research group and in the literature [67, 68], and higher concentrations were reported by Sánchez-Avila et al. [66].

4. CONCLUSIONS

Extraction using PES tubes has been proved to be a cheap, simple and precise alternative for the extraction of musk fragrances, pesticides, PEs, APs, hormones and BPA from WWTP effluents and estuary water samples using sample volumes of 150 mL. Small organic solvent volume consumption (300 μL) and low overall cost of the present method taking into account the reduced cost of the polymer (c.a. 0.05 €/unit), together with the scarcely affection of the yield in the

whole procedure by the type of water, are the main advantages of the present methodologies. It should also be underlined; the extraction procedure is carried out overnight with samples being simultaneously extracted in a 15 positions magnetic stirring plate.

In general terms, good apparent recovery (80-118 %) values were obtained for both non-polar and polar compounds. However, DEHP could not be measured in the present work due to its widespread presence including Milli-Q, and a procedural calibration which could not be built at the desired concentration range in the present work. Although, better LOD values were obtained in the case of LVI (2-21 ng/L) compared to split/splitless injection (1-76 ng/L), comparable MDL values were obtained in both cases (i.e. LVI-PTV and split/splitless injection) probably due to the increase in the lack of repeatability at low level when more real matrix is injected.

In addition, comparable results in terms of precision were obtained by PES-silylation-GC-MS and PES-LC-MS/MS for all the analytes except for NP mix (45 %) in the case of PES-LC-MS/MS. However, better MDLs (6-55 ng/L and 15-89 ng/L for WWTP and estuary, respectively) were obtained in the case of PES-silylation-GC-MS protocol. PES-LC-MS/MS method provided a shorter analysis time (no need of derivatisation) and an external calibration approach was enough for the accurate quantification of the target compounds. On the other hand, LC-MS/MS was not able to measure NP mix with good quality (in terms of precision) and, as a consequence, LC-MS/MS could not be employed in order to analyse NP mix at the low concentration levels considered in the present work. In this case, even if the sample preparation is a bit more tedious, GC-MS analysis may be used.

Finally, the developed method was satisfactorily applied to water samples collected at the effluent of a WWTP and in the estuary with concentrations ranging from MDL-347 ng/L.

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Determination of endocrine disrupting compounds and their metabolites in fish bile

Science of the Total Environment, 536 (2015) 261

1. INTRODUCTION

The presence of emerging contaminants (ECs) and persistent organic pollutants (POPs) is becoming one of the main concerns of the 21st century. On the one hand, POPs are often referred as legacy contaminants which remain intact for long periods of time. On the other hand, ECs are those chemical compounds whose presence and environmental concern have been raised recently. They are widely distributed throughout the environment, accumulated in the fatty tissues of living organisms and are toxic to both humans and wildlife [1, 2].

It has been pointed that wastewater treatment plants (WWTPs) act as a secondary source of many of these contaminants since they are not efficiently removed and therefore they are continuously discharged to the water bodies such as rivers, estuaries or sea [3-6]. Among these contaminants, endocrine disrupting compounds (EDCs) are becoming of increasing concern due to their possible adverse health effects in intact organisms and communities as a result of changes in the hormone homeostasis [7, 8]. Compounds including alkylphenols (APs), estrogens, bisphenol-A (BPA), some pesticides, phthalate esters (PEs) or synthetic musks are considered EDCs [9] and they have been included in different European legislations such as Water Framework Directive (WFD, 2013/39/EU), REACH (Registration, Evaluation and Authorisation of Chemical Substances EC1907/2006) and Stockholm convention (www.pops.int) and in the U.S. Environmental Protection Agency (EPA).

As shown in previous works, these compounds could be released to the environment at low or very low concentrations (ng/L level or lower) [10-12], however, this sublethal but continuous discharge causes a chronic exposure scenario that may cause different alterations in fish population such as feminisation, intersex, decreased fertility and fecundity and developmental abnormalities [9, 13-15].

Many of these ECs, usually the most lipophilic ones, are metabolised in the liver and excreted through the bile [9, 13, 16]. According to Pedersen and Hill [17], APs are accumulated in different organs as follows: bile > liver > gonads > blood~

gill ~ kidney > muscle. Although both, fish bile and liver seems to be proper target tissues for the analysis of EDCs, the high content of lipids found in liver increases the complexity of the analysis [18] and thus, fish bile has become an alternative target tissue.

Since the amount and variety of compounds that are analysed is growing up, the analytical strategies are evolving from the target approach to the non-target one as long as the instrumental capabilities can accommodate them. This leads to the so-called multi-residual or multi-screening methods, which allow the simultaneous determination of many organic pollutants in a single run [19]. For instance, the simultaneous analysis of different organic pollutant families has been reported for water samples [10, 20, 21] sediments [22-24] or biota [24-26]. To the contrary, in the analysis of fish bile, only a single family or two families of contaminants have been simultaneously analysed, as the case of polycyclic aromatic hydrocarbons (PAHs) [27], hormones [28], musk fragrances [29], BPA [28] or pharmaceuticals [30]. To the best of our knowledge, only Yang et al. [31], have analysed 2 APs, BPA and 2 hormones, becoming the method presented in this work, the only multiresidue method for organic compounds in fish bile.

Besides, due to the low concentration of the EDCs present in fish bile samples [28, 32-34] and the complexity of the matrix, it is necessary a preconcentration step such as liquid-liquid extraction (LLE) [9, 29], solid-phase extraction (SPE) [28, 33, 34] or solid-phase microextraction (SPME) [30]. In many cases, a further clean-up step is also required to get better results in terms of accuracy, repeatability and limits of detection (LODs) [35]. In addition to this, since many of these contaminants are subjected to metabolic transformation to ease the transport along the fish organs or their elimination [36], the metabolic by-products analysis is also required to obtain a real and relevant environmental data of these contaminants [37]. As consequence, not only the analytes must be studied but also their metabolites. Thus, there is a necessity of developing new methods for the simultaneous determination of different families of compounds and their metabolites in fish bile.

Therefore, in the framework of a broader study aiming to the assessment of the intersex condition and molecular markers of endocrine disruption [38], the objective of this work was the development of a multiresidue analytical method for the determination of different target analytes including 2 synthetic musk fragrances (tonalide (AHTN), galaxolide (HHCB)), 3 APs (4-*tert*-Octylphenol (4*t*OP), 4-*n*-octylphenol (4*n*OP), nonylphenol technical mixture (NP mix)), 2 hormones (17 β-estradiol (E2), 17 α-ethynilestradiol (EE2)), BPA, 10 pesticides (2,4-bis(p-chlorophenyl)ethane (2,4′-DDD), 2,2-bis(p-chlorophenyl)ethane (4,4′-DDD), 1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl] (2,4′-DDT), 1,1′-(2,2,2-trichloroethylidene)bis[4-chloro]), (4,4′-DDT), α-hexachlorocyclohexane (α-HCH), β-hexachlorocyclohexane (β-HCH), γ-hexachlorocyclohexane (γ-HCH), δ-hexachlorocyclohexane (δ-HCH), chlorpyrifos, chlorfenvinphos, the metabolites of chlorfenvinphos (α-2,4′-trichloroacetophenone (TCA)) and chlorpyrifos (3,5,6-trichloro-2-pyridinol (TCP)) and 3 PEs (butyl benzyl phthalate (BBP), bis(2-ethylhexyl)phthalate (DEHP), di-*n*-octyl phthalate (DOP) and their metabolites (mono benzyl ester (MBzP), mono-2-ethylhexyl ester (MEHP), mono-*n*-octyl ester (MOP)) in fish bile using SPE as extraction/preconcentration and clean-up steps after an enzymatic hydrolysis and a gas chromatography-mass spectrometry (GC-MS) analysis. The developed method was applied to the biomonitoring of thicklip grey mullets (*Chelon labrosus*) collected at five different estuaries and harbours along the Basque coast during the period of May-June 2012 and a deep study of the results obtained was carried out.

2. MATERIALS AND METHODS

2.1. Reagents and materials

Names, abbreviations and characteristics of the chemicals compounds used in this work are summarised in **Appendix I**. All the chemicals were > 91 % purity. All analytes were individually prepared at concentrations between ~ 1000-4000 ng/µL in methanol (MeOH, HPLC grade, Lab Scan, Dublin, Ireland) except for DEHP, which was directly obtained at 2000 ng/µL in MeOH, and [$^{2}H_{15}$]-MX, which was obtained at 100 ng/µL in cyclohexane. 10-100 ng/µL solutions were

weekly prepared according to the experiments and stored in amber vials at -20 °C.

Ethyl acetate (EtOAc, HPLC grade), acetone (Ace, HPLC grade), toluene (HPLC grade), 2,2,4-trimethylpentane (isooctane, HPLC) and *n*-hexane (Hex, HPLC grade) were obtained from Lab Scan (Dublin, Ireland). Acetonitrile (ACN, gradient grade for HPLC) was obtained from Teknokroma (Barcelona, Spain) and acetic acid (HAc, 99.7 %) was obtained from Merck (Darmstadt, Germany).

Bond Elute Plexa SPE cartridges (hydrophilic styrene divinilbenzene, 200 mg, 6 mL) were purchased from Agilent (Agilent technologies, Avondale, PA, USA) and 1-g LC-Florisil® cartridges from Sigma-Aldrich (Steinheim, Germany). The SPE protocol was performed with a Visiprep® SPE manifold obtained from Supelco (Bellefonte, PA, USA).

Potassium di-hydrogen phosphate (100 %, Panreac, Barcelona, Spain) and diammonium hydrogen phosphate (99 %, Merck, Darmstadt, Germany) were used to prepare 0.1 mol/L buffer solution (pH=6).

Anhydrous pyridine (Pyr, 99.5 %) was obtained from Alfa Aesar (Karlsruhe, Germany) and the derivatisation reagent, N,O-bis(trimethylsilyl)trifluoroacetamide with 1 % of trimethylchlorosilane (BSTFA + 1 % TMCS, Sylon BFT, 99:1) from Supelco.

Ethyl-4-aminobenzoate methasulfonate salt (benzocaine) was obtained from Fluka (Steinheim, Germany). β-glucuronidase type VII-A from *Escherichia Coli* (4,974.48 U), β-glucosidase from almonds (102.8 U) and sulfatase from aerobacter aerogenes (12.25 U/mL) were obtained from Sigma-Aldrich, dissolved in Milli-Q water (< 0.05 μS/cm, Millipore, Bedford, MA, USA), divided into 250 μL aliquots and stored at -20 °C in amber vials.

2.2. Bile analysis

2.2.1. Sample collection

Adult thicklip grey mullets larger than 20 cm were captured by traditional rod (n=12-30) during May-June 2012 in the estuaries of Ondarroa (+ 43° 19' 11.21", - 2° 25' 24.59") which is a fishing port, urban and industrial (cannery) effluents, Deba (Marina, + 43° 17' 40.38", - 2° 21' 20.99") and Gernika (+ 43° 19' 26.18", - 2° 40' 25.61") downstream primary treatment WWTP located at the Biosphere Reserve of Urdaibai with a 66,000 inhabitants population area. In the harbours of a high densely populated area of Santurtzi (+43° 19' 45.54", -3° 1' 44.80") fishing, industrial and commercial port with a high maritime traffic and urban and industrial discharges and Pasaia (+ 43° 19' 19.21", - 1° 55' 50.38") fishing and industrial port with urban and industrial discharges. All sites are located in the Basque Coast (South East Bay of Biscay) (see **Figure 4.1**) and **Appendix IV** in the supplementary material for gender, size and weight of the fish). After fishing, fish were immersed in a saturated solution of benzocaine in order to anesthetise them before being dissected [39]. The bile was extracted by means of a syringe and up to ~1 mL of bile fluid was collected in sterile cryogenic vials (Corning Incorporated, Mexico), and kept in liquid N_2 until laboratory arrival where they were stored at -80 °C until analysis. 13 bile samples from Gernika, 9 from Pasaia, 12 from Deba, 10 from Ondarroa and 7 from Santurtzi were analysed.

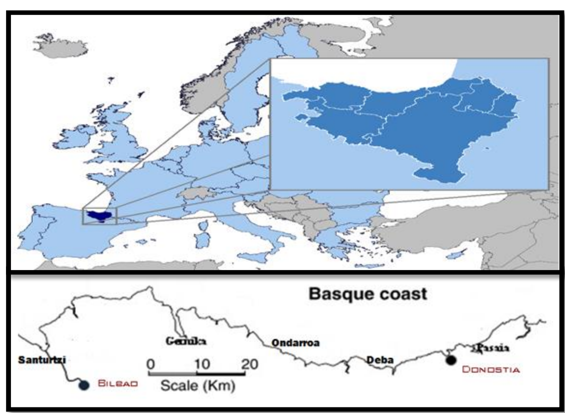

Figure 4.1: *Illustration of the sampling points along the Basque Coast.*

2.2.2. Extraction and clean-up of bile samples

Under optimum conditions, 100 μL of bile samples were hydrolysed using 1.5 mL of phosphate buffer (0.1 mol/L, pH=6), 800 μL of Milli-Q water and 200 μL of corresponding enzymes (1.000 U/mL for β-glucuronidase, 2 U/mL for sulfatase and 20 U/mL for β-glucosidase) according to Gibson et al. [32]. 100 μL of surrogate mixture ([$^2H_4$]-4*n*-nonylphenol ([$^2H_4$]-NP), [$^2H_3$]-17β-estradiol ([$^2H_3$]-E2) [$^2H_{16}$]-bisphenol-A ([$^2H_{16}$]-BPA), [$^2H_4$]-bis(2-ethylhexyl) phthalate ([$^2H_4$]-DEHP), [$^2H_{15}$]-musk xylene ([$^2H_{15}$]-MX) and [$^2H_8$]-4,4′-1,1′-(2,2,2-trichloroethylidene)bis[4-chloro]) ([$^2H_8$]-4,4′-DDT)) at 1000 ng/mL each one) were also added previous to the enzymatic hydrolysis step, which was carried out in an oven at 37 °C for 12 hours. Afterwards, 300 μL of HAc and 2 mL of Milli-Q water were added [34] to stop the hydrolysis. Thereafter, the hydrolysed fish bile was loaded into a 200 mg Plexa cartridge previously conditioned with 2 mL of MeOH and 2 mL of Milli-Q:HAc (99:1, v/v) solution. After sample loading, the cartridge was rinsed with 2 mL of Milli-Q:MeOH (95:5, v/v) for cleaning purposes and dried for 1 h under vacuum. Finally, the analytes were eluted with 4 mL of EtOAc and the extract was concentrated at approximately 1 mL using a gentle stream of N_2 (Turbovap® LV, Caliper, Life sciences, USA).

A clean-up of the extract was required after the extraction. The concentrated extract was loaded onto a 1-g Florisil cartridge, which had been previously conditioned using 5 mL of Hex. The target compounds were eluted in 3 mL of a mixture of Hex:EtOAc (90:10, v/v) (first fraction) followed with 3 mL of an EtOAc:MeOH (25:75, v/v) mixture (second fraction). The former was evaporated and reconstituted with 175 μL of Hex. Half of the fraction (APs, musks, phthalates, pesticides and TCA) was directly injected in the GC-MS system. However, TCP was eluted in the first fraction and it needed a derivatisation step prior to the analysis, thus, the other half of the fraction containing TCP was added to the second fraction and derivatised together. The second fraction containing hormones, BPA, phthalate metabolites (PEM) and TCP was derivatised as follows: 125 μL of ACN:Pyr (50:50, v/v) mixture and 50 μL of BSTFA + 1 % TMCS were added to the vials and the samples were heated at 65 °C for 45 min in an oven. The

derivatised aliquots were also finally analysed by means of GC-MS.

2.2.3. Water analysis

Water samples (superficial water) were collected at low tide at the same time fish were captured and 3 months after (see **Figure 4.1**) and extracted as described in chapter 3. Briefly, the analytes were extracted from 150 mL of sample (n=3) (containing 75 µL of deuterated compounds ([$^2H_4$]-NP, [$^2H_{15}$]-MX, [$^2H_4$]-DEHP, [$^2H_8$]-4,4′-DDT, [$^2H_{16}$]-BPA and [$^2H_3$]-E2) at 1000 ng/mL) using polyethersulfone tubes (PES, 0.7 mm external diameter, Membrane GmbH, Wuppertal, Germany). The PES tubes were immersed in 150 mL of the water sample previously filter through 0.45 µm cellulose filters (Whatman, Kent, UK) and extraction was performed at room temperature and 1200 rpm overnight. After the extraction, the tubes were removed from the water samples, cleaned with Milli-Q water and dried with a clean tissue. Afterwards, the analytes were desorbed from the tubes in an ultrasound bath for 16 min with 300 µL of EtOAc and musk fragrances, pesticides, APs and PEs were analysed by large volume injection-programmable temperature vaporization-gas chromatography-mass spectrometry (LVI-PTV-GC-MS) according to a previously developed method [12] and hormones and BPA by GC-MS after a derivatisation step [40].

2.2.4. GC-MS analysis

2 µL of samples were separately injected into a 6890N gas chromatograph (Agilent Technologies, Avondale, PA, USA) coupled to a 5973N electron impact ionisation mass spectrometer and a 7683 Agilent autosampler. The analysis was performed in the splitless mode for 1.5 min at 300 °C into a HP5-MS capillary column (30 m x 0.25mm, 0.25 µm, Agilent Technologies, Avondale, PA, USA). Two different oven temperature programs were used depending of the analysed extracts. The oven temperature for the analysis of the first fraction (i.e., non-derivatised compounds) was programmed from 60 °C (hold 1.5 min) to 170 °C at 10 °C/min and then until 300 °C at 15 °C/min where it was maintained for 5 minutes. The oven temperature program used for the analysis of the second fraction containing the derivatised compounds was as follows: start at 60 °C (hold

1.5 min), increase at 10 °C/min to 17 °C, increase at 15 °C/min to 200 °C, increase at 5 °C/min to 255 °C, increase at 15 °C/min to 285 °C maintaining it for 5 min and finally increased at 15 °C/min to 300 °C where it was maintained for 5 minutes.

Hydrogen (Hydrogen generator AD-1020, 99.9995 %, Cinel Strumenti Scientifici, Padova, Italy) was used as a carrier gas at constant flow of 1.3 mL/min. The MS transfer line temperature was maintained at 310 °C, and the ion source and quadrupole at 230 °C and 150 °C, respectively. Measurements were performed in SCAN (50-525 m/z) and SIM (select ion monitoring) modes and the m/z followed are listed in **Appendix II**.

3. RESULTS AND DISCUSSIONS

3.1. Optimisation of the derivatisation step

Among all the target analytes, the most polar ones (BPA, hormones, PEM and TCP) need to be derivatised before their analysis by means of GC-MS. According to our previous experience [10, 40], Pyr as solvent and BSTFA + 1 % TMCS as derivatisation reagent were used. However, in the present work, the derivatisation was not reproducible between days (relative standard deviations, RSDs > 35 %) for all the metabolites studied. In order to improve the reproducibility and based on the literature [41], the use of two different solvents was evaluated in the derivatisation step of BPA, hormones, PEM and TCP analytes: i) 125 µL of Pyr, and ii) ACN:Pyr (50:50, v/v) mixture. 50 µL of BSTFA + 1 % TMCS were used in both cases. As explained in the chapter 3 of the present thesis work, the direct analysis of the derivatised extract avoids loses of target analytes, but the presence of excess of derivatisation reagent can degrade the stationary phase of the column. Thus, two different ways to analyse the derivatised extracts were also evaluated: i) direct analysis of derivatised extracts in presence of derivatisation solvent and reagent, and ii) the derivatization of the extracts which were evaporated until dryness and reconstituted in 175 µL of Hex before GC-MS analysis.

As it is observed in **Figure 4.2** the best results were obtained when the sample was not submitted to a previous evaporation step for all the analytes excluding EE2, which showed no significant differences. In the case of ACN:Pyr (50:50, v/v) mixture and Pyr solvents, loses in the evaporation step were too high, and this step was then discarded. Regarding to the fraction not submitted to the evaporation step, the highest signals were obtained with Pyr was used as derivatisation solvent, but better reproducibility was achieved with the ACN:Pyr (50:50, v/v) solvent mixture (see **Figure 4.2**). Since the reproducibility of the results obtained in the case of Pyr was very variable we decided to use the mixture of ACN:Pyr (50:50, v/v) in further experiments. The possible formation of EE2 in E1 after the derivatisation step [42] was also studied here and non-oxidation of EE2 was observed when ACN:Pyr mixture was used.

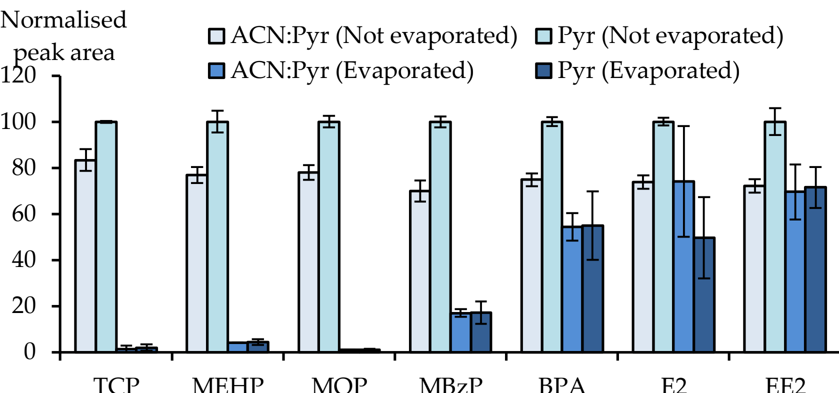

Figure 4.2: *Normalised average peak area (n=3) and their standard deviation using different eluent solvents.*

3.2. Optimisation of the solid phase extraction step

Both, the nature and volume of the elution solvent were firstly tested using Milli-Q water spiked with the hydrolysed compounds at 1000 ng/mL for all the analytes except for DEHP, NP mix, BPA and DOP (2000 ng/mL).

Three different solvents were evaluated: EtOAc, a mixture of EtOAc:Ace (50:50 v/v) and a mixture of EtOAc:Hex (50:50, v/v). Although, similar results in terms of chromatographic responses (see **Figure 4.3**) were obtained for most of the

analytes in the case of EtOAc and the mixture of EtOAc:Ace solvents, EtOAc rendered slightly better results in terms of repeatability (relative standard deviation, RSDs < 20 % and RSDs < 28 % for EtOAc and EtOAc:Ace, respectively). Thus, EtOAc was chosen as the optimum eluent solvent and used for further assays.

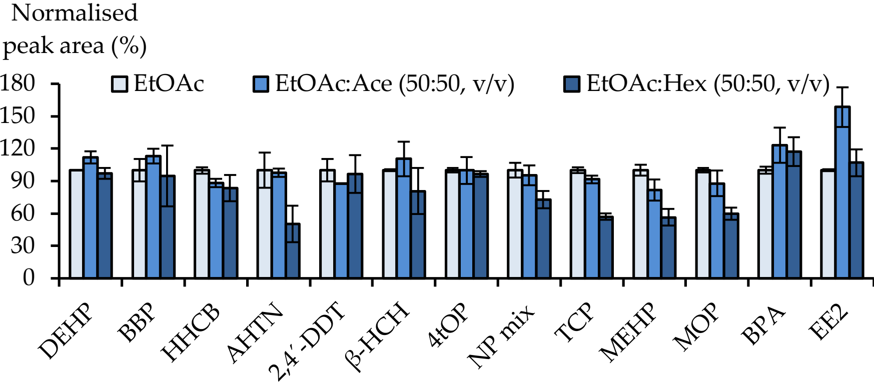

Figure 4.3: *Normalised average peak area (n=3) and their standard deviation using different eluent solvents.*

In the case of solvent volume and based on the elution profile of the studied compounds (see **Figure 4.4**), it was concluded that 4 mL were enough for the almost complete elution (> 96 %) of all the target analytes.

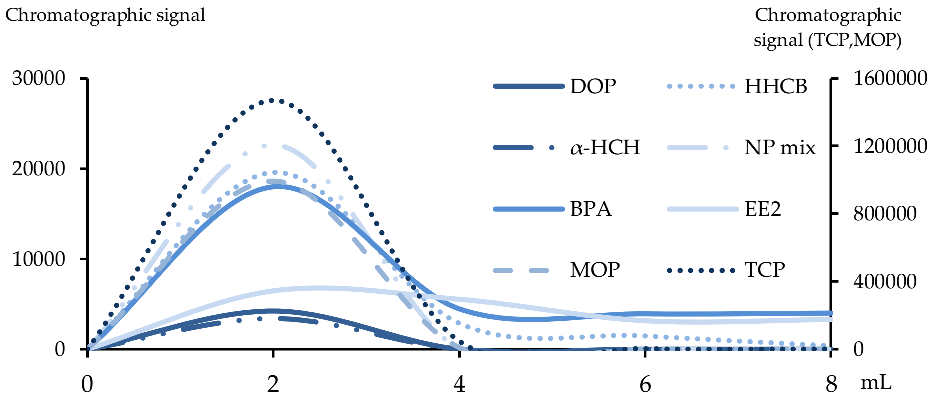

Figure 4.4: *Elution profile of the target analytes using EtOAc. Signals for TCP and MOP can be read in the right Y-axis, while the signals for the other compounds (one per family) are read in the left Y-axis.*

Finally, the optimised method was directly applied to the analysis of spiked bile samples (at 1000-2000 ng/mL range) but dirty chromatograms with many unknown peaks and chromatographic baselines irregularities were obtained. As a consequence, carryover effects were observed after 50 analyses, rendering recovery values between 11-226 % for all the target analytes. Consequently, further studies were required to assess the correction of matrix effect.

3.3. Study of the matrix effect and optimisation of the clean-up step

Different strategies can be used trying to overcome matrix effects. Matrix-matched calibration approach, sample dilution, an additional clean-up step or the correction of the target analytes signals with their deuterated analogues are the most applied alternatives. The dilution of the sample was not considered here owing to the expected target analytes low concentrations and the limits of detection. Matrix-matched calibration approach was considered, but the chromatograms obtained using cow bile as an analyte free matrix were not comparable with the fish bile chromatograms, thus, owing to the difficulties to have enough fish bile, the option of matrix-matched calibration was also discarded. Finally, the combination of a clean-up step using Florisil 1-g and the addition of deuterated surrogates was proposed to minimise the matrix effect.

Consequently, Milli-Q water was spiked at 1 ng/μL for all the analytes except for DEHP and NP mix (10 ng/μL) and DOP (2 ng/μL) and submitted to a clean-up step. The analyses were performed in triplicate (n=3). Loading solvent nature, elution solvent nature and elution solvent volume used in the clean-up step were optimised in these assays.

Target analytes were firstly extracted using the beforehand optimised conditions (see esperimental section 2.2.2), the extracts were evaporated until dryness and reconstituted in 1 mL of different loading solvents (i.e., EtOAc, Hex and Isooctane) in order to evaluate their effect in the clean-up step using 1-g Florisil cartridges. The extracts reconstituted in isooctane provided the dirtiest chromatograms. Better recovery values were obtained using Hex and EtOAc as loading solvents (80 - 120 %) and, finally, EtOAc was chosen as loading solvent

because, on the whole, it showed the best recoveries and no solvent change was required to perform before the clean-up step.

Once the EtOAc extract was loaded into 1-g Florisil, different eluents were also tested. In one hand, Hex, Hex:EtOAc (80:20, v/v), Hex:EtOAc (90:10,v/v) and Hex:Toluene (65:35, v/v) mixtures were used for the elution of APs, musk fragrances, pesticides and PEs. On the other hand, Hex:EtOAc (50:50, v/v) and EtOAc were tested for the elution of BPA and hormones. 3 aliquots (1 mL each) of the different solvents were tested in order to collect the non-derivatised analytes and 2 aliquots of 2 mL each to collect the analytes that should be derivatised before GC-MS analysis.

As it can be observed in **Figure 4.5**, the use of 3 mL of Hex:EtOAc (90:10, v/v) for non-derivatised analytes (first fraction containing musk fragrances, pesticides, APs and PEs) and 2 mL of EtOAc for derivatised analytes (second fraction containing BPA and hormones) was the best fractionation scheme. Due to practical reasons, 3 mL of EtOAc (instead of 2 mL) were employed in the second fraction, since no significant looses were occurring during the evaporation step.

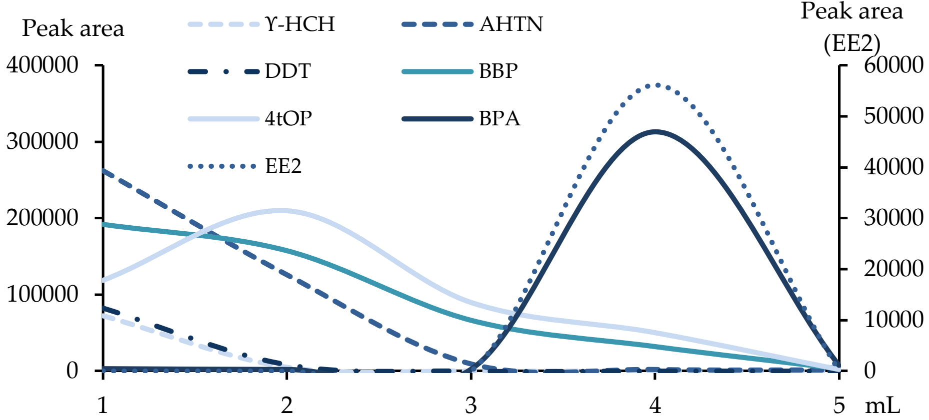

Figure 4.5: *Elution profile after the cleaning step with Florisil 1-g. Signals for EE2 can be read in the right Y-axis, while the signal for the rest of compounds (one per family) are read in the left Y-axis. 1-3 mL corresponds to Hex:EtOAc (90:10, v/v) and 3-5 mL to EtOAc.*

Regarding the metabolites, it was observed that pesticide metabolites (TCA and TCP) were quantitatively recovered in the first fraction (Hex:EtOAc; 90:10, v/v) but the use of 100 % of EtOAc was not strong enough for the complete elution of PEM (MEHP, MOP and MBzP). For this reason, different percentages (50 % and 75 %) of MeOH were tested for the elution of the metabolites and the use of EtOAc:MeOH (25:75, v/v) mixture yielded the higher extraction recoveries and it was chosen as optimum (see **Figure 4.6**).

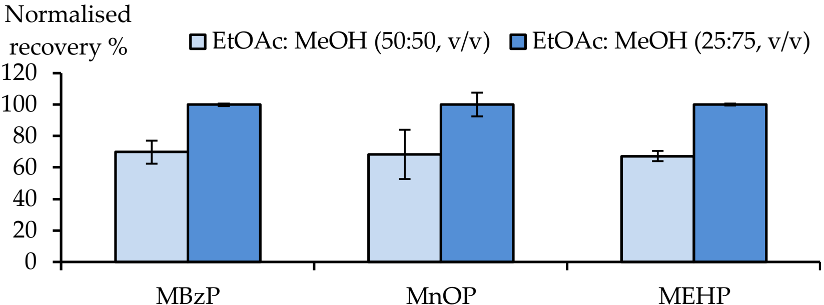

Figure 4.6: *Normalised recovery (n=3) and their standard deviation using different proportion of MeOH and EtOAc as elution solvents.*

Under optimal conditions, the compounds were sequentially eluted with 3 mL of two different solvent mixtures: Hex:EtOAc (90:10, v/v) for the first fraction and EtOAc:MeOH (25:75, v/v) for the second fraction. The first fraction was mainly constituted by non-polar compounds that can be directly analysed by GC-MS, except TCP metabolite which required to be derivatised prior to the analysis. The first fraction was evaporated until dryness, reconstituted in 175 µL of Hex and a half of the extract was directly analysed by GC-MS. The rest of the extract was added to the second fraction. The target compounds collected in the second fraction required to be derivatised prior to their analysis by GC-MS.

Matrix effect was finally evaluated using real fish bile sample. 100 µL of fish bile was spiked at 1 ng/µL for all the analytes except for DEHP and NP mix (10 ng/µL) and DOP (2 ng/µL) and was submitted to the overall optimised procedure. Clean chromatograms were also obtained for real fish bile sample extracts and the recoveries obtained were always above 81 %, except for MBzP (69 %) after their correction using deuterated analogues (see **Table 4.1**).

Table 4.1: *Quality parameters for bile analysis: apparent recoveries (%, n=3), relative standard deviation (RSD %, n=3), limits of detection (LODs, ng/mL, n=3) and matrix effect (%, n=3).*

Analyte family	Analyte	Recovery water (%)	Recovery bile (%)	Apparent recovery bile (%)	RSD (%)	LOD (ng/mL)
PEs[1]	BBP	84	86	119	24	22
	DEHP	105	86	116	7	205
	DOP	101	76	97	8	18
Musk[2]	AHTN	85	75	117	6	28
	HHCB	96	90	100	2	89
Pesticides[3]	2,4´-DDD	102	88	77	2	37
	4,4´-DDD	116	119	69	6	191
	2,4´-DDT	101	98	98	3	459
	4,4´-DDT	106	96	122	2	334
	α-HCH	107	41	101	1	28
	β-HCH	115	75	65	6	81
	γ-HCH	101	58	102	20	75
	δ-HCH	108	80	111	6	1
	Clorfenvinfos*	115	84	---	4	409
	Clorpyriphos*	113	80	---	1	71
APs[4]	4*t*OP	90	80	92	7	0.5
	4*n*OP	108	76	109	8	6
	NP mix	119	100	114	6	313
BPA[5]	BPA	84	51	94	3	7
Hormones[6]	E2	104	60	78	1	0.04
	EE2	88	63	63	4	1
PEM[4]	MBzP[3]	69	62	102	8	83
	MEHP	81	62	78	27	269
	MOP	104	50	99	5	21
Pesticides metabolites	TCA*	114	121	---	10	223
	TCP[5]	112	163	87	3	4

The analytes were corrected with: [1] $[^{2}H_{4}]$-DEHP, [2] $[^{2}H_{15}]$-MX, [3] $[^{2}H_{8}]$-4,4'-DDT, [4] $[^{2}H_{4}]$-NP, [5] $[^{2}H_{16}]$-BPA, [6] $[^{2}H_{3}]$-E2 and analytes marked with * were non corrected with any deuterated compound.

