Universidad del País Vasco
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Kimika Analitikoa Saila

Development of Analytical Methods for the Determination of Alkylphenols and Estrogens in Environmental Samples

Report to complete for the European PhD degree

Asier Vallejo

2010

Universidad del País Vasco Euskal Herriko Unibertsitatea

AUTORIZACION DEL/LA DIRECTOR/A DE TESIS

PARA SU PRESENTACION

Las Dras. Aresatz Usobiaga con N.I.F. 15385374-f y Olatz Zuloaga con N.I.F. 2903297-v como Directoras de la Tesis Doctoral: "*Development of Analytical Methods for the Determination of Alkylphenols and Estrogens in Enviromental Samples*" realizada en el Departamento de Química Analítica por el Doctorando Don Asier Vallejo, autorizamos la presentación de la citada Tesis Doctoral, dado que reúne las condiciones necesarias para su defensa.

En Leioa a 18 de Octubre del 2010

LAS DIRECTORAS DE LA TESIS

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Fdo.: Olatz Zuloaga

Universidad del País Vasco Euskal Herriko Unibertsitatea

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El Consejo del Departamento de Química Analítica en reunión celebrada el día 18 de Octubre del 2010 ha acordado dar la conformidad a la admisión a trámite de presentación de la Tesis Doctoral titulada: "*Development of Analytical Methods for the Determination of Alkylphenols and Estrogens in Environmental Samples*" dirigida por las Dras Aresatz Usobiaga y Olatz Zuloaga y presentada por Don Asier Vallejo ante este Departamento.

En Leioa a 18 de Octubre del 2010

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ACTA DE GRADO DE DOCTOR ACTA DE DEFENSA

DE TESIS DOCTORAL

DOCTORANDO: DON ASIER VALLEJO

TITULO DE LA TESIS: "*Development of analytical methods for the determination of alkylphenols and estrogens in environmental samples*".

El Tribunal designado por la Subcomisión de Doctorado de la UPV/EHU para calificar la Tesis Doctoral arriba indicada y reunido en el día de la fecha, una vez efectuada la defensa por el doctorando y contestadas las objeciones y/o sugerencias que se le han formulado, ha otorgado por_____la calificación de:

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VOCAL 1º,

Dr/a: _____

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Dr/a: _____

Fdo.:

EL/LA DOCTORANDO/A,

Fdo.: Asier Vallejo Ruiz

Bizitza kausalitatez beteta dagoela esan daiteke. 1998. urtean Juan Cruzen proposamena baztertu izan banu, azken urteotako esperientzia hauts izango litzateke. Azkeneko 4 urteetako pasatutako bi orritan laburtzea eta eskerrak ematea zaia egiten zait, orri faltagatik.

4 urteak laburtzea erraza da, esperientzia primerakoa, bere gauza on eta txarrekin, baina esperientzia guztietatik zerbait ikasi daiteke.

Eskerrekin hasteko, nola ez, lehenengo (eskerrak)^{n} nire bi (*super)*^{n}*jefaei*, beraien laguntzagatik, osagarriak izateagatik eta noski nirekin izan duten pazientziagatik, ez aldatu inoiz. Itzalean egon diren bi "*jefei*" (*super* titulua oso garesti dago) ere eskerrak eman behar dizkiet, beti laguntza eskatu dunanean hor egoteagatik (Gracias también a mis dos *jefes* en la sombra, el titulo de *super* esta caro, por ayudarme siempre que os lo he pedido, para que no te cabrees Luis). Eta lankideen beste nagusiei ere laguntza eskatu denean arazorik gabe laguntzeagatik.

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Eta orain bai, azkenik bihotz bihotzeko esker handiena Estiri, beti hor egoteagatik eta tesia nire moduan edo gehiago sufritzeagatik.

For Titxu and for you, mum, dad and Ainara

And for me and for all my colleagues

Just for today, do not worry

Just for today, do not anger

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Progress, action of going ahead, advance, improvement [1].

The last century can be called the “Century of Progress”. The lifestyle of humanity changed drastically in that time, from horses to cars, from letters to internet, from death at 40 to life at 80. New technologies and new pharmaceuticals have given humanity an easier and longer life. But what is the price that should be paid? Industry, transport, pharmaceuticals and so on, have an impact in the environment. Important environmental phenomena such as the influence of industry and transport in the CO_2 flows are already known [2-5]. However, the influence of micro-contaminants such as pharmaceuticals, degradation (products) of chemicals products or steroids in the environment is not so well known.

Traditional contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and others have been studied over the past. However, in the last two decades the interest in pharmaceuticals, degradation chemical products or

steroids has increased because of their endocrine disruptor function. An endocrine disrupting compound or chemical (EDCs) is defined by The United States Environmental Protection Agency (USEPA) as an "*exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour*" [6]. The European Commission (1997) defined the endocrine disrupter as "*an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function. A potential endocrine disruptor is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism*" [7].

EDCs are believed to interfere with the functioning of the endocrine system in at least three (possible) ways [8]:

(1) by mimicking the action of a naturally-produced hormones such as estrogens or testosterone, and thereby setting off similar chemical reactions in the body;
(2) by blocking the receptors in cells receiving the hormones (hormone receptors), thereby preventing the action of normal hormones;
(3) by affecting the synthesis, transport, metabolism and excretion of hormones, thus, altering the natural concentrations of the hormones.

For this reason, The European Commission included 94 emerging substances with potential endocrine disrupting effect in the Water Framework Directive (WFD) [9]. This list includes EDCs such as veterinary and human antibiotics, prescription and nonprescription drugs, other wastewater-related compounds, steroids and hormones. Other EDCs such as alkylphenols (APs), tributyltin (TBT), PCBs, pesticides or phthalates are included as organic priority pollutants. Figure 1 illustrates the classification of environmental pollutants according to their legal regulations [10].

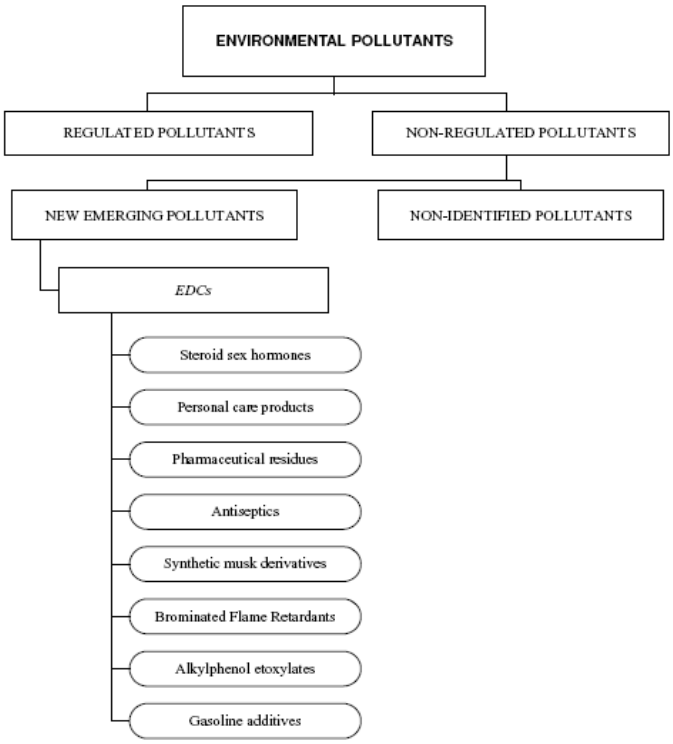


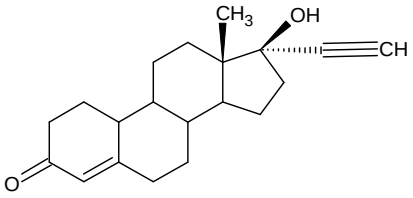
Figure 1: Classification of the environmental pollutants according to their legal regulations [10].

1. Importance of alkylphenols and estrogens in the environment

1.1. Estrogens

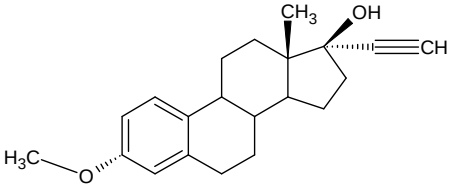
The 23rd of May of 2010, the 50th anniversary of one of the most important events that changed the life of many women, was celebrated in United States of America. The 9th of May of 1960 the United States Food and Drug Administrator (FDA) allowed the commercialization of a compound synthesized in 1951 by Luis Ernesto Miramontes in Mexico. This compound was the 19-norethindrone (Figure 2a). It was the first contraceptive pill. Although it was at first much criticized by doctors, religions or even banned in some countries, the pill has become the leading method for birth control and it is used by more than 100 million women over the world [11,12]. It was not until 1978 that it was commercially available in Spain and, although the condom is the most used contraceptive, the pill is situated in the second place (20.3 %) [13].

The active group of the contraceptive pills has changed from 1951. Mestranol (MeEE2, Figure 2b) was used after 19-norethindrone. This compound is demethylated and activated in the liver to produce 17α-ethynyl estradiol (EE2, Figure 2c). Nowadays, the most common contraceptive compound is EE2 in combination with different gestagenes (compounds that imitate the work of progesterone) [14].



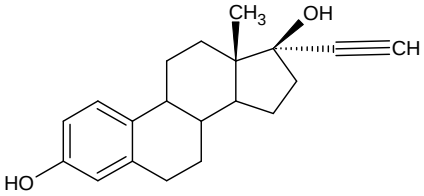
19-norethindrone

(a)



mestranol (MeEE2)

(b)



17α- ethynyl estradiol(EE2)

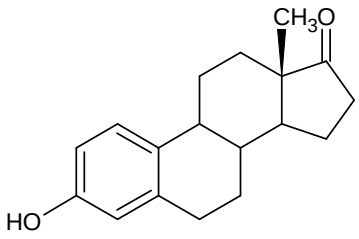
(c)

Figure 2: Structure of exogenous estrogens: (a) 19-norethindone, (b) mestranol and (c) 17α-ethynyl estradiol.

Although female exogenous hormones are used in contraceptive pills, the most useful application has been in animal farming as growth promoters and for the development of single-sex populations of fish in aquaculture, in the management of the menopausal and postmenopausal syndrome and in the treatment of prostatic and breast cancer [15-21].

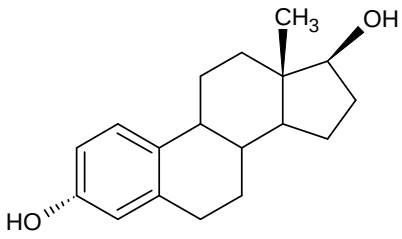
Apart from exogenous hormones, endogenous hormones are also included in WFD. The most important endogenous estrogens in

humans are estrone (E1), 17β-estradiol (E2) and estriol (E3) (Figure 3).



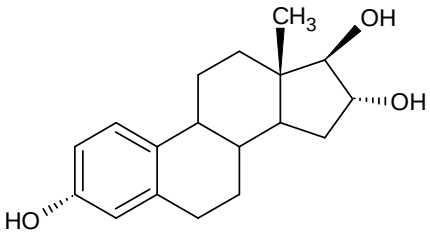
Estrone (E1)

(a)



17β- Estradiol(E2)

(b)



Estriol (E3)

(c)

Figure 3: Structure of endogenous estrogens: (a) estrone, (b) 17β-estradiol and (c) estriol.

E2, the most potent and important estrogen in non-pregnant women, is predominantly produced by the granulose cells of the active follicle from androgens delivered by the theca interna (a layer of the ovarian follicles) and circulates bound to albumin or to sex hormone-binding globulin. During pregnancy, E3, produced from androgenic precursors provided by the fetus and the mother, represents the major estrogen. E1 exists in metabolic equilibrium

with E2 due to the action of 17β-hydroxysteroid dehydrogenase. Table 1 summarizes some physical properties of estrogens.

Table 1: Different physical properties of endogenous and exogenous estrogens.

Abbrev.	CAS number	Mol Formula	PM (g/mol)	Melting Point (°C)	Water solubility (mg/L)	Log Kow
E1	53-16-7	$C_{18}H_{22}O_2$	270.37	260.2	30	3.13
E2	50-28-2	$C_{18}H_{24}O_2$	272.39	178.5	3.6	4.01
MeEE2	72-33-3	$C_{21}H_{26}O_2$	310.44	150.5	0.977	4.68
EE2	57-63-6	$C_{20}H_{24}O_2$	296.41	183	11.3	3.67
E3	50-27-1	$C_{18}H_{24}O_3$	288.39	282	441	2.45

Estrogens are biologically inactivated and excreted after glucosidation, sulfation or glucuronidation, respectively, allowing renal excretion of the inactivated steroids. Although considerable amounts of conjugated estrogens are excreted into the bile, only a small fraction appears in the feces. The majority of the conjugates are reabsorbed after hydrolysis by bacteria from the gastrointestinal tract. The amount of endogenous estrogens in female and male humans depends on the different stage of life [22].

As mentioned above, not all the dose administered in the treatment is metabolized by the body and, therefore, the analytes are excreted in urine as glucuronide, sulfate or glucoside derivates (Figure 4) and in feces as free compounds [23].

Although, women are one of the largest sources of estrogens, there is no doubt that farming is the largest one. Apart from women, E1, E2, E3 are one of the most important estrogens for vertebrates and the analysis of those compounds in urine or in manure have already been performed in order to determinate their impact in the environment [24-28].

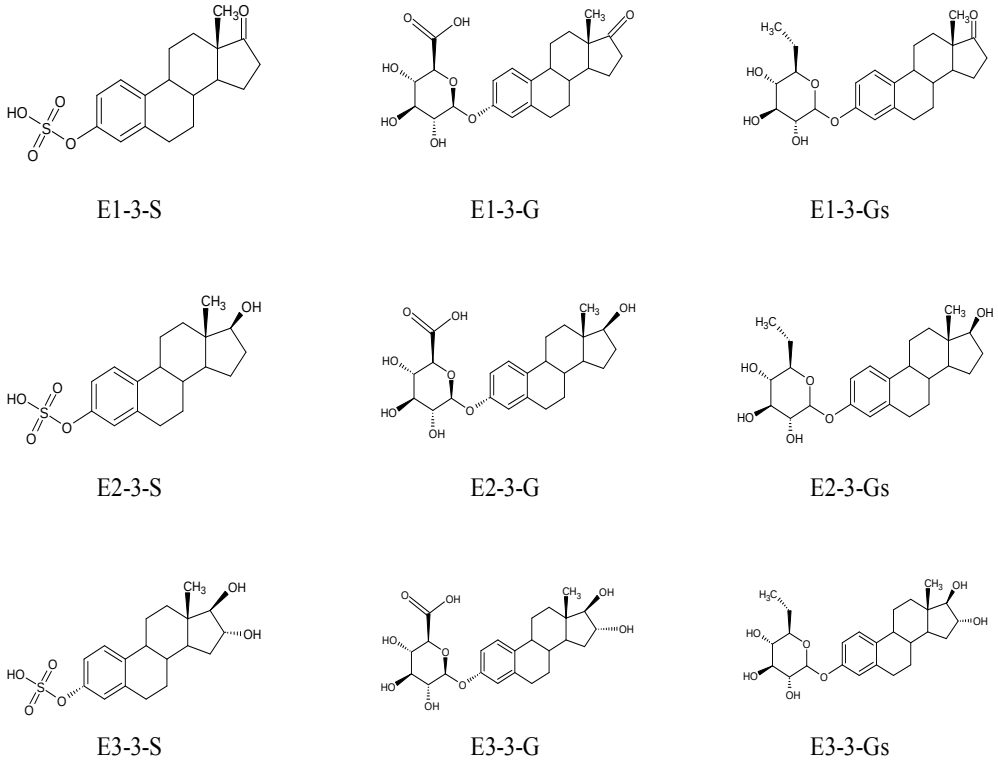


Figure 4: Examples of chemical structures of natural hormones in glucuronide, sulfate and glucoside form. E1-3-S: Estrone-3-sulfate; E1-3-G: Estrone-3 β-D-glucuronide; E1-3-Gs: Estrone-3-glucoside; E2-3-S: 17β estradiol-3-suphate; E2-3-G: 17β estradiol-3 β-D glucuronide; E2-3-Gs: 17β estradiol-3 β-D glucoside; E3-16-S: Estriol-16-sulfate; E3-3-G: Estriol-3 β-D glucuronide; E3-3-Gs: Estriol-3 β-D glucoside.

The conjugated forms are partially converted into the free compounds in sewer systems and in wastewater treatment plants (WWTPs), where the free and conjugate analytes are concentrated in the effluent [29-31] or the sludge [32-35]. Although new alternative techniques, such as activated sludge system for nitrification [36], use of brominated products in the ozonation [37], electrodialysis [38], mineralization [39], aerobic and anaerobic techniques [40] or ultrasounds [41] have been used in order to remove estrogens completely during wastewater treatment [42], WWTPs are the highest source of estrogens to the environment [15,43,44]. Figure 5 illustrates the different sources of estrogens in the environment [15].

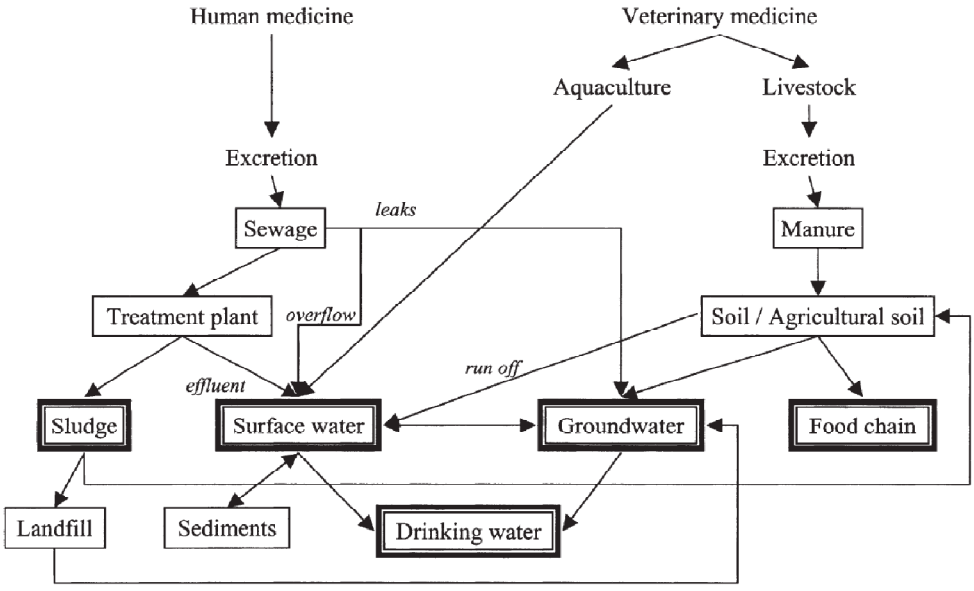


Figure 5: Different sources of estrogens in the environment [15].

Although these compounds are photo- [45,46] or biodegradated [47,48], their concentration in the effluents (free or conjugated form) is in the low ng/L range [49,50], high enough to disrupt the endocrine process. Feminization, intersex and reproductive performance problems are the most common processes that affect fish [51-69], amphibians [70], crustaceans [71-73], invertebrates [74,75], reptiles [76] and even mammals [77-79]. Normally, these compounds, after their oral or intra-peritoneallly insertion, are accumulated in bile [52,80-87], liver [64,88,89] or kidneys [90,91] in glucoside, glucuronide or sulfate form.

Apart from WWTP influents and effluents, estrogens have been determined in many matrices, such as sludge [15,43,92-94], ground water [95,96], sediments [15,43,94,97-101] and drinking water [23,102,103].

1.2. Alkylphenols

One of the other penalties that humanity must pay for progress is the ignorance of collateral effects. Obviously, when alkylphenol ethoxylates (APEOs) started to be used in industrial and household applications as detergents, emulsifiers, wetting and dispersing agents, antistatic agents, demulsifiers or solubilisers [104], nobody knew the effects of neither APEOs nor their degradation by-products. Among APEO degradation products we find nonyl - and octylphenols, NPs and OPs, respectively (see Table 2).

NPs and OPs are considered EDCs and has been designated as priority hazardous substances by the WFD [9]. Alkylphenols (APs) mimic the estrogen work and disrupt the endocrine system, femilizating males and producing abnormal reproductive performances in fish [105-107], oysters [108], rats [109], birds, plants, soil microorganisms [110,111] and mammals, humans included [109,112-114].

Once again, as in the case of estrogens, the main source of introduction of NPs and OPs to environment are the effluents of the WWTPs. Treatment plants degrade the APEOs to shorter chain NPs and OPs (Figure 6) [115], which are more stable and more toxic than their precursors [116]. APs have been determined in different matrices such as WWTPs effluents, rivers [117-120], ground water, drinking water [121,122] and even in baby food purees [123]. The concentration of these compounds in effluent or river water is higher than for estrogens, in the ng/L for OPs and μg/L level for NPs [117,120].

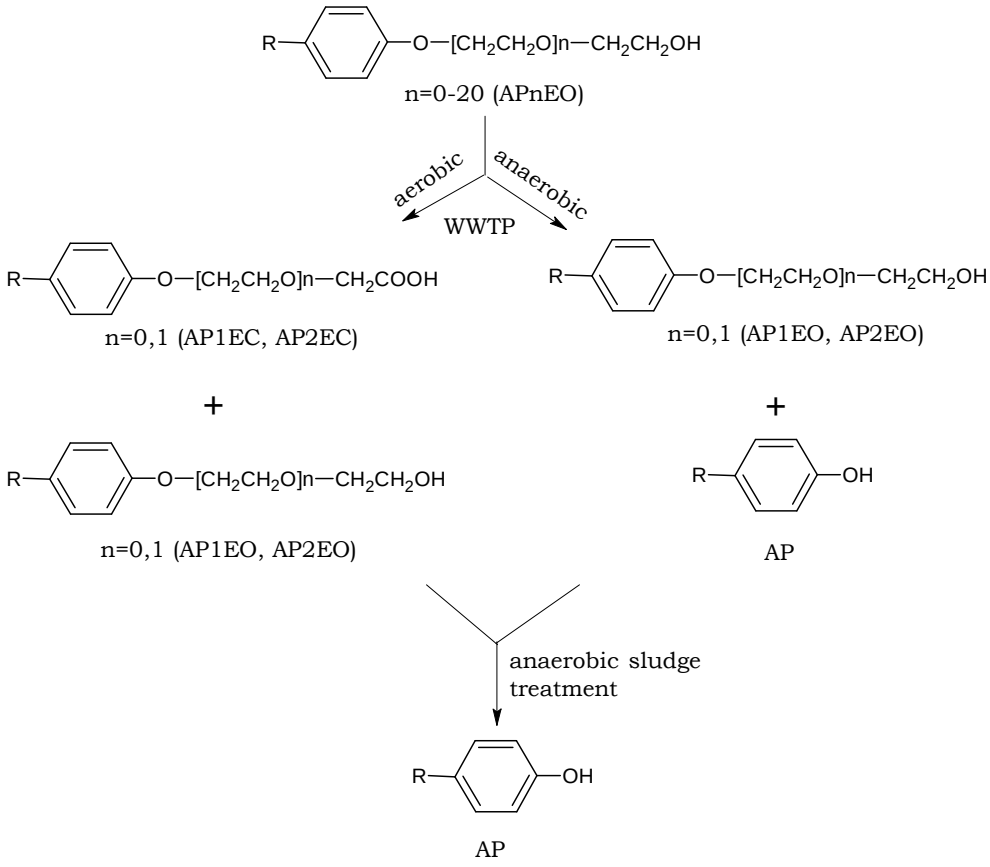
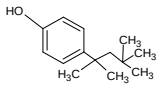
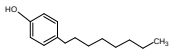
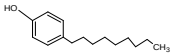
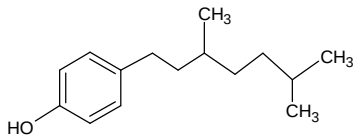


Figure 6: Aerobic and anaerobic biotransformation pathways of APEOs in WWTPs [115].

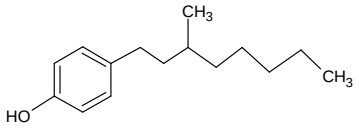
Table 2: Structure and physical properties of 4*tert*-octylphenol, 4n-octylphenol and 4-nonylphenol isomer mixture.

Compound	Abrev	Structrure	CAS-number	Mol Formula	PM g/mol	Melting Point °C	Water solubility mg/L	Log K_{ow}
4-*tert* octylphenol	4*t*OP		140-66-9	$C_{14}H_{22}O$	206.33	84.5	158	5
4n-octylphenol	4nOP		1806-24-4	$C_{14}H_{22}O$	206.33	44-45	12.6	4.12
4-nonylphenol Isomer mixture	4NP		72-33-3	$C_{15}H_{24}O$	220.36	295	-	5000

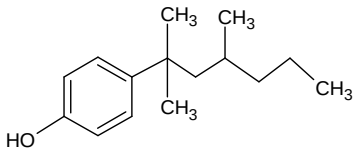
Commercially available NPs are constituted especially of *para*-substituted isomers. Although the total amount of *para*-nonylphenol isomers is around 550 [124], the number of branched isomers in *para*-nonylphenol technical mixtures is not clear, and it varies from 20 to 102 isomers depending on the authors [125-128]. Besides, it is already known that the estrogenicity of each isomer is different [125,129-134] and, thus, the identification and quantification of each single isomer is necessary. These single isomers are not commercially available therefore, the synthesis of these isomers is necessary. Some isomers of *para*-nonylphenol are shown in Figure 7.



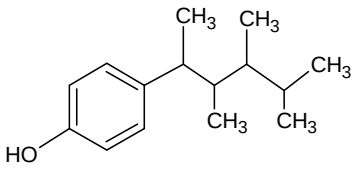
4-(3,6-dimethylheptyl)phenol



4-(3-methyl-octyl)phenol



4-(1,1,3-trimethylhexyl)phenol



4-(1,2,3,4-tetramethypentyl)phenol

Figure 7: Structure of four branched isomers of *para*-nonylphenol.

Due to the complexity of NP technical mixtures, the separation into each single isomer using one-dimension chromatography is not

possible (see Figure 8). In this sense, the synthesis [135] and chromatographic separation of these isomers using multidimensional separation techniques is still a great challlenge [125-128].

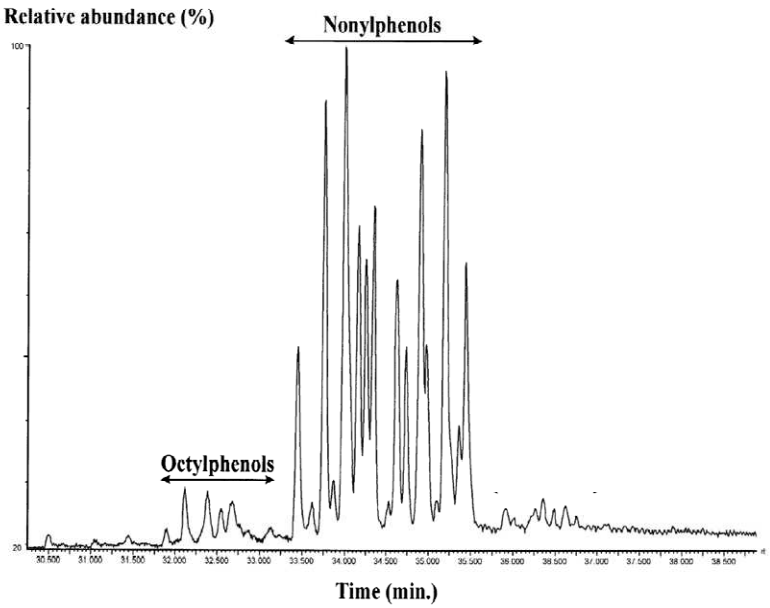
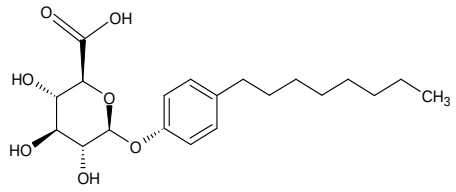


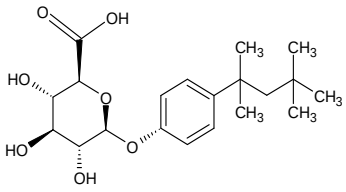
Figure 8: Typical one-dimension gas chromatogram of an octyl- and nonylphenol mixture.

APs are metabolized by enzymatic reactions such as oxidation, sulfation and glucuronidation (see Figure 9), in order to get more hydrophilic compounds and to obtain an easier excretion [136]. Glucuronidation is the major pathway for metabolism, it is carried out in the liver and the glucuronisated compounds are preferently accumulated in the bile [137,138], as it happens for estrogens. Many works are available in the literature where the metabolism of

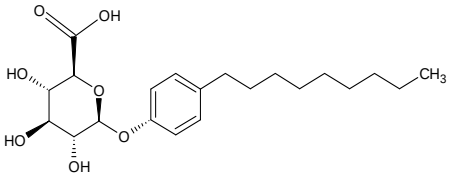
APs has been determined [138,139] and the analysis of glucuronide APs in bile has been carried out [81,84,86,140,141]. Although the conjugated form is less toxic than the free one, the bioaccumulation of these compounds in bile is a good biomarker of environmental fish exposure to APs.



4n-OP-G



4*tert*-OP-G



4n-NP-G

Figure 9: Structure of glucuronide form of 4n-octylphenol (4n-OP-G), 4*tert*-octylphenol (4*t*-OP-G) and 4n-nonylphenol (4n-NP-G).

2. Pre-treatment of water samples for the determination of APs and estrogens

As explained above, the main input for estrogens and APs are the effluents and influents of the WWTPs, where APs and estrogens have been observed in both the conjugated and free forms

[93,99,142]. The analysis of estrogens and APs in aqueous samples such as estuarine or WWTP samples requires of preconcentration and clean-up steps due to the low concentrations (ng/L-µg/L) observed, as well as to possible matrix effects. The most common preconcetration and clean-up step for estrogens and APs is solid phase extraction (SPE). SPE is based on the interactions of a solid sorbent and the liquid sample. Depending on the characteristics of the sorbent, the retention of the compound is different. In order to determine organic compounds in water samples, non-polar adsorbents (C_{18}, C_8, C_6, C_4, C_2, phenyl, cyclohexyl) or polymeric adsorbents such as, polystyrene–divinylbenzene (PS-DVB, Lichrolut® EN) or polystyrene–divinylbenzene N-vinylpyrrolidone (PS-DVB-NVP, OASIS® HLB) are commonly used [143,144]. Thus, different types of sorbents have been used to determine estrogens and alkylphenols, C_{18} [145-147], Lichrolut® [102] and the most popular OASIS® HLB [93,97,98,147-149], for instance.

In order to preconcentrate the analytes, other extraction techniques have been applied in the literature, such as immunoaffinity extraction [150,151], solid-phase microextraction (SPME) [152,153], stir-bar sorptive extraction (SBSE) [154-161], molecularly imprinted polymers (MIP) coated SPME fibers [162] or hollow-fibre microporous membrane liquid–liquid extraction (HF-MMLLE) [163].

In some cases, especially in the case of influent and effluent samples from WWTPs, a clean-up step is necessary once the preconcentration is carried out. Florisil, silica and alumina

cartridges and even gel permeation chromatography have been applied with this purpose [164].