3.4. Performance of the analytical method

The performance of the analytical method was carried out using 100 μL of thicklip grey mullet's fish bile previously kept and fed in a clean aquarium for two weeks to depurate thoroughly before extracting the bile. The concentration of the analytes in the clean bile was also calculated. In general, no analyte was found in the bile samples except for DEHP (0.68 ng/mL), BPA (0.24 ng/mL), NP mix (0.15 ng/mL), HHCB (0.15 ng/mL) and Chlorpyriphos (0.14 ng/mL). These values were taken in consideration for further corrections. As shown in **Table 4.1**, total recoveries, apparent recoveries, LODs and repeatability in terms of RSDs % were calculated.

First of all, the recovery was calculated spiking 100 μL of clean fish bile (n=3) at 1000 ng/mL for all the analytes except for DEHP and NP mix (10000 ng/mL) and DOP (2000 ng/mL) and submitted to the overall optimised procedure. In general, the recovery obtained for most of the analytes was between 75-125 %. However, 9 analytes out of 26 showed some matrix effect being positive for TCP and negative effect for α-HCH, γ-HCH, MEHP, MOP, MBzP, BPA, E2 and EE2 (see **Table 4.1**). Therefore and in order to correct this matrix effect the apparent recovery of every analyte was calculated using labelled standards corrections.

The apparent recovery was calculated after spiking the bile (n=3) at the mentioned concentrations according to an external calibration curve (10-5000 ng/mL for all the analytes except for NP mix and DEHP 1000-15000 ng/mL) (r^2 higher than 0.99 in all the cases). Apparent recoveries between 65-122 % were obtained for all the analytes (see **Table 4.1**). Similar values were reported in the literature [32-35] (see **Table 4.2**).

Table 4.2: *Review of the methods in fish bile for the studied compounds families.*

	Bile (µL)[a] Bile (mg)[b]	Extraction	Clean-up	Analysis	Recovery (%)	LOD (ng/mL)[a] LOD (µg/g)[b]	LOQ (ng/mL)[a] LOQ (ng/g)[b]	Ref.
APs	20-100[a]	SPE	No	GC-MS/MS	63-86	60[c]	nr	[28]
APs	20-30[b]	SPE	No	GC-MS	93-105	nd-0.01	nr	[43]
APs	100[a]	SPE	No	GC-MS	67-128	12-13[d]	28-35[d]	[34]
APs	100[a]	SPE	No	GC-MS	81	nr	nr	[32]
APs	100[a]	SPE	Florisil SPE	GC-MS	92-114	0.5-313[c]	nr	In this work
BPA	20-100[a]	SPE	No	GC-MS/MS	99	0.1[c]	nr	[28]
BPA	50-100[a]	SPE	No	LC-MS/MS	75-112	nr	6[c]	[33]
BPA	100[a]	SPE	Florisil SPE	GC-MS	94	7[c]	nr	In this work
Hormones	20-100[a]	SPE	No	GC-MS/MS	80-91	0.4[c]	nr	[28]
Hormones	100[b]	SPE	NH_2 SPE	GC-MS	68	0.002[d]	nr	[35]
Hormones	50-100[a]	SPE	No	LC-MS/MS	77-113	nr	6-13[c]	[33]
Hormones	100[a]	SPE	No	GC-MS	109	5[d]	15[d]	[34]
Hormones	100[a]	SPE	No	GC-MS	81	nr	nr	[32]
Hormones	100[a]	SPE	Florisil SPE	GC-MS	63-78	0.04-1[c]	nr	In this work
PEs	100[a]	SPE	Florisil SPE	GC-MS	97-119	18-205[c]	nr	In this work
Musk	100[a]	SPE	No	GC-MS	nr	nr	nr	[29]
Musk	100[a]	SPE	Florisil SPE	GC-MS	100-117	28-89[c]	nr	In this work
Pesticides	100[a]	SPE	Florisil SPE	GC-MS	65-122	1-459[c]	nr	In this work

[a]: µL; [b]: mg; [c]: ng/mL; [d]: µg/g. nr: not reported; nd: not detected.

Precision was calculated spiking the bile at the mentioned concentration levels and they were measured within a day. The RSD % values were below 20 % for all the analytes except for BBP (24 %) and MEHP (27 %). Although, some of the works found at the literature showed better precision (i.e., 5-12 %) [34] it should be highlighted, in the present work the simultaneous determination of 26 analytes was carried out.

Finally, LODs were calculated as the average signal (n=3) of blank samples plus three times their standard deviation or in the cases that no signal was observed in the analyte retention time, three times the S/N ratio was used. LOD values were at ng/mL level (1-459 ng/mL) for all the studied compounds, except for E2 (0.04 ng/mL) and 4tOP (0.5 ng/mL) for those LODs at ng/L levels were obtained. Comparable LOD values (ng/mL level) were pointed out in the literature for hormones [28, 35], BPA and APs [28]. Lower LODs were reported here compared with other works in the case of hormones [34, 44, 45], APs [34, 45] and BPA [45] (see **Table 4.2**).

3.5. Application to real samples

Average concentrations obtained for bile samples from the 5 different locations are illustrated in **Figure 4.7**. All the target analytes were measured at least in three of the studied locations. Water samples were also collected and analysed when fish were captured and three months later. In general, few pollutants were detected in water samples regardless the sampling campaign (see **Table 4.3**). Concentrations ranging from LOD to 0.3 ng/mL were obtained for most of the analytes except in the case of musk fragrances (up to 1500 ng/mL), chlorpyriphos (up to 1600 ng/mL) and DEHP (up to 1600 ng/mL). Regarding the water analysis, samples from the estuary of Gernika seem to be the most polluted ones. Similar ranges for all the studied families in effluent (ng/L range) and estuary (ng/L-ng/mL) samples were reported in previous works [10, 12, 40]. Although, Bizkarguenaga et al. [10] obtained similar concentration levels of PEs, APs, 4,4′-DDT and HCHs in Gernika, chlorpyrifos and chlorfenvinphos were not detected by the authors at that time. The analytes concentration levels reported by Blanco-Zubiaguirre et al. [12] and Ros et al. [40] in the estuary of Nerbioi-Ibaizabal were

similar to those obtained in the present work in Santurtzi.

Table 4.3: *Concentration (ng/L) for water analysis and the LODs (ng/L, in water). The rest of the compounds were below the LODs in the case of all the samples.*

May-June 2012			Sampling Points		
Analyte	Gernika	Ondarroa	Deba	Pasaia	LOD
BBP	19 ± 1	16 ± 3	20 ± 1	20 ± 3	8
DEHP	641 ± 195	350 ± 26	1595 ± 416	806 ± 380	32
HHCB	1528 ± 63	81 ± 1	82 ± 1	144 ± 4	9
AHTN	1089 ± 51	40 ± 6	37 ± 7	586 ± 29	12
α-HCH	280 ± 33	238 ± 117	< LOD	256 ± 102	64
δ-HCH	321 ± 38	115 ± 57	124 ± 26	< LOD	119
Clorpyriphos	134 ± 7	777 ± 73	1598 ± 319	136 ± 16	51
Clorfenvinfos	38 ± 19	53 ± 42	< LOD	< LOD	36
4*t*OP	na	na	na	na	12
NP mix	na	na	na	na	45
4*n*OP	na	na	na	na	15

August 2012			Sampling Points		
Analyte	Gernika	Ondarroa	Deba	Santurtzi	LOD
HHCB	227 ± 6	34 ± 1	44 ± 3	23 ± 1	9
AHTN	110 ± 1	< LOD	< LOD	< LOD	12
Clorpyriphos	1213 ± 203	< LOD	< LOD	< LOD	51
4*t*OP	41 ± 2	< LOD	< LOD	17 ± 2	12
NP mix	75 ± 12	< LOD	< LOD	< LOD	45
4*n*OP	22 ± 2	<LOD	< LOD	< LOD	15

na: not analysed

Concerning to APs (see **Figure 4.7**), 4*t*OP and 4*n*OP were detected at low ng/mL levels in fish bile whereas NP mix was found at higher concentrations (ng/µL) with the exception of Ondarroa and Pasaia, where no NP mix was detected. Similar concentrations were observed for the same contaminant within locations. Finally, APs levels were similar to those previously reported in thicklip grey mullets from the Basque Coast [46], but higher than the concentrations detected by Gibson et al. [32] in roach inhabiting UK rivers.

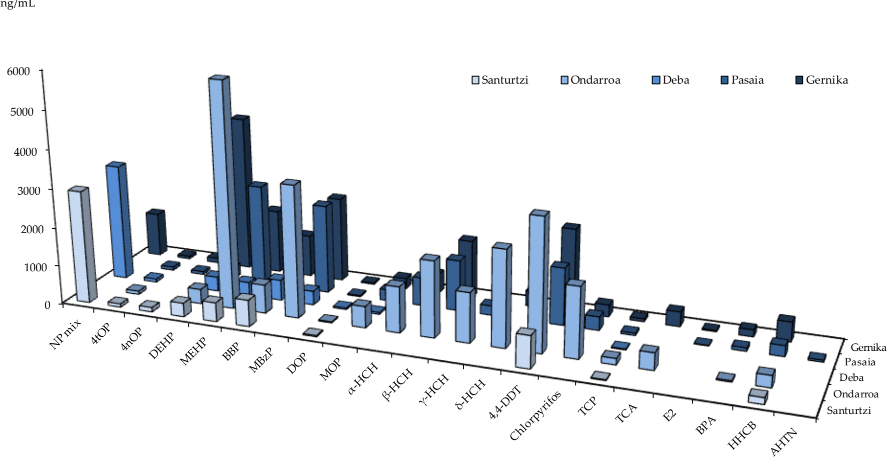

Figure 4.7: *Concentrations (ng/mL) for bile analysis. The rest of the compounds were below the LODs in the case of all the samples.*

In addition to this, PEs were present in all the studied mullet populations (see **Figure 4.7**). DOP, when present, was always found at low ng/mL concentrations, while BBP and DEHP were generally found at higher ng/mL or even at ng/µL levels for some individuals. It is worth mentioning that the highest concentration was 19226 ng/mL, in one fish captured in Gernika. In general, the concentrations found for PEs metabolites were higher than their parent analytes concentrations suggesting that PEs are metabolised to the monoester form. Therefore, both, parent and metabolites have to be analysed to determine efficiently the effects attributed to PEs. Furthermore, the concentration of DEHP in male fish is higher than in females (7-9 times higher) though the concentration ratio between males and intersex for DEHP and MEHP is quite similar, within 1.2-

1.5 and 0.7-0.9 respectively, a fact that may suggest a gender wise pattern. Valton et al. [47] estimated the ratio between MEHP and DEHP in the liver of roach (*Rutilus rutilus*) between 0.04 and 0.74, and in this work we raised up to 1.10. Thus, the ratio in liver and in bile is similar. Although, this suggests that the elimination of these compounds by bile maintains the ratio observed in the liver, deeper studies are needed to confirm this fact, as well as that DEHP is not totally metabolised in the fish. On the other hand, the ratio between MBzP/BBP was also determined. In this case, the ratio was calculated in the sampling locations where intersex fish were found (Pasaia, Gernika and Deba). In all the cases, the ratio was higher in intersex fish (see **Table 4.4**). This suggests a higher elimination of BBP metabolites through the bile in intersex fish than in male.

Table 4.4. *MBzP/BBP ratio in the sampling points where intersex fish were captured.*

	Pasaia		Gernika		Deba	
	Male	Intersex	Male	Intersex	Male	Intersex
MBzP/BBP	1-2.07	4.24	0.29-0.52	0.75-3.75	No metabolites	0.36-0.81

As it is observed in **Figure 4.7**, high concentrations of different pesticides and their metabolites were found in the locations of Ondarroa, Gernika and Pasaia, whereas Deba and Santurtzi only had measurable concentrations of one metabolite (TCP) and one pesticide (4,4'-DDT). In general terms, HCH isomers (ng/µL levels), 4,4'-DDT (high ng/mL ranges) and chlorpyrifos (ng/mL level) were the most predominant pesticides detected. Regarding the pesticide metabolites, in the case of chlorpyriphos, although deeper studies are required to determine the metabolism pathways in *Chelon Labrosus*, it must be highlighted that TCP is commonly observed in females and always at the same concentration level regardless to the water concentration. On the contrary, no chlorfenvinphos pesticide was found in any of the locations but its metabolite (TCA) was found at ng/mL level in Ondarroa and Gernika. This data may suggest the rapid metabolism process for chlorfenvinphos. Although a total hydrolysis of PEs, chlorpyrifos, 4,4´-DDT and HCHs could be expected, this study confirms that the parent compounds are not totally metabolised and they are excreted through the bile.

E2 was detected at low ng/mL levels in one specimen of Gernika and two of Pasaia (see **Figure 4.7**). Those concentrations are comparable to the levels reported previously in wild fish from the Basque Coast [34], Thames river in UK [28] and Puget Sound area in USA [33] but lower than those reported by Vigano et al. [45] in wild fish from Po river in Italy.

BPA showed measurable concentrations in few individuals of Ondarroa, Pasaia and Gernika, being the last one the most polluted site (see **Figure 4.7**). These concentrations are comparable to the levels reported in the literature [28, 33].

Finally, HHCB was detected at ng/mL levels in fish bile (see **Figure 4.7**) fished in Ondarroa, Gernika, Pasaia and Santurtzi whereas AHTN was only detected in Pasaia at low ng/mL levels. HHCB was always detected in water samples at ng/L levels except in Gernika (ng/mL) in June, regardless of the sampling location and campaign. AHTN was detected at ng/L levels in all the studied locations in the first sampling campaign whereas it was only detected in Gernika in August (see **Table 4.3**). The concentration levels of both HHCB and AHTN obtained in fish bile are comparable to the levels obtained in water samples (see **Table 4.3**), which could be attributed either to a lack of accumulation in fish bile or to the fast metabolism of those compounds. In addition, the lowest bioconcentration values (log BCF, log (C_{bile}/C_{water})) of this work were detected for HHCB (2.12) and AHTN (1.69). In the case of HHCB, and according to Fernandes et al. [29], this compound is actively transformed to its hydroxylated metabolite (HHCB-OH). When this study was carried out, HHCB-OH was not commercially available and thus, it was not included in the analysis. Unfortunately, to the best of our knowledge, there is not any published work reporting the metabolism of AHTN in fish bile.

The use of fish bile in biomonitoring shows the bioaccumulation of compounds that can be hardly measured in water samples. Therefore, the log BCF, was estimated in all possible cases (see **Appendix V**). The log BCF values range between 2.42 and 5.55 for all the sites and contaminants, with the exception of HHCB in Gernika (2.12) and AHTN in Pasaia (1.69). **Figure 4.8** illustrates the average BCF values for all the analytes in all the locations (See **Appendix V** for

individual values). BBP and NP mix have the highest BCF values. It is worth mentioning that the BCF values are higher in males than intersex and in intersex than female organisms. In fact, these values are 5-10 times higher in males and intersex than in females, regardless the size of the individuals. A relation between BCF and intersex condition was studied, however, no conclusive data was obtained and a deeper study would be necessary. Though this is a preliminary work with limited amount of samples and many samples with values below the LODs were obtained, this could be indicative of the high bioconcentration capacity of these compounds in fish bile.

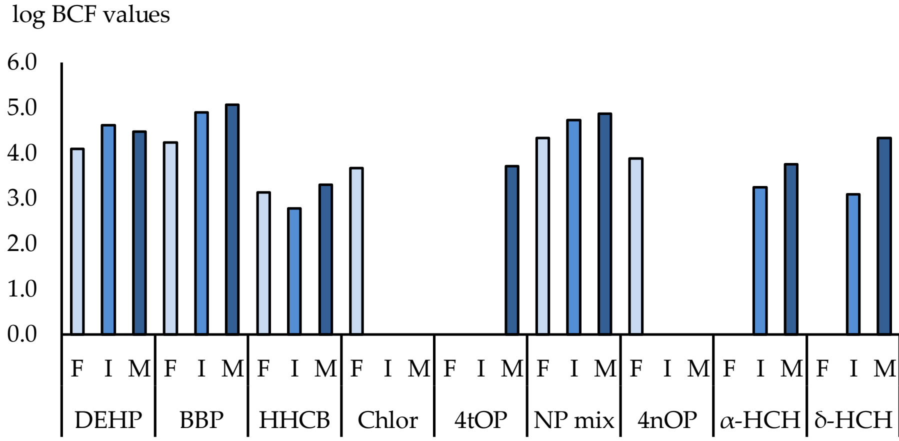

Figure 4.8: *Average Bioconcentration factor values (log BCF=log (C_w/C_{bile})) of the analytes.*

In addition to the analytical results, this work was part of a broader study aiming to the assessment of the intersex conditions and molecular markers of endocrine disruption [38]. Nevertheless, though the sample size was quite limited, the results obtained suggest a direct relationship between the occurrence of those contaminants and the prevalence of intersex condition. In fact, Gernika is the sampling point where the highest total concentrations were found and 13 % of the captured fish showed intersex conditions. On the contrary, it is hard to find any relationship between the uptake of EDCs in fish bile and the prevalence of intersex. As an example, a relative high number of intersex individuals were found (n=2) in Deba but the levels of EDCs were the lowest, and fishes from Ondarroa showed the highest sum of pollutants in bile, but no intersex was observed.

4. CONCLUSIONS

The method developed and validated in this work gives the opportunity to apply multiresidual analysis in fish bile samples, where parent contaminants and metabolites are found together. According to the results, it could be suggested that the sum of many compounds showing endocrine disrupting activity must be considered.

The sample treatment is based on a common technique such as SPE and the analysis is carried out by GC-MS, thus, most of the laboratories can apply this method without requiring sophisticate instrumentation. In general terms, the apparent recoveries obtained for the corrected analytes in bile samples were closed to 100 %. The repeatability was acceptable for the long process needed and the limits of detection for most of the analytes were at low ng/mL level. Those levels were sensitive enough for the analysis of field samples as demonstrated.

The method developed in the present work was successfully applied in wild populations of thicklip grey mullets from the Basque coast. Uptake of emerging contaminants with known endocrine disruption activity was demonstrated in five mullets populations from the Basque coast. Levels of contaminants in the bile of mullets were in the range of ng/mL-ng/μL. The BCF values were calculated, when possible, and in general, higher BCF values were calculated in males than in intersex and in females. This might indicate a gender wise tendency. However, no clear tendency was observed when the weight of the fish was considered. Log BCF values were between 2.42-5.55 for all the sites and analytes except in the case of musk fragrances which showed lower values. These values indicate that fish bile is suitable for biomonitoring purposes.

Finally, the results might indicate a relationship between the analytes concentrations reported in water samples and the number of intersex fish, as the case of Gernika. No clear correlation was observed between the intersex condition and the analyte concentration detected in the fish bile samples. Deeper studies have to be performed in this sense to get a firmly conclusion.

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Simultaneous enzymatic hydrolysis and extraction of endocrine disrupting compounds in fish bile using polyethersulfone polymer

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1. INTRODUCTION

Endocrine disrupting compounds (EDCs) are a wide and heterogeneous number of chemicals that can disrupt the hormonal control and homeostasis [1-3]. Many of these EDCs are found in a large variety of commodities and daily products and they are persistently released into the aquatic environment [1]. The EDCs presence has been associated with different alterations such as feminisation, intersexuality, decreased fertility and developmental abnormalities which have been observed in different fish species [2-6]. The ubiquity of EDCs together with the potential risks they may cause to environmental and human health are of growing concern for many scientists and international environmental agencies.

Among many other chemical compounds, alkylphenols (APs), estrogens, bisphenol-A (BPA) and phthalate esters (PEs) have been classified as EDCs [2, 7-9] and they have been included in different legislations such as the Water Framework Directive (WFD, 2013/39/EU). Although EDCs are usually found in environmental water samples at very low concentration levels (ng/L or lower) [10-12], this chronic sublethal EDC exposure of aquatic species seems to modify their endocrine system [2, 13, 14]. Metabolic reactions produce more stable and polar products which are easily excreted by urine or bile [15], and fish are able to conjugate the parental xenobiotics into the corresponding glucuronides [7, 8, 16], glucosides [17] or sulphates [7, 18] in order to eliminate many foreign compounds. As an example, it has been observed that some fish species can eliminate up to 90 % of the 17 β-estradiol (E2) as its glucuronide form (E2-G) [19]. Since these transformations take place in the liver and, as mentioned before, bile is one of the excretion routes, both are analytical target compartments to get insights about bioaccumulation of contaminants in fish [5, 20, 21]. Owing to the high lipid content of liver, which requires thorough and time consuming clean-up procedures, the use of fish bile is often preferred to estimate the bioaccumulation and biotransformation rates [5, 8, 18, 22].

Regarding the analysis of micro-organic pollutants in fish bile samples, most of the reported works are focused on the analysis of a single family such as polycyclic aromatic hydrocarbons (PAHs) [23], APs [8], synthetic hormones [8, 16], musk fragrances [22], BPA [16] or pharmaceuticals [24]. In addition to the large variety of organic chemical families, the low concentrations at which EDCs are present in fish bile and the complexity of the bile matrix, make necessary the use of specific procedures. In general, current analytical methods involve long (16–18 h) enzymatic hydrolysis [8, 16] followed by solid-phase extraction (SPE) [8, 16, 25] and even an additional clean-up step [25] before the chromatographic analysis, including in some cases both, the parental and the metabolic transformation products, such as glucuronides metabolites [26, 27]. However, as it has been previously reported in the literature [28, 29], the glucuronide metabolites often prove to be difficult analytes for chromatographic separations. First, in most instances, they are rather hydrophilic and therefore they tend to elute near the peak of the solvent or the hydrophilic matrix components. At the same time, the strongest ion suppression is often observed at the beginning of the liquid chromatographic run. Secondly, if more than one glucuronide structural isomers are present in a sample, the chromatographic separation may be difficult and requires the use of authentic glucuronide standards which are not always available.

The use of microextraction procedures is gaining a growing interest, especially solid phase microextraction (SPME) [30] and stir bar sorptive extraction (SBSE) [12, 31]. However, SPME lacks of the required low limits of detection and SBSE, usually performed with polydimethylsiloxane (PDMS) polymer, is still limited to the analysis of non-polar compounds [32, 33]. In this sense, it has been reported the suitable use of different polymeric materials to extract more polar compounds. However, the commercially available (Gerstel company) polyacrilate (PAc) and polyethylenglycol (PEG) mixture, known as acrylated twister, and PEG modified silicone known as EG Silicone twister are, according to the literature, not suitable enough for the analysis of polar compounds [34].

Recently, polyethersulfone (PES) [10, 35, 36] and polyoxymethylene (POM) [37-39] polymers have successfully been used for the extraction of hormones, APs, BPA, plasticisers and estrogens in environmental waters. In this sense, PES and POM allow the detection of slightly polar compounds at ng/L levels and their low cost favours their single use, avoiding carryover effects often reported in the literature [40] for commercial PDMS SBSE bars.

Within this context, the main aim of this work was the optimisation of a simple and fast simultaneous enzymatic hydrolysis and microextraction with PES and POM and the analysis by means of liquid chromatography-tandem mass spectrometry (LC-MS/MS). To the best of our knowledge, this is the first work that describes the simultaneous determination of 3 APs (4-*n*-octylphenol (4*n*OP); 4-*tert*-octylphenol (4*t*OP) and nonylphenol technical mixture (NP mix)), BPA, 2 estrogens (E2 and diethylbestrol (DES)) and a PEs metabolite (mono-2-ethylhexyl ester (MEHP)) in fish bile samples. In addition to this, the developed procedure was applied to the determination of target analytes in fish's bile (*Chelon labrosus*) collected close to a waste water treatment plant (WWTP) located in the Urdaibai estuary (Gernika-Lumo, South East Bay of Biscay, North of Spain).

2. MATERIALS AND METHODS

2.1. Reagents and materials

The characteristics of chemical standards used in this work are summarised in the **Appendix I**. Pooled male rat microsomes (Sprague-Dawley) from liver and magnesium chloride ($MgCl_2$, 98 %) were obtained from Sigma-Aldrich (Steinheim, Germany) and sodium cholate (≥ 96 %), tris HCl buffer and UDP-glucuronic acid trisodium salt (≥ 99 %) from Fluka (Steinheim, Germany).

All stock solutions were individually prepared at concentrations ranging from 1000-4000 ng/μL in methanol (MeOH, HPLC grade, LabScan, Dublin, Ireland) and stock solutions of 100 ng/μL were monthly prepared. The stock solutions were stored in amber vials at -20 °C. All the synthesised analytes were individually prepared at the concentration obtained after the synthesis (see **Table 5.1**) in MeOH.

According to the experiments, 1 ng/μL solutions were daily prepared.

Ethyl acetate (EtOAc, HPLC grade) was obtained from LabScan and acetic acid (HAc, 99.7 %) from Merck (Darmstadt, Germany).

The PES tubes used for extraction purposes were purchased (Membrana, Wuppertal, Germany) in a tubular format (0.7-mm external diameter, 1.43 g/mL density). Pieces of 1.5 cm each were cut using a sharp blade and soaked twice in EtOAc prior to their use. POM polymer was purchased in film format (1.41 g/mL density) from CS Hyde Company (Illinois, USA). Pieces of 1 x 1 cm were cut and soaked in EtOAc. Before using, PES and POM materials were removed from EtOAc and dried with a clean tissue.

MeOH (Optima, LC-MS quality) used as mobile phase eluent in LC-MS/MS was obtained from Fisher Scientific (Geel, Belgium). Ammonia (25 % as NH_3, Panreac, Reixac, Barcelona, Spain) was used for mobile phase modifications. Acetonitrile (ACN, < 99.9 %, gradient grade for HPLC) used as mobile phase in LC-DAD was obtained from Teknokroma (Barcelona, Spain).

Extracts were filtered before analysis with polytetrafluoroethylene (PTFE) hydrophilic filters (13 mm diameter, 0.2 μm pore size) obtained from Teknokroma.

Potassium di-hydrogen phosphate (KH_2PO_4, 100 %, Panreac) and di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$, 99 %, Merck, Darmstadt, Germany) were used to prepare 0.1 mol/L buffer solution (pH=6).

Ethyl-4-aminobenzoate salt (98 %, benzocaine) was obtained from Fluka. Regarding the use of enzymes, β–glucuronidase, type VII-A, from Escherichia Coli (25 KU) was obtained from Sigma-Aldrich, dissolved in Milli-Q water (< 0.05 μS/cm, Millipore, Bedford, MA, USA), divided into 250 μL aliquots and stored at -20 °C in amber vials.

2.2. Synthesis and separation of individual glucuronide compounds

All individual glucuronide compounds (4*t*OP, 4*n*OP, NP mix, EE2, BPA, DES and MEHP) except E2 which was directly obtained in the glucuronide form (see **Appendix I**), were individually synthesised according to Vallejo et al. [8] with some little modifications. Briefly, 10 µL of pooled male rat microsomes from liver and 400 µL of sodium cholate were incubated at room temperature for 30 min. 111 µL of each compound at 500 ng/µL were added together with the activated sodium cholate to a vial containing 400 µL tris-HCl buffer (50 mmol/L, pH=7.4), 1.5 mg UDP-glucuronic acid trisodium salt and 8 µL of $MgCl_2$ (800 mmol/L) and the mixture was incubated at 37 °C for one hour. To stop the process, 200 µL of ACN were added to the vial and the mixture was centrifuged at 4500 rpm for 10 min. The supernatant was evaporated until dryness and reconstituted in 200 µL of mobile phase (ACN:Milli-Q:HAc, 75:25:0.1) to be purified by means of gel permeation chromatography-liquid chromatography-diode array detector (GPC-LC-DAD).

The injection of 150 µL of the extracts was carried out into a Tosoh TSK gel 80TM (7.8 mm x 30 cm) reverse phase column placed in a high performance liquid chromatograph (Agilent Technologies, series 1100, Avondale, PA, USA) with a DAD detector, an automatic autosampler and an automatic fraction collector. The flow of the mobile phase was set at 1.5 mL/min and the separation of the target analytes was followed at the wavelength of 221 nm. Each glucuronide compound was separated from its parent analyte, and the fraction containing the glucuronide compound was evaporated until dryness and reconstituted in 2 mL of MeOH.

The concentration of each synthesised glucuronide compound was estimated from the mass balance shown in **equation 5.1**. For this purpose, the amount of each analyte not converted into the glucuronide form (i.e., non-glucuronide fraction) in the extract was quantified in the previous mentioned LC-DAD system using an external calibration built with target analytes (i.e., non-glucuronide compounds). The concentration of glucuronide compounds was determined using the **equation 5.1** and considering that the reaction stoichiometry within the glucuronide and non-glucuronide compounds is 1:1. The obtained

concentrations as well as the glucuronidation efficiencies are summarised in **Table 5.1**. Glucuronidation efficiencies higher than 64 % were obtained for all the analytes except in the case of EE2 (17 %). This low glucuronidation efficiency could be attributed to the low solubility of EE2 in aqueous media [41], and since the reaction has to be performed in water, EE2 was not included in further experiments.

$$\mathrm{mol_{glucuronide}} = \mathrm{mol_{total\ non-glucuronide}} - \mathrm{mol_{no\ reacted\ non-glucuronide}}$$

Equation 5.1

Table 5.1: *Studied analytes glucuronidation efficiency (%) and final concentration for glucuronide compounds.*

	Glucuronidation Efficiency (%)	Final glucuronide concentration (ng/μL)
4*t*OP	98	25.3
4*n*OP	71	19.3
NP mix	64	15.8
BPA	96	23.0
DES	71	32.8
E2	-[a]	---
EE2	17	3.5
MEHP	97	21.9

[a] Purchased from Sigma-Aldrich

2.3. Bile collection

Adult thicklip grey mullets (n=3) were captured in February 2013 by traditional rod nearby the WWTP of Gernika-Lumo, which is located at the Biosphere Reserve of Urdaibai (+43° 19' 26.18'', -2° 40' 25.61'') (South East Bay of Biscay, North of Spain). After fishing, fish were treated as described by Bizarro et al. [2]. Briefly, fish were immersed in a saturated solution of benzocaine in order to anaesthetise them before being dissected [42]. Afterwards, the bile was extracted by means of a sterile syringe, collected in sterile cryogenic vials and kept in liquid N_2 until laboratory arrival, where it was maintained at -80 °C until analysis.

2.4. Simultaneous enzymatic hydrolysis and extraction of bile samples

Under optimum conditions, 100 µL of fish bile were added to a 10 mL glass vessel containing 800 µL of Milli-Q water, 1.5 mL phosphate buffer (0.1 mol/L, pH=6), 200 µL of β-glucuronidase dissolved in Milli-Q (1000 U/mL) and 20 µL of a MeOH solution containing deuterated compounds ([$^2H_{16}$]-bisphenol-A ([$^2H_{16}$]-BPA), [$^2H_4$]-nonyl phenol ([$^2H_4$]-NP) and [$^2H_3$]-17 β-estradiol ([$^2H_3$]-E2)) at 5000 ng/mL). Five pre-cleaned PES tubes (1.5 cm each x 0.7 mm o.d.) or a 1 cm x 1 cm of POM film piece was introduced with a PTFE covered stirrer and the glass vessels were closed before performing the extraction at 37 °C for 3 hours in a stirring plate (RT 15 power, Ika Werkr, Staufen, Germany) at the scale of 5 (approx. 550 rpm). Once the combined extraction/hydrolysis procedure was over, the PES tubes or the POM film were removed, rinsed with Milli-Q to remove any residue, dried with a lint free tissue and placed into an amber eppendorf tube. Concerning to the target compounds desorption step, 500 µL of EtOAc were added and the materials soaked in an ultrasound bath (USB Axtor by Lovango) for 16 min. The EtOAc extract was quantitatively recovered and evaporated until dryness using a gentle stream of N_2 (Turbovap® LV, Caliper, 176 Life sciences, USA) and the extract was reconstituted in 250 µL of MeOH (Optima). Finally, the reconstituted extracts were filtered through a 0.2 µm PTFE filter before the LC–MS/MS analysis.

2.5. LC-MS/MS analysis

The samples were analysed in an Agilent 1260 series HPLC equipped with a degasser, a binary pump, an autosampler and a column oven coupled to an Agilent 6430 triple quadroupole mass spectrometer equipped with ESI source (Agilent Technologies). The quantification of the target analytes was performed in multiple reaction monitoring (MRM) mode. High purity nitrogen gas was used as nebuliser and drying (99.999 %, Messer, Tarragona, Spain) and collision gas (99.999 %, Air liquid, Madrid, Spain). MS/MS ionisation parameters (N_2 flow rate, the capillary voltage, the nebuliser pressure and the source temperature), fragmentor electric voltage and collision energy (CE) were optimised somewhere else [43]. The fragmentor and CE of MEHP were optimised in the negative mode in

this work (70-175 V and 5-45 eV ranges, respectively) by means of Agilent Source Optimizer program (see **Appendix III** for LC-MS/MS transitions and optimal conditions of all the analytes).

The separation parameters were optimised elsewhere [36]. Briefly, 5 μL of sample were injected into a partially porous Ace Ultra Core Super C_{18} core-shell (2.5 micron particle size) column at 30 °C and a flow rate of 0.3 mL/min. A binary mixture consisting on Milli-Q water containing 0.05 % NH_4OH (eluent A) and MeOH containing 0.05 % NH_4OH (eluent B) were used for gradient separation of target analytes. Linear gradient was as follows: 30 % B maintained for 4 min, increased to 60 % B in 3 min and to 80 % B in 10 min, where it was maintained constant for 10 min. Initial gradient conditions (30 % B) were then achieved in 3 min where it was finally held for another 10 min (post-run step). Instrumental operations, data acquisition and peak integration were performed with the MassHunter Workstation Software (Version B.06.00).

3. RESULTS AND DISCUSSIONS

3.1. Optimisation of sample preparation

Enzyme amount, polymer nature and amount, time profile and desorption solvent nature and time affecting both, the hydrolysis and the microextraction of the glucuronide compounds from bile samples, were studied (in triplicate) in order to get the optimum conditions for the simultaneous hydrolysis and extraction procedure.

Due to the difficulties to find fish bile without any target compound, 100 μL of Milli-Q water were used as sample during the hydrolysis and extraction optimisation. Hence, 200 μL of 1000 ng/mL of the compounds at glucuronide form or non-glucuronide form were added depending on the parameter to be optimised together with 200 μL of a solution containing 1000 U/mL of β-glucuronidase, 1.5 mL of phosphate buffer and 900 μL of Milli-Q water. Samples were stirred at ~550 rpm and at 37 °C for 24 h in order to make sure the complete hydrolysis and extraction, and the polymers were desorbed for 32 min using 500 μL of EtOAc.

3.1.1. Evaluation of polymeric material

The suitability of PES and POM polymeric sorbent materials during the extraction/hydrolysis combined procedure of the target analytes was evaluated for 24 h. According to our previous experience [10, 36], 5 PES pieces of 1.5 cm each (10 mg of total mass) and its equivalent in mass of POM (1 cm x 1 cm film) were used with this purpose.

Overall, lower extraction efficiencies (7-74 %) using POM as polymeric material comparing with PES (53-109 %) were achieved for all the studied compounds. This could be attributed to the low uptake kinetic reported in the literature for POM [44] and, thus, the time required to reach equilibrium might be longer than 24 h. Thus, PES was selected for further experiments.

In order to improve the extraction efficiency for the target analytes, the amount (5 and 10 pieces of 1.5 cm each) of PES material was evaluated. When higher amount of PES was used, the double quantity of buffer and Milli-Q water was added in order to assure the complete covering of PES material. The assays were only performed for non-glucuronide form of analytes since, at this step, extraction step was assessed and hydrolysis step was not considered.

As it is observed in **Figure 5.1**, the increase of the number of pieces did not bring any benefit for any of the studied analytes. In the case of DES and MEHP, besides, the use of 10 PES rendered lower extraction yields. This trend has already been reported by Camino-Sánchez et al. [45] who carried out the analysis of several organic compounds using other polymeric materials such as PDMS. Consequently, 5 PES tubes were selected as optimal sorbent amount and used in further experiments.

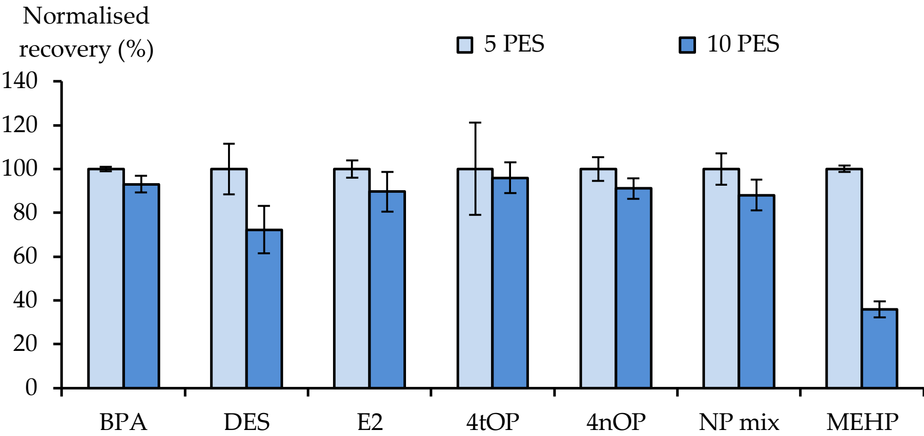

***Figure 5.1**: Normalised recoveries obtained (n=3) for the studied analytes using different amounts of PES (5 and 10 PES of 1.5 cm each) at 1000 ng/mL.*

3.1.2. Enzyme amount

Since the hydrolysis for some of the target compounds has not been reported in the literature, and in order to assure the complete hydrolysis of them, different amounts of β-glucuronidase enzyme were evaluated. In this sense, 200 µL, 400 µL and 600 µL of a solution containing 1000 U/mL of β-glucuronidase were tested.

As it is observed in **Figure 5.2** and according to the analysis of variance (ANOVA, p-level > 0.05), no significant differences (F_{exp}=1.3-2.6 < $F_{critical}$=9.6) were observed for all the studied compounds when different amounts of β-glucuronidase were used, thus, 200 µL of enzyme were used in further experiments. This value is in accordance with the amount of enzymes used by Gibson and co-workers [18] who used 200 U of β-glucuronidase for the hydrolysis of E2 and NP.

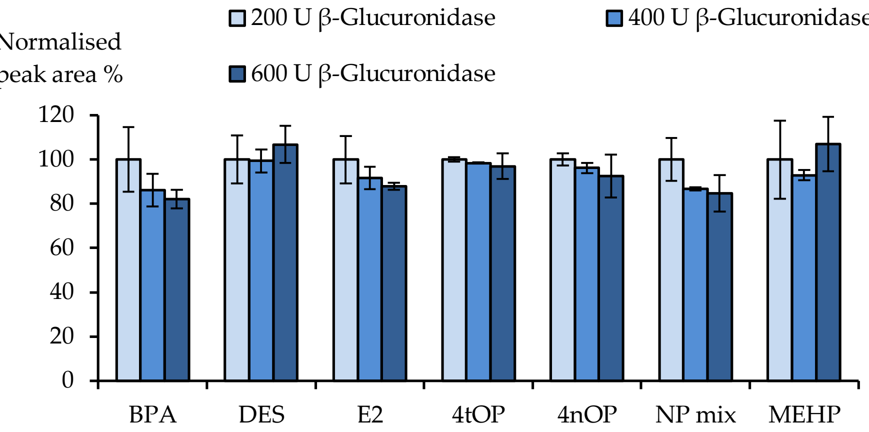

Figure 5.2: *Different amounts (200, 400 and 600 µL of 1000 U/mL) tested (n=2) for β-glucuronidase enzyme during the hydrolysis process.*

3.1.3. Time profile

The time profile was evaluated between 30 and 900 min, trying to find out the minimum time necessary for the simultaneous hydrolysis and extraction procedure. Samples were analysed after 30, 60, 360, 540 and 900 min.

As it is observed in **Figure 5.3**, 3 hours were enough to reach the equilibrium for all the analytes. This time is shorter than other methods reported in the literature which require times between 1.5 and 18 h only for the extraction [7, 16, 18, 46-49]. Although Vallejo and co-workers [8] proposed an alternative method for the hydrolysis of APs and some hormones using focused ultrasound assisted acceleration in 20 min, the methodology was overall time consuming because the samples had to be hydrolysed one by one, and more than 5 hours were necessary for the hydrolysis step of 15 samples.

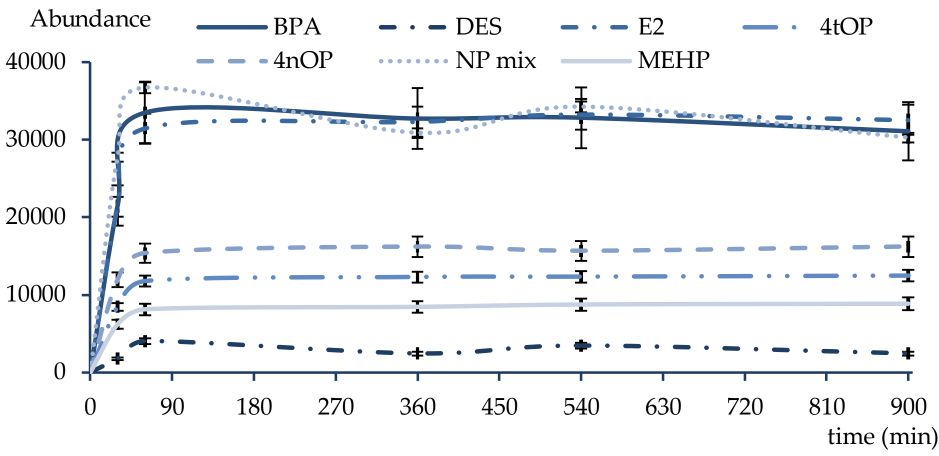

***Figure 5.3**: Extraction time profile (30, 60, 360, 540 and 900 min) for the studied analytes under optimum conditions (200 µL of β-glucuronidase and 5 PES tubes).*

3.1.4. Desorption solvent nature and time

Desorption step was studied in order to remove quantitatively the compounds from the PES tubes. In previous works [10, 36], EtOAc has been extensively used as desorption solvent in PES tubes for APs, BPA and hormones, but there is a lack of information about the behaviour of phthalate esters' metabolites. In this sense, the suitability of EtOAc and MeOH as desorption solvents was evaluated. The PES tubes were soaked with 500 µL of EtOAc and MeOH each, for 32 min. The results showed that EtOAc was slightly better than MeOH for the MEHP desorption (45 and 28 %, respectively (extraction + desorption steps)) and thus, EtOAc was maintained as desorption solvent.

With the aim of establishing the time needed for quantitative desorption of target analytes, chemical desorption of 5 PES was performed adding 500 µL of EtOAc to an eppendorf tube and soaking it for 8 min. The process was repeated 4 times recovering the solvent quantitatively and adding fresh EtOAc. As it is observed in **Figure 5.4**, 16 min (the sum of the 1st and 2nd fractions) were enough for the complete desorption (> 96 %) of all the compounds except NP mix (86 %). In the case of the NP mix, similar chromatographic responses were observed in the last desorption fractions, mainly attributed to the analysis blank problems. This

blank problems were minimised up to 91 % by changing the filters from polypropylene (PP) hydrophobic filters to PTFE hydrophilic filters, as it was also evidenced in a previously published work of our research group [43]. Thus, 500 μL of EtOAc and 16 min were the conditions chosen for the desorption of all the target analytes from PES tubes. Hydrophilic PTFE filters were used for further analysis.

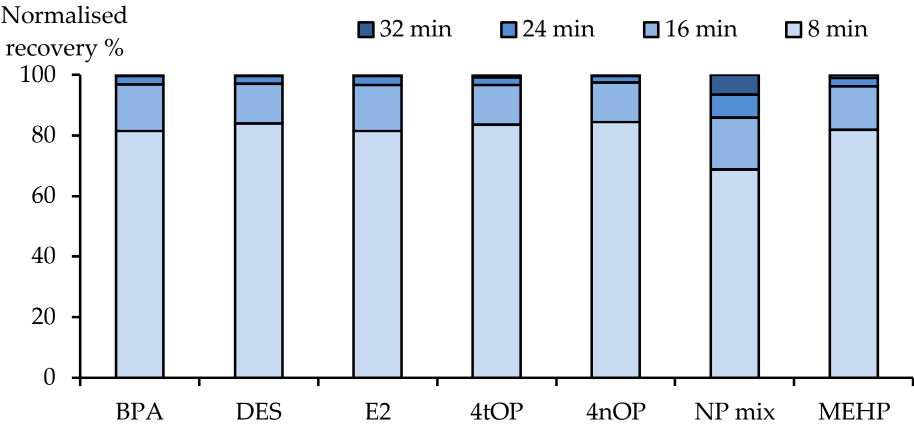

Figure 5.4: *Recoveries (%) obtained after the desorption of the PES tubes using EtOAc as solvent at different desorption times (8-32 min).*

3.2. Study of the matrix effect

Owing to the difficulty obtaining any matrix comparable to fish bile, some thicklip grey mullets were previously kept in an aquarium for 15 days with the aim to depurate them. These bile samples were used for method validation purposes.

First of all, matrix effect occurring at LC-MS/MS detection was evaluated by comparing the chromatographic responses obtained for bile samples spiked at 1000 ng/mL in extract and a standard solution of MeOH spiked at the same concentration. Non-spiked bile samples were also analysed and the chromatographic areas were subtracted to those obtained for spiked bile samples. In general, no matrix effect (recovery values close to 100 %) was observed for all the analytes (86-109 %) except for MEHP (210 %) which showed a signal enhancement during the detection step.

Afterwards, matrix effect that takes place during the extraction and hydrolysis step was estimated by comparing the chromatographic responses obtained for Milli-Q water and bile samples, which were spiked with the target analytes. Non-spiked bile samples were also analysed and considered for calculations. The analyses were performed in triplicate using 100 µL of fish bile. In this step, other three samples were processed in parallel adding 100 µL of deuterated compounds at 1000 ng/mL which were added as surrogate prior to the extraction. All the samples were processed under previously optimised conditions.

As observed in **Figure 5.5**, lower than 100 % extraction/hydrolysis efficiencies (23-66 %) were obtained for most of the analytes. This could be attributed to a common non quantitative polymeric material microextraction, an incomplete hydrolysis or a negative matrix effect. However, good apparent recovery values in the range of 97-125 % were obtained for all the target analytes, using deuterated compounds corrections. Anyway, while other published methods require an additional SPE clean-up step [25], the present method do not require any clean-up to get accurate results in less analysis time (i.e., 3 hours).

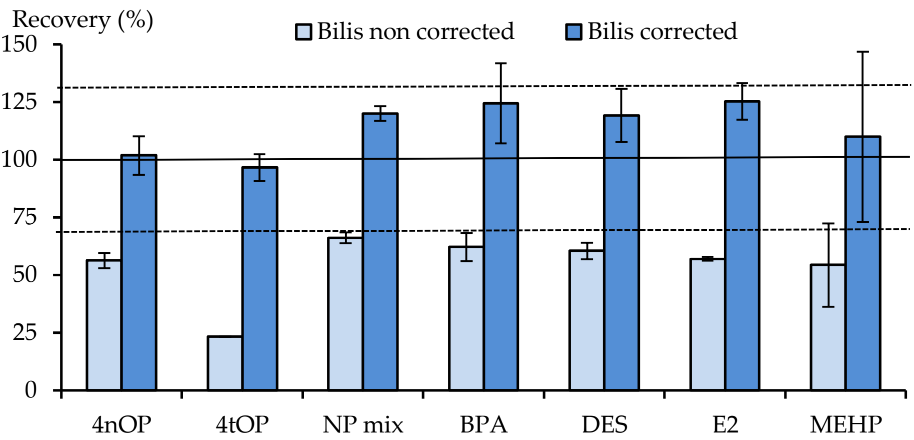

Figure 5.5: *Recoveries (%) obtained (n=3) for bile samples before and after the correction with the corresponding deuterated compound. The dotted lines indicated the high (130 %) and low limit (70 %) for the recommended recoveries [50].*

3.3. Performance of the analytical method

Calibration linear range, instrumental limits of detection (LODs) and quantification (LOQs), recovery and apparent recovery (%), repeatability in terms of relative standard deviation (RSD, %) and method detection limits (MDLs) using spiked real samples were determined using the previously depurated fish (see section 3.2) (see **Table 5.2**). Blank samples were always processed in parallel in order to guarantee the quality of the results.

The calibration curves were built in the range of 0.1-45 ng/mL in extract for all the analytes except in the case of MEHP, for which a calibration curve up to 7000 ng/mL concentration level was used. All the curves were linear at the mentioned range ($r^2 > 0.990$). LODs and LOQs were calculated using the lowest repeated calibration point (1 ng/mL for all the compounds and 8 ng/mL for NP mix). For the determination of the LOD, the average signal (n=3) of the mentioned calibration point plus 3 times the standard deviation was used and in the case of the LOQ, 10 times the standard deviation was considered. In general, the limits obtained were at low ng/mL level (0.7-3 and 1-9 ng/mL for LOD and LOQ, respectively) (see **Table 5.2**) in extract, except for MEHP (8 and 32 ng/mL, respectively) and NP mix (22 and 46 ng/mL, respectively). In the case of MEHP (8.4), although LODs were comparable to the rest of the analytes, the LOQ increases (32) due to a lack of repeatability at low concentrations (RSD ≤ 25 %). The low repeatability at low concentration levels could be attributed to the chromatographic conditions used during the analysis. According to the literature [51, 52], MEHP is always measured using an acid mobile phase. In this work, however, a basic mobile phase was used because it showed better peak shape and sensitivity for the rest of the compounds [9, 53]. The high LOD and LOQ obtained for NP mix could be attributed to the presence of different isomers forming the standard solution, as reported in other works [36], or attributed to the common blank problems. Those values are similar to the ones reported in the literature using LC-MS/MS [48], GC-MS or GC-MS/MS [8, 16, 25] for hormones, APs and BPA.

Table 5.2: *Data obtained for the method validation using PES as microextraction polymer in fish bile in terms of recovery (%, n=3), apparent recovery (%, n=3), relative standard deviation (RSD %, n=3), instrumental limits of detection (LODs, ng/mL), instrumental limits of quantification (LOQs, ng/mL), method detection limits (MDLs, ng/mL) and concentration values (in ng/mL) found in the analysed real samples. High level refers to 1000 ng/mL for all the compounds and low level refers to 5 ng/mL for 4tOP and DES, 12.5 ng/mL for 4nOP, 25 ng/mL for BPA, 100 ng/mL for E2 and 250 ng/mL for NP mix, which were selected according to the LOQs.*

	Recovery (%, n=3)	Apparent Rec. (%, n=3)		Repeatability RSD (%)		LODs	LOQs	MDLs	Real Samples (ng/mL, n=3)		
Analyte	*Low level*	*Low level*	*High level*	*Low level*	*High level*	*ng/mL (n=3)*	*ng/mL (n=3)*	*(α:95,n=6) ng/mL*	*Sample1*	*Sample2*	*Sample3*
4*n*OP[a]	109	140[a]	102[a]	6	8	0.65	1.0	1.1	< MDL	< MDL	< MDL
4*t*OP[a]	74	108[a]	97[a]	24	6	0.77	1.1	1.5	< MDL	< MDL	< MDL
NPmix[a]	108	121[a]	120[a]	16	2	21	46	61	< MDL	< MDL	< MDL
BPA[b]	75	96[b]	124[b]	17	12	1.5	2.8	9	11 ± 3	< MDL	< MDL
DES[b]	53	73[b]	119[b]	27	10	1.1	1.4	1.1	1.4 ± 0.2	< MDL	< MDL
E2[c]	91	77[c]	125[c]	1	6	3.4	8.9	14	< MDL	< MDL	< MDL
MEHP[a]	nc	nc	110[a]	nc	9	8.4	32	nc	2604 ± 312	1099 ± 212	975 ± 162

[a] corrected with $[^{2}H_{4}]$-NP, [b] corrected with $[^{2}H_{16}]$-BPA and [c] corrected with $[^{2}H_{3}]$-E2. nc: non-calculated values.

Recovery and apparent recovery values (after correcting the chromatographic signals with the corresponding deuterated analogue) were calculated at two concentration levels. Clean fish bile (n=3) was spiked at high concentration level (1000 ng/mL) for all the compounds and at different low levels (4*t*OP and DES at 5 ng/mL, 4*n*OP at 12.5 ng/mL, BPA at 25 ng/mL, E2 at 100 ng/mL and NP mix at 250 ng/mL). Those low concentration values were selected according to the LOQs and taking into account the compounds signals previously detected in the real bile samples.

Overall, the recoveries obtained for samples spiked at low level were in the 74-109 % range except for DES (53 %) (see **Table 5.2**). Those values are in good agreement with the values reported in the literature using classical techniques as SPE known for their quantitative capacity [8, 16, 18, 25]. However, those recovery values are higher than the previously reported ones for the PES microextraction of the target compounds in water samples using PES [10, 36]. Regarding the apparent recovery of the method, acceptable values (73-121 %) were obtained for all the compounds except for 4*n*OP (140 %) (see **Table 5.2**) in the case of low level and high level (97-125 %). Trueness, expressed as mean recovery, must be in the range of 70–130 % according to the Commission Decision 2002/657/EC [50]. The apparent recoveries were higher than those reported by Budzinski et al. (63 %) for E2 using SPE and GC-MS [25]. In the case of MEHP, recovery and apparent recovery were only calculated at high concentration level due to the presence of this analyte at high concentration levels in real samples.

The precision was estimated in terms of repeatability (RSDs %) at low and high concentration levels. As observed in **Table 5.2**, similar repeatability values were obtained at both levels, being less than 17 % in all the cases, except for 4*t*OP and DES at low levels (24 % and 27 %, respectively). Similar values (5-12 %) were reported in the literature for the determination of 4*t*OP, 4*n*OP and E2 [8] in fish bile. Besides, precision, expressed as RSD (inter-day precision) must be ≤ 30 % [50].

Finally, MDLs were also determined following MDL calculation guideline proposed by the U.S. Environmental Protection Agency (EPA) [54]. Briefly, 6 aliquots of clean fish bile were spiked at the same concentration used for the low

level. The signals obtained for non spiked samples were subtracted to the spiked samples before the statistical analysis, and the MDL was then calculated as: $MDL_{95} = t_{99,5} \times s$, where t corresponds to the Student's t-value for a 95 % confidence level and 5 degrees of freedom whereas s is the standard deviation of the replicate analyses. The values obtained (see **Table 5.2**) were at low ng/mL levels (1-14 ng/mL) in extracts for all the analytes except in the case of NP mix (61 ng/mL). However, it should be highlighted the isomeric mixture that forms the standard. The determination of MDL for MEHP was not possible since it was detected at relatively high concentration levels (ng/μL) in non-spiked bile samples.

3.4. Application to real samples

Finally, the method was applied to 3 fish captured at the same time (sample 1, sample 2 and sample 3) in the Gernika-Lumo, Urdaibai estuary and the analyses were performed in triplicate. Urdaibai estuary was selected in this work because previous studies demonstrated that relatively high levels of some EDCs were detected [2, 55, 56] in water samples collected at the same place where the fish were captured.

In this work, BPA and DES were detected in sample 1 (11 ± 3 and 1.4 ± 0.2 ng/mL, respectively) whereas MEHP was found in all the bile's at ng/μL levels (see **Table 5.2**). The rest of the compounds were below their MDLs. The concentrations obtained are similar to the values reported in the literature by Vallejo et al. [8] from the same estuary and Da Silva et al. [48] from samples collected at the Puget Sound Bay (USA), but lower than the values reported by Fenlon et al. [16] for the fish collected in the Thames river (UK).

4. CONCLUSIONS

A new relatively rapid, low cost and simple method was developed for the simultaneous enzymatic hydrolysis and microextraction, using PES as sorptive material, for APs, estrogens, BPA and a phthalate metabolite (MEHP) in fish´s bile samples. The use of deuterated compounds corrects the possible matrix effect that could produce the bile samples and no additional clean-up step was needed. The

present procedure is a good alternative to the traditional combination of SPE protocols and long enzymatic procedures usually employed to monitor EDCs in the aquatic environment. Finally, since the apparent recoveries (73-140 % and 97-125 % for low and high levels, respectively) and MDLs at 1-61 ng/mL levels are in good agreement with previously reported methods, this simple and fast method ensures the regulation compliance in environmental monitoring and surveillance programs.

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Determination of endocrine disrupting compounds in fish liver, brain and muscle using focused ultrasound solid-liquid extraction and dispersive solid phase extraction as clean-up strategy

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1. INTRODUCTION

Although a wide range of natural and synthetic chemicals are classified as endocrine disrupting compounds (EDCs), only a small fraction of the existing synthetic chemicals have been assessed for endocrine disrupting activity [1]. In this sense, the European Commission evaluated 553 compounds in relation with their disrupting activities [2]. Taking into account the European Commission's EDC classification, many substances considered as priority pollutants, including alkylphenols (APs), bisphenol-A (BPA), phthalate esters (PEs), some pesticides and emerging contaminants, such as pharmaceuticals and personal care products (PPCPs) and estrogens, are proven or suspected to disrupt the endocrine system [3-6]. However, nowadays, the presence of chemicals considered as EDCs is poorly regulated [1]. In fact, the lack of precise knowledge about the behaviour and fate of these xenobiotics in the environment, together with the increasing number of new products commercialised yearly, complicates the activity of international organisations to set concentration limits [7].

Although the EDCs are usually discharged to the environment at very low concentrations (ng/L levels) [3, 8, 9] making the biomonitoring challenging, they tend to accumulate in fish tissues and, thus, fish have been widely used for biomonitoring purposes [4, 10, 11]. Owing to these requirements, sensitive analytical methodologies are necessary for the determination of EDCs in fish tissues. Soxhlet extraction has been traditionally used for the extraction of organic compounds in many matrixes, including biota [10, 12]. However, the long time required and the large amount of solvent wasted, which is not only expensive to dispose off but which can itself cause additional environmental problems, are its most significant drawbacks. In order to overcome these problems, new extraction techniques such as microwave assisted extraction (MAE), accelerated solvent extraction (ASE) or ultrasound-assisted solid-liquid extraction (USE) have been developed and applied during the last years [13-16]. Among the techniques using ultrasound energy, the use of focused ultrasound solid-liquid extraction (FUSLE) is gaining interest in the recent years [17-19]. The energy applied during FUSLE is focused into the sample achieving 100 times higher energy compared to traditional

USE and hence, it enables efficient extractions in short time [20]. In a few words, FUSLE offers a simple extraction procedure requiring a low amount of sample (0.1–1.0 g), solvent (5-10 mL) and short extraction times (from seconds to few minutes) [13, 19].

However, since those extraction techniques are not selective, a clean-up step is usually necessary [13, 21]. Clean-up procedures based on solid phase extraction (SPE) [10, 22] or gel permeation chromatography (GPC) [13, 23] have been widely used during decades. As an alternative to those classical clean-up approaches, the use of dispersive solid phase extraction, (dSPE) which was firstly introduced by Anastassiades et al. [24] along with the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction method, is increasing nowadays. The main advantages of dSPE are the time saving, the small amount of organic solvents required and its low cost [6, 25]. The same conventional column-based SPE sorbents including primary secondary amine (PSA) to remove fatty acids, ENVI-CARB (ENVI) to eliminate sterols and pigments or octadecylsilane (C_{18}) used to remove non polar interferences [6, 18, 26] are used in dSPE.

Regarding to the analysis of EDCs in biological matrices, fish muscle and/or fish homogenate are the most analysed ones in the recent literature [6, 11, 14]. In fact, there are only a few works dealing with the development of analytical methodologies to determine EDCs in specific tissues such as liver [27] or muscle [15, 28]. Since EDCs are not equally distributed in the different fish compartments, several studies have demonstrated the necessity to assess the bioaccumulation patterns of EDCs in different tissues of fish, in order to get a better understanding of the xenobiotics effects inside the organism. For instance, according to Pedersen and co-workers [29] the bioaccumulation of APs in Cyprinic fish goes as follows: bile > liver > gonads > blood~gills~kidney > muscle and according to Liu. et al. [30] the phenolic EDCs are accumulated in liver followed by gill and muscle of Crucian Carp fish.

Within this context, the aim of the present work was to develop a method based on FUSLE coupled to dSPE protocol and liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis for the determination of 8 EDCs

including 3 APs (4-*tert*-octylphenol (4*t*OP), 4-*n*-octylphenol (4*n*OP) and nonylphenol technical mixture (NP mix)), BPA, 3 estrogens either natural or synthetic (17 β-estradiol (E2), 17 α-ethynilestradiol (EE2) and diethylstilbestrol (DES)) and one PEs metabolite (mono-2-ethylhexyl phthalate, MEHP) in different fish (*Chelon labrosus*) compartments (muscle, liver and brain matrices). The developed method was applied to the analysis of the target analytes in fish captured downstream a wastewater treatment plant (WWTP, estuary of Urdaibai, South-East Bay of Biscay) in order to evaluate the analytes fish tissue distribution.

2. MATERIALS AND METHODS

2.1. Reagents and materials

Names, abbreviations and characteristics of the chemicals compounds used in this work are summarised in **Appendix I** All the chemicals were > 94 % purity. All the analyte solutions were prepared in methanol (MeOH) at concentration ranging from 1000-4000 ng/μL and stored at -20 °C. 1-5 ng/μL stock solutions were daily prepared according to the experiments.

For the clean-up step, Florisil and ENVI graphitised carbon (100 m^2/g specific surface area, 120/400 mesh, Supelco), PSA (570 m^2/g specific surface area, Agilent Technologies, Palo Alto, CA, USA) and C_{18} (500 m^2/g specific surface area, Agilent Technologies) and PLEXA (hydrophilic styrene divinilbenzene, 450 m^2/g specific surface area, Agilent Technologies) were used as dispersive sorbents. Additionally, magnesium sulphate anhydrous ($MgSO_4$, extra pure, Scharlab, Barcelona, Spain) was also used in the dSPE step. A 24-Place centrifuge (5424) obtained from Eppendorf (Hamburg, Germany) was used.

Ethyl-4-aminobenzoate methasulfonate salt (benzocaine) used for anesthetise purposes was obtained from Fluka (Steinheim, Germany).

Acetone (Ace), MeOH, *n*-hexane (Hex) and acetonitrile (ACN) were purchased from Sigma Aldrich. All the reagents were HPLC grade, except the MeOH (UHPLC-MS) used for mobile phase purposes in the LC-MS/MS system

that was purchased from Scharlab. Ultrapure water (Milli-Q) was obtained using a Milli-Q water purification system (< 0.057 μS/cm, Millipore, Bedford, MA, USA). Ammonium (25 % as NH_4OH, Panreac, Reixac, Barcelona, Spain) was used for mobile phase modifications.

High purity nitrogen (N_2) gas (> 99.999 %) supplied by Messer (Tarragona, Spain) was used as collision gas and nitrogen gas (99.999 %) provided by AIR Liquid (Madrid, Spain) was used as both nebuliser and drying gas.

2.2. Sample collection and pretreatment

Adult thicklip grey mullets (n=3) (*Chelon labrosus*) were captured by traditional rod in December 2015 in Gernika. The sampling point (+43˚ 17′ 40.38″, -2˚ 40′ 25.61“) is located downstream a WWTP in the Biosphere reserve of Urdaibai.

Fish were treated as described in a previous work [31]. Briefly, fish were anesthetised using a saturated solution of benzocaine before being dissected, according to the guidelines for the Euthanasia of Animals [32]. Fish muscle without the skin, the liver and the brain compartments were placed in cryovials and kept in liquid N_2 until laboratory arrival. Afterwards, the brain was stored at -80 °C until analysis and the liver and the muscle were freeze dried for 48 hours and stored at 4 °C before analysis.

2.3. Sample extraction and clean-up

The extraction was done according to Mijangos et al. [18] with some modifications. In brief, 0.5 g (fish muscle and liver) or 0.1 g (brain) were weighed, placed into a tube and spiked with the deuterated analogues ([$^2H_4$]-4*n*-nonylphenol ([$^2H_4$]-NP), [$^2H_3$]-17β-estradiol ([$^2H_3$]-E2) [$^2H_{16}$]-bisphenol-A ([$^2H_{16}$]-BPA)) at 25 ng/g. 10 mL of Hex:Ace (50:50, v/v) were added to the sample and the samples were extracted for 5 minutes by means of FUSLE using a titanium probe (MS 73, Sonopuls HD 2070, 20 Hz, 70 W, Bandelin electronic GMBH & Co. KG, Berlin, Germany) at 33 % of power and pulse time on of 0.8 s and pulse time off of 0.2 s. Afterwards, samples were filtered through 0.45 μm pore polyamide (PA) filters

(Chromafil Ao, Macherey-nagel, Düren, Germany) and the solvent was evaporated until dryness under a gentle N_2 stream (Turbovap® LV, Caliper, 176 life sciences, USA). The extract was reconstituted in 1 mL of ACN and added to 1.5 mL-eppendorf tubes containing 100 mg of ENVI and 100 mg of $MgSO_4$. In the case of liver, 100 mg of PSA were also used. The tube was vortexed for 1 min, centrifuged for 5 min at 4000 rpm and the upper ACN layer was collected in a glass test tube. This process was repeated twice and the supernatants collected together, evaporated until dryness and reconstituted in 150 µL (brain) or 200 µL (liver and muscle) of MeOH (UHPLC-MS). Finally, all the extracts were filter through a 0.2 µm polytetrafluoroethylene (PTFE) hydrophilic filter (13 mm diameter, 0.2 µm pore size, Teknokroma, Barcelona, Spain) prior to LC-MS/MS analysis.