3. Pre-treatment of fish samples for the determination of APs and estrogens

Organic contaminants such as APs and estrogens are determined either in the bile, where they are mainly accumulated, or in what is called the fish homogenate.

As mentioned before, in the case of fish bile, APs and estrogens are accumulated in their glucoside, glucuronide or sulfate forms [137,138] and a hydrolysis step is necessary for their analysis. Commonly, enzymatic hydrolysis [82,83,165], acid hydrolysis [166,167] or alkaline hydrolysis [168] are used. Analyte losses can occur during acidic and alkaline hydrolysis due to the strong conditions used and, thus, enzymatic hydrolysis is recommended. However, enzymatic hydrolysis is time consuming (~16h) [81,86,166,169,170]. In order to accelerate this hydrolysis step, the use of ultrasound probes has been suggested during the determination of steroids in urine or selenium in biological samples [171-173]. This hydrolysis step is usually followed by a clean-up step using SPE, GPC [84] or liquid-liquid extraction (LLE) [84]. Common sorbents are OASIS® HLB [81,82] or C_{18} [140].

In the case of fish homogenate, a solid-liquid extraction step is necessary and different techniques such as Soxhlet extraction [174-176], accelerated solvent extraction (ASE) [177-179], microwave

assisted extraction (MAE) [180,181] or focused ultrasound solid-liquid extraction (FUSLE) [182-184] have been applied during the determination of a variety of organic pollutants such as APs, petroleum biomarkers or PAHs. This extraction step is followed by the clean-up of the extract that is usually performed using SPE [185,186] or GPC [174,175,187] to eliminate the most lipophilic compounds that can interfere during the chromatographic separation and analysis of the target analytes of interest.

Next, two of the extraction techniques used during the analysis of fish bile and fish homogenate will be described.

3.1. Ultrasound energy and ultrasound probe

Ultrasound energy has been long used in the analytical laboratory with sample preparation purposes. However, classical ultrasound assisted sample preparation lacks of efficiency and precision.

The recent advances in ultrasonic performance have led to the development of new commercially available devices. Commonly, a generator converts voltages to high frequency electrical energy. This generator is coupled with an ultrasound converter, which transforms electrical energy into mechanical vibrations of fixed frequency, normally 20 kHz. This converter is fitted with the standard or cup booster horn (Figure 10) [188].

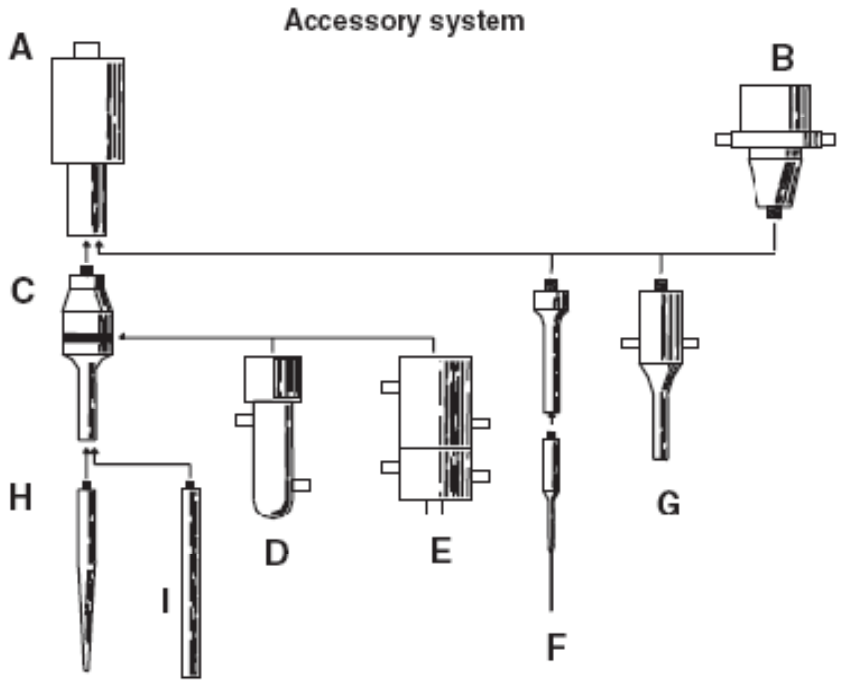


Figure 10: The Branson Sonifier II and accessories. A: converter; B: cup horn; C: standard horn; d: sealed atmosphere chamber with water jacket; E: continuous flow-cell attachment; F: special microtip with coupling section; G: flow-through horn; H: standard microtip; I: extender [188].

Ultrasounds (sound waves) produce a movement of vibration that is transmitted from one molecule to another. In liquids, particle oscillation takes place in the direction of the wave, producing new longitudinal waves. The expansion cycle produces a negative pressure that pulls molecules away from one another. If the ultrasound intensity is high enough, the expansion cycle can create bubbles or cavities in the liquid. The process where bubbles form,

grow and undergo implosive collapse is known as "cavitation" (Figure 11) [189].

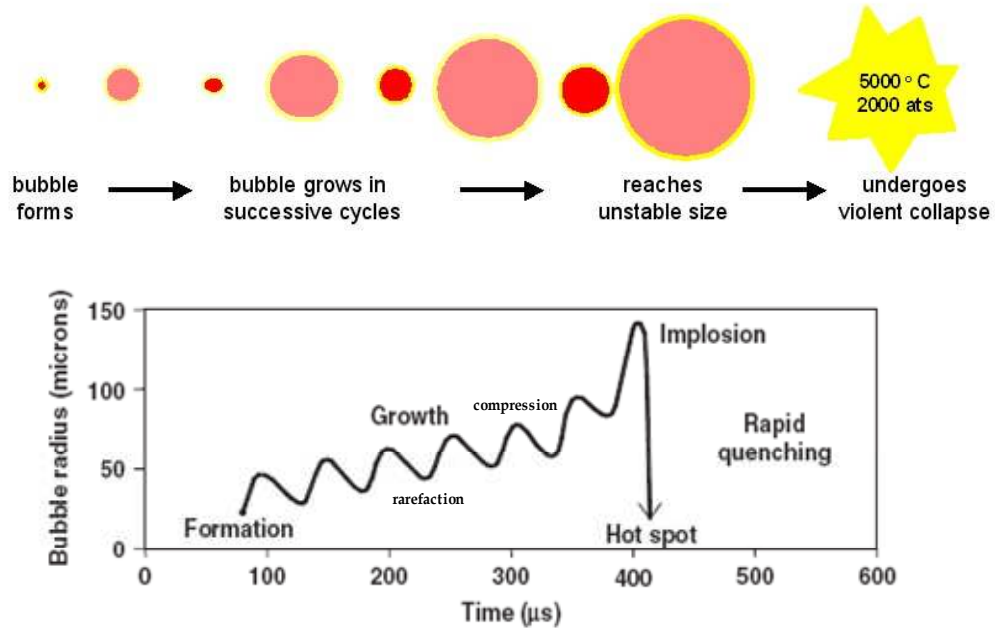


Figure 11: Development and collapse of cavitation bubbles [189].

The significance of cavitation to sonochemistry is what happens when bubbles collapse. At one time, the bubble cannot absorb more energy from the ultrasound and it explodes. The rapid adiabatic compression of gases and vapors produces extremely high temperatures (~ 5000 ºC) and pressures (~ 2000 atm). The size of the cavitation is so small in comparison with the whole liquid volume that the heat produced is rapidly scattered through the liquid. Thus, no appreciable change in the environmental conditions happens. When the ultrasound energy is chosen to improve any

chemical reaction, some conditions must be taken into account, such as solvent viscosity, solvent surface tension, solvent vapour pressure, external pressure, gas and particulate matter, applied frequency, temperature, intensity, field type, attenuation or type of ultrasound cavitation [189].

Two are the main differences when comparing the ultrasonic probe with the ultrasonic bath:

(1) The ultrasonic probe is immersed directly into the solution where the sonication takes place.
(2) The ultrasonic power provided by the probe is at least up to 100 times greater than the one supplied by the bath.

Those major differences make each system suitable for a different set of applications. There are dedicated probes for a given range of volumes. It should be stressed that the amplitude control of the probes allows the ultrasonic vibrations at the probe tip to be set to any desired level. Temperature is another factor that must be controlled. As the ultrasound is delivered into the solution, a slow but constant increase in the bulk temperature is achieved and, at one point, due to the change of the physical characteristics of the liquid media, the decoupling of the probe can occur and no more cavitation is achieved. When long sonication times are needed, the "pulse" mode is recommended. In this mode, the amplifier switches the power of the probe on and off repeatedly [188].

A critical factor to be considered for a correct ultrasonic probe application deals with the shape of the reaction vessel, which should be conical-type and with the diameter as small as possible in order to rise up the liquid level. This way the probe can be inserted more deeply into the processed sample, avoiding aerosoling and foaming, which has the effect of "de-coupling" the probe. Besides, the sides of the vessel should not touch the probe [188]. Table 3 illustrates the last applications of ultrasound energy.

Table 3: Last applications of ultrasound energy.

Extraction	- Sterols and fatty acids	[190]
	-Metals in sediments	[191]
	-PAHs in biota	[192]
	-Triterpenic fractions for olive leaves	[193]
Hydrolysis	- Protease-Catalyzed Hydrolysis of Selenium Bound to Proteins in Yeast	[171]
	-.Conjugated steroids in urine	[173]
Derivatization	-Sterols and fatty acids	[190]
	-Silylation for triterpenic fractions	[193]

3.2. Microwave assisted extraction

Microwave energy is used to heat solvents in contact with solid or liquid samples and to partition analytes from the sample matrices into the solvent. The principle of MAE is based on the direct effect of microwaves on molecules of the extracted system caused by two mechanisms: (1) ionic conduction and (2) dipole rotation [194].

Heat generation under a sample in the microwave field requires the presence of a dielectric compound. The greater the dielectric constant, the more thermal energy is released and the more rapid is the heating for a given frequency. Consequently, the effect of microwave energy is strongly dependent on the nature of both the solvent and the matrix. Most of the time, the solvent chosen has a high dielectric constant, in order to strongly absorb microwave energy. Polar molecules and ionic compounds (usually acids) absorb microwave energy strongly because they have a permanent dipole moment that is affected by microwaves. However, non-polar solvents do not heat up when exposed to microwaves. The extraction heating process may occur as follows:

(1) The sample is immersed in a single solvent or mixture of solvents that absorb strongly microwave energy.
(2) The sample is extracted in a combined solvent containing solvents with both high and low dielectric losses mixed in various proportions.
(3) Samples that have a high dielectric loss can be extracted with a microwave transparent solvent.

For microwave instrumentation opened and closed systems are available, although closed systems are mostly used (Figure 12).

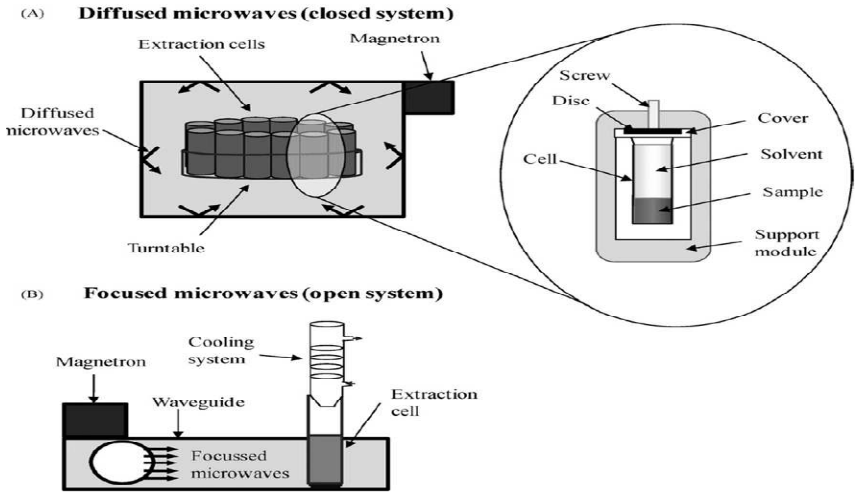


Figure 12: Schematic view of devices (A) pressurized MAE and (B) focused MAE [195].

Different variables that affect the extraction, such as solvent type, solvent volume, temperature, extraction time and microwave power, are usually optimized [194-197].

MAE has been widely used for the determination of organic pollutants in environmental samples and some of the most recent applications are included in Table 4.

Table 4: Last applications of microwave energy.

Extractions	-PAHs in soil	[198]
	-PCB in soil	[199]
	-Phenolic compounds in sediment	[198]
	-Bisphenol A in marine samples	[195]
Derivatization	-Estrogens silylation	[200]

4. Analysis of APs and estrogens

4.1. Derivatization of APs and estrogens

Different approaches have been found in the literature for the determination of estrogens and APs, such as immunoaffinity chromatography [151], proliferation test (E-screen assay) [201], immunoassays [202], liquid chromatography-mass spectrometry (LC-MS) [177,203-205], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [206,207], gas chromatography-mass spectrometry (GC-MS) [86,99,102,185,208,209] or gas chromatography-tandem mass spectrometry (GC-MS/MS) [146,210].

When GC is used for the separation and detection of APs and estrogens, a derivatization step is necessary in order to increase the volatility and stability of the analytes, improving the selectivity and the sensitivity of the signal. Different reactions are used to derivatize estrogens and APs, although silylation is mostly used. In this case, the hydroxyl group is substituted by a more apolar silyl group (see Figure 15). Different silylation reagents N,O-bis (trimethylsilyl) trifluoroacetamide with 1 % of trimethylchlorosilane (BSTFA + 1 % TMCS), N-tert-butyldimethylsilyl- N-methyltrifluoroacetamide (MTBSTFA) and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) are used in combination with different solvents but, when 17α-ethynyl estradiol (EE2) is analyzed, BSTFA or MSTFA with pyridine or formaldehyde must be

used in order to avoid the oxidation of EE2 to E1 and to obtain the silylation of both alcohol groups [211-217].

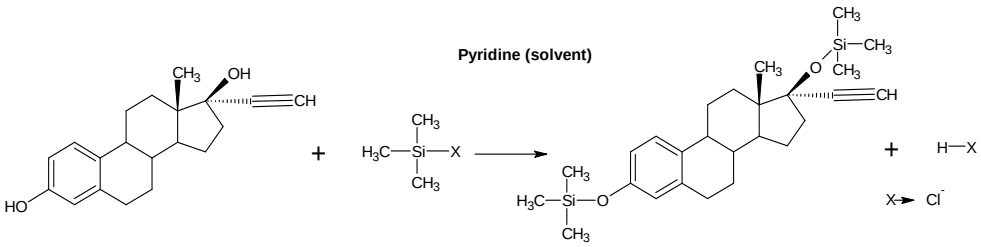


Figure 15: Trimethylsilyl derivatization reaction of EE2 in the presence of pyridine.

Apart from this silylation reaction, other derivatization reagents, such as pentafluorobenzyl bromide (PFBBr) [218,219], pentafluorobenzoyl chloride (PFBOCl) [120], pentafluoropropionic acid anhydride (PFPA) [50], heptafluorobutyric anhydride (HFBA) [210,220,221] or acetic acid anhydride [156] have been used.

The main problem of the derivatization reactions is the use of heat to increase the temperature (60-70 °C) of the mixture and to obtain the complete derivatization of the analytes, which consumes high periods of time (30-120 min) [99,147,208,222-224]. In order to accelerate this procedure, different energy sources have been used, such as microwaves [200] or ultrasounds [225,226], reducing the derivatization from 30-120 min to a few minuntes.

New ultrasonic devices prepared to handle low sample volumes as those used in silylation reactions are now commercially available. Cup booster devices offer an indirect sonication, that is, ultrasound waves need to cross the wall of the sample container but with

intensities higher than in traditional ultrasonic water baths. In the cup horn, the titanium probe is held within an acrylic cup filled with water (see Figure 16). Samples are placed within the cup, above the probe. The cavitation produced in the immersed samples is higher than the one given by a traditional ultrasonic baths but it is lower than the cavitation produced by direct immersion of the ultrasonic probe into the solution [188,226]. Cup boosters are smaller than conventional ultrasound baths (<12 mL) and are suitable for low samples volumes (<1.5 mL). Besides, samples can be placed in sealed tubes, eliminating aerosol formation and cross contamination.

Figure 16: The structure of ultrasound cup horn [227].

4.2. Large volume injection-programmable temperature vaporization

Preconcentrations obtained during sample preparation do not always provide limits of detection (LODs) low enough to perform

trace analysis. In such cases and when GC is used for analysis, large volume injection (LVI) can be an alternative in order to improve the LODs. One way to perform LVI is the use of the solvent vent mode of programmable temperature vaporization (PTV) inlets (see Figure 13). PTV inlets allow increasing injection volume from the 1-4 µL of common split/splitless injectors up to 1 mL sample.

The PTV injector is equipped with a temperature control function which can be rapidly heated or cooled during injection, while the conventional split/splitless injector is isothermal. Due to the temperature control, PTV has become the most popular LVI interface. LVI with PTV can be achieved in various modes, including splitless injection, vapor overflow and solvent-vent.

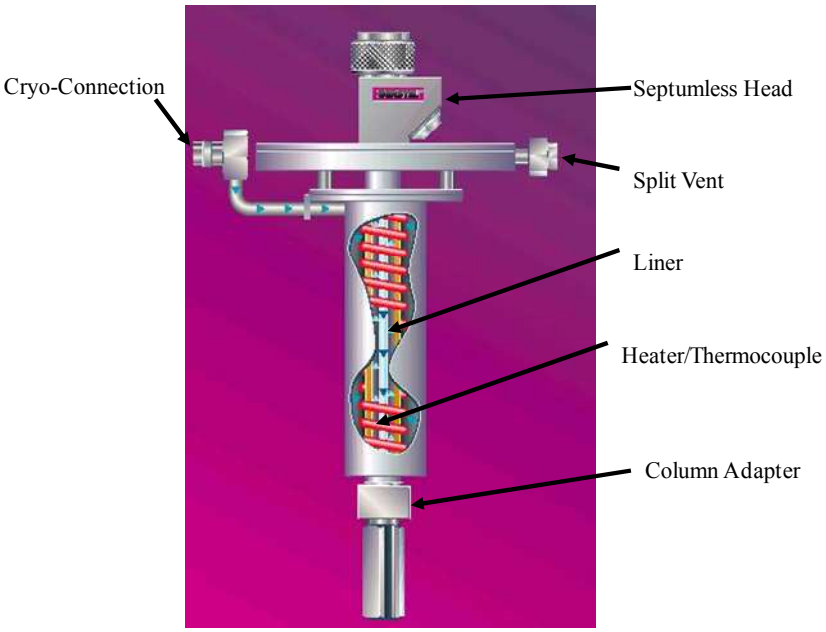


Figure 13: Schema of a commercial LVI-PTV [228].

Among these, solvent-vent injection has been the most widely used PTV technique in LVI applications. Briefly, the solvent-vent injection is carried out as follows (see Figure 14):

(1) Sample is introduced at a relatively low temperature, ~20 °C below the solvent boiling point.
(2) Solvent is eliminated via the split exit while the higher-boiling point analytes are retained in the liner.
(3) The PTV is rapidly heated and the retained analytes are transferred to the analytical column in splitless mode, keeping the oven temperature below solvent boiling temperature to refocus the analytes at the front of the column.
(4) After the splitless transfer, the split exit is re-opened to remove residual the solvent vapour and low-volatile matrix compounds from the inlet.

The sample introduction to a vaporizer can be carried out by three approaches: at once (one injection), multiple and speed controlled injections [229,230]. Speed controlled injections allows to control the sample injection speed in order to get the vaporization of the solvent while the injection is carried out.

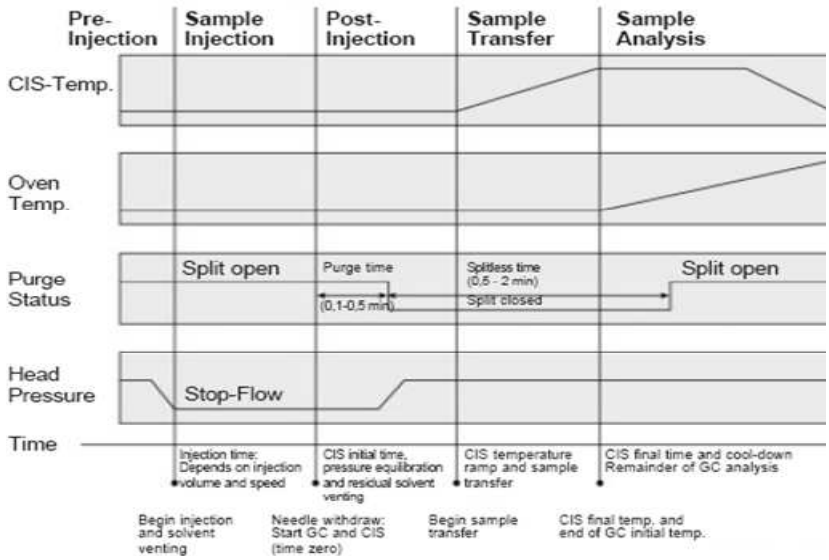


Figure 14: Schema of the LVI-PTV performance, Gerstel®: CIS-Temp.: Temperature of the injector; Head pressure: Head column pressure; Oven Temp.: Oven temperature.

The LVI-PTV injector has been widely used in order to diminish the LODs during the analysis of PAHs, pharmaceuticals and personal care products (PPCPs), phenolic endocrine disrupters, steroids and pesticides in sample matrices such as water and sediment [119,149,164,231-233].

4.3. Comprehensive two-dimensional gas chromatography

In the case of the determination of AP isomers, conventional one-dimension GC is inappropriate when single isomers are to be determined because of the impossibility to separate the complex mixture. In order to get the whole separation, comprehensive two-dimensional gas chromatography (GCxGC) can be a good alternative.

The main argument for using GCxGC is probably to separate components that co-elute on a single column. When such a group of co-eluting components are transferred from the first column to a second one, which has different separation characteristics, the co-eluents will be hopefully separated [234].

The first column can be a conventional capillary column, connected by means of a stream selection system (modulator) to the secondary column (see Figure 17).

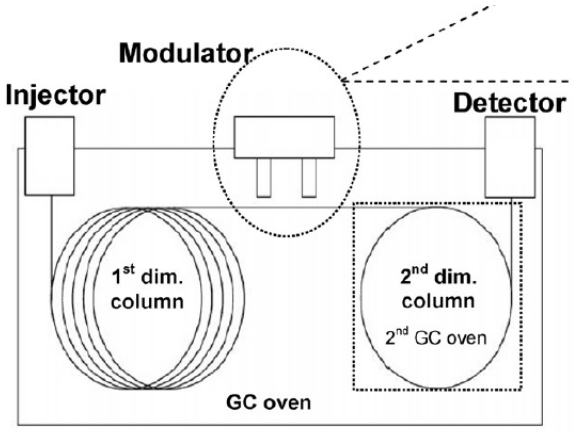


Figure 17: Schema of a GCxGC system [235].

In principle, all types of stationary phases can be used in the first dimension of GC×GC but, generally, non-polar phases are used and boiling-point separation occurs. This means that compounds eluting at similar retention times have similar vapour pressures [234].

The second column is operated very rapidly in comparison with the first column and in comparison with the temperature

gradient in the column oven. As a result, the temperature of the second column is only increased a few tenths of a degree per separation and, thus, the separations obtained on this column are essentially isothermal. The separation in the second dimension is, therefore, essentially determined by molecular interactions between the analytes and the stationary phase (polarity) of this column [234,235].

The second column generally contains a more-polar stationary phase and it is shorter (1-5 m). Thus, GC×GC offers virtually orthogonal separation mechanisms in the two dimensions, analyte volatility and analyte polarity. Since volatility and polarity are uncoupled now, it results in independent separations in the two columns (see Figure 18) [235-237].

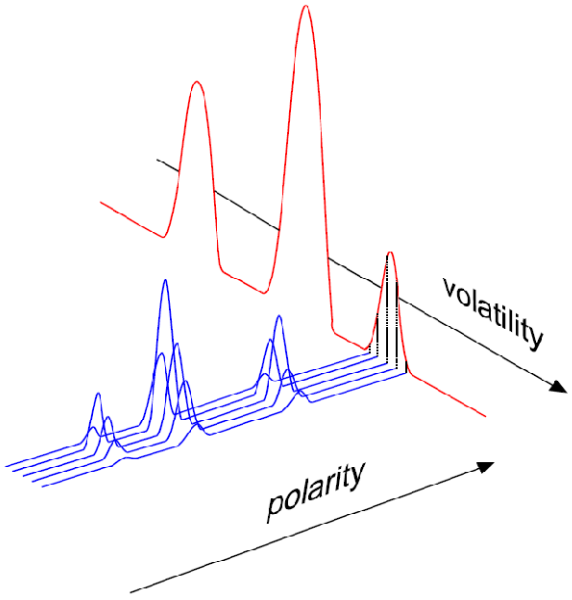


Figure 18: Orthogonality of GCxGC system [234].

The interface between the two GC columns, the so-called modulator, has three main functions [234]:

(1) It discretely samples small and adjacent parts from the first dimension effluent.

(2) It focuses these fractions into narrow pulses.

(3) It launches these narrow pulses into the second dimension.

The second-dimension separation should be finished before the release-injection of the next fraction

Three different modulation ways are available: valve-based modulation, thermal desorption modulation and cryogenic modulation. Although cryogenic modulation [218] and thermal desorption [126,128] are mostly used, a valve-based modulation will only be described.

In this case, the modulation period is divided in two steps: load and inject. In the load mode (see Figure 19), the analytes, which elute from the first column, are collected in the collection channel, while the H_2 gas blocks the pass through the second column.

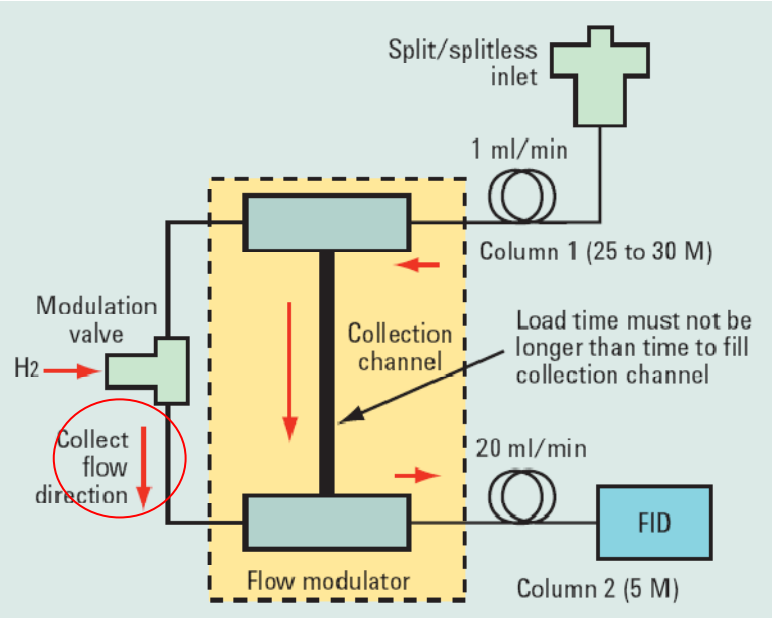


Figure 19: Modulation period: Load. FID: Flame ionization detector [238].

In the inject mode (see Figure 20), the modulation valve direction changes and the H_2 passes through the collection channel, transporting the analytes to the second column. Each cycle is called "the modulation period".

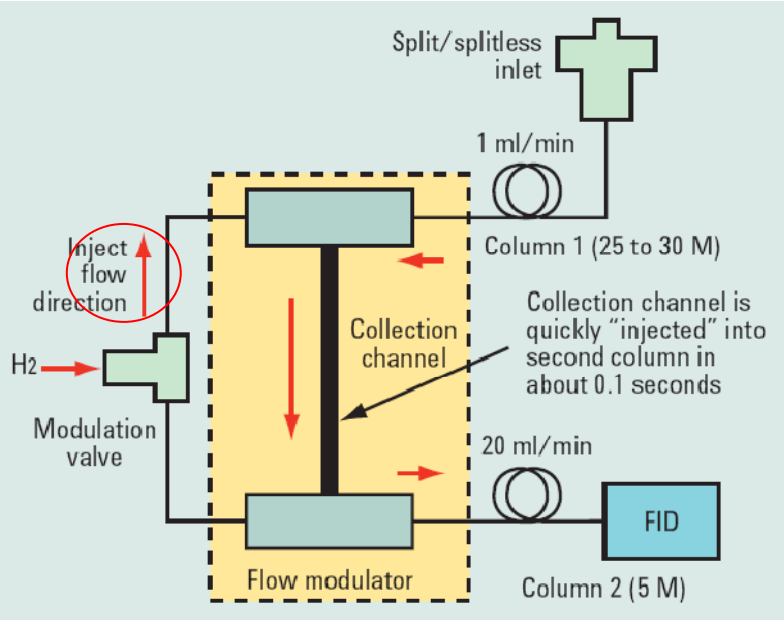


Figure 20: Modulation period: Inject. FID: Flame ionization detector [238].

As it is observed, the flow of the H_2 is too high to connect it directly to a MS and, thus, this modulator is commonly connected to a flame ionization detector (FID). In order to connect it to a MS, a splitter must be installed to divide the flows [239].

Detectors with high data processing are commonly used, such as FID, which can acquire data at frequencies of 200 Hz, time-of-flight-mass spectrometer (TOF-MS) which can acquire spectra at very high frequencies, new MS detectors that can acquire spectra at 24-36 Hz (depending of the scan range) [128] or electron capture detectors (ECD) [234,240].

A large series of high-speed second dimension chromatograms are collected and transformed in 2D chromatograms stacked side by side with the first dimension representing the retention on the first column and the other, the retention time on the second column. The peaks can be visualized by 2D colour plots, contour lines and 3D plots (see Figure 21) [235-237].

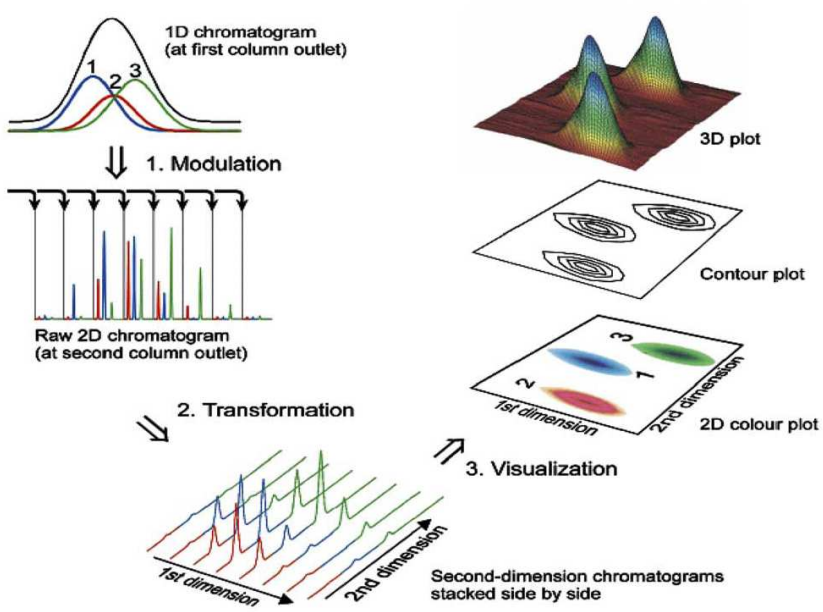


Figure 21: Schema of the GCxGC processing data [235].