2.4. LC-MS/MS analysis

The samples were analysed as described in a previous work [33] using a LC-MS/MS system (Agilent 1260 series HPLC coupled to an Agilent 6430 triple quadrupole mass spectrometer equipped with ESI source, Agilent Technologies). In brief, 3 µL (liver) or 5 µL (brain and muscle) of the extracts were injected into a partially porous Ace Ultra Core Super C_{18} core-shell (2.1 mm x 50 mm and 2.5 micron particle size) column set at 30 °C and a flow rate of 0.3 mL/min. A binary mixture consisting of Milli-Q:MeOH (95:5, v/v) (mobile phase A) and of MeOH:Milli-Q (95:5,v/v) (mobile phase B), both containing 0.05 % of NH_4OH were used for gradient separation of the target analytes. The chromatographic separation was done as follows: 30 % B maintained for 4 min, increased to 60 % B in 3 min and to 80 % B in 10 min, where it was maintained constant for 10 min. Initial gradient conditions (30 % B) were then achieved in 3 min where it was finally held for another 10 min (post-run step). Regarding the MS/MS ionisation parameters, they were set as follows: N_2 flow rate at 11 mL/min, a capillary voltage of 4000 V, a nebuliser pressure of 52 psi (358.5 kPa) and a source temperature of 325 °C. Finally, the samples were quantified using multiple reaction monitoring mode (MRM) and scan mode was also used in order to check the cleanliness of the

extracts. The transitions followed in the MS/MS mode as well as the fragmentor, collision energy (CE), polarity and cell voltage are summarised in **Appendix III**. Instrumental operations, data acquisition and peak integration were performed with the Masshunter Workstation Software (Version B.06.00, Agilent Technologies).

3. RESULTS AND DISCUSSIONS

3.1. Optimisation of the clean-up strategy

A clean-up strategy based on dSPE was studied with the aim to remove all the matrix interferences prior to LC-MS/MS analysis. The optimisation of the clean-up step was carried out in terms of different nature and amount of dispersive phases and the use of $MgSO_4$. Regarding the three different matrices studied in the present work, i.e. liver, brain and muscle, fish liver of monkfish (*Lophius piscatorius*) was selected for the clean-up step optimisation since this matrix has the highest lipid content and, thus, seemed to be the most challenging. In this sense, 0.5 g of freeze dried and grinded fish liver samples were extracted according to the previous experience of the group for similar target compounds [18]. Extraction was performed by means of FUSLE as it was previously described in section 2.3. After the extraction, the extracts were evaporated until dryness, reconstituted in 1 mL of ACN and spiked at 1 ng/µL with the target analytes before the clean-up step evaluation.

Different sorbents (ENVI, Florisil, PSA, C_{18} and PLEXA) [3, 6, 18, 34, 35] used either in the dSPE procedures or in classical SPE protocols were evaluated in the present work. In this sense, five different sorbent combinations were studied: (i) ENVI (100 mg) and $MgSO_4$ (100 mg), (ii) ENVI (100 mg), PSA (100 mg) and $MgSO_4$ (100 mg), (iii) ENVI (100 mg), C_{18} (100 mg) and $MgSO_4$ (100 mg), (iv) ENVI (100 mg), Plexa (100 mg) and $MgSO_4$ (100 mg) and (v) ENVI (100 mg), Florisil (100 mg) and $MgSO_4$ (100 mg). The suitability of the clean-up approaches was carried out by comparing them in terms of both, recovery of the target analytes and the cleanliness of the final extracts. Florisil combination (v) was in a first approach

(prior to the LC-MS/MS analysis) discarded due to the orange colour of the final extracts. Extracts were almost colourless in the case of the rest of sorbents combinations.

Figure 6.1 shows the recoveries obtained (%, n=3) using the other four (i-iv) different dSPE sorbents combinations. Low recoveries (7-28 %) were obtained in the case of E2, EE2, DES and 4*t*OP using all the sorbents combinations except the ENVI and PSA mixtures (53-64 %). On the contrary, when PSA was used, the recovery of BPA decreased almost to half (53 %) comparing to the rest of the combinations and MEHP was hardly removed (~3 %). However, the use of PSA was selected for further evaluation since recoveries higher than 50 % were obtained for all the compounds except for MEHP. Similar results were also reported in the literature for EE2 [36] and 4*n*OP and BPA [35] using QuEChERS method and PSA as sorbent during the clean-up step of liver samples.

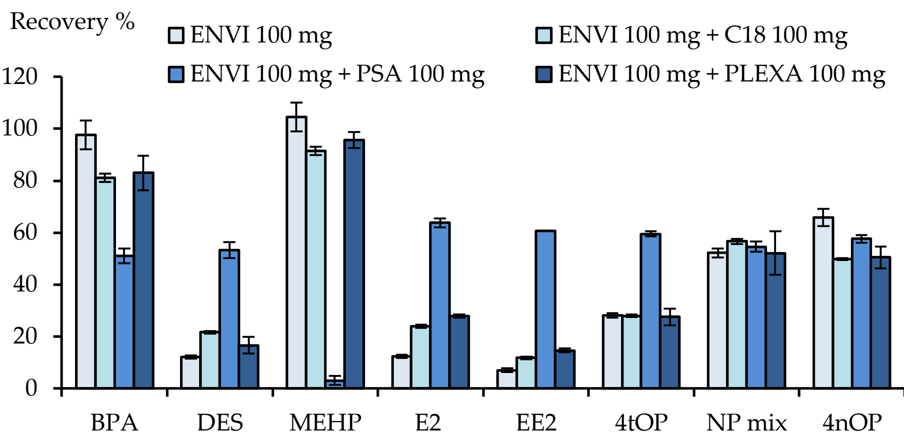

Figure 6.1: *Recovery obtained (%, n=3) when different dSPE sorbent phases were evaluated for the dSPE clean-up of fish liver extracts.*

In addition, all the extracts were also injected in SCAN mode to evaluate the extracts cleanliness and, in general terms, similar chromatograms (data not shown) were observed for any of the studied phase's combinations.

The amount (50-200 mg) of PSA was also evaluated in order to reach a compromise between the recoveries of the target analytes and the extracts

cleanliness. As depicted in **Figure 6.2**, low recoveries were obtained for E2, EE2, DES and 4*t*OP using the lowest (50 mg) amount of PSA and similar recoveries were obtained in the case of the majority of target analytes using PSA between 100-200 mg. 100 mg of PSA showed the best recoveries for all the analytes except for MEHP which showed low (< 15 %) recoveries in the case of all the PSA amounts tested. Comparing to BPA, APs or hormones [3, 33], MEHP is usually detected at higher concentrations [3, 31] in environmental samples, endorsing the decision to sacrifice the recovery of MEHP.

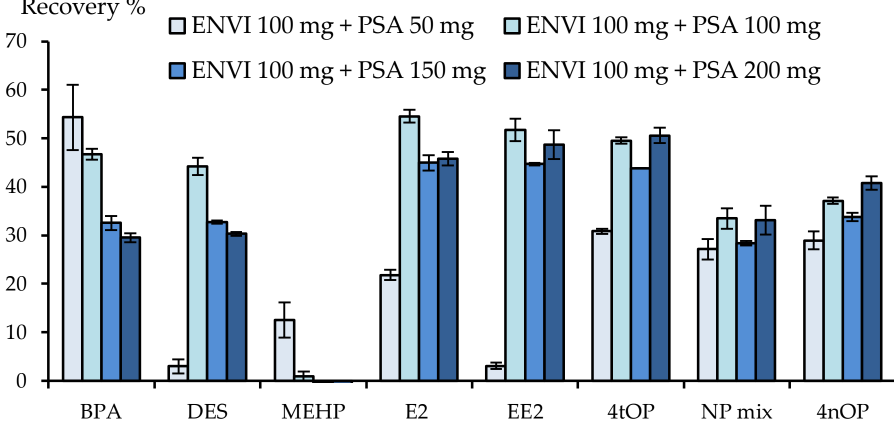

Figure 6.2: *Recovery obtained (%, n=3) when different amounts of PSA sorbent were used for the clean-up of fish liver extracts.*

Finally, the addition of $MgSO_4$ was also evaluated since this salt is widely employed when the samples are extracted and cleaned-up by means of QuEChERS-dSPE methodologies [15, 35, 36]. In this sense, 100 mg of ENVI and 100 mg of PSA previously selected as optimum clean-up combination, were mixed with 100 mg of $MgSO_4$ and the use of this salt improved the recoveries for DES (3 times), 4*n*OP (2 times) and E2 (1.5 times) and no significant differences ($F_{Experimental}$=1.0-3.8 < $F_{Critical}$=18.5) were observed for the rest of the studied compounds according to the analysis of variance (ANOVA). Thus, 100 mg of $MgSO_4$ were used in further experiments. Since the clean up based on dSPE is very matrix dependant, the optimum conditions were tested in a less fatty liver bought in a local supermarket (European Seabass, *Dicentrarchus labrax*). The apparent

recovery (79-110 % and 78-102 %) and repeatability (< 16 % and < 30 %) were comparable for European Seabass and monkfish, respectively, making the recently optimised clean-up approach suitable for the analysis of liver samples containing different amounts of fat. European Seabass was used for further optimisations.

Additionally, the dSPE based on the (i) and (ii) approaches were applied and compared in the case of muscle and brain matrices. Higher recoveries (72-100 % and 84-100 % for muscle and brain, respectively) when only ENVI sorbent was used compared to the addition of PSA (49-78 % and 42-99 % for muscle and brain, respectively) were obtained for both matrices and all the target analytes, except for MEHP which reported negligible recoveries in the case of PSA use. MEHP was quantitatively recovered from both matrices extracts without the PSA addition. In this case, the samples were also analysed in SCAN mode and similar to liver, no differences were observed in the chromatograms.

Thus, ENVI-PSA-$MgSO_4$ and ENVI-$MgSO_4$ dSPE approaches were selected as optimum clean-up procedures for fish liver and fish muscle and brain, respectively.

3.2. Optimisation of the extraction temperature and extractant nature

After the extracts clean-up procedures were fixed, the extraction temperature and the extractant nature during the FUSLE protocol were studied. With this purpose, 0.5 g (dry weight) of both fish liver and muscle matrices, once again selected as the target samples tissues, were extracted according to the method developed by Mijangos et al. [18]. Samples were spiked at 500 ng/g concentration level and the experiments were performed in both cases in triplicate.

During the FUSLE, the cavitations' phenomena of micron size gas bubbles and especially their violent collapses happened, induce extreme physical conditions (pressures up to tens of GPa, temperatures up to thousands °C) and, thus, looses of the more volatile analytes can occur [39, 40]. In this sense, the extraction step was performed and evaluated at both, room temperature and at ~0°C using an ice bath. Better results in terms of recovery (29-45 % versus 33-72 %

at room temperature and 0 °C, respectively) and precision (7-13 % versus 1-7 % at room temperature and 0 °C, respectively) were obtained for all the analytes at 0 °C, except in the case of NP mix which showed no significant effect. Thus, further experiments were carried out at 0 °C.

Afterwards, different extraction solvents including Hex, Ace or EtOAc, usually used in solid-liquid extraction protocols for the determination of several EDCs [37-39] were studied in this step. In this sense, different mixtures of Hex and Ace and pure EtOAc were evaluated. Regarding to liver samples (see **Figure 6.3a**), higher than 50 % of Ace amounts or pure EtOAc seem to be necessary to extract the MEHP from liver samples. A similar tendency was also observed in the case of EE2 to extract it quantitatively, and when low amounts of Ace or pure EtOAc were used, the repeatability (46 % and 56 %, respectively) was very poor. In the case of DES, EtOAc showed the lowest recoveries while the different combinations of Hex and Ace did not show significant differences. Finally, the use of a mixture of Hex:Ace (50:50, v/v) showed better recoveries for BPA and 4*n*OP. No significant differences were observed for the rest of analytes. Thus, Hex:Ace (50:50, v/v) was selected as optimum solvent since the target analytes recoveries were the highest, and acceptable repeatability (< 25 %) was achieved.

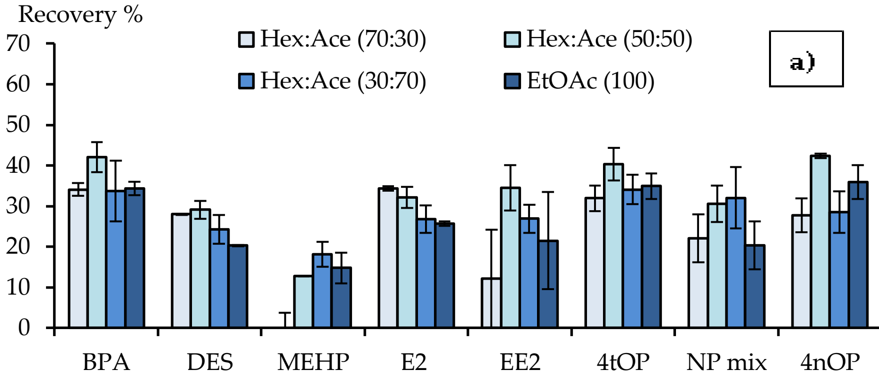

Figure 6.3: *Recoveries (%, n=3) obtained for* **a)** *fish liver and* **b)** *fish muscle when different extraction solvents were tested.*

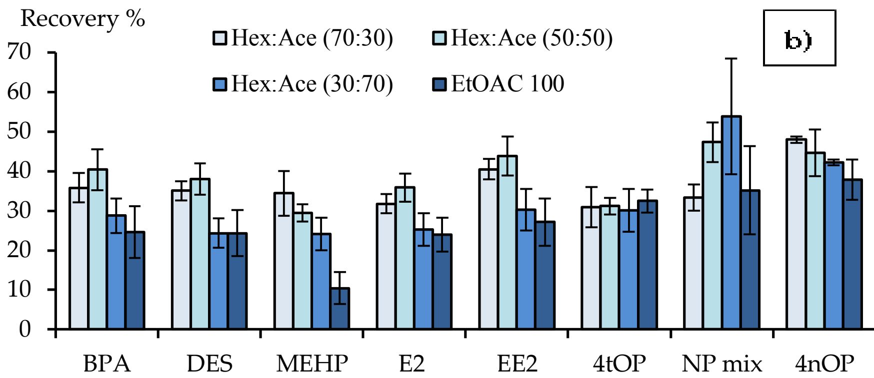

Figure 6.3: *Recoveries (%, n=3) obtained for* **a)** *fish liver and* **b)** *fish muscle when different extraction solvents were tested.*

In the case of fish muscle (see **Figure 6.3b**), a similar tendency was observed. In the case of DES and E2, a high percentage of Hex (higher than 50 %) showed the best recoveries. EtOAc seemed not to be a proper solvent for the extraction of MEHP from muscle, and no significant differences were observed for the rest of the analytes. Within the best two solvent mixtures (70:30 and 50:50 Hex:Ace, v/v) the one containing higher amounts of Ace was selected since the evaporation time is reduced and the repeatability was lower in general (< 13 %). Since the final selected extraction solvent was the same as the one used for the clean-up optimisation, a further evaluation of the clean-up protocol was not necessary at this method development stage. Different mixtures of Hex and Ace as extraction solvents were also reported in the literature for the extraction of several EDCs in fish tissue [38, 39] and mussels [40].

In the absence of a certified reference material (CRM) and in order to determinate whether exhaustive extraction was carried under optimised conditions, consecutive extractions were performed on the same samples using 10 mL of a clean solvent (Hex:Ace, 50:50, v/v). Recoveries lower than 10 % were obtained in the second extraction for both, fish liver and muscle matrices. Thus, only a single extraction was selected and carried out in further experiments.

3.3. Evaluation of the matrix effect

Matrix effect occurring at the LC-MS/MS stage was evaluated. Three samples were submitted to the whole analytical procedure and the extracts spiked at 1000 ng/mL before the LC-MS/MS analysis (5 μL were injected in all the cases) and compared with the analyte standards (in MeOH) spiked at the same concentration. Non spiked samples were also analysed and the signals obtained were subtracted to the spiked samples. The matrix effect was calculated as explained in **Equation 6.1**. A value close to 100 % indicates a lack of matrix effect.

$$Matrix\ Effect = \left(\frac{Signal\ of\ spiked\ sample}{Signal\ of\ standard\ solution}\right) \times 100$$

Equation 6.1

Although, slight or negligible detection matrix effect (69-111 %) was observed with the injection of 5 μL of liver extracts, after 10 consecutive analyses of these samples a decrease in the target analytes signals was observed. 3 μL as injection volume were also tested and no signal decreased was observed, in this case, after more than 10 consecutive injections. Thus, 3 μL injection volume was finally selected as optimum for liver extracts. No signal decrease was observed for the other two studied matrices when 5 μL were injected.

The results for the matrix effect are shown in **Tables 6.1, 6.2** and **6.3** for liver (3 μL), muscle (5 μL) and brain (5 μL), respectively. In general, no matrix effect in the detection was observed for any of the studied compounds and matrices with the exception of 4*t*OP in muscle (57 %) and NP mix in liver (72 %). However, the deuterated compound ([$^2H_4$]-NP) used for corrections in both cases showed the same tendency (67 % and 71 %, for muscle and liver, respectively) and the matrix effect in the detection step was satisfactorily corrected.

Table 6.1: *Data obtained (fish liver) for the method validation in terms of recovery (%, n=3), apparent recovery (%, n=3), relative standard deviation (RSD %, n=3), matrix effect (ME %, n=3), method detection limits (MDLs, ng/g) and concentration values (in ng/g) found in the analysed real samples.*

	Recovery (n=3) %		Apparent Rec. (n=3) %		Repeatability, RSD (%)		Matrix effect (%)	MDLs	Real Samples (ng/g, n=3)		
Analyte	*Low level 25 ng/g*	*High level 500 ng/g*	*Low level 25 ng/g*	*High level 500 ng/g*	*Low level 25 ng/g*	*High level 500 ng/g*	*(n=3) 1000 ng/mL*	*(α:99, n=7) ng/g 25 ng/g*	*Sample1*	*Sample2*	*Sample3*
4*n*OP	50	58	97[a]	101[a]	4[a]	7[a]	100 ± 7	26	< MDL	< MDL	< MDL
4*t*OP	92	67	104[a]	110[a]	19[a]	8[a]	99 ± 4	25	117 ± 6	672 ± 100	154 ± 24
NPmix	nc	42	nc	79[a]	nc	17[a]	100 6	11*	491 ± 72	629 ± 116	473 ± 94
BPA	124	41	116[b]	96[b]	15[b]	2[b]	93 ± 5	35	68 ± 11	97 ± 13	49 ± 12
DES	15	44	62[b]	105[b]	14[b]	2[b]	95 ± 2	10	< MDL	< MDL	< MDL
E2	34	64	128[a]	103[a]	12[a]	2[a]	90 ± 4	15	< MDL	< MDL	< MDL
EE2	34	64	132[a]	103[a]	16[a]	3[a]	72 ± 1	20	< MDL	< MDL	< MDL
MEHP	nc	4	nc	8	nc	16	91 ± 1	105*	171 ± 16	361 ± 85	160 ± 3

[a] corrected with [$^2H_3$]-E2 and [b] corrected with [$^2H_{16}$]-BPA. *values were calculated using the instrumental LOD values and expressed in terms of ng/g in sample. nc: non-calculated values.

Table 6.2: *Data obtained (fish muscle) for the method validation in terms of recovery (%, n=3), apparent recovery (%, n=3), relative standard deviation (RSD %, n=3), matrix effect (ME %, n=3), method detection limits (MDLs, ng/g) and concentration values (in ng/g) found in the analysed real samples.*

	Recovery (n=3) %		Apparent Rec. (n=3) %		Repeatability, RSD (%)		Matrix effect (%)	MDLs	Real Samples (ng/g, n=3)		
Analyte	*Low level 25 ng/g*	*High level 500 ng/g*	*Low level 25 ng/g*	*High level 500 ng/g*	*Low level 25 ng/g*	*High level 500 ng/g*	*(n=3) 1000 ng/mL*	*(α:99, n=7) ng/g 25 ng/g*	*Sample1*	*Sample2*	*Sample3*
4*n*OP	57	72	113[a]	126[a]	11[a]	3[a]	97 ± 1	9	< MDL	< MDL	< MDL
4*t*OP	49	54	90[a]	106[a]	11[a]	0.3[a]	88 ± 2	22	170 ± 9	209 ± 17	77±9
NP mix	nc	44	nc	70[a]	nc	4[a]	104 ± 5	11*	962 ± 202	761 ± 164	910 ± 195
BPA	42	54	132[b]	101[b]	7[b]	0.2[b]	85 ± 3	9	24 ± 4	20 ± 5	28 ± 2
DES	45	56	118[b]	105[b]	5[b]	2[b]	89 ± 1	5	< MDL	< MDL	< MDL
E2	51	60	129[c]	99[c]	3[c]	1[c]	57 ± 4	3	< MDL	< MDL	< MDL
EE2	55	57	96[c]	94[c]	12[c]	0.1[c]	112 ± 9	8	< MDL	< MDL	< MDL
MEHP	nc	38	nc	74[a]	nc	11[a]	87 ± 2	4*	73 ± 11	102 ± 17	75 ± 9

[a] corrected with [$^2H_4$]-NP , [b] corrected with [$^2H_{16}$]-BPA and [c] corrected with [$^2H_3$]-E2. *values were calculated using the instrumental LOD values and expressed in terms of ng/g in sample. nc: non-calculated values.

Table 6.3: *Data obtained (fish brain) for the method validation in terms of recovery (%, n=3), apparent recovery (%, n=3), relative standard deviation (RSD %, n=3), matrix effect (ME %, n=3), method detection limits (MDLs, ng/g) and concentration values (in ng/g) found in the analysed real samples.*

	Recovery (n=3) %		Apparent Rec. (n=3) %		Repeatability, RSD (%)		Matrix effect (%)	MDLs	Real Samples (ng/g, n=3)		
Analyte	*Low level 25 ng/g*	*High level 1000 ng/g*	*Low level 25 ng/g*	*High level 1000 ng/g*	*Low level 25 ng/g*	*High level 1000 ng/g*	*(n=3) 1000 ng/mL*	*(α:99, n=7) ng/g 25 ng/g*	*Sample1*	*Sample2*	*Sample3*
4*n*OP	37	48	70[a]	78[a]	12[a]	1[a]	108 ± 6	8	< MDL	< MDL	< MDL
4*t*OP	60	65	114[a]	106[a]	3[a]	2[a]	106 ± 2	3	10 ± 1	13 ± 2	11 ± 1
NP mix	nc	82	nc	89[a]	nc	36[a]	96 ± 16	53*	718 ± 42	816 ± 74	1115 ± 103
BPA	71	60	98[a]	97[a]	26[a]	4[a]	101 ± 2	23	46 ± 12	43 ± 12	31 ± 6
DES	31	57	66[a]	93[a]	11[a]	3[a]	104 ± 2	6	7 ± 1	8 ± 1	8 ± 2
E2	21	17	120[b]	103[b]	21[b]	1[b]	105 ± 2	20	< MDL	< MDL	< MDL
EE2	52	53	97[a]	86[a]	13[a]	2[a]	128 ± 9	10	< MDL	< MDL	< MDL
MEHP	nc	28	nc	70[c]	nc	21[c]	110 ± 3	21*	88 ± 13	76 ± 21	67 ± 9

[a] corrected with [$^2H_{16}$]-BPA, [b] corrected with [$^2H_3$]-E2 and [c] corrected with [$^2H_4$]-NP. *values were calculated using the instrumental LOD values and expressed in terms of ng/g in sample. nc: non-calculated values.

3.4. Performance of the analytical methods

Calibration linear range, recovery, apparent recovery, repeatability of the method in terms of relative standard deviation (RSD, %) and method detection limits (MDLs) were calculated in the present work in order to validate the developed procedures.

Calibration linear ranges in the range of 1-500 ng/mL for all the studied matrices and analytes, except in the case of NP mix and MEHP (10-1000 ng/mL) were used. All the calibration curves were linear ($r^2 > 0.999$) in the mentioned ranges.

Recovery and apparent recovery (after correcting the chromatographic signals with the corresponding deuterated analogue) were calculated at two different levels: 500 ng/g for liver and muscle and 1000 ng/g for brain (high level) and 25 ng/g for all the matrices and analytes (low level). Analyses were performed in triplicate and blank samples were always performed in parallel and considered for calculation. In the case of MEHP and NP mix, it was not possible to calculate the recovery and apparent recovery at the low level because those analytes were present in the matrix at high concentrations. Recovery was in the range of 15-124 % and 41-67 % for liver samples at low and high levels, respectively (see **Table 6.1**) for all the compounds except for MEHP (4 %) at the high level. As it has already been mentioned in section 3.1, the use of PSA to remove the lipids present in the liver rendered very low recoveries for MEHP. Regarding to the recovery obtained for muscle samples (see **Table 6.2**), similar values were obtained within the two levels (42-57 % and 38-72 %, for low and high levels, respectively). Finally, in the case of brain (see **Table 6.3**) recoveries in the range of 31-71 % and 28-82 % (low and high values, respectively) were obtained for all the analytes except for E2 (17-21 %).

In the case of liver, good apparent recoveries were obtained for all the studied analytes in the case of both levels (see **Table 6.1**) except for MEHP (8 %) at high level. Unfortunately, since any proper deuterated analogue was not available in our laboratory, it was decided to consider this value for the calculation of MEHP

real sample concentrations. Similar apparent recovery values have also been reported in the literature for BPA [41, 42] and APs [42] using ultrasound extraction and ASE methodologies. In the case of fish muscle (see **Table 6.2**), good apparent recoveries in the range of 90-129 % and 70-126 % for low and high concentration levels were obtained, respectively, for all the target analytes. Similar values were also reported in the literature for BPA [4, 30, 42], DES [43], APs [4, 28], hormones [28] and MEHP [44] using different extraction techniques such as ASE, ultrasound extraction, MAE or SPE. Finally, as observed in **Table 6.3**, good values were also obtained for fish brain matrix (66-120 % and 70-106 % for low and high levels, respectively). Similar values were reported for the extraction of BPA and APs for human brain [41].

Repeatability of the method was expressed as RSD at both levels (see **Tables 6.1, 6.2** and **6.3** for liver, muscle and brain, respectively). RSDs below 26 % were obtained for all the studied matrices and analytes. It has to be highlighted that the highest RSDs were obtained at the low level, but even though, acceptable values were achieved. Similar values were also observed in the literature for all the studied analytes in the present matrices using different methodologies [41-45].

Finally, MDL values were determined spiking the samples at 25 ng/g (n=7 for each matrix) and following the EPA guidelines for MDL calculations [46]. The signals obtained for blank samples were subtracted to the signals obtained for spiked samples. The MDL was then calculated as $MDL_{99} = t_{99,\,6} \times s$, where t = 3.71 corresponds to the Student's t-value for a 99 % confidence level and 6 degrees of freedom whereas s is the standard deviation of the replicate analyses. In the case of NP mix and MEHP, no MDL values were calculated because of the samples originally contained high concentration levels of the target analytes. Thus, MDL values were calculated using the instrumental LODs for these target analytes. For that purpose, the average signal of the lowest calibration level (10 ng/mL) plus 3 times the standard deviation was used to get the LOD value. In the case of MEHP, the low recovery value achieved in the case of liver was also considered for calculation. Low MDL values, in ng/g levels, were obtained for the three matrices (see **Table 6.1, 6.2** and **6.3** for liver, muscle and brain, respectively). However, it is

worth mentioning that the highest MDL values were obtained for liver, which is the highest lipid content matrix within the present studied matrices. As a consequence of the low recovery obtained for MEHP, the MDL value increases up to 105 ng/g. The values obtained here are higher than the MDL values reported by Lair et al. [44] for MEHP and Pojana et al. [40] and Jakimska et al. [6] for several EDCs, but it should be highlighted that they use a different MDL calculation protocol that is based on the S/N ratio.

3.5. Application to real samples

Grey mullet's (*Chelon labrosus*) liver, muscle and brain samples of Gernika from the Basque Coast were analysed in triplicate (see **Tables 6.1-6.3** for liver, muscle and brain, respectively). In general, 4*t*OP, NP mix, BPA and MEHP analytes were detected in all the fish and all the tissues whereas DES was only detected in brain tissue, even the values were very close to MDLs. The rest of the analytes concentrations were always below their MDLs.

Regarding the concentrations found for BPA, low ng/g concentration level was obtained for all the studied tissues. Similar concentrations were obtained for muscle (20-28 ng/g) and brain (31-46 ng/g) and the highest concentrations (47-97 ng/g) were found in liver. Lower concentrations were reported in this work comparing to the concentrations found in Iran [4]. Liu and co-workers [30] and Belfroid and co-workers [42] found similar concentrations of BPA in liver and muscle from China´s Crucian carp and bream and flounder from Netherlands. The same accumulation patterns were observed in different wild species (Rainbow trout, Crucian carp, Carp and Silvery minnow) [30, 42] and under laboratory conditions for Flounder and Bream [47]. To the best of our knowledge, no studies have been performed measuring the accumulation of the present target analytes simultaneously in liver, muscle and brain fish compartments. However, Nilsen et al. [48] analysed the distribution of several EDCs in fish tissue and reported that the highest concentrations were found in liver, followed by brain and muscle. In addition, the same tendency was also observed in humans according to Geens et al. [41].

NP mix was the most predominant AP found in the samples with high ng/g concentrations, whereas 4*t*OP was detected at low ng/g in brain and slightly higher ng/g values in liver and muscle. Lower concentrations were reported by Liu and co-workers in fish muscle collected in China for 4*t*OP [30]. Different accumulation patterns were observed for both APs. In the case of NP mix, the accumulation was as follows: liver > muscle > brain but on the case of 4*t*OP a different pattern was observed: brain > muscle > liver. Among the tissues studied in the present work, liver is the one presenting a central role in the fish metabolism and a major site for accumulation, biotransformation and excretion of the target compounds [49]. Thus, the highest concentrations should be expected in fish's liver.

MEHP was found at low ng/g in the studied 3 matrices. As it was pointed out before, the concentrations of MEHP presented here have been calculated considering the low apparent recovery (8 %) obtained for this analyte. The lowest concentrations were determined in brain (67-88 ng/g) and muscle (73-102 ng/g) whereas a double concentration was found in liver (160-361 ng/g). Once again, the highest concentrations were detected in liver which it could be related with compounds metabolisation, and in this case, the parent analyte (bis(2-ethylhexylphthalate), DEHP) is metabolised to its monoester form. Similar concentrations were also determined in fish tissue by Blair et al. [44] for fish caught in Canada. However, when bile samples from the same estuary were analysed, much higher concentrations were determined for MEHP (high ng/mL-ng/µL levels) [3, 33], since bile is the responsible for the transport of the target analytes before being excreted.

Finally, DES was only detected in brain samples at low ng/g level (7-8 ng/g), close to MDL values. No research works have been found where the accumulation of DES is measured in brain samples. However, Jiang and co-workers [43] found higher concentrations of DES (> 90 ng/g) in three different kind of fishes (cyprinoid, hairtail and herbivorous fish).

4. CONCLUSIONS

A new method was developed for the simultaneous analysis of several EDCs in different fish tissues (liver, muscle and brain). The use of FUSLE combined with dSPE provides good apparent recoveries for all the studied analytes except for MEHP in the case of liver. However, this contaminant is usually found at high concentrations in environmental samples and even the low recovery allowed the determination of MEHP in real samples. The obtained MDL values were at low ng/g level allowing the determination of the target EDCs in different tissues from fish captured in the estuary of Urdaibai, downstream a WWTP. In general, the liver was the tissue with the highest accumulation of contaminants followed by the brain and the muscle, almost at the same level. The analyte concentrations found in this work, together with other previously published works where the analysis of the target compounds in the same estuary was carried out [3, 31, 33, 50, 51], might indicate that the analysed contaminants have been present in the sampled area during a continuous period of time.

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Galindoko araztegiko ur-efluenteari esposatutako arrainen behazunean neurtutako disruptore endokrinoen biokontzentrazio-mailen determinazioa

1. SARRERA

Azken hamarkadetan, disruptore endokrinoen (EDCen) gaineko interesa piztu da, ingurumenean eta gizakiengan sor ditzaketen kalteak direla eta [1-3]. Ikerlariek, arrainak [4-7], moluskuak [8] eta anfibioak [9, 10] EDCetara esposatu dituztenean, intersexualitatea eta garapen- edo feminizazio-arazoak antzeman dituzte. EDCen artean, konposatu kimiko oso desberdinak topa daitezke, hormona naturalak zein sintetikoak, ftalato esterrak (PEak), alkilfenolak (APak), bisfenol-A (BPA), zenbait pestizida edo musketa fragantziak, adibidez. Konposatu horiek, ingurumeneko uretan oso kontzentrazio baxuetan egoten dira (ng/L mailan) orokorrean, eta iturririk nagusiena hondakin-ur araztegien irteera korronteak (efluenteak) dira, izan ere araztegiak ez dira gai konposatu guztiak arazteko [7, 11, 12].