GCxGC has been used to separate pesticide residues from grapes [241], during the identification of several PAHs, NPs and dialkylated benzenes [242], polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and PCBs [243], pesticides [244], esential oils [245], sterols [246,247] and APs [128,218].

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Hormones and alkyphenols (EDCs) have become important emerging contaminants due to their presence in environmental waters, the threat they suppose to drinking water source and the concern about their possible estrogenic effect among others effects. In that sense, the determination of these compounds in environmental samples such as estuarine water and influents or effluents of wastewater treatment plants is necessary and other biological samples, such as fish tissues, where these target analytes are accumulated.

Therefore, the main objective of this PhD thesis was the development of routine methods for the analysis of alkylphenols and estrogens in water and fish tissues that meet the environmental quality standards (EQSs) required by directives such as the European Water Framework Directive (WFD). In this study, nonylphenols (NPs) and octylphenols (OPs) were considered among the different alkylphenols. Also, estrogens such as 17β-estradiol (E2), 17α-ethynyl estradiol (EE2), estrone (E1), mestranol (MeEE2) and estriol (E3) were studied among the estrogens.

In order to develop robust and trustful methods for routine analysis, both the sample preparation and detection steps must be optimized. Besides, if both the consumption of solvents and the analysis time are reduced, the better. With the aim to reach the previous aims the following objectives were considered:

(1) *Development of ultrasound accelerated derivatization procedures for the analysis of alkylphenols and estrogens by GC-MS.*

When the analysis of target analytes is performed by GC-MS, a time consuming derivatization step is necessary. The present objective tried to reduce the time consumed using a cheap energy source such as an ultrasound cup booster.

(2) *Development of an improved enzymatic hydrolysis procedure for alkylphenols and estrogens in fish bile based on focused ultrasounds.* Once again, with the use of new devices such as ultrasound probes, the focused ultrasound assisted hydrolysis of target analytes in fish bile was optimized.

(3) *Comparison of microwave and focused ultrasound energy for the extraction and optimization of different clean-up steps in the analysis of alkylphenols and estrogens in fish homogenate*. Within this objective, some critical steps in the analysis of lipidic samples such as fish homogenate, the extraction and clean-up, were studied.

(4*) Optimization of large volume injection inlet parameters for the determination of alkylphenols and estrongens in environmental samples by GC-MS.* In this section the limits of detection (LODs) obtained in common split/splitless inlets by means of the large volume injection of sample extract in a programmable temperature vaporizers were improved.

On the other hand and, as mentioned in the introduction, research found in the literature shows that different octyl- and nonylphenol isomers show different estrogenicity and, thus, classical analysis techniques, where the total isomer concentration is measured, are no longer useful to answer such scientifical

requests. Within this framework two new sub-objectives were established:

(5) *The synthesis of individual isomers of octyl- and nonylphenols which are not commercially available.* The synthesized isomers were used for their identification and quantification in a commercially available technical mixture, as well as, their use in experiments where their estrogenicity will be studied (not included in the present work).

(6) *Optimization of the separation of octyl- and nonylphenols from a commercially available technical mixture by comprehensive two dimensional gas chromatography (GCxGC).* Since one-dimension GC is not capable of separating complex octyl- and nonylphenol mixtures, separation by comprehensive two-dimensional gas chromatography was studied using GCxGC-FID-qMS and a valve-based modulator.

Abstract

Cup horn boosters are miniaturized ultrasound baths that maximize efficiency and precision. The optimization of an ultrasonic-assisted derivatization step by means of a cup horn booster and the determination of estrone (E1), 17β-estradiol (E2), estriol (E3), 17a-ethynyl estradiol (EE2) and mestranol (MeEE2) was developed by GC-MS. Different derivatization reagents and solvents were studied for maximizing the di-derivarization of EE2 under ultrasound energy. Only BSTFA+1 % TMCS in pyridine gave satisfactory results and this mixture was further used in the optimization of the ultrasound assisted derivatization. The experiment designs included sonication time (1-10 min), sonication power (20-80 %), sonication cycles (1-9), derivatization reagent volume (25-125 µL) and solvent volume (25-125 µL). Once, the optimum conditions were fixed, the effect of organic matter and the frequency of the water bath change were studied. Finally, the validation of the analytical method was carried out using spiked natural and synthetic waters. Recoveries (natural, 138-70 %, and synthetic, 112-89 %), the limits of detection (0.35-1.66 ng/L), and quantification (1.16-5.52 ng/L), and the precision (0.2-5.3 %) of the method were studied. This is the first work in the literature where a cup horn booster is used with the aim of minimizing derivatization time during the determination of estrogenic compounds.

Keywords: Cup horn booster, Estrogens, GC-MS, Optimization, Ultrasonic derivatization

1. Introduction

Estrogens are a group of steroid hormones that regulate the development and growth in wildlife and humans by inducing cell proliferation and cell differentiation [1]. Until 1996, these compounds were indiscriminately used in animal farming as growth promoters [2], but the European Union banned their use in animal growing because of the risk to human health [3]. Additionally, estrogens are also used in human medicine as contraceptives, in the management of the menopausal and postmenopausal syndrome and in the treatment of prostatic and breast cancer [2].

In the case of the therapeutic use of estrogens, it is generally known that the dose of estrogens administered in the treatment is not fully metabolized in the body and, therefore, the analytes are excreted in urine as glucoside, glucuronide or as sulfate form, and, in feces, as the free compounds [4]. Once they are disposed, they are likely converted into the free compounds in sewer systems and in wastewater treatment plants (WWTPs), where the free analytes are concentrated in the effluent or the sludge [2,4,5]. Estrogen concentrations present in effluents can be considered chronic to disrupt the endocrine process of fishes and, as a consequence, cause the feminization of male fishes (imposex), promote abnormal reproductive processes and induce the development of testicular and prostate cancer [6].

As a consequence, the European Water Framework Directive (WFD) included estrogens in a list of emerging substances [7]. Five

of those estrogenic compounds are selected for this work, i.e, three natural female hormones (estrone (E1), 17β-estradiol (E2) and estriol (E3)) and two synthetic hormones (17α-ethynyl estradiol (EE2) and mestranol (MeEE2)) and the need of well improved analytical methods has been claimed in order to fulfill the requirements of the directive in natural waters [8].

Different strategies have been developed for the determination of estrogens, such as immunoassays, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and even tandem systems such as GC-MS/MS or LC-MS/MS [9-13]. When GC is used, a derivatization step is necessary to suit the chromatographic behavior of those analytes and, therefore, improve the selectivity and the sensitivity [14]. Two major problems arise during the derivatization of estrogens, both related to EE2 compound. Depending on the derivatization reagent and solvent, the oxidation of EE2 to E1 has been observed [15]. Besides, EE2 has two hydroxyl groups that are submitted to derivatization and one of the following scenarios can take place depending on the derivatization conditions: (i) both hydroxyl groups are derivatized (di-EE2), (ii) only one of the hydroxyl groups is derivatized (mono-EE2) or (iii) both the di-EE2 and mono-EE2 are observed in the chromatogram [15-19]. In order to improve the chromatographic separation, the formation of the di-EE2 compound should be maximized.

Derivatization of estrogens is usually carried out at high temperatures (60-75 °C) in sand, water or oil baths, in heater blocks or even in the ovens of gas chromatographs [6,15,17,20-21]. However, these derivatization procedures are time consuming (30-90 min) [21-23]. In order to accelerate this derivatization step Zuo et al. [24] used microwave energy and obtained optimum derivatization after 1 min. Fiamegos et al. [25] used traditional ultrasound baths for the acceleration of the derivatization of amino acids. However, although recent advances in ultrasonic performance have led to the development of new commercially available devices [26], most of the new applications found in the literature deal with the use of ultrasound probes for the extraction of metals [27], organometals [28] and organics [29-31] in solid samples. Among the new ultrasound devices, cup horns and sonoreactors are also found. Both devices offer indirect sonication, that is, the ultrasonic waves need to cross the wall of the sample container, in contrast to ultrasonic probes, which are directly immersed in the sample. In this sense, cup horns and sonoreactors are similar to ultrasonic water baths [26,32] but smaller (12 mL), more intense and suitable for handling low sample volumes (< 1.5 mL). In cup horns, the titanium probe is held within an acrylic cup filled with water and samples, which can be placed in sealed tubes that eliminate aerosol and cross-contamination, are placed within the cup (see Figure 16 in the introduction chapter). Besides, cup boosters are cheaper than microwave ovens used in analytical laboratories.

In this sense, the main aim of this work was to optimize the miniaturized ultrasound assisted derivatization of several estrogens. In this optimization process the derivatization reagent, the derivatization solvent, volumes of the derivatization reagent and of the derivatization solvent, the sonication power, the number of cycles and the sonication time were studied. The optimized derivatization step was applied to the determination of estrogens in spiked water samples previously extracted by solid-phase extraction (SPE) and analyzed by GC-MS. Besides, the influence of organic matter in the in SPE, recoveries, precision, limits of detection (LODs), limits of quantification (LOQs) and linearity were also studied.

2. Experimental

2.1. Reagents and Materials

Estrone (E1, Riedel-de Haën, analytical standard, 99.5 %), 17β-estradiol (E2, Sigma, Sigma Reference Standard), mestranol (MeEE2, Riedel-de Haën, analytical standard, 99.4 %), 17α-ethynyl estradiol (EE2, Riedel-de Haën, analytical standard, 99.4 %) and estriol (E3, Riedel-de Haën, analytical standard, 99.7 %), as well as the internal standard 17β-estradiol-16,16,17-d_3 (E2-d_3, Aldrich, 98 %) were individually dissolved in anhydrous methanol (Alfa Aesar, 99.9 %) at ~ 5000 mg/L concentration. Those standard solutions were stored at 4 ºC in the dark. 0.3 mg/L to 150 mg/L standards

were prepared in ethyl acetate (HPLC, Lab Scan analytical science, 99.8 %) or anhydrous methanol, according to experimentation, and were stored in amber vials at -20 ºC.

Dichloromethane (HPLC grade, 99.8 %), isooctane (HPLC grade, 99.5 %), ethyl acetate (HPLC grade, 99.8 %), *n*-hexane (HPLC grade, 95 %) and acetone (HPLC grade, 99.8 %) were all obtained from LabScan (Dublin, Ireland). Acetonitrile (HPLC, 99.9 %) was obtained from Panreac (Barcelona, Spain) and pyridine (99.5 %) from Riedel-de Haën (Seelze, Germany).

N,O-bis (trimethylsilyl) trifluoroacetamide with 1 % of trimethylchlorosilane (BSTFA + 1 % TMCS, Sylon BFT, 99:1) was obtained from Supelco (Walton-on-Thomas, UK) and N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA, derivatization grade) and N-methyl-N- (trimethylsilyl) trifluoroacetamide (MSTFA, derivatization grade) from Sigma-Aldrich (Milwaukee, WI, USA).

Humic acids were obtained from Fluka (ash 20 %) and 200-mg (6 mL) Oasis HLB cartridges from Waters (Milford, MA, USA).

Estuarine surface water samples were collected in Zorrotza (N 43º 16´ 0.41´´; W 02º 57´ 35.9´´) and Bilbao (N 43º 15´ 38.4´´; W 02º 55´ 30.4´´) (Biscay, Spain) in February 2009 in pre-cleaned glass bottles, transported to the laboratory in cooled boxes, filtered through 0.45 µm filters (Whatman, cellulose nitrate membrane filters) and analyzed within 48 hours.

2.2. Solid-Phase Extraction (SPE) from water samples

The preconcentration of the analytes of interest from water samples was performed using solid-phase extraction (SPE) according to Hernando et al. [33]. Briefly, a 100-mL aliquot of spiked (~ 1 µg/L each) Milli Q (< 0.05 µS/cm, Milli Q model 185, Millipore, Bedford, MA, USA) or natural water that contained 50 µL (~ 1 mg/L) of E2-d_3 was passed through a 200-mg Oasis HLB cartridge, which had been previously conditioned with 5 mL of ethyl acetate, 5 mL of methanol and 5 mL of Milli Q water. Then, the cartridge was washed with 5 mL of a (95:5) water:methanol mixture and dried under vacuum for 15 min before elution of the analytes in ethyl acetate (2x4 mL) at a flow rate of approx. 1 mL/min. The extract was evaporated to dryness under a gentle stream of nitrogen in a Turbovap LV Evaporator (Zymark, Hopkinton, MA, USA) before it was transfered to a 500-µL amber Eppendorf safe lock microtube and redissolved in 125 µL of pyridine.

2.3. Derivatization

During optimization 50 µL of standard solution of a mixture of all the analytes at 15 mg/L each in anhydrous methanol were added into a 500-µL amber Eppendorf safe lock microtube and evaporated to dryness under a gentle stream of nitrogen. 75 µL of different solvents (pyridine, dichloromethane, isooctane, ethyl acetate, *n*-hexane, acetone or acetonitrile) and 75 µL of the derivatization reagent (BSTFA + 1 % TMCS, MSTFA or MTBSTFA) were added, the mixture was shaken in a vortex and submitted to

ultrasound assisted derivatization in a Bandelin Sonoplus HD 2070 ultrasound system (Berlin, Germany) with a BR 30 Cup booster under constant conditions (1 min sonication at 50 % of power and 5 cycles).

Once the best derivatization reagent and solvent were fixed, other variables such as the volumes of the derivatization reagent and solvent, the sonication power, the number of cycles and the sonication time were studied by means of experimental designs.

In the case of sample extracts in ethyl acetate, SPE extracts were partially evaporated and dried in 500-µL amber Eppendorf safe lock microtubes, redissolved in 125 µL of pyridine and 25 µL of BSTFA + 1 % TMCS. The mixture was shaken in a vortex and sonicated at 80 % of power and 9 cycles for 10 min.

2.4. GC-MS analysis

The derivatized analytes were analyzed in a 6890N gas chromatograph (Agilent Technologies, Avondale, PA, USA) equipped with an Agilent 5973N electron impact ionization mass spectrometer and a 7683 Agilent autosampler. 1 µL of the derivatized extract was injected in the splitless mode at 280 ºC into a HP5 MS (30 m x 0.25 mm, 0.25 µm) capillary column. The following oven temperature program was used for the separation of the analytes: 95 ºC (1 min), temperature increase at 20 ºC/min to 150 ºC and a second increase of 10 ºC/min up to 280 ºC, where it was finally held for 15 min. Helium (99.9995 %, Carburos Metálicos, Barcelona, Spain) was

used as the carrier gas at a constant flow of 1 mL/min. The transfer line temperature was maintained at 290 ºC, and the ion source and quadrupole at 230 ºC and 150 ºC, respectively. Measurements were performed both in the scan (50-550 m/z) and in the SIM (Selected Ion Monitoring) modes. A typical chromatogram obtained in the scan mode can be observed in Figure 1. Ions followed in the SIM mode were 342/327 for E1, 416/285 for E2, 419 for E2-d_3, 367/382 for MeEE2, 425/440 for EE2 and 345/504 for E3. First ion was used as quantifier and the second one as qualifier.

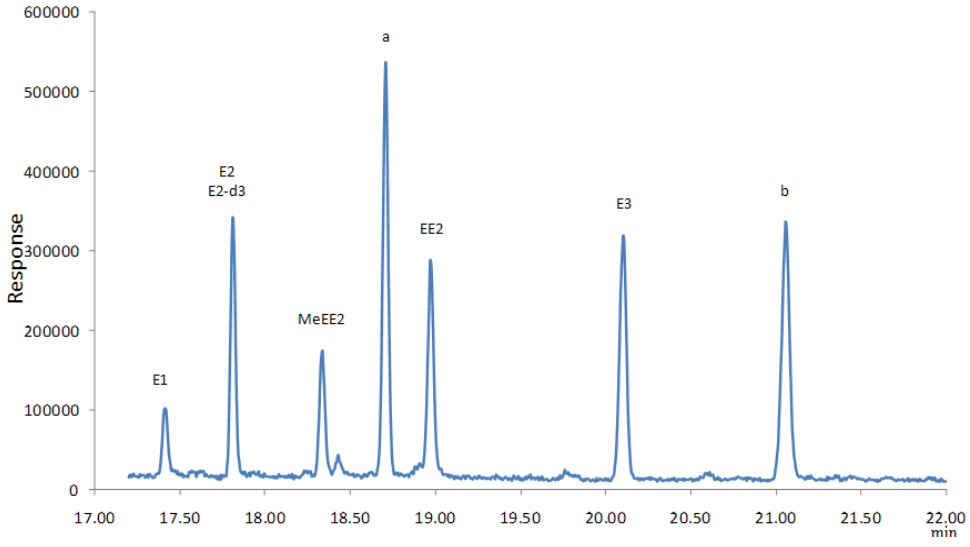


Figure 1: Scan mode chromatogram for E1, E2, E2-d_3, MeEE2, EE2 and E3. Peaks named a and b are due to the Eppendorf safe lock tubes used during sonication.

3. Results and Discussion

3.1. Optimization of the derivatization reagent and solvent

First of all, the optimization of the best solvent and derivatization reagent was performed in order to obtain the simultaneous derivatization of the estrogens.

As it has been mentioned before, two major problems arise during the derivatization of estrogens. On the one hand, during the derivatization step EE2 can be oxidized to E1 [15-19]. On the other hand, in some derivatization conditions, only one of the hydroxyl groups of EE2 is derivatized [15-19]. In this sense, the right choice of the derivatization reagent and solvent are necessary. Although these variables have already been studied in previous works, where the derivatization took place usually in a dry block heater or in a sand or water bath at high temperatures (45-105 ºC) and for long periods (30-60 min) [15,17,34,35], it was decided to study the effect of both variables when the derivatization took place under ultrasounds. In this sense, different solvents (dichloromethane, isooctane, ethyl acetate, *n*-hexane, acetone, acetonitrile and pyridine) and derivatization reagents (BSTFA+1 % TMCS, MTBSTFA and MSTFA) were studied under constant ultrasound conditions (1 min sonication at 50 % power and 5 cycles) and constant volumes (75 µL of both the solvent and the derivatization reagent).

Since the cup horn is able to handle three samples simultaneously, the experiments were organized as follows: one of the vials contained all the analytes except for EE2 (E1, E2, MeEE2 and E3), another vial contained only EE2 and the third one contained a blank sample. This way, the artifacts introduced by the different derivatization patterns of EE2 can be overcome.

Table 1 summarizes the presence of E1, mono-EE2 and di-EE2 under the different experimental conditions studied in experiments where only EE2 was present. As it can be observed from the results in Table 1, E1 and mono-EE2 were obtained when MTBSTFA was used no matter which solvent was used and, in that sense, the use of MTBSTFA was discharged for the simultaneous derivatization of estrogens. In the case of BSTFA + 1 % TMCS, only when pyridine was used the di-EE2 derivative was the only peak observed. In the case of MSTFA, E1 was observed together with di-EE2 with all the solvents, except for isooctane, when no signal of the derivatized analytes was observed at all. In the case of dichloromethane, the mono-EE2 derivative was also observed. For the rest of the estrogens, good results were obtained in all the cases.

Table 1: Peaks observed during the derivatization of EE2 with BSTFA+1 % TMCS, MTBSTFA and MSTFA and different solvents. YES= peak detected; nd=peak not detected.

	BSTFA+ 1 % TMCS			MTBSTFA		MSTFA		
	E1	mono-EE2	di-EE2	E1	mono-EE2	E1	mono-EE2	di-EE2
Pyridine	nd	nd	YES	YES	YES	YES	nd	YES
Acetonitrile	YES	YES	YES	YES	YES	YES	nd	YES
Ethyl Acetate	YES	YES	YES	YES	YES	YES	nd	YES
Dichloromethane	YES	YES	YES	YES	YES	YES	YES	YES
n-Hexane	YES	YES	YES	YES	YES	YES	nd	YES
Acetone	YES	YES	YES	YES	YES	YES	nd	YES
Isooctane	YES	YES	YES	YES	YES	nd	nd	nd

The results obtained for ultrasound derivatization fully agreed with the results previously obtained in the literature for classical derivatization [15] and, thus, BSTFA + 1 % TMCS in pyridine was used for further optimization of the simultaneous ultrasound derivatization.

3.2. Optimization of the ultrasound-accelerated derivatization

In a first approach, a fractional factorial design (Resolution III) was performed in order to establish the possible influence of different variables in the ultrasonic derivatization carried out in the cup horn booster. The variables and their intervals studied were: pyridine volume (25-125 μL), BSTFA + 1 % TMCS volume (25-125 μL), sonication time (0.5-1 min), sonication power (20-80 %) and sonication cycles (1-9), where the sonication cycle is the 1/10 of a

second that the ultrasound is sonicating. All the solutions were diluted to 250 μL in pyridine before injection in the GC-MS. The solvent and derivatization reagent volumes were define as a combination of the rest of variables in order to minimize the number of experiments. Since the cup horn device has three positions, one of the microtubes contained all the analytes except for EE2, a second one contained only EE2 and, finally, in the third position a blank sample was sonicated. The three samples were, therefore, sonicated simultaneously under the same instrumental conditions and, this way, it could be assumed that neither oxidation or mono-silylation of EE2 occurred under the different experimental conditions studied.

The fractional factorial design proposed (three replicates of the central point were performed) by The Unscrambler program (Camo, Norway) and the responses (as chromatographic peak areas) obtained for each analyte are shown in Table 2.

Table 2: The design matrix and the responses (as chromatographic peak areas x 10^6) obtained for the fractional factorial design.

Sonication cycles (1/s)	Sonication time (min)	Power (%)	$V_{pyridine}$ (µL)	V_{BSTFA} (µL)	E1	E2	MeEE2	EE2	E3
1	0.5	20	100	100	2.71	5.29	2.53	6.86	5.90
9	0.5	20	50	50	4.20	8.41	7.28	8.22	9.11
1	5	20	50	100	3.64	7.27	3.33	9.27	7.71
9	5	20	100	50	4.19	8.43	4.14	8.16	8.98
1	0.5	80	100	50	4.05	8.25	4.00	9.11	8.76
9	0.5	80	50	100	2.94	6.30	2.84	8.34	6.75
1	5	80	50	50	3.89	8.08	3.95	7.36	8.90
9	5	80	100	100	4.04	7.80	3.66	9.56	8.44
5	2.75	50	75	75	4.68	9.69	4.35	9.19	10.25
5	2.75	50	75	75	4.70	9.59	4.21	10.50	10.40
5	2.75	50	75	75	4.80	9.80	4.26	9.91	10.60

The data obtained was fitted to eq. 1 by means of multilinear regression (MLR) in The Unscrambler program, where x_A is the number of sonication cycles, x_B sonication time, x_C sonication power, x_D the volume of pyridine, x_E the volume of BSTFA+1 % TMCS and Bi and Bij the fitting parameters. The results obtained are summarized in Table 3.

$$Y=B_0 + B_A x_A + B_B x_B + B_C x_C + B_D x_D + B_E x_E + B_{BC} x_B x_C + B_{BE} x_B x_E \quad (1)$$

Table 3: Results obtained after the multilinear regression analysis (MLR) of the data obtained in the fractional factorial design center.

Variable	E1	E2	MeEE2	EE2	E3
Sonication cycles (B_A)	+	+	++	NS	+
Sonication time (B_B)	++	++	-	NS	+
Sonication power(B_C)	NS	NS	--	NS	NS
Vpyridine(B_D)	NS	NS	--	NS	NS
V_{BSTFA} (B_E)	--	--	---	NS	--
B_{BC}	NS	NS	++	NS	NS
B_{BE}	++	++	++	NS	+

NS = not significant, + = positive effect, ++ =high positive effect, +++ = very high positive effect, - = negative effect, -- = high negative effect, --- = very high negative effect

In the case of the number of cycles, the variable was fixed at its highest value since the parameter had a positive effect for all the analytes except for EE2 (not significant for this analyte). In the case of the sonication power, it was only significant for MeEE2 and two opposite effects were observed. While B_C parameter ($-3.56 \cdot 10^5$) was negative, B_{BC} ($3.9 \cdot 10^5$) was positive. When the effect of both parameters was simulated, the overall effect was an increase of the response when a higher sonication power was applied. Thus, in order to built the response surface by means of a central composite design (CCD), sonication power and sonication cycles were fixed at 80 % and 9, respectively and sonication time (0.5-10.5 min), pyridine volume (25-125 µL) and BSTFA+1 % TMCS volume (25-125 µL) were studied. The experiments were carried out as explained before and the central point was repeated in triplicate.

The experiments proposed by The Unscrambler program for the CCD and responses (as chromatographic peak areas) obtained are shown in Table 4. The data obtained were fitted to the multilinear equation shown in eq. 2 by means of an analysis of variance (ANOVA) performed by The Unscrambler program, where x_A is the derivatization time, x_B the volume of pyridine, x_C the volume of BSTFA+1 % TMCS and Bi, Bij and Bii the fitting parameters.

Table 4: The design matrix and the responses (as chromatographic peak areas x 10^6) obtained for the central composite design.

Sonication time (min)	$V_{pyridine}$ (µL)	V_{BSTFA} (µL)	E1	E2	MeEE2	EE2	E3
0.5	75	75	2.27	6.79	5.97	6.26	7.48
10.5	75	75	3.01	8.72	7.88	8.66	9.38
5.5	25	75	3.09	8.92	7.83	8.74	9.59
5.5	125	75	3.08	9.06	7.99	8.72	9.52
5.5	75	25	2.72	7.79	6.98	7.29	8.08
5.5	75	125	2.80	8.61	7.42	8.79	9.10
2.5	45	45	2.96	8.18	7.22	7.96	9.05
8.5	45	45	2.84	8.09	7.32	8.02	9.00
2.5	105	45	2.54	7.46	6.75	7.07	8.00
8.5	105	45	2.66	7.79	6.97	7.28	8.21
2.5	45	105	3.02	8.20	7.15	7.36	8.57
8.5	45	105	2.84	8.51	7.63	8.38	9.07
2.5	105	105	2.76	7.75	6.87	7.05	8.22
8.5	105	105	2.75	8.31	7.28	7.60	8.43
5.5	75	75	2.85	8.02	7.07	7.24	8.06
5.5	75	75	2.86	7.77	6.79	7.13	7.71
5.5	75	75	2.71	7.61	6.77	7.17	7.75

$$Y=B_0+B_Ax_A+B_Bx_B+B_Cx_C+B_{AB}x_Ax_B+B_{AC}x_Ax_C+B_{BC}x_Bx_C+B_{AA}x_A^2+B_{BB}x_B^2+B_{CC}x_C^2 \quad (2)$$

B-coefficients with a p-value lower than 0.05 were considered as significant. None of the variables studied was significant for E1, while time (B_A) and pyridine volume (B_{BB}) had a positive effect for the rest of the analytes. Figure 2 shows the response surface for E2. The rest of the compounds showed a similar behavior.

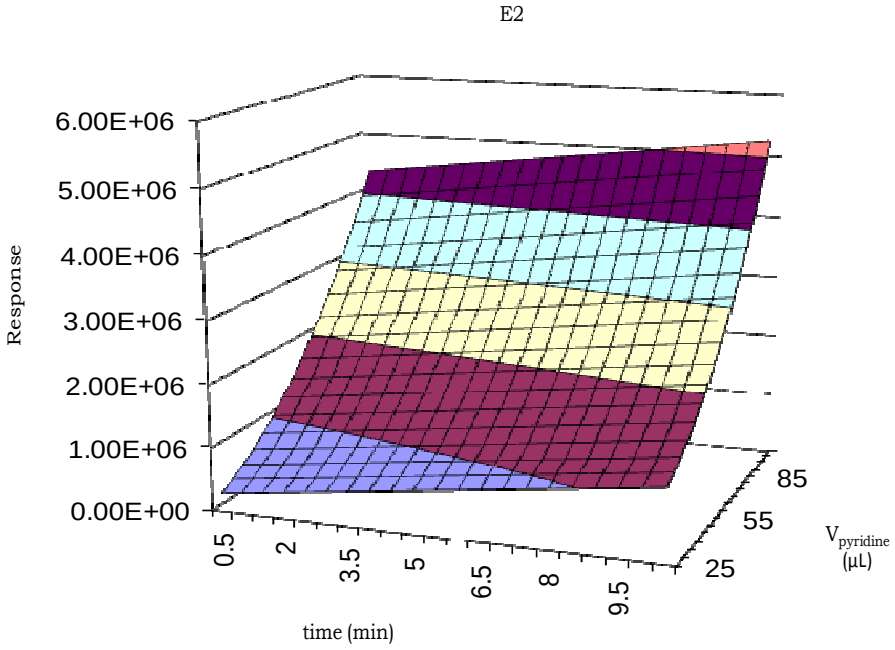


Figure 2: Response surface obtained during the ultrasound assisted derivatization in a cup horn booster for E2. Only significant parameters (p-value < 0.05) have been used in order to build the surfaces.

Thus, sonication time and the volume of pyridine were fixed at the highest values studied, 10 min and 125 μL, respectively. In the case of the volume of the derivatization reagent, it had no significant effect in the range studied (25 - 125 μL) and was, therefore, fixed at the lowest value studied (25 μL) in order to minimize derivatization reagent consumption.

As a summary, the ultrasonic assisted derivatization was performed with 125 μL of pyridine and 25 μL of BSTFA+1 % TMCS for 10 min under 80 % of power and 9 cycles in a cup booster ultrasound bath.

3.3. Optimization of the water change of the cup horn bath

The temperature of the sonication bath increases when a refrigeration system is not installed and this can affect the repeatability of the derivatization reaction. Since our sonication system does not have a refrigeration system, we decided to study how often the water bath should be changed. In this sense three consecutive ultrasound assisted derivatizations were performed without changing the water of the bath. Since the system has three positions, only three simultaneous derivatizations could be performed each time. The results obtained are summarized in Figure 3.

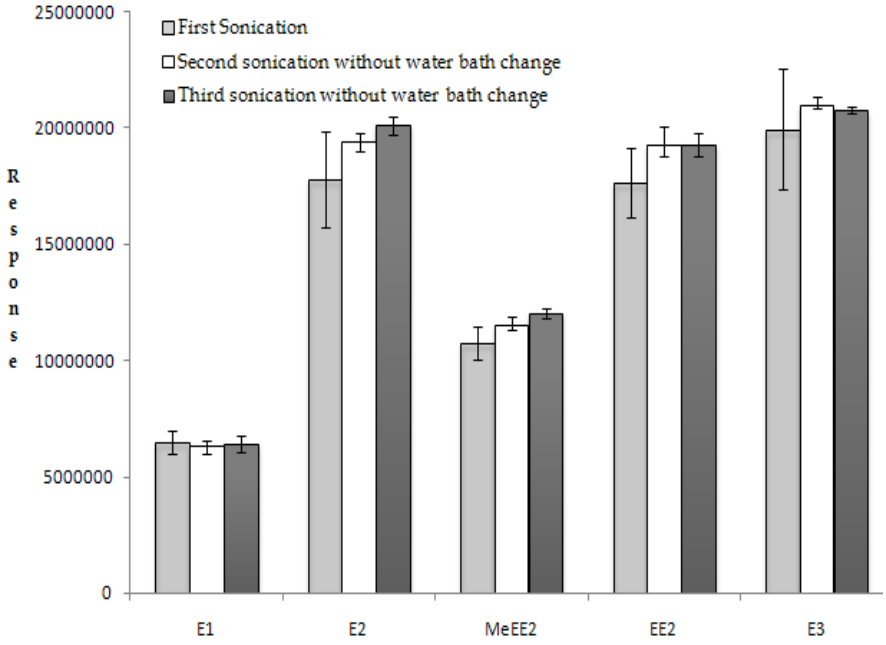


Figure 3: Average (n=3) and standard deviation of the chromatographic peak areas obtained for three consecutive derivatizations performed without changing the water of the cup horn bath.

One factor analysis of variance (ANOVA) was performed in order to know whether the values obtained were comparable. The results were comparable (p-value < 0.05) in all the cases except for MeEE2 (p-value > 0.05). Thus, it could be concluded that at least three consecutive derivatizations could be performed without any special care about bath overcoming.

3.4. Figures of merit of the developed method

Calibration curves were built in the scan and the SIM modes in the 200 ng to 7500 ng and 4 ng to 40 ng ranges, respectively. Good determination coefficients ($r^2 > 0.998$) were obtained in both cases for the analytes studied after correction with E2-d_3.

The whole analytical procedure was applied to three replicates of ~ 1 μg/L of spiked water to determinate the precision of the process. The relative standard deviations (RSD) after correction with E2-d_3 were within the 0.2-1.7 % range, except in the case of MeEE2 whose RSD value was up to 5.3 %.

Limits of detection (LODs) and quantification (LOQs) were estimated as three and ten times the average of the signal to noise ratio (3·S/N and 10·S/N), respectively, of the whole procedure (SPE preconcentration step + ultrasound assisted derivatization and GC-MS analysis) in the SIM mode after 1 min and 10 min of sonication time. Results obtained are included in Table 5. As expected and since sonication time had a positive effect on the derivatization step, better LODs were obtained after 10 min sonication. However, LODs obtained after only 1 min of sonication are similar to most of the LODs found in the literature (see Table 6), except if compared to the results obtained by Zuo et al. [24], who reported even lower LODs for GC-MS analysis.

Table 5: Limits of detection (LODs in ng/L) and limits of quantification (LOQs in ng/L) of each estrogen in the SIM mode. LODs were estimated after 1 min and 10 min sonication.

	SIM (1 min)	SIM (1min)	SIM (10 min)	SIM (10 min)
Analytes	LODs (ng/L)	LOQs (ng/L)	LODs (ng/L)	LOQs (ng/L)
E1	1.62	4.04	0.95	3.15
E2	0.37	1.24	0.35	1.16
MeEE2	6.10	15.26	1.66	5.52
EE2	1.40	3.51	1.00	3.34
E3	1.24	3.11	0.44	1.45

Table 6: Different absolute limits of detection (LODs, ng/L) observed in the literature.

Analytes	Sample	LODs (ng/L)	V_{sample} (L)	Reference
E1,EE2	Surface water	10-20	1	[21]
E1,E2,EE2	Wastewaters	4-17	0.1	[33]
E1,E2,EE2	Water	0.8-3.4	0.5	[6]
E1,E2,EE2	Effluent	0.3-3	1	[36]
E1,E2,EE2,E3,MeEE2	Water	0.3-8.0	1	[22]
E1,E2,EE2,E3,MeEE2	Water	0.02-0.1	0.05	[24]
E1,E2,EE2,E3,MeEE2	Spiked Water	0.37-6.1	0.1	This work (1 min)
E1,E2,EE2,E3,MeEE2	Spiked Water	0.35-1.66	0.1	This work (10 min)

In order to establish method recovery, Milli Q water and natural estuarine water, where the presence of the estrogens had not been observed, were spiked and submitted to the developed analytical procedure. Mean recoveries (n=6) and standard deviations obtained are summarized in Table 7.

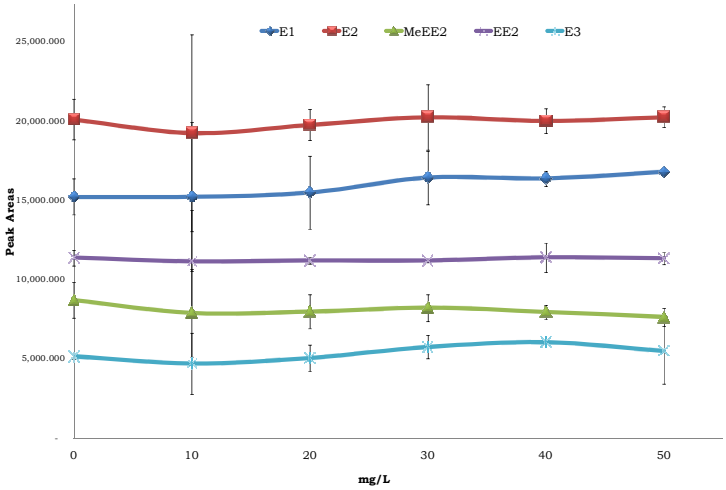
Table 7: Mean recoveries (%) (n=6) and standard deviations obtained for spiked Milli-Q and analyte-free estuarine water.

	Milli-Q (%)	Zorrotza (%)	Bilbao (%)
E1	108±3	115±2	128±3
E2	99±4	108±8	108±4
MeEE2	112±7	119±5	138±3
EE2	96±7	109±8	112±5
E3	89±1	102±2	70±4

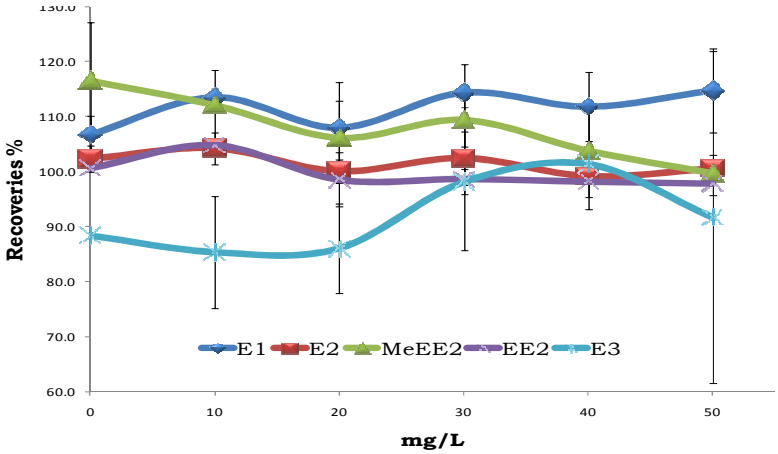
As can be observed, in general, good recoveries were obtained for all the analytes in both Milli Q and estuarine water. Two major conclusions can be draw: firstly, no interferences were observed and, secondly, the presence of organic matter up to ~ 30 mg/L (quantity observed in the estuarine samples) had little or none influence on the recoveries. In order to study more deeply the influence of organic matter on the method recovery, different aliquots of spiked (~ 1 µg/L) Milli Q water, which contained different amounts (0, 10, 20, 30, 40, 50 mg/L) of humic acids, were filtered through 0.45 µm filters, submitted to SPE preconcentration and

ultrasound assisted derivatization before GC-MS analysis. Two replicates were performed at each organic matter level in order to verify the previously observed lack of matrix effect. Results are shown as non-corrected chromatographic peak areas (Figure 4a) and as recoveries after correction with E2-d_3 used as surrogate (Figure 4b).

One factor analysis of variance (ANOVA, 95 % confidence level) of results in Figure 4a and 4b showed that, while no significant differences were observed for the non-corrected signals of none of the analytes, significant differences were observed for MeEE2 after signal correction with E2-d_3. Since the non-corrected signals did not show significant differences, this means that the presence of organic matter has no or little influence on the amount of analyte detected, as pointed out in the literature [6,37]. However, after correction with E2-d_3 significant differences were observed for MeEE2, which means that E2-d_3 is not an ideal surrogate for MeEE2. This lack of correction ability for MeEE2 is only significant when organic matter concentrations are higher than 50 mg/L.



(a)



(b)

Figure 4: Average responses (n=2) obtained for the different estrogens after addition of different amounts of humic acids as: (a) non-corrected chromatographic peak areas. Confidence range shown as two times the standard deviation (n=2). (b) Recoveries after correction with E2-d_3 used as surrogate. Confidence range shown as two times the standard deviation (n=2).

4. Concluding remarks

An ultrasound assisted derivatization procedure was successfully optimized in a cup horn sonication device that allows simultaneous derivatization of at least three samples in a short period of time (1 or 10 min). LODs obtained after 1 or 10 min ultrasound assisted derivatization are similar to those found in the literature for long derivatization procedures.

It could be concluded that this is the first work in the literature where a sonication device such as cup horn that is capable of handling small sample volumes is used with derivatization purposes in the analytical chemistry field.

Acknowledgements

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Abstract

According to the European Water Framework Directive (WFD), alkylphenols such as octyl- and nonylphenols and 17β-estradiol are considered as priority or emerging pollutants respectively, mainly due to their possible properties as endocrine disrupting compounds (EDCs). EDCs are accumulated in liver, fat, kidney and bile in the glucuronide, glucoside and sulfate form.

In order to determine the concentration of these compounds in bile, an enzymatic hydrolysis step is necessary. This step is usually long (~16 h) and, in this sense, ultrasound probes were studied as a possible alternative energy source to accelerate this process. Enzymatic hydrolysis was reduced to 20 min using ultrasound probe at 1 cycle and 10 % of amplitude. For validation of analytical procedure nonylphenol glucuronide (4NP-G), 4-tert-octylphenol glucuronide (4tOP-G) and 4-n-octylphenol glucuronide (4nOP-G) were synthesized while 17β-estradiol glucuronide (E2-G) was commercially available. Bile from thicklip grey mullets (Chelon labrosus) was spiked with known amounts of 4NP-G, 4tOP-G, 4nOP-G and E2-G, and submitted to the optimized procedure. Good recoveries (77-122 %), precision in the 5 % to 12 % range, and limits of detection ranging from the low ng/g level for 4tOP, 4nOP and E2 to the low µg/g level for NPs were obtained.

The optimized method was applied for the determination of alkylphenol in the bile of thicklip grey mullets fish bile from the

Urdaibai estuary (UNESCO reserve of the Biosphere, Bay of Biscay) and high concentrations (2.3-14.2 µg/g), such as those obtained in polluted areas, were measured. E2 was determined in the bile of thicklip grey mullets intraperitoneally injected with E2.

Keywords: Nonylphenols, octylphenols, 17β-estradiol, enzymatic hydrolysis, ultrasound probe, fish bile.

1. Introduction

The interest about endocrine disrupting compounds (EDCs) has increased during the last years due to their interference with the development and functioning of the endocrine system in aquatic animals. EDCs can act at several sites mimicking the occurrence of natural hormones, blocking their production or by inhibiting or stimulating the endocrine system [1-7]. Compounds such as estrogens, pharmaceuticals, phenols, organtins or phthalate esters are considered EDCs [8] and some of them have been classified as priority or emerging pollutants according to European Water Framework Directive (WFD, 2009) [9]. Pollutants such as EDCs are inefficiently eliminated in wastewater treatment plants (WWTPs) and WWTPs effluents are, therefore, one of the major sources of EDCs to the environment. In fact, effluents from several WWTPs have been reported to be estrogenic to fish [10-12].

Compounds such as plant sterols, estrogenic and androgenic synthetic hormones, alkylphenols, phthalates and pharmaceutical drugs are present in WWTPs and can disrupt the reproduction and development of exposed fish [1,13-15]. After metabolization, these compounds tend to accumulate in the bile of fish in the glucuronide form [16-20], although little is known about the concentration of these analytes in bile [11,21-23].

Determination of ECDs in bile is done after a hydrolysis step. Commonly enzymatic hydrolysis [10,22], acid hydrolysis [24,25] or alkaline hydrolysis are used. Analyte losses can occur during acidic

and alkaline hydrolysis due to the strong conditions used, whereas enzymatic hydrolysis of bile, urine and serum is time consuming (~16h) [23,25-27].

During the last years, new ultrasound devices such as ultrasound probes have been developed in order to improve the efficiency and precision of classical ultrasound baths. New ultrasound devices have been mainly used to extract metals [28] and organic compounds [29,30] from solid samples, but other miniaturized ultrasound devices have been applied with other purposes such as derivatization [31]. Recently, ultrasound probes have also been used for the enzymatic hydrolysis of female steroids [32]. Ultrasound probes are more efficient and reproducible than classical ultrasound baths and the equipment involved is relatively cheap if compared to other extraction devices such as microwave ovens or pressurized liquid extraction devices.

In the present work, the optimization and validation of the enzymatic hydrolysis using an ultrasound probe of nonylphenols (NPs), 4*tert*-octylphenol (4*t*OP), 4n-octylphenol (4nOP) and 17β-estradiol (E2) in fish bile was carried out. Variables such as sonication amplitude, sonication time and sonication cycles were studied by means of an experimental design approach. Nonyl- and octylphenol glucuronides (NP-G, 4*t*OP-G and 4nOP-G) in the glucuronide form were synthesized and isolated for validation purposes. The developed method was applied to the determination of NPs, 4*t*OP, 4nOP and E2 in different fish bile.

2. Experimental

2.1. Reagents and Materials

4*tert*-octylphenol (Supelco, Bellenfonte, PA, USA), 4-n-octylphenol (99 %, Aldrich, Steinheim, Germany), 4-nonylphenol (94 %, Riedel de Haën, Seelze, Germany), 17β-estradiol (E2, Sigma, Sigma Reference Standard, Steinheim, Germany), 2,3,5,6-d_4-4-nonylphenol (NP-d_4, min 97 atom % D, Isotec, Miamisburg, Ohio, USA), 16,16,17-d_3-17β-estradiol (E2-d_3, Aldrich, 98 %, Steinheim, Germany), D-(-)-salizin (Salicin, 98 %, Steinheim, Germany), potassium 4-nitrophenol sulfate (4NitroP-S, 98 %, Sigma, Steinheim, Germany), β-D-4-nitrophenol glucuronide (4NitroP-G, 98 % Sigma, Steinheim, Germany), 4-nitrophenol (4NitroP, >99 %, Fluka, Steinheim, Germany) and 2-hydroxybenzyl alcohol (2HBA, 99 %, Aldrich, Milwaukee, Wisconsin, USA) at 4000-5000 mg/L were prepared after dissolving the appropriate amount of the reagents in methanol (Alfa Aesar, 99.9 %, Karlsruhe, Germany).

β-glucuronidase type VII-A from E.Coli (4974.48 unit), β-glucosidase from almonds (102.8 unit) and sulfatase from aerobacter aerogenes (12.25 unit/mL) were obtained from Sigma (Steinheim, Germany), dissolved in Milli Q water (< 0.05 µS/cm, Milli Q model 185, Millipore, Bedford, MA, USA) and divided in 250 µL aliquots, which were kept at -20 °C in closed amber vials until use.

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

Sodium cholate (≥ 96 %), Tris-HCl buffer and UDP-glucuronic acid trisodium salt (≥ 99 %) were obtained from Fluka (Steinheim, Germany) and pooled male rat microsomes, magnesium chloride (98 %) and pyridine (99.8 %) from Sigma (Steinheim, Germany). N,O-bis (trimethylsilyl) trifluoroacetamide with 1 % of trimethylchlorosilane (BSTFA + 1 % TMCS, Sylon BFT, 99:1) was purchased from Supelco, (Walton-on-Thomas, UK).

Acetic acid (99.7 %) and acetonitrile (99.9 %) were obtained from Panreac (Reixac, Barcelona, Spain) and ethyl acetate (HPLC quality) from LabScan (Dublin, Irenland).

200-mg (6 mL) Oasis HLB cartridges were obtained from Waters (Milford, MA, USA).

Biles were obtained from thicklip grey mullets (*Chelon labrosus*) sampled in the estuary of Urdaibai (Basque Country, North of Spain) in April 2009. Some fishes were brought to the laboratory and maintained in 300 L aquaria at 18 ºC with continuous aeration. Animals were fed with dry-bread every day. Fishes were intraperitoneally injected [17] with two doses of 17β-estradiol (1 and 5 mg/Kg) dissolved in a mixture of acetone:corn oil (1:5). Bile samples were collected after 5 and 7 days and stored at

-80 °C until analysis. Cow bile used during optimization was obtained from a local butchery.

2.2. Focused ultrasound assisted enzymatic hydrolysis

100 µL of cow bile, 1.5 mL of phosphate buffer (0.1 mol/L, pH 6.0), 800 µL of Milli Q water, 50 µL of surrogate (E2-d_3 and NP-d_4, 2 mg/L) and 200 µL of corresponding enzymes (1000 units/mL for β-glucuronidase, 2 units/mL for sulfatase and 20 units/mL for β-glucosidase) were added to a 10 mL glass vial. Concentrations of enzymes were chosen according to Gibson et al. [10]. Next, the titanium microtip (MS73, diameter 3 mm, Bandelin, Berlin, Germany) coupled to a Bandelin Sonoplus HD 2070 ultrasound system (20 kHz, 70 W, Berlin, Germany) was immersed 1 cm in the mixture and the enzymatic hydrolysis was carried out at 10 % of amplitude and 1 cycle for 20 min. After hydrolysis 300 µL of acetic acid and 2 mL of Milli Q water were added previous to the solid phase extraction (SPE) clean up.

2.3. SPE clean-up and derivatization

The hydrolyzed bile was loaded onto a 200-mg Oasis HLB cartridge, which had been previously conditioned with 5 mL of methanol (MeOH) and 5 mL of 1 % (v/v) acetic acid solution in Milli Q water. The cartridge was rinsed with 2 mL of Milli Q water, dried under vacuum for 10 min and the target analytes were eluted in 8 mL of ethyl acetate. Finally, the extract was evaporated to dryness under a gentle stream of nitrogen in a Turbovap LV Evaporator

(Zymark, Hopkiton, MA, USA). The concentrated extract was redissolved in 125 µL of pyridine and 25 µL of BSTFA+1 % TMCS and submitted to derivatization for 10 min at 80 % of amplitude and 9 cycles in a Bandelin Sonoplus HD 2070 ultrasound system (20 kHz, 70 W, Berlin, Germany) coupled with a BR 30 Cup booster device as described in a previous work [31]. The extract was kept at -20 °C before GC-MS analysis.

2.4. GC-MS analysis

The derivatized analytes were analyzed in a 6890N gas chromatograph (Agilent Technologies, Avondale, PA, USA) equipped with an Agilent 5973N electron impact ionization mass spectrometer and a 7683 Agilent autosampler. 1 µL of the derivatized extract was injected in the splitless mode at 280 ºC into a HP5-MS (30 m x 0.25 mm, 0.25 µm) capillary column. The following oven temperature program was used for the separation of the analytes: 100 ºC (5 min), temperature increase at 20 °C/min to 200 ºC, a second increase of 1.5 ºC/min up to 240 ºC and a third one of 20 °C up to 300 °C, where it was finally held for 2 min. Helium (99.9995 %, Carburos Metálicos, Barcelona, Spain) was used as carrier gas at a constant flow of 1 mL/min. The transfer line temperature was maintained at 310 ºC and the ion source and quadrupole at 230 ºC and 150 ºC, respectively. Measurements were performed both in the scan (50-525 m/z) and in the SIM (Selected Ion Monitoring) modes. Ions followed in the SIM mode were 135/65 for 4NitroP, 106/78 for 2HBA, 207/278 for 4*t*OP, 416/285 for E2, 419 for E2-d_3,

292/193/179 for NPs, 179/278 for 4OP and 183/296 for NP-d_4. The first ion was used as quantifier and the second one as qualifier.

2.5. Synthesis of individual 4*t*OP-G, 4nOP-G and 4NP-G

4*t*OP-G, 4OP-G and 4NP-G were synthesized according to Kawaguchi et al. [33]. A mixture of 400 µL sodium cholate and 10 µL pooled male rate microsomes was incubated at room temperature for 30 min. 185 µL of 4*t*OP, 4nOP or 4NPs at 300 mg/L together with the cholate activated microsomes, 400 µL Tris-HCl buffer (50 mmol/L, pH=7.4), 1.5 mg UDP-glucuronic acid trisodium salt and 8 µL $MgCl_2$ (800 mmol/L) were incubated for 1h at 37 °C. Afterwards, 200 µL acetonitrile were added and centrifuged at 9 g for 15 min. The supernatant was evaporated to dryness and reconstituted in 200 µL of acetonitrile:water:acetic acid mixture (75:25:0.1, *v/v*). 150 µL were injected to a Tosoh TSKgel 80TM (7.8 mm-30 cm) reverse-phase column coupled to a high performance liquid chromatograph (Agilent Technologies, Series 1100, Avondale, PA, USA) with a diode array detector (DAD, Agilent, Technologies, Series 1100, Avondale, PA, USA), a fluorescence detector (FLD, Agilent, Technologies, Series 1100, Avondale, PA, USA), an automatic autosampler (Technologies, Series 1100, Avondale, PA, USA) and an automatic fraction collector (Technologies, Series 1100, Avondale, PA, USA). The mobile phase was acetonitrile:water:acetic acid (75:25:0.1) at 1.5 mL/min. The DAD was set at 221 nm while the FLD at 315 nm for excitation and 225 nm for emission. 4*t*OP-G, 4OP-G and NP-G were collected in the 6.8

to 10 min, 7.35 to 9 min and 7.1 to 8.6 min, respectively as can be observed in Figure 1. Finally, the fractions were evaporated to dryness and reconstituted in 1 mL of MeOH. In order to guarantee that pure glucuronides had been collected, the extracts were injected in a GC-MS and the mass spectra obtained (see Figure 2) confirmed that alkylphenols were in the glucuronide form.

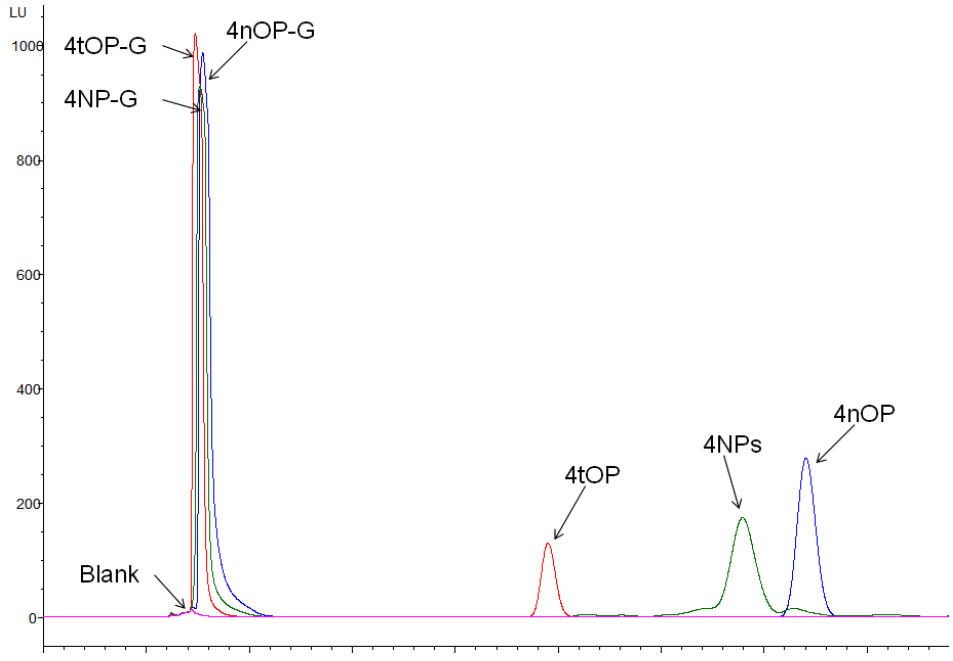

Figure 1: Overlaid chromatograms obtained from HPLC-FLD for 4*t*OP-G, 4nOP-G, 4NP-G and 4*t*OP, 4nOP, NPs.

A mass balance was used in order to calculate the concentration of the synthesized glucuronide derivatives and, with that aim, the concentration of the fraction of alkylphenols that were

not converted to the glucuronide form was calculated. The results obtained are included in Table 1.

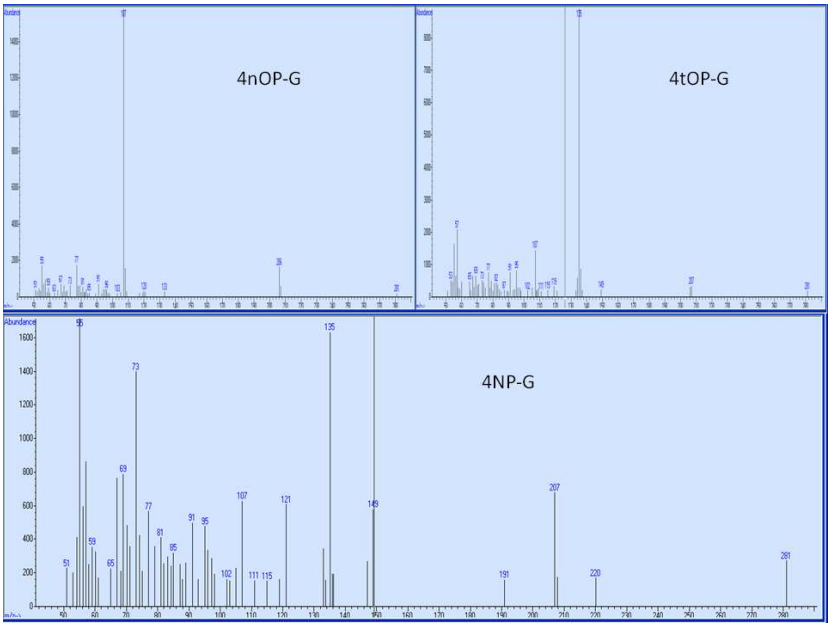

Figure 2: Mass Spectra of different glucuronide forms (4*t*OP-G, 4nOP-G, 4NP-G).

Table 1: Efficiency of the glucuronidation synthesis and concentrations (mg/L) obtained for 4*t*OP-G, 4NP-G and 4nOP-G.

Analyte	Efficiency (%)	mg/L
4*t*OP-G	98	74.9
4NP-G	98	77.2
4nOP-G	93	72.4

3. Results and discussion

3.1. Optimization of the focused ultrasound assisted enzymatic hydrolysis

A central composite design (CCD) was built in order to optimize the focused ultrasound assisted enzymatic hydrolysis of the target analytes in fish bile. The variables and ranges studied were the following: sonication amplitude (5-30 %), sonication cycles (1-9 1/s) and sonication time (1-20 min).

Due to the low volume of bile presented in fishes, cow bile was used during optimization. Besides, since alkylphenol glucuronides, glucosides and sulfates are not commercially available and in order to develop the optimization of the focused ultrasound assisted enzymatic hydrolysis, similar compounds commercially available, D-(-)-salizin, potassium 4-nitrophenol sulfate and β-D-4-nitrophenol glucuronide were used. The enzyme concentration was maintained constant as described by other authors [10].

The design matrix and the results are summarized in Table 2. The data was fitted to a multilinear equation and parameters with a p-value lower than 0.05 were considered significant. The response surface was built with the significant variables (see Figure 3). While none of the studied variables affected to 4-nitrophenol sulfate hydrolysis, sonication cycles (negatively) and the quadratic parameter of time (positively) affected the hydrolysis of D-(-)-salizin. In the case of potassium 4-nitrophenol sulfate and β-D-4-nitrophenol glucuronide, none of the variables affected significantly

the response. 20 min sonication, 1 cycle and 10 % of amplitude (in order to prolong the lifetime of the titanium microtip) were finally chosen as optimum conditions.

Table 2: Design matrix and responses (as chromatographic peak area) obtained for 4-nitrophenol and D-(-)-salizin during the focused ultrasound assisted enzymatic hydrolysis in fish bile.

Sonication time (min)	Sonication amplitude (%)	Sonication cylcles (1/s)	4-nitrophenol	D-(-)-salizin
1	18	5	$7.94 \cdot 10^5$	$1.22 \cdot 10^5$
20	18	5	$9.38 \cdot 10^5$	$3.60 \cdot 10^5$
10.5	5	5	$3.04 \cdot 10^5$	$5.29 \cdot 10^5$
10.5	30	5	$4.21 \cdot 10^5$	$2.53 \cdot 10^5$
10.5	18	2	$7.34 \cdot 10^5$	$2.77 \cdot 10^5$
10.5	18	8	$9.72 \cdot 10^5$	$1.43 \cdot 10^5$
5	10	3	$7.61 \cdot 10^5$	$1.88 \cdot 10^5$
16	10	3	$6.76 \cdot 10^5$	$1.59 \cdot 10^5$
5	25	3	$5.49 \cdot 10^5$	$3.11 \cdot 10^5$
16	25	3	$2.56 \cdot 10^5$	$2.11 \cdot 10^5$
5	10	7	$5.64 \cdot 10^5$	$3.86 \cdot 10^4$
16	10	7	$3.40 \cdot 10^5$	$2.41 \cdot 10^5$
5	25	7	$4.40 \cdot 10^5$	$5.98 \cdot 10^4$
16	25	7	$3.50 \cdot 10^5$	$4.51 \cdot 10^4$
10.5	18	5	$3.35 \cdot 10^5$	$5.02 \cdot 10^4$
10.5	18	5	$4.43 \cdot 10^5$	$5.16 \cdot 10^4$
10.5	18	5	$4.67 \cdot 10^5$	$4.87 \cdot 10^4$

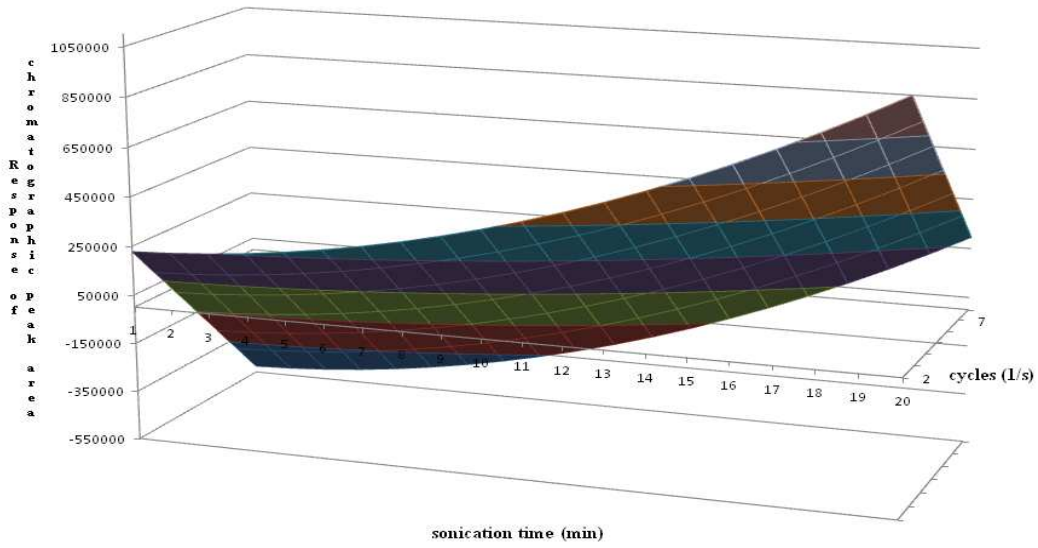

Figure 1: Response surface obtained for D-(-)-salizin using only the significant parameters ($p<0.05$, sonication cycles and the quadratic parameter of time). Sonication amplitude was fixed at 10 %.