Uretan ez bezala, arrainek duten metatze-ahalmena dela eta, arrainen atal desberdinetan EDCen kontzentrazio altuagoak (ng/mL edo ng/g) aurkitu dira [1, 6, 13-15]. Zentzu horretan, arrainaren behazuna eta gibela dira metatze-ahalmen handiena erakusten duten fluidoak/organoak [6, 16, 17]. Behazunaren eta gibelaren artean, behazuna nahiago izaten da analisi kimikoak egiteko, gibelak baino koipe gutxiago baitu [13, 18]. Hala ere, behazuna aztertzen duten metodo gutxi garatu dira [3, 6, 17], eta are gutxiago, biokontzentrazio-faktorea (BCF) determinatzen dutenak [1, 14]. BCF balioak arrain espeziearen arabera alda daitezke baita arrainen elikadura, mugimendu-ohiturak eta kate trofikoaren mailaren arabera ere [19]. Espezie berdinaren artean ere, BCF desberdinak kalkulatu dira arrainen tamaina, sexua eta ugalketa-egoeraren arabera [20]. Askotan, bibliografian aipatu diren BCF balioak behazunaren kasuan 5.000 (edo log BCF > 3,7) baino handiagoak dira [1, 13, 14], EDCak behazunean metatzeko gaitasun handia erakutsiz [21].

Arrainek eta muskuiluek daukaten metaketa-ahalmena, material polimerikoekin alderatzea izan da azken urteetan ikerlari askoren ekimena [2, 22]. Konparaketa horrek, erantzun positiboa izanez gero, ingurumenaren egoera determinatzeko arrainak eta muskuiluak erabili beharrean, material polimerikoak erabili ahalko ziren. Modu horretan, arrainak eta muskuiluak ez lirateke hil behar,

kutsatzaileen metatze-ahalmena eta maila determinatzeko edota espezie horiek ez lirateke jaso behar halako informaziorik lortzeko. Orain arte mintz erdi-iragazkorreko gailuak (SPMD) izan dira biomonitorizazio programetan gehien erabili direnak [22, 23] baina azken urteetan konposatu organiko polarren lagin-bilketa integraleko (POCIS) sistemak ere erabili dira arrainen eta muskuiluen metaketa-ahalmena simulatzeko [2].

Analisiari dagokionez, azken urteetan kimika berdearen ikuspuntutik mikroerauzketan oinarritzen diren metodoek garrantzia hartu dute. Bibliografian aurkitzen diren metodo gehienek konposatu organikoak ur-laginetan neurtzeko garatu dira [24-26]. Hala ere, badira konposatu organikoak arrainen behazunean aztertzeko mikroerauzketan oinarritzen diren metodoak; hala nola, fase solidoko mikroerauzketan (SPMEn) oinarritzen direnak [15] edo material polimeriko desberdinak erabiltzen dituztenak [27]. Polietersulfona (PES) polimeroak emaitza oparoak eskaini ditu log K_{ow} desberdina duten hainbat konposatu aldi-berean uretatik erauzteko [24, 28]. Horretaz gain, komertzialki saltzen diren mikroerauzketa-gailuen aldean merkeak dira, eta horrek jatorriz lagin-bilketa batek duen kostua murrizten laguntzen du. Guk dakigula, PES polimeroa ez da inoiz biomonitorizazio tresna moduan erabili.

Testuinguru horretan, Bizkaian dagoen Galindo (Espainiako iparraldean) araztegiko efluenteko urak erabiliz, heldugabeko lazunen (*Chelon labrosus*) behazunaren metaketa-ahalmena aztertu zen 11 EDCrentzat: 2 musketa fragantzia (galaxolide (HHCB) eta tonalide (AHTN)), 2 hormona (17 β-estradiola (E2) eta 17 α-etinilestradiola (EE2)), BPA, 2 PE (*n*-butilbentzil ftalatoa (BBP) eta bis(2-etilhexil) ftalatoa (DEHP)) eta 3 AP (4-*n*-oktilfenola (4*n*OP), 4-*tert*-oktilfenola (4*t*OP) eta nonilfenol nahaste teknikoa (NP mix)). Arrainak 10 egunez esposatu ziren araztegiko uren diluzio desberdinetara itsasoko ura eta araztegiko efluenteko ura erabiliz. Horrez gain, PES-tutuak eta mintzean gordetako geruza adsorbatzailea-polietersulfona (M-PES) ere, arrainekin batera eduki ziren ontzietan, horien metaketa-ahalmena arrainen behazunaren metaketa-ahalmenarekin alderatzeko.

2. MATERIALAK ETA METODOAK

2.1. Erreaktiboak eta materialak

Lan honetan erabili diren erreaktiboen ezaugarri kimikoak **I. eranskinean** laburbildu dira. Analito guztiak metanoletan prestatu ziren 1.000-4.000 ng/µL kontzentrazio-tarteetan (HPLC kalitatea, LabScan, Dublin, Irlanda). 100 ng/µL-ko disoluzioak hilero prestatu ziren eta 1-10 ng/µL tartekoak egunero. Prestatutako disoluzio guztiak anbarrezko ontzitan gorde ziren -20 °C-an.

Azetona (Ace), metanola (MeOH), azetonitriloa (ACN) eta *n*-hexanoa (Hex) Sigma Aldrich etxe komertzialak (Steinheim, Alemania) hornitu zituen eta etil azetatoa (EtOAc), Labscan etxeak (Dublin, Irlanda). Erreaktibo guztiak HPLC kalitatekoak ziren. Azido azetikoa (HAc), aldiz, (% 99,7) Merck etxeak hornitu zuen (Darmstadt, Alemania). Deribatizazioan erabilitako N,O-Bis (trimetilsilil)trifluoroazetamida 1% trimetil-klorosilanoarekin (BSTFA 1% TMCS, SilonBFT, 99:1) eta piridina (Pyr) Supelco eta Alfa Aesar (Karlsruhe, Alemania) etxe komertzialetatik lortu ziren, hurrenez hurren.

Behazun-laginen hidrolisia egiteko erabilitako disoluzio indargetzailea (0,1 mol/L, pH=6) prestatzeko potasio di-hidrogenofosfatoa (KH_2PO_4, % 100, Panreac) eta amonio hidrogenofosfatoa ($(NH_4)_2HPO_4$, % 99, Merck, Darmstadt, Alemania) erabili ziren. Etil-4-aminobentzoato gatza (% 98, bentzokaina) Fluka etxe komertzialari (Steinheim, Alemania) erosi zitzaion. Entzimei dagokienez, β–glukuronidasa VII-A motatakoa (*Escherichia Coli*, 4.974,48 U), β-glukosidasa (almendra, 102,8 U) eta sulfatasa (aerobacter aerogenes, 12,25 U) (Sigma-Aldrich, Steinheim, Alemania) Milli-Q uretan (Milli-Q ur purifikazio sistema < 0,057 µS/cm, Millipore, Bedford, MA, EEBB) disolbatu ziren, eta 250 µL-ko alikuotetan banatu ostean -20 °C-an gorde ziren.

Analitoen erauzketa ur-laginetan, tutu formako PES-hodiak (0,7 mm-ko kanpo diametroa, 1,43 g/mL dentsitatea), Membrana (Wupertal, Alemania) etxean erosi ziren. 1,5 cm luzerako zatiak moztu eta etil azetatotan garbitu ziren 24 orduz. Azkenik, PES-tutuak erabili aurretik, etil azetatotik atera eta paper garbi batekin

lehortu ziren.

Behazun-laginen fase solidoko erauzketa (SPE) egiteko erabilitako Bond-Elute Plexa (dibinilentzeno estireno hidrofobikoa) kartutxoak eta SPE garbiketa egiteko Florisil kartutxoak, Agilent etxeari (Agilent technologies, Avondale, PA, USA) eta Sigma-Aldrich etxeari erosi zitzaizkien, hurrenez hurren.

2.2. Arrainen esposizio esperimentuak

Araztegiko efluenteko urak Galindo araztegitik jaso ziren 2013. urteko otsailean. Galindoko araztegiak 350.000 m^3/eguneko lan-karga dauka eta Bilbo eskualdeko (~ 1.000.000 biztanleria) urak jaso eta arazten ditu. Irteerako urak 48 orduro (~1.000 litro) jaso ziren eta laborategira plastikozko ontzietan garraiatu ziren. Behin laborategian, araztegiko urak, 0,22 µm-ko iragazkitik iragazitako itsasoko urarekin diluitu ziren 75:25 (efluente:itsasoko ura, b/b, % 25 diluzioa), 50:50 (b/b, % 50 diluzioa) eta 25:75 (b/b, % 75 diluzioa) diluzioetara. Diluzio horiek, 60 L-ko ontzietara (polietilenozko ontziak) pasa ziren. Aipatutako efluenteko ur:itsasoko ur-diluzioez gain, kontroleko ontzi bat ere erabili zen itsasoko ura erabiliz. Ontzi guztietan gazitasuna konstante mantentzeko, itsasoko ura beti kopuru berdinean mantendu zen, eta araztegiko uraren doiketa ur gezarekin egin zen gazitasuna ‰ 10-an doitzeko.

Esperimentuan zehar erabilitako heldugabeko lazunak (*Chelon labrosus*) CIFP Maritino Zaporito institutoan (San Fernando, Cadiz) erosi ziren 2013. urteko urtarrilean. Arrainak itsasoko uretan mantendu ziren astebetez, laborategi girora moldatzeko. Aste horretan zehar uraren gazitasuna ‰ 10-an doitu zen, hori baita inguruko estuarioko urak gainazalean duen gazitasun-maila [29]. 20 arrain sartu ziren ontzi bakoitzean baldintza semi-estatikoetan. 10 arrain 3. egunean jaso ziren eta gainontzekoak, 10. egunean. Arrainei egunero jaten eman zitzaien ur-aldaketa baino bi ordu lehenago (ur guztiaren % 80-an berritzen zen egunero). 3. eta 10. egunetan, aldiz, arrainak 24 orduz eduki ziren baraualdian.

Supermerkatu baten erositako teontzietan 4 PES-tutu (7,5 cm, 7,3 µL) eta 4 M-PES (7,5 cm, 7,3 µL) sartu ziren, PES-tutuak eta M-PESak arrainengatik

babesteko. Teontzi bana arrainen ontzi berdinean eta kontrol-ontzian sartu ziren arrain guztiek estres-maila berdina izateko. 3. eta 10. egunean arrainak jasotzeko une berean teontziak ere jaso ziren. Teontzi bakoitzetik 2 PES-tutu eta 2 M-PES atera eta teontziak berriro ontzira sartu ziren gainontzeko arrainekin batera.

Behin laginak jasota, lazunak, disekzionatu aurretik, bentzokaina disoluzio asetuan sartu ziren anestesiatzeko[30]. Xiringa baten laguntzaz bilisa esterilizatutako ontzietan (Corning Incorporated, Mexiko) jaso zen (~1 mL) eta nitrogeno likidotan gorde zen laborategira heldu arte. Bertan, -80 °C-an gorde zen analisia egin arte.

2.3. Laginen tratamendua eta analisia

2.3.1. Ur-laginen azterketa

Ur-laginak, lan honen 3. kapituluan azaldu bezala aztertu ziren. Laburki, 150 mL ur (n=3) 0,45 μm-ko iragazkietatik iragazi ziren eta ez zitzaion inolako gatzik gehitu itsasoko urak jatorriz duen gazitasuna dela eta. Ontziari, ur-laginaz gain, aurretiaz EtOActan garbitutako 1,5 cm-ko 5 PES-tutu, PTFEz estalitako irabiagailua eta analitoen trazagarriak (([$^{2}H_{4}$]-4*n*-nonilfenola ([$^{2}H_{4}$]-NP), [$^{2}H_{3}$]-17 β-estradiola ([$^{2}H_{3}$]-E2), [$^{2}H_{16}$]-bisfenol-A ([$^{2}H_{16}$]-BPA), [$^{2}H_{4}$]-bis(2-etilhexil ftalatoa ([$^{2}H_{4}$]-DEHP) eta [$^{2}H_{15}$]-musk xilenoa ([$^{2}H_{15}$]-MX)) zituen disoluzioa gehitu zitzaion 1 ng/mL-ko kontzentrazioa izan arte. Erauzketarekin hasteko, ontziak itxi eta inguruko tenperaturan, laginak 1.200 bira/min-ko abiaduran (15 posiziotako plaka birakari magnetikoa, Gerstel, Mulheim an der Ruhr, Alemania) irabiatzen utzi ziren gau osoan zehar. Erauzketa-urratsa amaitu ostean, PES-tutuak atera, gatz arrastoak kentzeko Milli-Q uraz garbitu eta, azkenik, eskuzapi garbi batez lehortu ziren. PES-tutuak, eppendorf hodi baten sartu eta 300 μL EtOAc erabiliz, analitoak ultrasoinu-bainuan (USB Axtor, Lovango) erauzi ziren. Teontzietatik jasotako PES-tutuak eta M-PES tutuak, ur garbiarekin ondo garbitu eta 1,5 cm zituzten 5 zati berdinetan moztu ziren, eppendorf hodi batean sartu eta 300 μL etil azetatorekin analitoak erauzi ziren uren analisian egindako urrats bera jarraituz.

Analitorik ez-polarrenak, hau da, deribatizazio urratsik jasan behar ez duten

analitoak (musketa fragantziak eta PEak), bolumen handiko injekzioa-tenperatura programatutako baporizatzailea gas-kromatografia–masa espektrometriaren (LVI-PTV-GC-MS-aren) bidez neurtu ziren. Analitorik polarrenak, aldiz (BPA, hormonak eta APak), gas-kromatografia–masa-espektrometriaren (GC-MS-aren) bidez deribatizazio-urrats baten ondoren. Deribatizazio-urratsa egiteko, aurreko erauziak lehorreraino lurrundu ziren N_2 korronte baten pean (Turbovap® LV, Caliper, 176 life sciences, USA) eta 125 µL piridina eta 50 µL BSTFArekin berreratu ziren. Ondoren, disoluzio horiek 45 minutuz berotu ziren 65 °C-an, analitoen deribatizazioa gerta zedin.

Musketa fragantziak eta PEak LVI-PTV-GC-MS-aren bidez determinatu ziren 6890N gas-kromatografoari (Agilent Technologies) lotutako bolumen handiko injekzio (LVI) sistemaren bidez eta 5975N elektroien talkaren bidezko ionizazio-iturria duen masa-espektrometroaren bidez. 100 µL-ko xiringa bat erabiliz, 40 µL lagin injektatu ziren injekzio hotzeko sistema (CIS) batean (N_2 likidoa erabiltzen da sistema hozteko). LVI-PTV analisiaren parametroak sakonki optimizatu ziren beste lan baten [31]. Laburki azalduz, laginak 50 °C-an injektatu ziren eta purga-balbula 3 minutuz eduki zen zabalik 75 mL/min fluxua eta 2,9 psi-ko purga presioan. Horrela, splitless moduan (1,5 minutuz) analitoen fokatzea faboratu zen zutabearen burugunean. PTVaren injekzio portuko tenperatura 12 °C/s-ko abiaduran igo zen 300 °C-raino. Azkenik, atorra 50 mL/min fluxuarekin garbitu zen. Gas-kromatografia banaketa metodoari dagokionez, labearen hasierako tenperatura 60 °C-an mantendu zen minutu batez. Ondoren, tenperatura 170 °C-ra igo zen zen 10 °C/min-ko abiaduran eta gero 250 °C-ra 150 °C/min-ko abiadura, bertan 2 minutuz Tª hori mantendu zen. Azkenik, labea 300 °C-ra igo zen 15 °C/min-ko abiaduran eta tenperatura horretan 3 minutuz mantendu zen.

APen, BPAren eta hormonen 2 µL lagin 6890N kromatografoari (Agilent Technologies) lotutako 5973N elektroi-ionizazio masa-espektrometroan injektatu ziren. Analisia splitless injekzio sisteman 1,5 minutuz egin zen 300 °C-an. Analitoen bereizketarako labea 60 °C-an eduki zen 1,5 minutuz eta 170 °C-ra igo zen 10 °C/min abiaduran. Azkenik, 15 °C/min-ko abiaduran 300 °C-ra igo zen 5 minutuz mantentzeko.

GC-MS metodo bietan analitoen banaketa HP-5MS zutabe kapilarrean (30 m × 0,25 mm, 0,25 µm, Agilent technologies) egin zen eta erabilitako gas garraiatzaile H_2 (Hydrogen generator AD-1020, 99,9995 %, Cinel Strumenti Scientifici, Padova, Italia) izan zen bi kasuetan 1,3 mL/min fluxuan. MS transferentzia-lerroa, ionizazio-iturria eta kuadrupoloa 310 °C-an, 230 °C-an eta 150 °C-an mantendu ziren, hurrenez hurren. Neurketa guztiak scan (50-525 m/z) eta hautatutako ioi-monitorizazio (SIM) neurketa moduetan egin ziren. Neurketa bietan jarraitutako ioiak **II. eranskinean** daude laburbilduta.

2.3.2. Arrain-behazunaren azterketa

Lagin likidoak lan honen 4. kapituluan aipatu bezala aztertu ziren. Laburki azalduz, 100 µL arrain-behazuni (n=3) 1,5 mL fosfato disoluzio indargetzaile (0,1 mol/L, pH=6), 800 µL Milli-Q ura eta dagozkion entzimen 200 µL gehitu zitzaizkien (1.000 U/mL β-glukuronidasa, 2 U/mL sulfatasa eta 20 U/mL β-glukosidasa) hidrolisia aurrera eramateko Gibson eta lankideek [32] proposatu bezala. Hidrolisia egin aurretik, analito deuteratuen ([$^2H_4$]-DEHP, [$^2H_{15}$]-MX, [$^2H_4$]-NP, [$^2H_4$]-BPA eta [$^2H_3$]-E2) disoluzio-nahastearen 100 µL gehitu zen eta hidrolisia 37 °C-an egin zen 12 orduz. Hidrolisia gelditzeko, laginei 300 µL HAc eta 2 mL Milli-Q ur gehitu zitzaizkien [33]. Behin hidrolisia amaituta, laginak aurretiaz 2 mL MeOH eta 2 mL Milli-Q:HAc (99:1, b/b) nahastearekin egokitutako Plexa kartutxoetatik pasarazi ziren. Ondoren, kartutxoa 2 mL Milli-Q:MeOH (95:5, b/b) nahastearekin garbitu zen. Analitoak eluitzeko 4 mL EtOAc pasarazi ziren kartutxotik eta lortutako erauzia ~1 mL izateraino lurrundu zen N2 korronte baten pean. Garbiketa urratserako, 5 mL hexanorekin egokitutako 1 gramotako Florisil kartutxoak erabili ziren. Eluzio urratserako, 3 mL Hex:EtOAc (90:10, b/b) eta 3 mL EtOAc:MeOH (25:75, b/b) erabili ziren. Lehenengo frakzioa (APak, musketa fragantziak eta PEak) 175 µL hexanotan berreratu zen eta GC-MS-aren bidez aztertu. Gainontzeko frakzio erdia, bigarren frakzioarekin batera (BPA eta hormonak) 150 µL ACN:Pyr (50:50, b/b) eta 50 µL of BSTFA + 1 % TMCS erabiliz deribatizatu zen 45 minutuz 65 °C-an. Lagin horiek, GC-MS-aren bidez aztertu ziren.

Behazun laginen bi frakzioak 2 injekzio desberdinekin neurtu ziren 6890N kromatografoari (Agilent Technologies) lotutako 5973N elektroi-talkaren bidezko ionizazio duen masa-espektrometroan. 2 frakzioetan analitoen banaketa HP-5MS zutabe kapilarrean (30 m × 0.25 mm, 0.25 μm, Agilent technologies) egin zen eta erabilitako gas garraiatzaile H_2 izan zen 1,3 mL/min-ko fluxuan. MS transferentzia -lerroa, ionizazio-iturria eta kuadrupoloak 310 °C-an, 230 °C-an eta 150 °C-an mantendu ziren, hurrenez hurren. Neurketa guztiak scan (50-525 m/z) eta hautatutako ioi-monitorizazioan (SIM) egin ziren, eta monitorizatutako ioiak **II. eranskinean** daude laburbilduta.

Analitoen bereizketa egiteko labean 2 metodo desberdin erabili ziren. 1. frakzioaren 2 μL hurrengo metodo kromatografikoa erabiliz banatu ziren: labea 60 °C-an mantendu zen minutu batez, eta 170 °C-raino igo zen 10 °C/min-ko abiaduran. Jarraian 15 °C/min-ko abiaduran 250 °C-raino igo zen eta 2 minutuz mantendu, azken igoera bat izateko 300 °C-ra 15 °C/min abiaduran (3 min mantendu). 2. frakzioa, aldiz, hurrengo metodoaren bidez neurtu zen: labea 60 °C-an mantendu zen 1,5 minutuz eta 170 °C-raino igo zen 10 °C/min abiaduran. Jarraian 15 °C/min abiadurarekin 200 °C-raino igo eta 5 °C/min abiadurarekin 255 °C-raino. 15 °C/min abiaduran 285 °C graduan mantendu zen 5 minutuz, eta azkenik 300 °C-ra berotu zen labea 15 °C/min abiaduran. Metodoa amaitzeko, tenperatura horretan mantendu zen 5 minutuz.

2.4. Metodoaren kalitate-parametroak

Ur- eta behazun-laginak neurtzeko erabilitako metodoen kalitate-parametroak jarraian agertzen dira laburbildurik. % 78-117 (% RSD 1-21) eta % 63-122 (% RSD 1-27) izan ziren lorturiko berreskurapen zuzenduak ur- eta behazun-laginentzako, hurrenez hurren. Detekzio-mugei (LOD) dagokienez, 2-76 ng/L eta 1-313 ng/mL izan ziren ur-lagin eta behazun-laginentzako, hurrenez-hurren. Ur-laginen analisiari dagozkien kalitate-parametroak lan honen 3. kapituluan daude laburbildurik, eta behazun-laginei dagozkienak 4. kapituluan.

Laginen neurketetan zehar, zuri-laginak (Milli-Q) eta kontrol-laginak (kontzentrazio jakin batera dopatutako laginak) neurtu ziren. Zurien kasuan, LOD balioak baino txikiagoak lortu ziren, eta berreskurapen zuzenduak, aldiz, kalitate-parametro egokien tartean egon ziren.

2.5. Biokontzentrazio balioen estimazioa

BCF-faktorea, konposatu jakin batek behazunean duen kontzentrazioa eta uretan duen kontzentrazioaren arteko erlazioa bezala definitzen da (ikus **7.1 ekuazioa**). BCF-balioak 2.1 atalean azaldutako 3 diluzioentzako kalkulatu ziren. BCFak kalkulatzeko, lehenengo 3 egunetan neurtutako ur-kontzentrazioen batezbesteko balioak erabili ziren lehenengo bildu ziren arrain multzoarentzako (3. eguneko lagin-bilketa), eta 10 egunetan zehar neurtutako ur-kontzentrazioen batezbestekoa gainontzeko arrainentzako (10. eguneko lagin-bilketa). Uretan inolako analitorik neurtu ez zenean, baina bai behazun-laginetan, ur-laginen LOD balioa erabili zen BCFa kalkulatzeko.

$$\mathrm{BCF} = \frac{\mathrm{EDCen\ kontzentrazioa\ behazunean}\ \left(\frac{\mathrm{ng}}{\mathrm{mL}}\right)}{\mathrm{EDCen\ kontzentrazioa\ uretan}\ \left(\frac{\mathrm{ng}}{\mathrm{mL}}\right)}$$

7.1 ekuazioa

Arrainen behazunarekin egin bezala, PES-tutuetan BCF balioak kalkulatu ziren. Kasu honetan, PES-tutuetan ezin da "bio" terminoa aplikatu, eta ondorioz kontzentrazio-faktore (CF) bezala definitu behar da terminoa, eta **7.2 ekuazioan** laburbilduta dagoen bezala kalkulatzen da.

$$\mathrm{CF} = \frac{\mathrm{EDCen\ kontzentrazioa\ PES - tutuan}\ \left(\frac{\mathrm{ng}}{\mu\mathrm{L}}\right)}{\mathrm{EDCen\ kontzentrazioa\ uretan}\ \left(\frac{\mathrm{ng}}{\mu\mathrm{L}}\right)}$$

7.2 ekuazioa

3. EMAITZAK ETA EZTABAIDA

3.1. EDCen metaketa

Esposizio-esperimentuak iraun bitartean, ur-laginak 48 orduan behin aztertu ziren, ur-laginak esposizio tankean gehitu aurretik eta esposizioa bukatuta (48 h-ra) (ikus 2.2 atala). Orokorrean, ng/L-ng/mL mailako kontzentrazioak antzeman ziren (ikus **7.1 taula**) konposatu gehienentzako. Ez zen inolako igoera/jaitsiera nabarmenik antzeman analitoen kontzentrazioetan egunen artean. Salbuespena BPA izan zen, kontzentrazioen igoera detektatu baitzen esperimentua aurrera zihoan heinean. Kontroleko laginei dagokienez (itsasoko ura:ur geza) konposatu gehienak LOD balioetatik behera egon ziren NP mix eta BParen salbuespenarekin, LODtik gertu egon baitziren.

7.1 taula: *Araztegiko efluenteko ura:itsasoko ura nahaste desberdinetan (25:75 efluenteko ura:itsasoko ura (% 75eko diluzioa), 50:50 efluenteko ura:itsasoko ura (% 50eko diluzioa), 75:25 efluenteko ura:itsasoko ura (% 25eko diluzioa)) neurtutako analitoen kontzentrazioaren batazbestekoa, 3. eta 10. egunetan. Ur-laginen analisien desbideratze estandar erlatiboak % 1-21 bitartekoak dira.*

Analitoa	3. eguna			10. eguna		
	% 75eko diluzioa	% 50eko diluzioa	% 25eko diluzioa	% 75eko diluzioa	% 50eko diluzioa	% 25eko diluzioa
4*t*OP	< 12	< 12	< 12	< 12	< 12	< 12
4*n*OP	< 15	< 15	< 15	< 15	< 15	< 15
NP mix	29	57	85	28	55	83
E2	< 2	< 2	< 2	< 2	< 2	< 2
EE2	< 25	< 25	< 25	< 25	< 25	< 25
BPA	340	681	1.021	751	1.501	2.252
HHCB	295	589	884	345	689	1.034
AHTN	14	28	42	20	41	61
BBP	169	337	506	139	277	416
DEHP	1.452	2.905	4.359	1.331	2.664	3.996

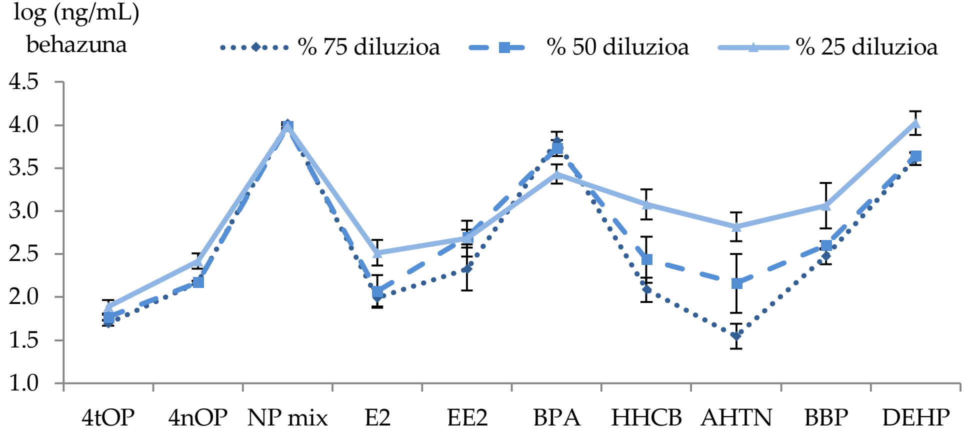
Behazun-laginei dagokienez, lan honetan azterturiko konposatu guztiak gutxienez ng/mL mailan antzeman ziren (ikus **7.2 taula**). Alde batetik, AHTN konposatua izan zen LODtik gertu antzeman zen konposatu bakarra (3. eguna, % 75 diluzioan) eta, beste alde batetik, uretan detektatu ez ziren hormonak (ikus

7.1 taula) behazun-laginetan antzeman ziren, literaturan askotan aipatu den behazunak duen metatze-gaitasuna baieztatuz [1, 6, 13, 19]. Esperimentuak iraun bitartean arrainen kontrol-laginak ere aztertu ziren (n=3) eta lortutako kontzentrazioak **7.2 taulan** daude laburbilduta. Aipatu beharra dago kontroleko uretan konposatuak LOD azpitik egonik, kontroleko arrainen behazunean antzeman diren kontzentrazioak altuak direla. Antzemandako konposatuen jatorria ezin izan da ziurtatu. EE2, HHCB, AHTN eta BBP konposatuen kasuan, kontroleko arrainetan neurtu ziren kontzentrazioak ontzi diluituenean (25:75 efluente:itsasoko ura) neurtutakoen parekoak edo handiagoak izan ziren. Toxikologian, Hormesis fenomenoa konposatuen dosiarekin erlazionatzen da [34, 35]. Zehatzago esanda, dosi baxuetan konposatuen kanporatzea estimulatzen da, eta aldiz, dosi altuetan kanporatze-fenomenoa inhibituta geratzen da. Hormesis fenomenoa izan liteke kontzentrazio baxuetara esposatutako arrainek kontrolekoek baino kontzentrazio baxuagoa izateko arrazoia.

7.2 taula: *araztegiko irteerako ura:itsasoko ura nahaste desberdinetara (25:75 efluenteko ura:itsasoko ura (% 75eko diluzioa), 50:50 efluenteko ura:itsasoko ura (% 50eko diluzioa), 75:25 efluenteko ura:itsasoko ura (% 25eko diluzioa)) esposatutako arrainen behazunean neurtutako analitoen kontzentrazioaren batazbestekoa, 3. eta 10. egunetan.*

Analitoa	Kontrola	3. eguna			10. eguna		
		% 75eko diluzioa	% 50eko diluzioa	% 25eko diluzioa	% 75eko diluzioa	% 50eko diluzioa	% 25eko diluzioa
4*t*OP	28 ± 2	50 ± 3	59 ± 4	70 ± 3	76 ± 6	79 ± 16	113 ± 5
4*n*OP	87 ± 5	148 ± 7	148 ± 8	293 ± 53	278 ± 2	292 ± 31	314 ± 6
NP mix	4.925± 199	10.343 ± 451	9.741 ± 394	9.734 ± 775	13.180 ± 743	11.132 ± 1849	14.287 ± 1130
E2	97 ± 29	100 ± 24	149 ± 23	339 ± 114	320 ± 7	389 ± 114	131 ± 40
EE2	266 ± 60	212 ± 20	443 ± 12	634 ± 2	419 ± 36	713 ± 89	297 ± 1
BPA	1.874 ± 244	6.647 ± 1619	4.833 ± 630	2.326 ± 118	2.762 ± 421	2.090 ± 183	4.471 ± 225
HHCB	291 ± 76	122 ± 3	390 ± 101	1.003 ± 284	539 ± 12	946 ± 102	1.419 ± 400
AHTN	142 ± 101	29 ± 1	168 ± 120	816 ± 147	139 ± 5	433 ± 47	682 ± 123
BBP	251 ± 7	268 ± 28	403 ± 43	820 ± 96	1.198 ± 21	421 ± 123	1.788 ± 208
DEHP	1.212 ± 125	4.118 ± 664	4.409 ± 337	12.687 ± 418	10.421 ± 1046	3.710 ± 1148	10.896 ± 350

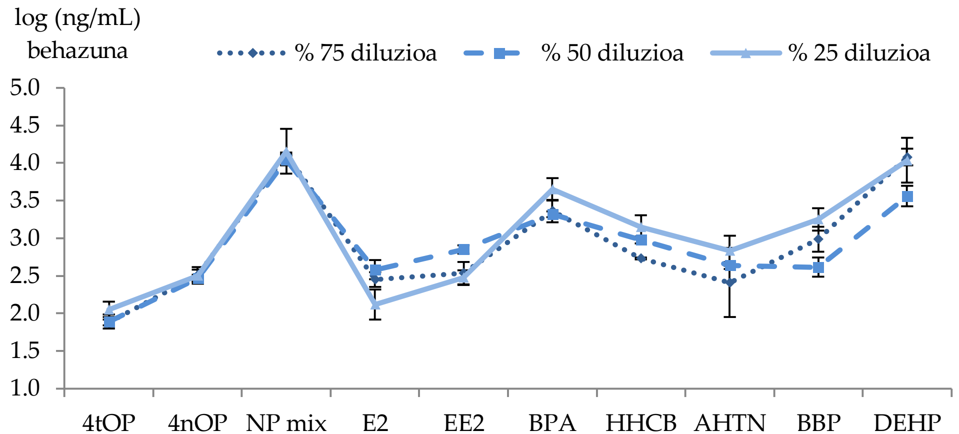
Diluzio desberdinetara esposatutako arrainen behazunean neurtutako kontzentrazioak (ng/mL) **7.1a** eta **7.1b irudietan** daude irudikatuta eskala logaritmikoan 3. eta 10. egunetarako, hurrenez hurren. 3. egunean (ikus **7.1a irudia**) lortutako kontzentrazioak arretaz behatzen badira, esan daiteke 4*t*OP, NP mix eta EE2 konposatuen kasuan aztertutako 3 diluzioetan pareko kontzentrazioak antzeman zirela, beraz, pentsa daiteke konposatu horien metaketa behazunean ez dela uretan dagoen kontzentrazioaren menpekoa. AHTNren kasuan (ikus **7.1a irudia**), aldiz, behazunean neurtutako konposatuen kontzentrazioa uretan dagoen kontzentrazioarekin zuzenki proportzionala da. Gainontzeko analitoen kasuan ez zen desberdintasunik ikusi (ikus **7.1a irudia)** bi ontzi diluituenetan, hau da, % 50eko eta % 75eko diluzioetan, baina bai ordea, konposatuak maila altuenean zeuden ontzian. Ontzi horretan neurtutako behazunetan, beste bi ontzietakoetan baino kontzentrazio handiagoak antzeman ziren 4*n*OP, E2, HHCB, BBP eta DEHP analitoen kasuan. Aldiz, kontrako joera ikusi zen BPAren kasuan, non analito gehiago zeuden ontzian, kontzentraziorik baxuena ikusi zen behazunean. Wu eta lankideek [19] antzeko konportamendua ikusi zuten 30 egunez BPAren kontzentrazio desberdinetara (0,1-1.000 µg/L) esposatutako heldugabeko zamo arruntetan (*Cyprinus carpio*) laborategi-baldintzetan.