3.2.Validation of the procedure

100-µL fish bile aliquots (n=3) were spiked with 50 µL of 4NP-G (~77 mg/L) and 50 µL of 4*t*OP-G, 4nOP-G and E2-G (~ 8 mg/L) and the proposed procedure was applied. Recoveries in the 77-121 % range were obtained (see Table 3). In order to find out whether the lower recoveries obtained for 4nOP were due to a lack of hydrolysis or not, fish bile was spiked with the free forms of analyte and, as it can be deduced from the results included in Table 3, the

low recoveries cannot be attributed to a non exhaustive hydrolysis of 4nOP-G.

Table 3: Recoveries of the whole process obtained after the ultrasonic accelerated enzymatic hydrolysis applied to glucuronide and free compounds.

	Analyte	Recovery (%)
Glucuronide compounds	4*t*OP	102
	NP	121
	4nOP	77
	E2	98
Free compounds	4*t*OP	101
	NP	128
	4nOP	67
	E2	109

Limits of detection (LODs) were estimated as the signal average of five blanks plus three times the standard deviation of the signal (S+3s) and limits of quantification (LOQs) as ten times the standard deviation (S+10s). LODs were 12.7 ng/g, 12.3 ng/g, 5.0 ng/g and 1.8 µg/g for 4*t*OP, 4OP, E2 and 4NPs, respectively and LOQs, 34.9 ng/g, 28.4 ng/g, 15.2 ng/g and 7.2 µg/g. The precision of the method range from 5 % to 12 %.

3.3. Application to different real samples

The method developed in the present work was applied in two different cases. First, alkylphenols were analyzed in 5 biles of thicklip grey mullets (*Chelon labrosus*) fished in the estuary of Urdaibai near the WWTP of Gernika (Biscay, North of Spain).

Concentrations in the μg/g range (see Table 4) were obtained for 4NPs, which are similar to those obtained in polluted zones [23]. Octylphenols and E2 were not detected. Although E2 has not been detected, there are different research articles in literature where E2 was determined in fish bile after enzymatic hydrolysis [7,11,12,19]. Concentrations in the 0.1-152 μg/mL, 4000-0.8 ng/mL and 8.2-0.2 μg/mL ranges have been found in fish bile for NPs, OPs and E2, respectively.

Table 4: Concentration of natural biles obtained from Thicklip grey mullets (*Chelon labrosus*) fished in the estuary of Urdaibai (Basque Country, North of Spain).

		μg/g			
		4*t*OP	NP	4OP	E2
Natural biles	1	nd	2.3	nd	nd
	2	nd	4.0	nd	nd
	3	nd	9.9	nd	nd
	4	nd	11.2	nd	nd
	5	nd	10.66	nd	nd

Afterwards, the procedure was applied to fish bile of thicklip grey mullets, which had been injected different concentrations (1 mg/kg and 5 mg/kg for 5 and 7 days) of E2, in order to observe the bioaccumulation of E2 and to stimulate the synthesis of estrogen dependent proteins vitellogenin and choriogenin (data not shown). As reported by Förlin et al. [17] 90 % of the E2 accumulated in fish bile is present in the glucuronide form. The results obtained are summarized in Table 5.

Table 5: Concentration of E2 in spiked bile of Thicklip grey mullets (Chelon labrosus) fished in the estuary of Urdaibai (Basque Country, North of Spain).

			µg/g
	Spiked concentration	Exposed time	E2
Spiked biles	*1 mg/kg*	*5 days*	11.2
	1 mg/kg	*7 days*	4.3
	5 mg/kg	*5 days*	35.8
	5 mg/kg	*7 days*	18.5

The higher the injected dose, the higher E2 concentrations accumulated in fish bile. Concentrations of E2 decreased 7 days after injection compared to the values obtained 5 days after injection for the two doses tested. This reduction could be related to a higher rate of E2 metabolism and excretion by fish as shown previously in rainbow trout injected with 17α-ethynyl estradiol [34].

4. Conclusions

A new methodology has been developed to accelerate the enzymatic hydrolysis of glucuronide alkylphenols and E2 in fish bile based on focused ultrasounds. Commonly consumed hydrolysis time has been reduced from 16 h to 20 min. The procedure has been successfully validated and applied to samples from different sources.

Acknowledgements

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Abstract

The determination of target analytes such as nonyl- and octylphenols and 17ß-estradiol in fish homogenate require of a solid-liquid extraction step. In this work microwave-assisted extraction (MAE) and focused-ultrasound liquid extraction (FUSLE) were studied as two different alternatives for extraction of the target compounds in zebrafish (Danio rerio) homogenate. In this work solid phase extraction (SPE) using 5-g and 10-g Florisil cartridges and gel permeation chromatography (GPC) were studied for the clean-up of the MAE and FUSLE extracts due to the non-selective extraction step. Although good recoveries were obtained both for SPE (106 % and 126 % range) and GPC (79 % and 100 % range) clean-up procedures, cleaner chromatograms were obtained after SPE and finally 5-g Florisil cartridges were tested since no improvement was observed when 10-g Florisil cartridges were used. Under optimized clean-up conditions, MAE and FUSLE provided comparable results for 4nOP and NP, while more accurate results were obtained for 4tOP and E2 after FUSLE. Finally, the method was applied to the determination of alkylphenols and 17ß-estradiol in zebrafish homogenate that had been exposed to known concentrations of the target analytes. In the case of alkylphenols two different isomers of nonyl- and octylphenol (4-(3',6'-dimethyl-3'hepthyl)phenol, 363-NP, and 4-(3´-methyl-3´-hepthyl)phenol, 33-OP) were studied.

Keywords: nonylphenols, octylphenols, 17β-estradiol, fish homogenate, focused ultrasound liquid extraction, microwave-assisted solvent extraction, clean-up, solid phase extraction, gel permeation chromatography

1. Introduction

The interest of analytes such as alkylphenols and estrogenic compounds has increased in the last decades due to their possible effects as endocrine disruptor compounds (EDCs) once they enter the environmental ecosystems. Actually, alkylphenols such as nonyl- and octylphenols (NPs and OPs, respectively) have been included as priority pollutants by the European Water Framework Directive (WFD) and estrogens such as 17β-estradiol (E2) as emerging pollutants [1].

NPs and OPs are important intermediates in the production and degradation of their polyethoxylate surfactants, which have a wide variety of industrial, agricultural and household applications [2-12]. Because of their properties as EDCs, the use and sale of products containing more than a 0.1 % of nonylphenol ethoxylates or NPs has been forbidden in the European Union (EU) since 2005 [13] and worldwide actions have been taken in order to restrict their use [2]. Not only are these alkylphenols interesting because of their endocrine disruptive properties but also due to their wide distribution and high concentrations in the environment [2]. Under the synthesis procedures used, the technical NP is a mixture of more than 20 isomers, especially *para*-substituted, with different alkylic chains [14-16]. Recently, several works have demonstrated that the estrogenic activity depends on the structural features of the isomers and, thus, it is necessary to study the specific activity of each isomer [17].

The use of steroid hormones such as E2 in the fattening of animals has been described since 1950 but such use was forbidden by the EU [18,19,20] due to the risks on human health [6,21,22].

The most important source of EDCs such as NPs, OPs and E2 to the environment is through urban or industrial inputs [2]. EDCs accumulate in wastewater treatment plants (WWTPs) due to their incomplete elimination during wastewater treatment [2-4,23,24]. This way, the water cycle has become a priority environmental issue [22]. Although EDC concentrations have often been measured in wastewater effluents, no conclusive association has been proven yet between environmentally relevant concentrations and estrogenic effects. In this sense, the measurement of those analytes *in vivo* experiments is necessary to understand their estrogenic activity [25]. Therefore, it is necessary to develop trustful and robust methods for the determination of such analytes in biological tissues such as fish homogenate.

The analysis of target analytes like the ones mentioned above requires of extraction and clean-up steps prior to chromatographic separation and analysis as reviewed elsewhere [26-29]. In the case of the extraction step, Soxhlet is often used [10,30] although it requires of high extraction volumes and long extraction periods. Recently, other extraction techniques such as sonication [31, 32] or accelerated solvent extraction (ASE) [33-35] have also been used for the determination of such target analytes from biota samples in order to minimize solvent consumption and analysis time. In a similar trend, microwave-assisted extraction (MAE) [36-42] or

focused ultrasound solid-liquid extraction (FUSLE) [43-46] have also been successfully applied to the extraction of organic pollutants from environmental matrices such as sediment or biota. However, none of the extraction techniques mentioned above is selective and a clean-up step is necessary in order to eliminate compounds, mainly lipids, that can interfere during the chromatographic separation and analysis of the analytes of interest [30-32,47]. Solid-phase extraction (SPE) using Flosiril, silica, alumina, aminopropyl silica or diol cartridges and/or gel permeation chromatography (GPC) have been mostly studied with these clean-up purposes [30-32,35,47].

The aim of the present work was to study two of the steps, the extraction and clean-up, of the analysis of NPs, OPs and E2 in zebrafish homogenate. This work is part of a project where the estrogenic activity of NP and OP isomers is being studied in both *in vivo* and *in vitro* experiments. Thus, the developed method was applied to the determination of (4-(3',6'-dimethyl-3'hepthyl)phenol, 363-NP, 4-(3´-methyl-3´-hepthyl)phenol, 33-OP) and E2 in zebrafish homogenate.

2. Experimental

2.1. Reagents and Material

4-*tert*-octylphenol (4tOP) was supplied by Supelco (Walton-on-Thames, UK); 4n-octylphenol (4nOP, 99 %), 2,3,5,6-d_4-4n-nonylphenol (4NP-d_4, 98 %) and 16,16,17-d_3-17β-estradiol (E2-d_3, 98 %) by Aldrich (Steinheim, Germany) and nonylphenol technical mixture (NPs, Pestanal®) by Fluka (Steinheim, Germany) and 17β-estradiol (E2, ≥ 98 %) by Sigma (Steinheim, Germany). 5000 mg/L stock solutions of analytes were individually prepared in methanol and stored in amber vials at -20 ºC. Dilutions of the stock solutions (50 mg/L) were performed for sample spiking and calibration purposes.

N,O-bis (trimethylsilyl) trifluoroacetamide with 1 % of trimethylchlorosilane (BSTFA + 1 % TMCS, Sylon BFT, 99:1) was purchased from Supelco (Walton-on-Thomas, UK) and pyridine (99.5 %) from Alfa Aesar (Karlsruhe, Germany).

Methanol (MeOH, HPLC grade, 99.9 %), ethyl acetate (EtOAc, HPLC grade, 99.8 %), dichloromethane (DCM, HPLC grade, 99.8 %) and acetone (HPLC grade, 99.8 %) were supplied by LabScan (Dublin, Ireland).

5-g and 10-g Florisil® cartridges were obtained from Supelco (Walton-on-Thames, UK).

Zebrafish homogenate was prepared as follows: after eliminating the tail and the fins of each zebrafish, samples of each

experimental group were homogenized adding 20 % ultra pure water in a Potter S homogenizer (B. Braun, Melsungen, Germany) held in an ice-water cooled bath with 4-5 strokes.

1 g of fish homogenate was fortified after addition of 10 µg of NPs and 2 µg of 4*t*OP, 4nOP and E2 and covered in acetone in order to obtain a slurry, which was stirred overnight. Acetone and not methanol was preferred for homogenization of the fortified sample since it is more easily evaporated. Acetone was gently evaporated at room temperature and in a hood. The fortified fish homogenate obtained was kept in the refrigerator at -20 °C for a month before extraction.

EtOAc and *n*-hexane used during the synthesis of individual isomers were supplied by Panreac (Barcelona, Spain) and anhydrous ligroin and anhydrous diethyl ether by Aldrich (Steinheim, Germany).

Magnesium, crystal of iodine, 1-bromobutane, 3-methyl-1-bromobutane, 2-butanone and BF_3-Et_2O complex were supplied by Aldrich.

Calcium chloride, ammonium chloride, anhydrous sodium sulfate were supplied by Panreac.

TLC silica gel sheets (0.040-0.063 nm) were supplied by Merck (Darmstadt, Germany).

2.2. Microwave-assisted extraction

MAE experiments were performed with a MDS-2000 closed microwave solvent extraction system (CEM, Matthews, NC, USA) equipped with a 12-sample tray and pressure feedback/control. The MAE procedure used was optimized before for the determination of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), phthalate esters (PEs) and nonylphenols (NPs) in sediment and biota samples [36,37]. Briefly, approximately 0.03 - 0.1 g of fish homogenate were accurately weighed and quantitatively transferred to the Teflon lined extraction vessel. 350 ng of 4NP-d_4 and E2-d_3 and 5 mL of acetone were added to the sample and the extraction vessel was closed. Extractions were performed at 21 psi for 15 min at 504 watt (80 % of the maximum irradiation power). When the irradiation period was completed, samples were removed from the microwave cavity and were allowed to cool to room temperature before opening. The supernatant was filtered through PTFE filters (25 mm, 5 µm, Waters), which had been previously washed with the extraction solvent. The extract was concentrated to ~ 0.5 mL using a nitrogen blow-down evaporation after the addition of ~ 1 mL of *n*-hexane. The concentrated extract was submitted to the clean-up step (SPE or GPC).

2.3. Focused ultrasound solid-liquid extraction

0.03 - 0.1 g of fish homogenate were accurately weighed and transferred to the Teflon lined extraction vessel. 350 ng of 4NP-d_4 and E2-d_3 and 5 mL of acetone were added and the mixture was

exposed to ultrasonic irradiation (Sonopuls HD 2070, 20 Hz, 70 W, Bandelin electronic GMBH & Co. KG, Berlin, Germany) under 45 % power for 2 min and 5 cycles, with the titanium tip (MS73, diameter 3 mm, Bandelein) of the probe immersed 1 cm from the upper surface of the slurry. Instrumental conditions were fixed according to a previous work of the research group [44]. The supernatant was filtered though PTFE filters (25 mm, 5 µm, Waters). The extract was concentrated to ~ 0.5 mL using a nitrogen blow-down evaporation after the addition of ~ 1 mL of *n*-hexane. The concentrated extract was submitted to the clean-up step (SPE or GPC).

2.4. Solid phase extraction

SPE cartridges (5-g or 10-g Florisil) were conditioned with 10 or 20 mL of *n*-hexane, respectively. Next, the extracts were loaded on top of the Florisil cartridges and the analytes were eluted with different volumes of ethyl acetate (see Results and Discussion) in order to minimize the elution of interfering compounds and maximize the recovery of alkylphenols and E2. Finally, the extracts were concentrated to dryness under a gentle stream of nitrogen, previous transfer to 2 mL amber vials, and submitted to a derivatization step before GC-MS analysis.

2.5. Gel permeation chromatography

GPC clean-up of the extracts was carried out in a HP 1100 Series liquid chromatograph (Agilent Technologies, Avondale, PA, USA) coupled to a diode array (DAD) and a fluorescence detector

(FLD) and equipped with an automatic injector and a fraction collector. 150 µL of fish homogenate extract or a solution of target analytes was injected into an Envirosep ABC (350 x 21.2 mm) gel permeation column (Phenomenex®, Torrance, CA, USA). Dichloromethane was used as mobile phase at a flow rate of 5 mL/min. The detectors were set at 254 nm for the DAD and 250 nm (excitation) and 410 nm (emission) for the FLD, respectively.

By means of the fraction collector, from 15.0 min to 18.0 min of the elution profile and at every 0.3 min, a fraction (~1.5 mL) was taken in a vial. The collected fractions were evaporated to dryness, previous transfer to 2 mL amber vials, before derivatization and GC-MS analysis.

2.6. Derivatization

Concentrated extracts were re-dissolved in 125 µL of pyridine and 25 µL of BSTFA + 1 % TMCS were added. The mixture was shaken in a vortex and sonicated at 80 % of power and 9 cycles for 10 min in a Bandelin Sonoplus HD 2070 ultrasound system with a BR 30 Cup booster [48].

2.7. Gas chromatographic-mass spectrometric detection

The derivatized analytes were analyzed in a 6890N gas chromatograph (Agilent Technologies, Avondale, PA, USA) equipped with an Agilent 5973N electron impact ionization mass spectrometer and a 7683 Agilent autosampler. 2 µL of the derivatized extract was injected in the splitless mode at 280 °C into a HP5 MS (30 m x 0.25

mm, 0.25 μm) capillary column. The following oven temperature program was used for the separation of the analytes: 100 ºC (5 min), temperature increase at 10 ºC/min to 200 ºC, a second increase of 5 ºC/min up to 240 ºC, and a final increase of 20 ºC/min to 300 ºC where it was finally held for 2 min. Helium (99.9995 %, Carburos Metálicos, Barcelona, Spain) was used as carrier gas at a constant flow of 1 mL/min. The transfer line temperature was maintained at 310 ºC, and the ion source and quadrupole at 230 ºC and 150 ºC, respectively. Measurements were performed both in the scan (50-525 m/z) and in the SIM (Selected Ion Monitoring) modes. The m/z values followed for each analyte are the following 4*t*OP (207/278), NPs (193/179), 4nOP (179/278), NP-d_4 (183/296), E2 (416/285), E2-d_3 (419/285). First ion was used as quantifier and the second one as qualifier.

2.8. Synthesis of 4-(3´-methyl-3´-hepthyl)phenol (33-OP) and 4-(3',6'-dimethyl-3'-hepthyl)phenol (363-NP)

Synthesis of 3-methylheptan-3-ol and 3,6-dimethylheptan-3-ol

3-Methylheptan-3-ol and 3,6-dimethylheptan-3-ol were synthesized according to Ruβ et al. [49]. Magnesium (2.01 g, 82.3 mmol), diethyl ether (10 mL) and crystal of iodine were put in a two-necked flask with reflux condenser, a calcium chloride tube and dropping funnel. In order to activate magnesium, the mixture was heated smoothly. 1-Bromobutane (8.9 mL, 82.3 mmol) or 3-methyl-1-bromobutane (10.72 mL, 82.3 mmol) dissolved in anhydrous diethyl ether (30 mL) was added slowly. When the addition was

finished, the mixture was heated to 40 ºC for 1 hour. Then, the reaction mixture was cooled to 0 ºC. A solution of 2-butanone (6.7 mL, 74.0 mmol) in anhydrous diethyl ether (20 mL) was added slowly. Then, the reaction mixture was heated to 40 ºC for 1 hour. Subsequently, the resulting octanol or nonanol was protonated at 0 ºC with a mixture crushed ice in water (20 mL) and ammonium chloride (30 mL, 10 %). The organic layer was separated, and the aqueous phase was extracted with diethyl ether (2 x 50 mL). The combined organic phases were washed with aqueous sodium bisulfite (3 x 50 mL, 40 %) and with saturated aqueous sodium bicarbonate (3 x 50 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated in vacuo. 3-Methylheptan-3-ol (9.2 g, 86 %) and 3,6-dimethylheptan-3-ol (10.26 g, 86 %) were obtained as oils (see Appendix A for NMR data).

Synthesis of 4-(3´-methyl-3´-hepthyl)phenol (33-OP) and 4-(3',6'-dimethyl-3'-hepthyl)phenol (363-NP)

In a dry two-necked flask, a solution of 3-methylheptan-3-ol (1.01 g, 7.8 mmol) or 3,6-dimethylheptan-3-ol (1.23 g, 8.52 mmol) and phenol (1.5 g, 15.9 mmol) in anhydrous ligroin (150 mL) was heated under argon at 60 ºC. Then, $BF_3.Et_2O$ complex (1.2 mL, 10.38 mmol) was added slowly and the resulting mixture was stirred for 1 hour at 60 ºC. Crushed ice and water (150 mL) were added and stirring for 30 min more. The organic layer was separated and was washed with water (7 x 30 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated in vacuum. Flash column chromatography (silicagel, *n*-hexane/AcOEt 15/1)

afforded 4-(3´-methyl-3´-hepthyl)phenol (33-OP) (1.12 g, 70 %) and 4-(3',6'-dimethyl-3'-hepthyl)phenol (363-NP) (1.32 g, 70 %) as oils (see Appendix A for NMR data).

2.9. Exposure of zebrafish to 363-NP, 33-OP and 17β-estradiol

Two exposure experiments were carried out. In both experiments, 5 days post fertilization (dpf) to 14 dpf fish were fed Sera Micron (Sera) three times daily. At 14 dpf, zebrafish were fed an alternating diet of live Artemia or Cyclop Eeze (Argent) and Sera Vipan (Sera) in the mornings and only Sera Vipan at midday and afternoons. Fish were maintained in a temperature-controlled room at 28 ºC with a 14-hour light/10-hour dark cycle. Ammonium concentrations were controlled daily.

The aim of the first experiment was to test different concentrations of commercial NP in order to select a sublethal dose for the second experiment. Newly fertilized eggs of zebrafish were collected and immediately transferred to Petri dishes (50 eggs per dish) containing embryo water (0.4 mg/L methylene blue and 0.4 mg/L ampicillin in 1500 µS water). At 1 day dpf eggs (50 per experimental group) were moved to aerated open-circuit 11 L glass tanks and exposed to different concentrations (50, 250, 500 µg/L) of commercial NP (Sigma-Aldrich, Seelze, Germany) for 4 weeks. Then, zebrafishes were kept in the same aquaria with clean water for additional 2 weeks. Concentrations of commercial NP were selected based on previous experiments with zebrafish [50,51]. E2 at 10 ng/L was used as positive control [52] and dimethylsulfoxide

(DMSO) at 0.01 % (v/v) was used as vehicle and as a negative control. All the eggs exposed to 250 and 500 µg/L NP died after one day exposure. The survival rate of fish exposed to 50 µg/L NP, E2 and DMSO were 48 %, 66 % and 91 %, respectively. Therefore, a 50 µg/L dose was selected for the second experiment with NP and OP isomers. At 6 weeks whole body samples were taken from E2 and DMSO control groups (10 and 18, respectively) for fish homogenate chemical analysis. All fish exposed to the commercial NP mixture were used for other analyses and therefore they were not included in the present study.

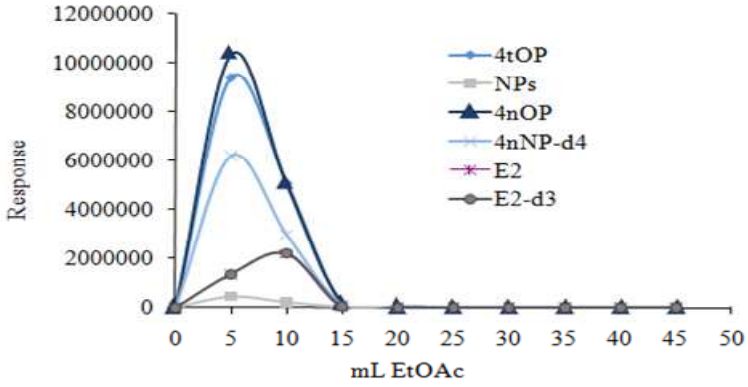
In the second experiment, newly fertilized eggs of zebrafish were collected and immediately transferred to Petri dishes (50 eggs per dish) containing embryo water. At 1 day post-fertilization (dpf) eggs (250 per experimental group) were moved to aerated 38 L glass tanks and exposed to 50 µg/L 363-NP and 33-OP and to DMSO at 0.01 % for 4 weeks and then for additional 2 weeks in clean water. Water (5 L) was changed every 24 h. At 6 weeks whole body samples were taken from 363-NP, 33-OP and DMSO groups (51, 34, and 40, respectively) for fish homogenate chemical analysis.

3. Results and Discussion

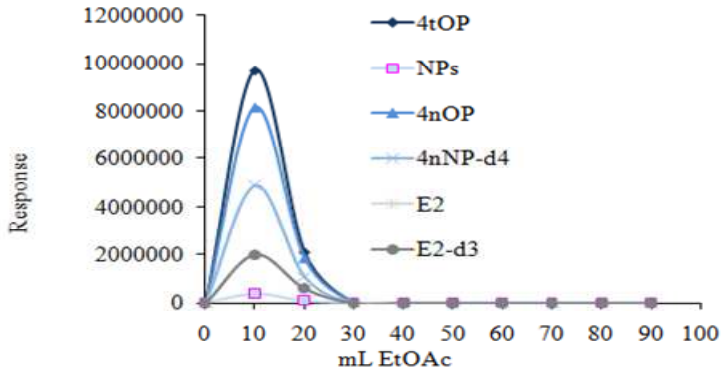
3.1. Optimization of the clean-up step

In order to optimize the clean-up step of the determination of alkylphenols and E2 in fish homogenate, two approaches were studied: SPE using Florisil cartridges and GPC.

Flosiril, silica, alumina, aminopropyl silica or diol cartridges have been mostly used during SPE clean-up of target analytes in biota samples [30-32,35,47]. In this work, 5-g and 10-g Florisil cartridges were studied due to previous experience of the research group [37]. In a first step, the elution volume necessary for the quantitative recovery of the target analytes was also studied. After conditioning with *n*-hexane, cartridges were loaded with 0.5 mL of *n*-hexane containing 350 ng of the target analytes and elution was performed with EtOAc based on previous results [36-53]. 5-mL and 10-mL fractions were collected in the case of 5-g and 10-g Florisil cartridges, respectively. Elution volumes up to 45 mL and 90 mL were studied for 5-g and 10-g Florisil cartridges, respectively. The study was repeated in triplicate for each of the cartridges used. As it can be observed from Figures 1a and 1b, volumes higher than 15 mL and 30 mL did not enhance the recovery of the target analytes from the 5-g and 10-g Florisil cartridges, respectively.



(a)



(b)

Figure 1: Elution profiles of target analytes and deuterated analogues during the elution with EtOAc from (a) 5-g Florisil and (b) 10-g Florisil cartridges.

In order to quantify the recoveries obtained during the clean-up step using 5-g and 10-g Florisil cartridges and GPC, non-fortified fish homogenate was extracted under FUSLE conditions and the extract was divided into three equal volume aliquots that, after evaporation to dryness using a gentle stream of N_2 and

reconstitution in an appropriate solvent, were fortified with 350 ng of the target analytes and submitted to SPE or GPC clean-up. Non-fortified FUSLE extracts were processed in parallel for blank correction. Figure 2 shows the recoveries (n=3) obtained for the target analytes after Florisil clean-up with 5-g and 10-g cartridges, as well as after GPC, after correction with the corresponding deuterated analogues (alkylphenols using 4NP-d_4 and E2 using E2-d_3) that had been added just after FUSLE extraction, correcting the evaporation and clean-up steps.

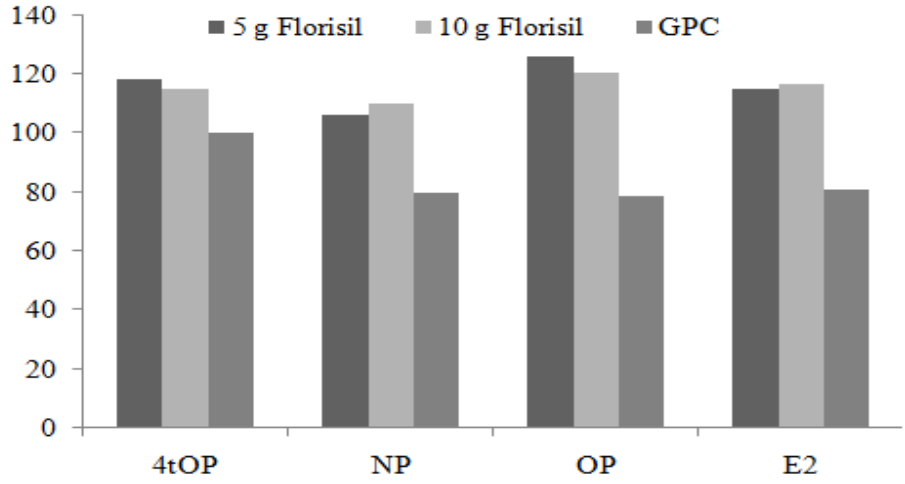


Figure 2: Recoveries obtained for a fish homogenate extracted fortified with target analytes after FUSLE extraction and submitted to different clean-up conditions: 5-g Florisil, 10-g Florisil and GPC.

As it can be observed, good recoveries were obtained for 5-g Florisil (106-126 %), 10-g Florisil (110-120 %) and GPC (79-100 %) clean-up. Relative standard deviations (RSD) lower than 15 % were obtained in all the cases. Blank samples of non-spiked fish homogenate showed no signal at the retention time of the target

analytes in the SIM mode, thus recoveries higher than 100 % could not be attributed to the presence of interferences.

Finally, the SIM chromatograms obtained for the extracts obtained after 5-g Florisil (Figure 3a), 10-g Florisil (Figure 3b) and GPC (Figure 3c) clean-ups show that Florisil based SPE provided cleaner chromatograms than GPC and that the chromatograms obtained for 5-g or 10-g Florisil extracts were similar. Besides, according to the analysis of variance of the results obtained for the 5-g and 10-g Florisil cartridges, no significant differences were obtained ($F_{calc}<F_{crit}=7.71$, for a 95 confidence interval). Therefore, 5-g Florisil cartridges were finally chosen.

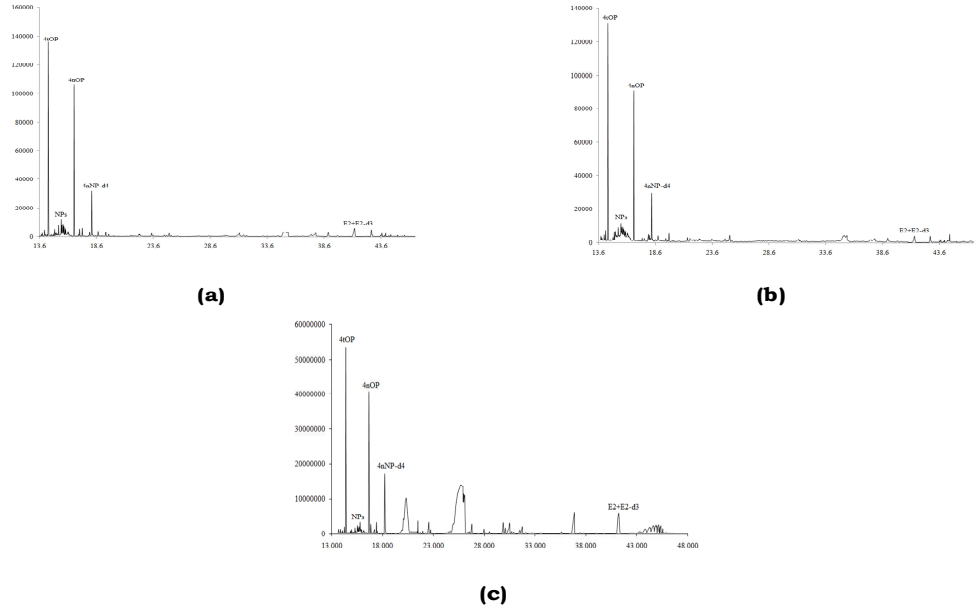


Figure 3a, 3b and 3c: SIM Chromatograms obtained for fish homogenate fortified with target analytes and surrogates after (a) SPE clean-up with 5-g Florisil cartridges, (b) SPE clean-up with 10-g Florisil cartridges and (c) GPC clean-up.