7.1a irudia: *3. egunean neurtutako konposatuen log (ng/mL) balioak disoluzio desberdinetarako.*

10. egunean jasotako arrainen kasuan (ikus **7.1b irudia**) AP (4*t*OP, 4*n*OP eta NP mix), BPA eta HHCB molekulen kasuan ez zen inolako desberdintasunik ikusi diluzioen artean, beraz, kasu hauetan ere, pentsa daiteke behazunean dagoen kontzentrazioak ez duela uretan dagoen analitoen kontzentrazioarekiko menpekotasunik. Gainontzeko konposatuentzako tendentzia desberdinak ikusi ziren. Alde batetik, HHCB konposatuak ontzirik diluituenean (% 75eko diluzioa) jasotako behazunetan kontzentraziorik baxuena neurtu zen eta gainontzeko bi diluzioetan ez zen desberdintasunik ikusi. E2 konposatuak kontrako joera erakutsi zuen non ontzirik kontzentratuenean (% 25eko diluzioa) konposatu gutxien metatu zen behazunean. Azkenik, EE2 hormonaren kontzentraziorik altuena % 50eko ontzian antzeman zen, eta kontrara, 2 PEen kontzentraziorik baxuena ontzi berdineko arrainetan kuantifikatu zen.

Orokorrean 4*t*OP eta NP mix izan ziren 3. eta 10. egunean tendentzia berdina erakutsi zuten analitoak. Bi kasuetan, ez zen inolako desberdintasunik antzeman diluzioen artean ere, beraz, pentsa daiteke konposatu horiek asetuta aurkitzen direla behazunean. Gainontzeko konposatuentzako ez zen tendentziarik ikusi, eta lan gehiago egin behar da ondorio garbiak atera ahal izateko.



7.1b irudia: *10. egunean neurtutako konposatuen log (ng/mL) balioak disoluzio desberdinetarako.*

Konposatu desberdinentzako uretan eta behazunean neurtu ziren kontzentrazioak, eta **7.1 ekuazioa** erabiliz, BCF-balioak kalkulatu ziren. **7.3 taulan**, BCFen balio logaritmikoak agertzen dira laburbilduta. Konposatu guztientzako log BCF-balioak 3,62-5,68 tartean egon ziren, beraz, metatzeko gaitasuna nahiko handia dela [21] esan daiteke Kanadako gobernuak ezartzen dituen mugen arabera (BCF 5.000 edo log BCF 3,7 baino handiagoa).

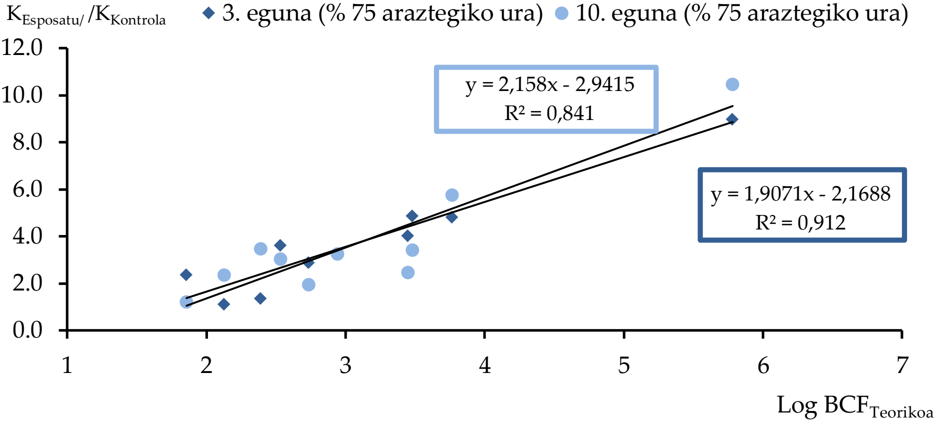
Alde batetik, 2. faseko (*phase II*) transformazioa jasan dezaketen konposatuek (APak, hormonak eta BPAk) log BCF-baliorik handienak zituzten. Bestetik, BCF-baliorik txikienak zituzten konposatuak 1. eta 2. faseko metabolismoa jasan dezaketenak dira, hau da, PEak eta musketa fragantziak, alegia. Gure ikerketa taldeko lan batean antzeko emaitzak argitaratu ziren [13] musketa fragantzien kasurako.

7.3 taula: *Aztertutako analitoen log BCF-balioak diluzio desberdinetara esposatutako arrainen behazunean, baita BCFWIN v2.17 bidez kalkulatuko log BCF balio teorikoak ere.*

Analitoa	BCF teorikoak	3. eguna			10. eguna		
		% 75eko diluzioa	% 50eko diluzioa	% 25eko diluzioa	% 75eko diluzioa	% 50eko diluzioa	% 25eko diluzioa
HHCB	3,48	2,62 ± 0,01	2,82 ± 0,11	3,05 ± 0,12	3,19 ± 0,01	3,14 ± 0,05	3,14 ± 0,04
AHTN	3,76	3,31 ± 0,09	3,78 ± 0,34	4,29 ± 0,08	3,84 ± 0,02	4,03 ± 0,05	4,05 ± 0,09
BBP	2,94	3,20 ± 0,05	3,08 ± 0,05	3,21 ± 0,05	3,94 ± 0,01	3,18 ± 0,13	3,63 ± 0,10
DEHP	5,78	3,45 ± 0,07	3,18 ± 0,03	3,46 ± 0,01	3,89 ± 0,04	3,14 ± 0,14	3,44 ± 0,11
4*t*OP	3,45	4,22 ± 0,03	3,99 ± 0,02	3,89 ± 0,04	4,40 ± 0,04	4,12 ± 0,09	4,10 ± 0,07
NP mix	2,74	5,56 ± 0,02	5,23 ± 0,03	5,06 ± 0,02	5,68 ± 0,02	5,30 ± 0,07	5,24 ± 0,08
4*n*OP	2,53	4,60 ± 0,02	4,29 ± 0,09	4,42 ± 0,01	4,87 ± 0,01	4,59 ± 0,05	4,45 ± 0,04
BPA	1,86	4,29 ± 0,11	3,85 ± 0,02	3,36 ± 0,07	3,57 ± 0,07	3,14 ± 0,04	3,30 ± 0,08
E2	2,39	5,30 ± 0,11	5,17 ± 0,15	5,35 ± 0,01	5,81 ± 0,01	5,59 ± 0,13	4,94 ± 0,08
EE2	2,13	4,53 ± 0,05	4,55 ± 0,05	4,53 ± 0,04	4,83 ± 0,04	4,76 ± 0,05	4,20 ± 0,06

3.2. Behazunaren metatze/eliminazio-gaitasunaren azterketa

Esperimentu osoan erabilitako 3 diluzio desberdinetako EDCen kontzentrazioa, kontroleko ontziko arrainen behazunean neurtutako kontzentrazioekin erlazionatu ziren ($K_{Esposatu}/K_{Kontrola}$). Lortutako erlazioa, log K_{ow} balioetan oinarritzen den BCF-balio teorikoekin (BCFWIN v2.17) erlazionatu zen. 25:75 eta 50:50 araztegiko ur:itsasoko ur diluzioen kasuan eraikitako modeloak ez ziren onak izan 0,5 baino txikiagoak ziren erregresio-koefizienteak (r^2) lortzen baitziren. Ondorioz, 2 diluzio horiek modelotik kanpo geratu ziren. 75:25 araztegiko ur:itsasoko ur diluzioaren kasuan, aldiz, r^2 > 0,84 baino balio handiagoak lortu ziren 3. eta 10. egunetarako **7.2 irudian** ikus daitekeen moduan. Log BCF-balio teorikoak altuak direnean (log K_{ow} balio altuak), kalkulatutako erlazioa ($K_{esposatu}/K_{kontrola}$) geroz eta handiagoa da. Erlazio horren arrazoia arrainak konposatu gehiago kanporatzearekin erlazioa izan dezake, konposatu arrotzen aurrean metabolismoa bizkortu egiten baita [34, 35]. Nahiz eta ikerketa gehiago egin behar diren horren inguruan, badirudi log K_{ow} handia duten konposatuek behazunaren bidez kanporatzeko joera handiagoa dutela. Hala ere, lan gehiago aztertu behar dira gai horren inguruan hipotesi guztiak baieztatu ahal izateko, baita log K_{ow} desberdinak dituzten konposatu gehiago ere.



7.2 irudia: *Log BCF teorikoaren eta $K_{Esposatu}/K_{Kontrolaren}$ arteko erlazioa.*

3.3. Material polimerikoen erabilera arrainaren behazunaren metaketa-ahalmena simulatzeko

Arrainak egon ziren ontzian jasotako PES-tutuak eta M-PES-ak aztertu ziren horiek arrainen behazunarekin duten metatze-ahalmena erkatzeko. Lehenik eta behin, araztegiko uren kontzentrazio baxuena zuen ontziko (% 75eko diluzioa) PES-tutuak eta M-PES-ak aztertu ziren. Lagin horietan lortu ziren kromatogramak oso zikinak ziren eta gainontzeko laginak ez aztertzea erabaki zen. PES-tutuen eta M-PESen arteko konparaketa BCF laginekin diluzio bakarrean egin zen.

Lan honetan zehar azterturiko konposatuen artean 4*t*OP, HHCB, AHTN, BBP eta DEHP konposatuak bakarrik determinatu ziren bai PES-tutuetan zein M-PESn, gainontzeko konposatuak ez baitziren adsorbatuta geratu. Beraz, behazunaren metatze-ahalmenarekin konparatzeko 5 konposatu horiek baino ez ziren erabili. PES-tutuen kasuan, % RSD egokiak (RSD < % 29) lortu ziren, baina ez ordea, M-PES laginen azterketan (RSD > % 50). Lortutako errepikakortasun eskasa literaturan askotan aipatu izan den mintzen "biofouling" efektuaren ondorioa izan daiteke [36, 37]. Beraz, PES-tutuak baino ez ziren kontuan eduki arrainen behazunarekin alderatzeko.

PES-tutuetan log CF-balioak kalkulatu ziren **7.2 ekuazioan** azaldutakoaren arabera. 3. eta 10. egunetarako lortutako balioak **7.4 taulan** laburbilduta daude. Aztertutako konposatu guztien kasuan BBParen salbuespenarekin, 3. egunean determinatu ziren log CF-balioak 10. egunean lortutakoak baino txikiagoak izan ziren. Beraz, pentsa daiteke PES-tutuak ez zirela orekara heldu 3. egunerako, eta 10. egunari dagokionez, aldiz, ezin da ziurtatu orekara heldu ziren edo ez. Zentsu horretan, esperimentu luzeago bat egin beharko litzateke orekan dauden edo ez ikusteko.

Behazun-laginetan kalkulatutako log BCF-balioak PES-tutuetan kalkulatutako log CF-balioekin alderatu ziren 3. eta 10. egunerako (ikus **7.4 taula**). 3. egunean kalkulatutako erlazioei dagokienez, aztertutako kasu guztietan lortutako balioak 1 baino txikiagoak izan ziren, beraz, behazunak metatze-ahalmen handiagoa du. Aipatu berri den moduan, badirudi PES-tutuak ez zirela orekara

heldu 3. egunerako, beraz, hori izan daiteke 1 baino balio txikiagoak lortzearen arrazoia. 10. egunean aldiz, tendentzia berdina ikusi zen 4*t*OP, HHCB eta AHTN analitoen kasuan (ikus **7.4 taula**) eta PEek kontrako joera izan zuten, hau da, 1 balioa baino handiagoak determinatu zen. Datu horiek azterturik, esan daiteke PES-tutuak 10. egunean ere ez zirela orekara heldu 4*t*OP, HHCB eta AHTN analitoen kasuan, baina bai PEen kasuan. Berriro ere, lan gehiago egin behar da horren inguruan, baina badirudi PES-tutuak egokiak izan daitezkeela etorkizunean biomonitorizazio tresna moduan erabiltzeko.

7.4 taula: *Log BCF balioak behazuna eta PES zuntzentzako 3. eta 10. egunetan, baita behazuna eta PES-tutuen arteko erlazio ere.*

	4*t*OP	HHCB	AHTN	BBP	DEHP
Log $BCF_{Behazuna}$, 3. eguna	4,22	2,62	3,31	3,20	3,45
Log $BCF_{Behazuna}$, 10. eguna	4,40	3,19	3,84	3,94	3,89
Log CF_{PES}, 3. eguna	4,84	3,46	4,37	3,71	3,62
Log CF_{PES}, 10. eguna	5,34	3,83	4,45	3,64	3,80
Log $BCF_{behazuna}$/log CF_{PES} 3. eguna	0,87	0,76	0,76	0,86	0,95
Log $BCF_{behazuna}$/log CF_{PES} 10. eguna	0,82	0,83	0,86	1,08	1,02

4. ONDORIOAK

Disruptore endokrinoak diren hainbat konposaturen metaketa-azterketa ikertu da araztegiko efluentearen diluzio ezberdinetara eta egun desberdinetan esposatutako lazunen behazunean. Orokorrean ezin izan da analitoen tendentzia garbirik ikusi diluzio eta egunen artean. Hala ere, aipatu behar da 3. Egunean diluzioen tendentzia nabariagoa ikusi zela eta 10. egunean aldiz, aztertutako 3 diluzioetan neurtutako kontzentrazioak antzekoagoak zian zirela. Beraz, pentsa liteke 3. egunean behazuna oraindik asetu gabe zegoela. Konposatuen kanporaketa maila propietate fisiko-kimikoekin (log K_{ow}arekin) erlazionatuta egon daitekeela

ikusi da, nahiz eta lan gehiago egin behar den gai honen inguruan.

Azkenik, material polimerikoekin lortutako emaitzekin lan handia dago oraindik egiteko, baina lehenengo ikerketek erakutsi dute PES polimeroa etorkizunean biomonitorizazio tresna moduan erabil litekeela ingurumeneko uren azterketa egiterako orduan. Lan honetan PES-tutuak behazunarekin guztiz konparagarriak ez direla ikusi den arren, 10. egunean lortu diren balioak behazunaren balioekiko hurbilagoak dira. Hori dela eta, pentsa liteke PES-tutuak ez zeudela oreka egoeran, eta horrek ateak zabaltzen ditu etorkizunean lan sakonago bat egiteko eta konparagarriak diren edo ez ikusteko.

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Disruptore endokrinoetara esposatutako lupien (*Dicentrarchus labrax*) organo desberdinetako metaketa-azterketa, kontrolpeko baldintzetan

1. SARRERA

Azken urteotan, disruptore endokrino (EDC) moduan ezagutzen diren konposatuen erabilera izugarri hazi da eta, horrekin batera, horien arriskuarekiko ardura ere. EDCak, sistema endokrinoan aldaketak sortzen dituzten substantzia exogenoak, edo horien nahasteak, dira. Konposatu horiek organismo osasuntsuetan edo bere ondorengoetan kalteak sor ditzakete [1]. Europar Batasunak (EU) EDC moduan joka dezaketen 553 konposatu ebaluatu ditu dagoeneko. Aztertutako konposatu guztietatik, 194 konposatu lehenengo multzoan sailkatu ditu organismo osasuntsuetan duten eragin nabarmenengatik [2]. Asko dira EDC moduan sailkatu diren konposatuak; alkilfenolak (AP, bisfenol-A (BPA), fungizidak, pestizidak, surfaktanteak, konposatu farmazeutikoak eta estrogenoak, besteak beste [3-5]. Ingurumenean maila altuenean topa daitekeen EDCa BPA da. BPA, epoxi, polikarbonato eta poliester erretxinen fabrikazioan erabiltzen da % 60, % 30 eta % 10 mailan, hurrenez hurren [6]. APak alkilfenol polietoxilatuen degradazioaren eratorriak dira gehienbat. Konposatu etoxilatuak araztegietan degradatzen dira batik bat eta beraien erabilera nagusiena industrian eta nekazaritzan aurkitzen da [5]. 17 β-estradiol (E2) estrogenoa, emakumeen ugalketa-zikloa doitzeaz gain, osteoporosiaren prebentzioan, prostata eta bular minbizien terapian eta menopausia-sintomak arintzeko erabiltzen da [7]. Diestilbestrola (DES) estrogeno sintetikoa da eta 1940-1970 urteen bitartean erabili zen haurdun zeuden emakumetan abortuak saihesteko gehienbat. Zenbait urte geroago frogatu zen, DESak ametan eta umeetan sor zitzakeen osasun-kalteak eta, horregatik, bere erabilera debekatu egin zen 1978. urtean EUn [8].

EDCak eguneroko bizitzan etengabe erabiltzen dira eta saneamendu-sarean zehar, araztegietara (WWTP) heldu eta bertan aurrekontzentra daitezke. Araztegiak ez dira gai konposatu horiek guztiz arazteko eta, ondorioz, araztegiaren irteera-korrontea bilakatu da konposatu horiek ingurumenera isurtzeko iturririk nagusiena. Araztegien irteera-korronteaz gain, abeltzaintza, nekazaritza, akuikultura eta industriak dira konposatu horien ingurumeneko beste isurketa iturri nagusiak [4, 9]. Orokorrean, μg/L mailan edo kontzentrazio baxuagoetan aurkitzen dira ingurumen-uretan [4, 10]. Hala ere, kontzentrazio-

maila horiek nahikoak dira bertan bizi diren organismo bizidunei kalteak sortarazteko. Eragindako kalteen artean, besteak beste, malformazioak, toxikotasun teratogenikoa, ugalketa-arazoak, espezieen tamainaren txikitzea edo intersexualitatea aurkitu dira arrainetan, anfibiotan, moluskuetan, narrastitan eta ugaztunetan [5, 11]. EDCek sistema endokrinoa kontrolatzen duten konposatu naturalak ordezkatzen dituzte eta haien ohiko funtzionamendua oztopatzen dute. Alkilfenolak eta BPA, adibidez, sistema endokrinoko molekulak imitatzeko gai dira [12]. Estrogenoak, aldiz, mekanismo molekularra blokeatzeko gai dira, bai errezepzioan zein garraioan [13]. Beraz, oso garrantzitsua da kutsatzaile organiko horiek arrainen gorputzaren atal desberdinetan metatzeko duten gaitasuna aztertzea, beraien eraginak hobeto ulertu ahal izateko.

Orain arte egindako lan gehienetan, EDCak arrainen muskuluan eta arrain osoan aztertu dira [4, 14, 15]. Hala ere, geroz eta gehiago dira arrainen atal eta organo desberdinak kontuan hartzen dituzten ikerketak [16-18]. Aurretiaz azaldu bezala, EDCak oso kontzentrazio baxutan egon ohi dira uretan eta askotan detektaezinak izaten dira egun ezagutzen diren teknika analitikoekin. Arrainek, aldiz, konposatu horiek organo desberdinetan metatzeko (kontzentratzeko) joera erakusten dute. Modu horretan, uretan detekta ezinak diren konposatu asko, arrainetan topa daitezke eta konposatu horietara esposatuak izan direla ziurtatu [3, 19]. Gibela da konposatu naturalak zein toxikoak metabolizatzeko ardura duen organoa. Metabolizazio-prozesu horretan, konposatuek fase bakarra edo bi jasan ditzakete, I fasea eta II faseko metabolismoa, hain zuzen ere. I fasean, konposatuak, orokorrean, polarragoak diren metabolitoetan transformatzen dira; modu horretan, II fasea erraztu egiten da. II fasean, metabolitoak glukuronido, glukosido edota sulfato talde batekin lotzen dira, kaleratze- zein barneratze-prozesua errazteko [3, 19, 20]. Konposatu ez-polarrenek bi faseak jasaten dituzten bitartean, talde polarrak dituztenek II fasea bakarrik jasaten dute. Bigarren multzo horretan alkilfenolak, estrogenoak eta BPA aurkitzen dira. Transformazio-pauso horren ondoren, konposatuak behazunera garraiatzen dira gorputzetik kanporatzeko asmoz. Hori dela eta, behazuna da konposatu horien kontzentrazio-maila altuena espero den fluidoa [20-22]. Arrainek konposatuak metatzeko duten ahalmena biokontzentrazio-faktorearen (BCF) bidez determinatzen da (ikus **8.1 ekuazioa**) eta

parametro hori oso erabilgarria da konposatu kimikoek ingurumenean izan dezaketen eragina aurresateko. BCF konposatuak arrainaren atal desberdinetan eta uretan duen kontzentrazioaren arteko erlazioa bezala definitzen da. Metaketa hori hainbat parametroren menpekoa da: arrain-espeziea, sexua, tamaina, elikadura-ohiturak eta ingurumeneko baldintzak, besteak beste [5, 14]. Yang eta lankideen arabera, AP eta BPA konposatuentzako BCF balioak, muskuluan 100 baino txikiagoak diren bitartean, behazunean 1.000-10.000 bitartekoak dira zamo arrunta (*Carassius carassius*) espeziearentzat [3]. Ildo berean, gure taldearen esperientziaren arabera, EDC desberdinen BCF balioak 300.000 izatera iritsi dira lazunen (*Chelon labrosus*) behazunean [23].

$$BCF = \frac{K_{analito\ ehunean}}{K_{analito\ uretan}}$$

8.1 ekuazioa

BCF balioak kalkulatzeko egindako lan gehienetan, ingurumenean arrantzatutako arrainak erabili izan dira [5, 24], baina kasu horietan ingurumen-baldintzak ezin dira kontrolpean mantendu eta zaila da ondorio garbiak lortzea. Arazo horri aurre egiteko, laborategian egiten diren kontrolpeko esperimentuak dira irtenbide bat. Bertan, arrainak kontzentrazio ezaguneko kutsatzaile bakarrera zein nahaste batera esposatzen dira. Horrez gain, posiblea da arrain-talde homogeneo batekin lan egitea, edo sexualki heldugabeko arrainekin, kasurako. Zenbait lanetan aipatzen da kutsatzaileen nahasteak gehitzen direnean, beraien efektua arrainean asko handitzen dela [21, 25, 26]. Brack eta langileen esanetan [27], adibidez, kutsatzaileak era indibidualean aztertzen direnean, beren toxikotasuna % 0,1 ingurukoa baino ez da. Ildo berean, Tang eta lankideek, era indibidualean eragin nabarmenik sortzen ez duten konposatuak nahasten direnean, eraginak sortzeko gai direla frogatu dute [28].

Lan honen helburua, beraz, efektu estrogenikoa duten familia desberdineko 4 EDCren nahasteak, (4-*tert*-oktil fenola (4*t*OP), BPA, DES eta E2) heldugabeko lupien (*Dicentrarchus labrax)* atal eta organo desberdinetan (muskuluan, plasman, gibelean, behazunean eta burmuinean) metatzeko duten joera aztertzea izan da.

2. MATERIALAK ETA METODOAK

2.1. Erreaktiboak eta materialak

Lan honetan erabili ziren erreaktiboen ezaugarri kimikoak **I. eranskinean** laburbildu dira. Analito guztiak banan-banan 1.000-4.000 ng/µL kontzentrazio bitartean prestatu genituen metanoletan (MeOHtan) (HPLC kalitatea, LabScan, Dublin, Irlanda) eta disoluzio diluituagoak (100 ng/µL) hilero. Horrez gain, 1-5 ng/µL tarteko disoluzioak, egunero prestatu genituen esperimentuaren beharrizanen arabera. Azkenik, arrainen esposizioa egiteko, 65 ng/mL disoluzio bat prestatu genuen etanol:itsasoko uretan (20:80, b/b) (EtOH, *super pure* kalitatea, Teknokroma, Bartzelona). Prestaturiko disoluzio guztiak anbarrezko ontzietan gorde genituen -20 °C-an.

Azetona (Ace), MeOHa, n-hexanoa (Hex) eta azetonitriloa (ACN) Sigma Aldrich etxe komertzialak (Steinheim, Alemania) hornitu zizkigun eta etil azetatoa (EtOAc), aldiz, Labscan etxeak (Dublin, Irlanda). Erabilitako erreaktibo guztiak HPLC kalitatekoak ziren, likido-kromatografia–tandem masa-espektrometria (LC-MS/MS) neurketetan erabilitako MeOHa izan ezik (UHPLC-MS, Scharlab, Bartzelona). Azido azetikoa (HAc, % 99,7) Merck etxeari (Darmstandt, Alemani) erosi zitzaion. Ur purua (Milli-Q), Milli-Q ura ur-purifikazio sistematik (< 0,057 µS/cm, Millipore, Bedford, MA, EEBB) eskuratu zen. LC-MS/MS-an erabilitako fase mugikorra basikotzeko (pH=10,5), amoniakoa (% 25 NH_4OH, Panreac, Reixac, Bartzelona, Espainia) erabili zen. Behazun laginen hidrolisia burutzeko erabilitako disoluzio indargetzailea (0,1 mol/L, pH=6) prestatzeko potasio di-hidrogeno fosfatoa (KH_2PO_4, % 100, Panreac) eta amonio hidrogenofosfatoa ($(NH_4)_2HPO_4$, % 99, Merck, Darmstadt, Alemania) erabili ziren. Etil-4-aminobentzoato gatza (% 98, bentzokaina) Fluka etxe komertzialari (Steinheim, Alemania) erosi zitzaion. Entzimei dagokienez, VII-A motatako β–glukuronidasa erabili zen Escherichia Colitik lortua (Sigma-Aldrich, Steinheim, Alemania). Entzima hori Milli-Q uretan disolbatu zen, 1000 U/mL kontzentrazioa lortzeko. Azkenik, Heparina Sigma-Aldrich etxe komertzialari erosi zitzaion.

Erauzketan erabilitako polietersulfona (PES) hodiak, tutu-forman (0,7 mm-ko kanpo diametroa, 1,43 g/mL dentsitatea) Membrana (Wupertal, Alemania) etxean erosi ziren. 1,5 cm luzerako zatiak moztu eta EtOActan garbitu ziren 24 orduz. Azkenik, PES-tutuak erabili aurretik, EtOActik atera eta paper garbi batekin lehortu ziren.

Lagin solidoen garbiketa urratsa egiteko, ENVI-CARB (ENVI, gainazal espezifikoa 100 m^2/g, 120/400 zulo sarea) Supelco etxeak (Steinheim, Alemania) hornitu zuen eta amina primarioa eta sekundarioa (PSA, gainazal espezifikoa 570 m^2/g) Agilent etxeak (Avondale, PA, USA). Horrez gain, fase solidoko erauzketa dispertsiboan (dSPE) magnesio sulfatoa ($MgSO_4$, extra purua, Scharlab) ere erabili zen. Azkenik, dSPE urratsean erabilitako zentrifuga (5424) Eppendorf etxearena (Hamburgo, Alemania) izan zen.

2.2. Lagin-bilketa

Heldugabeko (~20 g, 5 hilabete) lupiak (*Dicentrarchus labrax*) Tinamenor (Pesues, Kantabria, Espainia) arraintegian erosi ziren 2015eko apirilean. Esperimentua hasi aurretik, arrainak, laborategiko girora moldatzeko hilabetez eduki ziren itsasoko ura zuen akuario batean eta egunean behin eman zitzaien jaten (lupien pisuaren % 5 inguru, Mar Perla MPL, 1.5). Lupientzat erabilitako argi periodoa 12 ordukoa izan zen.

Egokitze-garaiaren ondoren, 340 lupia, 0,22 µm filtroetatik iragazitako itsasoko ur garbia zuen 250 L-ko bi ontzitara pasatu ziren. Ontzietako itsasoko ur hori, 8,5 L/h-ko emari konstante batekin aldatzen zihoan. Horrela, 48 orduz behin 250 L-ko ontzia urez berrizten zen. Ontzi batean (kontrol-ontzia deituko dena), ez zen inolako kutsatzailerik gehitu eta bestean (esposizio-ontzia), aldiz, 20 mL/h-ko emariarekin, EtOH:itsasoko (20:80, v/v) uretan disolbatuta zeuden BPA, DES, E2 eta 4*t*OP kutsatzaileak ponpatu ziren. Horrela, analito bakoitzeko 150 ng/L-ko kontzentrazioa nominala espero zen. Lupiak 10 egunez egon ziren kontzentrazio horretara esposatuak eta, ondoren, 12 egunetako arazketa (kutsatzailerik gabe) jasan zuten.

Esperimentua hasi eta 1., 3., 7. eta 10. egunetan, 10 lupia jaso ziren ontzi bakoitzetik. Esposizio-denbora amaituta, esposizio-ontzian geratutako arrainak, ontzi garbi batera eraman ziren arazketa urratsari hasiera emateko. Arrainak, arazketa hasi eta 4., 7. eta 12. egunen ostean jaso ziren.

Arrainak lan honen 4. kapituluan azaldu bezala tratatu ziren. Laburki, arrainak bentzokainaz saturatua zegoen ontzi txiki batean sartu ziren. Ondoren, arrain bakoitzaren luzera eta pisua jaso zen eta odola atera zitzaien heparina zuen xiringa baten laguntzaz. Arrainak dekapitazioz sakrifikatu ziren, UPV/EHUko Animali Esperimentalen Etika Batzordearen ebaluazio positiboa zuen protokoloa betez. Azkenik, zorroztutako bisturi batez lupia zabaldu eta gibela, muskulua, burmuina eta behazun-poltsa osoa jaso ziren esterilizaturiko krio-ontzietan. Odol-laginak berehala zentrifugatu ziren plasma eskuratzeko. Lagin guztiak nitrogeno likidotan (N_2) izoztu ziren. Plasma, behazuna eta burmuina -80 °C-an gorde ziren eta muskulua eta gibela, 48 orduz liofilizatu ostean, -20 °C-an.

2.3. Laginen azterketa

2.3.1. Lagin solidoen azterketa

Lagin solidoak (arrain-burmuina, gibela eta muskulua) lan honen 6. kapituluan deskribatu bezala aztertu ziren. Laburki, 0,1 g burmuin (n=3) eta 0,5 g muskulu (n=3) edo gibel (n=3) ontzi batean pisatu eta 1 ng/µL zuen trazatzaile-disoluzioaren ([$^2H_4$]-4*n*-nonilfenola ([$^2H_4$]-NP), [$^2H_3$]-17β-estradiola ([$^2H_3$]-E2), [$^2H_{16}$]-bisfenol-A ([$^2H_{16}$]-BPA)) 25 µL gehitu ziren. Ondoren, 10 mL Hex:Ace (50:50, b/b) gehitu eta lagina 5 minutuz erauzi zen ultrasoinu fokatuaren (FUSLE) bidez (Sonopuls HD 2070, 20 Hz, 70 W, Bandelin electronic GMBH & Co. KG, Berlin, Alemania). FUSLEaren lan-baldintzak, % 33-ko potentzia eta 0,8 s lanean eta 0,2 s itzalita izan ziren 5 minutuz. Ondoren, laginak 0,45 µm-ko poliamidazko (PA) iragazkitik (Chromafil Ao, Macherey-nagel, Düren, Alemania) pasarazi eta disolbatzailea lehorreraino lurrundu egin zen N_2 korronte baten pean (Turbovap® LV, Caliper, 176 life sciences, USA). Erauzia 1 mL ACNtan berreratu zen eta 100 mg ENVI eta 100 mg $MgSO_4$ zituen 1,5 mL-ko Eppendorf hodi batera pasatu zen. Gibelaren kasuan 100 mg PSA ere gehitu ziren. Eppendorf hodia, 1 minutuz

irabiatu ostean, 5 minutuz zentrifugatu zen 4.000 bira/min-ko abiaduran. Gaineko ACN geruza, saiodi batean jaso eta prozesu osoa birritan errepikatu zen. Azkenik, 2 ACN geruzak batu, lehortzeraino lurrundu eta burmuinaren kasuan, 150 µL MeOHetan berreratu ziren konposatuak eta, gibelaren eta muskuluaren kasuan, 200 µl-tan. Azkenik, erauziak, 0,2 µm politetrafluoroetileno (PTFE) iragazkitik (13 mm diametroa, 0,2 µm poro tamaina, Teknokroma, Espainia) iragazi ziren LC-MS/MS analisi aurretik.

2.3.2. Lagin likidoen azterketa

Lagin likidoak lan honen 5. kapituluan azaldu bezala aztertu ziren. Laburki azalduz, 100 µL arrain-behazun (n=1) edo plasma (n=2) 10 mL-ko ontzi batean pisatu, eta 800 µL Milli-Q, 1,5 mL fosfato disoluzio indargetzaile (0,1 mol/L, pH=6) eta 1 ng/µL zuen trazatzaileen disoluzioaren ([$^{2}H_{16}$]-BPA, [$^{2}H_{4}$]-NP eta [$^{2}H_{3}$]-E2) 25 µL gehitu ziren lagin bakoitzean. Behazunaren kasuan, 200 µL β-glukuronidasa (1.000 U/ml) ere gehitu ziren. Aurretiaz egokitutako 5 PES-tutu (1,5 cm-koa bakoitza) eta PTFEz estalitako irabiagailu bat gehitu ziren ontzi bakoitzean eta, ontziak ondo itxi ostean, erauzketa 3 orduz utzi zen 37 °C-an eta ~550 bira/min-ko abiaduran (RT 15 power, Ika Werkr, Staufen, Alemania). Behin erauzketa amaituta, PES-tutuak ontzitik atera eta Milli-Q uraz garbitu ostean, eskuzapi garbi batez lehortu ziren bertan gera zitezkeen hondar-arrastoak kentzeko. Jarraian, PES-tutuak 1,5 mL anbarrezko Eppendorf hodi batean sartu ziren eta ultrasoinu- (US) bainuan 300 µL MeOH erabiliz, analitoak 16 minututan erauzi ziren (USB Axtor by Lovango). MeOH erauziak lehortu arte lurrundu ziren, 200 µL MeOHetan berreratu eta 0,2 µm PTFE iragazkietatik iragazi ziren LC-MS/MS-aren bidez aztertu aurretik.

2.3.3. Ur-laginen azterketa

Ur-laginak lan honen 3. kapituluan azaldu bezala aztertu ziren LC-MS/MS erabiliz. 150 mL ur (n=3) 0,45 µm-ko iragazkietatik iragazi ziren eta, kasu honetan, ez zitzaion inolako gatzik gehitu itsasoko urak jatorriz duen gazitasuna dela eta. Ontziari, ur-laginaz gain, aurretiaz EtOActan garbitutako 1,5 cm-ko 5 PES-tutu, PTFEz estalitako irabiagailua eta analitoen trazagarriak ([$^{2}H_{16}$]-BPA, [$^{2}H_{4}$]-NP eta

[$^{2}H_3$]-E2) zituen disoluzioa gehitu zitzaizkion 200 ng/L-ko kontzentrazioa izan arte. Erauzketarekin hasteko ontziak itxi eta inguru tenperaturan, laginak 1200 bira/min-ko abiaduran (15 posiziotako plaka birakari magnetikoa, Gerstel, Mulheim an der Ruhr, Alemania) irabiatzen utzi ziren gau osoan zehar.

Erauzketa urratsa amaitu ostean, PES-tutuak atera, gatz arrastoak kentzeko Milli Q uraz garbitu eta, azkenik, eskuzapi garbi batez lehortu ziren. PES-tutuak, eppendorf hodi baten sartu eta 300 µL MeOH erabiliz, analitoak ultrasoinu-bainuan erauzi ziren. Azkenik, LC-MS/MSaren bidez aztertu aurretik, laginak 0,2 µm-ko PTFE-zko iragazkitik pasatu ziren.

2.4. LC-MS/MS-aren bidezko azterketa

Laginak lan honen 6. kapituluan deskribatu bezala aztertu ziren LC-MS/MS-aren bidez (Agilent HPLC 1260 seriea, Agilent 6430 masa-espektrometro kuadrupolo hirukoitza-elektroesprai-ionizazio iturri bati lotua). Laburbilduz, 5 µL erauzi (plasma, behazun, muskulu eta burmuinaren kasuetan) eta 3 µL gibelaren kasuan, partzialki porotsua den *Ace Ultra Core Super C_{18} core-shell* (2,1 mm x 50 mm eta 25 µm-ko partikula tamaina) zutabean injektatu ziren 30 °C-an eta 0,3 mL/min–ko fluxuan. Erabilitako fase mugikorrak Milli-Q:MeOH (95:5, b/b) (fase mugikor A) eta MeOH:Milli-Q (95:5, b/b) (fase mugikor B) izan ziren % 0,05 NH_4OH-tan. Bereizketa kromatografikoa hurrenkera honetan egin zen: % 30 B konposizioa 4 minutuz era isokratikoan mantendu zen eta % 60 B-raino igo zen 3 minuturen tartean. Ondoren, 10 minutu behar izan zituen % 80 B konposizioa lortzeko eta konposizio horretan 10 minutuz mantendu zen. Azkenik, hasierako baldintzetara bueltatu zen (% 30 B) 3 minututan eta beste 10 minutuz baldintza horietan mantendu zen (injekzio osteko denbora). LC-MS/MS-an erabilitako N_2 gasak (purutasun altua, % > 99,999) Messer-ek (Tarragona, Espainia) eta Air Liquid-ek (Madrid, Espainia) hornitu zituzten. Gas horiek talka-eragile moduan eta nebulizatzaile eta gas-lehortzaile moduan erabili ziren, hurrenez hurren. MS/MS-aren ionizazio-baldintzei dagokienez, hurrengo eran finkatu ziren: N_2 fluxua 11 mL/min-tan, kapilarraren boltaia 4.000 V-tan, nebulizazio presioa 52 psi-tan eta iturriaren tenperatura 325 °C-tan. Azkenik, laginak erreakzio anitzeko monitorizazioaren (MRM) bidez kuantifikatu ziren, eta analisian zehar jarraitutako

trantsizioak eta erabilitako talka-energia (CE), polaritatea eta gelaxka-boltaia (CV), **III. eranskinean** daude laburbilduta. Instrumentuaren kontrolerako eta datuen tratamendurako erabilitako programa Masshunter Workstation (B.06.00, Agilent) izan zen.

2.5. Kalitate-parametroak

Lan honetan aztertu diren 4 EDCak trazatzaileekin zuzendutako kanpo kalibratu baten bidez kuantifikatu ziren, detekzio-muga-(LOD) 2.500 ng/mL bitartean. Hala ere, kasu bakoitzean, laginetik gertuen zeuden balioak baino ez ziren erabili, beti ere 7 puntuko kalibratu bat mantenduz. Kasu guztietan lorturiko determinazio-koefizientearen (r^2) balioak 0.99 baino handiagoak izan ziren. Erauzketa metodo desberdinetan lorturiko berreskurapen zuzenduak (%), desbideratze estandar erlatiboak (RSD) eta metodoaren detekzio-mugak (MDL) **8.1 taulan** laburbilduta agertzen dira azterturiko analito guztietarako.

8.1 taula: *Lan honetan zehar erabilitako metodoen kalitate-parametroen laburpena: berreskurapen erlatiboa (%), % RSD eta MDL (ng/mL).*

	% Berreskurapen zuzendua	% RSD	MDL (ng/mL)
Gibela	62-108	2-19	10-35
Muskulua	90-132	1-11	3-22
Burmuina	66-120	2-26	3-23
Behazuna	73-125	1-24	1-15
Plasma	71-144	2-10	8-49
Ura	89-118	5-16	0,03-0,09

3. EMAITZAK ETA EZTABAIDA

3.1. Organoen neurriak eta itxura

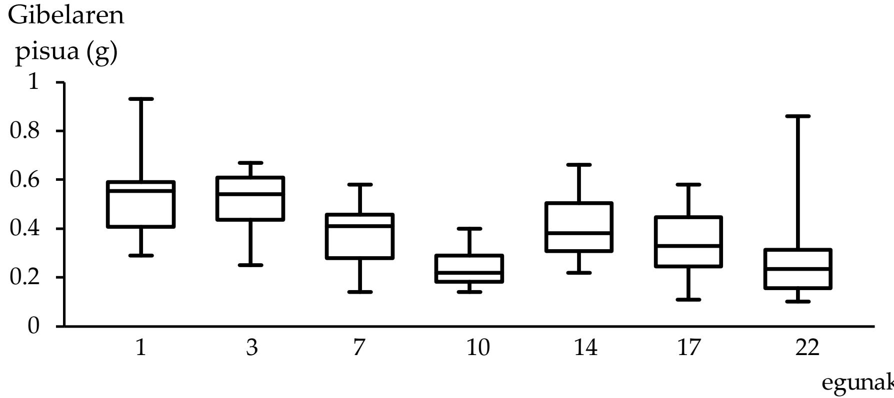
Esperimentuak iraun bitartean jasotako arrain guztien luzerak (cm), arrain osoaren pisuak (g) eta arrain bakoitzaren gibelaren pisuak (g) jaso ziren (ikusi **8.2 taula** arrainen multzo osoaren batezbestekoa eta desbideratze estandarra ikusteko). Luzerari dagokionez ez zen inolako desberdintasunik ikusi azterturiko

talde eta egunen artean ($F_{esperimentala}$=2,2-4,4 < $F_{kritikoa}$=4,5) ikus **8.2 taula**). Arrain osoaren pisua kontuan hartzen badugu, ez zen desberdintasun esanguratsurik egon ($F_{esperimentala}$=1,4-4,2 < $F_{kritikoa}$=4,5) lehenengo 7 egunetan, baina bai ordea 10. egunean ($F_{esperimentala}$=6,3 > $F_{kritikoa}$=4,45), esposizioko ontzian arrainen pisu galera bat ikusi zen (ikus **8.2 taula**). Arazketa-garaian, aldiz, arrainek pisua irabazi zuten hasierako balioetara helduz (ikus **8.2 taula**). Esperimentu osoan zehar arrainei jaten eman zitzaien arren, arazketa-egunetara heldu arte ez zuten gehiegi jan, emandako jana ontzien hondoan ikusten baitzen, eta hori izan zitekeen pisu-galera sortzeko arrazoietariko bat. Tancioni eta lankideek [30], aldiz, ezpain-estuko lasunen kasuan (*Liza ramada*) luzeraren eta pisuaren gorakada bat ikusi zuten araztegiko uretara esposatutako arrain-talde batean kontrolekoekin alderatuz.

8.2 taula: *Arrainen datu morfometrikoak eta gibelaren organometria (batezbestekoa ± desbideratze estandarra): luzera (cm), arrain osoaren pisua (g) eta gibelaren pisua (g).*

	Luzera (cm)		Arrainaren pisua (g)		Gibelaren pisua (g)	
	Esposatua	Kontrola	Esposatua	Kontrola	Esposatua	Kontrola
1.eguna	13,4 ± 0,9	12,8 ± 0,6	24,48 ± 5,41	22,61 ± 4,40	0,54 ± 0,18	0,43 ± 0,12
3.eguna	13,2 ± 0,9	13,3 ± 0,6	25,37 ± 4,69	24,92 ± 2,20	0,51 ±0,14	0,48 ± 0,19
7.eguna	13,4 ± 0,6	13,2 ± 1,6	24,89 ± 3,90	24,18 ± 6,56	0,38 ±0,14	0,49 ± 0,16
10.eguna	12,8 ± 0,6	13,6 ± 0,9	20,76 ± 2,51	25,13 ± 3,77	0,25 ±0,09	0,41 ± 0,14
14.eguna	13,3 ± 0,9	---	24,09 ± 4,33	---	0,41 ±0,16	---
17.eguna	13,3 ± 1,0	---	23,16 ± 4,91	---	0,34 ±0,15	---
22.eguna	13,1 ± 0,9	---	23,41 ± 6,08	---	0,28 ±0,22	---

Azkenik, gibelaren pisuari erreparatzen badiogu, esposizioko lehenengo bi egunetan pisua nahiko egonkor mantendu zen, baina 7. egunetik aurrera pisuak behera egin zuen (ikus **8.1 irudia**). Nahiz eta pisu galera 7. egunetik aurrera somatu, ez zen desberdintasun esanguratsurik nabaritu esposizioko eta kontroleko ontzien artean ($F_{esperimentala}$=2,1-4,2 < $F_{kritikoa}$=4,5) baina bai 10. egunean ($F_{esperimentala}$=9,1 > $F_{kritikoa}$=4,5). Behin arazketa egunetara helduta, bazirudien gibelaren pisua berreskuratuko zela (14. eguna), baina hurrengo egunetan berriro beherakada bat ikusi zen, esposizioko 7. eta 10. egunetako pareko balioak lortuz. Badirudi, EDCen ondorioz, gibela kaltetu zela, eta arrain osoaren pisuarekin gertatzen denaren kontrara, arazketa garaian ez zela gai izan hasierako balioak berreskuratzeko.



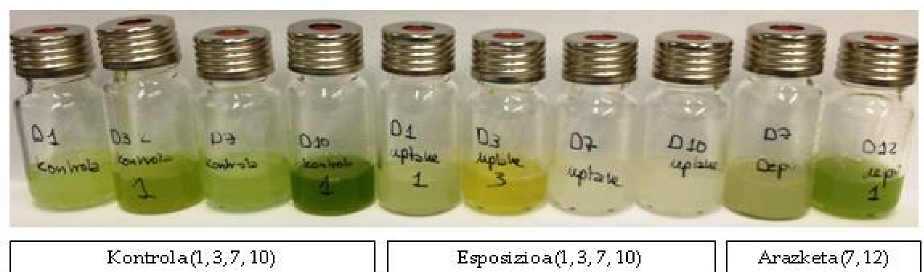
8.1. irudia: *EDC nahasketara esposatutako arrainen gibelen pisua (n=10). 1-10 egunak esposatutako garaiari dagozkio eta 14-22 egunak arazketa garaiari.*

Gibelaren koloreari eta testurari erreparatuz gero (ikus **8.2 irudia**), esan daiteke, egunak igaro ahala, liofilizatutako gibelaren kolorea iluntzen zihoala eta itxura geroz eta koipetsuagoa zela. Kolore aldaketa hori, lehenago aipatu berri den gibelaren pisuarekin zuzenki erlazionaturik dago, gibelaren pisua minimoa zen kasuetan, kolorerik ilunena lortu baitzen.

8.2. irudia: *Arrain-talde desberdinetan (n=10) jasotako gibelaren itxura liofilizatu ostean.*

Gibelarekin gertatu zen moduan, behazunean ere esposizio-egunen menpeko aldaketa nabariak ikusi ziren koloreari dagokionez (ikus **8.3 irudia**). Kontroleko arrainen behazunaren (Milli-Q ura eta fosfato tanpoiarekin diluitu ostean) kolorea berde mantendu zen 10 egunetan zehar, tonu-aldaketa batzuk gorabehera. Esposizio-ontziko arrainetatik jasotako behazunean, aldiz, egunak aurrera joan ahala, behazunaren kolorea aldatzen joan zen. Lehenengo hiru egunetan, behazunaren kolorea berdetik marroira pasatu zen. Esposizioko 7. eta 10. egunetan eta arazketako 4. egunean, aldiz, behazunaren itxura nabarmen

aldatu zen, behazun-xixkuan likido zuri eta koipetsu bat lortuz. Horrez gain, arazketako 4. egunean lortu zen likido zuriaren kantitatea hain zen txikia, ez zela nahikoa izan analisia egiteko. Behin arazketa-egunak aurrera joan ahala, behazunaren itxura hobetzen joan zen, hau da, behazunaren berde kolorea berreskuratu egin zen.



8.3 irudia: *Arrain-talde desberdinetan (n=10) jasotako behazunaren kolorea Milli-Q urarekin eta fosfato tanpoiarekin diluitu ostean.*

Gibelean eta behazunean gertatutako aldaketa fisikoen ondorioz, pentsa zitekeen EDCen eraginagatik gibela kalteturik zegoela eta ez zela gai behar bezala bere eginkizunak betetzeko. Gibelaren funtzioetako bat, azido biliarren sintesia da, bertan dagoen kolesteroletik (CHL) abiatuz [31]. Badirudi, esposaturiko lupinen gibela ez zela gai izan azido biliarrik sintetizatzeko, eta ondorioz, eraldatu gabeko kolesterola behazun-poltsan pilatu zela. Hipotesi hori baieztatzeko behazun laginen analisia egin zen CHL zela baieztatzeko. Esposatu gabeko ontzian, CHL antzeman zen, beti ere antzeko mailan, arrainak era naturalean duen CHL, hain zuzen ere. Esposizioko ontzian, aldiz, egunak igaro ahala, CHL-maila izugarri hazi zen. Nabariena esposizioko 7. eguna izan zen, bertan esposizioko ontzian, kontrolekoan baino ~2,5 aldiz CHL gehiago aurkitu baitzen. Laginak jaso ziren hurrengo egunean CHL-maila 3. eguneko antzeko balioetara jaitsi zen (kontrolekoa baino ~1,3 aldiz handiagoa), baina arazketa-garaiko 17. egunean beste gorakada bat ikusi zen. Azkenik, arazketako azken egunean CHL-mailaren beherakada bat ikusi zen hasierako balioak berreskuratuz. CHLaren azterketaren bidez, gure hipotesiak indarra hartu zuen. Hala ere, azterketa gehiago egin behar dira horren inguruan, hipotesia guztiz baieztatu ahal izateko.

3.2. Ur-laginak

Esperimentuak iraun bitartean, EDC desberdinen kontzentrazioak neurtu ziren kontrol eta esposizioko ontzietan. Azken ontzi horretan esposizio-garaiak eta arazketa-garaiak iraun bitartean jaso ziren ur-laginak. Kontroleko ontzian BPA izan zen antzeman zen konposatu bakarra 23-57 ng/L bitartean. BPA, plastikoen osagaietariko bat izanik, ohikoak izaten dira BPA konposatuarekin izaten diren kutsadura arazoak [32].

Esposatutako ontzian, aldiz, BPA (112 ng/L), E2 (102 ng/L) eta 4*t*OParen (101 ng/g) kontzentrazioak nominalen (150 ng/L) parekoak izan ziren 0. egunean, hau da, arrainik ez zegoen egunean, DESaren kasuan izan ezik, bere kontzentrazioa apur bat baxuagoa baitzen (68 ng/L). Esposizio-egunak aurrera joan ahala, eta arrainak ontzira sartzearekin batera, uretan muki itxura zuten zuntz antzekoak ikusi ziren. Muki horien agerpenarekin 4*t*OP, DES eta E2 analitoen kasuan kontzentrazioen beherakada bat ikusi zen urak 0,45 μm-tako iragazkietatik iragazten zirenean. BPAren kasuan 0. egunean neurtutako kontzentrazioen pareko balioak lortu ziren. Ur berdinen analisia egin zen, baina ura iragazi gabe eta lortu ziren kontzentrazioak 0. egunekoen parekoak izan ziren, beraz, gainontzeko egunen urak iragazi gabe egitea erabaki zen. Esperimentuak iraun bitartean neurturiko uren kontzentrazioa hurrengoa izan zen: BPA (97 ± 12), 4*t*OP (87 ± 24), DES (59 ± 12) eta E2 (78 ± 17). Batezbesteko balio hauek erabili dira beharrezkoak diren kalkuluak egiteko. Konposatuen beherakada hori ulertu nahian, azterturiko 4 EDCek uretan duten disolbagarritasuna kontuan hartu behar da. E2 (82 mg/L), 4*t*OP (4 mg/L) eta DES (3 mg/L) konposatuek oso disolbagarritasun baxua dute uretan, baina BPA (173 mg/L) uretan askoz disolbagarriagoa da, eta hori izan daiteke uretan konposatu hori bakarrik aurkitu izanaren arrazoietariko bat. Hala ere, hipotesi hori ezin izan da baieztatu mukiaren gaineko analisirik egiteko aukerarik ez baita egon. Esan beharra dago, konposatuak mukian adsorbatuta egoteak ez duela zertan esan nahi konposatu horiek arrainentzat bioeskuragarri ez daudenik, azken finean, arrainak zatirik txikienak jakiak bailiran jan baititzake.

Azkenik, behin arazketa garaira helduta, BPA izan zen antzeman zen konposatu bakarra 47-55 ng/L bitartean, balio horiek kontroleko ontzian neurtutakoen parekoak ziren, beraz, badirudi arrainek ez zutela inolako konposaturik kanporatu arazketa-egunetan zehar.

Aipatu den moduan, EDCen kontzentrazioen beherakada, ontzian agertutako zuntz-itxura zuten muki batzuen agerpenarekin batera etorri zen. Bibliografiako lanetan irakurritakoaren arabera [33], arrainek, mukia era naturalean sortzen duten defentsa mekanismoa da. Muki jario hori, bizitza osoan zehar gertatzen den fenomenoa da, ingurumenean dauden patogenoei eta estres egoerei aurre egiteko [33-35]. Horien artean, konposatu kimikoak, toxinak, mikroorganismoak eta estresa eragin dezaketen faktore fisikoak zein biologikoak daude. Mukiaren etengabeko eraketa horrek, kalteturiko ehunetatik ioien galera ekidin dezake [33]. Mukia, pisu molekular altuko glikoproteinaz eta karbohidratoz osatuta dago gehienbat [33] eta bere pisu lehorraren % 65a karbohidratoak dira [33].

3.3. Konposatuen metaketa arrainen organo desberdinetan

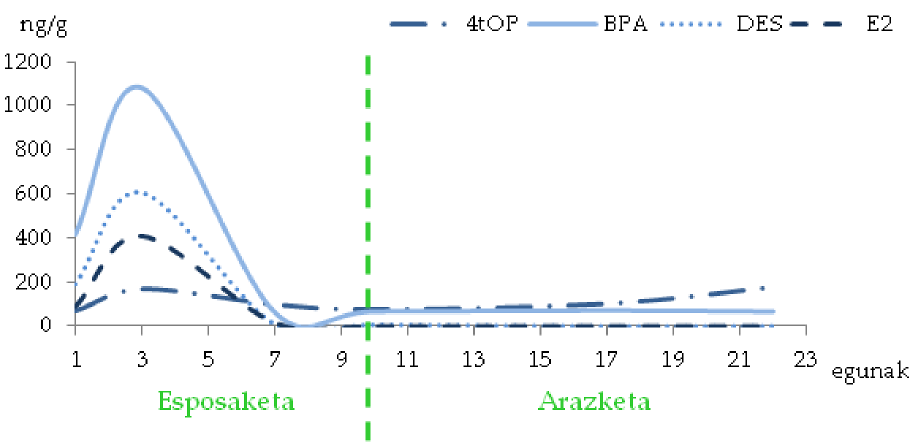
Arrainak, 4 EDC desberdinen 150 ng/L-ko kontzentrazio nominalera esposatuak egon ziren arren, 3.2 atalean ikusi den moduan, lehenengo 3 egunetan, lupiak 4 EDCetara esposatuak egon ziren eta ondorengo egunetan, badirudi BPA izan zela uretan disolbaturik zegoen konposatu bakarra, gainontzekoak (E2, DES eta 4*t*OP) mukian absorbatuta zeudela.

3.3.1. Analitoen metaketa behazunean

Lehenbizi arrainaren behazunari erreparatzen badiogu, esan daiteke 1. egunetik aurrera azterturiko 4 EDCak metatzen hasi zirela (ikus **8.4.irudia**) esperimentuko 3. egunera arte, non azterturiko 4 konposatuentzako maximo bat ikusten den. Esposizioko 7. egunetik aurrera ez zen konposatuen inolako metaketarik ikusi eta hemendik aurrerako egunetan ere ez zen inolako konposaturik antzeman. 3.1 atalean aipatu den moduan, 7. egunetik aurrera lortu zen likidoa, behazuna ez zelako susmoa geneukan, eta CHL izan zitekeela uste

zen. Egia da CHL-maila igo egiten dela baina hipotesi hori guztiz baieztatzeko, azterketa sakonagoak egin beharko litzateke. Hala ere, argi geratzen da arrainaren organismo barruan aldaketak gertatu zirela. Wu eta lankideek [36] heldugabeko karpak (C*yprinus carpio*) BPAren kontzentrazio desberdinetara (0,1-1.000 μg/L) esposatu ondoren, gibelaren metabolismoa kaltetu zelako susmoa zuten, gibelaren metabolismoa eta analitoen kanporatzea oztopatuz. Gure kasuan, lupiak BPAra esposatuak izateaz gain, beste hiru EDCtara esposatuak izan dira. Konposatuen nahasketei esposatuak izan diren arrainen kalteak handiagoak direla kontuan edukita, suposa daiteke BPAk, DES-ak, E2-ak eta 4*t*OP-ak kalteak sortu zituztela gibelean eta, ondorioz, behazunean metatzen diren kontzentrazio-mailan eragina eduki zutela. Hala ere, aurrekoetan esan bezala, hipotesi hori guztiz baieztatzeko azterketa sakonagoak egin behar dira.

8.4 irudian ikus daitekeen moduan, BPA da maila altuenean metatu zen konposatua, gero DES, E2 eta metatzeko joera gutxien zuen konposatua 4*t*OP da. Aipatu beharra dago, BPA izan dela uretan era askean egon den konposatu bakarra, eta hori izan daitekeela BPA kontzentrazio altuenean ikusteko arrazoietako bat. Egun guztien ur kontzentrazioen batezbestekoak kontuan edukiz gero, 3. eguneko BCF balioak kalkulatu ziren **8.1 ekuazioa** erabiliz. Aztertutako EDCen BCF balioak hurrengo hurrenkera izan zuten: BPA (11.144) > DES (10.280) > E2 (5.218) > 4*t*OP (1.902). Balio horiek ikusita esan daiteke konposatu guztiek, 4*t*OP izan ezik, BCF balioa 5.000 balio handiagoa zutela, eta ondorioz metatzeko gaitasun handia [37].

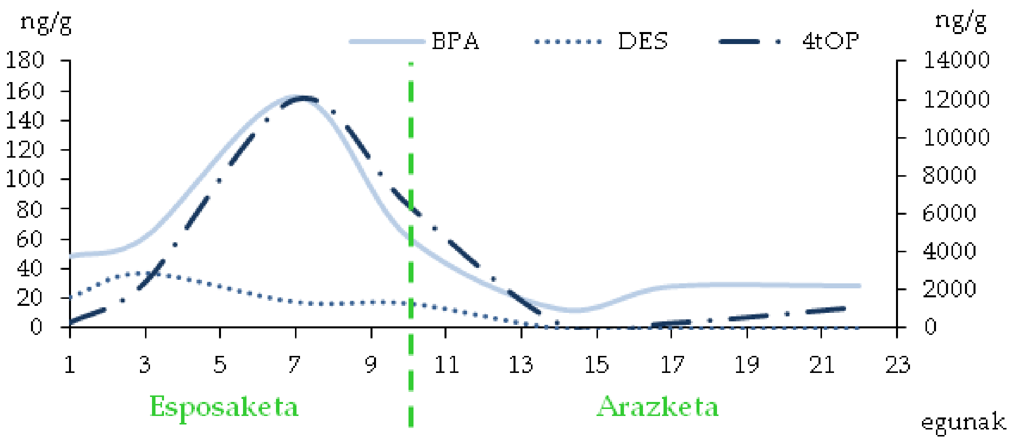


8.4. irudia: *Aarrainen behazunean ikusitako metaketa ng/g-tan. 1-10 egunak esposizio garaiari dagozkio eta 11-22 egunak arazketa garaiari.*

3.3.2. Analitoen metaketa gibelean

Gibel laginei dagokienez, DES analitoak metatze maximoa 3. egunean erakutsi zuen bitartean 4*t*OPk eta BPAk esposizioko 7. egunean izan zuten maximoa (ikus **8.5 irudia**). Badirudi gibelaren mekanismoa kaltetuz zihoala egunetan zehar eta konposatuak metabolizatu eta behazunaren bidez kaleratu beharrean, gibelean metatzen hasi zirela. Behazunean metaketa maximoa dagoenean, gibelean ez da maximorik ikusten; behazunean analitoen metaketarik ikusten ez denean aldiz, (3. egunetik aurrera), gibelean konposatuen metaketa maximoa ikusten da. Guzti hori ikusita, ondoriozta daiteke, EDCen eraginez, gibelaren itxura fisikoa aldatzeaz gain, gibelaren metabolismoa kalteturik egon zitekeela, eta ondorioz ez zen gai konposatuak metatzen jarraitzeko.

Arazketa garaiari erreparatzen badiogu, konposatuek 14. egunean zuten metatze txiki horrekin, gibelaren metabolismoa apur bat hobetu zelaren susmoa egon daiteke, hala ere, hipotesi hori baieztatu ahal izateko, azterketa gehiago egin behar dira. Gernhofër eta lankideen arabera [38], EDC desberdinetara (pestizidak eta hidrokarburo polizikliko aromatikoak (PAH)) esposatutako arrainek, 3 hilabete behar izan zituzten arazketa garaian gibela eta giltzurrunaren erabateko berreskurapena lortzeko.

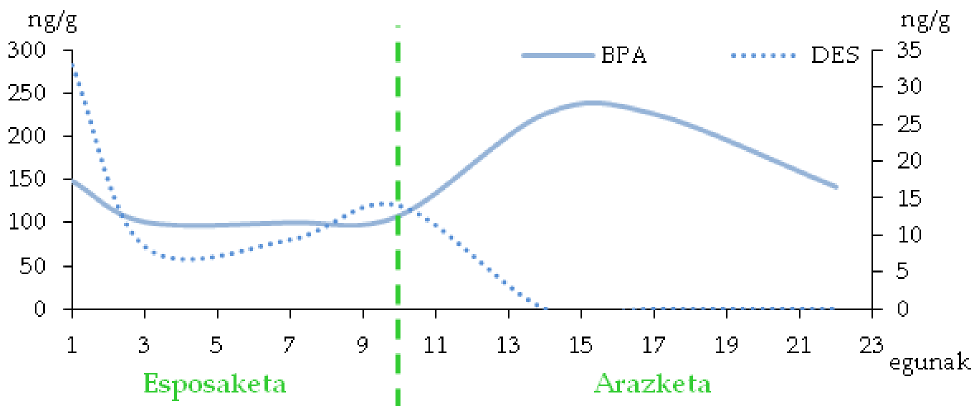


8.5. irudia: *Arrainen gibelean ikusitako metaketa ng/g-tan. 4tOP analitoa eskumako Y ardatzean dago irudikaturik eta DES eta BPA ezkerreko Y ardatzean. 1-10 egunak esposatutako garaiari dagozkio eta 11-22 egunak arazketa garaiari.*

Metaketa-maila, behazunarenarekin konparatuz, askoz txikiagoa da BPA (6,5 aldiz txikiagoa) eta DES (15 aldiz txikiagoa) kasuetarako. 4*t*OPren kasuan ordea, metaketa-maila handiagoa da gibelean (6,5 aldiz handiagoa) behazunean baino. E2aren kasua, kasu berezia da. Nahiz eta behazunean 3. egunera arte detektatu, egun horretatik aurrera ez da inon antzematen. Horren arrazoia ez dakigu eta azterketa sakonagoak egin beharko lirateke horren arrazoia adierazteko.

3.3.3. Analitoen metaketa plasman

Plasma laginei erreparatzen badiegu (**8.6. irudia**), BPA eta DES izan ziren antzemandako EDC bakarrak eta BPA izan zen kontzentrazio altuenean zegoen konposatua. Konposatu horren metaketa esposizioko lehenengo egunetik hirugarrenera jaitsi egin zen arazketa-garaia heldu arte konstante mantentzeko. Arazketa-garaian, BPAren kontzentrazioa plasman igo egin zen eta oso astiro jaitsi zen hasierako balioetara heldu arte. DESaren konportamendua antzekoa izan zen esposizio-garaian baina arazketa hasi zenean konposatua MDL balioetatik behera zegoen, BPA ez bezala.



8.6 irudia: *Arrainen plasman ikusitako metaketa ng/g-tan. BPA analitoa ezkerreko Y ardatzean dago irudikatuta eta DES eskumako Y ardatzean. 1-10 egunak esposatutako garaiari dagozkio eta 11-22 egunak arazketa garaiari.*

Arrainaren metabolismoak ondo funtzionatuko balu, konposatu arrotzak gibelean metabolizatu eta behazunaren bidez kanporatu beharko lirateke. Lehen aipatu den moduan, badirudi gibelak ez duela behar bezala funtzionatzen, eta gerta daiteke konposatuak ez metabolizatzea, atzera-egite bat egotea eta konposatu guztiak odolera igarotzea. Hala ere, berriro ere, hipotesi hori ziurtatzeko azterketa gehiago egin beharko lirateke.

3.3.4. Analitoen metaketa burmuinean eta muskuluan

Azkenik, burmuina eta muskuluan, orokorrean, ez zen inolako konposaturik ikusi, 4*t*OParen salbuespenarekin burmuinean. Esposizioko 1. egunean, kontrolean baino kontzentrazio apur bat altuagoa kuantifikatu zen, 1.291 (± 166) ng/g eta 1.011 (± 93) ng/g esposizioko tankean eta kontrolekoan, hurrenez hurren. Ferreira-Leach eta lankideen arabera [39], 4*t*OP konposatuaren metaketa-prozesua amuarrainaren (*Oncorhynchus mykiss*) muskuluan eta burmuinean geldoa zen eta ez oso esanguratsua. Amuarrainak, 4*t*OP konposatuaren 4 ng/mL-ko etengabeko kontzentraziora esposatu zituzten, eta hainbat ehunek (behazuna, plasma eta gibela, besteak beste) 4. egunerako oreka lortu zuten bitartean, burmuina eta muskulua ez ziren orekara heldu 10 egun igaro ostean. Gure kasuan, ia 30 aldiz

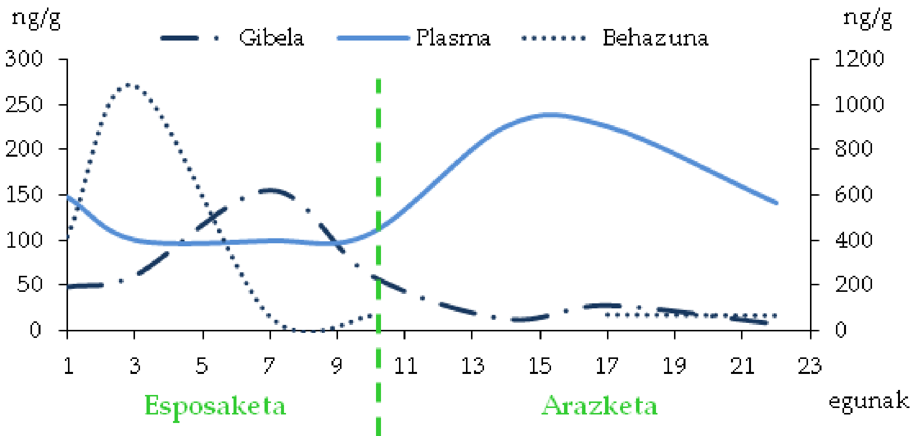
kontzentrazio baxuagoa izanik, ulertzekoa da konposatuak muskuluan eta burmuinean ez metatzea.