3.2. MAE versus FUSLE

MAE and FUSLE were studied as two different alternatives for the solid-liquid extraction of alkylphenols and E2 from fish homogenate samples. Working conditions were chosen from previously developed methods in our research group [36,37,44]. Fish homogenate was fortified with target analytes and aged for 1 month. Different aliquots were processed using FUSLE and MAE procedures. Both MAE and FUSLE extracts were submitted to SPE clean-up using 5-g Florisil cartridges and elution with 15 mL EtOAc as optimized before. Non-fortified fish homogenate was also processed in parallel for blank correction. Average recoveries (n=3) and standard deviations obtained after correction with the corresponding blanks and deuterated surrogates are included in Figure 4.

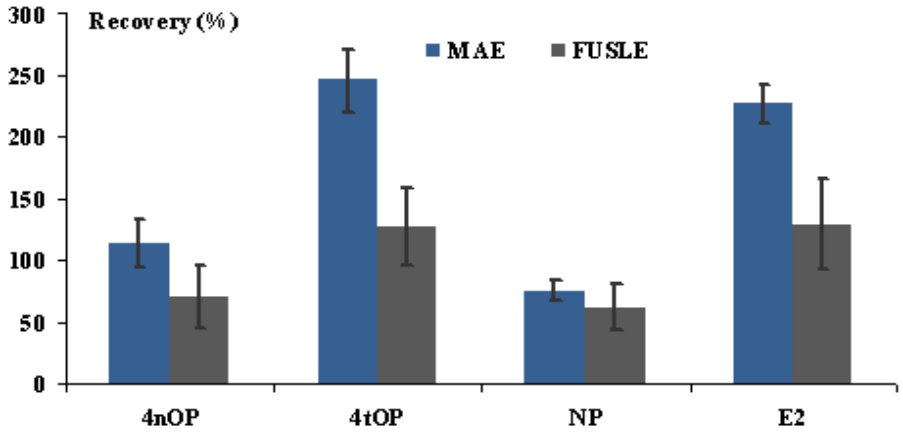


Figure 4: Comparison of the average (n=3) recovery percentages obtained for 4nOP, 4tOP, NPs and E2 after MAE and FUSLE of a fortified zebrafish homogenate.

Comparable (F_{calc}=1.5-5.9<39) relative standard deviations (RSD) were obtained for MAE (7-25 % range) and FUSLE (10-31 %). RSD values in the 20 % range are found in the literature for similar analyses [31,32]. According to Student t-test and for a 95 % interval of confidence, no significant differences were obtained for the recoveries obtained for 4nOP and NPs after FUSLE and MAE (t_{calc}=1.14-2.34<t_{crit}=2.78), while more accurate results were obtained for 4*t*OP and E2 after FUSLE. The results obtained for 4tOP and E2 after MAE exceed by far the 100 % value, indicating the possibility of some kind of interference for these two analytes. It should be kept in mind that the clean-up optimization was carried out using FUSLE extracts and it seems that MAE extracts needed further purification. Thus, the analysis of real samples was performed under FUSLE extraction.

Limits of detection (LODs) were calculated as the average signal (n=3) plus three times the standard deviation of reagent blank samples. The values obtained were 2 ng (4*t*OP), 827 ng (4NPs), 0.04 ng (4nOP) and 1.5 ng (E2), similar to those obtained in the literature [7,8,32,34,54].

Finally, the optimized method was applied to the determination of 363-NP, 33-OP and E2 in zebrafish homogenate. Concentrations of 1.8 μg/g (363-NP), 33 μg/g (33-OP) and 0.35 μg/g (E2) were obtained.

4. Conclusions

Two different alternatives both for extraction and clean-up of alkylphenols and E2 in zebrafish homogenate were studied. While SPE provided cleaner extracts than GPC for FUSLE, MAE provided recoveries that exceeded the 100 % extraction yield for certain analytes (4*t*OP and E2), probably due to inadequate clean-up of MAE extracts in 5-g Florisil cartridges. The developed method was applied to the determination of octyl- and nonylphenol isomers and E2 in zebrafish homogenate exposed to the cited endocrine disruptors.

Acknowledgements

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Appendix I

NMR data of 4-(3´-methyl-3´-hepthyl)phenol (33-OP) and 4-(3',6'-dimethyl-3'-hepthyl)phenol (363-NP)

4-(3´-methyl-3´-hepthyl)phenol (33-OP)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.66 (t, *J* = 7.4 Hz, 3H, C$\underline{H}_3$-CH_2-CH_2), 0.82 (t, *J* = 7.3 Hz, 3H, C$\underline{H}_3$-CH_2-C), 0.90-0.97 (m, 1H, C-C$\underline{H}_2$), 1.07-1.15 (m, 1H, C-C$\underline{H}_2$), 1.21 (s, 3H, C-C$\underline{H}_3$), 1.23-1.28 (m, 2H, CH_3-C$\underline{H}_2$-C), 1.44-1.49 (m, 2H, C-C$\underline{H}_2$-C$\underline{H}_2$), 1.50-1.71 (m, 2H, C-C$\underline{H}_2$-C$\underline{H}_2$), 4.45 (broad s, 1H, OH), 6.76 (d, *J* = 8.5 Hz, 2H, $H_{2,6}$), 7.13 (d, *J* = 8.5 Hz, 2H, $H_{3,5}$). ^{13}C NMR ($CDCl_3$) δ = 8.6 (-CH_2-CH_2-$\underline{C}H_3$), 14.0 (CH_2-$\underline{C}H_3$), 23.4 (-CH_2-$\underline{C}H_2$-CH_3), 23.6 ($\underline{C}H_3$-C), 26.4 (-$\underline{C}H_2$-CH_2-CH_3), 35.6 (CH_3-$\underline{C}H_2$-C), 40.3 (CH_3-CH_2-$\underline{C}$), 42.8 (C-$\underline{C}H_2$-CH_2), 114.6 ($C_{2,6}$), 127.6 ($C_{3,5}$), 140.3 (C_4), 152.9 (C_1).

4-(3',6'-dimethyl-3'-hepthyl)phenol (363-NP)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.66 (t, *J* = 7.4 Hz, 3H, C$\underline{H}_3$-CH_2), 0.80-0.83 (m, 6H, ($C\underline{H}_3$)$_2$CH), 0.84-0.88 (m, 1H, (CH_3)$_2$CH-C$\underline{H}_2$), 0.97-1.04 (m, 1H, (CH_3)$_2$CH-C$\underline{H}_2$), 1.21 (s, 3H, C-C$\underline{H}_3$), 1.38-1.45 (m, 1H, (CH_3)$_2$C$\underline{H}$), 1.47-1.51 (m, 1H, C-C$\underline{H}_2$), 1.52-1.57 (m, 1H, CH_3-C$\underline{H}_2$-C), 1.61-1.67 (m, 1H, C-C$\underline{H}_2$), 1.68-1.71 (m, 1H, CH_3-C$\underline{H}_2$-C), 4.62 (broad s, 1H, OH), 6.76 (d, *J* = 8.6 Hz, 2H, $H_{3,5}$), 7.13 (d, *J* = 8.6 Hz, 2H, $H_{3,5}$). ^{13}C NMR ($CDCl_3$) δ = 8.6 (C-CH_2-$\underline{C}H_3$), 22.6 (($\underline{C}H_3$)$_2$CH), 23.6 (C-$\underline{C}H_3$), 28.7 ((CH_3)$_2$$\underline{C}$H), 33.2 (($CH_3$)$_2$CH-$\underline{C}H_2$), 35.7 ($CH_3$-$\underline{C}H_2$-C), 40.3 ($CH_3$-$CH_2$-$\underline{C}$), 40.6 (C-$\underline{C}H_2$-$CH_2$), 114.7 ($C_{2,6}$), 127.6 ($C_{3,5}$), 140.3 ($C_4$), 152.9 ($C_1$).

Abstract

Large volume injection-programmable temperature vaporization-gas chromatography-mass spectrometry (LVI-PTV-GC-MS) was optimized for the determination of estrone (E1), 17β-estradiol (E2), 17a-ethynyl estradiol (EE2), mestranol (MeEE2) and estriol (E3) for their determination in environmental samples (estuarine water, wastewater, fish bile and fish homogenate) after derivatization with 25 µL (BSTFA + 1 % TMCS) and 125 µL of pyridine. Experimental designs such as Plackett-Burman (PBD) and central composite designs (CCD) were used to optimize the LVI-PTV variables (cryo-focusing temperature, vent time, vent flow, vent pressure, injection volume, purge flow to split vent, splitless time and injection speed). Optimized conditions were as follows: 45 µL of n-hexane extract are injected at 60 °C and 6 µL/s with a vent flow and a vent pressure of 50 mL/min and 7.7 psi, respectively, during 5 min; then the split valve is closed for 1.5 min and afterwards the injector is cleaned at 100 mL/min before the next injection. The method was applied to the determination of estrogenic compounds in environmental samples such as estuarine water, wastewater, and fish homogenate and bile. Limits of detection (0.04-0.15 ng/L for water samples, 0.04-0.67 ng/g for fish bile and 0.1-7.5 ng for fish homogenate) obtained were approx. ten times lower than those obtained by means of a common split/splitless inlet.

Keywords: environmental samples; estrogens; experimental design; large volume injection-programmable temperature vaporization; derivatization.

1. Introduction

The development of analytical methods for analytes such as hormones and steroids is of increased interest due to their possible adverse effects as endocrine disrupters in the aquatic environment, especially in fishes and amphibians [1-3]. These effects can cause feminization in male fishes (imposex) or to promote abnormal reproductive processes [4-6]. Therefore, in 2003, fourteen steroids and hormones were included in the list of emerging pollutants of concern (EPOCs) within the European Water Framework Directive (WFD, 2009) [7].

Estrone (E1), 17β-estradiol (E2), 17α-ethynyl estradiol (EE2), mestranol (MeEE2) and estriol (E3) are some of the analytes included in the previously mentioned list. Natural hormones, E1, E2 and E3, are synthesized by all species and sexes. Humans, livestocks and wildlife are the main sources of those compounds and the concentration excreted varies during the different stages of life [8-11]. Synthetic hormones such as EE2 and MeEE2 are supplied to females as contraceptives or used in different medical treatments [9].

These compounds are excreted in urine or excrement as glucuronide, glucoside or sulfate derivatives [12,13], they are partially deconjugated in sewer systems or in wastewater treatment plants (WWTPs) and finally accumulated in sludges and effluents [14-16]. Within this scenario, fish are exposed to doses of such analytes that are accumulated in bile preferentially in the glucuronide form [5,17-20].

Estrogens, such as those mentioned above, can be analyzed using different techniques such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), tandem systems of mass spectrometry (LC-MS/MS or GC-MS/MS), liquid chromatography with fluorescence detection (LC-FLD), or even by means of immunoassay techniques [21-25]. When GC is used, derivatization of the compounds is a necessary step in order to improve selectivity and sensitivity. Usually, this last step is carried out in heater blocks, sand baths or ovens [26-30] for 30-90 min at high temperatures (60-75 °C). However, this step can be accelerated using other energy sources such as microwave ovens [31] or ultrasound baths [32,33]. Although ultrasonic probes are the mostly used with extraction purposes [34-36], they can also be used for derivatization [37] or even using other new ultrasound devices such as ultrasonic cup boosters [38]. The latter are small ultrasonic baths (< 12 mL) which can handle low sample volumes (< 1.5 mL). Besides, since samples are introduced in safe lock microtubes, cross contamination is avoided.

The splitless inlet is the most commonly used device in GC when trace analysis is accomplished. However, it has several deficiencies since it is a hot vaporizing device. In order to improve it, in the late 1970s, the programmable-temperature vaporization (PTV) inlet was developed based on a splitless inlet. During the injection this inlet is kept cool and, using a temperature controlled program, it is heated up quickly to transfer the sample into the column. One of the most interesting aspects of PTV injection is the ability to

perform large volume injection (LVI) using the cold split/splitless solvent vent technique [39]. In this sense, LVI-PTV can improve the sensitivity in several orders of magnitude in comparison with common splitless inlets. LVI-PTV has already been used for the determination of several pollutants [40-42], including some estrogenic compounds [43] but with no previous derivatization. In this work, LVI-PTV-GC-MS conditions were optimized for the determination of estrogenic compounds (E1, E2, EE2, MeEE2 and E3) in environmental samples (estuarine water, wastewater, fish homogenate and fish bile). Variables such as cryo-focusing temperature, vent time, vent flow, vent pressure, injection volume, purge flow to split vent, splitless time and injection speed were evaluated by means of design of experiments. Limits of detection (LODs) obtained were compared with those obtained in a common split/splitless inlet.

2. Experimental

2.1. Reagents and Materials

Estrone (E1, 99.5 %), mestranol (MeEE2, 99.4 %), 17α-ethynylestradiol (EE2, 99.4 %) and estriol (E3, 99.7 %) were obtained from Riedel-de HaënSeelze, Germany. 17β-estradiol (E2, Sigma Reference Standard, Steinheim, Germany), and 16, 16, 17-d_3-17β-estradiol (E2-d_3, 98 %, Steinheim, Germany) were also purchased from Sigma and Aldrich, respectively. All of them were

dissolved individually in anhydrous methanol (Alfa Aesar, 99.9 %, Karlsruhe, Germany) at ~2000 mg/L concentration and the standard solutions were stored at 4 ºC in the dark. 3 µg/L to 150 mg/L standards were prepared in ethyl acetate (HPLC, Lab Scan analytical science, 99.8 %, Dublin, Ireland) and stored in amber vials at -20 ºC.

Acetone (HPLC grade) was supplied by LabScan (Dublin, Ireland) and *n*-hexane (HPLC grade) and acetic acid (99.7 %) were obtained from Panreac (Reixac, Barcelona, Spain).

β-glucuronidase, type VII-A, from Escherichia coli (4974.48 unit), β-glucosidase from almonds (102.8 unit) and sulfatase from aerobacter aerogenes (12.25 unit/mL) were obtained from Sigma (Steinheim, Germany), dissolved in Milli-Q water (< 0.05 µS/cm, Milli Q model 185, Millipore, Bedford, MA, USA) and divided in 250 µL aliquots, which were kept at -20 °C in closed amber vials until use.

Potasium di-hydrogenphosphate (RFE, USP-NF, BP, Ph.Eur., 100 %, Panreac, Reixac, Barcelona, Spain) and di-ammoniun hydrogenphosphate (99 %, Merck, Darmstadt, Germany) were used to prepare 0.1 mol/L buffer solution (pH=6).

Anhydrous pyridine (99.8 %) was obtained from Sigma-Aldrich (Steinheim, Germany) and the derivatization reagent, BSTFA + 1 % TMCS (Sylon BFT, 99:1) from Supelco (Walton-on-Thomas, UK).

200-mg (6 mL) Oasis HLB cartridges were obtained from Waters (Milford, MA, USA) and 1-g and 5-g Florisil® cartridges from Supelco (Walton-on-Thames, UK).

2.2. Sampling

Estuarine water samples were collected from Zorrotza (Basque Country, Spain) and influent and effluent water samples were collected from WWTPs of both Gernika and Bakio (Basque Country, Spain) in October of 2009 in pre-cleaned glass bottles, transported to the laboratory in cooled boxes, filtered through 0.45 μm filters (Whatman, cellulose nitrate membrane filters, Dassel, Germany) and analyzed within 48 hours.

Biles were obtained from thicklip grey mullets (*Chelon labrosus*) fished in the estuary of Urdaibai near the WWTPs of Gernika (Biscay, North of Spain).

Zebrafish (*Danio rerio*) homogenate was prepared eliminating the tail and the fins of each zebrafish. Samples of each experimental group were homogenized adding 20 % ultra pure water in a Potter S homogenizer (B. Braun, Melsungen, Germany) and held in an ice-water cooled bath with 4-5 strokes.

2.3. Water sample pre-concentration

In the case of estuarine and wastewater samples, target analytes were pre-concentrated using SPE according to Hernando et al. [44]. Briefly, a 100-mL aliquot of the water sample, which

contained E2-d_3 at ~10 ng/L, was loaded into a 200-mg Oasis HLB cartridge, which had been previously conditioned with 5 mL of ethyl acetate, 5 mL of methanol and 5 mL of Milli Q-water. Then, the cartridge was washed with 5 mL of a Milli Q-water:methanol mixture (95:5, *v/v*) and dried under vacuum for 15 min. Finally, the analytes were eluted using ethyl acetate (two portions of 4 mL). The extract was finally evaporated to dryness under a gentle stream of nitrogen in a Turbovap LV Evaporator (Zymark, Hopkinton, USA) previous to the derivatization step.

In the case of wastewater samples, the ethyl acetate extract was further cleaned-up using 1-g Florisil® cartridge as described by Guitart et al. [42]. The extract (8 mL of ethyl acetate) was evaporated to approx. 100 µL under a gentle stream of nitrogen and passed through a previously activated (5 mL *n*-hexane and 5 mL ethyl acetate) 1-g Florisil® cartridges. Finally, the analytes were eluted with 8 mL of a mixture of dichloromethane:ethyl acetate:methanol (40:40:20, *v/v*) and evaporated to dryness under a gentle stream of nitrogen previous to derivatization.

2.4. Fish homogenate: ultrasonic extraction and clean-up

The extraction was carried out according to the results obtained in a previous work [45]. Briefly, approx. 0.03 - 0.1 g of fish homogenate were weighed and transferred to a Teflon lined extraction vessel. 2 ng of E2-d_3 and 5 mL of acetone were added and the mixture was exposed to ultrasonic irradiation (Sonopuls HD 2070, 20 kHz, 70 W, Bandelin electronic GMBH & Co. KG, Berlin,

Germany) under 45 % power for 2 min and 5 cycles, with the titanium tip of the probe (MS73, diameter 3 mm, Bandelin, Berlin, Germany) immersed 1 cm. The supernatant was filtered through PTFE filters (25 mm, 5 µm, Waters) and the extract concentrated to ~ 0.5 mL using nitrogen blow-down evaporation after the addition of ~1 mL of *n*-hexane. The concentrated extract was then submitted to a SPE clean-up.

5-g Florisil® cartridge was conditioned with 10 mL of *n*-hexane. Afterwards the extract was loaded and eluted with 15 mL of ethyl acetate. Finally, the extract was evaporated to dryness under a gentle stream of nitrogen previous transfer to 2 mL of amber vial and derivatized as explained below (see Section 2.6).

2.5. Ultrasonic hydrolysis and SPE clean up of fish bile

Biles were obtained from thicklip grey mullets (*Chelon labrosus*) and stored at -80 ºC until analysis.

The ultrasonic hydrolysis was previously optimized [46]. In this sense, 100 µL of fish bile were accurately weighed and 1.5 mL of phosphate buffer (0.1 mol/L, pH 6.0), 800 µL of Milli-Q water, 2 ng of E2-d_3 and 200 µL of corresponding enzymes (1000 units/mL for β-glucuronidase, 2 units/mL for sulfatase and 20 units/mL for β-glucosidase) were added to a 10 mL glass vial. The titanium microtip coupled was immersed 1 cm in the mixture and the enzymatic hydrolysis was carried out at 10 % of amplitude and 1

cycle during 20 min. Then, 300 µL of acetic acid and 2 mL of Milli-Q water were added previous to the SPE clean-up step.

The hydrolyzed bile was loaded onto a 200-mg Oasis HBL cartridge, which had been previously conditioned with 5 mL of MeOH and 5 mL of 1 % (v/v) acetic acid solution in Milli-Q water. The cartridge was rinsed with 2 mL of Milli-Q water, dried under vacuum for 10 min and the target analytes were eluted in 8 mL of ethyl acetate. Finally, the extract was evaporated to dryness under a gentle stream of nitrogen.

2.6. Derivatization

The extracts were re-dissolved in 125 µL of pyridine and 25 µL of BSTFA + 1 % TMCS in 2-mL amber vials, shaken in a vortex and sonicated at 80 % of power and 9 cycles for 10 min in a Bandelin HD 2070 ultrasound cup booster (Berlin, Germany) [38]. The pyridine extract was evaporated to dryness under a gentle stream of nitrogen and reconstituted in 120 µL of n-hexane, which allowed 45 µL injection of the samples without bubble introduction in the PTV inlet.

2.7. LVI-PTV-GC-MS analysis

LVI of the extracts was performed in a CIS 4 PTV inlet (Gerstel GmbK & Co. KG, Mülheim an der Ruhr, Germany), which contains a septumless head and an empty baffled deactived glass liner kept cool using liquid nitrogen. A 45-µL aliquot of sample extract was injected using a 100-µL syringe in a MPS2 autosampler (Gerstel) at

60 ºC, while the vent valve was opened for 5 min, at a flow rate of 50 mL/min and a vent pressure of 7.7 psi. Afterwards, the vent valve was closed for 1.5 min and the PTV temperature was increased to 300 ºC at a 12 ºC/s rate and held at 300 ºC for 3 min. Finally, the inlet was further cleaned at a purge flow of 100 mL/min before further injections.

The derivatized analytes were introduced into a 6890 gas chromatograph (Agilent Technologies, Avondale, USA) equipped with an Agilent 5975 electron impact ionization mass spectrometer and with a HP5 MS (30m x 0.25 mm, 0.25 μm) capillary column. The following oven temperature program was used for the separation of the analytes: 50 ºC (6 min), temperature increase at 15 ºC/min to 200 ºC, a second increase of 1.5 ºC/min up to 240 ºC followed by a 20 ºC/min increase up to 300 ºC, where it was finally held for 2 min. Helium (99.9995 %, Carburos Metálicos, Barcelona, Spain) was used as carrier gas at a constant flow rate of 1 mL/min. The transfer line temperature was maintained at 310 ºC, and the ion source and quadrupole at 230 ºC and 150 ºC, respectively. Measurements were performed both in the scan (50-525 m/z) and in the SIM (Selected Ion Monitoring) modes. The following m/z fragment ions were monitored in the SIM mode: 342/327 (E1), 416/285 (E2), 419 (E2-d_3), 367/382 (MeEE2), 425/440 (EE2), 345/504 (E3). The first ion was used as quantifier and the second one as qualifier.

3. Results and discussion

3.1. Technical problems and improvement of blanks

In a first approach, all the derivatization experiments were carried out under sonication of the analytes in 500-µL amber Eppendorf safe lock microtubes and without evaporation of the pyridine used during derivatization with BSTFA + 1 % TMCS in the ultrasound cup booster. However, large peaks overlaid the signals of EE2 and MeEE2 and overpressure and failure of the EPC (Electronic Pressure Control) unit were observed.

On the one hand, Eppendorf microtubes were substituted by amber glass vials during derivatization and new tips for micropipettes from another supplier were obtained. As a result, all the previous undesirable chromatographic peaks were eliminated. Although an unknown peak was still observed, chromatographic separation from target analytes was possible.

On the other hand, pyridine was evaporated and the analytes were re-dissolved in 120 µL of n-hexane. No further overpressure or failure of the EPC unit was observed.

3.2. Optimization of the LVI-PTV parameters

Eight parameters of the LVI-PTV system were optimized: cryo-focusing temperature (T_{CIS}, ºC), vent time (t_{vent}, min), vent flow (F_{vent}, mL/min), vent pressure (P_{vent}, psi), injection volume (V_{inj}, µL), purge flow to split vent (F_{purge}, mL/min), splitless time ($t_{splitless}$, min) and

injection speed (v_{inj}, µL/s). An experimental design approach was chosen since maximum information can be obtained with a minimum number of experiments and interactions among variables are also considered.

First, a Plackett-Burman design was performed to establish which variables had a significant influence in the responses. The ranges studied were: cryo-focusing temperature (40-80 °C), vent time (0.4-5 min), vent flow (50-100 mL/min), vent pressure (2-7.7 psi), injection volume (25-45 µL), purge flow to split vent (30-100 mL/min), splitless time (0.5-1.5 min) and injection speed (2-6 µL/s). A Plackett-Burman design was built (see Table 1) and the responses were defined as the ratio of the chromatographic peak area and chromatographic peak width, in order to maximize peak area and minimize peak width. The design matrix and the responses obtained for the analytes of interest are also included in Table 1.

The effect of the variables studied was defined according to the p-values obtained (p-value > 0.05 no significant, p-value 0.01-0.05 significant, p-value 0.005-0.01 highly significant and p-value < 0.005 very highly significant) for a 95 % confidence level. According to the results obtained (Table 2) all the variables studied had a significant effect on one or another analyte. However, the apparently least significant vent pressure, vent time, vent flow, purge flow to split vent and splitless time variables were fixed. Due to its negative effect, vent flow was fixed at the lowest value studied (50 mL/min), while vent pressure, purge flow to split vent, splitless time and vent

time were set at the highest values studied (7.7 psi, 100 mL/min, 1.5 min and 5 min, respectively) since, when significant, they showed a positive effect. In the case of splitless time, although significant for all the analytes, we decided not to study it further since, according to the experience of our research group [41], higher values cause contamination when repeated injections are performed. In the case of purge flow high values also prevent from inlet contamination. The rest of the variables were further evaluated by means of a central composite design (CCD) in order to obtain the response surfaces. Thus, the variables were studied in the following ranges: T_{CIS} (40-80 °C), Vinj (25-45 µL), and vinj (2-6 µL/s).

Table 1: Design matrix and responses, defined as the peak area/peak width ratio (x 10^6), obtained for the Plackett-Burman design.

P_{vent} (psi)	T_{CIS} (°C)	t_{vent} (min)	F_{vent} (mL/min)	F_{purge} (mL/min)	V_{inj} (µL)	$t_{splitless}$ (min)	v_{inj} (µL/s)	E1	E2	E2-d_3	MeEE2	EE2	E3
7.70	80	0.4	100	100	45.0	0.5	2	0.55	1.58	1.09	12.55	3.84	1.36
2.00	80	5.0	50	100	45.0	1.5	2	1.48	3.77	2.52	15.58	5.29	1.47
7.70	40	5.0	100	30	45.0	1.5	6	2.40	6.5	4.19	24.29	8.24	2.83
2.00	80	0.4	100	100	20.0	1.5	6	0.32	0.94	0.66	8.13	2.71	1.01
2.00	40	5.0	50	100	45.0	0.5	6	1.97	4.88	3.18	18.56	7.23	2.11
2.00	40	0.4	100	30	45.0	1.5	2	0.61	2.03	1.41	15.27	4.16	1.65
7.70	40	0.4	50	100	45.0	1.5	6	0.89	4.19	2.79	27.82	6.76	2.12
7.70	80	0.4	50	30	45.0	0.5	6	0.63	2.29	1.53	15.76	5.02	1.65
7.70	80	5.0	50	30	20.0	1.5	2	0.61	1.67	1.16	7.1	2.17	0.73
2.00	80	5.0	100	30	20.0	0.5	6	0.89	2.17	1.43	7.44	3.22	0.95
7.70	40	5.0	100	100	20.0	0.5	2	0.66	1.66	1.14	5.87	2.49	0.74
2.00	40	0.4	50	30	20.0	0.5	2	0.16	0.7	0.5	5.67	1.89	0.7
4.85	60	2.7	75	65	32.5	1.0	4	0.92	1.97	1.25	10.84	3.85	1.06
4.85	60	2.7	75	65	32.5	1.0	4	1.07	2.07	1.44	12.14	4.22	1.7
4.85	60	2.7	75	65	32.5	1.0	4	1.01	2.07	1.37	11.37	4.27	1.3

Table 2: Results obtained after the linear regression analysis of the Plackett-Burman design. Significance testing method: center.

Variables	E1	E2	E2-d_3	MeEE2	EE2	E3
P_{vent}	NS	++	+	++	+	NS
T_{CIS}	-	---	--	--	--	-
t_{vent}	++	+++	++	NS	+	NS
F_{vent}	NS	--	-	-	-	NS
F_{purge}	NS	+	NS	+	+	NS
V_{inj}	++	+++	++	++	++	++
$t_{splitless}$	+	++	++	++	+	+
v_{inj}	++	+++	++	++	++	+

NS = not significant (p-value ≥ 0.05), + = positive effect (p-value: 0.01-0.05), ++ =high positive effect (p-value: 0.005-0.01), +++ = very high positive effect (p-value < 0.005),- = negative effect (p-value: 0.01-0.05), -- = high negative effect (p-value: 0.005-0.01), --- = very high negative effect (p-value < 0.005).

The design matrix and responses (peak area/peak width ratio) are included in Table 3. The data was fitted to equation (1), where y is the response, x_A, x_B and x_C correspond to the T_{CIS}, the V_{inj} and the v_{inj}, respectively, and Bi, Bij and Bii are the fitting parameters.

Table 3: Design matrix and responses, defined as the peak area/peak width ratio x 10^5, obtained for the central composite design.

T_{CIS} (°C)	V_{inj} (µL)	v_{inj} (µL/s)	E1	E2	E2-d3	MeEE2	EE2	E3
40	33	4	14.35	8.49	6.00	6.45	11.54	8.25
80	33	4	15.02	8.88	6.08	7.10	13.26	9.39
60	20	4	6.64	4.13	2.95	2.45	4.97	5.03
60	45	4	17.97	11.20	8.04	9.16	16.67	12.64
60	33	2	13.67	8.76	5.71	6.15	12.15	8.65
60	33	6	17.19	10.80	7.19	7.80	15.26	11.47
48	25	3	12.65	6.36	4.46	3.73	7.14	6.20
72	25	3	11.09	6.72	4.97	5.20	9.54	7.52
48	40	3	18.20	11.25	7.71	8.77	17.76	12.02
72	40	3	16.14	10.07	7.26	7.87	15.01	10.79
48	25	5	14.26	9.28	6.29	6.72	14.07	9.68
72	25	5	13.49	8.11	5.87	5.97	11.32	9.44
48	40	5	18.81	12.48	8.39	9.88	17.43	12.40
72	40	5	19.87	12.89	8.87	9.54	18.36	12.79
60	33	4	16.10	10.29	7.07	8.23	14.61	10.88
60	33	4	16.58	10.99	7.66	7.99	15.31	11.15
60	33	4	15.64	10.13	6.89	7.52	14.37	10.71

$$Y=B_0+B_Ax_A+B_Bx_B+B_Cx_C+B_{AB}x_Ax_B+B_{AC}x_Ax_C+B_{BC}x_Bx_C+B_{AA}x_A^2+B_{BB}x_B^2+B_{CC}x_C^2 \quad (1)$$

B-coefficients with a p-value lower than 0.05 were considered as significant and were further used in order to build the response surfaces. According to the results obtained T_{CIS} was not significant at the studied range and was fitted at 60 °C in order to minimize the N_2 consumption used during the cooling of the PTV. The influence of V_{inj} and v_{inj} can be observed for E2 in Figure 1 (T_{CIS} fixed at 60 °C). As it can be observed, the highest injection volume and speed

provided the best responses (peak area/peak width ratio) and were, therefore, fixed at 45 µL and 6 µL/s, respectively. The rest of target analytes showed a similar behaviour.