3.4. Konposatuen metaketa egunetan zehar

Konposatuen metaketa atal desberdinetan aztertu ostean, haien metaketa-egun desberdinetan zehar aztertu zen. 3.2. atalean aipatutakoaren arabera, BPA hartuko da eredutzat bera izan baitzen uretan disolbatuta egon zen konposatu bakarra esposizio-egun guztietan. **8.7 irudian** ikus daiteken moduan, BPA behazunean metatu zen lehenengo, hau baita konposatu arrotzak gorputzetik kanporatzeko bide nagusiena [21, 36]. Horretaz gain, kontzentraziorik altuenak ere hemen aurkitu ziren, plasma eta gibelean baino 6 eta 8 aldiz gehiago, hurrenez hurren. Esposizio egunak aurrera joan ahala, lehen aipatu den moduan, arrainaren metabolismoa kaltetzen joan zen eta ondorioz badirudi gibela ez zela gai izan EDCak behazunetik kanporatzeko eta ondorioz gibelean metatu ziren esposizioko 7. egunean maximo bat lortuz. Hala ere, aipagarria da kontzentraziorik baxuenak (behazun, gibel eta plasmaren artean) gibelean aurkitu izana. Bibliografiako lan askotan aipatu izan da, behazunaren ostean gibela dela konposatuak kontzentrazio altuenean metatzen dituen organoa [36, 39]. Gure esperimentuan, ordea, gibelaren metabolismoak ez zuen behar bezala funtzionatu eta ondorioz ez zen gai izan konposatuak behazunetik kanporatzeko. Hala ere, posiblea izan daiteke gibelak BPA metabolizatu izana eta nagusiki glukuronido moduan aurkitzea gibelean, hori baita kanporatze modurik nagusiena [23, 39]. Gibel-laginetan hidrolisia egitearen beharra, zenbait egilek [40, 41] eztabaidatu izan duten kontua da, eta hala ere, zaila da ondorio garbi batera heltzea. Bibliografian oinarrituz lan honetan inolako hidrolisirik ez egitea erabaki zen. Gibelean antzemandako metabolismo arazoak ikusita, agian, analitoak glukuronido eran egon zitezkeen, egoera arruntean baino frakzio handiagoan eta, horregatik, EDC askeen kontzentrazioa, espero zitekeena baino txikiagoa da. Berriro ere, hipotesi hori baieztatzeko, lan sakonago bat egin beharko litzateke.

Azkenik, behin esposizio egunak amaituta, eta arrainaren metabolismoa kaltetzen joan ahala, badirudi gibela ez zela gai izan konposatuen metaketarekin jarraitzeko eta plasmara igaro zirela, haien metatze-maximoa 14 eta 17 egunen

artean baitago. **8.7 irudia** zehaztasunez begiratuta, badirudi 7. egunean gibelean zegoen BPA guztia plasmara igaro zela 14 eta 17. egunetarako, BPAren batura plasman eta gibelean 7., 14. eta 17. egunetan oso antzekoa baita (240-255 ng/g).



8.7 irudia: *BPA konposatuaren metaketa esposizio eta arazketa-garaian zehar. Gibela eta plasma laginak ezkerreko Y ardatzean daude irudikaturik eta behazun lagina eskumako Y ardatzean. 1-10 egunak esposatutako garaiari dagozkio eta 11-22 egunak arazketa garaiari.*

4. ONDORIOAK

Disruptore endokrinoak diren 4 konposaturen metaketa aztertu zen heldugabeko lupien atal desberdinetan (behazuna, gibela, plasma, burmuina eta muskulua). Nahiz eta lan honetan erabilitako kontzentrazioa baxua izan, antzeko esperimentutan erabiltzen denaren balioekin alderatuta [36, 39, 42, 43], badirudi nahikoa izan zela azterturiko arrainetan hainbat kalte eragiteko, eta ondorioz analitoen metaketa ez zen espero zena izan. Posiblea da erabilitako konposatuen nahasketak toxikotasuna handitu izana eta, nahiz eta kontzentrazio baxuak izan, nahikoa izatea heldugabeko arrainetan kalteak eragiteko. Kalte horien ondorioz, badirudi arrainak euren defentsa mekanismoa martxan jarri zutela, eta horren ondorioz 3. egunetik aurrera arrainak ez zirela kontzentraziora teorikora (150 ng/L) esposatuak egon 4*t*OP, DES eta E2 analitoentzako, haien disolbagarritasuna uretan baxua dela eta.

Lan honen garapenean zehar hainbat arazo sortu ziren arren, hiru ondorio atera daitezke garbi. Lehenengo ondorioa esposizioko 1-3. egunei dagokie. Egun horietan zehar esperimentuak aurretiaz pentsatutakoaren arabera funtzionatu zuen eta argi ikusi zen behazuna dela EDCak metatu eta kanporatzeko ardura duen fluidoa.

Bigarren ondorioa, aldiz, 4. egunetik aurrera BPA konposatuarekin atera daitekeena da. Arrainaren metabolismoak ondo funtzionatuko balu, BPA behazunean metatuko litzateke lehenengo, eta ondoren gibelean. Gibelaren metabolismoa kaltetzen joan ahala, gibeleko BPA guztia plasmara igaro delako susmoa dago. Konposatu arrotzak kanporatzeko ohiko bideak ez badu funtzionatzen, arrainak beste mekanismo batzuk bilatu behar ditu.

Azkenik, lan honetatik ateratako 3. ondorioa arrainaren metabolismoaren kaltetzeari dagokio. Nahiz eta 150 ng/L oso kontzentrazio baxua iruditu, badirudi arrainaren metabolismoa kaltetzeko nahikoa izan daitekeela. Hainbat lanetan aipatu izan da konposatu organikoak ng/L-mg/L aurkitu izan direla ingurumenean [10, 44-46], beraz, oso garrantzitsua da ur-ekosistemak ahalik eta garbien mantentzea arrainen populazioetan kalteak saihesteko eta noski, arrainak kutsatzaileen esposizio jarrai batera esposatuak ez egotea.

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Chapter 9

Conclusions

This chapter summarises the main conclusions from the present PhD thesis.

Four analytical methods were optimised and validated for the determination of different endocrine disrupting compounds (EDCs), such as alkylphenols (APs), bisphenol-A (BPA), some hormones, musk fragrances, pesticides and phthalates esters (PEs) and some of their metabolites, in aqueous samples (estuary and wastewater treatment plant effluents, WWTP effluents) and biota liquid and solid (fish bile, plasma, muscle, liver and brain) samples.

Different extraction techniques based on sorptive extraction on polymeric sorbents were optimised, validated and applied to real samples during this thesis. Solid phase extraction (SPE) as a well-established technique and the use of low cost commercial polymers such as polyethersulfone (PES) which has received less attention, have been implemented here.

As an alternative to SPE, PES proved to be an excellent material for the microextraction of target analytes in different aqueous samples. Although, lower extraction efficiency values were obtained compared with the SPE-extraction efficiencies, the use of organic solvents was minimal (300-500 μL for desorption) and the robustness of the technique was demonstrated in several liquid (estuary, effluents and fish bile) matrices. Despite requiring long extraction times and the lack of an exhaustive procedure, its simplicity allows the simultaneous performance of multiple extractions at a very low cost. The scarcely affection of the yield in the whole procedure by the type of water, was also one of the main important advantages of the PES methods. In the case of fish bile matrix, the developed PES procedure was a good alternative to the traditional combination of classical SPE protocols and long (more than 16 hours) enzymatic hydrolysis procedures usually employed to monitor EDCs in the aquatic environment.

In addition, APs, BPA and hormones were quantified by means of gas chromatography coupled to mass spectrometry (GC-MS) due to its simplicity compared to liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) systems, and LC-MS/MS was not able to measure NP mix with good quality in terms of precision. Thus, even if the sample preparation was a bit more

tedious (need of a derivatisation step) GC-MS analysis was the best target analytes option here.

In the case of solid matrices, the use of focused ultrasound solid-liquid extraction (FUSLE) was selected since this exhaustive technique offered a simple extraction procedure requiring a low (0.1–0.5 g) amount of sample, solvent (10 mL) and a short extraction time (5 min). However, since this extraction technique is not selective a clean-up step was necessary. In this sense, as an alternative to the traditional SPE, dispersive solid phase extraction (dSPE) was applied for fish muscle, liver and brain extracts clean-up step. This low-cost clean-up strategy provided not only the time saving and the use of a small amount (1-2 mL) of organic solvents, but also the opportunity to mix in an easy way sorbents with different nature. The use of FUSLE combined with dSPE provides good apparent recoveries for all the studied analytes, except for mono-2-ethylhexyl phthalate (MEHP) metabolite in the case of liver. However, this contaminant was found at high concentrations in environmental samples and even the low recovery allowed us its determination in real fish samples.

Once the analytical methods were developed, a biomonitoring study of thicklip grey mullets (*Chelon labrosus*) collected at five different estuaries and harbours along the Basque coast was carried out. It has been demonstrated that not only effluents and estuary but also fish samples are clearly polluted with EDCs, particularly by those related to known endocrine disruption activity. The results might indicate a relationship between the analytes concentrations reported in water samples and the number of intersex fish present in some of the studied sampling sites. A relation between bioconcentration factors (BCFs) and intersex condition was also studied, however, no conclusive data was obtained and a deeper study would be necessary. Besides, uptake evaluation of several EDCs by different fish species (*Chelon Labrosus* and *Dicentrarchus labrax*) under laboratory conditions, using both natural contaminated WWTP effluents and fortified seawater (4-*tert*-octylphenol (4*t*OP), BPA, diethylstilbestrol (DES) and 17 β-estradiol (E2)) were also performed.

In one hand, the uptake of BPA, hormones, musk fragrances, APs and PEs was demonstrated in the bile. No clear conclusions were found between dilutions, and slightly higher concentrations were found on the day 10 comparing to day 3. Together with the fish uptake evaluation, different polymeric materials were also tested to assess the bioconcentration of fish. In the case of PES tubes, more studies are necessary until they can be fully used for biomonitoring purposes, but some promising results were achieved in the present work. The use of polymeric materials for uptake simulation could facilitate preliminary experiments since the uptake and monitorisation studies are laborious. Even, further evaluation is compulsory in order to predict the BCFs from living organism.

On the other hand, the uptake of several EDCs in European seabass demonstrated that the bile was the fluid with the highest accumulation followed by plasma and liver, and in a less extends muscle and brain. Even if EDCs concentrations of 150 ng/L seem to be a very low concentration level, it could be enough to damage the fish's metabolism. Even though, further studies are needed in order to confirm that hypothesis.

Appendix / Eranskinak

Appendix I: *Chemical characteristics of the target compounds including name, acronym, chemical structure, CAS number, supplier, purity and log k_{ow}.*

I. eranskina: *Konposatu kimikoen ezaugarri kimikoak: izena, laburdura, egitura kimikoa, CAS zenbakia, hornitzailea, purutasuna eta lok K_{ow}.*

	Name Izena	Acronym Laburdura	Chemical structure Egitura kimikoa	CAS Number CAS zenbakia	Supplier Hornitzailea	Purity Purutasuna	Log K_{OW} Log K_{ou}
Alkylphenols / Alkilfenolak	4-*tert*-octylphenol 4-tert-oktilfenola	4*t*OP	H_3C, CH_3, CH_3, CH_3, H_3C, HO	140-66-9	Supelco	99.4	5.28
	Nonylphenol technical mixture Nonilfenol nahastea	NP mix	CH_3, HO	104-40-5	Riedel-de Häen	94.0	5.76
	4*n*-octylphenol 4*n*-oktilfenola	4*n*OP	CH_3, HO	1806-26-4	Sigma-Aldrich	99.0	4.12
	[$^2H_4$]-nonyl phenol [$^2H_4$]-nonil fenola	[$^2H_4$]-NP	D, D, D, D, CH_3, HO	358730-95-7	Sigma-Aldrich	97.0	

Appendix I: *Continued* / **I. eranskinaren** *jarraipena.*

	Name Izena	Acronym Laburdura	Chemical structure Egitura kimikoa	CAS Number CAS zenbakia	Supplier Hornitzailea	Purity Purutasuna	Log K_{OW} Log K_{ou}
Musk fragrances / Musketa fragantziak	Galaxolide Galaxolidea	HHCB		1222-05-5	Dr. Ehrenstorfer	100	5.9
	Tonalide Tonalidea	AHTN		21145-77-7	Dr. Ehrenstorfer	100	5.7
	[$^2H_{15}$]-musk xylene [$^2H_{15}$]-musk xilenoa	[$^2H_{15}$]-MX		877119-10-3	Supelco	100	

Appendix I: *Continued* / **I. eranskinaren** *jarraipena.*

	Name / Izena	Acronym / Laburdura	Chemical structure / Egitura kimikoa	CAS Number / CAS zenbakia	Supplier / Hornitzailea	Purity / Purutasuna	Log K_{OW} / Log K_{ou}
Pesticides / Pestizidak	α–hexachloro cyclohexane α–hexakloro ziklohexanoa	α-HCH		319-84-6	Dr. Ehrenstorfer	99.0	3.72
	β-hexachloro cyclohexane β–hexakloro ziklohexanoa	β-HCH		319-85-7	Dr. Ehrenstorfer	99.0	3.78
	γ-hexachloro cyclohexane γ–hexakloro ziklohexanoa	γ-HCH		58-89-9	Dr. Ehrenstorfer	99.0	3.72
	δ–hexachloro cyclohexane δ–hexakloro ziklohexanoa	δ-HCH		319-86-8	Dr. Ehrenstorfer	99.0	4.14

Appendix I: *Continued* / **I. eranskinaren** *jarraipena.*

	Name / Izena	Acronym / Laburdura	Chemical structure / Egitura kimikoa	CAS Number / CAS zenbakia	Supplier / Hornitzailea	Purity / Purutasuna	Log K_{OW} / Log K_{ou}
Pesticides / Pestizidak	Chlorpyrifos Klorfirifos	Chlorpyrifos		2921-88-2	Fluka	99.9	4.96
	3,5,6-trichloro-2-pyridinol 3,5,6-trikloro-2-piridinola	TCP (Chlorpyrifos metabolite)		6515-38-4	Dr.Ehrenstorfer	99.0	3.21
	Chlorfenvinphos Klorfenbinfos	Chlorfenvinphos		470-90-6	Fluka	97.3	3.81
	α-2,4′-trichloro acetophenone α-2,4′-trikloro azetofenona	TCA (Chlorfenvinphos metabolite)		4252-78-2	Dr.Ehrenstorfer	99.0	3.22

Appendix I: *Continued* / **I. eranskinaren** *jarraipena*.

	Name Izena	Acronym Laburdura	Chemical structure Egitura kimikoa	CAS Number CAS zenbakia	Supplier Hornitzailea	Purity Purutasuna	Log K_{OW} Log K_{ou}
Pesticides / Pestizidak	2,4′-bis (p-chlorophenyl) ethane 2,4′-bis (p-klorofenil) etanoa	2,4′-DDD		53-19-0	Dr.Ehrenstorfer	99.0	5.87
	2,2′-bis (p-chlorophenyl) ethane 2,2′-bis (p-klorofenil) etanoa	4,4′-DDD		72-54-8	Dr.Ehrenstorfer	99.0	6.02

Appendix I: *Continued* / **I. eranskinaren** *jarraipena*.

	Name / Izena	Acronym / Laburdura	Chemical structure / Egitura kimikoa	CAS Number / CAS zenbakia	Supplier / Hornitzailea	Purity / Purutasuna	Log K_{OW} / Log K_{ou}
Pesticides / Pestizidak	1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl] 1-kloro-2-[2,2,2-trikloro-1-(4-klorofenil)etiloa]	2,4′-DDT		789-02-6	Dr.Ehrenstorfer	99.0	6.79
	1,1′-(2,2,2-trichloroethylidene) bis[4-chloro] 1,1′-(2,2,2-trikoroethilideno) bis[4-kloro]	4,4′-DDT		50-29-3	Dr.Ehrenstorfer	99.0	6.79
	[$^2H_8$]-1,1′-(2,2,2-trichloroethylidene) bis(4-chloro) [$^2H_8$]-1,1′-(2,2,2-trikloroetilideno) bis(4-kloroa)	[$^2H_8$]-4,4′-DDT		50-29-3	Supelco	100	

Appendix I: *Continued* / **I. eranskinaren** *jarraipena*.

	Name Izena	Acronym Laburdura	Chemical structure Egitura kimikoa	CAS Number CAS zenbakia	Supplier Hornitzailea	Purity Purutasuna	Log K_{OW} Log K_{ou}
Bisphenol-A /Bisfenol-A	Bisphenol-A Bisfenol-A	BPA	H_3C, CH_3, HO, OH	80-05-7	Sigma-Aldrich	99.0	3.32
	[$^2H_{16}$]-bisphenol-A	[$^2H_{16}$]-BPA	D_3C, CD_3, D, DO, OD	96210-87-6	Supelco	99.9	

Appendix I: *Continued* / **I. eranskinaren** *jarraipena*.

	Name Izena	Acronym Laburdura	Chemical structure Egitura kimikoa	CAS Number CAS zenbakia	Supplier Hornitzailea	Purity Purutasuna	Log K_{OW} Log K_{ou}
Estrogens / Estrogenoak	17 β-estradiol 17 β-estradiola	E2	HO, CH_3, OH	50-28-2	Sigma-Aldrich	100	4.01
	17 α-ethynilestradiol 17 α-etinilestradiola	EE2	HO, CH_3, OH	57-63-6	Riedel-de Häen	99.4	3.67
	Diethylstilbestrol Dietilstilbestrola	DES	HO, OH	56-53-1	Sigma-Aldrich	99.9	5.64

Appendix I: *Continued* / **I. eranskinaren** *jarraipena.*

	Name / Izena	Acronym / Laburdura	Chemical structure / Egitura kimikoa	CAS Number / CAS zenbakia	Supplier / Hornitzailea	Purity / Purutasuna	Log K_{OW} / Log K_{ou}
Estrogens / Estrogenoak	17 β-estradiol-glucuronide 17 β-estradiol-glukuronidoa	E2-G		15087-02-2	Sigma-Aldrich	100	2.29
	[2H3]-17 β-estradiol [2H3]-17 β-estradiola	[2H3]-E2		79037-37-9	Sigma-Aldrich	98.0	

Appendix I: *Continued* / **I. eranskinaren** *jarraipena.*

	Name Izena	Acronym Laburdura	Chemical structure Egitura kimikoa	CAS Number CAS zenbakia	Supplier Hornitzailea	Purity Purutasuna	Log Kow Log Kou
Phthalate esters / Ftalato esterrak	*n*-butyl benzyl phthalate *n*-butil bentzil ftalatoa	BBP		85-68-7	Supelco	96	4.73
	Mono benzyl ester Mono bentzill esterra	MBzP (BBP metabolite)		2528-16-7	Dr.Ehrenstorfer	91.0	3.07
	Bis (2-ethylhexyl) phthalate Bis (2-etilhexil) ftalatoa	DEHP		117-81-7	Supelco	99	7.60
	Mono-2-ethylhexyl ester Mono-2-etillhexil esterra	MEHP (DEHP metabolite)		4376-20-9	Dr.Ehrenstorfer	98.5	4.73

Appendix I: *Continued* / **I. eranskinaren** *jarraipena*.

	Name / Izena	Acronym / Laburdura	Chemical structure / Egitura kimikoa	CAS Number / CAS zenbakia	Supplier / Hornitzailea	Purity / Purutasuna	Log K_{OW} / Log K_{ou}
Phthalate esters / Ftalato esterrak	Di-*n*-octyl phthalate Di-*n*-oktil ftalatoa	DOP	O, O, CH3, CH3	117-84-0	Supelco	99	8.10
	Mono-*n*-octyl ester Mono-*n*-oktil esterra	MOP (DOP metabolite)	H3C, O, O, OH	5393-19-1	Dr.Ehrenstorfer	100	4.80
	[$^2H_4$]-bis-(2-ethylhexyl) phthalate [$^2H_4$]-bis-(2-etilhexil) ftalatoa	[$^2H_4$]-DEHP	D, D, D, D, O, O, CH3, CH3, CH3, CH3	93951-87-2	Sigma-Aldrich	98.9	

Appendix II: *Monitored ions in GC-MS analysis in SIM mod.*

II. eranskina: *GC-MS analisietan jarraituriko ioiak SIM moduan.*

Analyte Analitoa	Quantifier transition Kuantifikazio-trantsizioa	Qualifier transition Detekzio-trantsizioa
4*t*OP	135[a] / 207[b]	206[a] /208[b]
NP mix	135[a] /207[b]	107[a] /208[b]
4*n*OP	107[a] /179[b]	206[a] /278[b]
$[^2H_4]$-NP	111[a] /183[b]	---
HHCB	243	258
AHTN	243	258
$[^2H_{15}]$-MX	294	---
α-HCH	181	183
β-HCH	181	183
γ-HCH	181	183
δ-HCH	181	183
Chlorpyrifos	199	197
Chlorfenvinphos	267	323
2,4′-DDD	235	237
4,4′-DDD	235	237
2,4′-DDT	235	237
4,4′-DDT	235	237
TCP	256	254
TCA	173	188
$[^2H_8]$-4,4′-DDT	243	---
BPA	357	358
$[^2H_{16}]$-BPA	368	---
E2	416	285
EE2	425	440
$[^2H_3]$-E2	419	---
BBP	149	206
MBzP	91	179
DEHP	149	167
MEHP	104	149
DOP	149	207
MOP	223	239
$[^2H_4]$-DEHP	153	---

[a]: ions followed in non derivatised samples and [b]: ions followed in the derivatized samples. /

[a]: deribatizatu gabeko laginetan jarraitutako ioiak [b]: deribatzitatutako laginetan jarraitutako ioiak.

Appendix III: *Qualifier and quantifier transitions, fragmentor (V), collision energy (eV), polarity and cell voltage (V) followed in the LC-MS/MS method.*

III. eranskina: *LC-MS/MS analisietan erabilitako baldintzak: kuantifikazio eta identifikazio trantsizioak, fragmentorra (V), talka-energia(eV), polaritatea eta gelaxka-boltaia (V).*

Analyte Analitoa	Transition Trantsizioa	Fragmentor (V) Fragmentorra (V)	Collision energy (eV) Talka energia (eV)	Polarity Polaritatea	Cell voltage (V) Gelaxka-boltaia (V)
4tOP	205.2>133.1[a]	105	25	-	3
	205.2>134.2[b]	120	15	-	3
4nOP	205.2>106.0[a]	135	17	-	1
	205.2>119.0[b]	120	20	-	1
NP mix	219.0>133.0[a]	120	25	-	4
	219.0>147.4[b]	120	25	-	4
BPA	227.1>212.1[a]	100	13	-	5
	227.1>133.0[b]	100	25	-	5
DES	267.1>251.1[a]	135	21	-	4
	267.1>237.1[b]	135	25	-	4
E2	271.2>145.1[a]	135	41	-	3
	271.2>183.2[b]	135	41	-	3
EE2	295.2>199.1[a]	110	41	-	3
	294.9>269.0[b]	81	25	-	3
MEHP	277.1>134.0[a]	75	9	-	1
	277.1>127.1[b]	75	9	-	1
[$^{2}H_{4}$]-NP	223.2>110.1[a]	114	17	-	1
[$^{2}H_{16}$]-BPA	241.3>142.0[a]	120	25	-	3
[$^{2}H_{3}$]-E2	274.2>145.0[a]	150	41	-	1

[a]: quantifier transition and [b]: qualifier transition / [a]: kuantifikazio-trantsizioa eta [b]: identifikazio-trantsizioa

Appendix IV: Gender, size and weight of every fish captured along the Basque Coast.

IV. eranskina: Euskal kostaldean jasotako arrainen generoa, tamaina eta pisua.

						GERNIKA							
Fish / Arraina	M-1	M-2	M-3	M-4	I-1	I-2	I-3	I-4	F-1	F-2	F-3	F-4	F-5
Sex / generoa	Male Arra	Male Arra	Male Arra	Male Arra	Intersex Intersexa	Intersex Intersexa	Intersex Intersexa	Intersex Intersexa	Female Emea	Female Emea	Female Emea	Female Emea	Female Emea
Size / luzera(cm)	34	42.5	35.5	38	33	36	32	36	33	34.5	38	33	36
Weight / pisua (g)	563	929	530	672	480	604	454	562	469	637	751	426	580
						DEBA							
Fish / Arraina	M-1	M-2	M-3	M-4	M-5	I-1	I-2	F-1	F-2	F-3	F-4	F-5	
Sex / generoa	Male Arra	Male Arra	Male Arra	Male Arra	Male Arra	Intersex Intersexa	Intersex Intersexa	Female Emea	Female Emea	Female Emea	Female Emea	Female Emea	
Size / luzera(cm)	32.5	31	36	32.5	37	35	34.5	40.5	33	33	37.5	36.5	
Weight / pisua (g)	499	408	644	425	686	579	584	919	545	500	688	661	
						ONDARROA							
Fish / Arraina	M-1	M-2	M-3	M-4	M-5	F-1	F-2	F-3	F-4	F-5			
Sex / generoa	Male Arra	Male Arra	Male Arra	Male Arra	Male Arra	Female Emea	Female Emea	Female Emea	Female Emea	Female Emea			
Size / luzera(cm)	32	29.5	36	35.5	35	31	30.5	25.5	25.5	31.5			
Weight / pisua (g)	550	480	654	551	515	461	531	264	267	535			
						PASAIA							
Fish / Arraina	M-1	M-2	M-3	M-4	M-5	I-1	F-1	F-2	F-3				
Sex / generoa	Male Arra	Male Arra	Male Arra	Male Arra	Male Arra	Intersex Intersexa	Female Emea	Female Emea	Female Emea				
Size / luzera(cm)	34	31	31	31.5	26	22.5	23	32	38				
Weight / pisua (g)	677	562	573	541	314	241	258	596	1111				
						SANTURTZI							
Fish / Arraina	M-1	M-2	F-1	F-2	F-3	F-4	F-5						
Sex / generoa	Male Arra	Male Arra	Female Emea	Female Emea	Female Emea	Female Emea	Female Emea						
Size / luzera(cm)	34	35.5	37.5	37	32	39	37.5						
Weight / pisua (g)	563	558	618	668	441	775	745						

Appendix V: *Biococentration factor values log BCF (C_{bile}/C_{water}) of all the analytes in each location.*

V. eranskina: Kokaleku *bakoitzean jasotako arrainen log BCF ($K_{behazuna}/K_{ura}$) konposatu guztientzako.*

Location Kokalekua	Sample Lagina	Analyte / Analitoa										
		4*t*OP	NP mix	HHCB	AHTN	BBP	DEHP	α-HCH	δ-HCH	γ-HCH	β-HCH	Chlorpyrifos
Santurtzi	M.1	nd	4.94	nd	nd	5.11	4.09	nd	nd	nd	nd	nd
	M.2	nd	4.93	nd	nd	5.55	4.15	nd	nd	nd	nd	nd
	F.1	nd	nd	nd	nd	4.26	4.05	nd	nd	nd	nd	nd
	F.2	nd	nd	nd	nd	4.33	4.04	nd	nd	nd	nd	nd
	F.3	nd	nd	3.86	nd	4.35	4.05	nd	nd	nd	nd	nd
	F.4	nd	nd	nd	nd	4.54	4.01	nd	nd	nd	nd	nd
	F.5	nd	4.35	nd	nd	4.24	4.07	nd	nd	nd	nd	nd
Ondarroa	M.1	nd	nd	nd	nd	5.32	3.91	nd	nd	nd	nd	nd
	M.2	nd	nd	nd	nd	nd	3.88	3.94	4.59	nd	nd	nd
	M.3	nd	nd	nd	nd	4.69	4.04	nd	nd	nd	nd	nd
	M.4	nd	nd	3.58	nd	4.74	4.01	nd	3.65	nd	nd	nd
	M.5	nd	nd	nd	nd	nd	3.82	3.06	nd	nd	nd	nd
	F.1	nd	nd	nd	nd	3.97	3.03	nd	nd	nd	nd	2.64
	F.2	nd	nd	nd	nd	4.08	3.00	nd	nd	nd	nd	2.52
	F.3	nd	nd	nd	nd	4.23	3.07	nd	nd	nd	nd	2.62
Deba	M.1	nd	4.99	nd	nd	5.01	4.16	nd	nd	nd	nd	nd
	M.2	3.70	4.84	nd	nd	5.17	4.41	nd	nd	nd	nd	nd
	M.3	nd	4.70	nd	nd	5.04	4.18	nd	nd	nd	nd	nd
	M.4	3.78	4.79	nd	nd	4.89	4.28	nd	nd	nd	nd	nd
	M.5	nd	4.90	nd	nd	nd	4.40	nd	nd	nd	nd	nd
	I.1	nd	4.74	nd	nd	5.05	4.49	nd	nd	nd	nd	nd
	I.2	nd	4.73	nd	nd	4.79	4.62	nd	nd	nd	nd	nd
	F.1	3.88	nd	nd	nd	4.42	4.10	nd	nd	nd	nd	nd
	F.2	3.87	nd	nd	nd	4.33	4.10	nd	nd	nd	nd	nd
	F.3	3.88	nd	nd	nd	4.33	4.06	nd	nd	nd	nd	nd
	F.4	3.84	nd	nd	nd	4.45	4.05	nd	nd	nd	nd	nd
	F.5	3.87	nd	nd	nd	4.30	4.03	nd	nd	nd	nd	nd

nd: these BCFs are no calculated because the analytes were not detected in the samples / nd: BCF balio hauek ez zien kalkulatu analitoak ez zirelako laginetan antzeman..

Appendix V: Continued. *Biococentration factor values log BCF (C_{bile}/C_{water}) of all the analytes in each location.*

V. Eranskina: Jarraipena. *kokaleku bakoitzean jasotako arrainen log BCF ($K_{behazuna}/K_{ura}$) konposatu guztientzako.*

		Analyte										
Location Kokalekua	Sample Lagina	4*t*OP	NP mix	HHCB	AHTN	BBP	DEHP	α-HCH	δ-HCH	γ-HCH	β-HCH	Chlorpyrifos
Gernika	M.1	nd	nd	2.60	nd	4.74	nd	nd	nd	nd	nd	nd
	M.2	nd	nd	2.54	nd	5.18	4.48	nd	nd	nd	nd	nd
	M.3	nd	nd	2.65	nd	nd	nd	nd	nd	nd	nd	nd
	M.4	nd	nd	2.65	nd	nd	nd	nd	nd	3.02	nd	nd
	I.1	nd	nd	2.63	nd	5.04	nd	3.52	nd	nd	nd	nd
	I.2	nd	nd	2.76	nd	4.89	nd	2.42	3.18	nd	nd	nd
	I.3	nd	nd	2.46	nd	4.97	nd	nd	nd	nd	nd	nd
	I.4	nd	nd	2.69	nd	4.81	nd	nd	3.12	nd	nd	nd
	F.1	nd	nd	2.44	nd	3.93	2.56	nd	nd	nd	nd	3.31
	F.2	nd	nd	2.12	nd	4.22	2.51	nd	nd	nd	nd	nd
	F.3	nd	nd	2.14	nd	4.00	2.60	nd	nd	nd	nd	3.23
	F.4	nd	nd	2.24	nd	4.07	nd	nd	nd	nd	nd	3.48
Pasaia	M.1	nd	nd	3.60	nd	4.71	nd	nd	nd	nd	nd	nd
	M.2	nd	nd	2.95	nd	nd	nd	nd	nd	nd	nd	nd
	M.3	nd	nd	2.94	nd	4.79	nd	nd	nd	nd	nd	nd
	M.4	nd	nd	3.47	nd	4.98	nd	3.46	nd	nd	nd	nd
	M.5	nd	nd	3.30	nd	nd	nd	nd	nd	nd	nd	nd
	I.1	nd	nd	3.11	nd	4.65	nd	nd	nd	nd	nd	nd
	F.1	nd	nd	3.19	1.69	4.05	2.43	nd	nd	nd	nd	3.38
	F.2	nd	nd	nd	nd	4.01	2.37	nd	nd	nd	nd	3.40
	F.3	nd	nd	nd	2.06	3.86	2.80	nd	nd	nd	nd	3.46

nd: these BCFs are no calculated because the analytes were not detected in the samples / nd: BCF balio hauek ez zien kalkulatu analitoak ez zirelako laginetan antzeman.