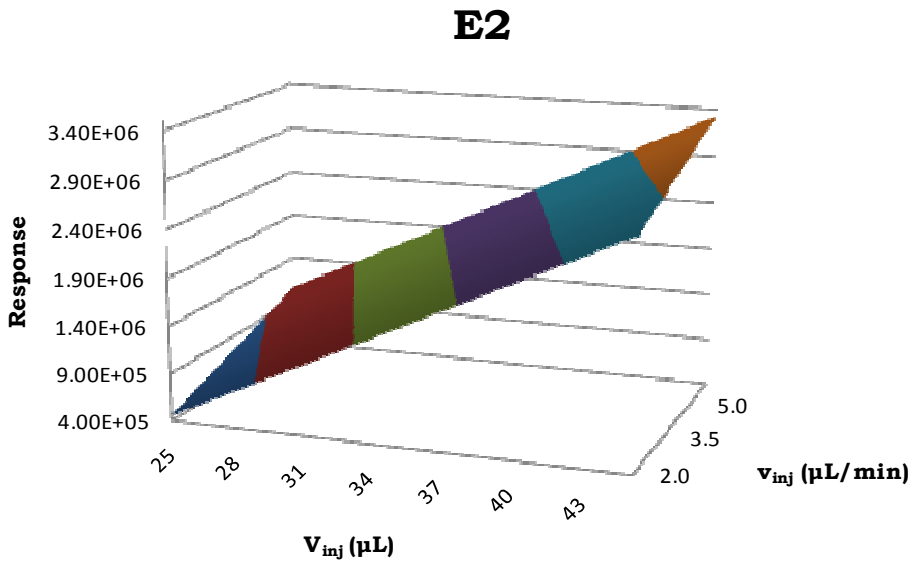

Figure 1: Response surface obtained for E2 using only the significant ($p<0.05$) parameters. Cryofocusing temperature 60 °C.

As a resume, while cryo-focusing temperature is maintained at 60 °C, 45 µL of the n-hexane extract are injected at 6 µL/s of speed. The solvent is vented at 50 mL/min and 7.7 psi pressure for 5 min. Afterwards, the vent valve is closed and the analytes are introduced to the column for 1.5 min. Finally, the vent valve is re-opened and the inlet is purged at 100 mL/min in order to avoid any carryover effect before the next sample injection.

3.3. Figures of merit of the developed method

Calibration curves were built in the SIM mode in 0.15-10 ng range. Correlation coefficients obtained after correction with E2-d_3 were up to 0.996 for all the analytes and the precision (n=5) was below 9 % in all the cases.

The optimized analysis method was applied to different environmental samples (estuarine water, wastewater, fish bile and fish homogenate) and some of the most important results are summarized below.

Estuarine water collected at the estuary of Bilbao (Spain) was spiked at ~10 ng/L and good recovery values (74-129 %, n=3) were obtained (see Table 4). Target analytes were not detected in the non spiked samples and precision was in the 2-8 % range.

Table 4: Recoveries (n=3) and standard deviation for spiked estuarine water from Zorrotza (Nerbioi-Ibaizabal estuary, Spain) and spiked effluent and influent water from the WWTP in Bakio (Basque Country, Spain).

	Zorrotza (estuarine water) Oasis HLB	Bakio (Effluent) OASIS HLB	Bakio (Effluent) Oasis HLB+Florisil	Bakio (Influent) Oasis HLB+Florisil	Standard additions Bakio (Effluent) Oasis HLB
E1	129±10	97±3	99±8	114±6	77±3
E2	105±5	106±6	83±2	74±2	94±8
MeEE2	94±2	181±17	90±4	85±2	105±7
EE2	102±2	230±9	99±4	92±2	76±4
E3	98±8	76±5	85±3	77±1	100±2

In the case of wastewater samples, in a first approach, the same method as that applied to estuarine samples was used but the

recoveries obtained for certain analytes (MeEE2 and EE2) exceeded by far the 100 %, even after correction with the non-spiked sample, as included in Table 4. In order to improve the previous values two alternatives were studied: the use of standard additions and a further clean-up of the ethyl acetate extract using 1-g Florisil® cartridges, as previously reported in the literature [42]. In the case of standard additions, 4 additions were performed to aliquots of previously spiked sample, before SPE preconcentration step. Additions of x/2 ng/L (where x was the spiked concentration) were performed.

The results obtained are summarized in Table 4. Both alternatives provided good results, although, in our opinion, the second approach is simpler since the use of standard additions requires a previous knowledge of the concentration of the analytes and at least 3-4 additions should be performed onto each sample, increasing the number of samples to be processed. Precision was in the 1-8 % and 2-9 % ranges for the method using a Florisil® clean-up of the extracts and for the standard addition method, respectively.

In the case of fish bile and fish homogenate, recovery values obtained are similar to those previously reported [34,46].

LODs, defined as three times the signal to noise ratio of the blanks, are included in Table 5. In the case of water samples, LODs were in the 0.02-6.07 ng/L range, similar to those obtained in the bibliography using LVI-PTV-GC-MS [43] or GC-MS after pre-column

trimethylsilyl derivatization [47] and better than those obtained after splitless injection followed by GC-MS, both in the electron impact ionization mode [38] or negative chemical ionization mode [48], and better than the results obtained by LC-MS/MS analysis [49].

In the case of fish bile, LODs are one order of magnitude better than those obtained in our research group using splitless injection onto a GC-MS [46], better than those obtained using GC-MS/MS [18] and much better than those obtained by means of GC-MS in the literature [50-52].

Finally, in the case of fish homogenate, LODs improved ten times compared to our previous results using splitless injection [45].

The optimized method was applied to influent and effluent samples from Gernika and Bakio (Basque Country, Spain) WWTPs. Results obtained are included in Table 6. Concentrations in the 0.8-682 ng/L (n=3) range were observed for influents while in the 2.1-28.2 ng/L range for the effluents. Problems in the precision of EE2 (RSD ~ 25 %) were observed for the determination of this analyte in the influent from the WWTP in Bakio. These values are similar to those found in a trickling filter/solid contact treatment plant in Canada (15-150 ng/L) [16], in a sewage treatment plant in Germany (not quantified-470 ng/L) [53,54] and effluents from Ulm WWTP in Germany (not quantified-13 ng/L) [55].

Table 5: Comparison between limits of detection (LODs) and limits of quantification (LOQs) obtained in this work for water, fish bile and fish homogenate after LVI-PTV-GC-MS and others found in the literature.

Water (LODs, ng/L, this work)			
Compounds	Estuarine	Effluent	Influent
E1	0.07	0.06	2.57
E2	0.05	0.02	0.06
MeEE2	0.15	0.02	0.04
EE2	0.04	0.02	0.05
E3	0.17	0.05	6.07

Water (LODs, ng/L)						
Compounds	LVI-PTV-GC-MS [43]	GC-MS [47]	GC-MS [38]	GC-NCI-MS [48]	GC-MS [27]	LC-MS [49]
E1	0.041	0.03	0.95	0.2	1.7	1
E2	0.046	0.03	0.35	0.3	3.4	2
MeEE2	---	---	1.66	---	---	---
EE2	0.031	0.07	1.00	---	0.8	2
E3	---	0.09	0.44	---	---	---

Bile (LODs and LOQs)							
Compounds	This work ng/g LODs	This work ng/g LOQs	ng/g LODs [46]	ng/mL LODs [18]	ng/g LOQ [50]	ng/g LOQ [51]	ng/g LODs [52]
E1	0.23	0.76	----	0.7	40	20	0.95
E2	0.05	0.16	5	0.4	40	30	3.04
MeEE2	0.14	0.45	----	-----	----	---	------
EE2	0.04	0.13	----	0.4	100	----	5.67
E3	0.47	1.63	----	----	----	----	3.67

Fish homogenate (LODs)		
Compounds	This work ng	ng LODs [45]
E1	0.1	----
E2	0.2	1.5
MeEE2	0.6	----
EE2	0.4	----
E3	7.5	----

Table 6: Average concentrations (n=3, ng/L) and standard desviation (s) of the analytes in the influent and effluent of the WWTP of Bakio and Gernika (Biscay, Spain), (n=3).

		TOC (mg/L)	E1	E2	MeEE2	EE2	E3
Bakio	Influent	286	152±5	56±3	43±2	0.8±0.2	682±127
	Effluent	203	<LOD	<LOD	<LOD	<LOD	<LOD
Gernika	Influent	145	121±20	130±16	11±1	2.2±0.4	101±13
	Effluent	78	28±2	18±1	3.7±0.1	2.1±0.6	12±1

4. Conclusions

LVI-PTV-GC-MS has been successfully optimized for the determination of estrogens (E1, E2, MeEE2, EE2 and E3) in environmental samples after derivatization with BSTFA + 1 % TMCS in an ultrasonic cup booster. Problems due to the use of plastic material during derivatization step and pyridine use during LVI-PTV injection arose, but they were solved by using glass vials during derivatization and pyridine exchange to n-hexane before LVI-PTV-GC-MS analysis.

LODs obtained with the developed method are in low ng/L level, better than those obtained with splitless inlets in GC-MS and better than those for tandem mass spectrometry coupled either to GC or LC.

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Abstract

Nonyl- and octylphenols have been widely analyzed for their endocrine disruption effect in the environment. Nevertheless, endocrine disrupting effect changes drastically depending on the nature of the para nonyl- or octylphenol isomer. The chromatographic separation of complex NP isomers cannot be achieved using traditional one dimension gas chromatography and other approaches such as comprehensive two-dimension gas chromatography (GCxGC) should be attempted. In the present work, the separation of complex nonylphenol technical mixture has been optimized using comprehensive two-dimensional gas chromatography coupled with a flame ionization detector and quadrupole mass spectrometer (GCxGC-qMS) and using valve-based modulator has been carried out. The optimization of GCxGC-qMS has been carried out using experimental designs and optimum separation was obtained at the following conditions: 1st column flow: 1 mL/min; 2nd column flow: 17.75 mL/min, oven temperature ramp: 1 °C/min, modulation period: 1.5 s and discharge time: 0.12 s. These values have been used to determinate previously synthesized 22OP, 33OP, 363NP and 22NP isomers in the NP technical mixture. Percentages obtained were as follows: 4.86 % for 22OP, 4.91 % for 33OP, 11.79 % for 363NP and 2.28% for 22NP. The values obtained for NP isomers are in good agreement with the literature.

Keywords: Nonyl- and octylphenol isomers, GCxGC, experimental design, valve-based modulator, nonylphenol technical mixture.

1. Introduction

Alkylphenols ethoxylates (APEOs) are surfactants that have been widely used as detergent, emulsifier and dispersing agents in industrial, commercial or household applications [1]. These compounds are degraded aerobically or anaerobically in wastewater treatment plants (WWTPs) and more toxic compounds, such as nonylphenols (NPs) and octylphenols (OPs), are obtained [2-4]. Although the main source of NPs and OPs is the degradation of APEOs, they have also been used in the production of plastics and phenolic oximes [5,6]. Commonly, NPs and OPs appear in a mixture of different isomers where the main isomers are *para*-substituted [7,8].

The interest in NPs and OPs has increased during the last decades due to their capacity to disrupt the endocrine system of fishes [9-12], oysters [13], microorganisms [14], mammals [10] and even humans [15]. For this reason, NPs and OPs have been included in the water framework directive (WFD) as priority hazardous substances [16].

Different works have shown that NP and OP isomers show different estrogenity [17-23]. However, due to the lack of commercially available isomers and the difficulty of separating the complex mixture of NPs and OPs using one-dimensional gas or liquid chromatography [24-26], traditionally the sum of the total concentration of the isomers is performed. At this point, the determination of all the isomers has become a great goal. Nevertheless, the synthesis and separation are not easy and the

quantity of the isomers changes depending on the author and the technical mixture. Guenther et al. [27] have characterized up to 211 possible constitutional isomers of NP according to a hierarchical and logical system, although it is thought that not all the isomers are included in the commercial technical mixture. Wheller et al. [8] were able to characterize 22 *para*–isomers using high resolution capillary gas chromatography-mass spectrometry (HRCGC-MS). Thiele et al. [28] separated 10 isomers using , as Wheller and co-workers, a 100 m capillary column and a GC-MS. However, the works that provide the maximum number of separated isomers are based in comprehensive two dimensional gas chromatography (GCxGC). Ieda et al. [29] separated 102 peaks and 13 compounds were characterized using comprehensive two-dimensional gas chromatography-mass spectrometry (GCxGC-qMS). Moeder et al. [30] separated 40 peaks using GCxGC coupled to time-of-flight mass spectrometry (GCxGC-TOF-MS) and, more recently, Eganhouse and co-workers [7] were able to separate from 153 to 204 peaks using a GCxGC-TOF-MS and around 59 to 66 were identified as *para*-NPs.

As illustrated above, GCxGC is an useful tool to separate a maximum number of compounds from the technical mixture. The main differences from one GCxGC to another, apart from the 1st column in the and 2nd dimensions, are the detectors and the modulator. Detectors with high data processing are commonly used, such as flame ionization detector (FID), which can acquire data frequencies of 200 Hz, TOF-MS, can acquire spectra at very high

frequencies, new quadrupole MS (qMS) that acquires spectra at 24-36 Hz (depending of the scan range) [29] or electron capture detector (ECD) [31,32]. Two main types of modulators can be found: thermal desorption modulators, including cryogenic and phase ratio modulator, and valve based modulators [33]. Although nowadays, thermal modulators are the most widely used [7,29,30], in this work, a valve-based modulator has been used. In this case, a three-way solenoid valve controls the fill of the collection channel and the discharge with H_2 at flows of 18-20 mL/min. Afterwards, a splitter restrictor divides the flow to the FID and the qMS in order to decrease the flow entering in the qMS detector.

In this work different variables (1st column flow, 2nd column flow, oven temperature ramp, modulation period and discharge time) affecting GCxGC separation of NP and OP isomers from a NP technical mixture have been optimized by means of an experimental design approach using GCxGC-FID-qMS and a valve based modulator. Symmetry, the blob volume and the blob number were chosen as the response in order to fit optimum conditions. Finally, two OP and two NP isomers have been synthesized and their identification and quantification has been performed.

2. Experimental

2.1. Reagents and Materials

Nonylphenol technical mixture (NPs, Pestanal®) was purchased from Fluka (Steinheim, Germany) and 2,3,5,6-d_4-4-nonylphenol

(NP-d_4, min 97 atom % D) from Isotec (Miamisburg, Ohio, USA). 4000 mg/L stock solutions of analytes were individually prepared in methanol (99.9 %, Alfa Aesar, Karlsruhe, Germany) and stored in amber vials at -20 ºC.

Ethyl acetate (EtOAc) and *n*-hexane used during the synthesis of individual isomers were supplied by Panreac (Barcelona, Spain) and anhydrous ligroin and anhydrous diethyl ether by Aldrich (Steinheim, Germany).

Magnesium, crystal iodine, 1-bromobutane, 1-bromopentane, 1-bromohexane, 3-methyl-1-bromobutane, 2-propanone, 2-butanone and BF_3-Et_2O complex were supplied by Aldrich.

Calcium chloride, ammonium chloride and anhydrous sodium sulfate were supplied by Panreac.

TLC silica gel sheets (0.040-0.063 nm) were supplied by Merck (Darmstadt, Germany).

2.2. GCxGC-FID-qMS detection

The analytes were analyzed in a 7890A gas chromatograph (Agilent Technologies, Avondale, PA, USA) equipped with an Agilent flame ionization detector (FID), an Agilent 5975C electron impact ionization quadrupole mass spectrometer (qMS) and a 7683B Agilent autosampler. 2 µL of the solution were injected in the splitless mode at 300 ºC into a primary HP5 MS (30 m x 0.25 mm, 0.25 µm) capillary column, coupled with a DB-17MS (5 m x 0.25 mm, 0.25 µm) capillary column. The following oven temperature

program was used for the separation of the analytes: 60 ºC (2 min), temperature increase at 20 ºC/min to 205 ºC, a second increase of 1 ºC/min up to 220 ºC, and a final increase of 30 ºC/min to 300 ºC, where it was finally held for 2 min. H_2 (Hydrogen generator AD-1020, Cinel Strumenti Scientifici, Padova, Italy) was used as carrier gas at a constant flow of 1 mL/min in the first column and 17.75 mL/min in the second. The flow was divided by a splitter to the FID with a tube of 70 cm length x 0.32 mm i.d., and to the MS with a tube of 45 cm length x 0.10 mm i.d. FID worked at 270 °C, 20 mL/min H_2 flow and 350 mL/min air flow (99.9992 %, Carburos Metálicos, Barcelona, Spain) and data acquisition was set at 200 Hz. qMS transfer line temperature was maintained at 310 ºC, and the ion source and quadrupole at 230 ºC and 150 ºC, respectively. Data acquisition was set at 36.14 Hz. Measurements were performed both in the scan (105-220 m/z) and in the SIM (Selected Ion Monitoring) modes. The m/z values followed for each analyte were the following: 22-OP (135/107), 33-OP (107/149), 363-NP (149/107), 22-NP (135/107) and NP-d_4 (111/110). First ion was used as quantifier and the second one as qualifier.

Valve based modulator parameters were settled as follows 1.5 s modulation period and 0.12 s discharge time according to the optimized results.

2.3. Synthesis of 2-methylheptan-3-ol, 3-methylheptan-3-ol, 2-methyloctan-3-ol and 3,6-dimethylheptan-3-ol

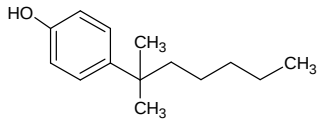
2-methylheptan-3-ol, 3-methylheptan-3-ol, 2-methyloctan-3-ol and 3,6-dimethylheptan-3-ol were synthesized according to Ruβ et al. [34]. Magnesium (2.01 g, 82.3 mmol), diethyl ether (10 mL) and crystal of iodine were put in a two-necked flask with a reflux condenser, a calcium chloride tube and a dropping funnel. In order to activate magnesium, the mixture was heated smoothly. 1-Bromopentane (10.2 mL, 82.3 mmol), 1-bromobutane (8.9 mL, 82.3 mmol), 1-bromohexane (11.6 mL, 82.3 mmol) or 3-methyl-1-bromobutane (10.72 mL, 82.3 mmol) dissolved in anhydrous diethyl ether (30 mL) was added slowly. When the addition was finished the mixture was heated to 40 ºC for 1 hour. Then, the reaction mixture was cooled to 0 ºC. A solution of 2-propanone (5.4 mL, 74.0 mmol) or 2-butanone (6.7 mL, 74.0 mmol) in anhydrous diethyl ether (20 mL) was added slowly. Then, the reaction mixture was heated to 40 ºC for 1 hour. Subsequently, the resulting octanol or nonanol was protonated at 0 ºC with a mixture of crushed ice in water (20 mL) and ammonium chloride (30 mL, 10 %). The organic layer was separated, and the aqueous phase was extracted with diethyl ether (2 x 50 mL). The combined organic phases were washed with aqueous sodium bisulphite (3 x 50 mL, 40 %) and with saturated aqueous sodium bicarbonate (3 x 50 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated in vacuum. 2-Methylheptan-3-ol (8.3 g, 86 %), 3-methylheptan-3-ol (9.2 g, 86 %),

2-methyloctan-2-ol (9.3 g, 86 %) and 3,6-dimethylheptan-3-ol (10.26 g, 86%) were obtained as oils (see Appendix A for NMR data).

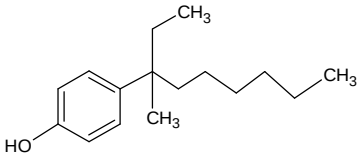
2.4. Synthesis of 4-(1,1-dimethylhexil)phenol (22OP), 4-(3´-methyl-3´-hepthyl)phenol (33-OP), 4-(1,1-dimethylhepthyl)phenol (22NP) and 4-(3',6'-dimethyl-3'-hepthyl)phenol (363-NP)

In a dry two-necked flask, a solution of 2-methylheptan-3-ol (1.11 g, 7.8 mmol), 3-methylheptan-3-ol (1.01 g, 7.8 mmol), 2-methyloctan-2-ol (0.37 g, 2.6 mmol) or 3,6-dimethylheptan-3-ol (1.23 g, 8.52 mmol) and phenol (2x alcohol mmol) in anhydrous ligroin (150 mL) were heated under argon at 60 ºC. Then, $BF_3.Et_2O$ complex (1.2 mL, 10.38 mmol) was added slowly and the resulting mixture was stirred for 1 hour at 60 ºC. Crushed ice and water (150 mL) were added and the mixture was stirred for 30 min more. The organic layer was separated and was washed with water (7 x 30 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated in vacuum. Flash column chromatography (silica gel, n-hexane/AcOEt 15/1) afforded 4-(1,1-dimethylhexil)phenol (22OP) (0.89 g, 70 %), 4-(3´-methyl-3´-hepthyl)phenol (33-OP) (1.12 g, 70 %), 4-(2´-methyl-2´-octhyl)phenol (22-NP) (0.95g, 70 %), 4-(3',6'-dimethyl-3'-hepthyl)phenol (363-NP) (1.32 g, 70 %) as oils (see Appendix A for NMR data).

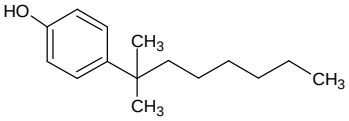
Chemical structures of the synthesized compounds are illustrated in Figure 1.



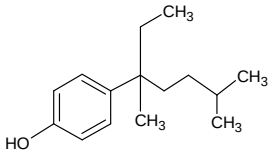
22OP



33OP



22NP



363NP

Figure 1: Structure of 22OP, 33OP, 22NP and 363 NP.

3. Results and Discussion

3.1. Optimization of the GCxGC-FID-qMS

In order to obtain the best separation of the OP and NP isomers from the NP technical mixture, a full factorial design (FFD) was developed using Statgraphics Centurion XV. ~1700 mg/L stock solution was injected and five variables were studied: 1st column flow (0.7-1.2 mL/min), 2nd column flow (18-25 mL/min), oven ramp (0.5-5 °C/min), modulation period (1.4-1.5 s) and discharge time (0.05-0.1 s). Modulation period is called to the time that elapses from the moment that the modulator channel starts filling with mobile phase until the modulator channel is emplied. Discharge

time is called the time consumed by the mobile phase from the moment that the modulator channel is filled until the modulator channel is emptied.

Three different responses were checked during the optimization: one dimension symmetry (symmetry) in three different points of the chromatogram (begging, middle, end) of the chromatogram, the blob number and the blob volume (see Table 1). Symmetry was qualitatively defined: 1 for good symmetry, 25 to acceptable symmetry and 50 to bad symmetry. Volume of 15 peaks around the chromatogram (see Figure 2) was checked and they were selected due to their high volume, which guaranteed their presence in all the experiments performed. Identification of these blobs was performed by means of their mass spectra. Blob number was quantified.

During the whole optimization process the nature of the columns were kept constant. The 2nd dimension column (DB-17MS) was chosen since it gives satisfactory results according to Ieda et al. [29].

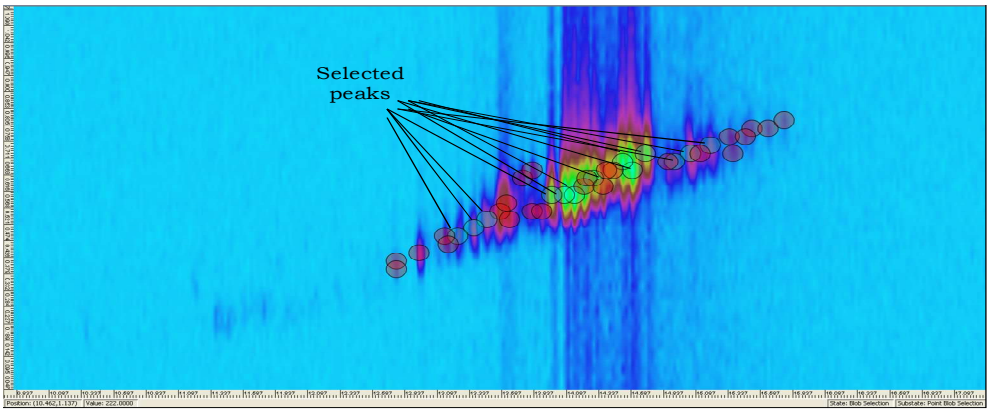


Figure 2: Peaks selected for the optimization of blob volume.

Table 1: Full factorial design built for the screening of significant variables during the optimization of the chromatographic separation.

1st column flow mL/min	2nd column flow mL/min	Oven ramp °C/min	Modulation period s	Descharge time s
1.2	18	5	1.4	0.1
0.7	18	0.5	1.5	0.05
0.7	25	0.5	1.5	0.05
0.7	25	0.5	1.4	0.05
0.7	18	5	1.4	0.1
0.7	18	5	1.4	0.05
1.2	25	5	1.5	0.05
0.7	18	0.5	1.4	0.05
1.2	18	0.5	1.4	0.1
0.7	18	5	1.5	0.1
1.2	18	5	1.4	0.05
1.2	25	5	1.4	0.1
0.95	21.5	2.75	1.45	0.075
1.2	25	5	1.4	0.05
1.2	25	0.5	1.5	0.05
1.2	18	0.5	1.5	0.1
0.7	25	5	1.4	0.05
1.2	25	5	1.5	0.1
1.2	25	0.5	1.4	0.05
1.2	25	0.5	1.4	0.1
0.7	25	0.5	1.4	0.1
0.7	25	0.5	1.5	0.1
1.2	18	0.5	1.5	0.05
0.7	25	5	1.5	0.1
1.2	18	5	1.5	0.1
1.2	25	0.5	1.5	0.1
0.95	21.5	2.75	1.45	0.075
0.95	21.5	2.75	1.45	0.075
0.7	25	5	1.4	0.1
0.7	18	5	1.5	0.05
0.7	18	0.5	1.5	0.1
1.2	18	5	1.5	0.05
0.7	18	0.5	1.4	0.1
0.7	25	5	1.5	0.05
1.2	18	0.5	1.4	0.05

The responses obtained for the FFD were analyzed by means of an analysis of variance and parameters with a p-value <0.05 were chosen as significant. Figure 3 shows the pareto-charts obtained for symmetry and it could be concluded that 2nd column flow, discharge time and the interactions between 1st column flow-discharge time and 2nd column flow-discharge time had a significant influence in the symmetry response.

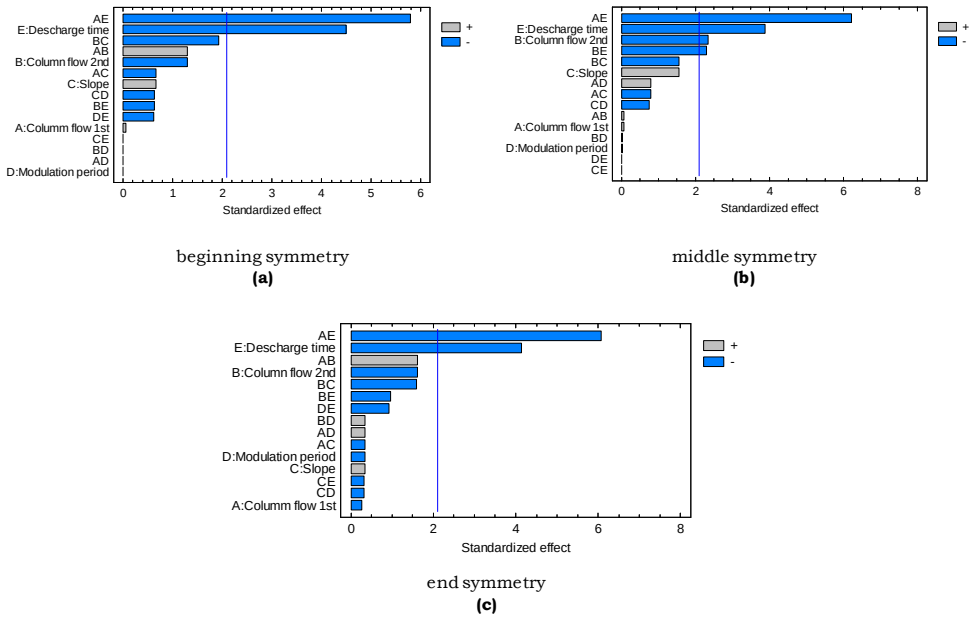


Figure 3: Pareto charts obtained in the FFD for symmetry at the beginning (a), at the middle (b) and at the end (c) of the chromatogram.

The pareto charts of 4 blobs have been illustrated as an example (see Figure 4). In the case of the blob volume, 1st column flow, 2nd column flow, discharge time and the interactions between

1st column flow and discharge time, affected significantly to most of the blobs chosen.

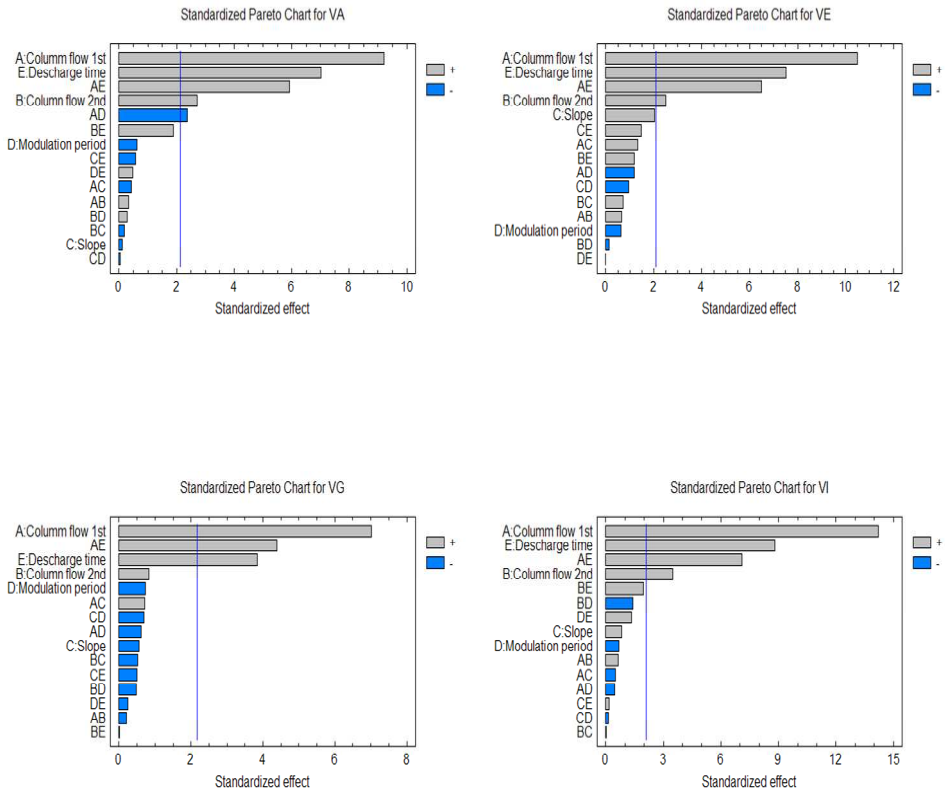


Figure 4: Pareto Chart obtained during the FFD for blob volume of four selected blobs.

Finally, the blob number was significantly affected by the 1st column flow, the 2nd column flow and discharge time (see Figure 5).

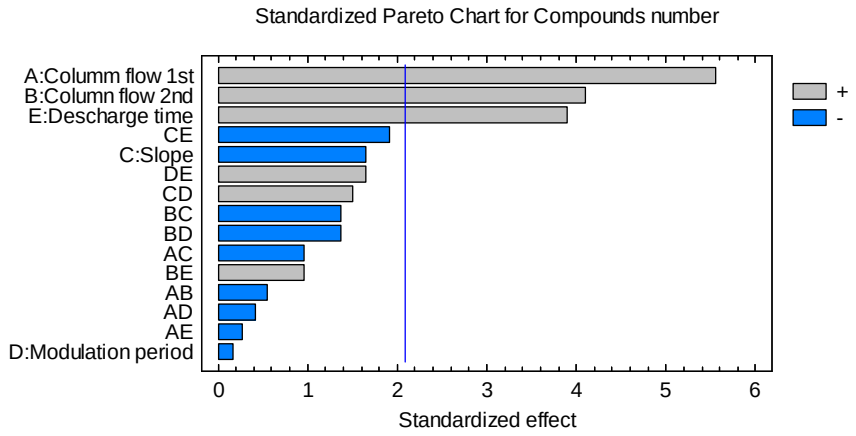


Figure 5: Pareto chart obtained during the FFD for the blob number.

This way, the 1st column flow, the 2nd column flow and the discharge time were analyzed in a central composite design (CCD), while the slope of the oven temperature ramp and the modulation time were fitted at a low (1 °C/min) and at the highest (1.5 s) values, respectively. In order to fit these values, we have taken in consideration that low values of oven temperature ramp are better for analytes separation. Some experiments were performed at low values and no significant differences were found between 0.5 and 1 °C/min. Thus, 1 °C /min was chosen in order to reduce analysis time. In the case of the modulation period, a high value was chosen since it is mostly used with this modulator.

A CCD was built with the rest of the variables: 1st column flow (0.53-1.37 mL/min), 2nd column flow (15.6-27.4 mL/min) and discharge time (0.033-0.12 s).

As for the FFD, the symmetry, the blob volume and the blob number were used as responses.

In order to optimize the symmetry a target value of 1 was set for optimization and according to the results obtained (see Figure 6), the discharge time, the quadratic value of discharge time and the quadratic value of the 1st column flow were significant for a 95 % confidence interval. According to the response surface obtained for symmetry, optimum values should be set at 1 mL/min for the 1st column flow and 0.11 s for the discharge time. The flow of the 2nd column was set at an intermediate value (17.75 mL/min) since it was not significant.

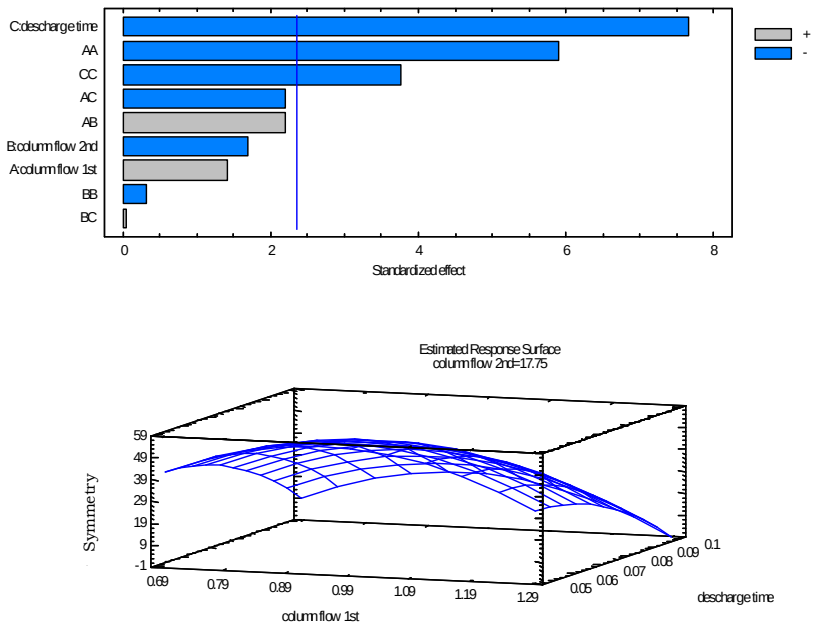


Figure 6: Pareto chart and the response surface obtained during the CCD for the symmetry. The flow of the symmetry column was set at 17.75 mL/min.

In the case of the blob volume the results obtained for two of the selected peaks are shown in Figure 7. The rest of the blobs studied showed a similar behavior. According to the results obtained in the CCD for the blob volume variables should be fitted at the highest value for the 1st column flow (1.37 mL/min) and at the highest value for discharge time (0.12 s). The 2nd column flow was fitted at an intermediate value (17.75 mL/min) since it was not significant.

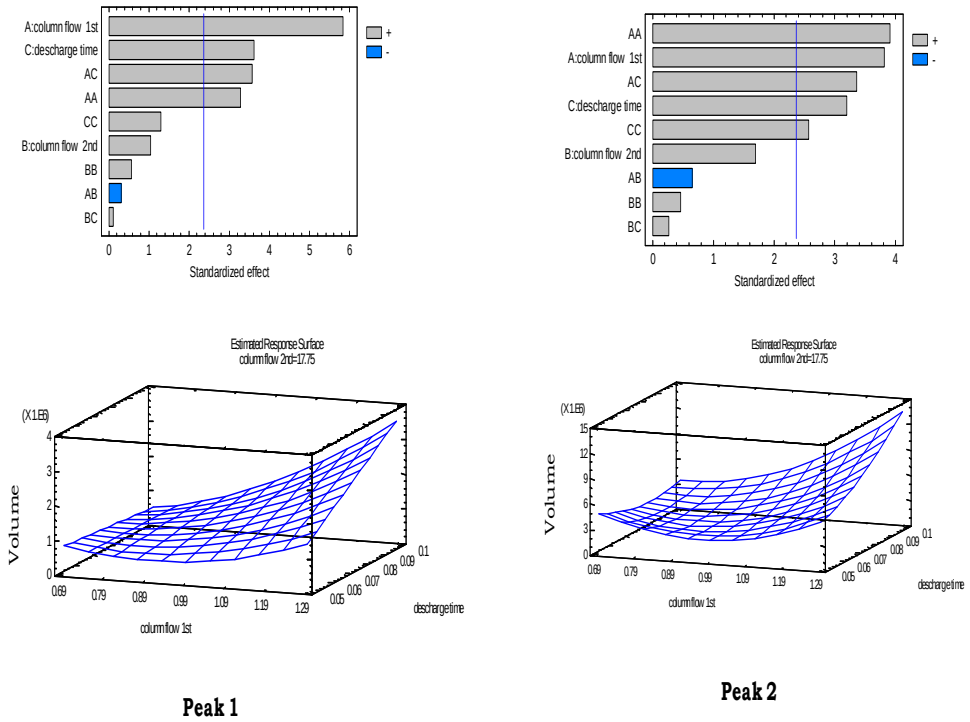


Figure 7: Pareto chart and the response surface obtained during the CCD for the blob volume. The flow of the symmetry column was fitted at 17.75 mL/min.

The results obtained for the number of blobs are shown in Figure 8 and it could be concluded that highest values for the 1st column flow and discharge time provided the highest number of blobs, while the flow of the 2nd column had not significant effect.

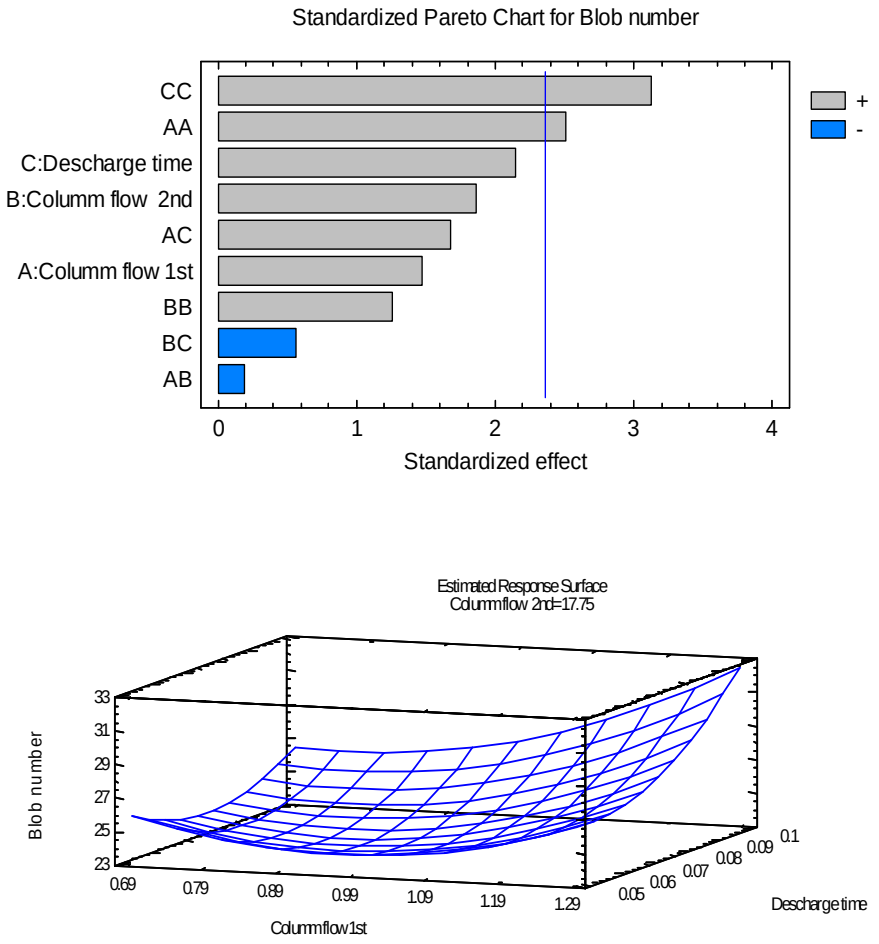


Figure 8: Pareto chart and response surface obtained during the CCD for the blob number. The flow of the symmetry column was fitted at 17.75 mL/min.

Table 2 summarizes the optimum values for the variables studied in the case of each of the response studied. The flow in the 2nd column was not significant and it was finally fitted at 17.75 mL/min intermediate value. In the case of the discharge time, the best response were obtained for discharge time in the 0.11-0.12 s range for the three responses considered and it was set at 0.12 s. Finally, in the case of the 1st column flow, the best values were obtained for 1.37 mL/min, while 1 mL/min was best for symmetry. Since the symmetry obtained at 1.37 mL/min was not good, 1 mL/min was finally chosen.

Table 2: Optimum values of optimized variables according to the variable studied.

	1st column flow mL/min	2nd column flow mL/min	Descharge time s
Symmetry	1.00	---	0.11
Blob volume	1.37	---	0.12
Blob number	1.37	---	0.12

Figure 9 and 10 show the chromatograms obtained under optimized conditions in the FID and qMS, respectively. Obviously, a higher number of compounds (up to 79) are detected in the FID, while only 38 were confirmed in the qMS as NP or OP isomers. The

signal obtained in the qMS was used for the identification of the isomers synthesized, while the FID signal for quantification.

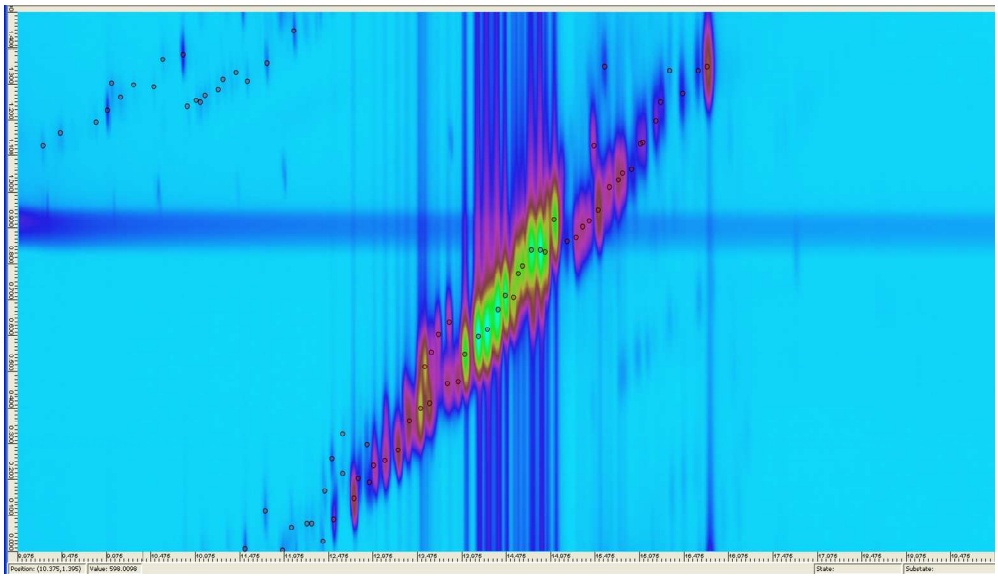


Figure 9: Two dimensional FID chromatogram.

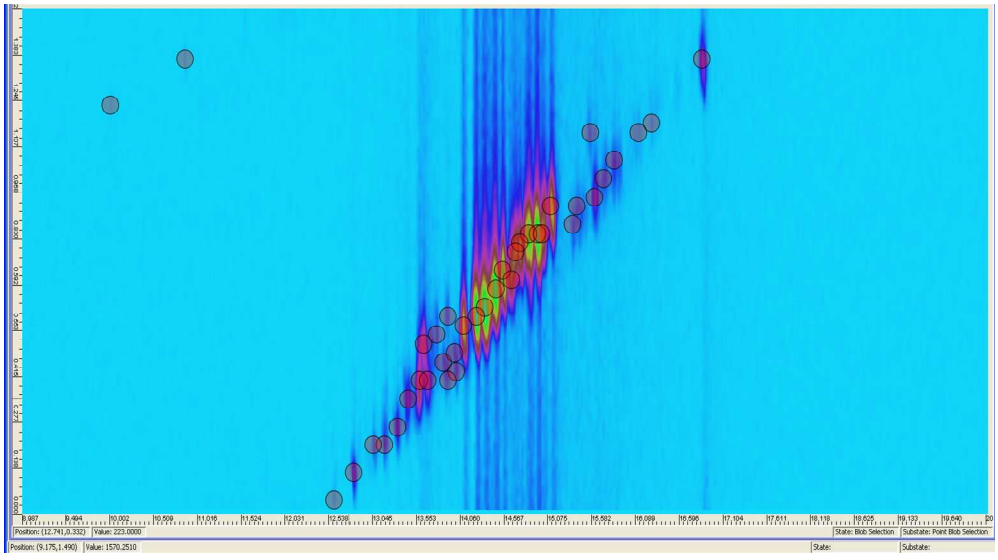


Figure 10: Two dimensional MS chromatogram.

Although an optimum condition has been settled after the optimization, the resolution of all OP or NP isomer has been impossible. In some cases the mass spectra obtained changes around the blob and in others the peak observed in the FID does not appear in qMS, thus the identification is not possible (see Figures 9 and 10).

3.2. Quantification of 22OP, 33OP, 363NP and 22NP in the technical mixture

In order to identify 22OP, 33OP, 363NP and 22NP isomers in the technical mixture, individual injections were performed and retention times and mass spectra of single isomers were obtained. Figure 11 shows the mass spectra of the target isomers.

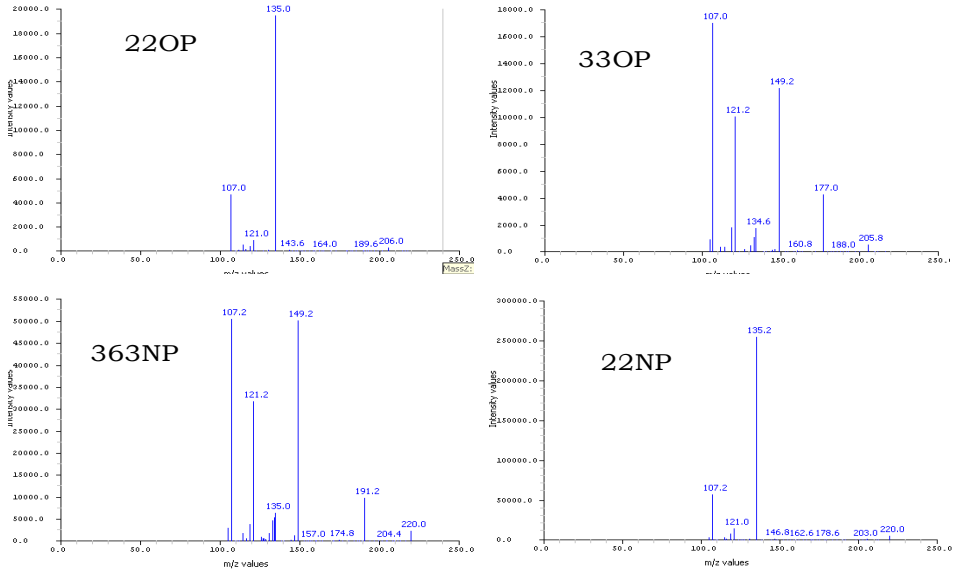


Figure 11: Mass spectra of 22OP, 33OP, 363NP and 22NP.

The synthesized isomers were identified in the NP technical mixture based on the retention time and mass spectra in the case of qMS and according to the retention times for the FID (see Figure 12).

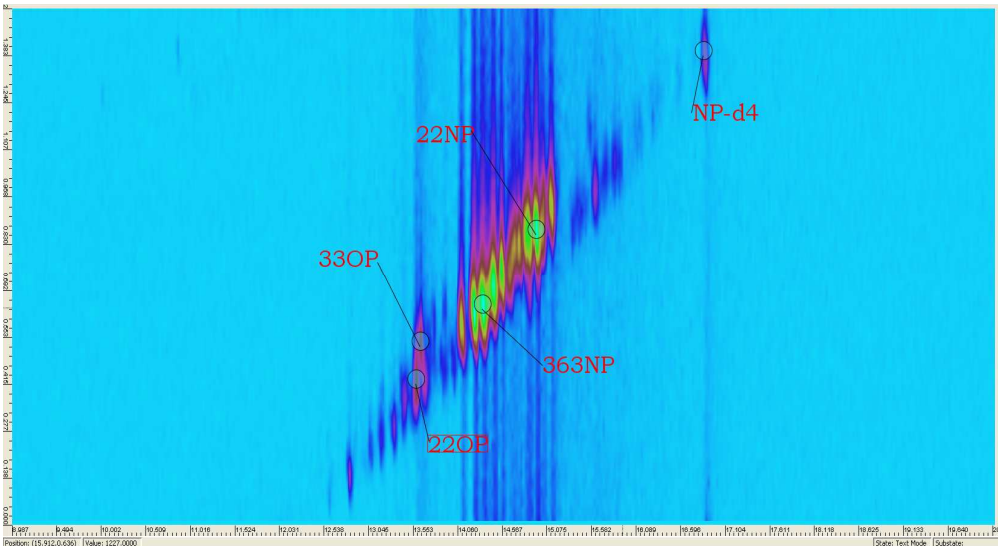


Figure12: Identification of 22OP, 33OP, 363NP, 22NP and NP-d_4 in the qMS chromatogram.

In the area corresponding to 22OP isomer blobs, two different mass spectra could be detected in the case of the technical mixture (see Figure 13). At one end of the blob, the mass spectra of 22OP isomer is observed while at the other edge of the blob an unknown NP isomer can be detected. That means, that 22OP isomer coelutes with an unknown NP isomer. In order to quantify, SIM mode could be an alternative, but the mass spectra are similar, except for 220 and 206 fractions, but which have a low abundance.

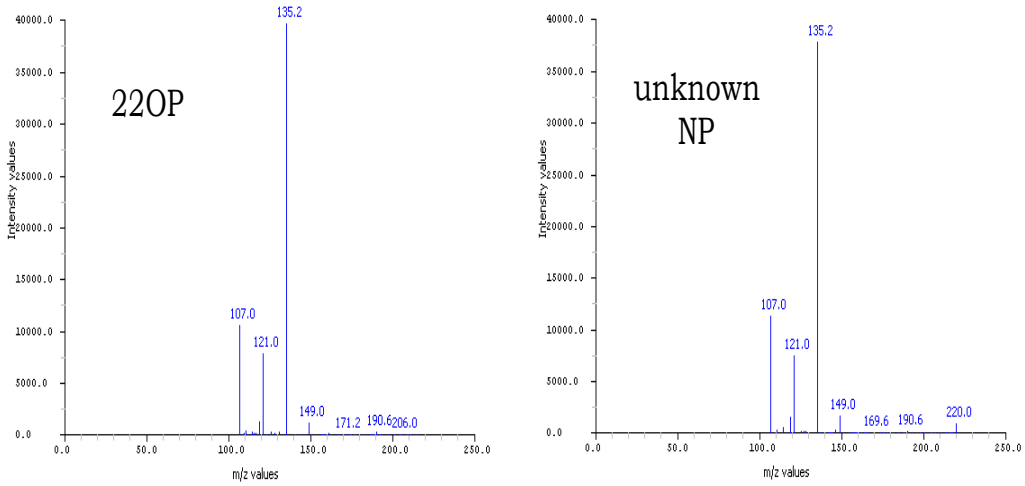


Figure 13: Mass spectra of 22OP and the unknown NP co-eluting.

Calibration curves were built for each analyte in 0.75-150 mg/L range, obtaining determination coefficients higher than 0.998 after correction with NP-d_4 for FID and higher than 0.990 for qMS. The precision (n=3) was below 7 % in all the cases for FID and below 13 % for qMS.

Table 3 shows the percentages of the synthesized isomers in the technical mixture.

Tabla 3: Percentage of isomers in a technical NPs mixture from Fluka.

Isomer	22OP	33OP	363NP	22NP
%	4.86±0.02	4.91±0.12	11.70±0.61	2.28±0.15

These values are similar to those obtained by Eganhouse et al. [7] by a GCxGC-TOF-MS, who obtained values of 10.6±1.0 % and 1.5±0.1 for 363NP and 22NP, respectively. No data has been obtained in the literature for OP isomers.

4. Coclusions

Separation of NP isomers using GCxGC-FID-qMS coupled with a valve-based modulator was optimized by means of experimental design. Up to 39 isomers were separated and identified using their mass spectra. 22OP, 33OP, 363NP and 22NP isomers were synthesized in the laboratory and were quantified in the technical mixture. The results obtained for NP isomers are in good agreement with the literature.

Acknowledgements

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Appendix I

NMR data of 4-(2´-methyl-2´-hepthyl)phenol (22-OP), 4-(3´-methyl-3´-hepthyl)phenol (33-OP, 4-(2´-methyl-2´-octhyl)phenol (22-NP). and 4-(3',6'-dimethyl-3'-hepthyl)phenol (363-NP)

4-(2´-methyl-2´-hepthyl)phenol (22-OP)

1H NMR (300 MHz, CDCl3): δ = 0.83 (t, J = 7.0 Hz, 3H, CH3-CH2), 1.04-1.08 (m, 2H, (-CH2(CH2)2CH3), 1.26 (s, 6H, (CH3)2C-), 1.16-1.24 (m, 4H, (-CH2-CH2-CH3), 1.53-1.57 (m, 2H, (CH3)2C-CH2-), 4.65 (broad s, 1H, OH), 6.76 (d, J = 8.7 Hz, 2H, H3,5), 7.19 (d, J = 8.7 Hz, 2H, H3,5). 13C NMR (CDCl3) δ = 14.0 (CH3-CH2), 22.6 (CH2), 24.4 (CH2), 29.1 (CH3)2C-), 32.6 (CH2), 37.1 ((CH3)2C-), 44.7 (CH3)2C-CH2-), 114.7 (C2,6), 127.0 (C3,5), 142.2 (C4), 153.0 (C1).

4-(3´-methyl-3´-hepthyl)phenol (33-OP)

1H NMR (300 MHz, CDCl3): δ = 0.66 (t, J = 7.4 Hz, 3H, CH3-CH2-CH2), 0.82 (t, J = 7.3 Hz, 3H, CH3-CH2-C), 0.90-0.97 (m, 1H, C-CH2), 1.07-1.15 (m, 1H, C-CH2), 1.21 (s, 3H, C-CH3), 1.23-1.28 (m, 2H, CH3-CH2-C), 1.44-1.49 (m, 2H, C-CH2-CH2), 1.50-1.71 (m, 2H, C-CH2-CH2), 4.45 (broad s, 1H, OH), 6.76 (d, J = 8.5 Hz, 2H, H2,6), 7.13 (d, J = 8.5 Hz, 2H, H3,5). 13C NMR (CDCl3) δ = 8.6 (-CH2-CH2-CH3), 14.0 (CH2-CH3), 23.4 (-CH2-CH2-CH3), 23.6 (CH3-C), 26.4 (-CH2-CH2-CH3), 35.6 (CH3-CH2-C), 40.3 (CH3-CH2-C),

42.8 (C-CH2-CH2), 114.6 (C2,6), 127.6 (C3,5), 140.3 (C4), 152.9 (C1).

4-(2´-methyl-2´-octhyl)phenol (22-NP)

1H NMR (300 MHz, CDCl3): δ = 0.85 (t, J = 6.9 Hz, 3H, CH3-CH2), 1.03-1.06 (m, 2H, (-CH2(CH2)3CH3), 1.26 (s, 6H, (CH3)2C-), 1.18-1.30 (m, 6H, (-CH2-CH2-CH2-CH3), 1.53-1.57 (m, 2H, (CH3)2C-CH2-), 4.69 (broad s, 1H, OH), 6.76 (d, J = 8.6 Hz, 2H, H3,5), 7.19 (d, J = 8.6 Hz, 2H, H3,5). 13C NMR (CDCl3) δ = 14.0 (CH3-CH2), 22.6 (CH2), 24.7 (CH2), 29.1 (CH3)2C-), 30.0 (CH2), 30.8 (CH2), 37.0 ((CH3)2C-), 44.7 (CH3)2C-CH2-), 114.7 (C2,6), 126.7 (C3,5), 142.2 (C4), 153.0 (C1).

4-(3',6'-dimethyl-3'-hepthyl)phenol (363-NP)

1H NMR (300 MHz, CDCl3): δ = 0.66 (t, J = 7.4 Hz, 3H, CH3-CH2), 0.80-0.83 (m, 6H, (CH3)2CH), 0.84-0.88 (m, 1H, (CH3)2CH-CH2), 0.97-1.04 (m, 1H, (CH3)2CH-CH2), 1.21 (s, 3H, C-CH3), 1.38-1.45 (m, 1H, (CH3)2CH), 1.47-1.51 (m, 1H, C-CH2), 1.52-1.57 (m, 1H, CH3-CH2-C), 1.61-1.67 (m, 1H, C-CH2), 1.68-1.71 (m, 1H, CH3-CH2-C), 4.62 (broad s, 1H, OH), 6.76 (d, J = 8.6 Hz, 2H, H3,5), 7.13 (d, J = 8.6 Hz, 2H, H3,5). 13C NMR (CDCl3) δ = 8.6 (C-CH2-CH3), 22.6 ((CH3)2CH), 23.6 (C-CH3), 28.7 ((CH3)2CH), 33.2 ((CH3)2CH-CH2), 35.7 (CH3-CH2-C), 40.3 (CH3-CH2-C), 40.6 (C-CH2-CH2), 114.7 (C2,6), 127.6 (C3,5), 140.3 (C4), 152.9 (C1).

According to the objectives previously established, the following main conclusions can be highlighted from the work presented:

1. The derivatization reaction for the determination of the target analytes was reduced from 60-90 min to 1-10 min using a mianturized ultrasound device. Compared to other alternatives in the literature where microwaves were used [1], the much lower prize of ultrasound devices should be highlighted. The limits of detection (LODs, 0.35-1.66 ng/L) obtained using miniaturized ultrasound assisted derivatization and GC-MS with splitless injection were similar to those obtained in the literature using classical derivarization by GC-MS [2] or GC-MS/MS [3]. The effect of organic matter present in environment water samples was negligible when solid phase extraction (SPE) is combined with the optimized derivatization and determination procedure. Thus, precise (<5.3 %) and accurate (70-138 %) results were obtained.

2. The focused ultrasound assisted enzymatic hydrolysis developed for the determination of the target analytes in fish bile reduced the sample preparation time from approx. 16 h [4] to 20 min. This enzymatic hydrolysis step combined with a SPE clean-up and followed by a GC-MS analysis provided accurate (77-121 %) and precise (5-12 %) results. LODs (in the 5.0 ng/g to 1.8 µg/g range) allowed the application of the developed method to real samples.

3. Focused-ultrasound liquid extraction (FUSLE) and microwave-assisted extraction (MAE) combined with a clean-up step

using SPE or gel permeation chromatography (GPC) were studied for the determination of alkylphenols and estrogens in fish homogenate. SPE provided cleaner extracts than GPC and 5 g Florisil cartridges were finally chosen. However, this clean-up procedure only yielded satisfactory results when combined with FUSLE, since recovery values for spiked samples using MAE exceeded 100 %. LODs (0.04-827 ng) were similar to those obtained in the literature [5]. This method was applied to the determination of previously synthesized 363-NP and 33-OP in homogenate from zebrafishes exposed to those isomers.

4. Large volume injection-programmable temperature vaporization (LVI-PTV) was optimized in order to improve the previously obtained LODs. Injection in a PTV inlet provided LODs approx. 10 times lower than those obtained in a classical split/splitless inlet in environmental samples such as water, bile and fish homogenate. These LODs were comparable or even better than those obtained in the literature using LC-MS/MS, GC-MS/MS or LVI-PTV-GC-MS [6-8]. When large volume injection was attempted, a clean-up step previous to the analysis was necessary in order to improve accuracy in waste water samples.

5. GCxGC-FID-qMS was optimized for the separation of octyl- and nonylphenol isomers from commercially available technical mixture using a valve based modulator. Optimization was performed using an experimental design approach and response variables such as symmetry, blob volume and blob number were studied. Up to 79 blobs were detected in the FID but only 38 where

confirmed using qMS. The lower sensitivity of the qMS is due to the splitter necessary previous to this detector. Previously synthesized isomers 22OP, 33OP, 363NP and 22NP were quantified in a technical mixture. The results obtained are similar to those observed in the literature.

It must be underlined that the use of a wide variety of sample preparation (FUSLE, MAE, SPE, GPC…) and analysis (GC-MS, PTV-GC-MS, GCxGC-FID-qMS, HPLC-DAD-FLD) techniques, together with the enzymatic or chemical synthesis of a variety of compounds (NP-G, OP-G, different octyl- and nonylphenol isomers) has been necessary for the accomplishment of the proposed objectives.

However, like many research work of this kind, it cannot be considered a finished work and other challenges have been opened for the future. Among other, the following can be mentioned:

i) The use of other sample preparation techniques such as stir bar sorptive extraction (SBSE) or membrane assisted solvent extraction (MASE) for the preconcentration of liquid samples in order to reduce the sample and organic solvent volume used in the sample treatment step.

ii) The use of LC-MS/MS as an alternative to GC-MS in order to avoid the derivatization step.

iii) The application of GCxGC-FID-qMS for the analysis not only of technical mixtures but of environmental sample extracts, which will hopefully lead to the understanding of the degradation pathways of alkylphenols in the environment.

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